An Iranian Velogenic Newcastle Disease Virus Genotype VII.1.1: Isolation, Plaque Purification, and Characterization as a Candidate Homologous Challenge Strain for Vaccine Efficacy Evaluation

Running Title: Iranian NDV VII.1.1 Candidate Homologous Challenge Strain

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Abstract

Background: Newcastle disease (ND) remains a threat to the poultry industry, with genotype VII strains causing outbreaks in vaccinated flocks. Genetic and antigenic differences between vaccine strains (genotypes I/II) and velogenic strains (genotype VII) call for homologous challenge strains to assess vaccine efficacy. **Objective:** This study aimed to isolate, plaque-purify and characterize velogenic Newcastle disease virus (NDV) isolates from Iran in order to propose a candidate homologous challenge strain for vaccine efficacy studies. **Methods:** Two velogenic NDV isolates from Iranian broiler farms, Ck/IR/ABD/2018 and Ck/IR/ABD/2023, were plaque-purified on Vero cells, yielding seven clones (three from the 2018 isolate and four from the 2023 isolate). The fusion (F) gene of each clone was amplified by RT-PCR, sequenced and analyzed phylogenetically. Pathogenicity was determined by mean death time (MDT) in embryonated SPF eggs (five clones) and the intracerebral pathogenicity index (ICPI) in day-old SPF chicks (three clones); the parental isolate Ck/IR/ABD/2018 and the reference strains Herts 33/56 and Texas GB were included for comparison. **Results:** All seven clones belonged to genotype VII, sub-genotype VII.1.1, and showed ≥99.9% F-gene nucleotide identity with their parental isolates (100% for clones vNDV-2 and vNDV-4 relative to Ck/IR/ABD/2018). The ^{112}RRQKRF^{117} cleavage-site motif, characteristic of velogenic viruses, was present in all clones. Clone MDT values ranged from 45.5 to 55.6 h (mean ± SD, 49.0 ± 3.9 h; n = 5) and ICPI values from 1.86 to 1.90 (1.88 ± 0.02; n = 3). The parental isolate Ck/IR/ABD/2018 (GenBank accession no. MN615882) had an MDT of 56 h and an ICPI of 1.97, close to that of Herts 33/56 (2.00) and higher than that of Texas GB (1.75). **Conclusion:** Plaque purification yielded genetically defined, velogenic clones that retained the genotype and virulence markers of the parental isolates. Ck/IR/ABD/2018 and its clones are proposed as a candidate

homologous (genotype VII.1.1) challenge strain for NDV vaccine evaluation in Iran, pending challenge-dose titration and protection studies.

Keywords: Newcastle disease virus, Avian paramyxovirus type 1, Genotype VII, Plaque purification, Challenge strain, Vaccine efficacy, Pathotyping, Phylogenetic analysis

1. Introduction

Newcastle disease (ND) is a highly contagious and economically important viral disease of poultry and many other bird species worldwide [1]. Its causative agent, Newcastle disease virus (NDV; avian orthoavulavirus 1, family *Paramyxoviridae*), has a single-stranded, negative-sense RNA genome of approximately 15.2 kb that encodes six structural proteins: nucleoprotein (NP), phosphoprotein (P), matrix protein (M), fusion protein (F), hemagglutinin-neuraminidase (HN) and the large polymerase protein (L) [2]. NDV isolates are divided into two classes; class II, which includes the virulent viruses responsible for most outbreaks, is subdivided into genotypes I–XXI [3].

The F protein is a major determinant of virulence, because it must be cleaved by host proteases to become fusion-active [4, 5]. Virulent strains carry multiple basic amino acids at the F0 cleavage site (motif ^{112}R/K-R-Q-R/K-R-F^{117}), which are cleaved by ubiquitous furin-like proteases and allow systemic spread, whereas avirulent strains have fewer basic residues (^{112}G/E-K/R-Q-G/E-R-L^{117}) and replicate mainly in the respiratory and enteric tracts [4, 5]. Genotype VII, particularly sub-genotype VII.1.1, has been responsible for the fourth panzootic and is now a predominant cause of ND outbreaks in Asia, the Middle East and Africa [6-8]. In Iran, VII.1.1 is the lineage most frequently detected in poultry, despite intensive vaccination [8].

