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Determination of Avian Influenza A Viruses in Some Avian Species in Van Lake Basin by Real Time-PCR, Isolation and Subtyping

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Abstract: In this study, feces samples collected during 37 months from February 2006 to March 2009 from 2013 animals consisting of 47 avian species covering irregular vagrant, transit migrant, winter visitor, migratory and native birds in the Van Lake Basin Turkey were tested by Real-Time PCR (RT-PCR) with respect to Avian Influenza (AI) type A virus M2 gene. Of them, 59 samples (2.9%) were found to be positive. RT-PCR positive samples were examined with the same method with respect to H5N1 and 4 samples (6.8%) were found to be positive. RT-PCR positive 59 samples were inoculated in Embryonated Chicken Egg (ECE) and AI type A virus was isolated from 12 samples (20.3%). Of the isolates, 3 were typed as H1N7, 2 as H7N9, 2 as H11N9 and 1 as H8N4 with Hemagglutination Inhibition (HI) and Neuraminidase Inhibition (NI) tests. About 4 isolates obtained from winter visitor *Anas cylpeata* which had been determined as H5N1 by RT-PCR and agarose gel electrophoresis, gave positive reaction by HI test both with H1 and H5 antisera and all were typed as N1 by NI-test. Feces samples found to be positive by RT-PCR belonged to avian species Anseriformes, Charadriiformes, Passeriformes, Gruiformes and Phoenicopteriformes orders. The highest positivity was determined in winter visitor Anas acuta (37.1%) and Anas penelope (22.5%) ducks. Of the RT-PCR positive 59 samples, 43 (72.9%) were determined in the samples collected during winter and spring of 2006-2009. Positivity was determined at a rate of 35.2% in respect of AI type A by RT-PCR in different species sharing the same time and place. With this study, the presence of AI type A viruses in various wild birds in the Van Lake Basin was determined for the first time in Turkey.

Key words: Avian influenza, wild birds, RT-PCR, isolation, subtyping, Turkey

INTRODUCTION

Avian Influenza (AI) viruses have been isolated from human, swine, horse, mink, cat, dog, several water mammals and various domestic and wild birds. Particularly, migratory water birds like duck, goose and swan play the major role in carrying the agent and spreading it to other continents (Sharp *et al*., 1993; Jucket, 2006; Happold *et al*., 2008).

Influenza viruses from Orthomyxoviridae family are agents of pleomorphic, sheeted, polarized negative and single thread RNA character. Influenza viruses are classified as A, B and C types according to their differences in the Nucleoprotein (NP) and Matrix (M) proteins which are two important internal structures (Ellis and Zambon, 2001; Alexander, 2008). In avian species, only the influenza A viruses cause natural infections. Influenza A viruses can be divided into 2 pathotypes; High Pathogenicity Avian Influenza (HPAI) and Low Pathogenicity Avian Influenza (LPAI) (Swayne, 2007). It is reported that both HPAI and LPAI viruses of some wild birds particularly of water birds are endemic carriers (Tumpey *et al*., 2005). Influenza A viruses when the antigenic structure of surface glycoprotein are taken into account, fall into Hemagglutinin (H) and Neuraminidase (N) subtypes. Currently 16 H (H1-16) and 9 N (N1-N9) subtypes have been determined (Kida *et al*., 1994; Alexander, 2008). It is reported that totally 144 subtypes have been isolated from various avian species the majority of which are wild birds (Tumpey *et al*., 2005; Olsen *et al*., 2006; Capua and Alexander, 2007; Swayne, 2007; Alexander, 2008). In various studies, it is reported that wild birds from Anatidae family in Anseriformes order and Charadriiformes order are natural host of influenza A

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viruses (Sharp *et al*., 1993; Jucket, 2006; Capua and Alexander, 2007; Happold *et al*., 2008). About 16 different hemagglutinin including H5 and H7 subtypes were isolated from migratory water birds. Although, these animals were the most important reservoir for AI type A viruses (Webster *et al*., 2006), waterbirds are not at the same degree, reservoir for all of AI type A virus subtypes (Sharp *et al*., 1993).

In determining AI type A viruses, conventional virologic culture methods, various tests applied for rapid viral antigen diagnosis researchers according to immunofluorescence methods (Playford and Dwyer, 2002; Alexander, 2008) different PCR methods used for determining genomic material (Playford and Dwyer, 2002; Spackman *et al*., 2002; Widjaja *et al*., 2004; Munster *et al*., 2005; Runstadler *et al*., 2007) and for indirect diagnosis, various serologic tests (Chen *et al*., 2008) are being used. In different PCR methods, specific primers are used largely for the M, H, NP and NS region of these viruses (Playford and Dwyer, 2002; Spackman *et al*., 2002; Widjaja *et al*., 2004; Nagy *et al*., 2007). In determining these viruses in various materials, mostly RT-PCR method is being preferred (Munch *et al*., 2001; Spackman *et al*., 2002; Spackman *et al*., 2006; Runstadler *et al*., 2007).

