Bulgarian Journal of Veterinary Medicine, 2022, **25**, No 1, 21–32
ISSN 1311-1477; DOI: 10.15547/bjvm.2020-0041

Original article

MOLECULAR CHARACTERISATION AND PHYLOGENETIC STUDY OF THE FUSION GENE OF NEWCASTLE DISEASE VIRUSES ISOLATED FROM BROILER FARMS OF IRAN IN 2018–2019

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Summary

Allahyari, E., M. Allymehr, A. Molouki, M. H. Fallah Mehrabadi & A. Talebi, 2022. Molecular characterisation and phylogenetic study of the fusion gene of Newcastle disease viruses isolated from broiler farms of Iran in 2018–2019. *Bulg. J. Vet. Med.,* **25**, No 1, 21–32.

Avian orthoavulavirus, commonly known as Newcastle disease virus (NDV) has been a constant threat for the poultry industry of Iran for decades. Recently, a couple of preliminary studies on backyard and commercial chicken suggested that a major subgenotype circulating in Iran may be VII(L) subgenotype, which is now known as VII.1.1 according to the new classification system. The unique subgenotype was not reported from other parts of the world and was slightly (≥3%) different from the closest group that was VIId. The study was conducted between July 2018 and March 2019 to determine the exact NDV genotypes/subgenotypes circulating in Iranian broiler poultry farms; five-hundred and forty chickens were sampled from thirty-six broiler farms located in eighteen provinces of Iran. As other genotypes/subgenotypes such as XIII and VI.2 are circulating in neighbouring countries, border provinces were also sampled. The F gene of the NDV isolates was sequenced and phylogenetic analysis was conducted. All the isolates clustered under the VII.1.1 group. The evolutionary analysis also revealed that the distances were between 0.0 and 0.7% meaning that the Iranian NDV circulating in broiler farms were not only of VII.1.1 sub-genotype, but also genetically very identical, indicating that the routine control measures for ND in Iran were not able to prevent the circulating NDVs. Although stricter biosecurity measures have been really effective in developed countries, surveillance of NDV to determine the circulating genotypes might also help us to implement better vaccination strategies in the future.

Key words: broilers, fusion gene, Iran, Newcastle disease virus, phylogenetic analysis, RT-PCR

INTRODUCTION

Newcastle disease virus (NDV) is an avian virus that is able to infect most species of birds (Dortmans *et al*., 2011; Miller & Koch, 2013). NDV spreads primarily through direct contact between infected and healthy birds. Together with avian influenza, NDV is one of the two major threats to the poultry industry of developing countries such as Iran. The first outbreaks of NDV were reported in Java, Indonesia, and Newcastle city in England, during the mid-1920s (Doyle, 1927). Within a few years NDV had spread throughout the world and became endemic in many countries (Dortmans *et al*., 2011). Now, the Newcastle disease (ND) has a global impact on poultry industry worldwide.

In the last five years, 109 of 200 member countries have reported the ND to the OIE (Dimitrov *et al*., 2019). Between 2006 and 2009, ND was ranked the 8th most important wildlife disease and the 3rd most important poultry disease (OIE, 2011). NDV was also formerly known as avian paramyxovirus 1 (APMV-1) but the International Committee on Taxonomy of Viruses (Walker *et al*., 2019) has recently renamed the virus to avian orthoavulavirus 1 (AOAV-1), a new species under the family of paramyxoviridae. Based on its pathogenicity, NDV has been categorised into five major pathotypes known as avirulent (asymptomatic enteric), mildly virulent (lentogenic), moderately virulent (mesogenic), and two types of very virulent (velogenic) strains with viscerotropic and neurotropic signs (OIE, 2018). This categorisation is mainly based on *in vivo* pathogenicity tests such as mean death time (MDT) and intracerebral pathogenicity index (ICPI) (OIE, 2018).

The genome of NDV is a non-segmented, single-stranded, negative sense RNA of 15186, 15192 or 15198 nucleotides in length, which contains six genes each encoding six structural proteins known as the nucleocapsid protein (NP), phosphoprotein (P), matrix protein (M), fusion protein (F), haemagglutinin-neuraminidase (HN), large polymerase protein (L). Additionally, two non-structural proteins known as V and W are produced as the result of RNA editing of the P gene (Alexander *et al*., 2008).

