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Determination of Newcastle disease virus among wild bird populations in Lake Van basin, Turkey

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Abstract: In this study, 540 feces samples taken from a total of 22 bird species, including transit migrant, winter visitor, migratory, and resident bird species, in Lake Van basin were examined by real-time polymerase chain reaction (RT-PCR) with respect to the Newcastle disease virus (NDV). All of the feces samples were cultured in specific pathogen-free embryonated chicken eggs (ECEs) for NDV isolation. The NDV isolates were analyzed for virulent fusion (F) protein by RT reverse transcription PCR (RRT-PCR). Of the 540 examined feces samples, 28 (5.18%) were found to be positive by RT-PCR. The same samples were cultured in ECEs and, of those, 9 (1.66%) were positive for NDV isolations. Of the isolates, 7 were obtained from the migrant waterfowls *Phoenicopterus ruber*, *Anas clypeata*, *Aythya ferina*, and *Aythya fuligula* and 2 were from the resident species *Columba livia*. The RRT-PCR F protein was determined in 1 isolate belonging to *Aythya ferina*; this strain was typed as mesogenic/velogenic avian paramyxovirus type 1 and the other 8 isolates as lentogenic. In this study, we investigated, for the first time, the presence of NDV in wild, seemingly healthy bird feces from areas in Lake Van basin, in Turkey.

Key words: Fusion protein, matrix protein, Newcastle disease virus, pathotype, RT-PCR, wild birds

1. Introduction

Newcastle disease (ND) is one of the most important diseases impacting international trade in poultry and poultry products. The causative agent, Newcastle disease virus (NDV), has been isolated from a variety of species of wild, domestic, and caged birds around the world (1,2). Major signs of the disease, which vary in severity and lethality, include respiratory distress, diarrhea, circulatory disturbances, and central nervous system impairment. The disease has been classified as an Office International des Epizootics "List A" disease. NDV isolates are characterized by virulence in chickens and may be categorized into 3 main pathotypes, depending on the severity of the disease. Lentogenic isolates of NDV are of low virulence and may cause mild respiratory or enteric infections. NDV strains of intermediate virulence that cause primarily respiratory disease are termed mesogenic, and those that cause high mortality are termed velogenic. Velogenic forms of NDV are further classified as neurotropic or viscerotropic, based on the pathological manifestation of the ND (2,3). NDV may infect humans as a result of contact with certain species of birds and often results in conjunctivitis (4,5).

NDV belongs to the genus *Avulavirus* of the subfamily *Paramyxovirine*, of the family *Paramyxoviridae*. It is designated as avian paramyxovirus type 1 (APMV-1) and is serotyped from 1 to 9 APMVs serotypes. The enveloped virus has a negative-sense, single-stranded RNA genome of 15.186 nucleotides, which encodes 6 proteins, including the nucleocapsid, phosphoprotein, matrix (M), fusion (F), hemagglutinin-neuraminidase, and RNA-directed RNA polymerase (2,3,6,7). The virulence of NDV isolates is known to be related to the amino acid sequence at the F protein cleavage site and the ability of specific cellular proteases to cleave the protein of different pathotypes. Fewer basic amino acids are present in the F protein cleavage site of lentogenic NDV isolates than in either the mesogenic or velogenic strains, which have similar cleavage site sequences. The Office of International Epizootes now accepts reporting of the F cleavage sequence as a primary virulence determinant (3,8).

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The ND and avian influenza (AI) A viruses are pathogens of social and economical importance known to be disseminated throughout the world by migratory birds. Many efforts have been made to control the introduction of these viruses into new environments, and complete world surveillance has yet to be achieved (9). As with AI, the majority of NDV strains isolated from wild birds, especially from waterfowl, are nonpathogenic (lentogenic) and cause no clinical diseases. However, a recent genetic comparison between the NDV strains in domestic poultry and feral waterfowl suggested that the velogenic viruses arise from avirulent strains, originating from wild birds. These findings indicate that it is important to survey for NDV strains harboring in wild waterfowls, as well as in avian influenza viruses (1). Waterfowls are considered important vectors for the introduction of AI viruses and APMV into poultry, causing outbreaks of disease with devastating outcomes when pathogenic strains are involved (10).

