Original Article

Investigation of Newcastle Disease Virus Infection in Pet Birds in the Southwest of Iran

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ABSTRACT

Newcastle disease virus (NDV) is a serious threat to the international poultry industry. Therefore, to determine the role of pet birds (Psittaciformes and Passeriformes) in its spread and epidemiology, the presence of this virus in these birds was investigated. In this study, fecal and cloaca swabs from 63 Psittaciformes and 37 Passeriformes, along with tissue samples of dead birds, including proventriculus, trachea, lungs, and intestine, were collected from breeding and sales markets as well as the birds referred to Faculty of Veterinary Medicine, Shahid Chamran University of Ahvaz, Ahvaz, Iran. Isolation of the virus was performed by injecting the suspension of the samples into the allantoic fluid of fertilized eggs, and NDV was detected in the achieved allantoic fluids by reverse transcription polymerase chain reaction. The NDV was detected in 13 allantoic samples. The partial F gene sequences of 10 positive samples were investigated, and their genetic relationship with each other as well as with other isolates in the gene bank was marked. Consequently, subgenotype VII.1.1 (VIId) was in the locus of all 10 viruses. By the amino acid cleavage site sequences of F protein, 10 isolates were determined as velogenic NDV. Moreover, all sequences were similar to each other and other Iranian isolates. Furthermore, the 112RRQKR/F117 pattern was the main amino acid (aa) sequence in the F-protein Cleavage site for VIId genotype isolates.

Keywords: F-protein, (VIId) genotype, RT-PCR, Psittaciforme, Passeriformes

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1. Introduction

One of the most significant avian disease viruses in the world is the Newcastle disease virus (NDV), which seriously affects poultry production (1). The disease has caused significant economic damage since its first formal report in 1926 in Newcastle city, through numerous epidemics with high mortality and prevalence rates. The structural proteins of NDV consist of nucleocapsid protein (N), phosphoprotein (P), matrix (M), fusion (F), hemagglutinin-neuraminidase (HN), RNA-dependent RNA polymerase (RNAP), and large polymerase (L) (2).

The NDVs are divided into four main pathotypes, namely velogenic, mesogenic, lentogenic, and asymptomatic enteric. Moreover, velogenic strains are divided into neurotropic and viscerotropic forms according to the main clinical signs (2). According to the F gene sequences, the strains of Avian paramyxovirus 1 are divided into two categories I and II. Low virulence viruses (isolated from wild birds) are in category I and have so far been divided into nine genotypes (genotypes 1-9). There are several genotypes of low virulence and most of the virulence NDV isolates are in Category II, which are isolated from domestic and wild birds. It should be mentioned that this category includes at least 18 genotypes (1 to 18) (3). The F and HN genes are the most important factors in the severity of Newcastle disease (4). The F Proteins (precursors) are broken down to F1 and F2 by cellular proteases (5). Different viruses have different amino acid (aa) sequences at the cleavage sites, and the variety of amino acids at this site determines the type of protease-to-fission F Protein (5).

In low virulent isolates, only trypsin-like proteases can break down the F0 protein into F1 and F2 in the extracellular respiratory and intestinal tissues, while in very virulent viruses, the furin-like proteases cleave the F0 protein that is available in the respiratory tract tissue cells (6). The main factor behind systemic replications and severe diseases caused by the acute virus is the variation of protease activation (1).

To successfully control ND, it is important to identify the factors that contribute to its localization. The spread of NDV among poultry species and wild birds has been previously reported (2). Pet birds have also been infected with acute strains of NDV (7). Sanitary control and quarantine of imported birds are important to inhibit the entrance of pathogenic viruses into industrial poultry farms as well as the spread of the Newcastle virus in pet bird rearing and sales centers, considering that no specific treatment for this disease has been known so far (2). As a result, it is important to diagnose and analyze NDV in poultry farms and bird sale centers for the epidemiology and more effective control of this disease. In this research, the NDV was isolated and identified by the reverse transcription polymerase chain reaction (RT-PCR) method based on the F gene protein.

2. Materials and Methods

2.1. Sampling

Fecal and cloaca samples were collected from 63 species of Psittaciformes and 37 samples of Passeriformes, along with tissue samples isolated from dead birds, including the proventriculus, trachea, lungs, and intestine samples. These samples were collected from rearing and sales markets, as well as cases that referred to Shahid Chamran Faculty of Veterinary Medicine in Ahvaz, Iran from June 2021 to March 2022. The recorded history, including the hygiene of the farm and the cage, communication or lack of communication with other birds, and information about the newly purchased birds was obtained. Moreover, the exact record of any diseases, like Newcastle disease, and analysis of the necropsy of the dead birds were collected (Table 1).

