TÜBİTAK

Turkish Journal of Veterinary and Animal Sciences

http://journals.tubitak.gov.tr/veterinary/

Research Article

Turk J Vet Anim Sci
(2016) 40: 750-756

doi:10.3906/vet-1601-117

Genetic analysis of avian paramyxovirus type I strains isolated from backyard poultry in Iran

Fereshteh SABOURI[1], Mehdi VASFI MARANDI[1,*], Vahid KARIMI[1], Mohammad MALEKAN[1], Mohsen BASHASHATI[2]

[1]Department of Avian Diseases, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran

[2]Department of Avian Disease Research and Diagnostics, Razi Vaccine and Serum Research Institute, Karaj, Iran

Received: 29.01.2016 • **Accepted/Published Online:** 26.04.2016 • **Final Version:** 15.12.2016

Abstract: Newcastle disease (ND) is one of the most serious diseases amongst many species of birds and causes huge economic losses in the poultry sector. Backyard poultry are the main source of food in rural areas of many developing countries and play a crucial role in the epidemiology of ND. The sequence of the F protein cleavage site is the major virulence determinant of Newcastle disease virus (NDV). The aim of this study was to understand the molecular characterization of F proteins of Iranian backyard poultry in NDVs. The complete open reading frame sequences of the F gene of six isolates were amplified and sequenced. All characterized NDVs had high similarities to Chinese isolates. The phylogenetic analysis revealed that the studied strains fell into genotype VII and grouped in a specific subgenotype. All tested NDVs have a cleavage site of ^{112}RRQKR$\downarrow$F^{117}, representing that all of them belonged to velogenic strains. Rearing methods of backyard poultry increase the chance of contact between a variety of poultry and wild birds; subsequently, new strains evolve and are transmitted between them. Therefore, continuous monitoring of NDVs in backyard poultry would help us to obtain more information about the evolution of NDVs and improve new control strategies in backyard poultry.

Key words: Newcastle disease virus, genetic characterization, F gene, backyard poultry, Iran

1. Introduction

Newcastle disease (ND) is a highly contagious viral disease that affects over 240 species of domestic poultry, aviary, and wild birds (1). This disease is caused by Newcastle disease virus (NDV), which belongs to the genus *Avulavirus*, family *Paramyxoviridae*, order *Mononegavirales* and is also designated as avian paramyxovirus-1 (AMPV-1). NDV is an enveloped virus that contains a negative-sense, nonsegmented, and single stranded RNA genome (2,3). The genome encodes six viral proteins, including nucleocapsid protein (NP), phosphoprotein (P), matrix protein (M), fusion protein (F), hemagglutinin-neuraminidase (HN), and RNA-depended RNA polymerase (L) (2). Two additional proteins, V and W, are generated by RNA editing during P gene transcription (4). According to the clinical signs, four NDV pathotypes are defined: (i) velogenic, which cause an acute lethal infection with high mortality at any age; (ii) mesogenic, which are less pathogenic with high mortality in young birds; (iii) lentogenic, which exhibit asymptomatic infections; and (iv) asymptomatic enteric, which do not show any visible disease (1,2).

In previous studies, two systems have been used to classify NDV isolates into lineages and genotypes based on the analysis of partial or complete F gene sequence. In the first system initially described by Aldous et al., NDVs were classified into seven lineages (1–7) and 20 sublineages (5–7). In the second system, NDVs were grouped into two major classes: class I and class II (3,8,9). Class I was divided into nine genotypes (1–9) (8,10) and class II consisted of eleven genotypes (I–XI) (3,8). Class I strains are found worldwide, generally isolated from waterfowl and shorebirds (3,8,10). Class II is composed of most virulent and some avirulent NDVs and is further divided into subgenotypes 1a, 1b, II, IIa, VIa–VIf, and VIIa–VIIh (11,12). Viruses of class II are generally found in wild birds and poultry species universally (8). Genotype I contains mainly avirulent isolates from wild waterfowl and poultry species of the world. Genotype II–IV viruses were responsible for the first panzootic before the 1960s, whereas genotype V was associated with the second panzootic during the 1960s and 1970s. The third panzootic was caused by genotype VIb in racing pigeons during the 1980s (4,7,8,17,18). Genotypes VIII and VII are respectively responsible for the fourth and latest pandemic that started in the late 1980s in the Far East, Europe, and South Africa (8,20–21). While genotype IX has been isolated in some regions of China since 1948, novel genotype X caused NDV infections in Taiwan in 1969 and 1981 (13,14). Recently, a new genotyping system

