

Molecular characterization and phylogenetic study of velogenic Newcastle disease virus isolates in Iran

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Abstract The pathogenicity and genetic characterizations of six Newcastle disease virus (NDV) isolates obtained from chicken farms in six different regions in Iran were carried out using conventional and molecular techniques. Based on the pathogenicity indices (MDT, ICPI, and IVPI), all of these isolates were found to be velogenic (highly virulent) strains. A sequence analysis of the full-length mRNA encoding the fusion glycoprotein precursor (F_0) of the NDV's fusion proteins F_1 and F_2 in these six isolates showed the presence of point mutations in form of nucleic acid substitutions at positions 82^(C→T), 83^(T→C), 736^(A→G), and 1,633^(G→A). However, the nucleic acid residues at positions 330–347 of the precursor F_0 gene, corresponding to the cleavage site of the F_0 protein, were found to have remained conserved among the six NDV isolates. A phylogenetic comparison between the six Iranian isolates and the NDVs whose F_0 gene sequences were previously deposited in GenBank Database showed that all of the newly characterized Iranian NDV isolates belonged to genotype VII.

Keywords Newcastle disease virus (NDV) · Velogenic · Phylogenetic · F gene

Introduction

Newcastle disease (ND) is a reportable “List A” disease defined by the world organization for animal health (Office International des Epizooties or OIE) as transmissible disease with serious socio-economic consequence. ND particularly leads to severe economic loss in commercial poultry industry. The virus that causes ND (i.e., NDV) is a single-stranded, negative-sense enveloped RNA virus. NDV is classified as a member of the genus *Avulavirus*, in the family *Paramyxoviridae type 1* [1, 2]. NDVs are further sub-classified based on their pathogenicity in the host. Conventionally, the pathogenicity of a newly identified NDV isolate or strain is assessed by methods including the intra-cerebral pathogenicity index (ICPI), the mean death time (MDT), and/or intravenous pathogenicity index (IVPI). Based on these indices, an NDV isolate may be categorized as an avirulent (lentogenic), a mildly virulent (mesogenic), or a highly virulent (velogenic) strain. The isolation of virulent NDVs, which exhibit an ICPI of ≥ 0.7 and an IVPI of ≥ 1.40 , must be reported to OIE [3, 4]. The velogenic strains of NDV are further sub-classified as viscerotropic velogenic (VV) and neurotropic velogenic (NV) based on clinical manifestation and the ND lesions in the host [5]. The NDV genome encodes six structural proteins including nucleoprotein (NP), phosphoprotein (P), matrix (M) protein, fusion (F) protein, hemagglutinin–neuraminidase (HN) protein, and the RNA polymerase (L) [5, 6]. Two additional proteins, V and W, are also produced by an RNA-editing event during the transcription of the P gene [7]. The F protein, which is synthesized as inactive precursor F_0 (66 kDa) is an important determinant of NDV pathogenicity. The inactive precursor F_0 protein is

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post-translationally cleaved by the host proteases into two subunits, C-terminal F_1 (55 kDa), and N-terminal F_2 (12.5 kDa). The consensus of the research community is that the genes for the F and HN proteins can be used for conducting phylogenetic and molecular epidemiology studies on NDVs [8].

The pathogenicity of NDV is highly dependent on the F_0 protein cleavage site and its amino acid makeup. The less virulent strains of NDV have an F_0 protein cleavage site with fewer basic amino acids, plus a leucine residue substituting a phenyl alanine at position 117. The highly virulent (v-NDV) strains, however, contain two pairs of basic amino acids, either lysine (K) or arginine (R), at amino acid positions 112–113 and 115–116, as well as a phenylalanine at position 117 [9]. It is believed that these basic amino acid residues in the v-NDV strains facilitate the cleavage of the F_0 protein into the F_1 and F_2 subunits by universal proteases found in most of the host tissues. The F_0 protein of low-virulence strains (e.g., APMV-1) is cleaved only in tissues containing unique trypsin-like enzymes. This tissue specificity limits the ND infection by low-virulence strains to only mucosal tissues of the host where the trypsin-like enzymes exist. Therefore, due to the importance of the F_0 protein cleavage site in conferring virulence, the amino acid sequence of this region is used as a molecular marker of virulence in NDVs [10].