The aim of this study was to determine, with RT-PCR and conventional methods, AI type A viruses in feces samples belonging to some avian species forming the avian fauna in the Van Lake Basin which has an important place among the aquatic regions located on the Northeast-South migratory routes and houses approximately 213 bird species and was to subtype the isolated viruses with specific antisera.

MATERIALS AND METHODS

Feces samples: In this study, feces samples collected during 37 months from February 2006-March 2009 from 2013 animals consisting of 47 avian species covering irregular vagrant, transit migrant, winter visitor, migratory and native birds in the Van Lake Basin, Turkey were shown in Table 1. The material was collected from 19 different stations to represent the whole Van Lake Basin. Of the materials, 541 were collected in 2006, 1082 in 2007, 337 in 2008 and 53 in 2009. During collecting feces samples no harm was given to the birds and nets were used to catch some bird species.

Sample collecting for RT-PCR: For this purpose, the collected fresh feces samples, 1/3rd from each were placed into tubes containing 3 mL phosphate buffer solution-PBS (8 g NaCl, 0.2 g Kcl, 1.15 g Na_2HPO_4, 0.2 g KH_2PO_4 L^{-1} distilled water-pH: 7.2) with sterile glycerol (20%) and antibiotics (penicillin G-2×10^6 U L^{-1}; streptomycin-200 mg L^{-1}; gentamycin-250 mg L^{-1}; nystatin -0.5×10^6 U L^{-1}). The samples were taken in cold chain and immediately to the Laboratory of Microbiology Department, Veterinary Faculty, Yuzuncu Yil University and stored at -80°C till the examination (WHO, 2002).

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

Status	No. of birds	No. of samples
Irregular vagrant	1	25
Transit migrant	2	111
Winter visitor	8	485
Migratory	15	352
Native	21	1040
Total	47	2013

Sample collecting for virus isolation: Fresh feces samples collected for virus isolation were placed into tubes containing 3 mL PBS with antibiotics (penicillin G-2×10^6 U L^{-1}; streptomycin-200 mg L^{-1}; gentamycin-250 mg L^{-1}; nystatin-0.5×10^6 U L^{-1}). The samples were immediately taken to lab in cold chain and stored at -80°C till examination (WHO, 2002).

Reference strain: In the laboratory studies in active H5N1 strain (A/Chicken/TR/47/Ekinlik/673/2006) provided by Bornova Veterinary Control and Research Institute, Ministry of Agriculture was used.

RNA isolation: For this purpose, 200 μL feces sample was used. Isolation was performed with high pure viral RNA isolation kit (Roche, 11 858 882 001) according to the prospectus.

cDNA synthesis: cDNA synthesis was performed on DNA thermal cycler (P ×2 Thermal Cycler, Thermo Electronic Corp., Milford, MA, USA) using transcriptor 1st strand cDNA synthesis kit (Roche, 04 896 866 001). The determination of AI type A matrix protein gene (M2) by RT-PCR: This was performed in microplates with 96 wells in Light Cycler (LC) 480 (Roche) using lightcycler faststart DNA master hybprobe kit (Roche, 03 003 248 001) and lightmix 480HT influenza A virus M2 (TIB Molbiol, 40-0277-96) test kits. All kits were used as suggested by the manufacturer. The analyses of AI type A M2 positive samples with respect to H5N1: Lightmix 480 HT for the detection of avian influenza A virus (subtype Asia) H5N1 (TIB Molbiol, 40-0311-96) and lightcycler faststart DNA master hybprobe kits (Roche, 03 003 248 001) were used as instructed in the prospectus. The test was performed in microplates with 96 wells and in Light Cycler (LC) 480.

Agarose gel electrophoresis: PCR products were made run in 1.5% agarose and dyed with etidium bromide (0.5 μg mL^{-1}). Gel was prepared with TBE (Tris-HCl, boric

acid, EDTA) buffer. The samples were loaded into vertical small DNA gel electrophoresis system in 5 μL volumes and were made run at 100 volts. In electrophoresis, standard marker (GeneRuler 100 bp DNA Ladder, Fermentas, Hilden, Germany) was used. Screening was performed with computer-based screening system (Spectronics Co., Model GL-5000, England).

Culture into embryonated chicken eggs: Culture into ECE was performed at Bornova Veterinary Control and Research Institute (Izmir, Turkey) the national reference laboratory in Turkey for avian influenza. The culture was performed in 9-11 day Specific-Pathogenic-Free (SPF) chicken eggs. The assessment of embryos was done according to WHO (2002) standard system. The allantoic fluid taken from dead embryos was tested with slide and micro-hemagglutination tests (WHO, 2002). Determination of avian influenza virus type A antigen with rapid test kit: For this process avian influenza virus type A antigen test kit (Symbiotic Corporation, USA) was used. The test was done according to the manual of the providing firm.

