

Newcastle Disease and Avian Influenza A Virus in Migratory Birds in Wetland of Boushehr-Iran

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Abstract: Wild birds are considered to be the natural reservoir of Newcastle Disease Virus (NDV) and Avian Influenza virus (AI) and are often suspected to be involved in outbreaks in domesticated birds. The objective of the present study was to determine ND and AI infection in migratory birds in the south of Iran in order to detect the possible source of these viruses to domestic poultry. A total of 443 fecal specimens (fresh dropping and cloacal swabs) were collected from migratory and wild resident birds in the Bushehr wetlands from October 2009 to June 2010. AI virus was isolated from 3 out of 443 samples processed for virus isolation and confirmed by reverse transcriptase chain reaction (RT-PCR). NDVs were isolated from 22 (fresh fecal) samples and were identified as avian paramyxovirus-1 by the results obtained from the HI test with NDV-specific antibodies and RT-PCR-method. Mortality related to NDV was reported in some chicken flocks in the south of Iran. These results, as well as other data from the literature indicate that wild birds play a minor role as a potential disseminator of NDVs and AIVS. This study is the first report of NDV and AIV isolation from migratory and resident birds in the wetlands of Boushehr-Iran. In addition, our findings support the notion that wild aquatic and migratory birds may function as a reservoir for AIV and NDV in the south of Iran.

Key words: Avian influenza virus, H9, Iran, migratory birds, newcastle disease virus, reverse transcriptase chain reaction

INTRODUCTION

Avian influenza (AI) and Newcastle disease (ND) are two of the most devastating diseases of poultry, domestic and migratory birds throughout the world and are caused by type A orthomyxoviruses and type 1 avian paramyxoviruses, respectively. (Alexander., 1995; Manvell *et al.*, 2000). Newcastle disease has a worldwide distribution and is caused by NDV, which is the sole member of avian *paramyxovirus* type 1 (APM-1) belonging to the *Avulavirus* genus of the *Paramyxoviridae* family (Choi *et al.*, 2008). Newcastle disease virus has a negative-sense, single-stranded RNA genome of about 15 kb (Choi *et al.*, 2008). NDV isolates are further categorized according to pathogenicity in chickens into velogenic, mesogenic and lentogenic strains (Liu *et al.*, 2008). ND has continued to cause serious losses to the poultry industry, and is defined as a list A disease by the office international des Epizootic A (Lee *et al.*, 2004). About 8000 species of birds seem to be susceptible to infection with Newcastle Disease Viruses (NDVs). A wide range of avian and non avian species act as reservoirs of NDV and transmit the disease to susceptible birds (Roy *et al.*, 1998). Although there is a

long history of NDV recovered from wildlife (Kawamura *et al.*, 1987; Linclon *et al.*, 1998; Ojeh and Okoro, 1992), most of the isolates have not been extensively characterized, except in the case where virulent NDV from migrating cormorants caused an outbreak in turkeys in North Dakota in 1992 (King, 1996). Recovery of low virulence NDV isolates from waterfowl have been reported from 1 to 5% of waterfowl sampled in Wisconsin from 1978 to 80 (Vickers and Hanson, 1982b) to 13% of teals sampled during 2002 (Hanson *et al.*, 2005; Douglas *et al.*, 2007). In Iran, eight teals (*Anas cercca*) died several days to lowing capture and NDV was isolated from all eight birds (Bozorgmehrfard and Keyvanfar., 1979). NDV was isolated from ostriches during a two year period from 2008 to 2010 in Iran (Momayez *et al.*, 2007).

