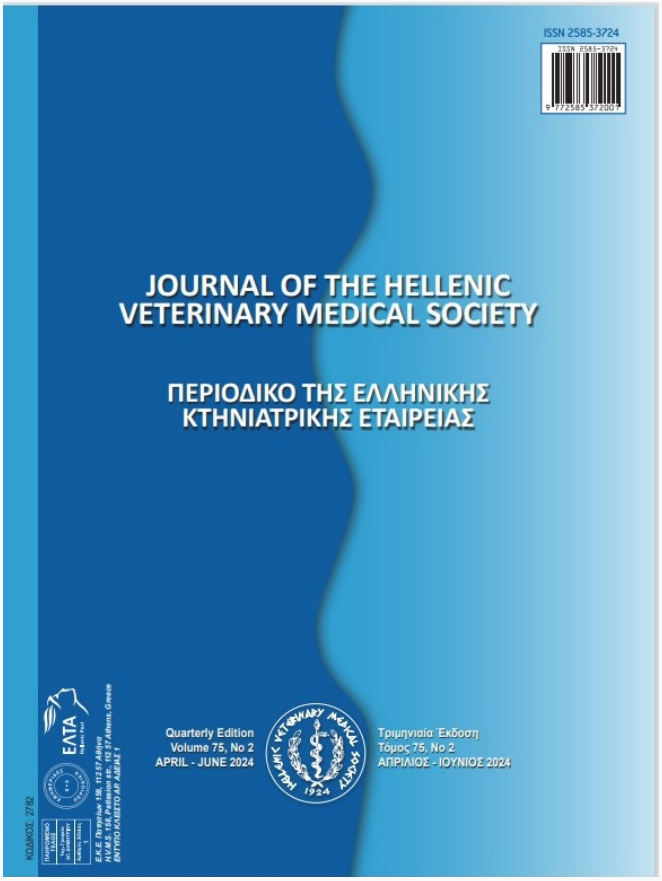
Journal of the Hellenic Veterinary Medical Society

Vol 75, No 2 (2024)



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doi: 10.12681/jhvms.34415

To cite this article:

Soltani, M., Shoushtari, A., Molouki, A., Babaeimarzangou, S., Abdoshah, M., Bashashati, M., & Fallah Mehrabadi, M. (2024). The simultaneous presence of three different Newcastle Disease Virus Genotypes in various bird species in a public bird garden. *Journal of the Hellenic Veterinary Medical Society*, *75*(2), 7351–7360. https://doi.org/10.12681/jhvms.34415

J HELLENIC VET MED SOC 2024, 75 (2): 7351-7360
ΠΕΚΕ 2024, 75 (2): 7351-7360

Research article
Ερευνητικό άρθρο

The simultaneous presence of three different Newcastle Disease Virus Genotypes in various bird species in a public bird garden

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ABSTRACT: Newcastle disease (ND) is one of the most important contagious bird diseases. Not only it continuously causes significant economic damage to the commercial poultry industry, but also it is of clinical importance as it occurs in a wide range of free-living and captive birds. Although different types of commercial vaccines are extensively used, the disease has not been eradicated. Between October 2018 and March 2019, three different outbreaks of ND were observed in three different captive bird species of pigeons, peafowls, and pheasants at the Saei public bird garden in Tehran. Genetic identification of viral isolates of AMMM116 (pheasant), AMMM122 (peafowl), and AMMM160 (pigeon) indicated the presence of genotypes VII.1.1, II, and VI.2.1.2, respectively. The virus isolated from the pheasants was closely related to the VII.1.1 subgenotypes obtained from commercial poultry farms in different parts of Iran. Moreover, the VI.2.1.2 isolate obtained from the ND outbreak in pigeons was related to the isolates obtained from pigeons in Nigeria and Kenya. Lastly, we isolated a genotype II NDV, identical to the common vaccine strains, from immature peacocks that had an acute death. The peacocks were not vaccinated, therefore, we speculated that the presence of genotype II could be because of the spillover of vaccine strains from commercial poultry flocks to wild birds that had visited the park and landed in the roofless aviary. The results of this study indicate the simultaneous circulation of different NDV genotypes in a small geographic location and emphasize the importance of imposing more restricted biosecurity measures.