ND control relies on live lentogenic vaccines derived from the LaSota and Hitchner B1 strains (genotype II) and on inactivated oil-emulsion vaccines [9, 10]. These vaccines generally protect against clinical disease and mortality, but protection against infection and shedding of heterologous genotype VII viruses may be incomplete [11-13]. Reasons proposed for vaccine failure include genetic and antigenic divergence between vaccine and field viruses, suboptimal vaccination practices and interference by maternally derived antibodies [10, 11, 14].

The World Organisation for Animal Health (WOAH) Manual describes the use of standard virulent challenge viruses such as Herts 33/56 (genotype IV) and Texas GB (genotype V) for the evaluation of NDV vaccines [6, 15]. These viruses were isolated several decades ago (Herts 33/56 in 1933 [16]) and are genetically distant from contemporary genotype VII viruses. Challenge with a heterologous virus may therefore not reflect vaccine performance against the viruses circulating in the field, particularly with respect to viral shedding [11, 12]. A well-characterized challenge virus that is homologous to local field viruses is therefore desirable. In Iran, Khabiri et al. proposed a VII.1.1 isolate as a challenge strain [17], and the velogenic strain Ck/IR/Beh/2011 (ICPI 1.81) has been characterized and used in challenge studies [2]; other genotypes, such as XIII.2.1, have also been detected [18].

Field isolates of RNA viruses are not genetically homogeneous but exist as quasispecies, i.e., populations of closely related variants [19, 20]. Passage of an uncloned isolate can change the composition of this population, which compromises the reproducibility of a challenge virus

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between seed lots, laboratories and time. Purification of the virus by plaque cloning is therefore the standard first step in virological work that requires a defined and reproducible virus stock, and a clonal master seed is the appropriate starting point for a seed-lot system that keeps later variation to a minimum. We therefore hypothesized that plaque purification of recent velogenic genotype VII.1.1 field isolates would give genetically defined clones that retain the genotype, the F0 cleavage-site motif and the velogenic phenotype of their parental isolates, so that a clonal stock of the most virulent isolate could serve as a homologous challenge virus.

The objectives of this study were to: (1) obtain clonal virus populations from two recent velogenic NDV isolates by plaque purification; (2) confirm the genotype of the clones by sequencing and phylogenetic analysis of the F gene; (3) determine the pathotype of the clones and the parental isolates using the standard in vivo indices MDT and ICPI; and (4) propose a suitable candidate as a homologous challenge strain for future vaccine efficacy studies.

2. Materials and Methods

2.1. Virus isolates

Two velogenic NDV isolates, designated Ck/IR/ABD/2018 (GenBank accession no. MN615882) and Ck/IR/ABD/2023, were obtained from the virus repository of the Department of Avian Disease Research and Diagnostics, Razi Vaccine and Serum Research Institute (RVSRI), Karaj, Iran. The isolates were originally recovered from broiler farms in Mazandaran Province (northern Iran, temperate climate) and Varamin, Tehran Province (hot and dry climate), respectively, and had been characterized previously by conventional virological methods. Virus stocks were propagated on Vero cell monolayers, aliquoted and stored at −70 °C until use.

2.2. Cell culture

Vero cells (ATCC CCL-81), derived from adult African green monkey (*Chlorocebus aethiops*) kidney epithelium, were maintained in Dulbecco's Modified Eagle Medium (DMEM; Gibco, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, USA), 100 IU/mL penicillin, 100 µg/mL streptomycin and 2.5 µg/mL amphotericin B. Cells were cultured at 37 °C in a humidified atmosphere containing 5% CO_2 (CO_2 incubator, Memmert, Germany), in which the cells formed a confluent epithelial-like monolayer within about 24–48 h and examined with an inverted microscope (Nikon, Japan). All cell and virus manipulations were performed in a class II biosafety cabinet (Beasat, Iran).

2.3. Plaque purification

Plaque purification was performed on Vero cell monolayers in 6-well plates according to Khabiri et al. [17] with modifications. Vero cells were seeded at 5×10^5 cells per well and incubated for about 48 h until the wells were confluent. Ten-fold serial dilutions (10^{-1} to 10^{-4}) of each virus isolate were prepared in DMEM, and 100 µL of each dilution was inoculated in duplicate wells; two wells served as uninfected cell controls. Virus was allowed to adsorb for 1 h at 37 °C, with gentle rocking every 20 min. A semi-solid overlay consisting of 1% agarose mixed with an equal volume of 2× DMEM (with 3% FBS, penicillin–streptomycin and amphotericin B) at 45 °C was then added (5 mL per well), and the plates were incubated at 37

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°C with 5% CO_2 for 5 days until distinct plaques were visible. Plaques were visualized by adding 0.5 mL of 0.1% neutral red solution per well; after about 30 min the excess dye was removed and the plates were kept in the dark for 4–5 h. Seven well-isolated plaques of three types (large hollow, large solid and small) were picked with sterile micropipette tips under a laminar-flow cabinet, resuspended in 200 µL of DMEM and each was propagated in a separate flask of Vero cells. When cytopathic effect (CPE) involved about 60–80% of the monolayer (day 4), the flasks were frozen, thawed once and clarified by centrifugation (2,000 × *g*, 20 min); the supernatants were aliquoted and stored at −70 °C as clonal stocks.

