ORIGINAL ARTICLE

Veterinary Research Forum. 2023; 14 (4) 221 - 228
doi: 10.30466/vrf.2022.548152.3373
Journal Homepage: vrf.iranjournals.ir

Veterinary Research Forum

Introduction of a Newcastle disease virus challenge strain (sub-genotype VII.1.1) isolated in Iran

Aliakbar Khabiri[1,2], Reza Toroghi[1]*, Mohammadreza Mohammadabadi[2], Seyed-Elias Tabatabaeizadeh[1]

[1] Razi Vaccine and Serum Research Institute, Agricultural Research, Education and Extension Organization (AREEO), Mashhad, Iran; [2] Department of Animal Science, Faculty of Agriculture, Shahid Bahonar University of Kerman, Kerman, Iran.

Article Info

Article history:

Received: 03 February 2022
Accepted: 29 August 2022
Available online: 15 April 2023

Keywords:

Challenge strain
Fusion gene
Hemagglutinin-neuraminidase gene
Newcastle disease virus
Sub-genotype VII.1.1

Abstract

Newcastle disease virus (NDV) sub-genotype VII.1.1 is the most common circulating NDV in Iran. In this study, a velogenic NDV isolate was plaque purified and then characterized according to Office International des Epizooties (OIE) standard protocols. The biological properties of the purified isolate named CH/RT40/IR/2011 were characterized using sequencing and phylogenetic analysis, measurement of pathogenicity indexes and challenge studies. The isolate was plaque purified on chicken embryo fibroblast cells for three rounds and then characterized using molecular and biological approaches. Phylogenetic and evolutionary distance analysis of fusion and hemagglutinin-neuraminidase genes classified the virus in sub-genotype VII.1.1. No mutation was observed in the glycosylation and neutralizing epitope sites of the fusion and hemagglutinin-neuraminidase proteins compared to other reported Iranian NDV VII.1.1 isolates. The presence of the 112RRQKRF117 motif in the fusion protein cleavage site together with mean death time, intracerebral pathogenicity index and intravenous pathogenicity index of 57 hr, 1.80 and 2.50 respectively, revealed that the RT40 isolate was a velogenic NDV. In the challenge study, all chickens were inoculated via eye drop, and intranasal route with RT40 isolate died within a week. While all chickens in the vaccinated and challenged group survived and showed no clinical signs. In conclusion, according to genetic analysis, pathotyping and challenge study, the RT40 isolate was similar to virulent NDVs in Iran and was a suitable candidate for a national standard challenge strain, vaccine trials and vaccine production in commercial levers.

Introduction

According to the latter category of the international committee on taxonomy of viruses (ICTV), Newcastle disease virus (NDV) belongs to avian genus avian orthoavulavirus 1 (AOAV-1), commonly known as avian paramyxoviruses 1 (APMV-1) or NDV.[1] Newcastle disease (ND) is a notifiable viral disease of poultry for Office International des Epizooties (OIE) because of the virus potential to cause significant economic losses in poultry industry.[2] The NDV, the causative agent of ND, is endemic in many developed and developing countries.[2,3] It is a negative-sense, single-stranded, non-segmented, an enveloped RNA virus that its genome encodes six structural proteins: nucleoprotein (NP), phosphoprotein (P), matrix protein (M), fusion protein (F), hemagglutinin-neuraminidase (HN) and RNA-directed RNA polymerase (L).[4]

Based on the three different pathogenicity, the indexes for NDVs included: Mean death time (MDT), intracerebral pathogenicity index (ICPI) and multiple basic amino acids at the fusion protein cleavage site (FPCS). The NDVs are classified into three main pathotypes: apathogenic (lentogenic), intermediate (mesogenic), and highly pathogenic (velogenic).[2,5] Although the molecular basis for NDV pathogenicity is a polygenic trait, the F gene plays the leading role in virulence.[6] Fusion and HN glycoproteins are located at the surface of the NDV envelope. Epitope mapping studies identified several antigenic sites on F and HN proteins related to antibody recognition.[7]

The latest NDV classification system for different genotypes and sub-genotypes was developed by Dimitrov *et al.*[1] The NDVs in genotype VII, especially the sub-genotypes of VII.1.1 and VII.2 are responsible for the fourth and fifth NDV panzootic.[1]

***Correspondence:**

Reza Toroghi. DVM, PhD
Razi Vaccine and Serum Research Institute, Agricultural Research, Education and Extension Organization (AREEO), Mashhad, Iran
E-mail: r.toroghi@rvsri.ac.ir

Several studies have been conducted in Iran for the genotyping of circulating virulent NDVs. A review of these studies showed that NDV sub-genotype VII.1.1 was the most prevalent sub-genotype in the country over the past years.[8,9] It is noteworthy that these viruses were mentioned under sub-genotypes of VIIb, VIId, VIIj, and VIIl before the new phylogenetic classification method was introduced.[8,10] With the updated phylogenetic classification system, these viruses were classified in NDV sub-genotype VII.1.1.[1] Despite the gradual evolution of NDV into new genotypes, the vaccine seeds have not been updated since the early ND vaccine development in the 1950s. However, the traditional vaccines (LaSota, B1 and V4 vaccines) still induce a significant immune response and protection against ND viruses genotype VII.[11,12] Although several challenge viruses are available world-wide to evaluate vaccines, the best challenge virus to assess vaccines in the case of immune responses and virus shedding is use of the virus circulating in the region.[13]

Considering that vaccination is essential in preventing the disease, this study was conducted to introduce a national challenge strain originating from common virulent NDV in Iran. The purified isolate can be used in vaccine trial studies for commercially available or newly introduced NDV vaccines.