NDV strains have been divided into two major classes based on the sequence of the F gene (Dimitrov *et al*., 2019). Class I strains are mainly avirulent and genetically less diverse while class II NDVs, are genetically more diverse and include viruses from all five pathotypes. Class II viruses are classified into 18 genotypes. The current virulent strains circulating in the Middle-East belong to genotype VII of Class II (Dimitrov *et al*., 2019). Recently, genotype VII.1.1 was identified as a possible dominant genotype in poultry farms of Iran (Molouki *et al*., 2019). The cleavage site on the F gene protein is known to be a major determinant for the virulence of NDV and presence of a multiple basic amino acids motif (112R/K-R-Q-K/R-R116), followed by a phenylalanine (F) at position 117 is indicative of a virulent virus (Peeters *et al*., 1999). In contrast, NDV with low virulence usually have a motif of 112 G/E-K/R-Q-G/E-R 116 followed by Leucine (L) at position 117 (Peeters *et al*., 1999). Moreover, the F glycoprotein of NDV contains six conserved potential N-linked glycosylation acceptor sites at residues 85, 191, 366, 447, 471 and 541. Four of these

sites present at residues 85, 191, 366 and 471 are functionally active and two of these residues at positions 191 and 471 are present within the heptad repeats HR1 and HR2, suggesting that N-glycosylation at these sites might play an important role in the fusion promotion (McGenns *et al*., 2001). The five major epitopes were mapped on the F of NDV(A1-A5), were involved in fusion inhibition and neutralisation (Toyoda *et al*., 1989). However, mutations in the neutralisation epitopes are considered to be the mechanism which involved in the evolution of NDV (Sakaguchi *et al*., 1989; Toyoda *et al*., 1989).

ND is endemic in Iran and many commercial poultry farms have been affected in recent years (Kiani *et al*., 2016; Sabouri *et al*., 2017; Ghalyanchilangeroudi & Hosseini, 2018; Jabbarifakhar *et al*., 2018; Molouki *et al*., 2019). The purpose of the current study was to investigate the phylogenetic relationship between NDVs circulating in across the commercial broilers farm in Iran in 2018–2019. Previous studies on NDV in Iran have been locally limited and the distribution of the industrial poultry population in Iran has not been very well addressed. In the current study, however, we provided more sampling and performed a more comprehensive phylogenetic analysis in order to give us a better picture of the distribution of the dominant NDV genotype/subgenotype in the industrial poultry population of Iran.

MATERIALS AND METHODS

Sample collection

Five-hundred and forty samples from thirty-six vaccinated broiler farms located in eighteen provinces of Iran were collected between July 2018 and March 2019 (Fig. 1). The provinces were selected for sampling based on existing disease records in the Iranian Veterinary Organiza-

Fig. 1. Samples were collected from broiler farms located in eighteen provinces of Iran, spanning the entire country and reaching the borders from all four cardinal directions.

tion (IVO) database. Moreover, the regions were selected according to their broiler farms density and history of previously reported NDV. Furthermore, from each geographical direction, at least one province that shared border with neighbouring countries was selected (North: East Azerbaijan, Ardabil and Golestan; South: Hormozgan; East: Razavi Khorasan; West: West Azerbaijan). Criteria for selection of broiler farms was incremental mortality for at least three consecutive days and also typical clinical symptoms of ND such as green diarrhoea, wing or neck paralysis and respiratory symptoms.

To calculate the minimum number of farms required for sampling, a 36.5% prevalence was assumed (Haji-abdolvahab *et al*., 2018). In each selected province, at least one farm suspected of ND was sampled. Furthermore, tissue samples such as lungs, trachea, spleen and cecal tonsils were obtained from 5–7 birds (with clinical sings or recently died) and brain tissue was sampled if neurological symptoms were observed (Brandon & Alexander, 2016).

Virus isolation

Virus isolation was carried out as previously described (Brandon & Alexander, 2016; OIE, 2018), Briefly, tissues were homogenised as a 10% (w/v) suspension in PBS containing antibiotics (streptomycin, penicillin) and centrifuged (2800 rpm for 10 min). Two-hundred microliter of supernatant was injected into 9-day-old specific pathogen-free (SPF) eggs via the allantoic sac route. The eggs were examined every 12 h for 3 days and the times of embryo deaths were recorded. After harvest, the allantoic fluid of either dead or live embryos was subjected to rapid hemagglutination test, as well as to HA and HI assays, according to OIE guidelines (OIE, 2018).

