Characterization of Newcastle Disease Virus in Broiler Flocks with Respiratory symptoms in some Provinces of Iran

Foroogh Makki

Shahid Chamran University of Ahvaz

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

Shahid Chamran University Faculty of Veterinary Medicine <https://orcid.org/0000-0002-7551-8352>

Mansour Mayahi

Shahid Chamran University of Ahvaz

Masoud Reza Seyfi Abad Shapouri

Shahid Chamran University of Ahvaz

Research Article

Keywords: Newcastle disease, respiratory symptoms, broiler flocks, Iran

Posted Date: August 9th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-750509/v1>

License: © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License. [Read Full License](#)

Abstract

Background: Newcastle disease, is one of the most important diseases of the poultry industry, has many economic losses. The aim of this study was to isolate and determine the molecular identity of Newcastle disease virus in 40 broiler flocks with respiratory symptoms in four provinces of Iran.

Methods and Results: Samples of farms with respiratory symptoms were collected from different regions of Isfahan, East Azerbaijan, Golestan, and Khuzestan provinces and inoculated into 9-day-old embryonated chicken eggs. The Reverse-transcription polymerase chain reaction (RT-PCR) was performed to detect the Newcastle disease virus on allantoic fluid. Of the 40 flocks, the virus was isolated and identified in 16 flocks. The PCR products of 16 isolates were sequenced and a phylogenetic tree was drawn. Accordingly, six isolates were in genotype II and ten isolates were in subgenotype VIId of class II.

Conclusion: Both genotypes were present in all four provinces. The isolates of Khuzestan province showed the greatest diversity compared to the other three provinces. The similarity of isolates belonging to genotype II in this study was observed with Pakistan, China, and Nigeria and other isolates were similar to previous isolates in Iran. Also, the highest amino acid sequence in the F-protein cleavage site was 112RRQKR/F117 for VIId genotype isolates and 112GRQGR/L117 for II genotype isolates.

Introduction

Newcastle disease (ND) is a common infectious disease in domestic and wild bird species that is important in many countries, including Iran, due to its harmful effects on the poultry industry [15]. The disease is characterized by respiratory and neurological symptoms and haemorrhage lesions in the gastrointestinal tract [3]. The causative agent of this disease is a virus with a single-stranded, negative-sense RNA, approximately 15 kbp, and enveloped, belonging to the paramyxoviridae family and serotype 1 (APMV-1) of the genus *Orthoavulavirus* [5–26,32]. The genome of this virus consists of encoding six structural proteins including nucleocapsid (N), phosphoprotein (P), matrix (M), fusion (F), hemagglutinin-neuraminidase (HN), and large polymerase (L) [18]. The pathotypes of Newcastle disease virus from high to low severity are high severity (velogenic), moderate severity (mesogenic), low severity (lentogenic), and asymptomatic intestines [13]. In low-virulent isolates, F0 protein is broken down into F1 and F2 only by extra-cellular trypsin-like proteases in the airways and gastrointestinal tract; In isolates causing acute disease, however, F0 protein is cleaved to F1 and F2 by furin-like proteases present in most cells of host tissues [9]. This difference in the activation of proteases is the main cause of systemic proliferation and severe disease caused by such kind of viruses [26]. Avian paramyxovirus serotype 1 strains are divided into two main classes I and II based on F gene sequence analysis. class I includes low-virulence viruses isolated from wild birds, and class II includes multiple genotypes of low-virulence and acute Newcastle disease viruses isolated from domestic and wild birds. The virus is also subdivided into genotypes based on the nucleotide sequence of the F gene, with class I containing one genotype and class II containing 18 genotypes (I–XVIII) [5–22,31].

In recent years, many cases of Newcastle disease have been reported in vaccinated flocks with high antibody levels [4], and on the other hand because the identification of Newcastle virus in many valid studies based on protein F0 [21], so in the present study, Newcastle disease virus was isolated from flocks with respiratory symptoms and F gene was detected by Reverse-transcription polymerase chain reaction (RT-PCR). The nucleotide and amino acid sequences of the F gene cleavage site were examined in the recovered isolates.

Materials And Methods

Sampling. Ten dead chicks from ten broiler flocks suspected of Newcastle disease from each of the provinces of Khuzestan, Isfahan, Golestan and East Azerbaijan in the period of spring 2019 to spring 2020, were collected. The tissue of tracheae,

lungs, and caecal tonsils were sampled. Flock information sheet for each sample was completed and vaccination schedule and flock age was recorded (Tables 1 and 2). Samples were stored at -70 ° C until the experiment.

Table 1

The broiler chicken farm history of Khuzestan (rows one to ten), and Isfahan (rows 11 to 20) provinces of Iran and their molecular results