* Correspondence: mvmarand@ut.ac.ir

has been designed according to full sequence of the F gene. In this system, NDVs are classified into two classes (class I and class II) with one and eighteen genotypes in each class, respectively (11,15).

Although other genes of APMV-1 are involved in the virulence of NDV, the F gene is a major virulence determinant in this disease (5). The F protein is synthesized as a precursor (F_0) in nonfunctional state that is cleaved by host proteases into two functionally active polypeptides (F_1 and F_2). Virulent strains of NDV contain basic amino acids at residue 113, 115, and 116 plus a phenylalanine at residue 117, while low virulent viruses have fewer than two basic amino acids plus a leucine at residue 117 in the same region (16). These basic amino acids at the cleavage site determine the kind of protease effects in this site. The F_0 of virulent strains is cleaved by ubiquitous furin-like proteases found in most tissues, whereas for low virulent viruses, cleavage can occur only with trypsin-like proteases in the respiratory and intestinal tracts (1).

In developing countries, backyard poultry are an important source of protein production in the form of eggs and meat. Due to the low level of biosecurity and the low rate or lack of vaccination in backyard poultry, ND is the main cause of mortality in these countries (17). In past decades, extensive vaccination in both commercial and backyard poultry has lessened the burden of ND in Iran. Nevertheless, this disease remains enzootic in poultry and other avian sectors. The role of backyard poultry in the epidemiology of ND still remains obscure. Therefore, the surveillance of NDVs in backyard poultry can provide useful epidemiological information of ND and determine links between backyard and commercial poultry. This study aimed to conduct molecular and phylogenetic analysis of the F gene of NDVs circulating in Iranian backyard poultry and compare them with previously published sequences in order to provide a better understanding of the epidemiology and pathogenicity of ND.

2. Materials and methods

2.1. Virus isolation

Six NDVs were isolated from dead chicken tissue samples including trachea and intestine submitted for diagnosis at the Department of Avian Diseases, Faculty of Veterinary Medicine, the University of Tehran, between 2011 and 2013. Virus isolation was performed in the allantoic cavity of 10-day-old embryonated chicken eggs (ECEs). The hemagglutination (HA) and hemagglutination inhibition (HI) assays of allantoic fluids harvested from inoculated embryonating eggs were used to recognize NDV-positive embryos (18). Virulent and nonvirulent NDV strains were differentiated by RT-PCR as previously described (19). Infectious allantoic fluids were harvested from ECEs and stored at –70 °C for further use.

2.2. Pathogenicity test

The pathogenicity of NDVs was determined by mean death time (MDT) assay. According to the MDT assay, velogenic, mesogenic, and lentogenic strains kill embryos in less than 60 h, in 60–90 h, and greater than 90 h, respectively (18).

2.3. RNA extraction and RT-PCR

Viral RNA was extracted from infectious allantoic fluids using RNX-plus solution (Cinnagen, Iran) according to the manufacturer's instruction. The RNA was reverse transcribed by RevertAid first strand cDNA synthesis kit (Thermo Scientific, USA) and Random Hexamer Primer. PCR was carried out using Pfu DNA polymerase (Thermo Scientific, USA). Two pairs of primers were designed for amplifying the complete coding sequence of F genes in two overlapping amplicons.