NDV isolation in embryonated chicken eggs (ECEs) followed by hemagglutination inhibition (HI) assays with NDV-specific antibodies represents the reference standard for virus detection. Although NDV isolation is a sensitive and specific method, it takes several days to make a routine diagnosis. More rapid pathotyping methods, based on reverse transcription polymerase chain reaction (RT)-PCR, followed by agarose gel electrophoresis, sequencing RT-PCR products, a heteroduplex mobility assay, restriction endonuclease analysis of amplification products, and the use of fluorogenic probes, have all been reported (11). RT-PCR and real-time (RT)-PCR (RRT-PCR) offer the high sensitivity afforded by conventional RT-PCR, with the advantage that a post-PCR processing step is avoided, which allows for savings in both time and material (3). The molecular basis for NDV pathogenicity is dependent on the F protein cleavage site amino acid sequence and the ability of specific cellular proteases to cleave the F protein of different pathotypes (11). Fewer basic amino acids are present in the F protein cleavage site of lentogenic NDV isolates than in either the mesogenic or velogenic strains, which have similar cleavage site sequences (12).

Lake Van basin in Turkey is one of the most important aquatic regions, located on the northeast-south migratory routes, and houses transit migrant, winter visitor, migratory, irregular vagrant, and resident birds, with a total of 213 bird species (13–15).

The aim of this study was to determine, with RT-PCR and conventional methods, the NDV in feces samples collected from wild bird species and to clarify its pathotypes.

2. Materials and methods

Feces samples: In this study, 540 feces samples collected from 22 bird species, covering transit migrant, winter visitor, migratory and resident birds, in the Lake Van basin, Turkey, were used (Table 1).

Collection of fecal samples: The location of the bird species from which the fecal samples were collected, the homogeneous bird species determined with telescopes, and the identification of the bird species were done according to the methods of Kiziroğlu (13,14).

Collection of the samples for RT-PCR and virus isolation: For this purpose, the collected fresh feces samples, one-third from each, were placed into tubes containing 3 mL of phosphate buffer solution (NaCl, 8 g; KCl, 0.2 g; Na_2HPO_4, 1.15 g; KH_2PO_4/L, 0.2 g; and distilled water, pH 7.2) with sterile glycerol (20%) and antibiotics (penicillin G, 2×10^6 U/L; streptomycin, 200 mg/L; gentamicin, 250 mg/L; nystatin, 0.5×10^6 U/L). The samples were taken and immediately transported in a cold chain to the laboratory of the Microbiology Department, Veterinary Faculty, Yüzüncü Yıl University, in the city of Van, and stored at –80 °C until examination (16).

Antisera: In the HI test, the APMV-1 NDV Ulster-2C, APMV-2/chicken/Yucaipa/56, APMV-3/Turkey/1087/82, and APMV-7/Dove 4/75 antisera provided by the Bornova Veterinary Control and Research Institute, Ministry of Agriculture, were used.

RNA isolation: For this purpose, a 200 µL feces sample was used. Isolation was performed using a High Pure Viral RNA isolation kit (Roche Applied Sciences, Germany), as described in the instructions.

Table 1. The status of the birds and the number of feces samples.

Status	Bird numbers	Sample numbers
Transit migrant	2	41
Winter visitor	5	115
Migratory	5	119
Resident	10	265
Total	22	540

cDNA synthesis: cDNA synthesis was performed on a DNA thermal cycler (Px2 Thermal Cycler, Thermo Electronic Corp., Milford, MA, USA) using a transcriptor first strand cDNA synthesis kit (Roche).