Determination of hemagglutinin subtypes: H subtypes of isolates were determined with antisera provided from Veterinary Laboratories Agency, VLA (Weybridge, England) (H1-H16) and from Avian influenza OIE/FAO Reference laboratory, IZSV (Padova, Italy) (H1-H15) by HI test. The test was done in V based microplates with 96 wells (Greiner) using 4 HAU antigen (WHO, 2002).

Determination of neuraminidase subtypes: N subtypes were determined by NI test. In NI test, antisera (N1-N9) provided from VLA (Weybridge, England) were used. The test was performed according to the standard method and assessed (WHO, 2002).

RESULTS AND DISCUSSION

Avian influenza type A M2 RT-PCR: Of the 2013 feces samples examined, 59 (2.9%) were found positive by RT-PCR with respect to AI type A M2 gene. RT-PCR positive samples were found in 59 (5.2%) of the 1128 feces material collected from 17 (36.1%) out of 47 avian species examined as shown in Table 1. Positivity with respect to M2 was determined in the feces of *Anas acuta*, *Anas clypeata*, *Anas crecca*, *Anas querquedula*, *Anas platyrhynchos*, *Anas penelope*, *Aythya ferina*, *Aythya fuligula*, *Himantopus himantopus*, *Larus michahellis*, *Larus ridibundus*, *Phoenicopterus ruber*, *Tadorna tadorna*, *Tringa totanus*, *Fulica atra*, *Pica pica* and *Passer domesticus*. Of them, 9 (52.9%) was from Anseriformes, 4 (23.5%) from Charadriiformes, 2 (11.8%) from Passeriformes, 1 (5.9%) from Gruiformes and 1 (5.9%) from Phoenicopteriformes Order as shown in (Table 2, Fig. 1). Of the positive samples, 43 (72.9%) were collected in the first period covering January-April of 2006-2009; 12 (20.3%) in the 3rd period covering September-December of 2006-2008; 4 (6.8%) in the second period covering May-August of 2008. When the positive samples were distributed according to the years in the

Table 2: Avian species, status, number of the samples and distribution of AI type A M2 positive samples by RT-PCR

Bird species	Status	Material numbers (n)	RT-PCR positive samples (%)	No.	Birds	Status	Material numbers (n)	RT-PCR positive samples (%)
Phalacrocorax carbo	Iv	25	0	25	*Sturnus roseus*	M	10	0
Philomachus pugnax	Tm	30	0	26	*Corracias garrulus*	M	2	0
Phoenicopterus rubber	Tm	81	2/2.5	27	*Podiceps nigricollis*	N	27	0
Cygnus cygnus	Wv	72	0	28	*Tachybaptus ruficollis*	N	23	0
Anas cylpeata	Wv	117	6/5.1	29	*Tadorna tadorna*	N	114	2/1.8
Anser albifrons	Wv	23	0	30	*Tadorna ferruginea*	N	96	0
Anas crecca	Wv	69	6/8.7	31	*Anas platyrhynchos*	N	115	3/2.6
Anas penelope	Wv	40	9/22.5	32	*Fulica atra*	N	102	1/1
Aythya fuligula	Wv	28	2/7.1	33	*Larus ridibundus*	N	70	1/1.4
Anas acuta	Wv	35	13/37.1	34	*Larus michahellis*	N	99	3/3
Aythya ferina	Wv, M	101	5/5	35	*Columba livia*	N	40	0
Nycticorax nycticorax	M	44	0	36	*Alauda arvensis*	N	20	0
Ciconia ciconia	M	11	0	37	*Motacilla alba*	N	25	0
Himantopus himantopus	M	48	2/4.2	38	*Pica pica*	N	22	1/4.5
Tringa totanus	M	50	1/2	39	*Corvus monedula*	N	40	0
Vanellus vanellus	M	45	0	40	*Corvus frugilegus*	N	25	0
Merops apiaster	M	1	0	41	*Sturnus vulgaris*	N	27	0
Oxyura leucocephala	M	20	0	42	*Passer domesticus*	N	27	1/3.7
Hirundo rustica	M	6	0	43	*Galerida cristata*	N	10	0
Riparia riparia	M	38	0	44	*Alectoris chukar*	N	20	0
Motacilla flava feldegg	M	27	0	45	*Gallus domesticus*	N	58	0
Chlidonias leucopterus	M	20	0	46	*Meleagris* sp.	N	25	0
Sterna hirundo	M	20	0	47	*Anser anser-domesticus*	N	55	024
Anas querquedula	M	10	1/10	-	-	-	-	-
Total	-	-	-	-	-	-	2013	59/2.9

Iv: Irregular vagrant; Tm: Transit migrant; Wv: Winter visitor; M: Migratory and N: Native