2.4. Gene sequencing and phylogenetic analysis

PCR products were purified with the GeneJET gel extraction kit (Thermo Scientific, USA). Sequencing was performed by a DNA service company (Source Bioneer, Korea) in both directions. The sequences were compiled and aligned using BioEdit Package (version 7.2.5) (20). The Blast analysis was used to retrieve the homologous sequences for the analyzed sequences. An unrooted phylogenetic tree was constructed by the maximum likelihood method using MEGA software (version 6.06) (21) and criteria described in previous studies (11,15). The robustness of the trees was evaluated by 1000 bootstrap replicates. Nomenclature of genotypes and subgenotypes was based on Diel et al.'s classification (11,15). To define NDV genotypes, the evolutionary distance between different populations at the nucleotide level was estimated using the maximum composite likelihood model. New genotypes should have the cut-off value of >10% (0.1), while the cut-off value of 3 (0.03) and 10% (0.1) was used to separate different subgenotypes (11,15).

2.5. Accession numbers of nucleotide sequences

The six nucleotide sequences for the F gene of NDV isolates were deposited in GenBank under accession numbers KU201408–KU201413.

3. Results

3.1. Pathogenicity analysis

Both RT-PCR and pathogenicity test (MDT), which were used to determine the virulence of NDV isolates, suggested that our isolates were velogenic strains (Table 1).

3.2. Homology analysis

The open-reading frames (ORFs) of the F genes were sequenced and analyzed. All of the studied isolates contained 1662 nucleotides encoded for 553 amino acids. The identity of nucleotide and deduced amino acid sequences of F genes amongst the tested strains was from 99.0% to 99.9% and 98.6% to 99.6%, respectively (data not

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

Sample name	Accession number	Cleavege site	MDT
Ck/IR/SMV-1/11	KU201408	^{112}RRQKR↓F^{117}	49.9 h
Ck/IR/SMV-2/11	KU201409	^{112}RRQKR↓F^{117}	42.0 h
Ck/IR/SMV-3/11	KU201410	^{112}RRQKR↓F^{117}	50.1 h
Ck/IR/SMV-4/12	KU201411	^{112}RRQKR↓F^{117}	47.0 h
Ck/IR/SMV-5/12	KU201412	^{112}RRQKR↓F^{117}	48.2 h
Ck/IR/SMV-6/12	KU201413	^{112}RRQKR↓F^{117}	51.9 h

shown). Comparison of nucleotide and deduced amino acid sequences between studied isolates and other Iranian published commercial poultry isolates ranged from 90.1% to 90.6% and 93.9% to 94.8%, respectively (Table 2). All the studied viruses were closely related to MDk/CN/FP1/02 and Go/CN/WF00G/00 with nucleotide and deduced amino acid sequence homologies of 96.6%–97.2% and 96.7%–97.5%, respectively (Table 2). Therefore, the F genes of tested NDVs displayed the highest nucleotide sequence similarities to Chinese isolates (97%), which suggests that the source of these strains may have come from China.

3.3. Phylogenetic analysis

Phylogenetic analysis was performed based on the ORF sequence (1–1662) of the F genes according to a previous study (11). The Figure shows the phylogenetic tree of the studied F genes and other representative NDVs published in GenBank. These results suggest that all the tested isolates in our study were classified as NDV genotype VII and a new subgenotype based on previous studies (11,15), while other Iranian isolates from commercial poultry were grouped in genotype XIIIa (22).

Table 2. Nucleotide and deduced amino acid sequence homology between studied isolates and other representative strains.