Broiler flocks number	Age of broiler flocks (day)	Vaccine program (method and age by day number)					RT-PCR results (positive Sample)			Isolated genotype
		Vitapest	Avinew	B1	Clone30	Lasota	Trachea	Lung	Cecal Tonsil	
1	33	-	23- Drinking	6-Eye drop	12&18- Drinking	-	6/10	7/10	8/10	VIIId
2	35	-	7- Drinking	12&29- Drinking	18- Drinking	-	4/10	-	-	VIIId
3	37	1- Drinking	-	7-Eye drop	12- Drinking	20&27- Drinking	9/10	8/10	5/10	VIIId
4	32	-	-	10- Drinking	18- Drinking	25- Drinking	-	-	-	-
5	19	-	-	2&8- Drinking	14- Drinking	-	-	-	-	-
6	34	2-Spray	-	7-Eye drop	12&18& 24- Drinking	30- Drinking	-	-	-	-
7	42	-	3- Drinking	32- Drinking	10&17&25- Drinking	-	-	-	-	-
8	29	-	3- Drinking	9- Drinking	17- Drinking	-	9/10	8/10	3/10	VIIId
9	20	-	-	-	10- Drinking	19- Drinking	5/10	-	2/10	II
10	37	1-Spray	-	7&23- Drinking	12&18& 30- Drinking	-	-	-	-	-
11	42	1-Spray	-	7-Eye drop	12&28- Drinking	18&36- Drinking	-	-	-	-
12	7	1-Spray	-	6-Eye drop	-	-	5/10	-	-	II
13	17	-	-	7-Eye drop	-	-	-	-	-	-
14	39	-	-	8-Eye drop	14&22- Drinking	-	6/10	3/10	5/10	VIIId
15	25	1-Spray	-	-	12&20- Drinking	-	-	-	-	-
16	17	-	-	6-Eye drop	12- Drinking	-	-	-	-	-
17	24	-	7- Drinking	2&12- Drinking	19- Drinking	-	-	-	-	-
18	32	-	3- Drinking	8-Eye drop	17- Drinking	-	7/10	5/10	4/10	VIIId

Broiler flocks number	Age of broiler flocks (day)	Vaccine program (method and age by day number)					RT-PCR results (positive Sample)			Isolated genotype
		Vitapest	Avinew	B1	Clone30	Lasota	Trachea	Lung	Cecal Tonsil	
19	18	2-Spray	-	9-Eye drop	14- Drinking	-	-	-	-	-
20	9	-	-	7-Eye drop	-	-	5/10	-	-	II

Table 2

The broiler chicken farm history of Golestan (rows one to ten), and East Azerbaijan (rows 11 to 20) provinces of Iran and their molecular results

Broiler flocks number	Age of broiler flocks (day)	Vaccine program (method and age by day number)					RT-PCR results (positive sample)			Isolated Genotype
		Vitapest	Avinew	B1	Clone 30	Lasota	Trachea	Lung	Cecal Tonsil	
1	23	-	-	26-Spray	19-Drinking	-	-	-	-	-
2	27	-	-	2-Drinking & 7-Eye drop	18-Drinking	-	6/10	-	5/10	VIld
3	16	1-Spray	-	8-Eye drop	-	-	-	-	-	-
4	38	1-Spray	-	7-Eye drop & 22-Drinking	13&30-Drinking	-	6/10	3/10	5/10	VIld
5	39	1-Spray	-	-	10&16-Drinking	28&35-Drinking	-	-	-	-
6	27	-	7-Drinking	-	13-Drinking	19-Drinking	-	-	-	-
7	9	-	-	7-Eye drop	-	-	8/10	-	-	II
8	10	-	2-Drinking	9-Drinking	-	-	9/10	-	-	II
9	38	1-Spray	-	6-Eye drop & 17-Drinking	11&24-Drinking	33-Drinking	-	-	-	-
10	20	2-Spray	-	-	11-Drinking	-	-	-	-	-
11	36	1-Spray	-	-	12&20-Drinking	31-Drinking	-	3/10	6/10	VIld
12	19	-	-	7-Eye drop	13-Drinking	-	-	-	-	-
13	35	-	9-Drinking	2-Drinking	14&25-Drinking	-	5/10	6/10	8/10	VIld
14	33	-	7-Drinking	-	18-Drinking	-	-	-	-	-
15	18	1-Spray	-	6-Eye drop	-	-	-	-	-	-
16	29	-	2-Drinking	7-Eye drop	12&18-Drinking	-	-	-	-	-

Broiler flocks number	Age of broiler flocks (day)	Vaccine program (method and age by day number)					RT-PCR results (positive sample)			Isolated Genotype
		Vitapest	Avinew	B1	Clone 30	Lasota	Trachea	Lung	Cecal Tonsil	
17	37	-	3-Drinking	8-Eye drop & 17-Drinking	27-Drinking	35-Spray	6/10	-	-	II
18	13	1-Spray	-	7-Drinking	-	-	-	-	-	-
19	26	-	-	7-Eye drop	12&17-Drinking	-	-	-	-	-
20	40	-	33-Drinking	-	12&18-Drinking	-	-	-	-	-

Virus isolation. Tissue samples were separately homogenized in phosphate buffer solution (PBS) containing penicillin 10000 IU / ml, streptomycin 10000 µg/ml, gentamicin 5 µg/ml and amphotericin B 5 µg / ml. The centrifuge was then used and the supernatant was used as inoculum. Then 200 microlitres of the samples were inoculated separately into the allantoic cavity of 9-day-old embryonated chicken eggs. Allantoic fluid was harvested and after the RT-PCR test, negative samples were serially passaged up to three times.

RNA Extraction. To extract total RNA, 200 µl of allantoic fluid was added separately to 1 ml of RNX-plus Solution (manufactured by Sinagen Company, Iran) and the extraction was performed according to the manufacturer's instructions. Then 50 µl of DEPC water was added to the extracted RNA and stored in the - 70 ° C.

cDNA synthesis. The cDNA was synthesized from the extracted RNA using the cDNA Synthesis Kit (YektaTajhizAzma Company, Iran). For this purpose, General primers (random Hexamer and Oligo DT) were used, and the synthesized cDNA was stored at -20°C.