Keywords: *Newcastle disease virus; Genotype; Diversity; Avian; Bird Garden; Tehran*

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Date of initial submission: 06-05-2023
Date of acceptance: 09-10-2023

INTRODUCTION

Virulent Newcastle disease virus (NDV), the causative agent of Newcastle disease (ND), is a severe and often fatal viral agent in naïve chickens. The virus is a threat to commercial and rural poultry industries worldwide (Dimitrov et al. 2016). NDV, previously known as avian paramyxovirus 1 (APMV-1), is currently classified as a species of Avian orthoavulavirus 1. In fact, all types of avian paramyxoviruses were substituted by Avian orthoavulavirus types 1 to 13 (Dimitrov et al. 2016). Based on the intensity of pathogenicity, NDVs are divided into five groups namely asymptomatic, lentogenic, mesogenic, neurotropic velogenic, and viscerotropic velogenic (Miller, Decanini, & Afonso 2010). Intracerebral pathogenicity index (ICPI) in day-old chickens is one of the methods used to characterize the virulence of NDV; briefly, isolates with indices 0.7 to 2.0 are considered virulent. Infections caused by NDV of low virulence will not lead to evident clinical signs. However, in the presence of other secondary factors such as co-infecting viruses or suboptimal environmental conditions, they can still lead to respiratory clinical disease (Miller et al. 2007).

Structurally, NDV is made from six constructional proteins, made from six major genes (3'-NP-P-M-F-HN-L-5'). Presence of multiple basic amino acids of [(112)(R/K)-R-Q-(R/K)-R↓F-I(118)] at the cleavage site of the F protein of NDV indicates a possible virulent virus (Diel et al. 2012).

Despite extensive vaccine application, endemic ND is a prominent problem across many parts of the world, including Asia, Africa, and Central America (Dimitrov et al. 2016). NDV has been isolated from 236 species of birds, but it is believed that all species of birds can be infected with the virus. It should also be noted that the severity of clinical signs and the required dose of infection do not always depend on the bird species, as it may depend on the isolate genotype as well (Miller, Decanini, & Afonso 2010). Since the first identification of ND in Iran in the 1950s (Sohrab 1973), numerous reports about the enzootic status of the virus and its economic impact on the commercial poultry business have been published (Allahyari et al. 2022; Babaeimarzangou et al. 2023a; Molouki et al. 2019; Soltani et al. 2019). Similar reports have been published for backyard poultry (Sabouri, Vasfi Marandi, & Bashashati 2018) as well as companion and aviator birds (Babaeimarzangou et al. 2023b; Madadgar et al. 2013). Moreover, the enzootic status can also be seen in the pigeon population of the country as severe clinical signs and high mortalities have been reported for several years, which are the result of a different group known as pigeon-origin NDV, or pigeon paramyxovirus type 1 (PPMV-1) (Molouki et al. 2019; Rezaei Far et al. 2017). Therefore, because of the widespread dispersion of the virus in Iran, it is important to identify other hosts and reservoirs and investigate their role in this vast spread across the country, to be able to mitigate this deadly disease.

In the current study, three different NDV genotypes isolated from three different captive bird species will be pathologically and molecularly characterized. All the birds were captive in their own aviaries and died as the result of three separate outbreaks that occurred at Saei Park located in central Tehran within just 6 months.

MATERIALS AND METHODS

Outbreaks history and sampling

Between October 2018 and March 2019, outbreaks of ND occurred in 3 different bird species, including rock pigeon (Columba livia domestica), Indian peafowl (Pavo cristatus), and golden pheasant (Chrysolophus pictus) in the Saei public bird garden in Tehran, the capital city of Iran. The clinical findings observed in each of these outbreaks were markedly different; in October 2018, in an aviary where three male and five female golden and ring-necked pheasants were confined, two male golden pheasants showed clinical symptoms of fatigue, polyuria, and acid uric in feces that subsequently resulted in their death. The two pheasants were not vaccinated against NDV and were newly added to the aviary. Other unvaccinated pheasants in the aviary were unaffected. Bleedings across the intestine, such as those in the cecal tonsil and proventricular glands, were the major macroscopic findings during the autopsy.

In a different outbreak in December 2018, two premature Indian blue peafowls confined in a roofless aviary died after showing severe NDV-like symptoms such as green diarrhea, neck or wing paralysis, and respiratory signs. These three-month-old peafowls were found unresponsive early in the morning, although they had shown no noticeable signs the day before. In the aviary, there were two other premature peafowls as well as eight mature peafowls, of which 4 were male and 4 were female, but no other bird showed clinical symptoms. Post-mortem analysis did not show any sign of wound or scar from a possible

fight with other birds, and necropsy only showed a slight inflation of liver and kidneys.

Moreover, in late March 2019, 42 of 53 confined pigeons died during a three-day outbreak after showing the first clinical symptoms. Fatigue, depression, polyuria, and inappetence were the signs before death. In the autopsy, only swelling of liver and kidney was recorded as abnormal.