Table1. Pet bird species, Number of positive results

species	Number of samples	Positive results
Cockatiels	20	7
Grey parrots	20	3
Finches	20	1
Canaries	17	1
Different species of parrots	12	0

2.2. Virus Isolation

Tissue samples and swabs were individually crushed and mixed in normal saline containing antibiotics (for inhibition of bacterial and fungal infections). The samples were centrifuged at 1,000 g for 10 min and then the supernatants were collected. After 48 h, 200 µl of the samples were injected into the allantoic fluid of 9-day-old fertilized eggs. It should be noted that harvested allantoic fluids were examined by RT-PCR.

2.3. RNA Extraction

The RNX-plus solution (CinnaGen, Iran) was used for RNA extraction according to the instructions (Sinagen, Iran).

2.4. cDNA Synthesis

YektaTajhizAzma (Iran) kit was used to make cDNA using a random hexamer as a general primer.

2.5. Polymerase Chain Reaction Assay

The volume and concentration of substances participating in the test were: 10 µl of Master Mix 2X with 1.5 mM MgCl2 (Amplicon, Canada), primers of F gene (10 pmol/µl), ndvf (TTG ATG GCA GGC CTC TTG C), and ndvr (GGA GGA TGT TGG CAG CAT T) [5], 3 µl of cDNA, and distilled water up to 20 µl. The steps with regard to temperature were 3 min at 95 °C (early denaturation) and 40 cycles, including 30 s at 95 °C (denaturation), 60 s at 55 °C (annealing), and 60 s at 72 °C (elongation), and the final step, 10 min at 72°C (final elongation). In total phases, Diethyl pyrocarbonate water was used as a negative control and RNA of a live vaccine was used as a positive control.

2.6. Polymerase Chain Reaction Product Evaluation

Electrophoresis of the PCR product was performed in 1% agarose gel and safe stain, and the positive samples with specific primers were sent to the Tekapouzist Company for purification and sequencing by the BIONEER Company (South Korea). The obtained sequences were aligned with those found in Iran, neighboring countries and NDV reference isolates at the National Center for Database Information Biotechnology. The phylogenetic relationship and tree were designed by one-click software at HTTP://www.Phylogeny.fr/ and the topological stability of the tree was assessed by 1,000 replications of the bootstrap (Figure 1).

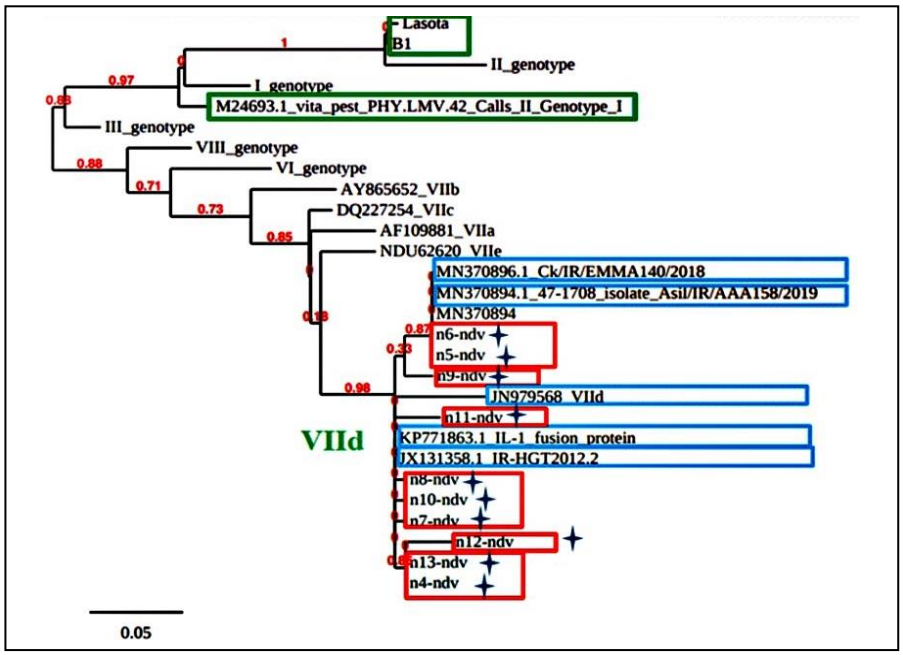

Figure 1. Phylogenetic tree based on the nucleotide sequence of partial F gene of Newcastle disease virus Isolates of this study. (Marked with an asterisk in the image). With other isolates in genbank like common live vaccines (Green boxes), standard genotypes and, isolates of genotype VIId (blue boxes). The branch spacing indicates the measure of sequence differences