PCR reaction. The factors involved in the reaction include Master Mix 2X with 1.5 mM MgCl₂ (Amplicon,Canada)10µl, specific primers of F gene, ndvf (TTGATGGCAGGCCTCTTGC), and ndvr (GGAGGATGTTGGCAGCATT) each 10 pmol/ µl [16], DNA template 3 µl, water 6 µl in final volume 20 µl and using a thermocycler device and temperature conditions of 95 ° C for 3 minutes (early denaturation), 40 cycles including denaturation at 95 ° C for 30 seconds, annealing at 55°C for 60 seconds, and extension at 72° C for 60 seconds, followed by Final Extension at 72 ° C for 10 minutes. It should be noted that at all stages of the PCR reaction, a negative control sample (DEPC water instead of the template DNA) and a positive control sample (RNA extracted from B1 and Lasota vaccines) were placed.

PCR product analysis. PCR products were electrophoresed in 1% agarose gel with a safe stain for 45 minutes at 100 volts then exposed to UV light. The DNA marker used was 100bp (manufactured by Sinagen Company, Iran). Fifty µl of Positive PCR product with 10 µl of each specific primers were sent to Takapozist company for purification and sending to the BIONEER company (South Korea) for sequencing. Nucleotide sequences of partial F gene Isolates were compared with previous isolates from Iran, neighboring countries, and NDV reference sites at the National Center for Database Information Biotechnology (<http://ncbi.nlm.nih.gov>) using nBLAST (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) and ClustalW2 software. The phylogenetic relationship was calculated by one-click software at http://www.phylogeny.fr/simple_phylogeny.cgi. The phylogenetic tree was obtained using the Neighbor-joining method and MEGA 6.0 software. The topological stability of the tree was evaluated by 1000 replications of bootstrap.

Results

Out of a total of 1,200 samples inoculated into the allantoic fluid, 190 positive results from 16 flocks were obtained by RT-PCR and were as follows in each province:

Isfahan province 40 positive cases in four flocks, East Azarbaijan province 34 positive cases in three flocks, Golestan province 42 positive cases in four flocks, and Khuzestan province 74 positive cases in five flocks; and finally a positive sample from each flock was sent for sequencing.

The results showed that Nd1 (MT047268), Nd2 (MT047269), Nd3 (MT047270), Nd4 (MT047271), Nd5 (MT047272), Nd6 (MT047273), Nd7 (MT047274), Nd8 (MT047275), Nd15 (MT047282), and Nd16 (MT047283) isolates were more than 90% similar and all belonged to the VIIId subgenotype; On the other hand, the same percentage of similarity was observed between the isolates of Nd9 (MT047276), Nd10 (MT047277), Nd11 (MT047278), Nd12 (MT047279), Nd13 (MT047280), and Nd14 (MT047281), which were members of genotype II; but there was no significant similarity between the two groups.

It should be noted that isolates of both genotypes were found in all four provinces. According to the phylogenetic tree (Fig. 1), it was found that subgenotype VIIId isolates in this study were most similar to Iranian isolates.

Isolates Nd9 (MT047276), Nd10 (MT047277), and Nd13 (MT047280) were more than 99% similarity to the Pakistani, Nigerian, and Chinese strains. Also, similarity among isolates Nd11 (MT047278), Nd12 (MT047279), Nd14 (MT047281), and Pakistani, Nigerian and Chinese strains were observed.

Nd10 (MT047277) was similar to B1 and Nd14 (MT047281) showed the highest similarity to Lasota.

Amino acid sequence. Based on the information in Table 3, the amino acid sequence of the F gene in the cleavage site and its comparison with Table 4 [32] and Fig. 2 [27], it was determined that isolates Nd1 (MT047268), Nd2 (MT047269), Nd4 (MT047271), Nd6 (MT047273), Nd7 (MT047274), Nd8 (MT047275), and Nd15 (MT047282) were similar to velogenic isolates including Essex70, Beaudette, Kvuzat-Yavne/50–826, Niger/1377-7/06, US(CA)/ 1083Fontana/72, CN/ZJ-1/00, NA-1 and AF2240. Examination of the amino acids in the F cleavage site of Nd3 (MT047270) and Nd5 (MT047272) isolates showed that both isolates follow the pattern of virulence viruses. Nd16 (MT047283) isolate was also similar to velogenic isolates including 617/83 and IT-227/82. The six isolates Nd9 (MT047276), Nd10 (MT047277), Nd11 (MT047278), Nd12 (MT047279), Nd13 (MT047280), and Nd14 (MT047281) were similar to lentogenic isolates including DE-R49 99, Lasota/46, and B1/46.

Table 3
The amino acid sequence of F
protein cleavage site of isolates
used in the present study

Sample no.	Amino acid seq.
Nd1	TSGGRRQKRFIG
Nd2	TSGGRRQKRFIG
Nd3	TSGGRKQKRFIG
Nd4	TSGGRRQKRFIG
Nd5	TSGGRKQKRFIG
Nd6	TSGGRRQKRFIG
Nd7	TSGGRRQKRFIG
Nd8	TSGGRRQKRFIG
Nd9	TSGGGRQGRLIG
Nd10	TSGGGRQGRLIG
Nd11	TSGGGRQGRLIG
Nd12	TSGGGRQGRLIG
Nd13	TSGGGRQGRLIG
Nd14	TSGGGRQGRLIG
Nd15	TSGGRRQKRFIG
Nd16	TSGGGRQKRFIG

Table 4
The amino acid sequences of F protein cleavage site of some previous isolates [32].