In the park, overall twenty-two avian species from the families of Psittaciformes, Passeriformes, Galliformes, and Columbiformes were housed and displayed in separate aviaries, of which only those for the peafowls and some of the pigeons were roofless. In other words, from the three species showing clinical symptoms only the pheasants were confined in roofed aviaries, and as a result, the peafowls and pigeons were in close contact with free-flying birds that mostly consist of passerines and columbiformes. On the other hand, vaccination of the pigeons and juvenile peafowls against NDV was not conducted before the outbreaks, but the mature peafowls and some of the pheasants were previously injected with inactivated NDV vaccines.

During the autopsy of the dead birds, all the tissue, including the brain, trachea, and lungs, were transferred on ice to the Department of Avian Disease Research and Diagnosis of Razi Vaccine and Serum Research Institute located in Karaj, Alborz, for pathogenicity and molecular analyses.

Virus isolation

All the procedures including preparation, inoculation, and isolation were performed as previously described (Molouki et al. 2019). Briefly, all the homogenized tissues were suspended in the PBS buffer containing antibiotics (streptomycin and penicillin) to make a 20% suspension. In the end, three brain and three trachea and lung suspensions were prepared. After 1 hour of incubation, the supernatant of centrifuged suspensions was inoculated in 9-day-old embryonated SPF eggs. The allantoic fluid was extracted and preliminary tests such as HA and HI, as well as the pathogenicity tests of MDT and ICPI were also conducted as previously described (Molouki et al. 2019) (Table 2). All animal work was also permitted by Razi Animal Ethical Committee and performed under their guideline.

RNA extraction and PCR

Viral RNA was extracted using the High Pure Viral RNA kit (Roche, Germany) and first-strand cDNA was made using RevertAid® (Thermo, USA) according to the manufacturer's protocol. Later, PCR was run using primers specific for the F gene of NDV (Molouki et al. 2019) (Table 1) and Pfu DNA polymerase (Vivantis, Malaysia). Bands with the right size were gel extracted and sequenced using the same PCR primers as well as two additional primers designed only for sequencing (Table 1).

Phylogenetic analysis

Phylogenetic analyses were conducted according to the latest classification system proposed by the international taxonomy consortium for NDV (Dimitrov et al. 2019). For this purpose, the latest datasets prepared by the consortium were downloaded from their GitHub page (Dimitrov et al. 2019). The ORF of F gene sequences of the current study were added to the files and the phylogenetic tree was computed by MEGA X (Kumar et al. 2018) using Maximum Likelihood with a bootstrap of 1000. For this purpose, the General Time Reversible model with a

Table 1 Primers sequences used in this study

Primer	Sequence	Position	PCR product size (bp)
F-Fwd	5'-YTGCTTATAGTTAGTTYACCTGTC-3'	4462-4485	1871 bp
F-Rev	5'-ACCCGTGTATTGCTYTTYGG-3'	6313-6332	1871 bp
F-Fwd-middle	5'-GCAACCAATGAAGCTGTGCATGA-3'	5027-5049	770 bp
F-Rev-middle	5'-ACAGCTTCTCCATAATTTTGCGA-3'	5774-5796	770 bp

Table 2 F glycoprotein cleavage site in 3 studied NDV isolates.

Isolate	Gene Bank Accession No.	F Glycoprotein Cleavage Site	MDT[a]	ICPI[b]
Ph/IR/AMMM116/2018	MT627321	111G-R-R-Q-K-R-F117	41.6	1.91
Pf/IR/AMMM122/2018	MT919259	111G-G-R-Q-G-R-L117	54.4	1.76
Pg/IR/AMMM160/2019	MN422262	111G-R-R-Q-K-R-F117	49.3	1.87

[a]MDT: Mean Death Time, [b]ICPI: Intracerebral pathogenicity index.

Gamma Distributed option was selected according to the criteria by Dimitrov et al. 2019 (Dimitrov et al. 2019). Additionally, evolutionary distances were computed using Maximum Composite Likelihood and a bootstrap of 1000.

RESULTS

The isolates from a pigeon (AMMM160) and pheasant (AMMM116) were isolated from the brain, but the peafowl-derived virus (AMMM122) was isolated from the trachea and lungs. After PCR and sequencing of 1662 nt F gene ORF (see Table 2 for GenBank accession numbers), the respective sequences were aligned in MEGA software and the phylogenetic tree was built (Fig 1) as described above. According to the tree, the isolate AMMM116 grouped with subgenotype VII.1.1 viruses that were previously isolated from the backyard (Sabouri, Vasfi Marandi, & Bashashati 2018) and commercial chickens (Molouki et al. 2019). After this, a separate tree for several Iranian VII.1.1 NDV was built to study their relations (Fig 2). Viruses from free-flying birds, such as a magpie (GenBank: MK659700.1) isolated recently were also seem to group with our isolate. Additionally, the genetic and amino acid distances using the consortium datasets were computed and the maximum and minimum distances with other genotypes/subgenotypes are shown in Table 3. AMMM116 showed the nearest genetic distance with the group of subgenotype VII.1.1 (95.2%), and the farthest distance genotype II (77.1%).

