Molecular characterization and phylogenetic study of the fusion genes of Newcastle disease virus from the recent outbreaks in Ahvaz, Iran

Zahra Boroomand¹ · Ramezan Ali Jafari¹ · Mansour Mayahi¹

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Abstract Newcastle disease (ND) is an acute and highly contagious disease affecting many domestic and wild species of birds. Its effects are most notable in domestic poultry due to their high susceptibility and the potential for severe impacts of an epizootic on the poultry industry. In this study, partial sequences of fusion genes of three Newcastle disease virus (NDV) isolates collected during 2013–14 outbreaks from the vaccinated commercial broiler chicken farms with high mortality around Ahvaz city (Southwest of Iran) were characterized. All three isolates showed the amino acid sequence 112RRQKRF117 at the C-terminus of the F2 protein and phenylalanine at the N-terminus of the F1 protein residue 117. These amino acid sequences were identical to a known virulent motif. The phylogenetic analysis revealed that the Iranian ND isolates in this study are closely related to the genotype VIIId of class II NDV strains. Our results specified that there are velogenic NDV circulating in Ahvaz commercial broiler flocks and causing outbreaks in poultry industry.

Keywords Chicken · NDV · Phylogenetic analysis · Fusion protein · Iran

Newcastle disease refers to an infection of birds caused by virulent virus of avian paramyxovirus serotype 1 (APMV-1). Newcastle disease virus remains a serious impediment for poultry production in Asia and Africa, and remains a control problem throughout the world [8]. Newcastle disease virus (NDV) has approximately a 15 kb RNA genome

encoding six proteins, including nucleo-protein (NP), phosphoprotein (P), matrix protein (M), fusion protein (F), hemagglutinin-neuraminidase (HN), and large protein (L), respectively [4, 11]. Entry of NDV into host cells requires the activation of viral envelope fusion glycoprotein through cleavage of the precursor glycoprotein F0 into F1 and F2. Only virus with a cleaved F0 can mediate the fusion of the viral and cellular membrane, which is required for infection of a cell. The post-translational cleavage is mediated by nonviral proteases and the types of proteases capable of cleaving F0 depends on the amino acid sequence motif around the cleavage site. The amino acid sequence of the F0 precursors in NDV of low virulence (loNDV) are characterized by a monobasic amino acid sequence motif at the C-terminus of the F2 protein and a leucine at the N-terminus of the F1 protein, 112G-R/K-Q-G-R-L117. The F0 precursors of loNDV are cleaved only extracellularly by trypsin-like proteases present in the respiratory and intestinal tract. Virulent NDV (vNDV) have a multibasic amino acid sequence motif at the C-terminus of the F2 protein, and a phenylalanine at the N-terminus of the F1 protein, 112R/G/K-R-Q/K-K/R-R-F117 and that are cleaved intracellularly by ubiquitous furin-like proteases found in most host tissues. This difference in protease activation is the major determinant of systemic replication associated with severe disease of vNDV [8]. Based on sequence analysis of the fusion (F) gene, NDV strains are divided into two distinct classes (I and II). Class I viruses are mostly loNDV found in wild birds, and contain nine genotypes (1–9), but class II viruses were originally broken into multiple genotypes representing loNDV and vNDV. Almost all virulent NDV strains isolated from wild and domestic birds belong to class II, which can be further subdivided into at least 18 genotypes from I to XVIII [12].

✉ Zahra Boroomand
z.boroomand@scu.ac.ir

¹ Department of Avian Medicine, Faculty of Veterinary Medicine, Shahid Chamran University of Ahvaz, Ahvaz, Iran

In recent years, the frequent occurrence of ND outbreaks in vaccinated flocks with high hemagglutination-inhibiting antibody levels raises questions about the causative agent. The main objectives of the present study are to show the phylogenetic relationships among the NDV isolates circulating in Iran and other parts of the world, and to analyze the F gene nucleotide sequences in NDV isolates from Ahvaz, Iran.

This study was performed on three NDV isolates collected in different ND outbreaks (2012–2013) from 4- to 6-week-old broiler chicken flocks located around Ahvaz city (Southwest of Iran). The flocks had been vaccinated with NDV killed and live vaccines before, and experienced high mortality when affected. From each flock, five daily mortalities and or moribund birds showing respiratory, nervous or digestive symptoms were examined at the onset of the disease. Samples were taken from trachea, lung, kidney, ceecal tonsil and ceacum, and virus propagation was performed in 10-day-old embryonated chicken eggs as described previously [2].