3. Results

Based on the results, 13 (13%) out of 100 allantoic fluids were positive by the RT-PCR test as follows: 7 out of 27 Cockatiels birds, 3 out of 24 Grey Parrots birds, 1 out of 23 Finches birds, and 2 out of 17 Canaries birds as well as 12 samples from different species of parrots were completely negative (Table 1). Finally, 13 positive cases were sent for sequencing, but only 10 samples were successfully sequenced (Table 2). These sequences were compared with the sequences of other NDV isolates that were previously identified in Iran and other countries. Moreover, the percentage of nucleotide similarity was also determined. In addition, all the sequences were similar to each other and other previous sequences of Iran.

The results showed that the Nd4 isolate was the most similar to IR-HGT2012.2 and IL-1 (99.63%) and Nd5 isolate was the most similar to CK/EMMA140/2018, CK IR / EMMA121/2018, and Asil/IR/AAA158/2019 (99.69%). Moreover, Nd6 isolate was the most similar to CK/EMMA140/2018 and CK/IR/EMMA121/2018 and Asil/IR/AAA158/2019(99.69%). In addition, Nd7 was the most similar to IR- isolates HGT2012.2 and IL-1 (99.73%) while the Nd8 isolate was the most similar to IR-HGT2012.2 and IL-1 isolates (99.68%). Besides, the Nd9 isolate was the most similar to IR-HGT2012.2 and IL-1 (98.75%) and the Nd10 isolate was the more similar to IR-HGT2012.2 and IL-1 isolates (99.37%). Furthermore, the Nd11 isolate was the most similar to IR-HGT2012.2 and IL-1 isolates (98.9%) and the Nd12 isolate was the most similar to IR-HGT2012.2 and IL-1 isolates (96.55%). Finally, the Nd13 isolate was the most similar to IR-HGT2012.2 and IL-1 isolates (99.67%). According to the phylogenetic tree (Figure 1), all isolates were classified in subgenotype VIId (Figure 1). These isolates were mostly related to the VIId genotype isolates of Iran.

Table 2. The bird's history and their molecular results

Isolated genotype	Isolate number in the gene bank	sample code	Symptoms of necropsy	Clinical signs	Species
VIId	ON007323	Nd4	Tracheal and pulmonary hyperemia	Ataxia , lethargy, paralysis, respiratory discharge	Cockatiel
VIId	ON007322	Nd5	Proventriculus hemorrhage, cerebellar congestion	Paralysis Respiratory secretions	Cockatiel
VIId	ON007326	Nd6	Intestinal hyperemia	Ataxia, lethargy	Cockatiel
VIId	ON007325	Nd7	Intestinal and Tracheal hyperemia	Sudden death	Cockatiel
VIId	ON007324	Nd8	Tracheal and pulmonary hyperemia	Anorexia and Ataxia	Cockatiel
-	-	-		-	Cockatiel
-	-	-		-	Cockatiel
VIId	ON007321	Nd9	No Clinical signs	No Clinical signs	Grey parrot
VIId	ON007320	Nd10	No Clinical signs	No Clinical signs	Grey parrot
VIId	ON007327	Nd11	No Clinical signs	No Clinical signs	Grey parrot
VIId	ON007319	Nd12	No Clinical signs	No Clinical signs	Finch
VIId	ON007318	Nd13	Lung and tracheal hyperemia	Ataxia	Finch
-	-	-	Lung and tracheal hyperemia	Ataxia	Finch

3.1. Amino Acid Sequence

The cleavage sites amino acid sequences of our isolates are summarized in table 3 and their comparison with figure 2 and table 4 showed that Nd4 (ON007323), Nd5 (ON007322), Nd6 (ON007326), Nd7 (ON007325), Nd10 (ON007320), and Nd13 (ON007318) were similar to Essex70, Beaudette, Kvuzat-Yavne/50-826, Niger/1377-7/06, US (CA)/1083Fontana/72, CN/ZJ-1/00, NA-1, and AF2240 that are velogenic isolates, respectively. However, Nd8 (ON007324), Nd9 (ON007321), Nd11 (ON007327), and Nd12 (ON007319) were dissimilar to Essex70, Beaudette, Kvuzat-Yavne/50-826, Niger/1377-7/06, US (CA)/1083Fontana/72, CN / ZJ-1/00, NA-1, and AF2240.