RNA extraction and PCR

Viral RNA was extracted from virus-infected allantoic fluid using RNX-plus™ solution (Cinnagen, Iran) according to manufacturer's protocol. Reverse transcription was conducted with a commercial cDNA synthesis kit (Cinnagen, Iran) using random primers. A preliminary PCR to confirm the presence of NDV by a 202-

Table 1. List of specific primers used in this study

Gene	Primer	Primer sequence	Primer binding locations	Reference
F	Creelan-F	5′-GGTGAGTCTATCCGGARGATACAAG-3′	4835-4859	Creelan *et al*., 2002
F	Creelan-R	5′-TCATTGGTTGCRGCAATGCTCT-3′	5016-5037	Creelan *et al*., 2002
F	F-Fwd	5′-YTGCTTATAGTTAGTTYACCTGTC-3′	4462-4485	Molouki *et al*., 2019
F	F-Rev	5′-ACCCGTGTATTGCTYTTYGG-3′	6313-6332	Molouki *et al*., 2019
F	F-Fwd-middle	5′-GCAACCAATGAAGCTGTGCATGA-3′	5027-5049	Molouki *et al*., 2019
F	F-Rev-middle	5′-ACAGCTTCTCCATAATTTTGCGA-3′	5774-5796	Molouki *et al*., 2019

bp product was carried out as previously described (Creelan *et al*., 2002). At the next step, a 1871-bp fragment containing the entire F gene was PCR amplified (2m at 94 ^{o}C, and 35 cycles of 35 s at 93.5 ^{o}C, 35 s at 50 ^{o}C, and 160 s at 72 ^{o}C, followed by an additional 7 m at 72 ^{o}C) using F-Fwd, and F-Rev specific primers (Table 1) binding to regions flanking the F genes of mostly virulent strains (Molouki *et al*., 2019). The gel-purified products were subjected to Sanger sequencing.

Phylogenetic analysis

The phylogenetic tree based on the sequence of the F gene was constructed and interpreted according to the latest classification system for NDV published by the NDV consortium (Dimitrov *et al*., 2019). For this purpose, the latest FAST files, i.e. the large and the pilot datasets, were downloaded from the NDV consortium's GitHub webpage and then the F gene sequence of our isolates were added to the files for analysis. However, the complete F gene sequence of only thirteen of the isolates are represented in this report as representatives for all the isolates (Table 2). This was because the F genes of the remaining isolates were partially sequenced and therefore they were not suitable for analysis as recommended by the NDV consortium's guidelines (Dimitrov *et al*., 2019).

The tree construction and the evolutionary distance analysis were both carried out using MEGA 7 (Kumar *et al*., 2016) and MEGA X (Kumar *et al*., 2018) software. Phylogenetic tree was constructed using maximum likelihood method with the optimum nucleotide method (GTR + G + I) and a 1000 bootstrap replicates.

The evolutionary distance was inferred using maximum composite likelihood model, with rate variation among sites that was modeled with a gamma distribution (shape parameter = 1). Complete deletion was selected in order to eliminate missing data for both analyses.

As recommended by the recent NDV classification system, distances of more than 10 and 5–10% must be used to assign genotypes and subgenotypes, respectively (Dimitrov *et al*., 2019).

RESULTS

The preliminary PCR analysis showed that 450 of 540 (> 83%) samples tested positive for ND (30 of 36 broiler farms and 16 of 18 provinces were positive). The positive samples belonged to the provinces of Isfahan, Yazd, Mazandaran, West Azerbaijan, East Azerbaijan, Gilan, Zanjan, Fars, Golestan, Kurdistan, Hormozgan, Semnan, Kerman, Arak, Qom and Hamadan.