Virus strain	Virulence ^a	ICPI	Cleavage site AA 111-118 ^b
Herts33	High	1.88	G-R-R-Q-R-R↓F-I
Essex '70	High	1.86	G-R-R-Q-K-R↓F-V
135/93	High	1.30	V-R-R-K-K-R↓F-I
617/83	High	1.46	G-G-R-Q-K-R↓F-I
34/90	High	1.81	G-K-R-Q-K-R↓F-I
Beaudette	High	1.46	G-R-R-Q-K-R↓F-I
Karachi/SPV/33	High	1.85	G-R-R-Q-R-R↓F-I
Kvuzat-Yavne/50-826	High	1.89	G-R-R-Q-K-R↓F-I
Australian isolates			
Peats Ridge	Low	0.41	G-R-R-Q-G-R↓L-I
QV4	Low	0.39	G-K-R-Q-G-R↓L-I
Somersby 98	Low	0.51	G-R-R-Q-R-R↓L-I
Dean Park	High	1.60–1.70	G-R-R-Q-R-R↓F-I
PR-32	Low	0.64	G-K-R-Q-G-R↓F-I
African isolates			
Chicken/MG/'92	High	<i>c</i> _—	G-R-R-R-R-R↓F-V
Niger/1377-7/06	High	1.84	G-R-R-Q-K-R↓F-I
Nigeria/228-7/06	High	1.90	G-R-R-Q-R-R↓F-I
Chicken/Mali/'07	High	<i>c</i> _—	G-R-R-R-K-R↓F-V
Burkina Faso/2415-580/08	High	1.69	G-R-R-R-K-R↓F-I
South Africa/08100426/08	High	1.91	G-R-R-R-K-R↓F-I
<i>a</i> Virulence for chickens.			
<i>b</i> ↓ = cleavage point. Basic amino acids in bold. Note that all virulent viruses have phenylalanine (F) at position 117 (the F1 N terminus).			
<i>c</i> Unknown ICPI.			

Discussion

The importance of Newcastle disease is such that even developed countries, have costs to prevent and control the disease and the economic damage caused by it or to achieve disease-free conditions after the outbreak of the disease. Also Newcastle disease virus is endemic in many developing countries and therefore as an important limiting factor in the development of the poultry industry in these countries [32]. In this study, Newcastle disease virus was isolated from 16 (40%) out of 40 broiler flocks in four provinces of the country. From each of the 16 positive flocks, one sample was sequenced, which showed that ten isolates were in subgenotype VIId and six isolates were in genotype II of class II Newcastle disease virus. The predominant subgenotype in this study was VIId, which had the highest isolation from trachea, cecal tonsils and

lungs, respectively, and the highest isolation was from Khuzestan, Isfahan, East Azerbaijan, and Golestan provinces, respectively.

Genotype VII is a large group (class II of Newcastle disease virus) with high genetic diversity, the first reports of which date back to the late 1980s and early 1990s. Viruses belonging to this genotype first originated in the Far East (East Asia) emerged and then spread to Europe, South Africa, and South America. Based on the amino acid sequence, it is predicted that all viruses of this genotype are highly pathogen. They are among the velogenic viruses and are associated with the sudden spread of the disease in Eastern Europe, the Middle East, and Asia, as well as the spread of the disease in Africa and South America [6–22]. Subgenotype VIId was quickly isolated from different parts of the world and became known as one of the most prevalent subgenotypes in circulation until the early 21st century. Viruses belonging to this subgenotype in China (1998–2013), South Korea (2000–2005), and Colombia (2006–2010) have been isolated. Also, there have been sporadic reports of the VIId subgenotype in birds in some countries, including South Africa, Ukraine, and Venezuela during 2004–2009. Isolation of viruses of this subgenotype from wild birds with clinical signs also occurs in some regions during the year 2006–2007, indicates the high capacity of these viruses to spread and rotate around the world, as well as the potential for their transmission between domestic and wild birds [6]. After that, Ebrahimi et al. (2012) analyzed the F gene of 51 Asian Newcastle disease virus isolates from poultry during 2008–2011, they showed that genotype VII was still predominant among domestic birds in Asia and it was circulating subgenotypeVIIb in Iran and Indian sub-continent, while the subgenotypeVIId was present in the Far East [7]. Rostamali et al. (2014), in the phylogenetic study of the Newcastle disease virus in a severe epidemic of this disease during 2010 and 2011 in Iran, stated that their study isolates, which were subgenotypeVIId of class II, were different with the previous isolates found in Iran (subgenotypeVIIb) but similar to the Chinese isolates. In fact, in some cases, such differences in the F gene cleavage site do not affect the severity of the virus but can be due to mutations or the arrival of the virus from other countries [29]. Ghalyanchi et al. (2014), in a phylogenetic study of Iranian Newcastle disease virus isolates during 2010–2012, recognized six isolates of virus (which were isolated from the sudden outbreak of this disease in broiler, broiler breeder and ostrich farms of Gilan, Mazandaran, Alborz, Isfahan, and Tehran provinces). These isolates were in subgenotypeVIId [11].

The molecular and epidemiological studies of Newcastle disease virus from 1995 to 2016 in Iran by Mayahi and Esmaelizad (2017), showed subgenotypesXIIId, XIIIa, VIg, VIj, VIIj andVIIdhad been circulating among Iranian commercial poultry farms in these 21 years [19].

