First detection and characterisation of sub-genotype XIII.2.1 Newcastle disease virus isolated from backyard chickens in Iran

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Abstract

Background: Newcastle disease (ND) is an economically significant poultry disease worldwide. During field surveillance for ND in 2010 in Iran, a backyard chicken flock showed clinical signs of ND with 100% mortality.

Objectives: We aimed to characterise genetically, biologically and epidemiologically an exotic virulent ND virus (NDV) detected in Iran.

Methods: After observing high mortality, dead birds were sampled and then disposed of by burial, and the chicken house was disinfected. Tissue samples were molecularly tested for NDV. The genetic homogeneity of the isolate RT30/2010 was tested by plaque assay, and then a large virus plaque was used for the second step of plaque purification. Fusion and matrix complete genes were sequenced and used for genotyping and epidemiological tracing. We tested biological pathotypes using mean death time (MDT) and intracerebral pathogenicity index (ICPI) assays.

Results: The isolate formed heterogeneous plaques in chicken embryo fibroblast cells. The second step of plaque purification produced homogeneous and large plaques. Phylogenetic analysis using both genes classified the virus into sub-genotype XIII.2.1. Nucleic acid and amino acid identities of RT30/2010 fusion gene with the closest available isolate SPVC/Karachi/NDV/43 are 97.95% and 98.73%, respectively. Isolate has \textsuperscript{112}RRRKRF\textsuperscript{117} motif at the fusion cleavage site, and pathogenicity tests showed MDT of 56.4 h and ICPI of 1.85.

Conclusions: This study presents the first detection and characterisation of a velogenic NDV of sub-genotype XIII.2.1 from Iran. Our follow-up surveillance for ND shows that timely virus detection and carcass management have led to the cessation of virus transmission in Iran.

Keywords
chicken, Iran, Newcastle disease virus, sub-genotype XIII.2.1, velogenic

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Newcastle disease (ND) is a viral disease of poultry that, despite a century since its first description in 1926 in Indonesia, is still associated with significant economic losses in the world poultry industry. ND virus (NDV) or avian paramyxovirus 1 as the causative agent of ND is a virus of the genus Orthoavulavirus and infects a wide range of wild and domestic birds worldwide (ICTV, 2021; Suarez et al., 2020). NDV is an enveloped virus and has a single-stranded, negative-sense and nonsegmented RNA genome. The genome with a size of about 15.2 kb encodes six structural proteins: nucleocapsid protein (N), phosphoprotein (P), matrix (M), fusion (F), haemagglutinin-neuraminidase (HN), large polymerase (L) (Suarez et al., 2020). Pathotypes of NDV in increasing order of virulence include asymptomatic enteric, lentogenic, mesogenic and velogenic. While asymptomatic enteric and lentogenic NDV strains have become widespread worldwide, mesogenic and velogenic pathotypes of virulent NDV are responsible for ND spread in poultry (OIE, 2022a). Virulent NDV is highly contagious and can cause devastating ND, especially in immunologically naïve poultry. The presence of basic amino acids at the F protein cleavage site of virulent NDV is a determining factor for the pathogenic potential of NDV in chickens (Alexander et al., 2012).

The virus can infect at least 28 different species of birds and is transmitted primarily through aerosol or oral routes. Domestic poultry is a resource for meat and egg production; therefore, the disease impact is economically significant in this case. Although waterfowls and poultry are, respectively considered reservoirs of avirulent and virulent NDV strains, viruses can be transmitted and exchanged between the two reservoir hosts (Jindal et al., 2009; Snoeck et al., 2013).

Live or inactivated vaccines have been used worldwide over the past decades and have reduced the economic burden of mortality and egg production losses in the poultry industry. Despite this, virulent NDV outbreaks occur worldwide, even in the population of vaccinated chickens. Accordingly, during the last years, ND was reported by 109 of 200 World Organisation for Animal Health (OIE) member countries (OIE, 2021b).