In order to conduct phylogenetic analysis, the entire F gene sequence of thirteen samples were selected (Table 2), aligned and studied (all samples were sequenced but because of shorter lengths some were not represented in here; see Materials and Methods for further explanation). As shown on Fig. 2, all of the selected viruses grouped with other VII.1.1 subgenotype members. Furthermore, the evolutionary distance was calculated to show high similarity between nucleotide sequences revealing the isolates to be closely related. As shown in Table 3, in fact, no other genotype/subgenotype was detected in our study as even the shorter sequences (not named here) had a low distance score (max difference 0.7%). In addition, the evolutionary distance between our isolates as a group and other VII sub-genotype groups revealed the isolates belonged to VII.1.1 sub-genotype (Table 4). At least four sequences are

Table 2. Information for isolates and preliminary test results. All thirteen isolates were very virulent.

Name	GenBank accession number	Date of isolation	Province	MDT
CK/IR/MAM18/2017	MN242824	Jul 2017	West Azerbaijan	<48 h
CK/IR/EMMA121/2018	MN370895	Oct 2018	Hamedan	<48 h
CK/IR/EMMA140/2018	MN370896	Nov 2018	West Azerbaijan	<48 h
CK/IR/EMA152/2019	MN481192	Feb 2019	Golestan	<48 h
CK/IR/EMA111/2018	MN481193	Jul 2018	Fars	<48 h–72 h
CK/IR/EMA150/2019	MN481194	Jan 2019	East Azerbaijan	<48 h
CK/IR/EMA130/2018	MN481195	Oct 2018	West Azerbaijan	<48 h
CK/IR/EMA151/2019	MN481196	Feb 2019	East Azerbaijan	<48 h
CK/IR/EMA132/2018	MN481197	Dec 2018	Gilan	<48 h
CK/IR/EMA136/2018	MN481198	Nov 2018	Semnan	<48 h–72 h
CK/IR/EMA133/2018	MN481199	Jun 2018	Kerman	<72 h
CK/IR/EMA128/2018	MN481200	Jun 2018	Mazandaran	<48 h
CK/IR/EMA148/2019	MN481201	Jan 2019	Gilan	<48 h

Table 3. The number of base substitutions per site (1044 positions) among the 13 isolates' sequences using the Maximum Composite Likelihood model (evolutionary analyses were conducted in MEGA X)

	18	111	121	128	130	132	133	136	140	148	150	151
18												
111	0.006											
121	0.004	0.004										
128	0.004	0.004	0.002									
130	0.007	0.007	0.005	0.005								
132	0.005	0.005	0.003	0.003	0.006							
133	0.006	0.006	0.004	0.002	0.007	0.001						
136	0.005	0.005	0.003	0.003	0.006	0.000	0.001					
140	0.004	0.004	0.000	0.002	0.005	0.003	0.004	0.003				
148	0.005	0.005	0.003	0.003	0.006	0.000	0.001	0.000	0.003			
150	0.006	0.006	0.004	0.004	0.003	0.005	0.006	0.005	0.004	0.005		
151	0.006	0.006	0.004	0.004	0.003	0.005	0.006	0.005	0.004	0.005	0.000	
152	0.005	0.005	0.001	0.003	0.006	0.004	0.005	0.004	0.001	0.004	0.005	0.005

needed to identify a new sub-genotype (Dimitrov *et al*., 2019). In this study thirteen were used.

Furthermore, all the NDV isolates represented the amino acid sequence of 112RRQKRF117 for the F0 cleavage site, an indicator for very virulent strains (Alexander *et al*., 2008).

DISCUSSION

NDV has been a major problem for poultry industry around the world and great hard works have been done to study its epidemiology and virology (Alexander *et al*., 2012). Iran has been dealing with ND for decades but it was first identified here in the 1950's (Sohrab, 1974). In fact,

Table 4. Estimates of evolutionary divergence between the 13 isolates sequences and other groups that have either been used as vaccine (genotype I and II) or been previously detected virulent genotypes in Iran and neighboring countries (genotypes VII and XIII), using both old and new nomenclature systems (Diel *et al*., 2012; Dimitrov *et al*., 2019).