The determination of the NDV M protein gene by RT-PCR: This was performed in 96-well microplates, with a LightCycler LC 480 (Roche), using LightCycler TaqMan Master (Roche, Cat. No. 04 535 286 001), specific primers and probe sets (M+4100** forward primer 1054964; M–4220** reverse primer 1054966; and M+4169** matrix probe 1054965, TibMolbiol, Berlin, Germany) of APMV-1 M protein test kits. All of the kits were used according to the manufacturers' instructions.

Agarose gel electrophoresis: PCR products were run in 1.5% agarose and dyed with ethidium bromide (0.5 µg/mL). The gel was prepared with a Tris-HCl/boric acid/ethylenediaminetetraacetic acid buffer. The samples were loaded into a vertical small DNA gel electrophoresis system, in 5 µL volumes, and were run at 100 V. For electrophoresis, a standard marker (Low range DNA ladder, Fermentas, SM1191, Hilden, Germany) was used. Screening was performed with a computer-based screening system (Spectronics Co., Model GL-5000, England).

NDV isolation into the ECEs: Culture into the ECEs was performed at the Bornova Veterinary Control and Research Institute (İzmir, Turkey). The isolation was done in 9–11-day specific pathogenic-free (SPF) chicken eggs. The assessment of the embryos was done according to the World Health Organization (16) standard system. The allantoic fluid taken from the dead embryos was tested with slide and microhemagglutination tests (16,17).

HI test: Allantoic fluids showing hemagglutinating activity were subjected to an HI test with APMV-1 NDV Ulster-2C, APMV-2/chicken/Yucaipa/56, APMV-3/Turkey/1087/82, and APMV-7/Dove 4/75 specific antisera. The test was conducted by the conventional microtiter method. Positive and negative controls were run simultaneously with the test samples to validate the test (16,17).

RRT-PCR: The presence of virulent F protein in the APMV-1 positive isolates grown in the ECEs was investigated by RRT-PCR test, according to the method suggested by Wise et al. (2004), at the Bornova Veterinary Control and Research Institute, İzmir, Turkey, using specific primers and probe sets (F+4829 forward primer; F+4894 Probe-1 (VFP-1) virulent fusion; and F–4939 reverse primer, TibMolbiol) of APMV-1 F protein test kits. A Qiamp Viral RNA kit was used for the RNA isolation and a 1-step RT-PCR kit for cDNA synthesis (both from Qiagen, Hilden, Germany). At all stages, the kits were prepared according to the manufacturer's instructions. The process was performed using LightCycler 2.0 (Roche).

3. Results

Of the 540 examined feces samples, 28 (5.18%) were found to be positive for the NDV M protein gene by RT-PCR. Of the 28 RT-PCR NDV positive samples, 7 (25%) were determined in transit migrant, 6 (21.43%) in winter visitor, 5 (17.85%) in migratory, and 10 (35.72%) in resident birds, and of these 28 NDV positive samples, 20 (71.42%) were detected in the feces of waterfowls. The highest positivity was seen in *Phoenicopterus ruber* (Greater flamingo) (Table 2, Figure 1). The amplicons obtained from the 28 NDV positive samples by RT-PCR were run on agarose gel electrophoresis and a band specific to NDV (121 bp) was observed (Figure 2).

The same samples were inoculated into ECEs, with 9 (1.66%) being positive for NDV. It was observed that all of the isolates from the ECEs had hemagglutination (HA) activity and their titers varied from 1/32 to 1/1024 ($\log_2 2^5$–$\log_2 2^{10}$). It was also determined that all of the viruses were inhibited by APMV-1 antisera in an HI test. While 7 of the isolates were isolated from the feces of transit migrant, winter visitor, and migratory waterfowls belonging to *Phoenicopterus ruber*, *Anas clypeata*, *Aythya ferina*, and *Aythya fuligula*, 2 were isolated from a resident species, *Columba livia* (Table 3).

By RRT-PCR, the F protein gene was determined in 1 isolate belonging to *Aythya ferina* (Pochard) and this isolate was defined as virulent mesogenic/velogenic APMV-1. In this strain, the mean death time, 50% egg infective dose, and effective lethal time to kill 50% values were 72 h, 8.50/0.2 mL, and 6.50/0.2 mL, respectively (this was performed at the Bornova Veterinary Control and Research Institute and the data are not shown). The other 8 isolates were typed as lentogenic APMV-1.