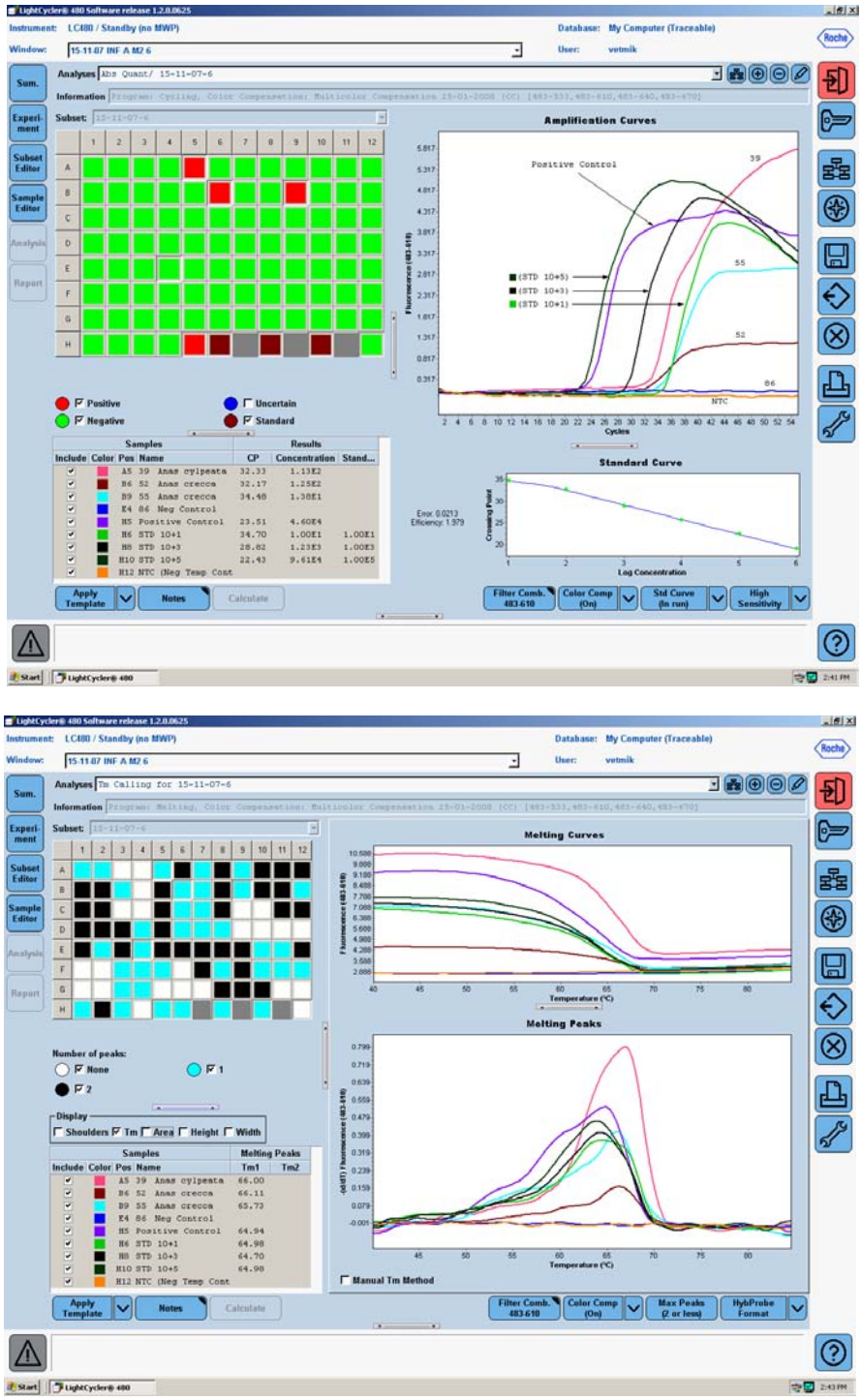

Fig. 1: Some positive RT-PCR avian influenza type A M2 samples

first period of 2009 the positivity was found to be >(10.6%) compared to 2008 which was found to be <(8.2%). That was because sample collecting did not continue the whole year in 2009. This rate was 5% in 2007 and 3% in 2006. Of the 59 RT-PCR AI type A M2 positive samples, 36 (61%) were found in feces samples collected from 5 winter visitor species, 12 (20.3%) from 7 native species, 4 (6.8%) from 3 migratory species, 5 (8.5%) from

Table 3: RT-PCR AI type M2 positive species, status, distribution of feces samples with respect to periods and year