NDV genotype/ subgenotype	Evolutionary distance between the isolates (n=13) and other groups
I (I) (n=4)	0.18
II (II) (n=6)	0.22
VII.1.1 (VII b) (n=5)	0.04
VII.1.1 (VII d) (n=5)	0.03
VII.1.1 (VII e) (n=4)	0.05
VII.1.1 (VII j) (n=8)	0.06
VII.1.1 (VII l) (n=11)	0.01
VII.1.2 (VII f) (n=4)	0.05
VII.2 (VII h) (n=5)	0.09
VII.2 (VII i) (n=5)	0.09
VII.2 (VII k) (n=5)	0.11
XIII.1.1 (XIII a) (n=4)	0.11
XIII.1.2 (XIII a) (n=5)	0.12
XIII.2 (XIII b) (n=6)	0.16
XIII.2.1 (XIII b) (n=4)	0.13
XIII.2.2 (XIII b) (n=4)	0.15

NDV is endemic in Iran, however, the genotypes circulating in the Iran are not well studied. Recently, Molouki *et al*. (2019) suggested that the current dominant NDV genotype circulating in the poultry farms of Iran may be VII.1.1. However, the number of samples, as well as the provinces selected for sampling, were limited in that study. In other words, the selection did not cover the entire country. In this study, not only more provinces were sampled, but also border provinces were selected (Fig. 1). As already mentioned, the idea was to have at least one province with shared border selected from every cardinal direction.

The phylogenetic tree grouped the isolates in the same cluster with other VII.1.1 NDV viruses isolated previously (Fig. 2). As a group, the evolutionary distance analysis between our isolates and other VII sub-genotypes showed more than 5% distance (Table 4). As described by Dimitrov, group distances below 5% are considered same subgenotype (Dimitrov *et al*., 2019). Moreover, the evolutionary analyses between all the isolate of this study showed between 0.0 to 0.7% distance (Table 3), further proving that the recent dominant NDV strains circulating in Iranian poultry farms are not only VII.1.1, but also very much identical. In other words, there is no phylogenetic difference between circulating NDV in different provinces. Furthermore, the amino acid sequence of the F0 cleavage site for all the isolates was found to be 112RRQKRF117, which is an indicator for virulent strains.

Genotype XIII isolates were previously reported from Iran (Ebrahimi *et al*., 2012; Soltani *et al*., 2019). Recently, a VII.2 isolate was also reported from Tehran, Iran (Ghalyanchilangeroudi & Hosseini, 2018). However, no genotype/subgenotype other than VII.1.1 was isolated from the broiler poultry farms of our study conducted between 2018 and 2019, This means that VII.1.1 is dominant in Iranian broiler poultry farms.

Genotypes XIII and VII.2 NDV are regularly reported from neighboring countries (Fuller *et al*., 2017; Wajid *et al*., 2018). Although, import and export of poultry is very limited in Iran, it is not far from expectation that these groups can be detected in Iran as well. But very interestingly, we only detected VII.1.1 in our study. This probably means that the uni-