Strain	Genotype	Studied isolates											
		SMV1		SMV2		SMV3		SMV4		SMV5		SMV6	
		nt	aa	nt	aa	nt	aa	nt	aa	nt	aa	nt	aa
Ck/CN/FMW/06 [(a)]	VIIb	96.1	96.0	96.2	96.4	95.9	96.2	96.1	96.0	96.0	95.7	96.0	95.8
MDk/CN/FP1/02	VIId	**97.1**[(b)]	**97.1**	**97.2**	**97.5**	**96.9**	**97.3**	**97.2**	**97.1**	**97.1**	**96.7**	**97.1**	**96.7**
Go/CN/WF00G/00	VIId	**96.8**	**97.1**	**96.9**	**97.5**	**96.6**	**97.3**	**96.8**	**97.1**	**96.6**	**96.7**	**96.8**	**96.7**
Ck/CN/Guangxi2/00	VIIe	95.5	96.0	95.7	96.2	95.5	96.4	95.6	95.8	95.5	95.7	95.5	95.8
Ck/CN/CH-A7/96	VIIf	94.4	94.9	94.5	95.1	94.3	95.3	94.5	94.8	94.3	94.6	94.4	94.8
Ck/CN/SRZ03/03	VIIg	91.3	91.9	91.4	92.0	91.2	91.9	91.5	91.7	91.3	91.5	91.3	91.9
Ck/ID/bali/020/10	VIIh	91.6	94.2	91.8	94.6	91.6	94.2	91.7	94.2	91.6	93.9	91.7	94.0
Ck/PK/Lahore/43/11	VIIi	91.6	94.8	91.8	95.1	91.8	94.9	91.7	94.8	91.6	94.4	91.6	94.4
Ck/IR/EMM-4/10	XIIIa	90.3	94.2	90.4	94.6	90.1	94.2	90.4	94.0	90.3	93.9	90.3	94.0
Ck/IR/EMM-5/11	XIIIa	90.4	94.4	90.6	94.8	90.2	94.4	90.5	94.2	90.4	94.0	90.4	94.2
Ck/PK/BYP/Lahore/10	XIIIb	89.4	93.7	89.5	93.9	89.2	93.3	89.3	93.3	89.2	93.1	89.3	93.3

(a) Abbreviations: Av, Avian; Bz, Buzzard; Ck, Chicken; Dk, Duck; Fo, Fowl; Go, Goose; Ma, Mallard; MDk, Muscovy Duck; Pg, Pigeon; Po, Poultry; AU, Australia; CN, China; IN, India; ID, Indonesia; IR, Iran; IE, Ireland; IT, Italy; MG, Madagascar; MY, Malaysia; MX, Mexico; PK, Pakistan; PE, Peru; US, United States.
(b) The highest percentages of homology are shown in bold.

