Serological Monitoring of Avian Influenza in Migratory Birds of Iran

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Summary

Although migratory waterfowl is well known to be a major reservoir for avian influenza virus (AIV), there are only few recently published reports about the seroprevalence of AIV in this group of birds. To investigate the AIV antibody status in migratory waterfowl of Iran, we collected 217 serum samples from 25 different species of waterfowl during 2003 and 2004. These serum samples were tested by a competitive ELISA. 77 samples from 14 different species were positive (35.5%). Interestingly, the seroprevalence of antibodies against type A influenza viruses was significantly higher in Anseriformes (64%) than in Non-Anseriformes (12%) and in total birds (35.5%). Our results show furthermore that mallards which winter in Iran in large numbers and show 87.5% positive reactions might play an important role in the epidemiology of influenza virus in this region.

Key words: Migratory birds, Avian Influenza Virus (AIV), Serology, Influenza

Introduction

Wild birds, especially ducks, geese and swans of the order Anseriformes, are the natural reservoir for avian influenza viruses (Stallknecht 1997). Avian influenza

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virus (AIV) belongs to influenza virus type A, which can be classified further by 16 haemagglutinin (HA) and 9 neuraminidase (NA) subtypes. All of them have been detected in different combinations in wild birds (Hinshaw *et al* 1980). Many infected birds react with the development of antibodies but remain without clinical symptoms. Most influenza A viruses from wild birds are low pathogenic for domestic poultry. Nevertheless, AIV-infected wild birds may be a real threat for poultry and the poultry industry. If they come in contact with e.g. free-ranging poultry, they may spread the infection to poultry by virus-contaminated excretions. Infections of poultry with even low pathogenic avian influenza (LPAI) viruses can produce economic losses by causing egg drop syndrome and reduced weight gain. Furthermore, there is a great danger for virus mutations caused by fast and repeated animal passages. After introduction of H5 or H7 AIV subtypes into the poultry population, virus mutations can lead to highly pathogenic forms of the infection (HPAI, fowl plague). Transmission of LPAIV of these two subtypes into regions with a high poultry density by wild birds and subsequent mutation to HPAIV have occurred in different countries around the world over the past few years. Such epidemics were observed in Italy in 1999, in the Netherlands in 2003, in Canada in 2004 and in many countries during 2003 to 2005 with predominantly fatal consequences for economy and the human population of the country.

The aim of our study was therefore to investigate the occurrence of AIV infections in wild birds in the Western Asian countries and Middle East region, where availability of this information is very limited. The results of such investigations are very important for risk management and the protection of especially free-ranging poultry.

Materials and Methods

Sample collection. During autumn/winter of 2003 and spring of 2004 a total of 217 blood samples were collected from 7 different orders and 25 species including

Anseriformes: mallard (*Anas platyrhynchos*), wigeon (*Anas penelope*), teal (*Anas crecca*), pintail (*Anas acuta*), pochard (*Aythya ferina*), gadwall (*Anas strepera*), shoveler (*Anas clypeata*), greylag goose (*Anser anser*); **Gruiformes:** coot (*Fulica atra*); **Charadriformes:** black-headed gull (*Larus ridibundus*), slender-billed gull (*Larus genei*), little gull (*Larus minutus*), yellow-legged gull (*Larus michahellis*), avocet (*Recurvirostra avocetta*), black-tailed godwit (*Limosa limosa*), redshank (*Tringa totanus*); **Ciconiiformes:** little egret (*Egretta garzetta*), grey heron (*Ardea cinerea*); **Pelecaniformes:** great cormorant (*Phalacrocorax carbo*); **Phoenicopteriformes:** greater flamingo (*Phoenicopterus ruber*) and **Podicepediformes:** great-crested grebe (*Podiceps cristatus*), little grebe (*Podiceps ruficollis*), black-necked grebe (*Podiceps nigricollis*). Serum samples were stored at −20°C until tested.

Area description. Sampling areas were the most important wetlands of Iran, most of them Ramsar wetlands of international importance (Figure 1). They represent important wintering sites for migratory waterfowl in Iran and are located in 7 different provinces.

Figure 1: *Map of Iran with main sampling areas (blue circles).*

cELISA. Recombinant baculovirus-derived nucleoprotein (RBV-NP) of avian influenza virus was used as antigen. The antigen was diluted, applied on 96-well microtiter plates and incubated overnight at 4°C. Wells were then washed three times with PBS containing 0.05% Tween 20 (PBST). Positive and negative control sera and wild bird sera diluted 1:11 in PBST, were placed in duplicate wells and incubated for 60 minutes at 37°C. Following the washing of the plates, the monoclonal antibody (HB 65) supplied from ATCC diluted in PBST, was added and plates were incubated again under the same conditions. Wells were washed and then the second antibody (goat anti-mouse conjugate, POD-labelled) was added for 60 minutes at 37°C. After final washing, substrate o-phenylendiamine (OPD) in citric acid buffer containing 10 µl H_2O_2 per 10 ml solution was used to develop the colour reaction. The reaction was stopped by 2N H_2SO_4 after 30 minutes, and the optical density of all reactions was measured at 492 nm. Thirty percent inhibition of the monoclonal antibody reaction alone was estimated to be the cut-off value.

