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

1M.J. Mehrabanpour, 2P.D. Fazel, 1A. Rahimian, 1M.H. Hosseini, 3H. Moein and 2M.A. Shayanfar
1Department of Virology, Razi Vaccine and Serum Research Institute Shiraz, Iran
2Islamic Azad University Jahrom Branch, Jahrom, Iran
3Bushehr Enviroment Protection Office, Bushehr, Iran

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 paramyxomyxovirus-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 I(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 cerca) died several days to lowing capture and NDV was isolated from all eight birds (Bozorgmehrifard 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

<table>
<thead>
<tr>
<th>Family</th>
<th>Species</th>
<th>Location</th>
<th>No. of samples</th>
<th>Assays results</th>
<th>RT-PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anatidae (Ducks)</td>
<td>Shelduck <em>(Tadorna tadorna)</em></td>
<td>Mond &amp; Helleh</td>
<td>65</td>
<td>Cloacal swab</td>
<td>+</td>
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<tr>
<td></td>
<td>Wigeon <em>(Anas penelope)</em></td>
<td>Mond &amp; Helleh</td>
<td>65</td>
<td>Cloacal swab</td>
<td>+</td>
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<tr>
<td></td>
<td>Mallard <em>(Anas platyrhynchos)</em></td>
<td>Mond &amp; Helleh</td>
<td>65</td>
<td>Cloacal swab</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Common teal <em>(Anas crecca)</em></td>
<td>Mond &amp; Helleh</td>
<td>65</td>
<td>Cloacal swab</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Common pochard <em>(Aytha ferina)</em></td>
<td>Mond &amp; Helleh</td>
<td>65</td>
<td>Cloacal swab</td>
<td>+</td>
</tr>
<tr>
<td>Ardeidae</td>
<td>Great white Egret <em>(Casmerodius albus)</em></td>
<td>Mond &amp; Helleh</td>
<td>10</td>
<td>Cloacal swab</td>
<td>+</td>
</tr>
<tr>
<td>Scolopacidae</td>
<td>Bar-tailed Godwit <em>(Limosa lapponica)</em></td>
<td>Bushehr</td>
<td>10</td>
<td>Cloacal swab</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Eurasian Curlew <em>(Numenius arquata)</em></td>
<td>Bahman area</td>
<td>10</td>
<td>Cloacal swab</td>
<td>+</td>
</tr>
<tr>
<td>Rallidae</td>
<td>Eurasian Coot <em>(Fulica atra)</em></td>
<td>Mond &amp; Helleh</td>
<td>24</td>
<td>Cloacal swab</td>
<td>+</td>
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<tr>
<td>Otulidae</td>
<td>Houbara <em>(Chlamydotis undulata)</em></td>
<td>Mond &amp; Helleh</td>
<td>23</td>
<td>Cloacal swab</td>
<td>+</td>
</tr>
<tr>
<td>Burhinidae</td>
<td>Stone Curlew <em>(Burhinus oedicnemus)</em></td>
<td>Mond &amp; Helleh</td>
<td>87</td>
<td>Cloacal swab</td>
<td>+</td>
</tr>
<tr>
<td>Laridae</td>
<td>Slender-billed Gull <em>(Larus genei)</em></td>
<td>Bandargah</td>
<td>43</td>
<td>Cloacal swab</td>
<td>+</td>
</tr>
<tr>
<td>Sterniidae</td>
<td>Caspian Tern <em>(Sterna caspia)</em></td>
<td>Nakhil</td>
<td>10</td>
<td>Cloacal swab</td>
<td>+</td>
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<tr>
<td></td>
<td>Great Crested Tern <em>(Sterna beringi)</em></td>
<td>Omolkharm, Bandargah</td>
<td>25</td>
<td>Cloacal swab</td>
<td>+</td>
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<tr>
<td></td>
<td>Lesser Crested Tern <em>(Sterna bengalensis)</em></td>
<td>kharkoo islands</td>
<td>25</td>
<td>Cloacal swab</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Bridled Tern <em>(Sterna anaethetus)</em></td>
<td>Mond &amp; Helleh</td>
<td>25</td>
<td>Cloacal swab</td>
<td>+</td>
</tr>
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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 Swanye et al. (1998). The aminoallantoic fluids were harvested and analyzed for HA (Swanye 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 (Swanye 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 10⁻⁸. For each
To identify NDV by RT-PCR, a set of primers according to Mohamed et al. (2005) were used. The expected size of the PCR product was 356 bp.

**Polymerase chain reaction for NDV**: To identify NDV by RT-PCR, a set of primers according to Mohamed et al. (2005) were used. 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 titran enzyme mix and 23 µL of ddH2O were pipette in a 0.5 PCR tube. The tube was then incubated at 50ºC for 30 min for reverse transcription, then cycled 40 times at 94ºC for one min, 52ºC for one min at 68ºC for one min, and finally incubated at 68ºC for 10 min.

**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 agaros 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 Wetland (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 measured 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 birds 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). Feridouni reported that avian influenza viruses can easily reach the wetlands of Iran from Siberia, and that these viruses could perpetuate in waterfowl 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 finding 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 is 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 viruses 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 to 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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