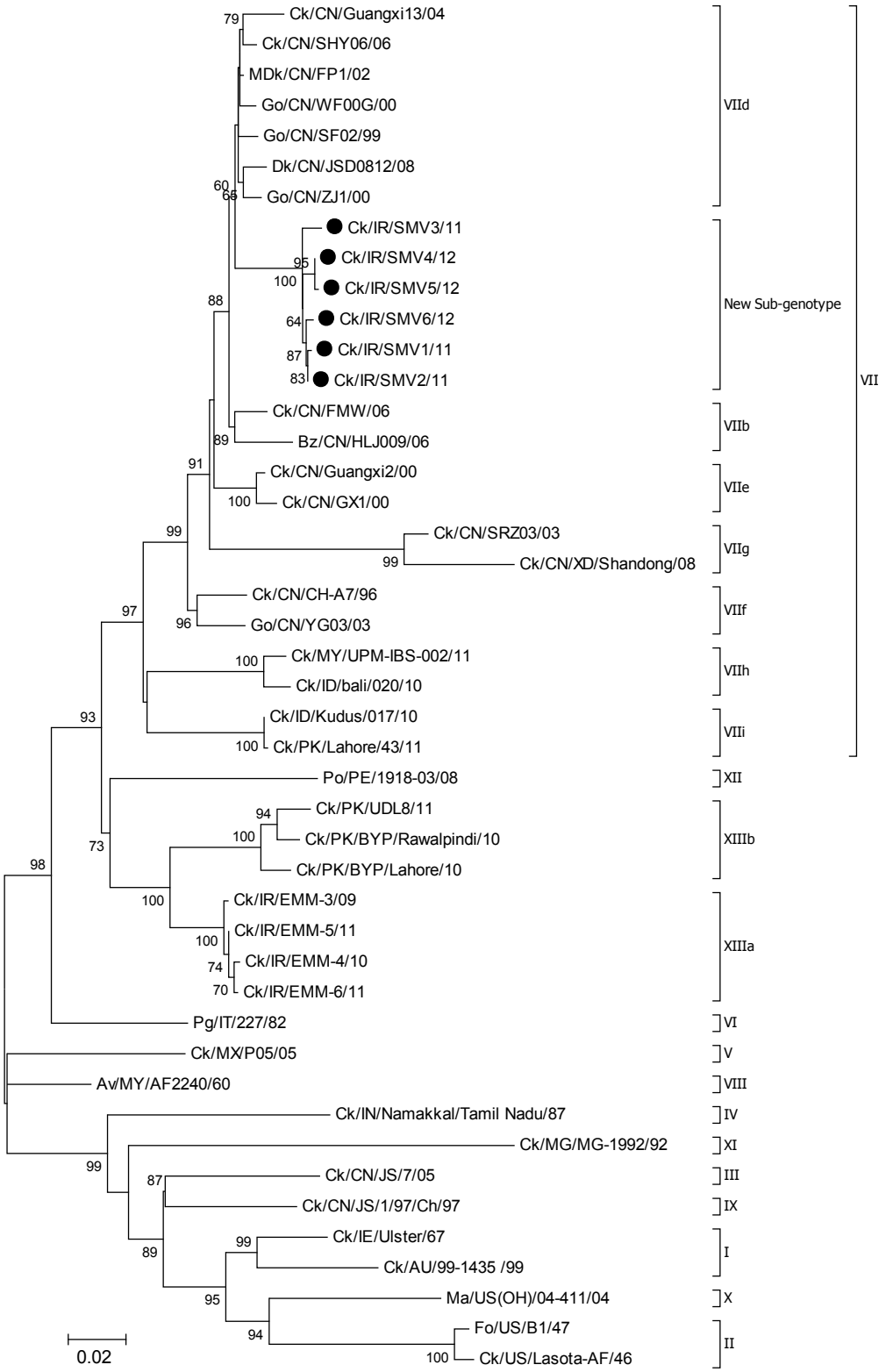

Figure. Phylogenetic analysis of F genes of Iranian NDVs based on the ORF sequence. This tree displays only class II. The phylogenetic tree was generated by using maximum likelihood method with MEGA 6.06 program. Number near the nodes indicates the percentage of bootstrap values of 1000 replicates. Bootstrap values more than 60% are displayed in this figure. The classification system in this figure is according to Diel et al. (11). Viruses characterized in this study are marked with a circle. Abbreviations can be found in the footnote of Table 2.

3.4. Characteristics of F gene

The deduced amino acid sequence of the F protein at the cleavage site was characteristic of virulent viruses. Analysis of the cleavage motif of NDV F_0 protein indicated that all NDV isolates characterized in this study have the same sequence in cleavage site (^{112}RRQKR$^{\downarrow}$F^{117}) (Table 1). This motif is characteristic of highly virulent NDVs (16).

4. Discussion

Since outbreak of ND in 1926, ND has spread widely in many countries and caused huge economic losses. The evidence of ND has been documented in over 240 species, but it seems that NDV can likely infect all avian species (1). The role of backyard poultry in NDV transmission has been explained between wild birds and poultry in both directions (23). Backyard poultry are kept in the rural areas of many developing countries as a source of income and food. Due to the low level of biosecurity and low rate of vaccination, ND can widely occur in backyard poultry (17) and therefore can be considered a source of infection to jeopardize commercial poultry. Furthermore, despite extensive vaccination against ND in Iran, this disease still causes high mortality in commercial broilers and layers. Little is known about the specific circulating genotypes of NDVs in the backyard poultry of Iran. To the best of our knowledge, the data of molecular characterization and phylogenetic analysis of F gene of NDVs circulating in Iranian backyard poultry are not fully reported. Sequence analysis and phylogenetic study were carried out by comparing them with previously published sequences obtained from GenBank. These results provide useful epidemiological information on ND and can be used as a means to design preventive strategies for both backyard and commercial poultry.

Homology between tested isolates was 99.0%–99.9%, suggesting that identical isolates are circulating in the backyard poultry population. Furthermore, the studied isolates shared homology of about 90% with other Iranian full length coding sequences of the F gene recovered from commercial chickens in 2008–2011 (22). These results suggest that tested isolates were distant from viruses that are circulating in commercial poultry.