In order for the Newcastle disease virus to enter the host cell, the precursor glycoprotein F0 must be broken down into F1 and F2, and only the membranes of the viruses whose F0 is cleaved have the ability to enter with the host cell [9–25]. Cleavage is performed by non-viral proteases, and the ability of the protease to cleave F0 depends on the amino acid sequence around the cleavage site. In this sequence in the lentogenic viruses, at the carboxy end of the F2 protein, there is a basic amino acid and at the amino end of the F1 protein, there is leucine (112G-R/K-Q-G-R↓L117). The velogenic Virus strains have several basic amino acid sequences (presence of at least three basic amino acids) at the carboxy terminus of F2 protein and the amino terminus of F1 protein contains phenylalanine amino acids (112R/G/K-R-Q/K-K/R-R↓F117) [25–32]. Inspection of amino acid sequences of F0 protein cleavage site of subgenotype VIId of this present study showed that the highest sequence in seven isolates was 112RRQKR/F117, of two isolates was 112RKQKR/F117, and one isolate was 112GRQKR/F117. Also, the presence of phenylalanine amino acid at position 117 of F0 protein is evident in all isolates subgenotype VIId of this study. On the other hand, the amino acid sequence of the F0 protein cleavage site of six isolates according to genotype II of this study, they were all similar to 112GRQGR/L117. In these six isolates, the amino acid leucine is located at position 117 of F0 protein. In the following, the sequences found in the present study are compared with other studies in this field. Kianizadeh et al. (2002), in the study of nine isolates of Newcastle disease virus obtained from different regions of Iran [17]; Fathi et al. (2008), by studying viral isolates of Newcastle disease virus in broiler and Commercial layer flocks, and Indigenous chickens [8] and Samadi et al. (2014), with molecular and phylogenetic Study of six velogenic isolates of Newcastle Disease Virus of different geographical regions in Iran where these isolates were included in genotype VII [30], all presented the amino acid pattern of 112RRQRR/F117 for the F0 protein cleavage site of the studied isolates. According to

Munir et al. (2012) from Pakistan, all of the velogenic isolates studied by them showed the amino acid pattern of 112RRQKR/F117 in the F0 protein cleavage site [24]. Also, the sequencing of viral isolates of Newcastle disease in the study of Rostamali et al. (2014) in Iran showed a similar pattern to the above finding [29]. Mehrabanpour et al. (2014), in a molecular survey of Newcastle disease virus in farms of Fars province, stated that six viruses isolates with 112RRQKR/F117 pattern at the cleavage site of F0 protein were in the velogenic group and four isolates with pattern 112GRQGR/L117 in this area were classified as lentogenic. All isolates of their study were in genotype III of class II Newcastle disease virus. Besides, the amino acid sequence for lentogenic isolates is similar to the sequence of the isolates in the present study [21]. Evaluation of amino acid sequence of F0 protein cleavage site of isolates with velogenic Newcastle disease virus (subgenotype VIIb) in Ahmadi et al.'s (2016) study [1] and Velogenic isolates from the study of Boroomand et al. (2016) in Ahvaz also followed the pattern of 112RRQKR/F117 in terms of the amino acid sequence of F0 protein cleavage site [4].

Ghalyanchi et al. (2018) showed that the acute and emerging subgenotype VIIi also follows the pattern of 112RRQKR/F117 in the mentioned area [10]. The Isolation sequencing in Jabbarifakhar et al. (2018) 's study, also indicated that these velogenic isolates followed the 112RRQKR/F117 amino acid pattern in the F0 protein cleavage site [14].

In this study, among the four studied provinces, the highest isolation of Newcastle disease virus belonged to Khuzestan province. Five cases out of ten studied flocks (50%) were positive and the isolates from four flocks were placed subgenotype VIId class II and 1 isolate was genotype II from class II. According to the results, the isolates of this province showed high similarity with the Iranian isolates (subgenotype VIId) in the gene bank; but one of the isolates was in genotype II. Due to the phylogenetic tree, this isolate is shown to be close to the Lasota strain, and according to the history of the flock that is from received the Lasota vaccine at 19 days of age and was sampled at 20 days of age, therefore, it seems that this isolate is from a vaccine preparation. Previous studies in this province also confirm the presence of different isolates with different intensities. Mayahi et al. (2015) isolated and identified the Newcastle disease virus from broiler farms in Khuzestan province. In this regard, sampling was done from 30 broiler farms in the province and after the RT-PCR test. Six isolates were obtained. These isolates were recovered from brain tissue. These were not related to the vaccine virus [20]. Boroomand et al. (2016), by determining the molecular identity of Newcastle disease virus in the prevalence of this disease in Khuzestan province, showed that three isolates from vaccinated broiler flocks with high mortality and respiratory symptoms were in class II and subgenotype VIId [4].

In the Isfahan province, the Newcastle disease virus was isolated from four flocks (40%). The isolates from two flocks were placed in genotype II of class II and according to the phylogenetic tree, similar to the B1 strain. Given that both flocks received the B1 (eye drop) vaccine at six and seven days of age, respectively, it is likely that these isolates were related to the vaccine strain. The other two isolates found in this province were very close in terms of percentage similarity (98.63%) and both were subgenotype VIId of class II virus and were similar to the Iranian isolates of the Newcastle disease virus. Rahimian et al. (2011), by identifying and phylogenetic analysis of the Newcastle disease virus in broilers in Isfahan, determined the genetic similarity of the F gene of the virus in Iran and compared it with other countries. Comparison of the nucleotide sequences determined in their study with the known sequence of F gene in other countries showed the existence of a percentage (1.4–27.3%) of genetic diversity, among which the closest resemblance to the nucleotide sequence of F gene in India, the greatest difference with the known nucleotide sequence of this gene was observed in the United States [28].

The results of ten flocks in Golestan province showed that the Newcastle disease virus was isolated from four flocks (40%). Of these, two isolates belonged to genotype II and two isolates belonged to subgenotype VIId of class II. Isolates belonging to genotype II in this province are also possible due to the phylogenetic tree and the vaccination history of flocks No. 7 and 8 in this province (receiving the B1 vaccine at seven and nine days, respectively) are related to the vaccine strain. Alamian et al. (2019), in the serological study of Newcastle disease virus antibodies in rural birds in the northern provinces of Iran (Golestan, Gilan, and Mazandaran) from the beginning of 2014 to the end of 2015, examined 70 villages from three provinces. It was considered as an epidemiological unit and 67 villages (96%) were serum positive. Among them, 30 villages belonged to Golestan province, of which 28 villages (93.3%) were serum positive. According to the results of their study, the serum contamination reported in these birds was high, which can be a major threat to industrial poultry farms. Since about

48.98% of broiler flocks and 19.85% of broiler breeder farms in Iran are located in the northern provinces, so the need for integrated vaccination programs in rural poultry and compliance with protection issues is doubled. The northern region of Iran has several important roles in the epidemiology of infectious poultry diseases. Among the high density of industrial poultry farms in the northern provinces of the country is such that about 30% of poultry meat is produced in these provinces and about 60% of day-old chicks from the northern regions are sent to other parts of the country. Accordingly and the prevalence of any disease in industrial poultry in these areas has a great impact on poultry in other parts of Iran [2].