On the other hand, AMMM122 grouped with genotype II viruses that were isolated from many different countries such as India, China, Nigeria, and Colombia (Fig 3). Moreover, the viruses in this group were isolated from different bird species such as poultry, duck, and free-flying birds. This group also included the vaccinal strains like clone IR12 (GenBank: MH247189.1), which is locally produced with a distance of 99.7%. According to Table 3, AMMM122 showed the lowest distance with genotypes XIV at 72.8%.

Isolate AMMM160, on the other hand, showed to be the closest to a few African pigeon-origin isolates (Fig 4) that were recently re-grouped as VI.2.1.2 (Dimitrov et al. 2019). The genetic distance with this group was 94.3% (Table 3). Moreover, the farthest distance for AMMM160 was the genotype XI viruses, with a score of 75.7%. In addition, AMMM160 showed a unique TAA stop codon that is different from the conserved TGA codon of the other two isolates. Also, it should be mentioned that this genotype has never been reported from Iran or Asian countries, and therefore it is the first of its kind.

Furthermore, from all the NDV sequences submitted to GenBank, the isolates Ck/IR/MAM72/2018, HB/China/1/2016/Duck, and Pigeon/Nigeria/NIE07-063/2007 showed the highest similarities to the isolates AMMM116, AMMM122, and AMMM160, respectively (Table 4).

Table 3 Nucleotide and amino acid distances between the three NDV isolates of this study and the different genotypes under class II. The lowest scores are in light grey and the highest in deep grey.

Genotype		G I	G II	G III	G IV	G V	VI.2.1.1.1	VI.2.1.1.2.1	VI.2.1.1.2.2	VI.1	VI.2.2.2	VI.2.2.1	VI.2.1.2	VII.1.1	VII.1.2	VII.2	VIII	IX	X	XI	XII	XIII	XIV	XVI	XVII	XVIII	XIX	XX	XXI
AMMM116	nt	79.6	77.1	81.1	84.3	82.9	83.6	84.6	83	86.3	84.6	84.9	84.3	95.2	93.3	89.2	84.9	81.7	78.4	74.5	86.4	85.7	82.2	81.8	83.8	85.6	80.7	86.9	83.5
	aa	88	85.1	87.1	90.4	90.5	90.3	92.1	91.4	92.2	90.9	90.9	91.5	96	94.6	93.3	90.4	88.2	87.8	83.2	92.1	91.8	89.2	88.2	90.7	92.2	87.4	92.9	89.3
AMMM122	nt	85.2	98.3	84.9	84.7	79.6	78	78.6	77.1	79.8	77.7	77.4	77.8	77.4	78.8	77.1	82.3	86.5	87.3	76.2	75.8	77.4	72.8	79.4	76.2	77.7	76.9	79.9	76.6
	aa	92.1	98.3	88	89.9	86.8	85.7	87.5	86.8	87.8	86.2	85.8	87.3	85.9	87.3	86.9	88.9	90.8	92.2	84.2	86.1	86.6	84.1	86.3	85.3	86.8	85	88	84.8
AMMM160	nt	78.4	77.1	80.3	82.4	83.9	91.5	92	91.2	92	90.7	91	94.3	84.7	86	84.5	87.4	81.1	77.8	75.5	86.1	84.6	82	83.5	83	86	81.6	89.2	86.7
	aa	91.6	88.4	91.2	92.3	93.5	95.9	97.7	97.1	96.8	94.5	95.9	98.2	94	95.3	94.1	94.6	92.8	91.5	85.3	94.1	94.2	92.1	91.5	92.8	93.6	90.5	96.4	93.4

Table 4 The percentage of similarity of the F gene sequence of the three isolates of this study with the closest matches obtained by BLAST and evolutionary distance analysis. In every cell, the left score belongs to the nucleotide distance, and the right score belongs to amino acid distance.