All backyard poultry strains were more closely related to Chinese isolates mainly species of the family *Anatidae*, showing 97% homology. While waterfowls are recognized as potential reservoirs of NDVs (23), they show few or no clinical signs. Furthermore, most NDV isolates recovered from waterfowls are classified as lentogenic viruses; therefore, virulent strains were isolated rarely in these birds (24). Previous studies have shown that the evolution of the NDV from low to high virulence takes place with only a few point mutations (25,26). Moreover, direct contact between backyard poultry and migratory birds provides an opportunity to transfer avian viruses to new hosts and increase the risk of viral evolution (27). Since 1997, outbreaks of NDVs with apparent clinical signs and high mortality have been reported from waterfowls in China. The pathogenicity indices and cleavage site of these isolates were typical of velogenic strains (28). Since backyard poultry are usually reared free-range, the chance of contact between domestic poultry and migratory birds increases and recombination and subsequent transmission of the new variants occur in backyard poultry (29).

Due to the large genetic diversity observed within subgenotypes of NDVs and the lack of objective criteria to define taxonomic groups, a unified classification system has been designed by Diel et al. (11). In this system, by using phylogenetic topology and evolutionary distances within and between clades of complete F gene sequences, NDVs have been divided into two classes (I and II) (11). Class I is made up of only a single genotype and class II is divided into 18 genotypes (30). Because of high diversity in genotypes I, II, V, VI, and VII, these groups have been divided into subgenotypes (8,10,14). Genotype VII comprises the most diverse NDVs amongst all genotypes and this genotype was related to outbreaks of ND in the Middle East and Asia (8). In this classification system, genotype XIII NDV viruses were isolated in Russia, Iran, and Pakistan between 1995 and 2008, whereas these viruses previously belonged to genotype VII (11). After the outbreak of genotype XIII in Pakistan between 2009 and 2011, this genotype was replaced by subgenotype VIIi, which has been responsible for ND outbreaks since 2012 (15). In our study, all characterized viruses were classified as a novel subgenotype in genotype VII. In a previous study based on ORF sequencing of the F gene, Iranian isolates grouped in subgenotype VIIb, while in this study, these NDVs fell into subgenotype XIIIa (22). In another study in Iran, molecular analysis of 6 NDVs showed that all isolates were velogenic according to sequence of cleavage site (^{112}RRQRR$^{\downarrow}$F^{117}). Phylogenetic analysis indicated that all Iranian NDVs isolated between 1996 and 2004 were related to genotype VII (31). This classification was conducted according to previous classification and sequencing of the hypervariable region of the F gene (31). These dissimilarities between our results and the other studies may be explained by different sets of criteria used in different classification systems.

The major determinant of virulence or pathogenicity of NDVs is correlated to the sequence of the F protein cleavage site. The cleavage site of the velogenic and mesogenic strains is ^{112}(R/K)RQ(R/K)R$^{\downarrow}$F^{117}, whereas the cleavage motif of the lentogenic strains is ^{112}(G/E)(K/R)Q(G/E)R$^{\downarrow}$L^{117}. These differences between NDV cleavage sites prepare different substrates for different types of cellular protease. The F protein of lentogenic viruses

is cleaved only by trypsin-like proteases found in the respiratory system and gastrointestinal tract. The sequence of virulent viruses can be cleaved by a variety of cellular proteases in different organs, resulting in wider systemic infection (32). All characterized NDVs in this study have typical motif of velogenic strains (^{112}RRQKR$^{\downarrow}$F^{117}); therefore, these strains are grouped in velogenic strains, as expected from the MDT test.

In summary, this study is the first report on characterization of the complete coding sequence of F genes in backyard poultry in Iran. The results of this study indicated that the tested isolates have a high level of similarity to Chinese isolates, suggesting a possible source of Iranian NDVs. Moreover, a new subgenotype circulating in backyard poultry was identified that has not been recognized in poultry so far. Due to the rearing method of backyard poultry, the chance of contact between a variety of poultry and wild birds increases, and, as a consequence, new strains could be evolved and transferred between them. Therefore, continuous monitoring of NDVs in poultry is necessary to obtain information about the evolution of NDVs and emergence of panzootic strains. Finally, the present study highlights the need to implement control strategies and use efficient vaccines in backyard poultry.