4. Discussion

ND is a virulent infectious disease that affects the poultry industry and causes significant economic losses (2,8). Avian paramyxoviruses have been isolated from wild, captive, caged, and domestic birds, and the distribution of these viruses appears to be worldwide (18–21). For primary isolation, ECEs are most commonly used. In the identification and differentiation of ND disease, various serological, antigenic, molecular-genetic, and immunological tests are used (6). RT coupled to PCR is one of the methods used to amplify the F protein gene sequences of various NDV strains (12). The considerable size of the wild bird population, the absence of frontiers, and the free movement of migratory birds lead to this population being considered an extremely important vector for virus dissemination (22).

A great many studies have been carried out on NDV isolation and characterization of isolates from wild birds, particularly from waterfowls. Wobeser et al. (23)

Table 2. Bird species, status, number of samples, and distribution of NDV positive samples by RT-PCR.

No.	Bird species	Status	Sample numbers (n)	RT-PCR positive samples/%
1	*Philomachus pugnax* (Ruff)	Tm	20	0/0
2	*Phoenicopterus ruber* (Greater flamingo)	Tm	21	7/33.33
3	*Anas clypeata* (Shoveler)	Wv	42	3/7.14
4	*Anser albifrons* (Greater white-fronted goose)	Wv	20	0/0
5	*Anas crecca* (Teal)	Wv	20	0/0
6	*Aythya fuligula* (Tufted duck)	Wv	10	1/10
7	*Aythya ferina* (Pochard)	Wv	23	2/8.69
8	*Nycticorax nycticorax* (Night heron)	M	19	0/0
9	*Himantopus himantopus* (Black-winged stilt)	M	20	0/0
10	*Tringa totanus* (Redshank)	M	40	5/12.5
11	*Vanellus vanellus* (Lapwing)	M	20	0/0
12	*Riparia riparia* (Sand martin)	M	20	0/0
13	*Tadorna tadorna* (Shelduck)	R	20	0/0
14	*Tadorna ferruginea* (Ruddy shelduck)	R	20	0/0
15	*Anas platyrhynchos* (Mallard)	R	40	0/0
16	*Fulica atra* (Coot)	R	18	1/5.55
17	*Larus michahellis* (Yellow-legged gull)	R	40	1/2.5
18	*Columba livia* (Rock dove)	R	40	1/2.5
19	*Pica pica* (Magpie)	R	20	1/5
20	*Corvus frugilegus* (Rook)	R	20	5/25
21	*Sturnus vulgaris* (Starling)	R	20	0/0
22	*Passer domesticus* (House sparrow)	R	27	1/3.70
		Total	540	28/5.18

Tm: transit migrant; Wv: winter visitor; M: migratory; R: resident.