AIV is a member of the family *Orthomyxoviridae* which includes influenza A, B, C and Thogoto virus (Douglas *et al.*, 2007). AI is a contagious viral disease and is worldwide in distribution. It is believed that wild living water birds, particularly wild waterfowls, are natural reservoirs of all avian influenza viruses (Račnik *et al.*, 2008; Wallensten, 2006). All known subtypes of influenza A viruses have been isolated from wild birds living in

Table 1: List of birds' family, species, location of sampling and assay results

Family	Species	Location	Assays results							
			No. of samples		HA positive		RT_PCR			
			Fecal	Cloacal swab	No	sample	H5	H7	H9	NDV
Anatidae (Ducks)	Shelduck (<i>Tadorna Tadorna</i>)	Mond & Helleh	65	42						
	Wigeon (<i>Anas Penelope</i>)									
	Mallard (<i>Anas Platyrhynchos</i>)				1	Cloacal swab				+
	Common teal (<i>Anas Crecca</i>)									
	Common pochard (<i>Aythya ferina</i>)									
Ardeidae	Great white Egretta (<i>Casmerodius albus</i>)	Mond & Helleh		10						
Scolopacidae	Bar-tailed Godwit (<i>Limosa lapponica</i>)	Mond &		14						
	Eurasian Curlew (<i>Numenius arquata</i>)	Bushehr								
Rallidae	Eurasian Coot (<i>Fulica Atra</i>)	Kabgan area	24							
Otididae	Houbara (<i>Chlamydotis undulata</i>)	Mond & Helleh	23	25						
Burhinidae	Stone Curlew (<i>Burhinus oedicnemus</i>)	Mond & Bordekhoon	87							
Laridae	Slender-billed Gull (<i>Larus genei</i>)	Bandargah	43	10	2	Fecal				+
Sternidae	Caspian Tern (<i>Sterna caspia</i>)	Nakhilo,								
	Great Crested Tern (<i>Sterna bergii</i>)	Omolkarm,								
	Lesser Crested Tern (<i>Sterna bengalensis</i>)	kharkoo islands	80	17	22	Fecal				+
	Bridled Tern (<i>Sterna anaethetus</i>)									

aquatic environment. The first documented outbreak of Highly Pathogenic Avian Influenza (HPAI) in the wild bird population was in 1961, when an outbreak in common Terns (*sterna hirundo*) killed about 1600 birds in South Africa (Becker, 1966). This indicates that migratory birds surveillance for influenza A virus can be of major value as a sentinel system to prevent outbreaks in domestic poultry (Wallensten, 2006). The influenza A virus, including all its subtypes and most of their subtype combinations, is commonly found in aquatic birds such as ducks, geese, gulls, and shorebirds, while only a limited number of subtypes have been found in non avian hosts. Therefore, waterfowl, in particular wild dabbling ducks, are believed to constitute the main natural viral reservoir for Low Pathogenic Avian Influenza Virus (LPAIV), from which strains occasionally arise that are transmitted to other species, including humans and poultry. The objective of the present study was to determine NDV and AIV infection in migratory and resident birds in the wetlands of Boushehr province of Iran, which is close to some major broiler and layer breeder areas during the avian migratory season from October 2009 to June 2010.

MATERIALS AND METHODS

The samples of present research collected from wetlands of Boushehr province in south of Iran in 2009-2010 (Table 1).

A total of 443 fecal specimens (212 fresh droppings from migratory birds and 110 fresh droppings from wild resident birds and 121 cloacal swabs from migratory birds) were collected. Some birds were trapped and for bird species that could not be trapped, fresh dropping samples were collected from the ground at locations where large numbers of birds congregate. Fecal samples of both migratory and resident birds were collected from several sites in Boushehr province, which is known for the arrival of migratory birds during the avian migratory season (Table 1). Only fresh and wet samples were collected. Cloacal swabs and fresh dropping samples were

collected using cotton swabs and immediately put in vials containing virus transport media (Hanks balanced salt solution containing 0.5% lactalbumin, 10% glycerol, 200 U/mL penicillin, 200 µg/mL streptomycin, 200 µg/mL polymyxin B sulfate, 250 µg/mL gentamycin, and 50 U/mL nystatin on wet ice and then frozen to -70°C). Virus isolation followed standard procedures for AIV and NDV as described. All of the samples were processed for virus isolation in embryonated Specific Pathogen Free (SPF) chicken eggs and were tested by RT-PCR.