The RNA extraction from allantoic fluid was performed with the Accuzal™ (BioNeer Corporation, South Korea) according to the manufacturer's protocol. Appropriate amount of allantoic fluids (250 µL) were used for the extraction. Isolated RNAs were directly used for the RT-PCR or stored at -80°C . The partial F gene including the cleavage site sequence was amplified with the use of a pair of specific primers. Primer sequences are TT GAT GGC AGG CCT CTT GC and GG AGG ATG TTG GCA GCA TT [7]. The cDNA was synthesized using BioNeer RT PreMix kit (BioNeer Co., South Korea) according to the manufacturer's instruction. The PCR assay was carried out in a 20 µL reaction volume consisting of 2 µL 10× PCR buffer, 0.2 µL 10 mM dNTPs, 1 µL of each primer (20 pmol/mL), 0.2 µL Taq DNA polymerase (5 U/mL), 0.6 µL 50 mM MgCl_2 , 10 µL of distilled water, and 5 µL of cDNA dilution, and was programmed in the following condition: 95°C for 3 min followed by 40 cycles of 95°C for 30 s, 55°C for 60 s, 72°C for 60 s, and a final extension at 72°C for 10 min. The PCR products (330 base pairs) were separated by electrophoresis with the use of 1.5 % agarose gel. The RT-PCR products were cut from the gel, purified using the PCR AccuPrepH PCR Purification Kit (BioNeer Co., South Korea) according to the manufacturer's protocol, and then sequenced in forward direction. Sequencing reactions were performed by BioNeer Co., South Korea. The nucleotide sequences of the F protein gene determined in this study were compared to the NDV sequence data available in the National Center for Biotechnology Information database (<http://ncbi.nlm.nih.gov>) by using nBLAST (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>), and the phylogenetic relationship was established by http://www.phylogeny.fr/simple_phylogeny.cgi

[3]. All of the sequences were aligned with the use of CLUSTAL W. Distance-based neighbor joining trees were constructed with the use of the approximate likelihood-ratio test (aLRT) available in http://www.phylogeny.fr/simple_phylogeny.cgi. The virus isolates used in this study were submitted to the GenBank sequence database and were assigned the accession numbers NDa:KP347437, NDb:KP347438 and NDd:KP347439.

RT-PCR with degenerate primers resulted in the amplification of a 330 bp product, as expected. The predicted amino acid sequences of the NDV isolates were compared. A 97–99 % of nucleic acid sequence identity of F gene and 97–98 % of amino acid sequence identity of F protein exists between newly identified isolates with the previously known Iranian isolates of vNDV. From the pathotype prediction based on the cleavage site of the F protein, all the isolates were placed into the velogenic group with the motif 112RRQKRF117. A phylogenetic tree (Fig. 1) was constructed based on the nucleotide sequences of the F gene hypervariable region in all field isolates of NDV and the corresponding regions of the other 35 NDV

Fig. 1 Nucleic acid-based phylogenetic relationships of fusion gene of Newcastle disease virus isolated from Ahvaz, Iran. The sequences were obtained from GenBank

strains retrieved from GenBank (Table 1). The field isolates and reference strains were classified as genotypes I–IX. All of the three field isolates were classified into genotype VII, and subclassified into sub genotype VIId. As mentioned above, this nucleic acid region codes for an amino acid sequence that includes the F0 protein cleavage site. A phylogenetic analysis showed that these NDV isolates studied here were closely related to the previously reported NDV isolates from Iran.

Sequence analysis of the F protein cleavage site can be used to predict potential pathogenicity of NDV which is consistent with the conventional methods such as mean death time [10]. The presence of these characteristic

patterns of amino acid demonstrated that the isolates could be considered as virulent. Three isolates in this study possessed the 112RRQKRF117 motif. Hosseini et al. [5] have detected an RRQKRF motif in the cleavage site of the F protein of the nine virulent isolates through their works on NDV isolates from Iranian commercial farms. Phylogenetic analysis revealed that these isolates were located in VIIId subgenotype. In another study, nine NDV isolates from ND outbreaks in different regions of Iran were characterized at molecular level, which eight of them had the same amino acid sequence as VOL95, a Russian NDV isolate from 1995 and all isolates had 112R-R-Q-R-R-F117 sequences at the cleavage site in the F protein gene [6].