Table 3. The amino acid sequences of the F protein cleavage site in this study

Sample no.	Amino acid seq.
Nd4	T S G G R R Q K R F I G
Nd5	T S G G R R Q K R F I G
Nd6	T S G G R R Q K R F I G
Nd7	T S G G R R Q K R F I G
Nd8	T S G G R R L K R F I G
Nd9	S T S G G R R Q R L I G
Nd10	T S G G R R Q K R F I G
Nd11	T S G R R R Q K R F I G
Nd12	T S G G G R R Q R F I G
Nd13	T S G G R R Q K R F I G

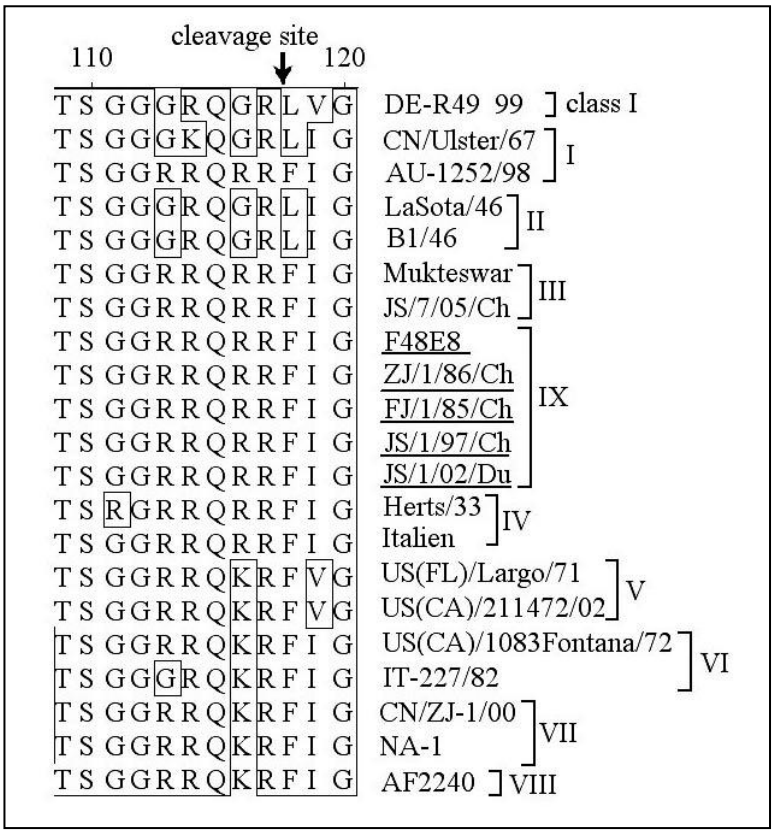

Figure 2. The amino acid sequence of the F protein cleavage site (class I and II genotypes) (8)

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

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	[c]	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	[c]	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

a Virulence for chickens
b ↓ = cleavage point. Basic amino acids in bold. Note that all virulent viruses have phenylalanine (F) at position 117 (the F1 N terminus)
c Unknown ICPI

4. Discussion

Despite widespread vaccination, Newcastle disease is endemic and a major problem all over Asia, Africa, and Central America. The presence of wild viruses in commercially vaccinated birds (9) and their continuous evolution over time are other factors that lead to the spread of this disease (10).

The existence of a high environmental viral load causes the proliferation of wild strains of Newcastle virus in the environment. To successfully control this disease, it is essential to identify the factors that contribute to its locations. Bilateral transmission of Newcastle disease between poultry and wild bird species has been previously reported (11). Exotic and pet birds can be infected with wild ND strains (2) and are considered a threat to the biosecurity of domestic and commercial poultry. The genetic correlation between circulating viruses in different bird species and the rearing systems is important since it may contribute to Newcastle disease endemicity. This study is one of the few studies that isolated and identified avian paramyxovirus 1 in pet birds (Psittaciformes and Passeriformes). A phylogenetic comparison of the results to the strains and isolates that existed in Iran and other countries was performed in this study. Furthermore, it should be noted that this study was one of the few studies performed in this regard in Iran.