Materials and Methods

Virus. The virus was selected from the virus bank in the Razi Vaccine and Serum Research Institute (RVSRI). The virus, named CH/RT40/IR/2011, was isolated from brain samples of a broiler flock with high mortality during a provincial project to characterize NDV in northeast Iran during 2011 - 2013. At that time, it was shown that the RT40 isolate belonged to NDV sub-genotype VIId based on phylogenetic analysis using the partial F gene.[14] The isolate was sub-cultured once in the allantoic fluid of 10day-old specific pathogen-free embryonated chicken eggs (SPF-ECE; Venkateshwara Hatcheries, Bangalore, India). The allantoic fluid with hemagglutination (HA) activity was pooled and stored at – 80.00 ˚C for further analysis.

Chick embryo fibroblast cell culture. The NDV can be infected and grown on many cell lines, however, host cells are the best cell to replicate for any virus. Chick embryo fibroblasts cells derived from 11 day-old SPF-ECE were grown in 75.00 cm^2 flasks containing Dulbecco's modified Eagle medium (DMEM; Gibco, Grand Island, USA), 10.00%, fetal bovine serum (FBS), 0.30% Tryptose phosphate broth (Difco, Rockville, USA), 100IU mL^{-1} penicillin, and 100 mg mL^{-1} streptomycin (Gibco). The cells were grown at 37.00 ˚C in a 5.00% CO_2 incubator. The cells were sub-cultured in the six-well plates for plaque purification assay. The project was found to be in accordance to the ethical principles and the national norms and standards for conducting animal research in Iran and animals were maintained according to the guidelines set by the Iranian Council of Animal Care (Guide to the Care and Use of Experimental Animals, 1. IUT, Iran).

Plaque purification. A six-well plates with 90.00% cell confluency were washed three times with Hank's buffer. The RT40 was diluted with DMEM (10^{-4} - 10^{-8}) and each well was infected with 0.50 mL of each dilution. One h was allowed for virus absorption with rocking at 15 min intervals in a 37.00˚C incubator with 5.00% CO_2. Following the viral suspension removal, the cells were rinsed with Hank's buffer three times. Then, 5.00 mL of melted (50.00˚C) overlay medium with 3.00% FBS and 0.30% purified agarose (Roche, Mannheim, Germany) was poured into each well. The overlay medium was allowed to solidify for 20 min at room temperature before keeping in a humidified 37.00˚C incubator with 5.00% CO_2 for 5 days. The plaque assay was carried out for three rounds by the plaque-to-plaque method. The picked-up virus from the third round was diluted in 1x sterile phosphate buffered saline (PBS) and was propagated in 10day-old SPF-ECE. The harvested virus was stored at – 80.00 ˚C as a stock virus for future experiments.

Mean death time. For MDT examination, 25 embryonated SPF eggs were inoculated with 0.10 mL of a series of 10-fold dilutions (10^{-5} - 10^{-9}) of fresh infective allantoic fluid of RT40. The same procedure was done eight hours later. As well, 10 eggs were inoculated with 0.10 mL sterile PBS as the control group. The eggs were incubated in a hatching machine and examined three times daily for seven days. The highest dilution that killed all embryos was considered the minimum lethal dose. MDT was the meantime in hours for the minimal lethal dose (MLD) to kill embryos.[2,5]

Intracerebral pathogenicity index. To determine ICPI, ten one-day-old SPF chicks were injected intra-cerebrally with 0.05 mL of a 1:10 dilution of filtered fresh infective allantoic fluid having more than 24 HA units. The chicks were examined daily for clinical signs and mortality for eight days. Each chick was scored zero if normal, one if sick and two if dead at each observation. For negative control, 10 chicks were injected with 0.05 mL PBS and separately housed. The ICPI was determined as the mean score per bird over the eight days.[2,5]

Intravenous pathogenicity index. For IVPI assay, ten 6-week-old SPF chickens were injected intravenously with 0.10 mL of freshly collected infective allantoic fluid with HA titer of more than 24 and diluted 1:10 in sterile PBS. Then, the chickens were examined daily and scored zero if normal, one if sick, two if paralyzed and three if dead at each observation. IVPI was the mean score per bird per observation within 10 days.[2]

Reverse transcription polymerase chain reaction (RT-PCR) of F and HN whole genes. Viral RNA was extracted using the high pure viral nucleic acid kit (Roche). A reverse transcription reaction was performed using viral