Virus isolation and characterization: Virus isolation and characterization of the samples collected were performed at the Razi Vaccine and Serum Research Institute, Shiraz, Iran. 200 µL of the original specimens were inoculated into the allantoic cavity of 9 to 11-day-old SPF chicken eggs according to the method described by Swaney *et al.* (1998). The aminoallantoic fluids were harvested and analyzed for HA (Swaney *et al.*, 1998). When the HA titers were negative the allantoic fluids were passaged once again in embryonated chicken eggs. Hemagglutination inhibition (HI) assay was used on HA positive allantoic fluids for virus isolates subtyping, and was performed using H5, H7 and H9 subtypes specific reference influenza and ND anti-sera obtained from Istituto Zooprofilattico Sperimentale delle Venezie (Swaney *et al.*, 1998). Samples were collected from different species with the majority of the samples originating from ducks, geese, gulls, Ardeidae and common fowls (Table 1).

Pathogenicity test: Avian paramyxovirus-1 isolates were tested for pathogenicity by Mean Death Time (MDT) in embryonated SPF eggs according to the guidelines of the standard procedures provided the World Organization for Animal Health (OIE).

Mean Death Time (MDT) in eggs: Fresh and sterile allantoic fluids were diluted in sterile saline to give a tenfold dilution series between 10⁻⁶ and 10⁻⁹. For each

dilution, 0.1 mL was inoculated into the allantoic cavity of each of five 10-day-old embryonated SPF fowl eggs, which were then incubated at 37°C. Each egg was examined twice daily for 7 days and the times of any embryo deaths were recorded. The MDT is the mean time in hours for the minimum lethal dose to kill all the inoculated embryos and has been used to classify NDV strains into the following groups: velogenic (taking under 60 h to kill); mesogenic (taking between 60 and 90 h to kill); and lentogenic (taking more than 90 h to kill).

RNA extraction: RNA was extracted from all amnio-allantoic fluid samples using viral Gene-spin™ viral DNA/RNA extraction kit (Vetek, Korea) following manufacturer's instructions.

Polymerase chain reaction for NDV: To identify NDV by RT-PCR, a set of primers according to Mohamed *et al.* (2005) nd1 sequence of: 5-GCAGCTCGA GGGATTGTGGT-3 nucleotide position 158-177, nd2 have the reverse sequence of :5-TCTTTGAG CAGGAGGATGTTG-3 nucleotide position 513-493, that flanks the region encompassing the cleavage site of the fusion protein gene (F), was used in the study (Mohamed *et al.*, 2005). The expected size of the PCR product was 356 bp.

RT-PCR amplification: Titan one tube enzyme mix system was used to prime the synthesis of the first strand cDNA and to perform PCR in one step. The reaction mixture (5 µL of the sample RNA extract, 1 µL from each primer of the first pair, 2 µL of dNTPs, 2.5 µL DDT, 0.5 µL. Rnase inhibitor, 10 µL of 5x PCR buffer, 4 µL magnesium chloride, 1 µL titan enzyme mix and 23 µL of ddH₂O were pipette in a 0.5 PCR tube. The tube was then incubated at 50°C for 30 min for reverse transcription, then cycled 40 times at 94°C for one min, 52°C for one min at 68°C for one min, and finally incubated at 68°C for 10 min.

Polymerase chain reaction for AIV: To identify avian influenza virus by RT-PCR two primers based on the conserved sequence of the NP the gene of the viruses influenza were used (Lee *et al.*, 2001). The NP specific primers used were: NP1200 (forward): 5-CAG (A/G) TACTGGGC (A/T/C) ATAAG (A/G) AC-3 and NP1529 (reverse): 5- CATTGTCTCCGAAGAAATAAG-3 (Lee *et al.*, 2001). Each positive reaction was tested for H9, H7 and H5. To detect specific subtypes of AIV by RT-PCR, 3 sets of primers, each based on conserved sequences of a single HA subtype were used (Lee *et al.*, 2001). Samples were amplified by one-step RT-PCR. RT-PCR was carried out as previously described (Lee *et al.*, 2001) in a reaction mixture (25 µL) containing 2.5 µL of 10-times the reaction buffer (Promega, Madison, WI), 2.5 µL

dNTP blend (2.5 mM each of four dNTPs, Promega), 0.2 µL AMV reverse transcriptase (9 units/µL, Promega), 0.3 µL RNase inhibitor (40 units/µL, Promega), 0.5 µL Taq DNA polymerase (9 units/µL, Promega), 1 µL of each primer (10 pmol each), 1 µL of RNA template (about 1 ng), and 17 µL of water. The PCR condition for the amplification of H5, H7 and H9 was 42°C for 45min (reverse transcription), 95°C for 3 min, 35 cycles of 95°C for 30 s (denaturation), 50°C for 40 s (annealing) and 72°C for 40 s (extension), followed by 72°C for 10 min (final extension) (Lee *et al.*, 2001).