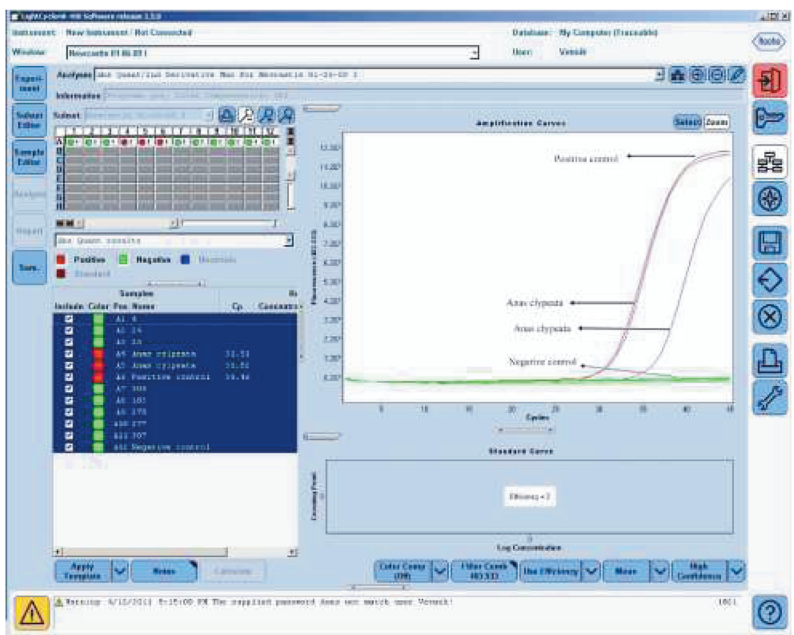

Figure 1. Some NDV positive samples in RT-PCR.

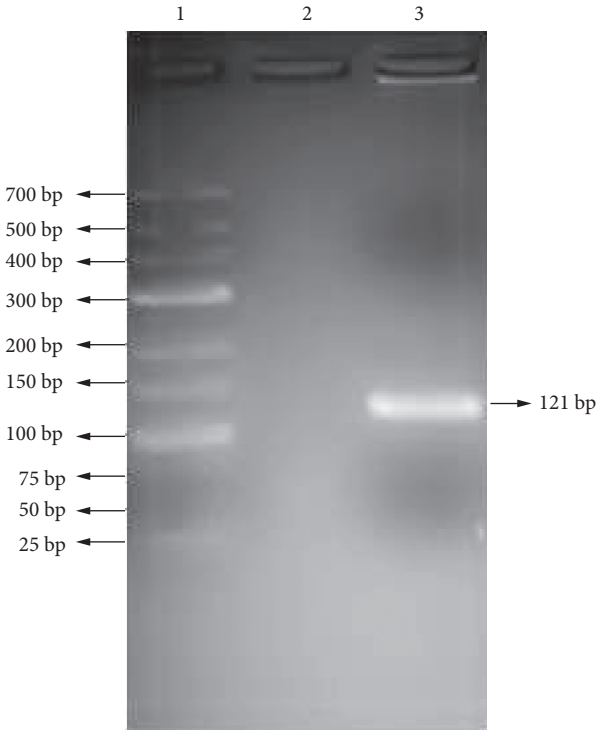

Figure 2. Picture of the RT-PCR NDV positive samples on 1.5% agarose gel electrophoresis: 1, low range DNA ladder (Fermentas, SM1191); 2, negative control (DNase, RNase free distilled water); and 3: ND positive strain.

conducted a study to isolate the NDV from various bird species living in different regions where high bird mortality was observed. They isolated the NDV from 1 (33.3%) out of 3 gull feces samples and the isolate was determined to be of velogenic character. In a study carried out in Japan (24), the HA virus was isolated in 244 (5.81%) out of 4196 feces samples obtained from various bird species during a 6-year period, and, of those, 47 (1.12%) were determined to be serologically NDV by HI test. Of the isolates, 39 belonged to ducks, 7 to geese, and 1 to a gull. Of the 47 strains tested, 29 were characterized as velogenic, and F protein was determined in 11 of the samples. Shengqing et al. (1) examined migratory waterfowl birds, in a 4-year period, with respect to myxoviruses. They isolated 4 (1.28%) NDV strains in the feces samples of 317 *Anas acuta* (pintails) and 5 (5.43%) NDV strains in the feces samples of 92 *Anser albifrons* (white-fronted geese). They also reported that the feces samples obtained from 89 *Anas platyrhynchos* (mallards) were negative with respect to the NDV. All of the isolates were found to be of lentogenic character. Stanislawek et al. (10) isolated APMV-1 in 10 (2.89%) of 346 feces samples obtained from *Anas platyrhynchos* (mallards) and all of the isolates were found to be of lentogenic character. Peroulis and O'Riley (25) isolated APMV-1 in 2 (0.70%) of 284 wild duck feces samples and found that all 133 of the feces samples of pigeon origin were negative with respect to APMV-1.