Names	AMMM116	AMMM122	AMMM160
Ck/IR/MAM72/2018	99.7 and 99.8%	*	*
HB/China/1/2016/Duck	*	99.4 and 99.7%	*
Pigeon/Nigeria/NIE07-063/2007	*	*	95.7 and 98.9%

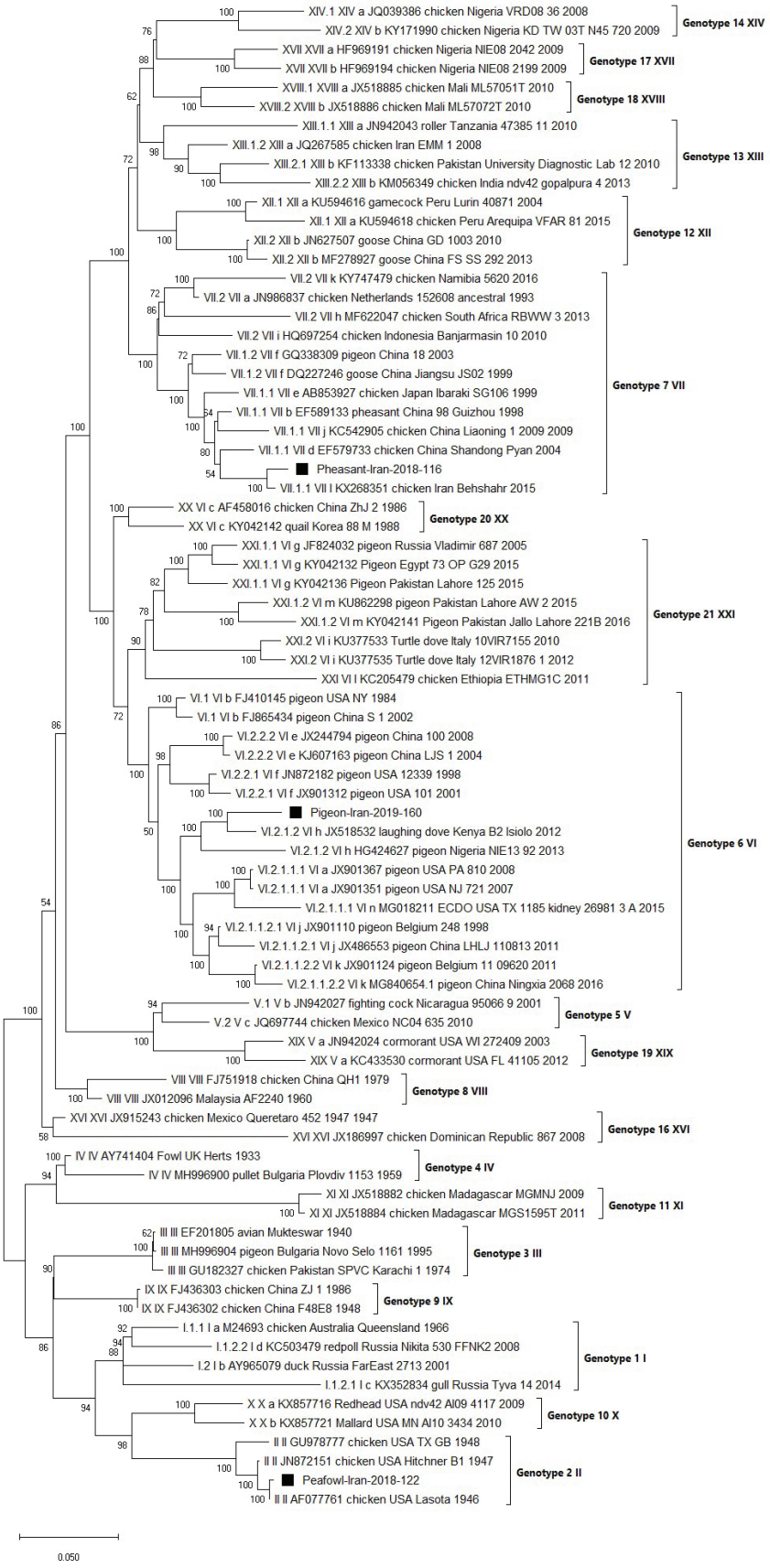

Fig. 1 The phylogenetic tree based on the sequence of the F gene of NDV isolates of this study. The tree was made with a bootstrap of 1000 using several ND isolates derived from GenBank.