2.4. Virus titration

Virus titers were determined as the 50% cell culture infectious dose ($CCID_{50}$/mL) in Vero cells by the Kärber method [21]. Vero cells were seeded into 96-well plates and incubated overnight. Ten-fold serial dilutions (10^{-1} to 10^{-7}) of the virus stocks were prepared in cold DMEM, and 100 µL of each dilution was added to five replicate wells. Plates were incubated at 37 °C with 5% CO_2 and examined daily for CPE with an inverted microscope; the final reading was made after 7 days. The titer was calculated as $\log_{10} TCID_{50} = x_k + (d/2) - (d \times \Sigma r)/n + \gamma$, where x_k is the $\log_{10}$ of the highest dilution showing 100% CPE, d is the log dilution factor, Σr is the sum of negative well ratios, n is the number of replicates per dilution, and γ is the $\log_{10}$ of the reciprocal of the inoculum volume.

2.5. Molecular characterization

2.5.1. RNA extraction: Viral RNA was extracted from 200 µL of infected cell culture supernatant using the High Pure Viral Nucleic Acid Kit (Roche, Germany) according to the manufacturer's protocol. RNA of uninfected Vero cells (negative control) and of an NDV isolate (positive control) was extracted in parallel. RNA was eluted in 50 µL of elution buffer and stored at −70 °C.

2.5.2. RT-PCR and sequencing: The F-gene coding region of each clone was amplified with a one-step RT-PCR kit (Biotechrabbit, Germany) using two primer sets (Table 1) that have been described previously [7, 22]. The RT-PCR mixture (50 µL) contained 25 µL Master Mix, 2.5 µL reverse transcriptase, 3 µL of each forward and reverse primer (10 µM), 4 µL RNA template and 12.5 µL nuclease-free water. Reactions were run in a gradient thermal cycler (Eppendorf, Germany) under the following conditions: 50 °C for 20 min (reverse transcription), 95 °C for 2 min (initial denaturation), 35 cycles of 95 °C for 10 s, 50 °C for 10 s and 72 °C for 90 s, and a final extension at 72 °C for 7 min. PCR products were analyzed by electrophoresis on 1% agarose gels (Bio-Rad, USA) and visualized on a UV transilluminator (UVP, UK). Amplicons were purified with a PCR product purification kit (Biotechrabbit, Germany), their concentration was measured with a NanoDrop spectrophotometer (Eppendorf, Germany), and they were sequenced bidirectionally by the Sanger method with the amplification primers at the sequencing facility of the Department of Epidemiology, RVSRI (Karaj, Iran), using (Model: Applied Biosystems 3500).

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Table 1. Primers used for RT-PCR amplification and sequencing of the F gene of NDV.

Primer set	Primer	Sequence (5′→3′)	Amplicon (bp)	Reference
P1	NDV-F1	TTGCTTATAGTTAGTTCGCCTGTC	1335	[7]
P1	NDV-R1	ACCCGTGTATTGCTCTTTGG	1335	[7]
P2	NDV-F2	GCAACCAATGAAGCTGTGCATGA	1306	[22]
P2	NDV-R2	ACAGCTTCTCCATAATTTTGCGA	1306	[22]

2.6. Phylogenetic analysis

Nucleotide sequences were assembled and edited using MEGA version 7 [23]. Sequence identity was determined by BLAST analysis against the NCBI GenBank database, and multiple sequence alignment was performed with ClustalW using default parameters. A phylogenetic tree was constructed with the neighbor-joining method and 500 bootstrap replicates to assess branch support; evolutionary distances were calculated with the Kimura 2-parameter model [24]. Reference sequences of genotype VII viruses were retrieved from GenBank (accession numbers are given in Figure 3) and included the Iranian reference genome Ck/IR/Beh/2011 (MF417546.1) [2]. Sub-genotypes were assigned according to the unified classification system [3].