		Distribution of positive samples with respect to years and periods/ positive samples										
		2006			2007			2008			2009	
Bird species (order)	Status	I	II	III	I	II	III	I	II	III	I	Total/ positive samples (%)
RT-PCR AI type A M2 positive bird species by RT-PCR												
Phoenicopterus ruber (Phoenicopteriformes)	Tm	-	-	20	-	20	32	-	5/1	-	4/1	81/2/2.5
Anas cylpeata (Anseriformes)	Wv	20	-	20/4	20	-	45/1	-	-	-	12/1	117/6/5.1
Anas crecca (Anseriformes)	Wv	-	-	20/3	-	-	30	10/3	-	6	3	69/6/8.7
Anas penelope (Anseriformes)	Wv	-	-	-	20/9	-	-	20	-	-	-	40/9/22.5
Aythya fuligula (Anseriformes)	Wv	-	-	-	-	-	-	20/2	-	8	-	28/2/7.1
Anas acuta (Anseriformes)	Wv	-	-	-	20/12	-	-	15/1	-	-	-	35/13/37.1
Aythya ferina (Anseriformes)	Wv/M	-	20	-	30/2	-	5	25	3/1	3	15/2	101/5/5
Himantopus himantopus (Charadriiformes)	M	-	20	-	-	18	-	10/2	-		-	48/2/4.2
Tringa totanus (Charadriiformes)	M	40	-	-	-	-	-	10/1	-		-	50/1/2
Anas querquedula (Anseriformes)	M	-	-	-	-	-	-	5	5/1		-	10/1/10
Tadorna tadorna (Anseriformes)	N	-	-	20	35	20	29/1	-	-	7	3/1	114/2/1.8
Anas platyrhynchos (Anseriformes)	N	20	-	20	60/3	-	-	5	-	10	-	115/3/2.6
Larus ridibundus (Charadriiformes)	N	-	-	-	70/1	-	-	-	-	-	-	70/1/1.4
Larus michahellis (Charadriiformes)	N	40/2	-	-	20	-	10	10	9/1	-	10	99/3/3
Fulica atra (Gruiformes)	N	17	-	-	60	-	20	-	-	5/1	-	102/1/1
Pica pica (Passeriformes)	N	-	-	-	20	-	-	-	-	2/1	-	22/1/4.5
Passer domesticus (Passeriformes)	N	25	-	-	-	-	-	-	-	2/1	-	27/1/3.7
Total/positive samples	162/2	40	100/7	355/27	58	171/2	130/9	22/4		43/3	47/5	
The number of total/positive samples/percent	302/9/3				584/29/5			195/16/8.2			47/5/ 10.6	1128/59/5.2

I: January, February, March, April; II: May, June, July, August; III: September, October, November, December; Tm: Transit migrant; Wv: Winter visitor; M: Migratory and N: Native

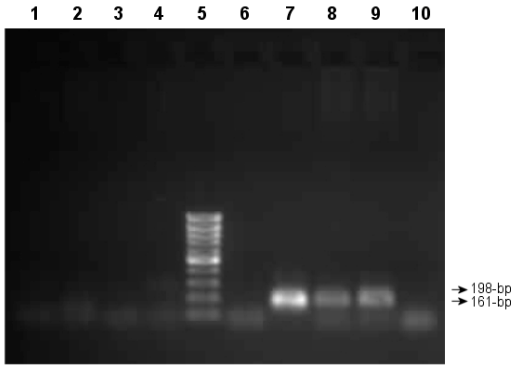

Fig. 2: The picture of H5N1 positive samples on 1.5% agarose gel electrophoresis. 1-6: Negative samples; 5: Marker (100 bp DNA ladder plus, SM0241, Fermentas); 7: Positive control (Reference H5N1 strain, A/Chicken/TR/47/Ekinlik/673/2006); 8 and 9: Positive samples (H5 161 bp, N1 198 bp); 10: Negative control (DNase, RNase free distilled water)

1 species considered to be both winter visitor and migratory and 2 (3.4%) from one transit migrant bird species. When the status of birds is taken into consideration, the highest positivity was determined in *Anas acuta* (37.1%) and *Anas penelope* (22.5%) duck species (Table 3). Of the 59 positive samples, 47 (80%) were determined in transit migrant, winter visitor and migratory bird species and 12 (20%) in native bird species.

RT-PCR positivity was determined in 31 (35.2%) of 88 feces samples collected from different avian species sharing the same place and time: 12 samples from 20 *Anas acuta* and 9 from 20 *Anas penolope*; 2 from 10 *Himantopus himantopus* and 1 from 5 *Tiringa totanus*; 1 from 5 *Phoenicopterus rubber* and 1 from 3 *Aythya ferina*; 1 from 2 *Passer domesticus* and1 from 2 *Pica pica;* 1 from 12 *Anas cylpeata,* 1 from 5 *Aythya ferina* and 1 from 4 *Phoenicopterus ruber*. As shwon in Table 3.