It has been shown that an amino acid sequence $^{112}R/K-R-Q-K/R-R^{116}$ (at the C terminus of the F_2 protein) and a phenylalanine residue at position 117 (at the N terminus of the F_1 protein) in the F_0 protein is a marker of high NDV virulence whereas the sequence $^{112}G/E-K/R-Q-G/E-R^{116}$ and a leucine at position 117 is a marker of low NDV virulence in chickens [11, 12].

Because the F_0 protein in highly virulent NDVs is highly susceptible to cleavage by a wide range of universal proteases present in host tissues, these NDVs are capable of causing multi-organ and fatal systemic infections in the host.

ND is endemic in Iran and its recurrent outbreaks are being reported in various parts of the country. Though, efforts have been made to prevent and control the spread of this disease, to date authors are unaware of any efforts being made to characterize the virulence based on the molecular and/or the genetic analysis of the NDV strains circulating the country. The aim of the present study was to conduct genetic and phylogenetic studies to determine the pathogenicity of six NDV strains isolated in Iran in recent years.

Materials and methods

Virus isolation

Six NDV isolates were recovered from samples taken from ill or dead poultry at six geographical regions A–F in Iran

Table 1 The NDV strains isolated from six different regions in Iran for this study

NDV strain	Accession no.	Origin	Poultry age (day)	Collection year
KND/20	AY928934	Qazvin (A)	25	1998
MK13/75	AY928933	Torbate-jam (B)	60	1996
KND/30	AY928935	Kermanshah (C)	24	1998
KND/35	AY928936	Ahwaz (D)	17	1999
KND/40	AY928937	Karaj (E)	36	2004
KND/45	AY928938	Nishapur (F)	45	1995

(Table 1; Fig. 1). After preparation, samples were inoculated into 9-day-old specific-pathogen-free (SPF) embryonated eggs and the allantoic fluids including viruses were collected and stored at $-70\text{ }^{\circ}\text{C}$ until use.

MDT test

For this test, the frozen sample for each viral isolate was thawed and diluted 10 times with saline (PBS). For each NDV sample, five 10-day-old embryonated eggs were inoculated. Each inoculation was carried out by carefully injecting 0.2 mL of the diluted sample in the allantoic cavity of the embryonated egg. Additional eggs were inoculated with saline to act as controls. After 24 h, the eggs were examined for embryo mortality at 2 h intervals for 96 h.

ICPI test

Briefly, 0.05 ml of 10-fold diluted virus containing allantoic fluid was injected intra-cerebrally into 10 day-old SPF chickens, and the chickens were observed for 8 days following the injection. In this period, healthy or control chickens were scored as 0, apparently or slightly sick chickens were scored as 1, and dead chickens were scored as two (Table 2). The ICPI values shown in Table 2 represent an average of the ICPI scores from ten chickens monitored for each NDV strain. Conventionally, an ICPI of below 0.7 represents the presence of a lentogenic strain of NDV, an ICPI of between 0.7 and 1.2 represents the presence of a mesogenic strain of NDV, and an ICPI of above 1.2 represents the presence of a velogenic strain NDV.

IVPI test

IVPI was carried out in 6-week-old chickens. The birds were inoculated intravenously with 0.1 ml of 10-fold

Fig. 1 A schematic illustrating geographical regions A–F in Iran, from which the NDV isolates of the present study were obtained



Table 2 The pathogenicity indices and molecular characterization of NDV isolates studied here

Strains	MDT	ICPI	IVPI	Fusion protein cleavage site
KND/20	39.6	1.9	2.55	$^{112}\text{RRQRR}^{116}\text{*F}^{117}$
MK13/75	47.4	1.97	2.32	$^{112}\text{RRQRR}^{116}\text{*F}^{117}$
KND/30	41.4	1.96	2.34	$^{112}\text{RRQRR}^{116}\text{*F}^{117}$
KND/35	49	1.97	2.55	$^{112}\text{RRQRR}^{116}\text{*F}^{117}$
KND/40	40.6	1.92	2.46	$^{112}\text{RRQRR}^{116}\text{*F}^{117}$
KND/45	44.2	1.91	2.37	$^{112}\text{RRQRR}^{116}\text{*F}^{117}$

diluted virus containing allantoic fluid and then were observed for 10 days. The healthy-looking birds were scored as zero, sick birds as one, paralyzed birds as two and dead birds as three (Table 2). The IVPI values shown in Table 2 represent an average of the IVPI scores from ten chickens monitored for each NDV isolate.