2.7. Pathogenicity indices

Pathogenicity was determined according to the WOAH Manual [15]: MDT in 9- to 10-day-old embryonated SPF eggs (five eggs per dilution) and ICPI in ten day-old SPF chicks per virus. The clones were first propagated in 10-day-old SPF embryonated eggs (0.2 mL into the allantoic cavity); allantoic fluid was harvested after chilling the dead embryos for 6–8 h, and hemagglutination (HA) and hemagglutination-inhibition (HI) tests were performed on it.

2.7.1. Mean Death Time (MDT): Fresh infectious allantoic fluid with an HA titer $>2^4$ was diluted 10^{-7}, 10^{-8} and 10^{-9}. Groups of five 9- to 10-day-old embryonated SPF chicken eggs were inoculated with 0.1 mL of each dilution via the allantoic cavity. Eggs were candled twice daily (morning and afternoon, about 9 h apart), and embryo mortality was recorded. MDT was calculated as the mean time (in hours) required for the minimum lethal dose (the highest dilution that caused 100% mortality) to kill all embryos. An MDT <60 h indicates velogenic, 60–90 h mesogenic and >90 h lentogenic strains [1, 15].

2.7.2. Intracerebral Pathogenicity Index (ICPI): Fresh allantoic fluid with an HA titer $>2^4$ was diluted 1:10. Ten 1-day-old SPF chicks were inoculated intracerebrally with 0.05 mL of the diluted virus and kept in isolators (Bell Isolation Systems, Scotland). Birds were observed daily for 8 days and scored as follows: 0 = normal, 1 = sick, 2 = dead. The ICPI was calculated as the mean score per bird per observation. An ICPI >1.5 indicates velogenic, 0.7–1.5 mesogenic and <0.7 lentogenic strains [1, 15].

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2.8. Statistical analysis

Mean ± standard deviation (SD) was calculated in GraphPad Prism version 8.0 for the MDT (n = 5 clones) and ICPI (n = 3 clones) values of the plaque-purified clones. Because each index was determined once per virus (MDT as the mean of five eggs per dilution; ICPI from one group of ten chicks) and the values of Herts 33/56 and Texas GB are reference values, no inferential tests (e.g., t-tests) were applied and comparisons with the reference strains are descriptive.

2.9. Ethics statement

All animal experiments were conducted in accordance with the guidelines of the Iranian Animal Ethics Committee and approved by the Institutional Animal Care and Use Committee of the Razi Vaccine and Serum Research Institute (approval number: RVSRI.REC.98.024).

3. Results

3.1. Virus propagation and plaque purification

Inoculation of Vero cells with the NDV isolates resulted in characteristic CPE within 48–72 h post-infection, including cell rounding, syncytium formation and detachment (Figure 1). Virus titers of 10^5 $TCID_{50}$/mL were obtained.

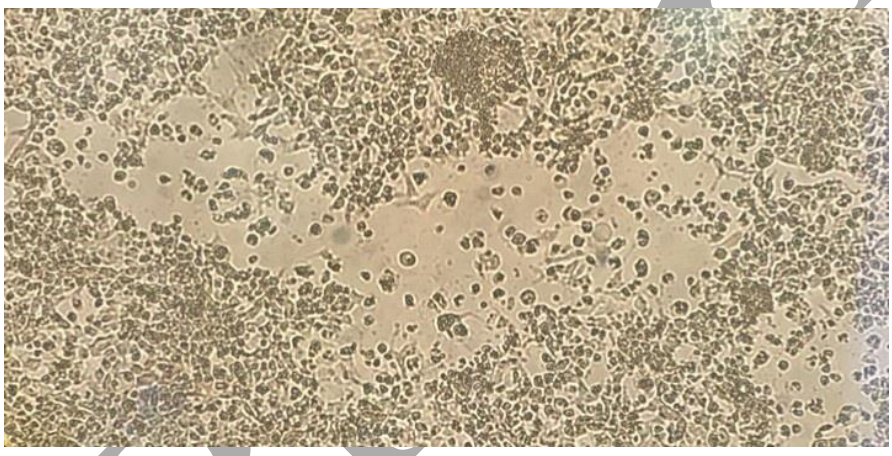


Figure 1. Cytopathic effect of Newcastle disease virus genotype VII.1.1 (isolate Ck/IR/ABD/2018) in Vero cells at 4 days post-infection (inverted microscope, ×100). Cell rounding, syncytium formation and focal detachment of the monolayer are visible.