Acknowledgment

This study was financially supported by a grant (No. 7508001/6/15) from the research council of Tehran University.

References

1. Miller PJ, Koch G. Newcastle disease. In: Swayne DE, Glisson JR, McDougald LR, Nolan LK, Suarez DL, Nair V, editors. Diseases of Poultry. 13th ed. Oxford, UK: Wiley-Blackwell; 2013. pp. 394-458.
2. Alexander DJ, Senne DA. Newcastle disese. In: Saif YM, Fadly AM, Glisson JR, McDougald LR, Nolan LK, Swayne DE, editors. Diseases of Poultry. 12th ed. Ames, IA, USA: Blackwell; 2008. pp. 75-100.
3. Czeglédi A, Ujvári D, Somogyi E, Wehmann E, Werner O, Lomniczi B. Third genome size category of avian paramyxovirus serotype 1 (Newcastle disease virus) and evolutionary implications. Virus Res 2006; 120: 36-48.
4. Steward M, Vipond IB, Millar NS, Emmerson PT. RNA editing in Newcastle disease virus. J Gen Virol 1993; 74: 2539-2547.
5. Aldous EW, Mynn JK, Banks J, Alexander DJ. A molecular epidemiological study of avian paramyxovirus type 1 (Newcastle disease virus) isolates by phylogenetic analysis of a partial nucleotide sequence of the fusion protein gene. Avian Pathol 2003; 32: 239-256.
6. Cattoli G, Fusaro A, Monne I, Molia S, Le Menach A, Maregeya B, Nchare A, Bangana I, Maina AG, Koffi JN et al. Emergence of a new genetic lineage of Newcastle disease virus in West and Central Africa—implications for diagnosis and control. Vet Microbiol 2010; 142: 168-176.
7. Snoeck CJ, Ducatez MF, Owoade AA, Faleke OO, Alkali BR, Tahita MC, Tarnagda Z, Ouedraogo JB, Maikano I, Mbah PO et al. Newcastle disease virus in West Africa: new virulent strains identified in non-commercial farms. Arch Virol 2009; 154: 47-54.
8. Miller PJ, Decanini EL, Afonso CL. Newcastle disease: evolution of genotypes and the related diagnostic challenges. Infect Genet Evol 2010; 10: 26-35.
9. Seal BS, King DJ, Bennett JD. Characterization of Newcastle disease virus isolates by reverse transcription PCR coupled to direct nucleotide sequencing and development of sequence database for pathotype prediction and molecular epidemiological analysis. J Clin Microbiol 1995; 33: 2624-2630.
10. Kim LM, King DJ, Suarez DL, Wong CW, Afonso CL. Characterization of class I Newcastle disease virus isolates from Hong Kong live bird markets and detection using real-time reverse transcription-PCR. J Clin Microbiol 2007; 45: 1310-1314.
11. Diel DG, da Silva LH, Liu H, Wang Z, Miller PJ, Afonso CL. Genetic diversity of avian paramyxovirus type 1: proposal for a unified nomenclature and classification system of Newcastle disease virus genotypes. Infect Genet Evol 2012; 12: 1770-1779.
12. Perozo F, Merino R, Afonso CL, Villegas P, Calderon N. Biological and phylogenetic characterization of virulent Newcastle disease virus circulating in Mexico. Avian Dis 2008; 52: 472-479.
13. Tsai HJ, Chang KH, Tseng CH, Frost KM, Manvell RJ, Alexander DJ. Antigenic and genotypical characterization of Newcastle disease viruses isolated in Taiwan between 1969 and 1996. Vet Microbiol 2004; 104: 19-30.
14. Wang Z, Liu H, Xu J, Bao J, Zheng D, Sun C, Wei R, Song C, Chen J. Genotyping of Newcastle disease viruses isolated from 2002 to 2004 in China. Ann N Y Acad Sci 2006; 1081: 228-239.
15. Miller PJ, Haddas R, Simanov L, Lublin A, Rehmani SF, Wajid A, Bibi T, Khan TA, Yaqub T, Setiyaningsih S et al. Identification of new sub-genotypes of virulent Newcastle disease virus with potential panzootic features. Infect Genet Evol 2015; 29: 216-229.
16. Collins MS, Bashiruddin JB, Alexander DJ. Deduced amino acid sequences at the fusion protein cleavage site of Newcastle disease viruses showing variation in antigenicity and pathogenicity. Arch Virol 1993; 128: 363-370.
17. Alexander DJ. Gordon Memorial Lecture. Newcastle disease. Br Poult Sci 2001; 42: 5-22.
18. Terregino C, Capua I. Conventional diagnosis of newcastle disease virus infection. In: Capua I, Alexander DJ, Editors. Avian Influenza and Newcastle Disease. 1st ed. Milan, Italy: Springer-Verlag Mailand; 2009. pp. 123-125.