Virus RNA preparation

The total RNA of NDVs (grown in allantoic fluid) was extracted using RNA Extraction Kit[®] (Roche, Germany).

RT-PCR

Two primers, FU66F: 5'-ACGGGTAGAAGATTCTGGATCC-3' and KF200R: 5'-GATACCTCCGCCTCTCATCT-3' were synthesized (using BioEditTM) based on the published

nucleotide sequences of the NDV fusion proteins and were used for RT-PCR. These primers were used to amplify a 935 bp fragment of the F₀ gene—corresponding to the amino acid residues encompassing the cleavage site of the F₀ protein—in each of the NDV isolates. For first strand cDNA synthesis, AMV (10 U/μl; Promega), and RNasin (40 U/μl) were used and the reaction mixture was incubated at 42 °C for 2 h. For each sample, the PCR amplification reaction was conducted under the following PCR parameters: 35 cycles of 94 °C for 1 min, 58 °C for 1 min, 72 °C for 1 min, and a final hold at 72 °C for 10 min.

Nucleotide sequencing

The amplified DNA fragments were purified by AdvantageTM PCR-Pure Kit (High Pure PCR Product Purification) and then sequenced with an ABI 3100 DNA sequencer (Applied Biosystems Inc., CA). MEGA5 program was used for comparing the nucleic acid and amino acid information obtained for this study. Based on the 935 bp region of the F₀ gene, which was amplified and sequenced for each of the NDV isolates and similar sequences obtained from Genbank for NDV strains from Iran and other countries, a phylogenetic tree was prepared (Fig. 1). The GenBank accession numbers for the six isolates sequenced in this study are AY928933–AY928938 (Table 1). The GenBank accession numbers of other identified NDV strains from Iran and other countries, which were used for comparison purposes in this study, are provided in Table 3.

Table 3 The NDV strains previously identified

NDV isolates	Genotype or subgroup	Accession no.	Country
QUE/66	I	M24693	Japan
V4	I	AF217084	Australia
JL01	I	EF464163	China
D26/76	I	M24692	Japan
Japan/Ishii/62	I	AB465607	Japan
Ulster	I	D00243	UK
LAS/46	II	M24696	Japan
NDV027344	II	EU258665	China
D58/India	II	EU330230	India
SP13	II	AY861659	China
Italian	IV	M17710	Italy
Herts/33	IV	AY741404	Netherlands
HER/33	IV	M24702	Japan
Australia-Victor	III	M21881	Australia
AUS/32	III	M24700	Australia
JO3	IX	DQ227244	China
F48E9	IX	AY508514	China
LuoY	IX	AY341061	China
TrenqueLauquen	VIII	AY734534	Argentina
AF2240	VIII	AF048763	Malaysian
Ck/Mexico(Morelos)/458/1988	V	EU518680	Mexico
Anhinga/USA(FL)44083/93	V	AY288989	USA
Chicken/USA(CA)/1083(Fontana)/72	VI	AY288992	USA
s/1/China	VI	FJ865434	China
YN/P1	VI	AY325798	China
STP96	VI	DQ417113	China
Ch/98/1	VI	AF358785	Taiwan
SPVC/Karachi/NDV/26/2005	VII	GU182329	Pakistan
JSO2	VII	DQ227246	China
BP01	VII	DQ080015	China
SG/Liaoning/2009	VII	FJ882014	China
Chicken/BYP/Pakistan/2010	VII	JN682210	Pakistan
Chicken/Iran-EMM/7/2011	VII	JQ267579	Iran
Chicken/Iran-EMM/6/2011	VII	JQ267580	Iran
NR-15	VII	KC161988	Iran
NR-12	VII	GU228496	Iran
NRO-350	VII	HM460892	Iran
NRO-268	VII	HM460890	Iran
46-NR	VII	GU228503	Iran

Results

Viral pathogenicity

The pathogenicity indices that were obtained in this study indicated that the six NDV isolates obtained for this study were all highly virulent or velogenic. As shown in Table 2, all of the strains studied had MDT scores of less than 50 h, ICPI scores of greater than 1.2, and IVPI scores of greater 2.3.