After plaque purification, distinct plaques of varying sizes (1–4 mm in diameter) became visible after 5 days of incubation under the agarose overlay (Figure 2). A total of seven plaques were harvested and propagated: four from isolate Ck/IR/ABD/2023 (clones vNDV-1, vNDV-5, vNDV-6 and vNDV-7) and three from isolate Ck/IR/ABD/2018 (clones vNDV-2, vNDV-3 and vNDV-4). On propagation in Vero cells, all clones showed CPE in 60–80% of the monolayer at day 4.

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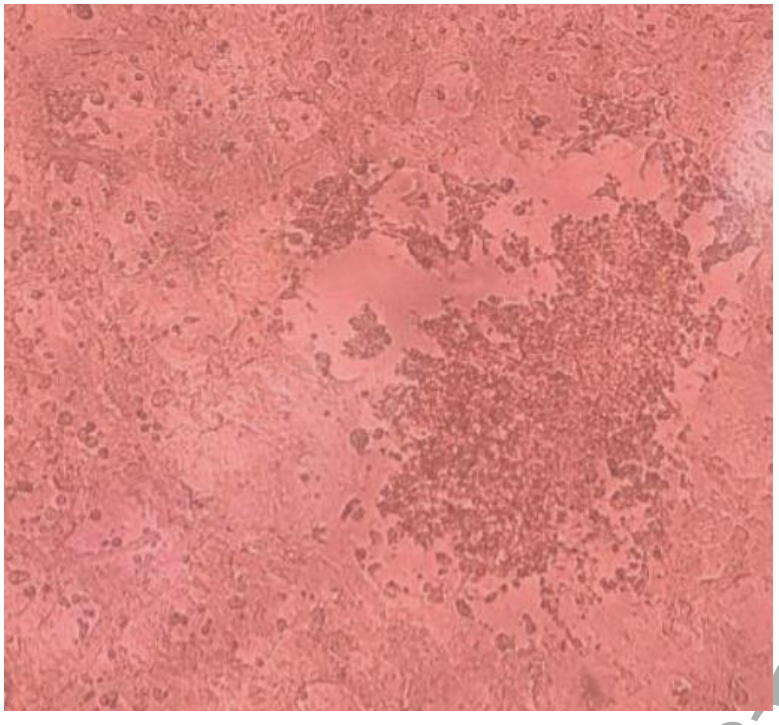


Figure 2. A well-isolated NDV plaque on a Vero cell monolayer under agarose overlay (×100). A distinct clear zone of cell lysis surrounded by viable cells is visible. Plaques were stained with 0.1% neutral red at 5 days post-infection.

3.2. Molecular characterization

3.2.1. RT-PCR confirmation: All seven clones yielded the expected amplicons of approximately 1335 bp and 1306 bp, corresponding to F-gene fragments, confirming their identity as NDV; the negative control (uninfected Vero cells) gave no product.

3.2.2. Sequence analysis: Sequencing of the F-gene fragments revealed ≥99.9% nucleotide identity between the parental isolates and their corresponding clones. The parental isolate Ck/IR/ABD/2018 showed 100% nucleotide identity with clones vNDV-2 and vNDV-4 and >99.9% identity with clone vNDV-3. The parental isolate Ck/IR/ABD/2023 showed >99.9% identity with clones vNDV-1, vNDV-5, vNDV-6 and vNDV-7. The deduced amino acid sequence at the F0 cleavage site (positions 112–117) was ^{112}RRQKRF^{117} for all clones, confirming the presence of multiple basic amino acids characteristic of velogenic NDV. No mutations were observed in glycosylation or neutralizing-epitope sites of the F protein compared with other Iranian VII.1.1 isolates [8].

3.2.3. Phylogenetic analysis: Phylogenetic analysis based on the F gene confirmed that the isolates and all seven clones belong to genotype VII, sub-genotype VII.1.1 (Figure 3). The 2018 isolate and its clones vNDV-2 and vNDV-4 grouped together with the Iranian sequence Ck/IR/EMMA140/2018, whereas clone vNDV-3 was placed on a different branch with moderate bootstrap support, consistent with its few nucleotide differences from the parental isolate. The 2023 isolate and its clones vNDV-1, vNDV-5, vNDV-6 and vNDV-7 formed a separate, well-supported cluster that also contained Egyptian sequences. The Iranian reference genome Ck/IR/Beh/2011 (GenBank MF417546.1), isolated in Behshahr, Mazandaran Province, was originally described as sub-genotype VII(L) [2] and is now classified within

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VII.1.1 under the unified system [3]; it was included in the tree as a regional reference sequence. Genetic distances ranged from 1.4% to 6.4% compared with other VII.1.1 strains in the International NDV Taxonomy Group reference file, and the isolates showed 99–100% similarity to sequences reported from Iran by Allahyari et al. [22] and Molouki et al. [8].