RNA, a gene-specific primer and superscript II RT mix (Invitrogen, Paisley, UK). One pair of specific PCR primers was designed to amplify each whole gene based on the available NDV sequences from GenBank (F: 5'-GAGGCATG CCATTGCCATTGCTAAAT -3' and R: 5'-CCTCATTCTCCA GCACGACT-3' for F gene, F: 5'-ATCCGTTCTACCACATCACC-3' and R: 5'-TCTTCCATTGCCTGCTGATA-3' for HN gene). Complete open reading frame (ORF) of F and HN genes were amplified by Speed STAR HS DNA polymerase (Takara Bio, Otsu, Japan) with the following thermocycling conditions: Initial denaturation of 5 min at 94.00 ˚C, followed by 37.00 cycles of 15 sec at 94.00 ˚C, 15 sec at 55.00 ˚C, 2 min at 72.00 ˚C and a final extension step of 10 min at 72.00 ˚C. PCR products were analyzed by electrophoresis with 1.00% agarose gel and purified from the gel using a high pure PCR product purification kit (Roche).

Cloning and nucleotide sequencing. The purified PCR products were ligated into pTZ57R/T cloning vector by T/A cloning strategy (Thermo Fisher Scientific, Bremen, Germany).*Escherichia coli* DH5α competent cell preparation and transformation steps were followed as described by Sambrook and Russell methods.[15] After transforming the recombinant vectors into competent *E. coli* DH5α cells, the bacterial clones harboring recombinant plasmid DNA were screened based on colony-PCR. The positive colonies were propagated in liquid LB media. The plasmids were purified using the high pure plasmid isolation kit (Roche) and confirmed by restriction sites enzyme digestion before sequencing. Sequencing was performed using the Sanger method (Bioneer, Daejeon, South Korea) in both directions using M13F, M13R and two pairs of overlap-specific primers designed for each gene. The achieved sequences from both genes were assembled using the software package CLC workbench (version 5.5; Qiagen, Hilden, Germany) and Bioedit (version 7.0; Ibis Therapeutics, Carlsbad, USA). The nucleotide sequence of the F and HN genes were submitted to GenBank® (Accession Number: MZ463065 and MZ463066).

Phylogenetic analysis, evolutionary distance estimation, glycosylation site and neutralizing epitopes comparison. The nucleotide sequences were used to find similar sequences using Basic Local Alignment Search Tool (BLAST). The substantiality of the genetic grouping and topology of a phylogenetic tree was affirmed by comparing F and HN whole gene nucleotide sequences of RT40 and published sequences relevant to class II NDVs that are available in GenBank. Iranian NDVs of sub-geno-types VII and LaSota vaccine that is highly used in Iran were analyzed in a phylogenetic tree. The phylogenetic tree for the F (1,662 nt) gene was constructed based on the ORF based on the new classification[1] using MEGA Software (version 6.0; Biodesign Institute, Tempe, USA). The Maximum-likelihood method based on the general time-reversible (GTR) model was employed. The codon positions included were 1st, 2nd, 3rd and noncoding. All positions containing gaps and missing data were eliminated. Also, another phylogenetic tree for HN (1,716 nt) gene was constructed based on the ORF using the same software and same analysis program. The glycosylation site[16] and neutralizing epitopes[17,18] were analyzed for F and HN proteins compared to other viruses of class II similar to RT40. To confirm the result of the phylogenetic tree, evolutionary distance values of the F gene between RT40 and NDVs of different genotypes were determined (Table 1).

Table 1. Estimate genetic distance between F nucleotide sequences (1,662 bp) of RT40 and other known NDV strains in class II. Values within the parentheses are the number of analyzed sequences.

Genotypes	Genotypes																			
	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII	XIV	XVI	XVII	XVIII	XIX	XX	RT40
I (n=109)																				
II (n=13)	0.115																			
III (n=4)	0.099	0.120																		
IV (n=6)	0.114	0.130	0.089																	
V (n=35)	0.162	0.175	0.144	0.135																
VI (n=230)	0.177	0.185	0.162	0.145	0.142															
VII (n=728)	0.172	0.195	0.156	0.144	0.145	0.130														
VIIII (n=4)	0.142	0.153	0.125	0.113	0.115	0.120	0.128													
IX (n=5)	0.102	0.113	0.077	0.092	0.144	0.162	0.157	0.123												
X (n=20)	0.106	0.104	0.118	0.129	0.177	0.186	0.185	0.155	0.114											
XI (n=11)	0.180	0.198	0.165	0.135	0.194	0.214	0.221	0.185	0.154	0.199										
XII (n=18)	0.176	0.201	0.162	0.149	0.140	0.122	0.111	0.127	0.164	0.188	0.222									
XIII (n=57)	0.172	0.193	0.161	0.148	0.144	0.136	0.115	0.129	0.158	0.186	0.214	0.106								
XIV (n=63)	0.203	0.232	0.199	0.177	0.165	0.160	0.138	0.151	0.202	0.213	0.257	0.126	0.131							
XVI (n=4)	0.187	0.192	0.169	0.158	0.088	0.159	0.157	0.135	0.167	0.197	0.220	0.164	0.161	0.185						
XVII (n=68)	0.160	0.180	0.148	0.131	0.141	0.154	0.161	0.124	0.148	0.171	0.211	0.157	0.157	0.184	0.166					
XVIII (n=13)	0.164	0.195	0.162	0.150	0.143	0.140	0.123	0.134	0.159	0.191	0.207	0.109	0.110	0.120	0.163	0.167				
XIX (n=29)	0.172	0.195	0.162	0.149	0.143	0.129	0.116	0.131	0.158	0.187	0.210	0.105	0.107	0.122	0.163	0.161	0.094			
XX (n=15)	0.151	0.168	0.139	0.124	0.121	0.089	0.115	0.103	0.137	0.172	0.196	0.115	0.121	0.149	0.144	0.135	0.122	0.117		
XXI (n=46)	0.177	0.194	0.165	0.151	0.148	0.104	0.135	0.129	0.163	0.194	0.219	0.138	0.144	0.165	0.166	0.163	0.150	0.141	0.095	
RT40	0.176	0.197	0.159	0.144	0.148	0.135	0.054	0.113	0.160	0.184	0.223	0.113	0.123	0.149	0.159	0.166	0.130	0.123	0.117	0.138