The first report of ND in Iran dates back to the 1950s (Sohrab, 1973). Currently, ND is endemic in Iran and is one of the most important viral diseases that has affected the poultry industry despite the widespread use of mass vaccination. Studies in Iran during the last decade have shown that sub-genotype VII.1.1 NDV is prevalent in the backyard and commercial broiler flocks (Allahyari et al., 2020; Molosuki et al., 2019, 2021; Sabouri et al., 2018). With much less frequency than VII.1.1, viruses of sub-genotypes VII.2 and XIII.2 have been detected in Iran by the older NDV classification system that had been formerly named VII and VIIb, respectively (Dimitrov et al., 2019; Ebrahimi et al., 2012; Ghalyanchilangeroudi et al., 2018).

Among Iran’s neighbours, outbreaks of sub-genotype XIII.2.1 NDV were reported in Pakistan from 2007 to 2015, while recent studies indicate the predominance of sub-genotype VII.2 NDV in Pakistan (P. J. Miller et al., 2015; Wajid et al., 2021). Studies over the past decade in Bangladesh and India show that sub-genotype XIII.2 NDV has been circulating and evolving in South Asia (Barman et al., 2017; Nath & Kumar, 2017; Nooruzzaman et al., 2021).

Iran is located in southwestern Asia and borders several countries, and it can serve as a transit point for migratory birds (Fereidouni et al., 2005). Accordingly, the implementation of regular ND monitoring programmes is essential for the quick identification and control of exotic virulent NDV strains. Viruses of genotype XIII.2 have been circulating in South Asia; therefore, the transmission of a virus of this genotype into Iran is constantly probable.

Phylogenetic, pathotype and epidemiological studies are essential when an exotic virulent NDV with a high mortality rate is detected in a country. While matrix protein is relatively conserved among virus types of the paramyxovirus family, the NDV matrix gene has the majority of synonymous nucleotide sequence substitutions (Seal et al., 2000). Accordingly, we prepared a matrix gene database with NDV of known genotypes and used this database along with the fusion gene for phylogenetic analysis of the isolate.

In the present study, we aimed to characterise, molecularly and biologically, an exotic virulent NDV strain from Iran and explore its relationships to other viruses from different geographic locations.

## 2 MATERIALS AND METHODS

### 2.1 Virus isolation and plaque purification

The NDV examined in this study, RT30/2010, was isolated from a backyard chicken flock in Khorasan Razavi Province, northeastern Iran, in January 2010 (Toroghi, 2014). The flock size was 15 chickens, and the mortality rate was 100%. Before dying, chickens suffered from respiratory disorders, lethargy, anorexia and had green diarrhoea.

We observed necrosis and haemorrhagic lesions in the intestinal tract, especially Peyer’s patches and cecal tonsils at necropsy. Proventricular tissue was necrotic and haemorrhagic. Catarrhal and haemorrhage exudates were present in the trachea lumen.

Due to the high mortality rate, the carcasses of the backyard chickens were sampled and then disposed of by burial, and the chicken house was thoroughly disinfected.

We used a brain tissue pool sample of three chickens for virus isolation in specific pathogen free (SPF) embryonated chicken eggs (ECE). Following the virus isolation in SPF ECE, we tested the genetic homogeneity of RT30/2010 by plaque assay. Briefly, primary cells of chicken embryonic fibroblast (CEF) were prepared using 11-day-old SPF ECE according to the standard protocol (Hernandez & Brown, 2010). CEF was grown in T75 flasks containing Medium 199 (M199; Sigma–Aldrich) supplemented with 10% fetal bovine serum (FBS) and antibiotics at 37°C/5% CO₂. The cells were then subcultured into the same medium in six-well culture flasks. The next day, the culture flasks with 90% cell confluency were infected with virus dilutions of 10⁻² up to 10⁻⁹. After an hour of infection, the virus dilutions were removed, and the wells were washed twice with phosphate buffered saline. Each well was then overlayed with M199 plaque medium,
supplemented with 3% FBS, antibiotics and 0.3% agarose and incubated at 37°C/5% CO₂. During a 5-day incubation, the flasks were examined macroscopically and microscopically for plaque formation.