In this study, 13 out of 100 samples were positive, which were sent for sequencing. The sequencing of 10 samples was successful. These isolates were in subgenotype VIId. The large cluster of NDV class II with great genomic variation is genotype VII. These viruses first appeared in East Asia and then spread to other continents. Almost all strains of this genotype are pathogenic based on the amino acid sequence of F protein and most of them have been identified from

unexpected outbreaks with high mortality rates in Eastern Europe and Asia. The prevalence of VII is also reported in Africa and South America (9, 12). Genotype VII is divided into VIIh, VIIg, VIIf, VIIe, VIId, VIIb, and VIIi. This virus is related to the fourth ND panzootic, which spread to East Asia in 1985 and since then has spread to Asia, Europe, Africa, and South America (13).

Madadgar, Karimi (14) investigated the occurrence of NDV contamination and its pathogenicity in caged birds. They collected 335 species of fecal samples and cloaca swabs from 2009 to 2010 in Tehran, Iran. In the aforementioned study, 38 samples were positive for Newcastle disease, and 27 out of these 38 samples were diagnosed with the acute type of Newcastle virus. Finally, five positive samples were sent for sequencing. All five samples were very similar to the NDV in the gene bank but their sequences did not show enough identity to Iranian isolates. However, in general, they looked like viruses with genetic differences that have been circulated in the environment of Iran for many years by migrants, wild birds, domestic poultry, and captive fowls (14).

Martins, Paulillo (15) investigated the presence of NDV and the role of lovebirds in the epidemiological program in terms of their potential source of NDV contamination after a challenge of Newcastle viscerotropic strain (intracerebral pathogenicity index [ICPI]=1.78) in these birds via oculonasal route. Moreover, these birds were placed near specified pathogen-free (SPF) chicks, and cloacal swabs were collected from these two species. The Newcastle virus genome was detectable by RT-PCR at 14, 9, and 21 days after the challenge. Moreover, 100% of SPF chicks placed next to infected lovebirds had clinical signs and injuries, while lovebirds showed no clinical signs of Newcastle disease (15).

Leow, Shohaimi (16) detected NDVs in imported birds from Malaysian quarantine unite (cloaca and tracheal swabs) by RT-PCR. They found different wild NDV genotypes from different origins in imported birds and isolated viruses belonging to genotypes VIa and VIIi (16). In 2014, Abdelrahim and Elhag collected Newcastle virus from lovebirds with depressive symptoms, cyanosis of the crown, diarrhea, and fever, which were kept near a poultry farm. Tracheal, lung, and intestinal tissue samples were collected to isolate the NDV.

Samples were inoculated into 10-day-old embryonic eggs. The NDV was detected in the allantoic fluid using hemagglutination and inhibition of hemagglutination tests with specific antiserum. Pathogenic evaluation of isolates was performed based on ICPI and mean death time (MDT) tests and the virus was classified as a lentogenic pathotype. They also showed that these birds could carry the Newcastle virus, and highlighted the epidemiological importance of these birds as a potential source of Newcastle virus infection (17).

In 1975, during an outbreak of velogenic strains of Newcastle disease in California, Pearson and McCann (18) investigated the role of wild and domestic bird species in the spread of the virus in quarantine areas. Very virulent NDv was isolated from 0.04% of 9446 free-range, 0.76% of 4367 semi-domesticated, and 1.01% of 3780 exotic birds (18). Panigrahy, Senne (19) studied the outbreak of Newcastle viscerotropic velogenic disease in parrots in six states of the USA, namely Illinois, Indiana, Michigan, Texas, California, and Nevada. The velogenic virus was isolated from the cloaca, tracheal swap, and various tissues of the lung, trachea, end of the intestine, and spleen organ. The virus was identified as a velogenic strain based on MDT tests. The virus was isolated from quarantined birds that were intended to be imported into the United States. Furthermore, it has been reported that birds (exotic birds) enter the country illegally; therefore, they are not tested pathologically before and are potential sources of viscerotropic velogenic NDV, which could be a serious threat to domestic poultry and other pet birds (cage birds) (19). To discover the possible source of NDV in domestic poultry, Boroomand, Jafari (20)

investigated the presence of NDVs in migratory birds from southern Iran with hemagglutination and RT-PCR tests. The virus was detected in about 5% of these birds. In addition, the genetic diversity of the virus in Iran has been reported in most of the studies performed in this country (20).