Challenge experiment. Twenty SPF chickens hatching from SPF chicken embryonated eggs (Venkateshwara Hatcheries, India) were divided into two groups of 10 and kept in separate rooms in a laboratory animal facility with *ad libitum* feeding and animal handling based on laboratory animal care guidelines. Vaccination for one of the groups was performed using an inactivated ND vaccine containing the LaSota strain (Razi Vaccine and Serum Institute, Karaj, Iran) at the age of 21 days. The RT40 was diluted in PBS to obtain an EID50 titer of 107 in 1.00 mL. Then, for both groups, 100 µL of RT40 per bird was inoculated through the eye and nose at the age of 42 days and the chickens were monitored until 76 days of age.

Results

Plaque purification. Virus plaques were observable with an inverted microscope in 10× magnification on the second day and naked eyes on the four-day post-infection. Four days post-infection, the segregated plaques were formed in three different sizes of 0.50, 1.00 and 2.00 mm diameter at dilutions of 10^{-6} - 10^{-8}. A large plaque (~2.00 mm diameter) was selected and resuspended in 0.50 mL PBS and then diluted to 10^{-4} - 10^{-8}. All plaques emerged in the second and third purification rounds were in the same size (2.00 mm diameter). After replicating the virus resulting from the third round of plaque purification in SPF ECE, the viral HA titer in the allantoic fluid was 1:512.

Pathogenicity tests of RT40. The results of pathogenicity tests including MDT, ICPI and IVPI were 57.00, 1.80 and 2.50, respectively. These values indicated that RT40 was velogenic.

Phylogenetic and evolutionary distance analysis of RT40. The sequence of the F protein cleavage site contained the typical virulence motif 112RRQKRF117. Phylogenetic tree construction was performed using the F whole gene nucleotide sequence of RT40 and NDV strains belonging to class II (available in the GenBank database). It was found that RT40 belonged to sub-genotype VII.1.1 of genotype VII and was located in a sister branch with isolate SMV-3 (KU201410), (Fig. 1). In another phylogenetic tree, RT40 was compared to other viruses isolated from different provinces of Iran and neighboring countries (Fig. 2A). The result of the second tree showed that RT40 had the highest similarity (98.90 - 99.30%) with the other Iranian NDV strains of sub-genotype VII.1.1 (Fig. 2A). The result from the phylogenetic tree construction of the HN whole gene showed that RT40 belongs to sub-genotype VII.1.1 (Fig. 2B). The F gene based evolutionary distance analysis between RT40 and 1,477 NDV strains from all genotypes I- XXI (genotype XV is a recombinant genotype and was not included in this analysis) was performed (Fig. 1). It was observed that there was a genetic distance of less than 10.00% only with genotype VII, which confirmed the presence of RT40 in this genotype. It was found that the genetic distance based on the F whole gene between the RT40 (genotype VII) and common vaccine strains (genotype I, II) was 82.90 - 83.40%.

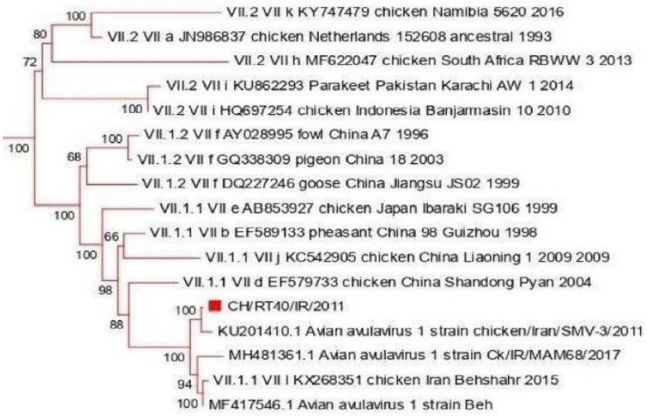

Fig. 1. Phylogenetic analysis based on NDV F whole genes from all genotypes (1,662 bp). All sequences were used for tree construction represent class II NDV genotypes (n = 117). ML method based on the GTR model with 1,000 bootstrap replicates was employed for tree construction.