We performed the second step of plaque purification to obtain a homogeneous virus population. The collected virus from the largest plaque size was used for the second step of plaque purification.

We replicated the virus obtained by two plaque purification steps in a 10-day-old SPF ECE and preserved the allantoic fluid at −80°C for later analysis.

### 2.2 Viral RNA extraction, reverse transcription-polymerase chain reaction (RT-PCR) and molecular cloning

According to the manufacturer’s instructions, the NDV genomic RNA was isolated from the harvested allantoic fluid by the High Pure Viral RNA kit (Roche). The quality and quantity of viral RNA were evaluated using NanoDrop 2000c (Thermo Scientific), and then RNA was stored at −80°C for later use. Two primer pairs were designed to amplify the complete coding sequences of fusion and matrix genes (Table 1). RT reactions were performed on viral RNA using the forward primers and Superscript II reverse transcriptase (Life Technologies). PCR reactions were performed at 50 µl total volume using a mixture of 0.5 µl of PrimeSTAR HS DNA polymerase (Takara Bio), 10 µl of 5X PrimeSTAR Buffer (Mg²⁺ plus), 4 µl of deoxyribonucleoside triphosphate mixture (2.5 mM each), 2 µl (10 µM) of each forward and reverse primer, 2 µl of the template DNA and 29.5 µl of sterile purified water. A three-step PCR protocol was used for both genes and included 30 cycles of 98°C for 10 s, 55°C for 15 s and 72°C for 2 min.

PCR products were gel purified (High Pure PCR product purification kit, Roche Diagnostics GmbH) and ligated to the pTZ57R/T cloning vector by the Thymine-Adenine (TA) cloning method (Thermo Scientific).

### 2.3 Sequencing and phylogenetic analysis

Bidirectional sequencing of the PCR products was done by Bioneer using the plasmid universal and the designed gene-specific sequencing primers.

Phylogenetic analysis of RT30/2010 was performed using 1678 fusion genes of class II NDVs using the method provided by Dimitrov et al. (2019). Fast Fourier transformation algorithm (MAFFT v7.450) was used for multiple sequence alignment of fusion genes (Katoh & Standley, 2013). Maximum-likelihood tree based on the general time-reversible (GTR) model constructed using RaxML with 1000 bootstrap replicates through the Cyberinfrastructure for Phylogenetic Research (CIPRES) Science Gateway (Dimitrov et al., 2019; M. A. Miller et al., 2010).

The ClustalW algorithm, implemented in BioEdit software version 7.5.2, was used for nucleotide alignment of matrix genes (Hall et al., 2011). Phylogenetic analysis of RT30/2010 was performed using 422 matrix complete genes in MEGA X software (Kumar et al., 2018). After finding the best models for tree construction, the maximum likelihood statistical method, GTR and G substitution models and phylogeny test by bootstrap method with 1000 replications were used.

### 2.4 Estimation of evolutionary distances between RT30/2010 and class II NDVs

The 1664 fusion genes of different genotypes and sub-genotypes were employed to estimate evolutionary distances. According to the criteria for the classification of NDV isolates, the average distance per site for different genotypes should be above 10% (Dimitrov et al., 2019). The mean nucleotide distance between groups is inferred as the number of base substitutions per site from averaging all sequence pairs between groups using MEGA X software. The maximum composite likelihood method was used for analysis (Tamura et al., 2004). The rate variation among sites was modeled with a gamma distribution (shape parameter = 1). Codon positions included were 1st+2nd+3rd+Noncoding. Ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 1662 positions in the final dataset.