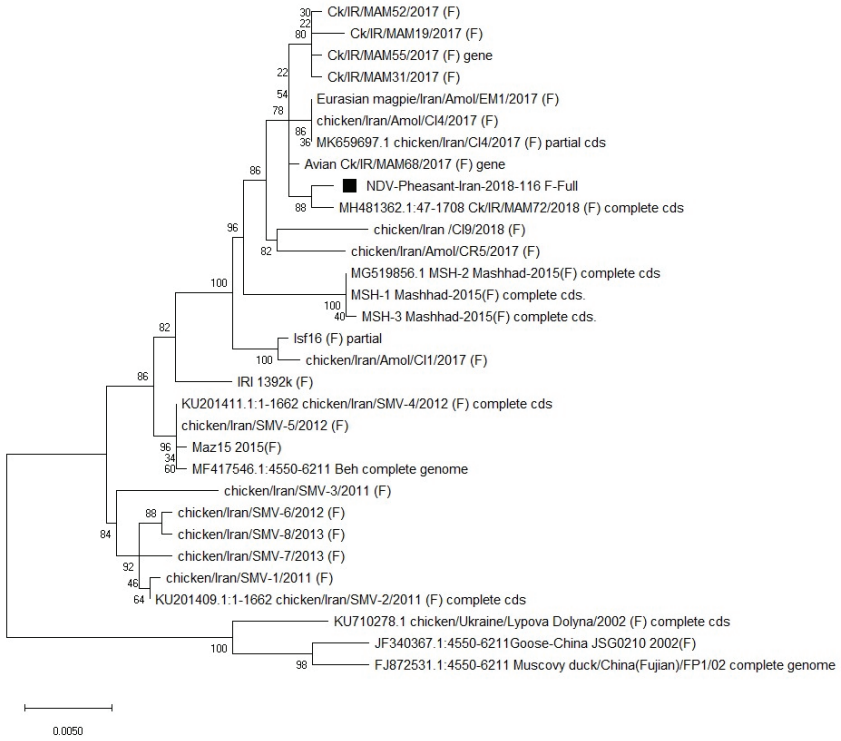

Fig. 2 The F gene of NDV isolate AMMM116 was used in phylogenetic analysis using several Iranian isolates derived from GenBank.

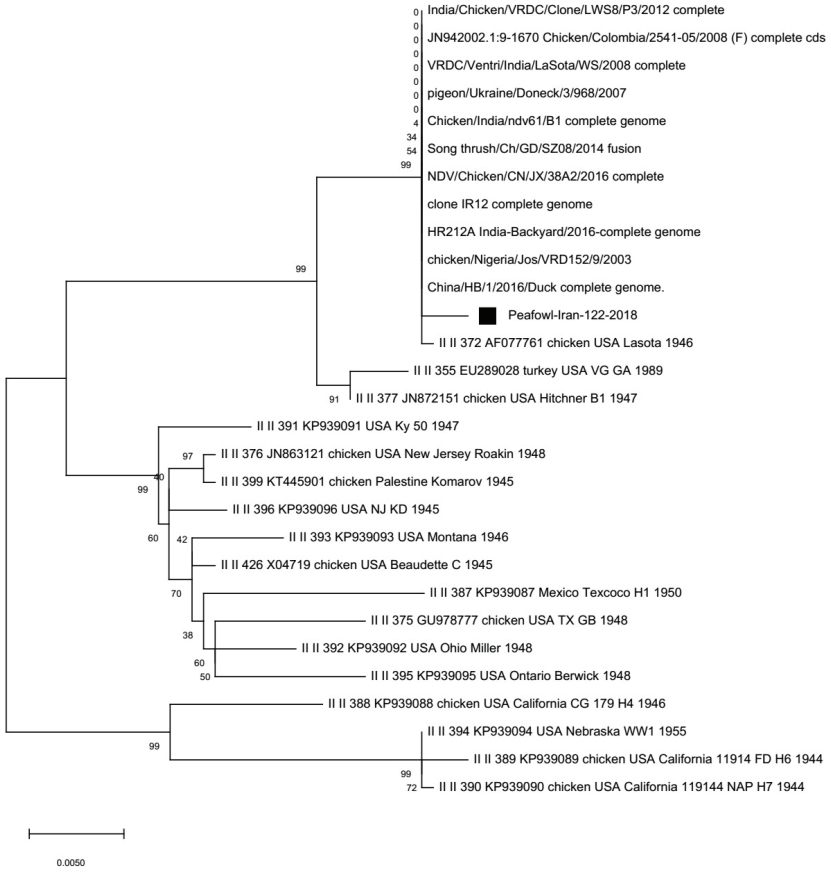

Fig. 3 Phylogenetic tree for NDV isolate AMMM122 along with several genotype II isolates derived from GenBank.