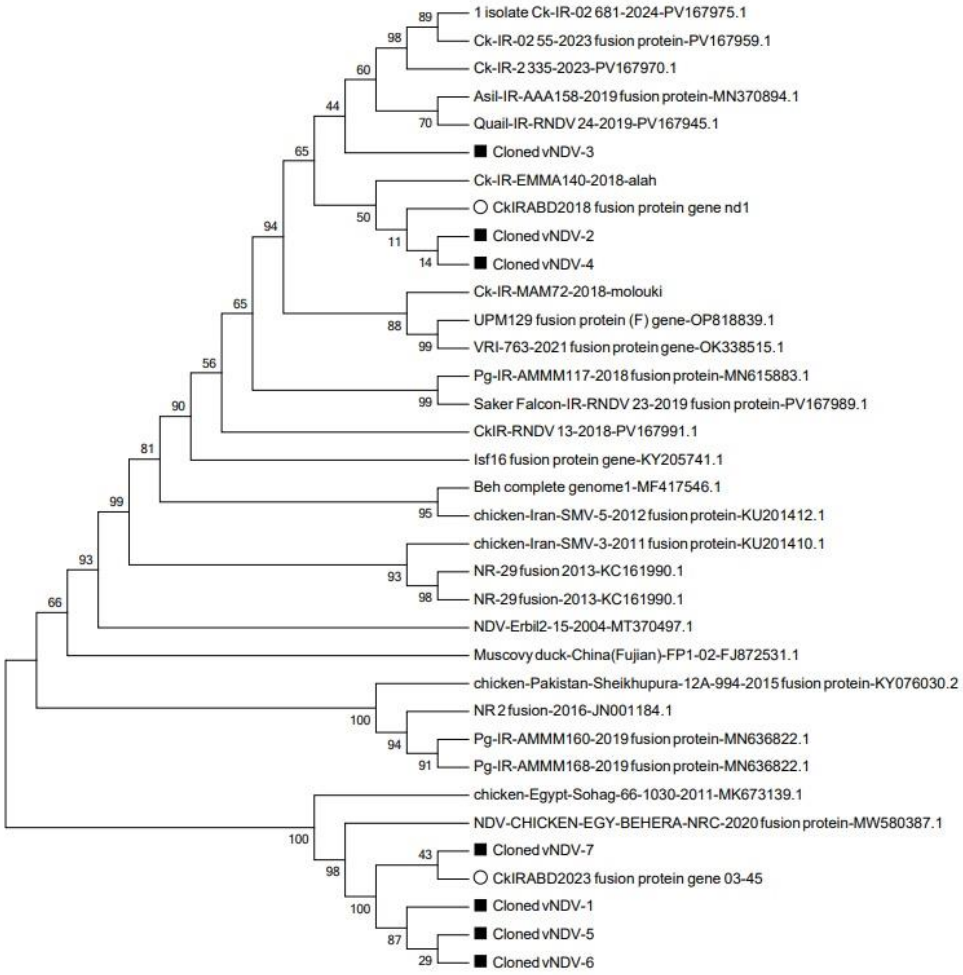


Figure 3. Phylogenetic tree based on the F gene of the NDV isolates and clones of this study and selected genotype VII sequences from GenBank (accession numbers are shown). The tree was constructed with the neighbor-joining method in MEGA 7; bootstrap values (500 replicates) are shown at the nodes. Black squares, plaque-purified clones (vNDV-1 to vNDV-7); open circles, parental isolates (Ck/IR/ABD/2018 and Ck/IR/ABD/2023).

3.3. Pathogenicity indices

The pathogenicity indices of the parental isolate Ck/IR/ABD/2018 and of the plaque-purified clones are presented in Table 2. Of the seven clones, five (vNDV-1, vNDV-2, vNDV-3, vNDV-4 and vNDV-6) were tested for MDT and three (vNDV-1, vNDV-2 and vNDV-4) for ICPI; the parental isolate Ck/IR/ABD/2023 was not tested in vivo. All tested viruses were classified as velogenic.