### 2.5 Mean death time (MDT) and intracerebral pathogenicity index (ICPI) assays

According to OIE guidelines (OIE, 2021a), the isolate pathogenicity was determined by MDT in 10-day-old SPF ECE and ICPI in 1-day-old SPF chicks. Briefly, MDT assay was performed by preparation of 10-fold dilution series of fresh allantoic fluid in sterile saline. For each dilution, 0.1 ml was inoculated into five 9 to 10-day embryonated SPF chicken eggs via the allantoic cavities, then the eggs were incubated at 37°C.

### Table 1 The oligonucleotide primers used in the amplification of fusion and matrix genes

| Target gene | Oligonucleotide | Sequence (5’ - 3’) | Position in Newcastle disease virus (NDV) genome |
|-------------|----------------|--------------------|-----------------------------------------------|
| Fusion      | NDV1-F3f       | GAGGCATGCGATTGCTAAAT | 4351 to 4370                                 |
|             | NDV1-F3r       | CGCCATGTGTTCTTGCTT  | 6464 to 6482                                 |
| Matrix      | NDV1-F2f       | ATCATGACAGCGAAGGAG  | 2440 to 2459                                 |
|             | NDV1-F2r       | GGATCCAGACTCTTCTACC | 4506 to 4526                                 |
FIGURE 1  Plaque assay of RT30/2010 infected chicken embryonic fibroblast monolayer. Following infection of six-well culture flasks with RT30/2010, plaques were formed at wells infected with 10^{-5}, 10^{-6} and 10^{-7} dilutions.

The eggs were observed twice daily for 7 days. The times of any embryo death were recorded. The minimum lethal dose was considered for the highest virus dilution that caused 100% mortality. The MDT was the mean time in hours for the minimum lethal dose to kill all the inoculated embryos. The MDT has been used to classify NDVs into three groups: velogenic (under 60 h to kill), mesogenic (taking 60–90 h to kill) and lentogenic (taking more than 90 h to kill) (Hanson & Brandly, 1955; Suarez et al., 2020).

For the ICPI assay, fresh allantoic fluid with a virus hemagglutination (HA) titer >16 was diluted 10-fold with sterile saline, and then 0.05 ml of the dilution was injected intracerebrally into 10 1-day-old SPF chickens. The chickens were observed every 24 h for 8 days. At each observation, the chickens were scored: 0 if normal, 1 if sick and 2 if dead (OIE, 2021a). The ICPI is the mean score per bird per observation over the 8 days. According to the ICPI values, pathotypes are defined: lentogenic viruses, 0.0–0.7; virulent mesogenic strains, 0.7–1.5; velogenic viruses, 1.5–2.0 (Suarez et al., 2020).

3  RESULTS

3.1  Heterogeneous plaque sizes produced by RT30/2010

Three days after the virus inoculation into the wells of cell culture flasks, the virus plaques formed at wells infected with the virus dilutions of 10^{-5}, 10^{-6} and 10^{-7} (Figure 1). Heterogeneity in plaque size was more clearly visible at the well infected with 10^{-6} dilution of RT30/2010, where three plaque sizes with diameters of approximately 1, 1.5 and 2 mm formed.

At the second round of plaque purification, homogeneous plaques formed that were all the same size as the large plaque of the first plaque purification step.

3.2  Phylogenetic analysis based on the fusion and matrix complete genes

The nucleotide sequencing of the fusion gene and the deduced 553 amino acid sequence showed a multibasic cleavage motif, RRRKRF, characteristic of a virulent NDV.