F and HN proteins characterization. Analysis of F protein functional domains of Iranian NDV isolates and LaSota vaccine was performed. A high degree of similarity was observed between RT40 and other Iranian NDV strains in the amino acid sequence of functional domains. However, there was a significant difference between the LaSota vaccine and NDV isolates circulating in Iran. The F gene amino acid sequence of RT40 was compared to other Iranian NDV isolates in 85, 191, 366, 447, 471 and 541 positions for glycosylation sites and 72, 74, 75, 78, 79, 157-171 and 343 for neutralizing epitopes. It was shown that all isolates had conserved amino acids in these sites. The HN protein of RT40 comprises 571 amino acids, the protein length found in high virulence NDVs. Moreover, the amino acid position of neutralization epitopes and the potential glycosylation sites of RT40 and other Iranian NDVs were compared. The results showed that all of the Iranian isolates had conserved motifs in these regions (Table 2). The HN protein of RT40 comprises 571 amino acids, the protein length found in high virulence NDVs. The amino acid position of neutralization epitopes and the potential glycosylation sites of RT40 and other Iranian NDVs were compared. The results showed that all of the Iranian isolates had conserved motifs in these regions (Table 2).

Challenge study by RT40 inoculation. The clinical signs of ND, including decreased water and food intake, depression, green diarrhea and neurological signs were observed in chickens that did not receive the vaccine. In contrast, all chickens in the vaccinated group were clinically protected and survived. All of the chickens in the unvaccinated group died by day 7.

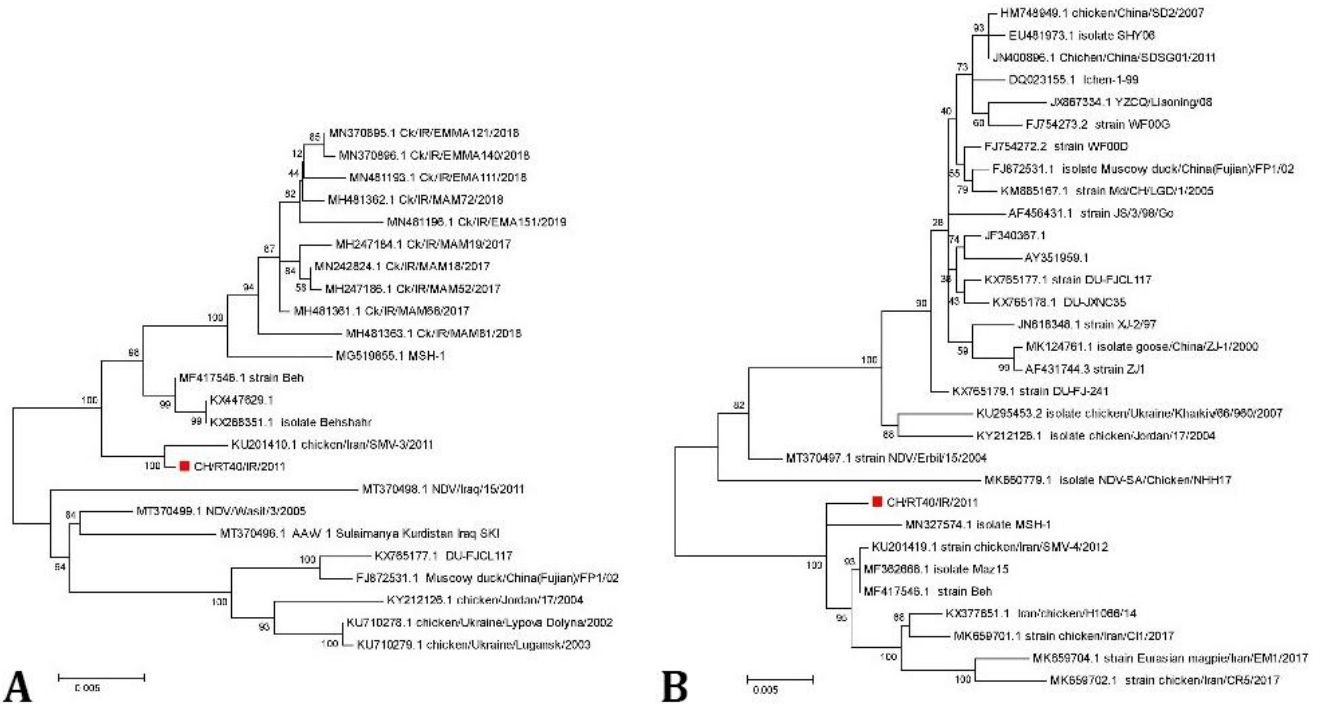

Fig. 2. A) The RT40 F whole gene (1,662 nt) was compared to the previously reported Iranian NDV strains and similar sequences were found through BLASTing. The ML method based on the GTR model with 1,000 bootstrap replicates was utilized for tree construction. **B)** Phylogenetic tree of NDV HN whole gene sequences (1,716 bp) of Iranian NDV isolates and similar sequences was found through BLAST. The ML method based on the GTR model with 1,000 bootstrap replicates was utilized for tree construction.