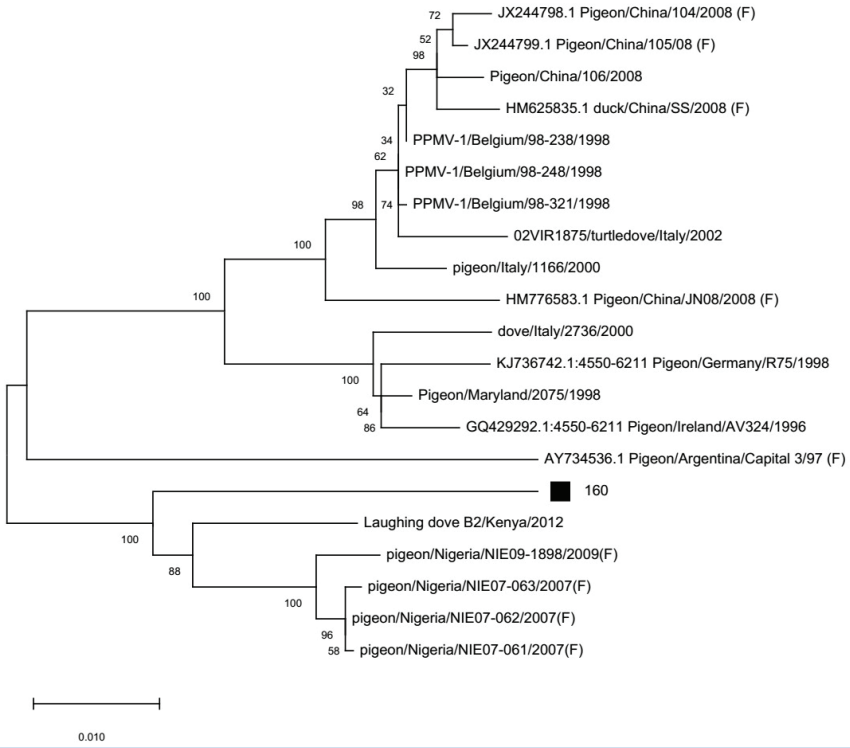

Fig. 4 A phylogenetic tree using the NDV isolate AMMM160 was made along with highest matching isolates derived from BLAST. Several VI.2.1.2 isolates can be seen in the tree.

Moreover, the cleavage sites of the three F gene sequences were compared (Table 2). The polybasic cleavage sequence of AMMM116 and AMMM160 showed that they belong to virulent strains of NDV. On the other hand, the non-poly basic sequence of AMMM122 showed that it might belong to non-virulent strains. Additionally, studying the N-linked glycosylation sites of 85 (NRT), 191 (NNT), 366 (NTS), 447 (NIS), 471 (NNS), and 541 (NNT) (Yusoff and Tan 2001) revealed that, except for position 191 (NKT in AMMM120), the conserved sites existed in all the three F gene sequences. Furthermore, the neutralizing epitopes at locations 72, 74, 75, 78, 79, 157-171 and 343 (Neyt et al. 1989) also showed to be conserved; however, AMMM160 bared amino acid N instead of D at location 170. Moreover, the GC content of the three sequences varied between 44.5 to 45.8%.

DISCUSSION

In the current study, three different NDV genotypes from three different species of birds were isolated from three different outbreaks at the Saei public bird garden in Tehran between autumn 2018 and winter 2019. It has been demonstrated that the genome of viruses must be monitored for all genetic changes (Babaeimarzangou et al. 2023c; Molouki et al. 2022). The result showed that different genotypes are circulating simultaneously in the park and similar events might be occurring in different parts of the country as Iran has been battling NDV for decades.

A similar incident to our report had occurred for captive African penguins and small owls in a zoo in Isreal in 2013 (Haddas et al. 2014). Hadas and colleagues detected NDVs that were very similar to viruses isolated from commercial poultry in the country. Similarly, the zoo birds were not regularly vaccinated against NDV. Interestingly, typical respiratory or neurological symptoms were not observed in some of the birds. In addition, two other small owls that lived next to the infected owls did not show any symptoms. Similar observations were made in our report; the AMMM116 virus isolated from pheasants showed a high similarity to subgenotype VII.1.1 viruses isolated from Iranian commercial poultry. This could mean that this dominant subgenotype (Allahyari et al. 2022) is present in both the Iranian free-flying birds and poultry and can be transmitted between both populations. Infected bird seeds and the use of sewage water for washing the aviary or its surroundings could be reasons for such outbreaks; however, the presence of infected free-flying birds such as crows and sparrows could be another route of infection at

Saei Park. In fact, Khosravi and colleagues have recently submitted to GenBank the F gene sequence of a VII.1.1 virus isolated from a Euroasian magpie (MK659700) (Khosravi, Seifi, & Tazeh 2021). Such identifications are important as many birds might be resistant to the virus, but shed the virus through their feces. In fact, similar observations in some species of sparrow have been reported (Roy et al. 1998). In a different report, Snoeck and colleagues discovered that ducks, turkeys, and guineafowls of a live bird market were more resistant or less sensitive to NDV infection than backyard poultry, and therefore, might have been completely asymptomatic (Snoeck et al. 2013). Although shedding of the virus by these exotic birds could be less likely than poultry, they may strongly be involved in the spread of the virus.