According to the fusion gene phylogenetic tree with 1000 bootstrap, RT30/2010 is classified in sub-genotype XIII.2.1 within genotype XIII (Figure 2). RT30/2010 grouped with isolates from Pakistan and formed a monophyletic branch with SPVC/Karachi/NDV/43 (GU182323). Nucleic acid and amino acid identities of RT30/2010 fusion gene with the closest available isolate SPVC/Karachi/NDV/43 are 97.95% (1628/1662) and 98.73% (546/553), respectively. At the phylogenetic tree, genotype XIII is divided into six sub-genotypes XIII.1, XIII.1.1, XIII.1.2, XIII.2, XIII.2.1 and XIII.2.2 (Figure 2). Sub-genotype XIII.1.1 includes viruses isolated in Africa, Sweden, Russia and India between 1995 and 2015, while sub-genotype XIII.1.2 includes viruses isolated in Iran between 2008 and 2011. Over the past decade, viruses isolated in Pakistan and India have formed sub-genotypes XIII.2.1 and XIII.2.2, respectively (Figure 2). Some viruses detected in India were assigned to the lower order branch XIII.2 (Figure 2).

With the addition of three viruses isolated in Kazakhstan in 2016, the suboclade structure of sub-genotype XIII.1.1 NDV was changed.

Although most of the viruses of genotype XIII have been detected in chicken, there are some viruses detected in other birds such as red lori (KX061544), Japanese quail (KF740478), cockatoo (JN942041), Sterna albifrons (AY865652), ostrich (JN942034), roller (JN942043), pelican (MN9091912), pigeon (MT362716), cormorant (MK693036), Dalmatian pelican (MK693039) and great black-headed gull (MK693037) (Figure 2).

Phylogenetic analysis of the matrix gene showed similar clustering patterns to that found with fusion gene phylogenetic analysis (Figure 3). Accordingly, the virus genotype and sub-genotype in the matrix gene tree were assigned the same as the ones in the fusion gene tree. Phylogenetic analysis using the matrix full gene placed RT30/2010 at the clade of Pakistani isolates within sub-genotypes XIII.2.1. Nucleic acid and amino acid identities of RT30/2010 matrix gene with the closest available isolate SPVC/Karachi/NDV/43 are 97.53% (1068/1095) and 98.63% (359/364), respectively.

3.3  Inferred genetic distances between RT30/2010 and genotypes of class II NDVs

Comparing the genetic distances of RT30/2010 and genotypes of class II NDVs showed that RT30/2010 has the lowest genetic distance (less than 10%) to genotype XIII viruses (Table 2).

The genetic distances analysis between RT30/2010 and different sub-genotypes of genotype XIII showed that RT30/2010 has the lowest (5.91%) and highest (12.61%) distances to sub-genotypes XIII.2.1 and XIII.2, respectively. Several Iranian isolates in sub-genotype XIII.1.2 have a distance of 7.29% to RT30/2010 (Figure 2 and Table 3).
Maximum likelihood phylogenetic tree for fusion gene. Phylogenetic analyses using the full fusion gene of 1678 class II Newcastle disease viruses (NDVs). The evolutionary history was inferred by using RaxML and utilising the maximum likelihood method based on the general time reversible model with 1000 bootstrap replicates. The subtree of genotype XIII is shown. A discrete gamma distribution was used to model evolutionary rate differences among sites, and the rate variation model allowed for some sites to be evolutionarily invariable. RT30 is indicated by a closed circle. The trees are drawn to scale, with branch lengths measured in the number of substitutions per site.
FIGURE 3  Evolutionary analysis by maximum likelihood method for the matrix gene. Phylogenetic analyses using the full matrix gene of 422 class II NDVs. The evolutionary history was inferred using the maximum likelihood method and general time reversible model with 1000 bootstrap replicates. The genotype XIII subtree is presented. Genotypes indicated for each virus are based on the full fusion gene phylogenetic analysis. A discrete gamma distribution was used to model evolutionary rate differences among sites and the rate variation model allowed for some sites to be evolutionarily invariable. RT30 is indicated by a closed circle. The trees are drawn to scale, with branch lengths measured in the number of substitutions per site.
### Table 2