Table 2. HN protein glycosylation site and neutralizing epitope comparison between RT40 and Iranian NDVs of sub-genotype VII.1.1.

Virus strain	Glycosylation site					Neutralizing epitopes									
						193-201	263	287	321	332-333	345-353	356	494	513-521	569
CH/RT40/IR/2011	119	341	433	481	144	LSGCRDHS	K	D	K	GK	PDQQDYQIR	K	D	RVTRVSSSS	D
CH/Beh/IR/2011	119	341	433	481	144	LSGCRDHS	K	D	K	GK	PDQQDYQIR	K	D	RVTRVSSSS	D
CH/ Maz15/IR/2015	119	341	433	481	144	LSGCRDHS	K	D	K	GK	PDQQDYQIR	K	D	RVTRVSSSS	D
CH/ MSH-1/IR/2015	119	341	433	481	144	LSGCRDHS	K	D	K	GK	PDQQDYQIR	K	D	RVTRVSSSS	D
CH/IR/H1066/2014	119	341	433	481	144	LSGCRDHS	K	D	K	GK	PDQQDYQIR	K	D	RVTRVSSSS	D
CH/IR/SMV-4/2012	119	341	433	481	144	LSGCRDHS	K	D	K	GK	PDEQDYQIR	K	D	RVTRVSSSS	D

Discussion

The NDV causes the highest mortality rate in unvaccinated chicken farms (up to 80.00%), thus, implementing strict biosecurity measures and effective vaccination is required for ND control.[12]

In recent years, the number of reports has shown that NDV is circulating in different provinces of Iran.[8,19,20] However, there is no report on the viral genotype circulating in northeastern Iran. This study was mainly performed for two requirements. First, this study completed and finalized the chain of previous studies by proposing the first report of NDVs circulating in the northeast of the country to find the origin NDVs circulating in Iran.[8] Second, To introduce a virus strain as a sample of all isolated ND viruses of sub-genotype VII.1.1 in the country for use as a national NDV challenge strain to evaluate different vaccines. Following two main goals, to raise the immunity level in chickens and to reduce the virus shedding after vaccination. To help prevent the chicken industry from more economic losses and prevent the virus from spreading as least as possible, choosing a suitable ND vaccine is critical.[21]

The RT40 isolate is a velogenic NDV belonging to genotype VII and subgenotype VII.1.1 which was isolated from brain samples of a broiler chicken flock during a provincial monitoring program (2011 - 2013) in northeast Iran. The virus was undergone the three-round plaque purification and was then analyzed for the complete F and HN genes. To date, several methods have been developed for virus purification, among these ultracentrifugation is a quick method.[22,23] In ultracentrifuge clarification, the virus population also consists of different subpopulations. Plaque purification was developed to subtract subpopulations by creating a virus clone from one subpopulation.[24] Each plaque that originated was derived from one single virus

particle and practically possessed the same biological uniformity. Also, the pathogenicity of subpopulations has been associated with the morphology of plaques.[25]

It has been shown that the cloned viruses derived from the third round of plaque purification had the same morphological, serological and pathogenic properties.[25] On the other hand, there is more chance to get uniform and reproducible results in challenge studies when a plaque purified challenge virus is used.

Following virus purification, it was a necessity to determine the virulence characteristics of RT40. According to the OIE protocol, the ICPI, MDT, IVPI and characterization of amino acid motifs in the FPCS of a new ND virus are necessary for introducing a new strain pathotype. Pathotyping tests and cleavage site motif confirmed that RT40 was virulent for chicken. Compared to other Iranian isolates, the results showed that the pathogenicity of all NDVs isolated in Iran was in the same range.[8,26] The FPCS motif contained 112RRQKRF117 also, which was a feature associated with a virulent NDV strain.

The entire F and HN genes were sequenced and analyzed by the phylogenetic tree and distance matrix to classify and find the position of this strain among the Iranian and worldND viruses. The complete F gene (1,662 bp) of RT40 was compared to all genotypes class II F genes. This strain was located in a branch of genotype VII and subgenotype VII.1.1 (Fig. 1). In the last decade, NDVs were reported from different Iran provinces grouped to subgenotype VII.1.1.[8,9,27] Comparing the RT40 among ND viruses reported from neighboring countries, it was very close to an Iraqi isolate (MT370498). This fact confirmed our previous guess that the recent ND epidemic in Iran was most probably originated in Iraq in 2009, then the virus was crossed from province to province and covered west to east of Iran within a year.[20] It seems that the government needs to implement more strict restriction rules for poultry and poultry products trade not only for foreign borders but also for provincial borders. The prevalence of other genotypes such as genotype XIII and VII.2 have been reported from our neighboring countries.[28,29] The same scenario might happen if the biosecurity measures is not implemented across country borders.

Another phylogenetic tree drawn with the entire HN gene (1,716 bp) also showed that the RT40 was located in sub-genotype VII.1.1. The HN protein of the RT40 strain has 571 amino acids length that has been found in velogenic strains.[30] This phylogenetic tree also confirms the F phylogenetic tree and shows that the RT40 isolate belongs to genotype VII.1.1.