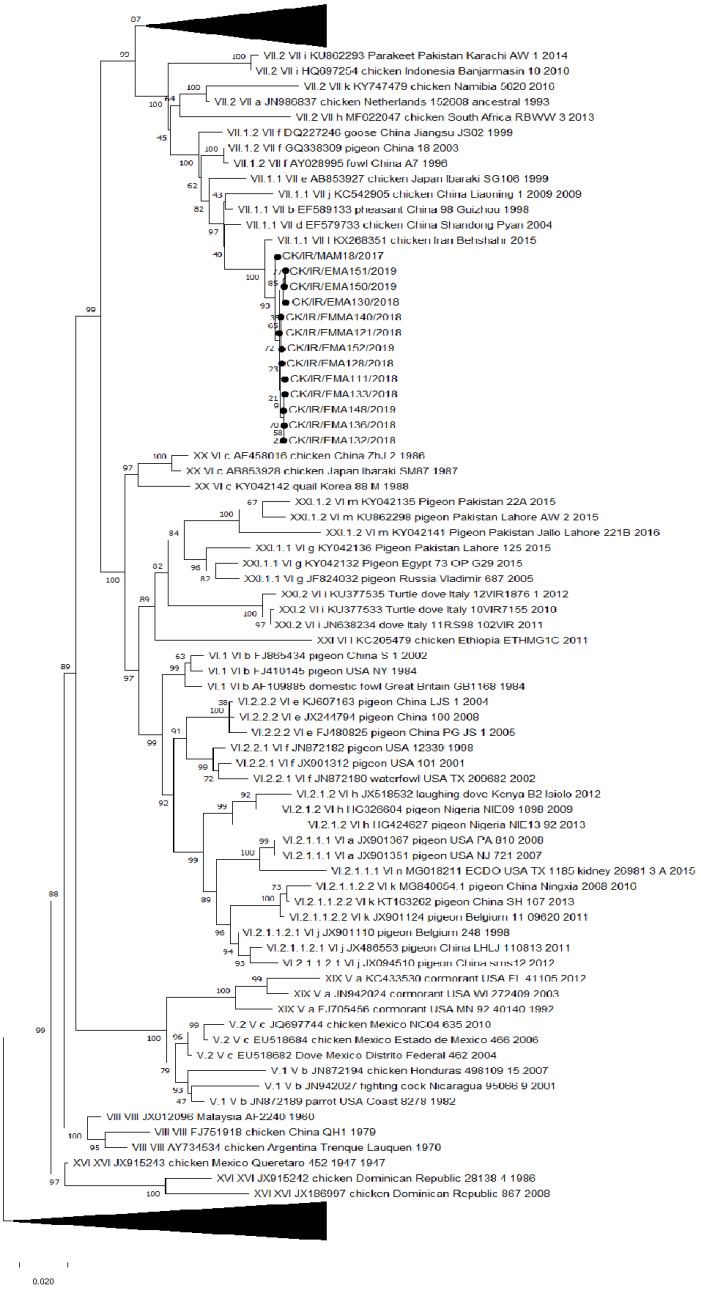

Fig. 2. Molecular phylogenetic tree based on F gene sequence of 13 NDV isolates by Maximum Likelihood method (1000 bootstrap replication). This analysis involved 138 nucleotide sequences.

que Iranian VII.1.1, which is not reported from other countries, is being spread within the country mainly through human factors such as transportation and trading.

Sabouri *et al*. (2017) suggested that the Iranian VII(L) sub-genotypes might have been derived from VIId subgenotype. The rapidly spreading subgenotype VIId strains were considered as one of the major circulating subgenotypes in many parts of the globe (Zhang *et al*., 2014). However, whether Iranian sequences are unique to Iran or not (Molouki *et al*., 2019), the new classification system has grouped the entire VII (b+d+e+J+L) as one group assigned as VII.1.1 (Dimitrov *et al*., 2019). Therefore, the previous nomenclature does not matter anymore.

As the routine vaccination programmes against Newcastle disease have not been really effective in Iran, therefore virulent NDV are still a big threat for Iranian poultry industry. We are optimistic that the results of our comprehensive study on the genotyping and epidemiology of ND viruses circulating across Iranian commercial broiler industry could be helpful in establishment of more effective vaccination strategies. For example, previous studies have shown that compared to broilers vaccinated with heterologous vaccines such as LaSota, broilers vaccinated with homologous vaccines (against subgenotype VII) had lower mortality and morbidity when challenged with virulent NDV (Hu *et al*., 2009).

Homologous vaccines also significantly reduced virus shedding from the vaccinated broilers and recently, it has been shown that the use of inactivated homologous vaccines against genotype VII provided better clinical protection during challenges with the field virus (Sedeik *et al*., 2018). Therefore, the use of homologous vaccines is indeed more effective in controlling ND, and this is not surprising. However, the finger of blame for the current devastating situation in poultry farms of Iran and other developing countries must not be pointed at the current vaccinal strains (Liu *et al*., 2018), In fact, one of the most important factors in controlling the disease is considered to be stricter biosecurity measures and eradication of affected farms. Although NDV is endemic in many developing countries, it is not considered a major threat in many economically advance countries such as Canada and many European countries. This includes other pathogens such as avian influenza virus (AIV) and infectious bronchitis virus (IBV) as well. Perhaps lessons must be learned from such developed and advanced systems to take these destructive and deadly diseases under control.

In conclusion, the results of this comprehensive study showed the dominant genotype/sub-genotypes of NDV with their geographical distribution in Iran and considering the high similarity of NDVs, they are recommended to produce more effective vaccines in controlling Iranian circulating NDVs.

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Paper received 17.02.2020; accepted for publication 03.05.2020

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