MDT values: The MDT values of the clones ranged from 45.5 h (clone vNDV-2) to 55.6 h (clone vNDV-1), all below the 60-h threshold for velogenic strains (mean ± SD, 49.0 ± 3.9 h;

n = 5). The parental isolate Ck/IR/ABD/2018 had an MDT of 56.0 h. The reference values for Texas GB and Herts 33/56 were 55.0 h and 48.0 h, respectively.

ICPI values: The ICPI values of the clones were 1.86 (vNDV-2), 1.88 (vNDV-4) and 1.90 (vNDV-1) (mean ± SD, 1.88 ± 0.02; n = 3), all above the threshold of 1.5 for velogenic strains. The parental isolate Ck/IR/ABD/2018 had an ICPI of 1.97, which is close to that of Herts 33/56 (2.00) and higher than that of Texas GB (1.75). The three clones derived from Ck/IR/ABD/2018 had shorter MDT values (45.5–48.8 h) and slightly lower ICPI values (1.86–1.88) than their parental isolate (56.0 h; 1.97).

Table 2. Pathogenicity indices of the NDV parental isolates and plaque-purified clones compared with the standard challenge strains Herts 33/56 and Texas GB.

No.	Isolate / strain	Virus	MDT (h)	ICPI	Pathotype
1	Ck/IR/ABD/2023	Parental isolate	ND	ND	ND
2	Ck/IR/ABD/2023	Clone vNDV-1	55.6	1.90	Velogenic
3	Ck/IR/ABD/2023	Clone vNDV-6	48.8	ND	Velogenic
4	**Ck/IR/ABD/2018**	**Parental isolate**	**56.0**	**1.97**	**Velogenic**
5	Ck/IR/ABD/2018	Clone vNDV-2	45.5	1.86	Velogenic
6	Ck/IR/ABD/2018	Clone vNDV-3	46.5	ND	Velogenic
7	Ck/IR/ABD/2018	Clone vNDV-4	48.8	1.88	Velogenic
8	Texas GB	Reference strain	55.0	1.75	Velogenic
9	Herts 33/56	Reference strain	48.0	2.00	Velogenic

MDT, mean death time (five embryonated SPF eggs per dilution); ICPI, intracerebral pathogenicity index (ten day-old SPF chicks per virus); ND, not determined. Clones vNDV-5 and vNDV-7 (Ck/IR/ABD/2023) were not tested in vivo. Pathotype was assigned as velogenic when MDT <60 h and/or ICPI >1.5 (for clones tested by one index only, by that index). Values for Texas GB and Herts 33/56 are reference values shown for comparison.

4. Discussion

In this study, plaque purification of two recent velogenic NDV isolates yielded seven clones that were characterized genetically and biologically. F-gene analysis placed all clones in genotype VII, sub-genotype VII.1.1, the lineage that predominates in Iran and is associated with the fourth panzootic [6, 8]. All clones carried the ^{112}RRQKRF^{117} cleavage-site motif, the molecular hallmark of virulent NDV, which allows cleavage of F0 by ubiquitous furin-like proteases and systemic spread [4, 5]; the same motif is found in other Iranian VII.1.1 viruses such as Ck/IR/Beh/2011 [2]. The absence of mutations in glycosylation or neutralizing-epitope regions of the F protein suggests that these viruses are antigenically conserved. The isolates from 2018 and 2023 occupied different positions in the phylogenetic tree, indicating that more than one VII.1.1 lineage circulates in Iran.

Both MDT (<60 h) and ICPI (>1.5) classified all tested viruses as velogenic [1, 15]. The ICPI of the parental isolate Ck/IR/ABD/2018 (1.97) was close to the maximum value of 2.0 and to

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that of Herts 33/56 (2.00), and higher than that of Texas GB (1.75) and than the values reported for Ck/IR/Beh/2011 (ICPI 1.81) [2] and for a XIII.2.1 isolate from Iran (ICPI 1.85, MDT 56.4 h) [18]. Its MDT (56 h) was similar to that of Texas GB (55 h) and longer than that of Herts 33/56 (48 h), whereas the MDT values of its clones were shorter (45.5–48.8 h). These comparisons are descriptive: each index was derived from a single assay and the values of the reference strains are reference values, so no statistical inference was drawn and the apparent differences should be regarded as indicative only.