| Genotype (number of analysed sequences) | No. of base substitutions per site (%) |
|----------------------------------------|---------------------------------------|
| I (n = 120)                            | 20.41                                 |
| II (n = 17)                            | 22.46                                 |
| III (n = 6)                            | 18.55                                 |
| IV (n = 8)                             | 17.35                                 |
| V (n = 47)                             | 16.72                                 |
| VI (n = 265)                           | 15.95                                 |
| VII (n = 772)                          | 13.82                                 |
| VIII (n = 6)                           | 14.99                                 |
| IX (n = 6)                             | 18.08                                 |
| X (n = 22)                             | 21.35                                 |
| XI (n = 14)                            | 25.30                                 |
| XII (n = 23)                           | 12.54                                 |
| XIII (n = 70)                          | 8.26                                  |
| XIV (n = 77)                           | 14.77                                 |
| XVI (n = 4)                            | 17.61                                 |
| XVII (n = 85)                          | 13.31                                 |
| XVIII (n = 17)                         | 12.86                                 |
| XIX (n = 38)                           | 18.71                                 |
| XX (n = 17)                            | 13.91                                 |
| XXI (n = 51)                           | 16.95                                 |

*Inferred from the complete nucleotide fusion gene sequences.

Compared to other sub-genotypes, RT30/2010 has the lowest evolutionary distance of 6.46% to sub-genotype XIII.1, which includes a virus isolated from cockatoo in India in 1982 (UN942041). This virus is an ancestor virus for genotype XIIIa.

Phylogenetic topology and genetic distances analysis between genotypes classified RT30/2010 in sub-genotype XIII.2.1 within the genotype XIII.

### Table 3

| Sub-genotype (number of analysed sequences) | No. of base substitutions per site (%) |
|--------------------------------------------|---------------------------------------|
|                                            | XIII.1  | XIII.1.1 | XIII.1.2 | XIII.2  | XIII.2.1 | XIII.2.2 |
| XIII.1 (n = 1)                             | 4.93     |           |          |         |          |          |
| XIII.1.1 (n = 11)                          | 4.01     | 4.15     |          |         |          |          |
| XIII.1.2 (n = 7)                           | 8.73     | 11.85    | 11.27    |         |          |          |
| XIII.2 (n = 9)                             | 6.22     | 9.68     | 6.37     | 12.35   |          |          |
| XIII.2.1 (n = 15)                          | 7.11     | 10.53    | 7.75     | 13.13   | 6.63     |          |
| XIII.2.2 (n = 27)                          | 6.46     | 10.59    | 7.29     | 12.61   | 5.91     | 7.51     |
| RT30/2010                                  |         |          |          |         |          |          |

### 3.4 High pathogenicity of RT30/2010

We used the MDT and the ICPI assays to determine the pathogenicity of the isolate. In the MDT assay, 10^(-8) was the highest virus dilution that caused the death of all inoculated embryos and was considered the minimum lethal dose. In this dilution, embryos were alive for 24 h after virus inoculation. However, 48 and 62 h after virus inoculation, two and three embryos were dead, respectively. Accordingly, the MDT was 56.4 h. Since this time is less than 60 h, RT30/2010 has a high virulence.

In the ICPI assay, all the inoculated chickens showed clinical signs during the first 24 h, and all were dead up to 3 days after inoculation (Table 4). Clinical signs were depression, muscle tremors, twisted head and neck and diarrhoea. According to the information in Table 4, the ICPI value of 1.85 indicates that RT30/2010 is a velogenic strain.

### 4 DISCUSSION

Our studies performed over the past 15 years in northeastern Iran have shown the role of NDV as a significant viral pathogen causing respiratory disease and mortality in broiler and layer chicken flocks (Fadaee et al., 2012a; Toroghi, 2009, 2014; Toroghi et al., 2020). In 2010, during annual surveillance for ND in northeastern Iran, we found a backyard chicken flock showing clinical signs of ND and 100% mortality. Backyard chickens are the most important source of meat and egg production, especially in rural areas of a developing country like Iran. However, due to the lack of biosecurity measures and poor vaccination status, there is always the possibility of transmitting exotic virulent NDVs from carrier birds to backyard chickens, which could pave the way for the transmission of new viruses to commercial chicken flocks (Conan et al., 2012; Wajid et al., 2017).