H5N1 RT-PCR: In the analysis of 59 RT-PCR M2 positive samples with respect to H5N1, positivity was determined in 4 feces samples (6.8%) collected from *Anas cylpeata* species. These feces samples were collected on 15 September 2006 from Karagunduz Village by Ercek lake. The amplicons obtained from 4 samples which were found H5N1 positive with RT-PCR were run on agarose gel electrophoresis. Bands specific to H5 (161-bp) and N1 (198-bp) were shown in Fig. 2.

Virus isolation, HA and avian influenza type A rapid tests: RT-PCR positive samples were cultured in ECE and from 12 (20.3%) feces samples AI type A virus was isolated. All of the isolates were found positive with slide HA and AI type A rapid test and in micro HA test titers were determined ranging from 1.32-256. Isolates were obtained from *Anas crecca*, *Anas clypeata* and *Aythya ferina* belonging to Anseriformes order.

HI and NI tests: The results of HI and NI tests performed to determine H and N subtypes of isolates are shown in Table 4. About 4 isolates determined as H5N1 in RT-PCR and agarose gel electrophoresis gave positive reaction both with H1 and H5 antisera by HI test and all typed as N1 by NI-test. Isolates typed as H7 were determined in feces samples of *Anas crecca* collected from Cakirbey town by Van lake on 12 March 2008. Some subtypes of AI type A viruses can spread from animals to humans causing deadly infections. Recently, the interest in these agents with high mutation ability to exceed the species resistance among the living organisms and the infections they cause has increased. In many parts of the world, numerous studies are being carried on influenza A viruses (Ellis and Zambon, 2001; Gaidet *et al*., 2007; Nagy *et al*., 2007; Runstadler *et al*., 2007).

In transferring influenza A virus suspected materials to the labs, different medium and antibiotics are being used (Stallknecht *et al*., 1990; Alfonso *et al*., 1995; Fouchier *et al*., 2005; Munster *et al*., 2005; Gaidet *et al*., 2007). The composition, pH, rate of salt and transfer temperature of the medium are important for transferring the material in suitable conditions. Among the transport medium are Phosphate Buffer Saline (PBS), PBS and Hanks' balanced salt transport medium containing glycerol at different rates; Among antibiotics are penicillin G, streptomycin sulfate, gentamicin sulfate, polymyxin B, mycostatin, ofloxacin and sulfamethoxazole.

In this study in collecting, transferring and storing of feces materials, the transport medium suggested by World Health Organization (WHO, 2002) was used. The fresh feces samples put into tubes containing transport medium at a rate of 1.3 were immediately transferred in cold chain to lab and stored at -80°C till examination. The transport medium which was freshly prepared and used during 37 months of the project was concluded that it can be used in similar studies and be recommended to the researchers because of its low cost of being easily and quickly prepared, the antibiotics and antifungal it contains.

Table 4: Typing of isolates by HI and NI tests and HI titers

Isolates	Bird species	HI subtype / HI titer	NI sub-type
1	*Anas clypeata*	H1: 1/512, H5: 1/64	N1
2	*Anas clypeata*	H1: 1/64, H5: 1/8	N1
3	*Anas clypeata*	H1: 1/128, H5: 1/16	N1
4	*Anas clypeata*	H1: 1/512, H5: 1/512	N1
5	*Anas crecca*	H1: 1/128	N7
6	*Anas crecca*	H1: 1/64	N7
7	*Anas crecca*	H1: 1/32	N7
8	*Anas crecca*	H7: 1/128	N9
9	*Anas crecca*	H7: 1/128	N9
10	*Aythya ferina*	H11: 1/256	N9
11	*Aythya ferina*	H11: 1/128	N9
12	*Aythya ferina*	H8:1/512	N4

In order to determine the agent in various clinical materials and diagnosing the disease, virus isolation and PCR methods are being used (Stallknecht *et al*., 1990; Alfonso *et al*., 1995; Fouchier *et al*., 2005; Munster *et al*., 2005; Gaidet *et al*., 2007; Alexander, 2008). As materials, tracheal swabs and feces samples from living animals, lung, liver, air vesicles, trachea, brain and pancreas from dead animals are being used (Chen *et al*., 2005; Munster *et al*., 2005; Capua and Alexander, 2007; Gaidet *et al*., 2007; Runstadler *et al*., 2007; Alexander, 2008). In this study fresh feces samples were used as material. Cattoli *et al*. (2004) compared one-step RT-PCR, real-time reverse transcriptase-polymerase chain reaction (RRT-PCR) and conventional virus isolation methods which they used in a study carried on 232 trachea swab samples collected from experimentally infected turkeys and chickens and turkeys during H7N3 outbreaks in Italy between October 2002 to September 2003. In both PCR methods, extraction commercial kits were used and M gene was targeted. Positivity was determined in 50 (21.6%) animals by one step RT-PCR and in 45 (19.4%) animals by RRT-PCR method. By culturing in embryonated eggs they isolated AI type A virus in 45 (19.4%) of the material. In typing with HI and NI tests, all isolates were determined as H7N3 subtypes. Wallensten *et al*. (2006) analyzed cloacal swab samples taken from 358 *Anas platyhynchos* and 203 *Tadorna tadorna* in spring of 2003 in Sweden by RT-PCR in order to determine the prevalence of AI type A viruses and found 3.4% positivity in the samples. Munster *et al*. (2006) collected over 27000 cloacal swabs from 1997 onwards, tested them by RT-PCR with respect to AI type A virus and determined 2.1% positivity.