Table 3. Test results of isolates with 1, 2, and 3 passages in ECEs, micro HA, and with APMV-1 positive antisera HI.

No.	Bird species	Status	Passages in ECEs			HA titers of isolates	HI test with APMV-1 antisera
			I. Passage	II. Passage	III. Passage		
1	*Phoenicopterus ruber*	Tm	Positive	Positive	Positive	1/256	+
2	*Anas clypeata*	Wv	Negative	Positive	Positive	1/64	+
3	*Anas clypeata*	Wv	Negative	Positive	Positive	1/128	+
4	*Anas clypeata*	Wv	Positive	Positive	Positive	1/1024	+
5	*Aythya ferina*	Wv	Weakly positive	Positive	Positive	1/32	+
6	*Aythya ferina*	Wv	Positive	Positive	Positive	1/64	+
7	*Aythya fuligula*	Wv	Negative	Negative	Positive	1/128	+
8	*Columba livia*	R	Weakly positive	Positive	Positive	1/512	+
9	*Columba livia*	R	Weakly positive	Positive	Positive	1/ 256	+

Tm: transit migrant; Wv: winter visitor; M: migratory; R: resident.

The researchers stated that both strains were of lentogenic character. In a study carried out in order to determine the NDV in 916 feces samples obtained from healthy wild birds consisting of gulls, penguins, geese, crows, swans, flamingos, and pigeons (22), HA activity showing viruses was determined in 26 (2.83%) samples and the HA titers varied from 1/16 to 1/512. In the HI tests of the 26 isolates, 7 (0.76%) isolates showed a positive result with anti-NDV serum and were identified as NDV. Of the isolates, 3 were of duck, 3 of swan, and 1 was of flamingo origin. In the pathogenicity test, the duck and swan isolates were found to be avirulent, while the flamingo isolate was classified as a lentogenic strain. The NDV flamingo strain was grouped as being closely related to the NDV La Sota strain, which is usually included in vaccines, showing 99.9% identity in the nucleotide sequences and 100% identity in the aa sequences. Hanson et al. (19), in a study carried out on ducks of different species (*Anas crecca*, *Anas cyanoptera*, *Anas discors*, *Anas fulvigula*, and *Anas acuta*) in 2001, found that all 96 duck feces samples examined were negative with respect to APMV-1. They isolated APMV in 11 (12.64%) of 87 mottled duck (*Anas fulvigula*) feces samples, collected in August 2002, and stated that all of the isolates were APMV-1. They also isolated APMV in 24 (32%) of 75 feces samples obtained from blue-winged teal (*Anas discors*), collected in January of the same year, and typed 23 of the isolates as APMV-1, while 1 was typed as APMV-7. In a study carried out in Bulgaria (6), feces samples obtained from 168 free living wild birds of different species, consisting of 59 *Anser albifrons*, 54 *Anas platyrhynchos*, 28 *Anser anser*, 21 *Fulica atra*, 2 *Anas penelope*, 1 *Anas clypeata*, 1 *Anas crecca*, 1 *Aythya ferina*, and 1 *Tadorna tadorna*, were examined with respect to APMV-1. Viruses with HA activity were isolated in only 5 (2.97%) samples belonging to ducks of the species *Anas platyrhynchos*. In that study, the HA titers of the isolates varied from 1:16 to 1:256. The lentogenic character of the isolated strains and their similarity to the vaccine strain used in Bulgaria was found to be significant. In a study (8) carried out on different duck species (*Anas platyrhynchos*, *Anas crecca*, *Anas acuta*, *Anas fabalis*, and *Aythya ferina*), 6 (5.21%) out of 115 cloacal samples were positive by RT-PCR, and APMV-1 was isolated in 4 (3.47%) samples. Of the RT-PCR positive samples, 5 belonged to *Anas crecca* and 1 belonged to the *Aythya ferina* duck species. Of the 4 APMV-1 isolates, 3 belonged to *Anas crecca* and 1 belonged to *Aythya ferina*. Racnik et al. (26) collected 670 cloacal swabs from healthy migratory passerine birds during a 4-year period and isolated APMV-2 in only 1 (0.14%) sample. They reported that all of the samples were negative by RT-PCR with respect to AMPV. In a study performed in Japan (21), APMV-1 (NDV) was isolated in 11 (0.46%) out of 2381 feces samples collected from clinically healthy northern pintail (*Anas acuta*) duck species. They concluded that the presence of the NDV and other viruses of HA characteristic circulated among the duck population that the samples were subclinically collected from, and that these birds played a significant role in the dissemination of NDVs transcontinentally.