Considering the necessity of safe disposal of chicken carcasses and contaminated materials for virus spread prevention (Toroghi et al., 2020; Wilkinson, 2007), following sampling, the dead birds were disposed of by burial, and the chicken house was disinfected.

While low virulent NDV strains like the LaSota virus require the addition of exogenous trypsin for cell-to-cell spread and cytopathic...
TABLE 4  Information regarding the health status of SPF chickens inoculated with RT30/2010 for intracerebral pathogenicity index (ICPI) assay

| Clinical signs | Days after virus inoculation | Total | Total score |
|----------------|-----------------------------|-------|-------------|
| Normal         | 0  0  0  0  0  0  0  0  0  0 | = 0   | = 0         |
| Sick           | 10 2 0 0 0 0 0 0 12 | = 12  | = 12        |
| Dead           | 0 8 10 10 10 10 10 10 68  | = 136 | = 148       |

Note: According to clinical signs, chickens received scores of 0, 1 and 2. By dividing the total scores (148) by the total number of observations (80), the ICPI value is 1.85.

FIGURE 4  The known distribution of genotype XIII NDV in Southern, Western and Central Asia. The locations of RT30/2010 and the closest available isolate (SPVC/Karachi/NDV/43) are indicated by a red circle and asterisk, respectively. In countries where genotype XIII has been detected, the sub-genotypes are marked in red under the name of each country. At present, there is no information on the presence of NDV genotypes in Iran’s neighboring countries such as Afghanistan, Turkmenistan, Tajikistan and Uzbekistan. Due to the detection of the genotype XIII viruses in chickens, ornamental and migratory birds, there is always the possibility of entering new exotic virulent NDV into Iran following illegal imports and through the migration of birds from countries in the region. Retrieved from https://www.google.com/maps/@31.4086078,68.6123076,4.75z.

effect (CPE) formation in cell culture (Hanson, 1975). RT30-infected CEF showed CPE without the need for exogenous trypsin.

A characteristic of velogenic NDV is the formation of heterogeneous plaques on cell culture due to the mixed genetic population (Hanson & Brandly, 1955; Reeve & Poste, 1971; Schloer & Hanson, 1968). In the first step of RT30/2010 plaque purification, three different sizes of virus plaque formed on CEF. The presence of plaques with diameters of about 2 mm and the plaque size heterogeneity were similar to the observations in previous studies for velogenic viruses (Hanson & Brandly, 1955; Schloer & Hanson, 1968). Since the plaque size of
NDV can positively correlate to the virulence of the virus (Hanson & Brandy, 1955; Reeve & Poste, 1971), we used the largest plaque size for the second step of RT30/2010 plaque purification, and consequently homogeneous plaques formed. This result was consistent with a previous study that cloning of small or large plaque sizes of Eng-Herts NDV led to the formation of homogeneous plaques (Schloer & Hanson, 1968).

The in vivo test results for the pathogenicity assessment of RT30/2010 confirmed the observations obtained by plaque assay regarding the velogenic nature of the isolate. Inoculation of RT30/2010 into SPF ECE and day-old SPF chickens resulted in relatively rapid death, with MDT and ICPI values of 56.4 h and 1.85, respectively.

RT30/2010 in both fusion and matrix trees was placed in the branch of sub-genotype XIII.2.1. Estimation of genetic distances as a criterion for NDV classification confirmed the results of the inferred trees (Dimitrov et al., 2019). Based on the results of this study and a previous study (Khan et al., 2010), the matrix gene, along with the fusion gene, has the potential to be considered a candidate for NDV genotyping.