The strain RT40 and all Iranian isolates were compared in neutralizing epitopes and glycosylation motif (Table 2). No mutation was found in neutralizing epitopes of the fusion protein (D72, E74, A75, R78, A79 and 157-SIAATNEAVHEVT-171 L343) and six glycosylation motifs at positions 85,191, 366, 447, 471 and 541. Fortunately, there has been no significant change and critical mutation in functional points of the F and HN genes of Iranian ND viruses in recent years. These viruses might have had a common ancestor.

Rapid diagnosis, extensive use of vaccines, strict quarantine and biosecurity implementation seem necessary to keep ND under control in each country. Genotype VII is responsible for the global epidemics and this is a big question why most countries use common vaccines which mostly belong to genotypes II and I. Many studies showed that if the used vaccine was more genetically similar to the circulating ND virus, it would create a higher level of immunity and lesser virus shedding.[13,31] Like most other vaccines, NDV vaccines are unable to prevent the bird from the NDV infection and subsequently, virus shedding. Although the amount of virus shedding depends upon various factors such as the host's immunity, the amount and virulence of the challenged virus, time between vaccination and challenge, the dose and type of ND vaccine, the homology between NDV and the used vaccine play a critical rule.[32] Many studies confirmed that using a more homologous vaccine with a circulating virus effectively reduced viral shedding.[11,33,34] Future studies for the best vaccine focus on individual immunity, reducing clinical signs and mortality, and special attention to virus shedding following vaccination, which is an important consideration in countries with endemic NDV. It seems that the more homogeneity between challenge virus and circulating ND viruses, the more chance to select the best vaccine antigen match to reduce viral shedding and raise the immune system of chickens.

In each vaccine trial study, the availability of a plaque purified challenge strain is a critical step. The lack of availability of such strains in the country initiated us to introduce a fully characterized pure ND virus as a challenging strain to the scientific community and vaccine industry.

In conclusion, the results of this study (genetic analysis, pathotyping and challenge study), indicated that the RT40 isolate was a representative of circulating virulent NDVs in Iran and was a suitable candidate for a national standard challenge strain.

Acknowledgments

The authors would like to thank all staff of the Research Department in the Vaccine and Serum Research Institute of the Mashhad Branch, Mashhad, Iran who participated in this study.

Conflict of interest

The authors declare that they have no conflict of interest.