Results

The overall seroprevalence against type A influenza viruses of all sampled birds was 35.5% (i.e. seventy seven samples were positive out of total 217 birds). Orders and species with positive samples include Anseriformes (mallard, teal, shoveler, pochard, pintail, greylag goose); Charadriformes (black-headed gull, avocet, redshank); Ciconiiformes (great egret, little egret); Phoenicopteriformes (greater flamingo); Pelecaniformes (great cormorant); Gruiformes (coot). In total, birds from fourteen out of 25 species carried antibodies against AIV (Table 1).

Table 1. *The frequency of influenza A antibody in different migratory birds in Iran during 2003-2004.*

Test result / Bird	Tested	Positive	Negative
Avocet	1	1	0
Black-headed Gull	10	4	6
Black-necked Grebe	1	0	1
Black-tailed Godwit	2	0	2
Coot	66	1	65
Great Cormorant	7	1	6
Greater Flamingo	3	3	0
Gadwall	1	0	1
Greylag Goose	2	2	0
Grey Heron	4	0	4
Great Egret	2	1	1
Great-crested Grebe	3	0	3
Lapwing	5	0	5
Little Egret	2	1	1
Little Grebe	4	0	4
Little Gull	1	0	1
Mallard	32	28	4
Pintail	11	5	6
Pochard	4	1	3
Redshank	4	2	2
Shoveler	6	5	1
Slender-billed Gull	1	0	1
Teal	41	22	19
Wigeon	2	0	2
Yellow -legged Gull	2	0	2
Total	217	77	140

The seroprevalence of antibodies to influenza A viruses was significantly higher in Anseriformes than in birds in total (64% *versus* 35.5% respectively) and in Non-Anseriformes (12%). Among the Anseriformes, mallards are the species with the highest percentage of antibody-positive animals (85.7%), followed by teals (53.7%) (Table 2).

Table 2. *Number of tested serum samples and negative and positive results for total birds, Anseriformes, non-Anseriformes and the 2 most important species of ducks.*

Test results / Birds	No. Samples	Negative	Positive	% Positive
Total birds	217	140	77	35.5
Anseriformes	99	36	63	63.6
Non- Anseriformes	118	104	14	11.9
Mallard	32	4	28	87.5
Teal	41	19	22	53.7

Discussion

The role of wild birds, especially of waterfowl, as main reservoir for avian influenza viruses of all subtypes has been well known for a long time (Alexander 2003, De Marco *et al* 2003). However, no really global data are available on the incidence of AIV-infections among wild birds, and their distribution among different orders and species. There are also different papers and different opinions about the best and most reliable method for evaluation of antibodies in these birds (Arenas *et al* 1990, Astorga *et al* 1994, De Macro *et al* 2003, Fouchier *et al* 2003, Graves 1992, Pfitzer *et al* 2000, Sala *et al* 2003, Shafer *et al* 1998). Based on preliminary studies (Starick, unpublished data) it is known that agar gel precipitation (AGP) test is not sensitive enough to detect antibodies in the Anseriformes and shore birds. Only limited information is available about the sensitivity of the haemagglutination inhibition (HI) assay in these species. In a recent publication, only 16.9% of wild duck sera tested positive in a double antibody sandwich blocking ELISA also were positive in HI assay (De Marco *et al* 2004). Competitive ELISA on the basis of recombinant AIV-NP antigen seems to be a valid and reliable method for testing different

wild bird serum samples for avian influenza infection (Shafer *et al* 1998, Zhou *et al* 1998).

In our investigation, the overall seroprevalence of antibodies to influenza virus was 35.5% with a seroprevalence rate of 64% in Anseriformes. Indeed, sixty three out of 77 positive birds belong to this order. This data shows the possible importance of these birds in the epidemiology of avian influenza. Interestingly, in a recent investigation on the avian influenza seroprevalence in Italian waterfowl, 52.2% (ranging from 39.2% to 73.7% in different years) of the ducks and 7.1% (ranging from 1.3% to 17.7% in different years) of the coots were positive (De Marco *et al* 2003, De Marco *et al* 2004). In our investigation presented here, mallards with 85.7% and teals with 54% positive reactions were the species with the highest rate of positive animals. Although the sample size for three other members of this order was small, the results are interesting. Two out of 2 greylag geese, five out of 6 shovelers and five out of 11 pintails were antibody-positive.

Although positive samples among Non-Anseriformes (only 12%) were found in 5 different orders, some species such as greater flamingo (3 tested /3 positive) and black-headed gull (10 tested / 4 positive) showed more noteworthy results.

The aim of our study was to obtain first information about the occurrence of AIV-antibodies in resident and migratory wild birds in Iran. The identified overall seroprevalence of 36.5% for all birds and the predominant role of Anseriformes with 85.7% seropositive animals correlate well with the latest data from Europe. The high percentage of seropositive mallards but also teals should be discussed considering that they represent a high percentage of the migratory wild bird population wintering in Iran. These first results, together with further investigations on the serological and virological status of wild birds in the region contribute to increasing the knowledge on AIV infections and also to improving the risk assessment with regard to the poultry industry.

In conclusion, Wild waterfowl, especially mallards and teals, may play a major role in the epidemiology of avian influenza in Iran, West and Central Asia and Middle East. Besides these two species, several others such as shoveler, flamingo, greylag goose and black-headed gull showed very strong reactions in our tests. For a more detailed understanding of their role, however, more samples from these species are required.

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