Isolations of NDV genotypes II, VII, and XII from captive peacocks have been reported in many countries (Chumbe et al. 2015; Khulape et al. 2014; Kumar et al. 2013) including Iran (Broomand et al. 2015), although the Iranian study did not reveal the molecular characteristic and genotype of the isolated virus. Most of the above-cited studies report the isolation of virulent strains that are found in commercial poultry, strongly suggesting the spillover of virulent strains from poultry to these captive bird species through different routes of infection. In the current study; however, isolation of vaccinal NDV genotype II from unvaccinated captive immature peafowls indicated the possible spillover from vaccinated birds to these birds. As mentioned earlier, contaminated food or even the park personnel could be to blame for this incident. However, the peafowl aviary was roofless and therefore, the spread of the virus from free-flying birds that landed in the aviary could also be a strong possibility. Such spillovers of NDV from vaccinated poultry to wild or free flying birds have been frequently reported (Ayala et al. 2016; Cardenas Garcia et al. 2013; Vidanović et al. 2011; Welch et al. 2019; Xiang et al. 2017).

The NDV isolate AMMM160 was isolated from a severely affected pigeon aviary that had a high mortality rate. The molecular analysis showed that the virus belonged to VI.2.1.2 (Dimitrov et al. 2019) viruses that were previously known as VIh (Diel et al. 2012). This group was first isolated in Argentina in 1997 (Benson et al. 2015) but has also been isolated from pigeons and doves in Nigeria and Kenya (Snoeck et al. 2013). Overall, not many such isolates have been identified and therefore, the origin of this subgenotype is not entirely known. However, the closest matching sequence to AMMM160 showed a 4.3% distance. Since no such subgenotype has been isolated from Iran before then it is possible to witness the identification of this group in the future again.

CONCLUSION

Genotypes VI, VII, and XIII have been reported from Iranian poultry and pigeon frequently (Molouki et al. 2019; Rezaei Far et al. 2017; Soltani et al. 2019). All three genotypes have existed in different parts of the world and possibly they spread to Iran through bird trading or migration. Iran has a large poultry population of 1 billion chicks and unfortunately, this industry has often reported outbreaks. On the other hand, the virus is endemic in backyard poultry (Sabouri, Vasfi Marandi, & Bashashati 2018; Soltani et al. 2019) and pigeons (Rezaei Far et al. 2017). Together, it can be concluded that the virus has been continuously circulating and passaging in these Iranian bird populations over the years. In addition, because of the extent of the poultry business, water and soil contamination with pathogens is of special importance and a big challenge to contain. In fact, NDV can remain active in water and soil for 90 days; therefore, this characteristic provides a dangerous ground for the virus to remain endemic. Moreover, since NDV remains in the carcass and feathers of infected birds for months, the spread of the disease to wild or exotic birds through this route can be considerably worrying (Dimitrov, Manvell, & Goujgoulova 2010). Therefore, because of the presence of the virus in the country, identification of at-risk species or possible reservoirs is of special importance because consequently, better biosecurity measures can be implemented and more successful protection of birds can be achieved. Additionally, as avian food components can affect the survival and output of avian species (Ghaniei, Eslami, & BabaeiMarzango 2018), the utilization of different supplements containing natural remedies cannot be disregarded as a solution to combat viruses (Babaeimarzangou et al. 2015; Hosseini et al. 2013; Mikaili et al. 2013).

Protecting captured birds from pathogens and infectious agents, especially in public zoos, is a difficult task. This is mainly because of the following reasons: 1- airborne viruses such as NDV can freely spread between dense bird aviaries. 2- personnel and human factors may physically spread infectious agents between aviaries. 3- the possibility of contact between visitors and birds 4- the spread of the virus

as the result of contact between free-flying birds and captured birds 5- the stress of birds because of unsuitable aviary conditions which may lower immunity against infectious agents (Dimitrov, Manvell, & Goujgoulova 2010; Haddas et al. 2014). On the other hand, the use of contaminated water for washing aviary and other areas In the Saei public bird park could be considered as an additional risk factor. Therefore, by implementing better vaccination programs and biosecurity measures it is possible to provide better conditions for sensitive species such as golden pheasants and pigeons.

CONFLICT OF INTEREST

The authors declare they have no conflict of interest.

Research Involving Human Participants and/or Animals: The present study did not involve any human subject. Animal handling procedures were performed in line with the national animal welfare regulations. The Veterinary Ethnics Committee of Urmia university approved all animal experiments (Permit Code: IR-UU-AEC 2381/DP/3).

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