Detection of PCR products: PCR products were separated in 1.5% agarose gel in 1×TAE buffer stained with ethidium bromide, compared with the molecular mass marker and visualized by ultraviolet (UV) transillumination.

RESULTS AND DISCUSSION

From October 2009 to June 2010 fresh dropping samples and cloacal swabs were collected from migratory and resident birds in the wetlands of Boushehr in Iran, and were tested for the presence of NDV and AIV by virus isolation and RT-PCR. According to virological and molecular study NDV and AIV were isolated from 22 and 3 samples respectively in the wetlands and islands of Boushehr province which is situated in the south of Iran. All NDVs were obtained from fecal samples in Omolkarm island which, is situated to the south of Boushehr province. NDVs were isolated from 22 (fresh fecal) samples and were identified as AMPV-1 by the results obtained from the HI test with NDV-specific antibodies and RT-PCR-method. All 22 SPF chicken embryo NDV isolates pathotyped in this study had MDT that put them within the range of lentogenic strains (>100 h). The specific 356 bp PCR products visualized on ethidium bromide-stained agarose gel were obtained from 22 infective allantoic fluids samples from migratory birds. Efforts were made to ensure the majority of these samples were collected at different locations in the Boushehr province. Avian influenza virus was isolated from 3 out of 443 samples processed for virus isolation and confirmed by RT-PCR. Positive HI results were shown only by the specific serum against H9. Specific serum against H5 and H7 were unable to inhibit HA activity of the virus. RT-PCR was done with H5, H7 and H9 and expected amplification of 488 bp with H9 subtype specific viruses of the H9 subtype were obtained from two resident birds and the other from migratory birds. The first and second H9 positive case was a stenderbilled Gull (*Larvs genei*) from a resident bird in the Helleh wetland, and the third case was a Mallard (*Anas platyrhynchos*) hunted on the Helleh Welland (Mullarney *et al.*, 2010). No samples were found positive for H5 and H7 by the cloacal swabs

in any of the wetlands in Boushehr province. This study is the first report of NDV and AIV isolation in migratory and resident birds in the South of Iran. The susceptibility of migratory birds to infection with NDV and AIV has resulted in speculation about the role of these free flying birds in the origin and transmission of the viruses infection (Vickers and Hanson, 1982a), although the mobility of migratory birds and their population size makes them an important and not easily controllable vector of NDV dissemination (Zenetti *et al.*, 2005).

The NDV infection in wild birds, especially aquatic species, is often asymptomatic and they are therefore considered to be a reservoir of the virus in the wild (Oreshkova *et al.*, 2009) so wild birds are often suspected of being involved in outbreaks in domesticated birds. Oreshkova stated that, out of the 4527 submitted samples from wild and domestic bird during 2005-2007 in Bulgaria, NDV has been detected in 160 of them (Oreshkova *et al.*, 2009). This is the first report of lentogenic NDV in wild birds in the south of Iran. Although outbreaks of NDV in wild birds and aquatic birds are rare, but in recent years mortality related to NDV has been reported in some chicken flocks in the south of Iran. These results, as well as other data from the literature indicate that wild birds play a minor role as a potential disseminator of NDVs (Krapež *et al.*, 2010). Waterfowl are often regarded as a potential reservoir of NDV infection. Although NDV strains are infectious to waterfowl, they cannot cause clinical symptoms. Most of the NDV strains isolated from migratory waterfowl were proven to be lentogenic strains, and even some virulent strains were proven to be avirulent for the original hosts (geese) (Liu *et al.*, 2008). The role of AIV reservoirs in wild aquatic birds is not fully understood and measures to control the spread of AIV are very difficult to monitor in the aquatic bird populations; therefore, understanding the ecology of AIV in these population is necessary to minimize the impact of future AI outbreaks. Oslen stated that LPAI viruses can be found in numerous bird species (Oslen *et al.*, 2006), but it is unclear in which of these species influenza viruses are endemic and in which the virus is a temporary pathogen. All influenza virus subtypes and most HA/NA combinations have been detected in the bird reservoir and poultry, whereas relatively few have been detected in other species. Although many wild bird species may harbor influenza viruses, birds of wetlands and aquatic environments constitute the major natural LPAI virus reservoir. LPAI viruses have been isolated from at least 105 wild bird species of 26 different families (Oslen *et al.*, 2006). Influenza A H9N2 viruses have been detected worldwide in poultry, and currently are endemic poultry in Asia (Li *et al.*, 2005; Xu *et al.*, 2005). During 1998-2000, H9N2 viruses were reported in Middle Eastern countries and were responsible for widespread and serious disease