In East Azarbaijan province, in three out of ten flocks (30%), the presence of Newcastle disease virus isolates was determined. Thus, two isolates belonged to subgenotype VIId and one isolate belonged to genotype II of class II. Considering the vaccination schedule of the flock (receiving clone 30 and Lasota vaccines on days 27 and 35, respectively) and considering the phylogenetic tree, it is likely that this isolate is related to the vaccine. Ahmadi et al. (2016), in a study of ten isolates of Newcastle disease virus with high virulence obtained from the outbreak of this disease in northwestern Iran, after phylogenetic analysis of the F gene, these ten isolates were placed in subgenotype VIIb and very similar to some isolates In Iran, Russia and Sweden. They stated that due to the wide geographical borders with three other countries in northwestern Iran, the possibility of transmitting different isolates of Newcastle virus from these countries to Iran, especially the provinces of areas are increasing [1].

It should be noted that due to the similarity of isolates belonging to genotype II in this study with Pakistani, Nigerian, and Chinese isolates of Newcastle virus and because our country is an importer in some cases and has wide geographical borders, more control and monitoring of these interactions is doubly necessary.

Haji-Abdolvahab et al. (2019) examined the prevalence of influenza, Newcastle, and infectious bronchitis viruses in broiler flocks with respiratory complex syndrome during 2015–2016 in Iran. According to the results, out of 85 positive flocks in terms of the presence of Newcastle disease virus, 63 cases were reported as velogenic. The statistics of infected flocks in provinces similar to the present study are as follows: Khuzestan province three out of nine flocks, Isfahan province five out of 22 flocks, Golestan province one out of four flocks, and East Azarbaijan province two cases Of 11 flocks [12].

Finally, according to the results of the present study and other researches in this field and the possibility of changes in the genome of the Newcastle disease virus, periodic monitoring is important for these isolates and their prevalence. In general, phylogenetic analysis of isolates can be used to evaluate the status of the Newcastle disease virus and improve the design of vaccination programs according to the needs of the regions. Comparison of the nucleotide acid sequence of some isolates studied with Common vaccines in the poultry industry, including B1 and Lasota, show differences. The above vaccines are in genotype II of class II and genetically, they show a large distance from these isolates. Understanding this may encourage the need to develop vaccines that are more genetically related to field viruses [23]. The present study showed that in all four studied provinces, the isolates were of both genotypes II and VII; although no two isolates were completely similar, the isolates related to each genotype showed a high percentage of similarity with each other. Meanwhile, Khuzestan province showed more diversity of isolates (especially subgenotype VIId) compared to the other three provinces and the province Golestan, Isfahan, and East Azarbaijan were in the next ranks in terms of diversity in the isolates, respectively. It can be used to improve vaccination programs and the type of vaccine used in broiler flocks more efficiently.

Declarations

COMPLIANCE WITH ETHICAL STANDARDS

The authors declare that they have no conflict of interest. This article does not contain any studies involving animals or human participants used by any of the authors.

FUNDING

The authors would like to express their thankfulness to the Vice-Chancellor for Research of the Shahid Chamran University of Ahvaz for providing financial support for this work.

COMPLIANCE WITH ETHICAL STANDARDS

The authors declare that they have no conflict of interest. This article does not contain any studies involving animals or human participants used by any of the authors.