References

1. Dimitrov KM, Abolnik C, Afonso CL, et al. Updated unified phylogenetic classification system and revised nomenclature for Newcastle disease virus. Infect Genet Evol 2019; 74: 103917. doi: 10.1016/j.meegid. 2019.103917.
2. OIE, Manual of diagnostic tests and vaccines for terrestrial animals. 8th ed. Paris, France: World Organization for Animal Health 2017.
3. Miller PJ, Haddas R, Simanov L, et al. Identification of new sub-genotypes of virulent Newcastle disease virus with potential panzootic features. Infect Genet Evol 2015; 29: 216-229.
4. Peeters B, Verbruggen P, Nelissen F, et al. The P gene of Newcastle disease virus does not encode an accessory X protein. J Gen Virol 2004; 85(Pt 8): 2375-2378.
5. Suarez DL, Miller PJ, Koch G, et al. Newcastle disease, other avian paramyxoviruses, and avian meta-pneumovirus infections. In: Swayne DE, Glisson JR, McDougald LR, et al (Eds). Diseases of poultry. 13th ed. Iowa, USA: Wiley-Blackwell 2013; 89-138.
6. Heiden S, Grund C, Röder A, et al. Different regions of the Newcastle disease virus fusion protein modulate pathogenicity. PLoS One 2014; 9(12): e113344. doi: 10.1371/journal.pone.0113344.
7. Liu J, Zhu J, Xu H, et al. Effects of the HN antigenic difference between the vaccine strain and the challenge strain of Newcastle disease virus on virus shedding and transmission. Viruses 2017; 9(8): 225. doi: 10.3390/v9080225.
8. Molouki A, Mehrabadi MHF, Bashashati M, et al. NDV subgenotype VII (L) is currently circulating in commercial broiler farms of Iran 2017-2018. Trop Anim Health Prod 2019; 51(5): 1247-1252.
9. Allahyari E, Allymehr M, Molouki A, et al. 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 2022; 25(1): 21-32.
10. Ahmadi E, Pourbakhsh SA, Ahmadi M, et al. Phylo-genetic characterization of virulent Newcastle disease viruses isolated during outbreaks in northwestern Iran in 2010. Arch Virol 2016; 161(11): 3151-3160.
11. Dortmans JC, Peeters BP, Koch G Newcastle disease virus outbreaks: vaccine mismatch or inadequate application? Vet Microbiol 2012; 160(1-2): 17-22.
12. Dimitrov KM, Afonso CL, Yu Q, et al. Newcastle disease vaccines-A solved problem or a continuous challenge? Vet Microbiol 2017; 206: 126-136.
13. Miller PJ, Afonso CL, El Attrache J, et al. Effects of Newcastle disease virus vaccine antibodies on the shedding and transmission of challenge viruses. Dev Comp Immunol 2013; 41(4): 505-513.
14. Toroghi, R. Isolation and nucleotide sequencing of the fusion protein gene cleavage site of Newcastle disease viruses contributing in respiratory syndromes of commercial chicken flocks in Khorasan Razavi province. Project report No 45939. Razi Vaccine and Serum Research Institute, Agricultural Research, Education, and Extension Organization, Iran 2014.
15. Sambrook J, Russell DW, et al. The condensed protocols from molecular cloning: An laboratory manual. 1st ed. Heidelberg, Germany: Heidelberg University Library 2006; 10-23.
16. Umali DV, Ito H, Shirota K, et al. Characterization of complete genome sequence of genotype VI and VII velogenic Newcastle disease virus from Japan. Virus Genes 2014; 49(1): 89-99.
17. Neyt C, Geliebter J, Slaoui M, et al. Mutations located on both F1 and F2 subunits of the Newcastle disease virus fusion protein confer resistance to neutralization with monoclonal antibodies. J Virol 1989; 63(2): 952-954.
18. Yusoff K, Nesbit M, McCartney H, et al. Location of neutralizing epitopes on the fusion protein of Newcastle disease virus strain Beaudette C. J Gen Virol 1989; 70(Pt 11): 3105-3109.
19. Ghalyanchilangeroudi A, Hosseini H, Jabbarifakhr M, et al. Emergence of a virulent genotype VIIi of Newcastle disease virus in Iran. Avian Pathol 2018; 47(5): 509-519.
20. Toroghi R, Salamatian I, Bassami MR, et al. Implementation of high-level biosecurity measures can reduce the baseline antibody titers of Newcastle disease in non-integrated layer flocks in northeast Iran. Worlds Poult Sci J 2020; 76(4): 757-766.
21. Bello MB, Mahamud SNA, Yusoff K, et al. Development of an effective and stable genotype-matched live attenuated Newcastle disease virus vaccine based on a novel naturally recombinant Malaysian isolate using reverse genetics. Vaccines 2020; 8: 270. doi:10.3390/ vaccines8020270.
22. Zhou Y, McNamara RP, Dittmer DP. Purification methods and the presence of RNA in virus particles and extracellular vesicles. Viruses 2020; 12(9): 917. doi: 10.3390/v12090917.
23. Théry C, Witwer K, Aikawa E, et al. Minimal information for studies of extracellular vesicles 2018 (MISEV2018): a position statement of the International Society for Extracellular Vesicles and update of the MISEV2014 guidelines. J Extracell Vesicles 2018; 7(1): 1535750. doi: 10.1080/20013078.2018.1535750.
24. Sardjono B. Plaque assay of Newcastle disease virus. Bul Penelit Kesehat 1989; 17(2): 207-215.
25. Schloer GM, Hanson RP. Relationship of plaque size and virulence for chickens of 14 representative Newcastle disease virus strains. J Virol 1968; 2(1): 40-47.
26. Sabouri F, Vasfi Marandi M, Bashashati M. Characterization of a novel VIIl sub-genotype of Newcastle disease virus circulating in Iran. Avian

Pathol 2018; 47(1): 90-99.
27. Esmaelizad M, Mayahi V, Pashaei M, et al. Identification of novel Newcastle disease virus sub-genotype VII-(j) based on the fusion protein. Arch Virol 2017; 162(4): 971-978.
28. Khan TA, Rue CA, Rehmani SF, et al. Phylogenetic and biological characterization of Newcastle disease virus isolates from Pakistan. J Clin Microbiol 2010; 48(5): 1892-1894.
29. Wajid A, Dundon WG, Hussain T, et al. Pathotyping and genetic characterization of avian avulavirus-1 from domestic and wild waterfowl, geese and black swans in Pakistan, 2014 to 2017. Arch Virol 2018; 163(9): 2513-2518.
30. Scanlon DB, Corino GL, Shiell BJ, et al. Pathotyping isolates of Newcastle disease virus using antipeptide antibodies to pathotype-specific regions of their fusion and hemagglutinin-neuraminidase proteins. Arch Virol 1999; 144(1): 55-72.
31. Miller PJ, Estevez C, Yu Q, et al. Comparison of viral shedding following vaccination with inactivated and live Newcastle disease vaccines formulated with wild-type and recombinant viruses. Avian Dis 2009; 53(1): 39-49.
32. Courtney SC, Susta L, Gomez D, et al. Highly divergent virulent isolates of Newcastle disease virus from the Dominican Republic are members of a new genotype that may have evolved unnoticed for over 2 decades. J Clin Microbiol 2013; 51(2): 508-517.
33. Cardenas-Garcia S, Diel DG, Susta L, et al. Development of an improved vaccine evaluation protocol to compare the efficacy of Newcastle disease vaccines. Biologicals 2015; 43(2): 136-145.
34. Kapczynski DR, King DJ. Protection of chickens against overt clinical disease and determination of viral shedding following vaccination with commercially available Newcastle disease virus vaccines upon challenge with highly virulent virus from the California 2002 exotic Newcastle disease outbreak. Vaccine 2005; 23(26): 3424-3433.