Effective sources in the transmission and spread of NDV include movements of live birds (wild, domestic, and game birds), communication with other animals, transfer of poultry equipment and products, and polluted water, air, vaccines, and poultry feed (2).

Based on the sequence of the amino acid (aa) cleavage site (^{112}RRQKR/F^{117}), our isolates belonged to subgenotype VIId. Moreover, the sequence obtained from this research confirmed the existence of phenylalanine at situation 117 of subgenotype VIId isolates. In general, the condition for the NDV to pass in the host cell is to break down the F glycoprotein into F1 and F2 (3). The host proteases usually break down the cleavage sites, and the susceptibility of F protein to break down and be influenced by the aa sequence in cleavage sites. There is a monobasic aa sequence at the C-terminus of the F2 protein of low-virulence NDV and leucine at the N-terminal of the F1 protein. In virulence NDVs, the C terminal of the F2 protein contains a polybasic aa sequence and the N terminal of F1 contains phenylalanine (3).

The authors of the present research reviewed the comparison between the sequences found in this study and those found in other studies in this field. Kianizadeh, Aini (21) investigated nine NDV isolates from diverse parts of Iran and Samadi, Kianizadeh (22) studied six velogenic isolates of NDV from six different topographical areas of Iran, which were in genotype VII with ^{112}RRQRR/F^{117} pattern of amino acid (aa) sequence of F protein. Rehmani, Wajid (7) also presented an equal sequence to the above-mentioned results in Iran.

Virulent NDV isolates (subgenotype VIIb) according to sequencing amino acid F0 cleavage sites by Ahmadi, Pourbakhsh (23) and the obtained velogenic NDV from the study done by Boroomand, Jafari (20) in Ahvaz which both studies followed ^{112}RRQKR/F^{117} pattern. Ghiamirad, Pourbakhsh (24) detected the subacute and newfound genotype VIIi similarly following ^{112}RRQKR/F^{117} pattern in the mentioned region.

In 2021, Makki, Boroomand (8) examined the characteristics of NDV strains in 20 broiler flocks that were suspected of Newcastle disease with respirational syndrome in Khuzestan and Isfahan provinces, Iran, using RT-PCR. In total, 9 flocks in the 20 studied farms were NDV-positive. Three of the nine isolates were in genotype II and six were in genotype VIId. Moreover, there were isolates of both genotypes in both provinces. The review of the cleavage site amino acids of their isolates showed ^{112}RRQKR/F^{117} sequence for isolates under VIId genotype in Khuzestan province and ^{112}RKQKR/F^{117} sequence for isolates of this subgenotype in Isfahan province (8).

Boroomand, Jafari (20) in their research determined the molecular identity and studied the phylogeny of the F gene NDV in Ahvaz, Iran. Three viral isolates were obtained from their sampling which were isolated from commercial and immunized broiler chickens with respirational signs that were classified in subgenotype VIId of this virus (20). In a study performed by Rahimian, Zamani (25), the NDV was detected and identified in a broiler flock in Isfahan province, Iran. They documented the genome identity of the F genes of Iranian isolates and matched it to other countries. It should be noted that the percentage of genetic diversity was 1.4-27.3% (25).

The present study showed that there is a dissimilar variety of pet birds with predispositions to NDV. These pets are able to be the significant cause of NDV spread in Iran. Moreover, the two isolated Nd4 and Nd13 were completely similar. Due to the annual occurrence of this disease and the mortality which occurs in domestic and commercial poultry farms, it is important to identify the factors that contribute to the endemic of this disease. Moreover, molecular identification of NDVs in rearing farms, sales centers of pet birds, and imported birds is very valuable as it can be helpful in epidemiological studies and in dealing with the spread of the disease.

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Authors' Contribution

Study concept and design: E. K.
Acquisition of data: E. K.
Analysis and interpretation of data: Z. B.
Drafting of the manuscript: M. M.
Critical revision of the manuscript for important intellectual content: Z. B.
Statistical analysis: M. R. S. A. S.
Administrative, technical, and material support: E. K.

Ethics

We hereby declare all ethical standards have been respected in preparation of the submitted article.

Conflict of Interest

The authors declare that they have no conflict of interest.

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