in commercial chickens in Pakistan (Naeem *et al.*, 1999), Iran (Nilli and Asasi, 2002; 2003), the United Arab Emirates (Manvell *et al.*, 2000) and Saudi Arabia (Banks *et al.*, 2000). Numerous infections of poultry and other birds with the subtype H9 during 1995 originated from separate introductions from feral and migratory birds (Banks *et al.*, 2000). In the north of Iran five subtypes of avian influenza virus, H3N8, H7N3, H8N4, H9N2 and H10N7 were isolated from migratory birds during a surveillance campaign in 2003-2004 (Fereidouni *et al.*, 2005). In the autumn and winter 2003 and 2004, 472 fecal samples were collected from migratory birds in the north of Iran, and avian influenza viruses were detected from the samples (Fereidouni *et al.*, 2005). Fereidouni reported that avian influenza viruses can easily reach the wetlands of Iran from Siberia, and that these viruses could perpetuate in water fowl in this region during autumn and winter (Fereidouni *et al.*, 2005). So Iran is one route of migratory wild and local wild and feral birds. Holdings where wild birds and domestic birds share the same habitat due to agricultural practices are at the highest risk for outbreaks (Gillbert *et al.*, 2006), suggesting that wild bird transmission is the most common route. An important result is that wild bird surveillance may be a tool for obtaining strains of the influenza virus that can be used for vaccine development as well as diagnostic tests and reagents, as they are indeed similar to outbreak strains. Although HPAI can threaten public health, fortunately, no HPAI were isolated during the period of this study. But a LPAI outbreak in a poultry farm could cause a large economic loss for the poultry industry, so it is not unreasonable to expect that LPAI would be transmitted to poultry farms in Iran. The current increased interest in influenza virus surveillance in wild and domestic birds provides a unique opportunity to increase our understanding not only of HPAI epidemiology, but also of the ecology of LPAI viruses in their natural hosts, at the same time and for the same cost. This is the first study on migratory and resident birds in the south of Iran. Although our findings support a circulation of H9N2 subtype in migratory birds, it seems further samples of migratory birds are necessary to more fully understand the ecology of influenza virus in migratory birds. In conclusion, our findings demonstrate that Orthomyxoviruses and paramyxoviruses are present in the sampled population of wild aquatic birds in the Boushehr province and might act as a reservoir for these viruses.

CONCLUSION

In this study, ND viruses of low virulence and AI viruses of nHPAI isolated from migratory and resident aquatic wild birds in wetlands of Boushehr province. All AI virus isolates were identified H9. The HPAI viruses previously found in north of Iran were not detected in this

study. The isolated ND viruses and AI viruses of strains may be use for development of vaccines and diagnostic test. The results indicated that mallard, and Gulls which possesses huge population size and world wide distribution, could be considered one of the most important natural carrier of AIV and NDV and may have more important ecological significance on viruses transmission than other species of wild and domestic birds. The current increase interested in influenza virus surveillance in aquatic wild birds a unique opportunity to increase our understanding not only of HPAI epidemiology but also the ecology of LPAI viruses in their natural hosts, at the same time and cost.

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