A central question is why cloning was performed if the proposed challenge strain derives from an original isolate. Virus stocks recovered from the field are not homogeneous: NDV, like other RNA viruses, exists as a quasispecies, i.e., a cloud of closely related genomes that can shift in composition on passage through cells or eggs [19, 20]. A challenge virus that is used for years by different laboratories and manufacturers must be genetically defined and reproducible, and in virology a defined starting stock is obtained by purification, ideally to a clone, before a seed-lot system is established. Our data show that plaque purification did not change the genotype or the virulence markers of the parental isolates: clones vNDV-2 and vNDV-4 had 100% F-gene identity with Ck/IR/ABD/2018 and all other clones had ≥99.9%. At the same time, the clones of the 2018 isolate gave MDT values up to about 10 h shorter and ICPI values slightly lower than the parental isolate, although their F genes were almost identical. This suggests that subpopulations of a field isolate can differ phenotypically, possibly through changes outside the F gene, which were not examined here, and underlines the need for a clonal master seed rather than reliance on an uncloned stock whose composition may drift. Cloning therefore served both to verify the genetic homogeneity of the isolate and to provide the defined starting material that a standardized challenge virus requires [19, 20].

Herts 33/56 (genotype IV) and Texas GB (genotype V) remain the standard challenge viruses [15], but genotype VII viruses differ genetically and antigenically from them and from the genotype II vaccine strains. The efficacy of LaSota-induced hemagglutination-inhibiting antibodies against heterologous genotype VII and IX challenge viruses [13] and the better protection afforded by genotype VII.1.1-matched vaccines against field isolates [12] indicate that vaccine–challenge genotype mismatch can influence the outcome of vaccination, whereas other work shows that a mismatched vaccine can still protect significantly [25]. A homologous challenge virus allows both aspects to be assessed, including viral shedding and transmission, and provides a more stringent test against viruses of the genotype that currently causes outbreaks. Previously proposed Iranian candidates include the VII.1.1 isolate described by Khabiri et al. [17] and Ck/IR/Beh/2011 [2]; the present work adds a plaque-purified, clonally defined virus characterized by F-gene sequencing and by MDT and ICPI, with an ICPI approaching that of Herts 33/56. On this basis, we propose the clonal stock of Ck/IR/ABD/2018 (clones vNDV-2 and vNDV-4, 100% F-gene identity with the parent) as a candidate homologous challenge strain. Its use would also reduce dependence on heterologous reference viruses and allow the evaluation of genotype-matched vaccines [12]. Because NDV continues to evolve, challenge viruses should be reviewed periodically on the basis of surveillance of circulating genotypes [8].

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This study has limitations. Only two isolates from two regions of Iran were examined, and only the F gene was analyzed; sequencing of other genes or whole genomes may reveal additional determinants of the phenotypic differences observed among clones, and would also allow recombination to be excluded. Clonality was verified by F-gene sequencing only. Each pathogenicity index derives from a single assay, so no statistical comparison was possible, and not all clones or the 2023 parental isolate were tested in vivo. Finally, challenge studies were not performed: the suitability of the candidate strain as a challenge virus, including the challenge dose, the reproducibility of disease and shedding in vaccinated and unvaccinated SPF chickens and its performance under field-like conditions, remains to be demonstrated.

5. Conclusion

Plaque purification of two recent Iranian velogenic NDV isolates (Ck/IR/ABD/2018 and Ck/IR/ABD/2023) gave seven clones of genotype VII, sub-genotype VII.1.1, that retained the ^{112}RRQKRF^{117} cleavage-site motif and the velogenic phenotype of their parental isolates. The parental isolate Ck/IR/ABD/2018 (MDT 56 h, ICPI 1.97) was the most virulent virus tested, with an ICPI close to that of Herts 33/56. Because field isolates are quasispecies and a challenge seed must be genetically defined and reproducible, we propose the plaque-purified clonal stock of Ck/IR/ABD/2018 as a candidate homologous challenge strain for NDV vaccine evaluation in Iran. Before routine use, its challenge dose and its protection and shedding profile should be confirmed in vaccinated and unvaccinated SPF chickens.

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Data Availability Statement

The F-gene sequence of isolate Ck/IR/ABD/2018 is available in the GenBank database under accession number MN615882. All other data supporting the findings of this study are available from the corresponding authors upon reasonable request.

Conflict of Interest Statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Author Contributions

ZG and ML performed the laboratory experiments. ML and MMA contributed to the study design and data interpretation. SK and AA participated in virus isolation and characterization. MS and AY performed the molecular analyses. MA and ML supervised the research. ZG drafted the manuscript. All authors reviewed and approved the final manuscript.

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