Apart from RT30/2010, there is no report of a sub-genotype XIII.2.1 NDV from Iran. Fortunately, during a 12-year follow-up surveillance for ND, we have not redetected any virus of sub-genotype XIII.2.1 in northeastern Iran (Fadaee et al., 2012; Toroghi, 2014, 2022), and this was the case for studies that were performed in other parts of Iran (Allahyari et al., 2020; Ebrahimi et al., 2012; Ghalyanchilangeroudi et al., 2018; Mayahi & Emami, 2017; Sabouri et al., 2018).

Although viruses of sub-genotype XIII.2.1 were prevalent in Pakistan from 2007–2015 (P. J. Miller et al., 2015), there has been no report of genotype XIII NDV from other Iran’s neighbouring countries (Figure 4). The genetic distance of more than 2% between RT30/2010 and its closest available isolate, SPVC/Karachi/NDV/43, shows an evolutionary gap between the two viruses of sub-genotype XIII.2.1. It seems that sub-genotype XIII.2.1 viruses have been circulating in Iran’s neighbouring countries; however, they have not been detected due to weak or lack of systematic surveillance. It is especially the case for countries like Afghanistan, Turkmenistan, Tajikistan and Uzbekistan, where epidemiological information on circulating NDV is not available.

Genotype XIII viruses are currently predominantly circulating and evolving in South Asia (Barman et al., 2017; Nath & Kumar, 2017; Nooruzzaman et al., 2021). Considering different hosts of genotype XIII viruses, various bird species such as chicken, ornamental and migratory birds are possible carriers to introduce genotype XIII viruses into Iran. Detection of genotype XIII NDV (MK693039, MK693037) in migratory birds in Kazakhstan, Central Asia, in 2016 suggests that migratory birds may also serve as carriers for transmitting the virus to West Asian countries and vice versa (Figure 4).

Lack of biosecurity, poor vaccination, illegal and uncontrolled trade of backyard chickens, pigeons and ornamental birds with neighbouring countries are risk factors that can expose backyard chickens in Iran to new virulent NDV.

Although we have been investigating ND for the years before 2010 (Toroghi, 2009, 2022), we had not detected any sub-genotype XIII.2 NDV. It raises the possibility that RT30/2010 entered Iran from neighbouring countries.

Continuous surveillance by us and others after RT30/2010 detection shows that a virus of sub-genotype XIII.2.1 has not spread in Iran. However, the geographical location of Iran and genotype XIII.2 prevalence in South Asia make the entrance of a virus of this genotype into the country possible.

As in the case of RT30/2010, we can detect exotic virulent NDV strains by continuous surveillance. This study shows that by the timely implementation of detection and control measures, including infected carcass management, one of the primary routes for an exotic velogenic NDV spread in the country has been blocked.

AUTHOR CONTRIBUTIONS
Data curation, formal analysis, investigation, visualisation: Zahra Hejazi. Conceptualisation, data curation, formal analysis, funding acquisition, investigation, methodology, project administration, resources, supervision, validation, writing—original draft preparation, writing—review and editing: Seyed-Elias Tabatabaeizadeh. Conceptualisation, data curation, investigation, methodology, resources, supervision, validation, writing—review and editing: Reza Toroghi. Conceptualisation, methodology, resources, validation, writing—review and editing: Hamide Reza Farzin. Methodology, validation, writing—review and editing. Parvaneh Saffarian.

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CONFLICT OF INTEREST
The authors declared that there are no conflicts of interest.

DATA AVAILABILITY STATEMENT
The data that support the findings of this study are openly available in GenBank at https://www.ncbi.nlm.nih.gov/genbank/, reference number MT755375 and MW446900.

ETHICS STATEMENT
The authors confirm that the ethical policies of the journal, as noted on the journal’s author guidelines page, have been adhered to and the appropriate ethical review committee approval has been received. The animal experiments were approved by the Ethics and Animal Welfare Committee of Razi Vaccine and Serum Research Institute (Approval No. RVSRI.REC.98.009).

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