References

1. Ahmadi E, Pourbakhsh SA, Ahmadi M, Mardani K, Talebi A (2016) Phylogenetic characterization of virulent Newcastle disease virus isolated during outbreaks in northwestern Iran in 2010. *Arch Viro* 161:3151–3160. <https://doi.org/10.1007/s00705-016-3021-6>
2. Alamian A, Pourbakhsh SA, Shoushtari A, Keivanfar H (2019) Seroprevalence investigation of Newcastle disease in rural poultries of the northern provinces (Golestan, Gilan, and Mazandaran) of Iran. *Archives of Razi Institute* 74:365–373. <https://doi.org/10.22092/ari.2017.116669.1175>
3. Banakar A, Sadeghi M, Shoushtari A (2016) An intelligent device for diagnosing avian diseases: Newcastle, Infectious Bronchitis, Avian Influenza. *Computers and Electronics in Agriculture* 127:744–753. <https://doi.org/10.1016/j.compag.2016.08.006>
4. Boroomand Z, Jafari RA, Mayahi M (2016) Molecular characterization and phylogenetic study of the fusion genes of Newcastle disease virus from the recent outbreaks in Ahvaz, Iran. *Virus Disease, Indian Journal of Virology* 27:102–105. <https://doi.org/10.1007/s13337-015-0299-z>
5. Dimitrov KM, Abolnik G, Afonso GL, Albina E, Bahl J, Berg M, Briand FX, Brown IH, Choi KS, Ghvala I, Diel DG, Durr AP, Ferreira HL, Fusaro A, Gil P, Goujgoulova GV, Grund C, Hicks JT, Joannis TM, Torchetti MK, Kolosov S, Lambrecht B, Lewis NS, Liu H, Liu H, McCullough S, Miller PJ, Monne I, Muller CP, Munir M, Reischak D, Sabra M, Samal SK, Almeida RS, Shittu I, Snoeck CJ, Suarez DL, Borm SV, Wang Z, Wong FYK (2019) Updated unified phylogenetic classification system and revised nomenclature for Newcastle disease virus. *Infection, Genetics and Evolution* 74:Article 103917. <https://doi.org/10.1016/j.meegid.2019.103917>
6. Dimitrov KM, Ramey AM, Qui X, Bahl J, Afonso CL (2016) Temporal, geographic and host distribution of avian paramyxovirus 1 (Newcastle disease virus). *Infection, Genetics and Evaluation* 39:22–34. <https://doi.org/10.1016/j.meegid.2016.01.008>
7. Ebrahimi MM, Shahsavandi S, Moazenijula G, Shamsara M (2012) Phylogeny and evolution of Newcastle disease virus genotypes isolated in Asia during 2008–2011. *Virus Genes* 45:63–68. <https://doi.org/10.1007/s11262-012-0738-5>
8. Fathi E, Pourbakhsh SA, Jafarian M (2008) Phylogenetic analysis and characterization of Newcastle disease virus by molecular methods in Chaharmahal-e-Bakhtiari province, Iran. *Vet J* 5:50–55.
9. Garten W, Berk W, Nagai Y, Rott R, Klenk HD (1980) Mutational changes of the protease susceptibility of glycoprotein F on Newcastle disease virus: effects of pathogenicity. *J Gen Virol* 50:135–147. <https://doi.org/10.1099/0022-1317-50-1-135>
10. Ghalyanchilangeroudi A, Hosseini H, Jabbarifakhr M, Fallah Mehrabadi MH, Najafi H, Ghafouri SA, Mousavi FS, Ziafati Z, Modiri A (2018) Emergence of a virulent genotype VIII of Newcastle disease virus in Iran. *Avian Pathology* 47:509–519. <https://doi.org/10.1080/03079457.2018.1495313>
11. Ghalyanchi Langroudi A, Hosseini H, Karimi V, Madadgar O, Hashemzadeh M, Ghafouri SA, Bagheri Ghadikolaei SS, Vahedi SM (2014) Phylogenetic study based on the phosphoprotein gene of Iranian Newcastle disease viruses (NDV) isolates,

2010–2012. *Iranian Journal of Veterinary Medicine* 8:73–77. <https://doi.org/10.22059/IJVM.2014.51403>

12. Haji-Abdolvahab H, Ghalyanchilangeroudi A, Bahonar A, Ghafouri SA, Vasfi Marandi M, Fallah Mehrabadi MH, Tehrani F (2019) Prevalence of avian influenza, Newcastle disease, and infectious bronchitis viruses in broiler flocks infected with multifactorial respiratory disease in Iran, 2015–2016. *Tropical Animal Health and Production* 51:689–695. <https://doi.org/10.1007/s11250-018-1743-z>
13. Hanson RP(1980) Newcastle disease. In: Hitchner SB, Domermuth CH, Purchase HG, Williams JE (eds) *Isolation and Identification of Avian Pathogens*, 2nd edn. American Association Avian Pathologists, New York, TX, pp 63–66.
14. Jabbarifakhar M, Mousavi FS, Ziafati Z, Rezaee H, Hosseini H, Fallah MH, Esmaealzade Dizaji R, Ghalyanchilangeroudi A (2018) Emergence of genotype VIIg of velogenic Newcastle disease virus in Iran, 2018: The first report. *Iranian Journal of Virology* 12:40–46.
15. Kaleta EF, Baldauf C (1988) Newcastle Disease in Free-Living and Pet Birds. In: Alexander DJ (ed) *Newcastle Disease*, 1st edn. Kluwer Academic Publishers, Boston, MA, pp 197–246.
16. Kant A, Koch G, Van Roozelaar DJ, Blak F, Ter Huurne A (1997) Differentiation of virulent and non-virulent strains of Newcastle disease virus within 24 hours by polymerase chain reaction. *Avian Pathology*, 26:837–849.
17. Kianizadeh M, Aini I, Omar AR, Yusoff K, Sahrabadi M, Kargar R (2002) Sequence and phylogenetic analysis of the fusion protein cleavage site of Newcastle disease virus field isolates from Iran. *Acta Virol* 46:247–251.
18. Lamb R, Parks GD (2007) Paramyxoviridae: The viruses and their replication. In: Fields B, Knipe DM, Howley PM (eds) *Field virology*, 5th edn. Lippincott, Williams, and Wilkins, Philadelphia, pp 1449–1496.
19. Mayahi V, Esmaeliazad M (2017) Molecular evolution and epidemiological links study of Newcastle disease virus isolates from 1995 to 2016 in Iran. *Arch Virol* 162:3727–3743. <https://doi.org/10.1007/s00705-017-3536-5>
20. Mayahi M, Seyfi Abad Shapouri MR, Jafari RA, Mohammadian Ghaleh Joughi B (2015) Isolation and identification of Newcastle disease agent from broiler farms in Khuzestan province. *Journal of Veterinary Microbiology* 11:101–108.
21. Mehrabanpour MJ, Khoobyar S, Rahimian A, Nazari MB, Keshtkar MR (2014) Phylogenetic characterization of the fusion genes of the Newcastle disease viruses isolated in Fars province poultry farms during 2009–2011. *Vet Res Forum* 5:187–191.
22. Miller PJ, Haddas R, Simanov L, Lublin A, Rehmani SF, Wajid A, Bibi T, Khan TA, Yaqub T, Setiyaningsih S, Afonso CL (2015) Identification of new sub-genotypes of virulent Newcastle disease virus with potential panzootic features. *Genetics and Evolution* 29:216–229. <https://doi.org/10.1016/j.meegid.2014.10.032>
23. Miller PJ, King DJ, Afonso CL, Suarez DL (2007) Antigenic differences among Newcastle disease virus strains of different genotypes used in vaccine formulation affect viral shedding after avirulent challenge. *Vaccine* 25:7238–7246. <https://doi.org/10.1016/j.vaccine.2007.07.017>
24. Munir M, Cortey M, Abbas M, Qureshi ZU, Afzal F, Shabbir MZ, Khan MT, Ahmed S, Ahmad S, Baule C, Stahl K, Zohari S, Berg M (2012) Biological characterization and phylogenetic analysis of a novel genetic group of Newcastle disease virus isolated from outbreaks in commercial poultry and from backyard poultry flocks in Pakistan. *Infect Genet Evol* 12:1010–1019. <https://doi.org/10.1016/j.meegid.2012.02.015>
25. Nagai Y, Klenk HD (1977) Activation of precursors to both glycoproteins of Newcastle disease virus by proteolytic cleavage. *Virology* 77:125–134. [https://doi.org/10.1016/0042-6822\(77\)90412-3](https://doi.org/10.1016/0042-6822(77)90412-3)