For each of the NDV isolates, a 935 bp fragment of the F_0 gene was amplified and sequenced. A study of the F_0 gene sequence showed that there were no deletions or insertions in nucleotides 330–347 and that this site was identical and reminded conserved in all the six isolates. The nucleotides 330–347 of the F_0 gene correspond to amino acid residues 112–117 of the F_0 protein cleavage site. The predicted amino acid sequences of the F_0 protein in the NDV isolates from Iran demonstrated that all of them carry the high virulence marker (i.e., $^{112}R/K-R-Q-K/R-R^{116}-F^{117}$) at their F_0 protein cleavage sites (Table 2). This finding further confirmed the velogenic nature of the six NDV isolates characterized in this study.

Phylogenetic analysis

The phylogenetic relationships among the six isolates studied here and other previously identified NDV strains (from Iran and other countries, Table 3) were established using the 935 bp sequence of the F_0 gene. As mentioned above, this nucleic acid region codes for an amino acid sequence that includes the F_0 protein cleavage site. A phylogenetic analysis showed that the Iranian NDV isolates studied here were closely related to the previously reported NDV isolates from Iran and were classified as group VII among genotype groups I–IX (Fig. 2).

Although there were similarities between the Iranian NDV isolates and their worldwide counterparts as shown in the phylogenetic tree, the newly characterized Iranian NDVs still contained several nucleotide substitutions unique to these isolates. For example, nucleotide substitutions were found at positions 82 (C replaced by T), 83 (T replaced by C), 736 (A replaced by G), and 1,633 (G replaced by A) in the mRNA sequence of the F_0 protein in these isolates. Based on the nucleotide sequence analysis, a unique amino acid substitution of “M to V” at position 246 was found in all six newly characterized NDV isolates. In addition, it was determined that there were two cysteine residues missing from positions 27 and 514 of the F_0 protein in these isolates (data not shown).