They reported that most of the samples were collected from different regions of Netherlands and Sweden that the samples were taken from over 250 avian species the majority of which were ducks, geese, gulls and shorebirds and that positivity was found only in *Anser albifrons*, *Anser anser*, *Anas platyrhynchos*, *Anas penelope*, *Anas clypatea*, *Anas acuta*, *Anas crecca*, *Larus ridibundus* and *Uria alge* species. In another study (Gaidet *et al*., 2007), cloacal swabs collected from waterbirds in Nigeria, Chad, Mali, Malawi, Mauritania, Senegal, Kenya, Mozambique, Morocco, Sudan and Egypt were analyzed by RT-PCR and positivity was determined with respect to influenza A virus in 159 (3.5%) of the 4553 animals; positivity was found in 134 (4.6%) of 2864 ducks, 6 (1.4%) of 409 shorebirds, 3 (0.6%) of 438 rails, 14 (3.8%) of 366 gulls, 2 (1.2%) of 159 terns. All of 317 *Phalacrocorax carbo* were found to be negative. Nagy *et al*. (2007) in a study, carried out in

Czechoslovakia in 2006 in order to determine AI type A viruses, 2101 samples were collected from 61 bird species and analyzed by RT-PCR and culturing method by inoculating into ECE. Of the animals 12 (0.6%) were found RT-PCR positive and influenza A virus was isolated from 10 (0.5%) animals. In a study carried out in Canada on wild ducks in order to determine HPAI type A virus, cloacal swabs were collected from 4268 animals living in 56 different areas of 6 different geographical regions. 1572 (37%) of the ducks were found to be positive with respect to M1 gene by RRT-PCR analysis. Of the M1 positive samples tested by RRT-PCR with respect to H5 and H7 subtypes, 208 (4.9%) were positive with respect to H5 subtype but no positivity was determined with respect to H7 subtype. The highest positivity was determined in samples taken from *Anas platyrhynchos* with respect to both M1 and H5 (Parmley *et al*., 2008).

In this study, feces samples were collected during 37 months from 2013 animals consisting of 47 avian species in the Van Lake Basin, Turkey and 59 (2.9%) were found positive with respect to AI type A virus M2 by RT-PCR. When the number of positive samples and rates were considered the data obtained from this study confirmed with the results of some studies (Munster *et al*., 2006; Wallensten *et al*., 2006) the number and rate of positivity was higher than some (Nagy *et al*., 2007) and lower than some studies (Cattoli *et al*., 2004; Parmley *et al*., 2008). The reason the data were lower than some literature was that when Cattoli *et al*. (2004) carried out their study, there were frequent AI type A virus outbreaks in Italy and the flock they took samples from were infected. As to Parmley *et al*. (2008), they might have worked on wild ducks, the natural reservoir for the agent, reported to have been carrying a high rate of asymptomatic AI type A viruses (Sharp *et al*., 1993; Juckett, 2006; Capua and Alexander, 2007). In this study, the highest positivity determined in duck species Anas acuta (37.1%) and Anas penelope (22.5%) confirmed with the finding of the workers (Sharp *et al*., 1993; Juckett, 2006; Capua and Alexander, 2007).

On the other hand of the 17 avian species from which RT-PCR positive feces samples were collected 9 (52.9%) belonged to ducks of Anseriformes order, 2 (11.8%) to shorebirds of Charadriiformes order and one (5.9%) to Phoenicopterus ruber of Phoenicopteriformes order. This result was found to be significant with regard to indicating migratory birds role in carrying AI viruses (Ellis *et al*., 2004; Chen *et al*., 2006; Capua and Alexander, 2007).

Stallknecht *et al*. (1990) collected 1389 cloacal and tracheal swabs from ducks (605 *Anas discors*, 75 *Anas fulvigula*, 375 *Anas strepera* and 334 *Anas crecca*) between 1986-1987 in Louisiana and reported that the prevalence rates in ducks during September, November and December-January were 3.1, 2 and 0.4%, respectively and that the differences in prevalence were due to the seasonal and age group factors. In a study, carried out between February 2001 to February 2002 in Texas (Hanson *et al*., 2005) in February 2001, 2 (6.8%) AI viruses from 29 *Anas crecca*, 1 (50%) from 2 *Anas cyanoptera* and 7 (21.8%) from 32 *Anas discors* were isolated but AI virus was not isolated from 3 *Anas fulvigula* in August 2001 1 (3.3%) from 30 *Anas acuta* and in February 2002 11 (14.6%) from 75 *Anas discors* were isolated. The prevalence was reported to be 11 and 15% according to years. Wallensten *et al*. (2006) reported that they analyzed feces samples collected during the spring of 2003 in a survey on AI viruses in wild birds, from 358 *Anas platyrhynchos* and 203 *Tadorna tadorna* living in South Sweden. They determined 3.4% virus in samples collected during April-June and 6.5% in May from *Anas platyrhynchos* and the prevalence was below 1% in samples from *Tadorna tadorna*.