26. Panda A, Huang ZH, Elankumaran S, Rockemann DD, Samal SK (2004) Role of fusion protein cleavage site in the virulence of Newcastle disease virus. *Microb Pathogenesis* 36:1–10. <https://doi.org/10.1016/j.micpath.2003.07.003>
27. Qiu X, Sun Q, Wu S, Dong L, Hu S, Meng C, Wu Y, Lui X (2011) Entire genome sequence analysis of genotype IX Newcastle disease viruses reveals their early-genotype phylogenetic position and recent-genotype genome size. *Virology Journal* 8:Article 117. <https://doi.org/10.1186/1743-422X-8-117>
28. Rahimian MD, Zamani Moghaddam A, Momtaz H, Niazi MH (2011) Detection and phylogenetic analysis of Newcastle disease virus based on molecular techniques in broiler in Isfahan province. *Journal of Veterinary Microbiology* 7:25–36.
29. Rostamali T, Shoostari AH, Charkhkar S, Bozorgmehri Fard MH (2014) Phylogenetic analysis of F gene of Newcastle virus isolated in Iran during 2010 and 2011. *Journal of Comparative Pathobiology* 10:1065–1070.
30. Samadi S, Kianizadeh M, Fathi Najafi M, Mousavi Nasab SD, Hosseinnia Davatgar AM, Royaee A, Pilvar P (2014) Molecular characterization and phylogenetic study of velogenic Newcastle disease virus isolates in Iran. *Virus genes* 48:290–295. <https://doi.org/10.1007/s11262-013-1015-y>
31. Snoeck CJ, Owoade AA, Hyman EC, Alkali BR, Okwen MP, Adeyanju AT, Komoyo GF, Nakoun E, Le Faou A, Muller CP (2013) High genetic diversity of Newcastle disease virus in poultry in West and Central Africa: cocirculation of genotype XIV and newly defined genotypes XVII and XVIII. *J Clin Microbiol* 51:2250–2260. <https://doi.org/10.1128/JCM.00684-13>
32. Suarez DL, Miller PJ, Koch G, Mundt E, Rautenschlein S (2019) Newcastle Disease, Other Avian Paramyxoviruses, and Avian Metapneumovirus Infections. In: Swayne DE, Boulianne M, Logue CM, McDougald LR, Nair V, Suarez DL, Wit S, Grimes T, Johnson D, Kromm M, Prajito TY, Rubinoff L, Zavala G (eds) *Diseases of Poultry*, 14th edn. Wiley Blackwell, New York, pp 109–166.

Figures

Figure 1

The phylogenetic tree based on the partial F gene nucleotide sequence of isolates obtained from this study. Isolates from this study are marked with *. Common live vaccines are marked with red lines. The distance between the branches indicates similarity/dissimilarity

110	cleavage site	120	
	↓		
T S G G G R Q G R L V G			DE-R49 99] class I
T S G G G K Q G R L I G			CN/Ulster/67] I
T S G G R R Q R R F I G			AU-1252/98] I
T S G G G R Q G R L I G			LaSota/46] II
T S G G G R Q G R L I G			B1/46] II
T S G G R R Q R R F I G			Mukteswar] III
T S G G R R Q R R F I G			JS/7/05/Ch] III
T S G G R R Q R R F I G			<u>F48E8</u>] IX
T S G G R R Q R R F I G			<u>ZJ/1/86/Ch</u>] IX
T S G G R R Q R R F I G			<u>FJ/1/85/Ch</u>] IX
T S G G R R Q R R F I G			<u>JS/1/97/Ch</u>] IX
T S G G R R Q R R F I G			<u>JS/1/02/Du</u>] IX
T S R G R R Q R R F I G			Herts/33] IV
T S G G R R Q R R F I G			Italien] IV
T S G G R R Q K R F V G			US(FL)/Largo/71] V
T S G G R R Q K R F V G			US(CA)/211472/02] V
T S G G R R Q K R F I G			US(CA)/1083Fontana/72] VI
T S G G G R Q K R F I G			IT-227/82] VI
T S G G R R Q K R F I G			CN/ZJ-1/00] VII
T S G G R R Q K R F I G			NA-1] VII
T S G G R R Q K R F I G			AF2240] VIII

Figure 2

The amino acid sequence of the F protein cleavage site (class I and II genotypes) [27].