19. Kant A, Koch G, Van Roozelaar DJ, Balk F, Huurne AT. Differentiation of virulent and non-virulent strains of Newcastle disease virus within 24 hours by polymerase chain reaction. Avian Pathol 1997; 26: 837-849.

20. Hall TA. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucl Acids Symp Ser 1999; 41: 95-98.

21. Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Mol Biol Evol 2013; 30: 2725-2729.

22. Ebrahimi MM, Shahsavandi S, Moazenijula G, Shamsara M. Phylogeny and evolution of Newcastle disease virus genotypes isolated in Asia during 2008-2011. Virus Genes 2012; 45: 63-68.

23. Schelling E, Thur B, Griot C, Audige L. Epidemiological study of Newcastle disease in backyard poultry and wild bird populations in Switzerland. Avian Pathology 1999; 28: 263-272.

24. Alexander DJ, Campbell G, Manvell RJ, Collins MS, Parsons G, McNulty MS. Characterisation of an antigenically unusual virus responsible for two outbreaks of Newcastle disease in the Republic of Ireland in 1990. Vet Rec 1992; 130: 65-68.

25. Chong YL, Padhi A, Hudson PJ, Poss M. The effect of vaccination on the evolution and population dynamics of avian paramyxovirus-1. PLoS Pathog 2010; 6: e1000872.

26. Kattenbelt JA, Stevens MP, Gould AR. Sequence variation in the Newcastle disease virus genome. Virus Res 2006; 116: 168-184.

27. Lee HJ, Kwon JS, Lee DH, Lee YN, Youn HN, Lee YJ, Kim MC, Jeong OM, Kang HM, Kwon JH et al. Continuing evolution and interspecies transmission of influenza viruses in live bird markets in Korea. Avian Dis 2010; 54: 738-748.

28. Liu H, Wang Z, Wang Y, Sun C, Zheng D, Wu Y. Characterization of Newcastle disease virus isolated from waterfowl in China. Avian Dis 2008; 52: 150-155.

29. Siddique N, Naeem K, Abbas MA, Ali Malik A, Rashid F, Rafique S, Ghafar A, Rehman A. Sequence and phylogenetic analysis of virulent Newcastle disease virus isolates from Pakistan during 2009-2013 reveals circulation of new sub genotype. Virology 2013; 444: 37-40.

30. Snoeck CJ, Owoade AA, Couacy-Hymann E, Alkali BR, Okwen MP, Adeyanju AT, Komoyo GF, Nakouné E, Le Faou A, Muller CP. High genetic diversity of Newcastle disease virus in poultry in West and Central Africa: cocirculation of genotype XIV and newly defined genotypes XVII and XVIII. J Clin Microbiol 2013; 51: 2250-2260.

31. Samadi S, Kianizadeh M, Najafi MF, Nasab SD, Davatgar AM, Royaee A, Pilvar P. Molecular characterization and phylogenetic study of velogenic Newcastle disease virus isolates in Iran. Virus Genes 2014; 48: 290-295.

32. Glickman RL, Syddall RJ, Iorio RM, Sheehan JP, Bratt MA. Quantitative basic residue requirements in the cleavage-activation site of the fusion glycoprotein as a determinant of virulence for Newcastle disease virus. J Virol 1988; 62: 354-356.