In this study of the 59 positive samples, 43 (72.9%) were collected in the first period covering January, February, March and April of 2006-2009; 12 (20.3%) in the third period covering September, October, November and December of 2006-2008; 4 (6.8%) were collected in the second period covering May, June, July and August of 2008. The highest positivity was determined in the samples collected during the winter and spring of 2006-2008 and this confirms with the view that the viruses mostly appear and spread in cold regions and seasons (Stallknecht *et al*., 1990; Hanson *et al*., 2005; Wallensten *et al*., 2006). PCR based methods are also used in order to determine the presence of H5 and H7 subtypes considered as HPAI viruses in various materials in their typing and sequence (Munch *et al*., 2001; Spackman *et al*., 2002, 2006; Nagy *et al*., 2007; Parmley *et al*., 2008). Ellis *et al*. (2004) reported HPAI H5N1 outbreaks in chicken and other gallinaceous poultry in 1997, 2001, 2002 and 2003 in Hong Kong and high rate of mortality. In a study carried out on dead ducks collected from a duck breeding farm in South Korea, the cause of mortality was found to be H5N1 virus (Kwon *et al*., 2005). Chen *et al*. (2006) reported H5N1 outbreaks among the wild birds in lake Quinghai-Chine between April-June 2005. The disease was reported to have spread to other bird populations of the lake. The sequence analysis of 15 viruses isolated from 6 avian species collected at different times during the outbreaks showed the presence of 4 different H5N1 genotypes. Parmley *et al*. (2008) in a study carried out in Canada in

2005 to determine the presence of HPAI viruses in wild ducks with RT-PCR, collected 4268 cloacal swab samples; 37% were found to be M1 protein positive, 5% H5 positive. They reported that the samples were not examined with respect to H7 gene. In this study of the M2 positive 59 samples 4 (6.8%) were found positive with respect to H5N1 by RT-PCR. H5N1 positivity was found in *Anas cylpeata,* a winter visitor. It confirmed with the results of the researchers (Chen *et al*., 2006; Parmley *et al*., 2008). Since, the determination was done from feces a clinical assessment was not made in *Anas cylpeata*.

In order to determine the subtypes of AI type A viruses in avian species, mostly conventional HI and NI tests are being used (Cattoli *et al*., 2004; Wallensten *et al*., 2006; Runstadler *et al*., 2007; Parmley *et al*., 2008). It is reported that all hemaglutinin subtypes including H5 and H7 were isolated from migratory birds, particularly from Anseriformes order (Spackman, 2008; Webster *et al*., 2006; Kida, 2008).

In this study also, typing was done by HI and NI tests using antisera provided from two different reference labs. All of the isolates belonged to ducks from Anseriformes order and 3 were typed as H1N7, 2 as H7N9, 2 as H11N9 and 1 as H8N4. Isolates typed as H7 were determined in feces samples of *Anas crecca.* And 4 isolates isolated from *Anas clypeata* gave positive reactions both with H1 and H5 antisera and all were typed as N1. These, 4 isolates determined as H5N1 in RT-PCR and in agarose gel electrophoresis. Therefore further studies were concluded to be done about these isolates including pathogenicity tests. In addition, this confirmed with the results of the researchers who have indicated that isolates can not always be typed by HI and NI tests (Alfonso *et al*., 1995; Fouchier *et al*., 2005; Runstadler *et al*., 2007).

CONCLUSION

In this study of the 2013 feces samples collected from 47 avian species covering irregular vagrant, transit migrant, winter visitor, migratory and native birds living in Van Lake Basin an important region for migratory birds, 59 (2.7%) were AI type M2 positive with RT-PCR. When the status of the animals is considered, the highest positivity was in winter visitors and lowest positivity in transit migrant birds. When the season is considered, winter and spring were found to be the most risky periods with respect to the spreading of the agent. On the other hand, positivity was determined by RT-PCR in avian species sharing the same place and time. When the literature is examined it will be seen that the presence of these subtypes in duck species has been determined for the first time in Turkey. As a result, this study is the first serious comprehensive study carried out concerning AI type A viruses in Turkey and is expected to contribute to similar studies to be done in the future at home and abroad in various ways, particularly epidemiological studies.

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