In Turkey, studies concerning ND, the isolation of the virus, and its characterization have generally been performed on chickens, turkeys, pigeons, and doves (18,20,27). No studies concerning the determination of the NDV in wild birds are recorded in the literature. Çöven et al. (18) examined the organ materials taken from a flock of 9 pigeons showing disease symptoms, living in İzmir and Manisa, and from 3 dead doves with respect to NDV. They isolated the HA virus from all of the animals and typed all of the isolates as APMV-1. In the further identification of these isolates at the Central Research Labs in the UK using monoclonal antibodies, they were determined to be PMV-1 (PPMV-1), a so-called pigeon variant of the NDV. All of the isolates were pathotyped as velogenic. In their study, Dakman et al. (20) isolated and identified 39 NDV strains and determined their pathogenicity. They reported that all 28 of the NDV strains isolated from backyard poultry were velogenic; of the 4 NDV strains isolated from domestic pigeons, 3 were mesogenic and 1 was lentogenic; of the 5 NDV strains isolated from wild pigeons, 2 were velogenic and 3 were mesogenic, and 2 of the NDV strains isolated from wild doves were mesogenic. The researchers stated that the isolation of velogenic strains from wild pigeons could indicate that the wild birds were an important source for the circulation of the virus.

In our study, the determination of 5.18% positivity by RT-PCR from the feces samples of wild bird and a 1.66% isolation rate were similar to the results of many studies (1,6,8,10,24), higher than some (21,22,25,26), and lower than others (19,23). The results of the study may vary according to the countries, seasons, and the migration routes of the country studied. When compared with similar studies (1,6,10,22,25), in our study, except for 1 mesogenic/velogenic strain from *Aythya ferina* (pochard), the isolation of 8 lentogenic strains was not surprising. There is no record of studies carried out in Turkey on wild bird species, except for on doves and pigeons. Therefore, the results of this study were not compared and discussed in detail with the literature. In both this study and the other 2 studies (20,27) carried out on domestic and wild pigeons, the determination of the presence and pathogenicity of the virus was found to be important when the pigeon population and the transmission of the virus to humans are considered.

Turkey, with its aquatic regions, hosting migratory and native bird species, is located on the important Eurasian migration routes (13,14). AI type A H5N1 outbreak

occurrences between 2005 and 2008 in Turkey seriously affected the backyard/village poultry living in aquatic regions on the routes of migratory birds (28). AI and ND are always a great risk for the native avian population (backyard/village poultry) living on the routes of migratory birds. In a study carried out by Boynukara et al. (15), 2.9% AI type A M2 positivity was determined by RT-PCR in the feces of wild birds living in Lake Van basin. In this study, 5.18% NDV positivity was determined by RT-PCR. Water birds, particularly wild ducks, have been proven to carry microorganisms like NDV, AI, and Enterococci (15,29) in this basin. The results of these studies have been evaluated as important with respect to the environment and public health. Therefore, we recommend that regular detailed studies should be carried out by scientists and ornithologists in this region to take precautions against any likely outbreaks.

In conclusion, in this study, for the first time in Turkey, AMPV-1 was isolated from wild birds such as *Phoenicopterus ruber*, *Anas clypeata*, *Aythya ferina*, and *Aythya fuligula*. It is expected that the results of this study will guide and contribute to further molecular and phylogenetic studies to elucidate the epidemiology of NDV strains.

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