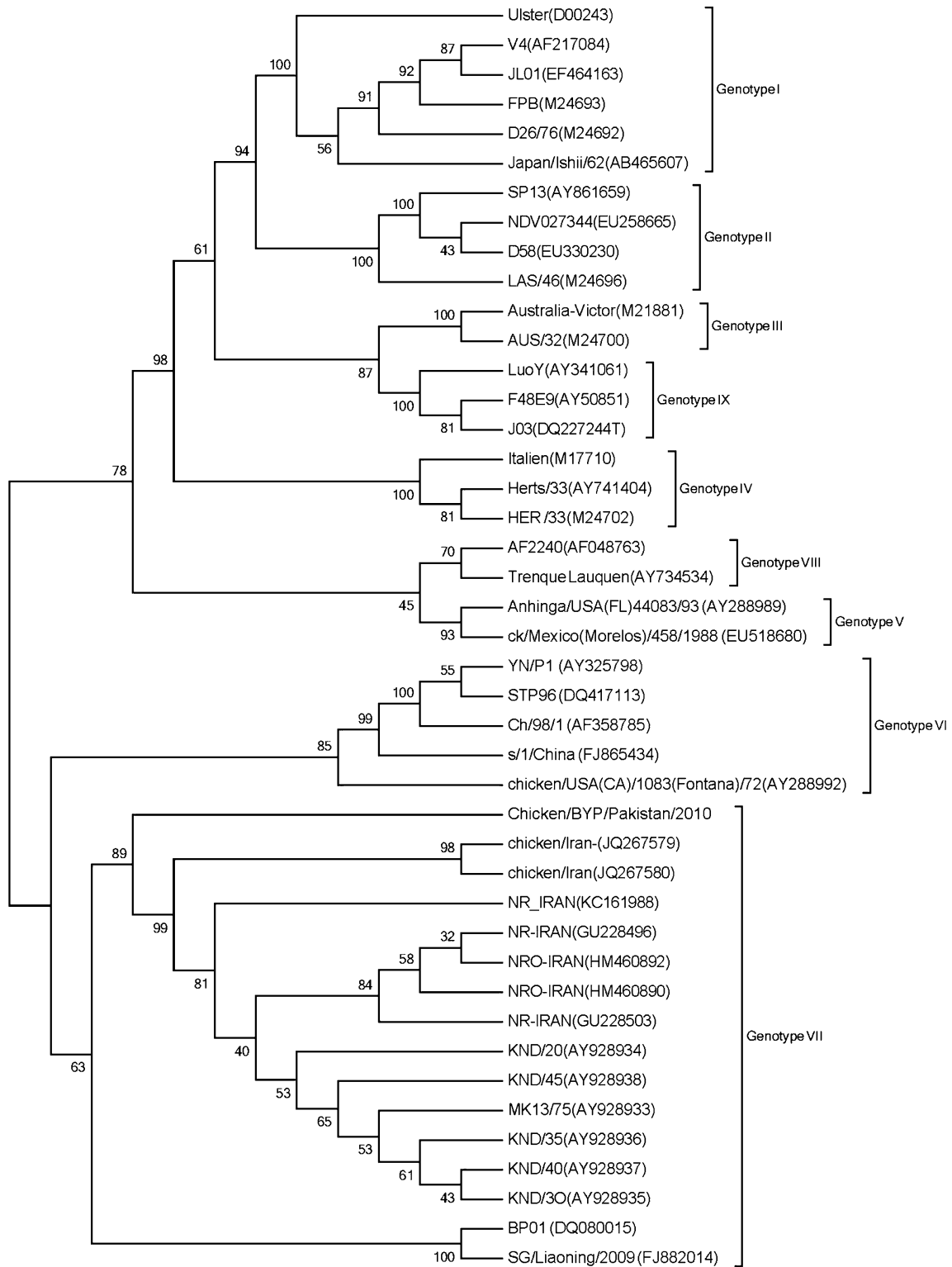


Fig. 2 Phylogenetic tree of the NDV isolates based on the 935 bp nucleotide sequences of the F0 genes in these viruses. The designations of the genotypes are indicated on the *right*

Discussion

ND is a highly contagious viral disease of chickens. The fatality of the disease depends mainly on the pathogenicity characteristics of the virus. The virulence of NDV is principally assessed by the ICPI and/or the presence or absence of amino acid sequence $^{112}\text{R/K-R-Q-K/R-R}^{116}\text{-F}^{117}$ at the cleavage site of the F_0 protein [13]. In this study, the molecular analyses of the six NDV isolates obtained from regions A–F in Iran (Fig. 1) indicated that the isolated strains were all velogenic with high ICPI scores and contained the $^{112}\text{RRQRR}^{116}/\text{F}^{117}$ sequence at the cleavage site of their F_0 proteins. It was also found that the nucleotides 330–347 corresponding to amino acid residues 112–117 of the F_0 protein in these isolates remained conserved. This information suggests that identical or quite similar velogenic strains of NDVs have spread through regions A–F (Fig. 1) of Iran.

The phylogenetic analysis conducted in this study showed that all the newly characterized strains of NDV, which were collected between 1996 and 2004 in Iran (Table 1), belonged to the genetic group designated as VII. Genotype VII was first emerged in Far East in 1990s and gradually spread to Europe and Africa over the next two decades [14–16]. Thus, the present study shows that similar strains of NDVs have spread to Iran as well. Authors are unaware of any other publications that discuss the initial isolation of NDV strains of genotype VII (NDV-VII) in Iran. The only other published work in this area is by Ebrahimi et al. [17] who discuss isolation of seven NDV strains of genotype VII in Iran between 2008 and 2011 without specifying the geographical location(s) from which these strains were obtained. Therefore, the present study not only identifies an earlier point in time, as early as 1995, at which NDV-VII was present in Iran but also provides a map (Fig. 1) that shows the extent by which NDVs-VII had already spread in Iran from 1995 to 2004.

As discussed above, the F_0 gene sequence of the newly characterized NDV isolates in Iran, in comparison with other identified NDV isolates, contained several point mutations that were unique to these isolates. Several factors or selective pressures may have contributed to these mutations. For example, the host immune response is one of the likely selective pressures that may have caused NDVs to mutate or to go through a forced evolution. Other factors contributing to such mutations may be the environmental conditions in Iran or the cross-transfer of the NDVs between the commercial poultry and the migratory

birds or the village chickens. A future study to classify the Iranian NDV strains based on these point mutations may be useful in determining the rate by which the Iranian NDVs evolve or undergo changes.

In conclusion, the present study demonstrates that genetic analysis is a valuable tool in tracking the spread of NDVs. Here, it was shown that highly virulent strains of NDVs have existed in Iran since 1995, and that by 2004 the virus had spread in many parts of the country from North East to South West of Iran. Authors believe that genetic analysis is an ideal tool for investigating the future outbreaks of ND in Iran. An accurate identification of NDVs enhances our understanding of the pattern by which these viruses spread and allows us to take appropriate measures to limit the spread or impact of ND.

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