Table 1 GenBank accession numbers of our newly-assigned NDV isolates and other NDV isolates previously identified

Isolate	Accession number	Genotype
BJ2013 clone NDa	KP347437	VIIId
BJ2013 clone NDb	KP347438	VIIId
BJ2013 clone NDd	KP347439	VIIId
IL-1	KP771863	VIIId
IR-HGT2012.2	JX131358	VIIId
IR-HGT2012.1	JX131357	VIIId
NR_97	JX129808	VIIId
NR_94	JX129806	VIIId
NR_87	JX129801	VIIId
IRI 1392k	KJ176996	VIIId
IR-HGT2011.1	JX131355	VIIId
IR-HGT2010.2	JX131353	VIIId
IR-HGT2010.1	JX131352	VIIId
IR-HGT2012.4	JX131360	VIIId
IR-HGT2012.3	JX131359	VIIId
IR-HGT2011.2	JX131356	VIIId
NR_98	JX129809	VIIId
NR_95	JX129807	VIIId
NR_92	JX129805	VIIId
NR_90	JX129803	VIIId
NR_88	JX129802	VIIId
Lasota	DQ195265	II
B1	AF309418	II
APMV1/chicken/Japan/Miyadera/51	AB070383	II
APMV1/chicken/Japan/Sato/30	AB070382	III
APMV1/chicken/Japan/Ishii/62	AB070385	I
APMV1/chicken/Japan/Chiba/81	AB070388	VI
APMV1/chicken/Japan/Kagoshima/91	AB070414	VIII
Ulster	JN872152	I
Herts/33	AY741404	IV
Sterna/Astr/2755/2001	AY865652	VIIb
16-1677	NDU62620	VIIe
DE 143/95	AF109881	VIIa
SWS03	DQ227254	VIIc
owl/Israel/645-6/2012]	JN979568	VIIId

According to the study of Fathi et al. [4] on NDV isolates of local and commercial broiler and layer farms, all isolates (even of local origin) have GRRRQRRF motif (virulent pattern) in cleavage site. Also, their isolates have high similarities with Italian and Russian NDV strains. In an earlier work on Iranian NDV isolates in different regions of Iran, sequence analysis revealed that the isolates shared two pairs of arginine and a phenylalanine at the N-terminus of the F protein cleavage site [6]. The results of the study performed by Mehrabanpour et al. [7] showed that six of the Fars province (South of Iran) isolates carried the 112PRQKRF117 motif, whereas four of the isolates carried 112GRQGRL117 motif which were assigned as velogenic and lentogenic motifs, respectively. Abdoshah [1] has detected an RRQRRF motif in the cleavage site of the F protein through his works on NDV isolates from Iranian commercial farms. Phylogenetic analysis revealed that these isolates were located in VIIb subgenotype. In Pakistan (the neighboring country of Iran), Munir et al. [9] reported that all Pakistani NDV isolates were considered velogenic with the motif RRQKRF. This analysis clearly demonstrated that PAK strains and former lineage 7 are closely related to lineage 5, and these are not distinct enough to be regarded as new lineages.

Based on phylogenetic analysis, the three vNDV isolates in the present study are placed into genotype VIId. The previous works also indicate that genotype VII isolates have been the most prevalent in Iran during the recent years. Genotype VII of NDV in Asia has traced back to 1984 in Taiwan and to 1985 in Japan [13]. The B1 and LaSota are the most common strains used as live and or inactivated vaccines in Iran. The B1 and LaSota strains belong to genotypes II, which are different from our isolates identified genotype VIId. Evidence from our molecular epidemiology investigation shows that genotype VIId virus has become the predominant pathogen in poultry in Iran. In spite of good biosecurity and vaccination practices in recent years, Iranian chicken farmers fail to deal with virulent ND outbreaks. Molecular epidemiology in China has revealed that genotype VIId was responsible for an ND outbreak in vaccinated chickens [11]. Our results specified that there are velogenic NDV circulating in Ahvaz commercial flocks and causing outbreaks in poultry industry. Phylogenetically, the similarity of NDV of our study with other Iranian strains indicated that the new strains of NDV are present in Iran, and these isolates have being continued.

In conclusion, the emergence and identification of new sublineages provide an insight into the high rate of genetic drift occurring in NDV strains in Iran, and raise many concerns about the efficacy of current ND control measures in the country.

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