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Surveillance of Avian Influenza Viruses in Wild Birds in Areas Adjacent to Epicenter of an out Break in Federal Capital Territory of Pakistan

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Abstract: Influenza is a highly contagious acute respiratory disease of avian origin that has caused epidemics and pandemics in humans for centuries. Among these avian influenza viruses (AIV), only serotypes H5 and H7 are considered highly pathogenic in poultry. However, serotype H9N2 has also been found to produce severe respiratory and reproductive tract infections in chickens. The previous studies have suggested that movement of poultry and wild birds play a major role in the spread of influenza viruses to distant areas in a country. The present study was carried out to monitor the prevalence of AIV in wild birds in certain areas of the Pakistan, which were free from infection during the outbreak of November 2003. In this regard, 7 wild bird species were selected and their blood, cloacal swabs and tissue samples were collected both for serological evaluation and virus isolation. The results indicated that antibodies to AIV serotype H9N2 were present in 10% of wild birds, whereas the virus was itself isolated only from 6.72% of the samples. These data provide the evidence regarding the wild birds as one of the major carrier of the AIV infection.

Key words: Avian influenza, serotype H9N2, wild birds

Introduction

Avian influenza (AI) is caused by type A Orthomyxoviruses. Only type A influenza viruses are known to cause natural infection both in birds and human. On the basis of different combination of 15 haemagglutinin and 9 neuraminidase antigens present on the surface of the avian influenza viruses, 256 combinations of Avian Influenza Virus (AIV) may exist in nature. Influenza A viruses infecting poultry can be divided into two distinct groups on the basis of their ability to cause disease. The very virulent viruses, named as highly pathogenic avian influenza viruses (HPAI), can cause heavy mortality and drastic decline in production. This group of viruses includes subtypes H5 and H7, although not all viruses of these subtypes cause HPAI-associated disease. All other viruses cause a milder, primarily respiratory disease, which may be exacerbated by other infections or environmental conditions. HPAI viruses are rarely isolated from wild birds. However, extremely high isolation rates of viruses of low virulence for poultry have been reported in surveillance studies in which 15% for ducks and geese and about 2% of all other species have been shown to be the carriers of AIV. Influenza viruses have been shown to affect all types of domestic or captive birds in all areas of the world, but the frequency with which primary infections occur in any type of bird depends on the degree of contact with feral birds. Secondary spread is usually associated with human involvement, probably by transferring infective feces from infected to susceptible birds (Alexander, 2003).

Avian influenza of HPAI type was first reported in Pakistan in 1995 (Naeem and Hussain, 1995). The disease caused by serotype H7N3 produced high mortality among the affected flocks especially in the broiler breeder rearing areas of the country. Another influenza outbreak in northern areas of Pakistan was reported in 1999, which resulted in 10-20% mortality with decrease egg production from 10 to 75%. It was found to be H9N2 subtype and was named as A/Chicken/Pakistan/3/99(H9N2) (Naeem *et al*., 1999). Since then the disease has been repeatedly reported from various poultry rearing areas at different locations throughout the country. In November of 2003 a new outbreak of AIV involving serotype H7N3 and H9N2 was reported from the coastal areas of Karachi, only affecting commercial layer flocks and also in broiler breeder rearing region in the north. The disease caused high mortality in these regions. This disease confined to the affected areas but there was a possibility of its spread to other poultry raising area in adjacent towns. Although very strict biosecurity measures were introduced in the affected areas, there was a possibility of AIV transmission to the northern part of the country through wild bird population, as there is heavy wild bird population in this area.

Some earlier studies of avian influenza A viruses carried out in Eastern Germany during 1977-89 showed virus isolation directly from feral ducks and other wild birds (Suss *et al*., 1994). The wild ducks are also known to be a major reservoir for avian influenza viruses but there are few recent published reports of surveillance directed at

Table 1: Seroprevalence of avian influenza virus serotype H9N2 in wild birds populations as determined by Haemagglutination Inhibition assay

Bird Type	Number of Serum Samples	GMT	Titre Range
Eagles	2	16	2-128
Myna	23	1	2
Crows	23	3	2-64
Ducks	10	2.3	2-8
Goose	13	3.5	2-256
Water Fowls	6	4	8-32
Doves	7	2	2

this group. Predominant AIV hemagglutinin (HA) subtypes reported in one of such studies of ducks in North America included H3, H4, H6, H5, H7, and H9 subtypes (Hanson *et al.*, 2003).

The present study was undertaken to assess the role of different wild birds in harboring both H7 and H9 serotypes of avian influenza virus in the federal capital territory of Pakistan that is close to some major broiler-breeder and layer rearing areas in the country.

Materials and Methods

Specimen collection: A number of wild birds were either caught, or shot at each of the three designated location from December 2003 to May 2004. These locations were Lake of Bhera, Chakshehzad, Islamabad (which is a thickly populated poultry area); banks of River Sindh near Attock and Wild Life Park of Lohi Bher. All these areas are located within 100 km of the epicenter of AI outbreak. The types of specimens collected during this study are described below:

a. **Blood:** Blood was taken from the following species of wild birds: Crows, Common Myna, Mallard ducks, Doves, Common Goose, Fowls and Eagles. From each bird 1-2ml of blood was drawn and allowed to clot. The serum was removed after centrifugation at 800xg for ten minutes. Sera were subsequently stored at -20°C until used.

b. **Cloacal swabs:** Cloacal swabs were taken from the wild birds and collected aseptically with soft Dacron tipped applicators in 1.5 ml vial containing virus transport medium (BHI broth with Penicillin, Streptomycin, Gentamycin). The samples were promptly transported in ice to the laboratory where they were stored at -20°C.

c. **Clinical specimens:** Trachea and lung specimen were collected from each bird for virus isolation. The samples were placed in transport media and then kept at -20°C till further processing. The tissues were minced, freeze-thawed thrice, and centrifuged at 800xg for 10 mins and the supernatant was collected for *in ovo* inoculation.

Virus isolation: Each tube-containing swab in virus transport medium was vortexed, and the swab carefully removed after squeezing. It was centrifuged and supernatant was filtered through 0.2µm filter for *in ovo* inoculation. Both the tissues homogenates and cloacal swab preparations were inoculated in 9-day-old embryonated eggs, via allantoic route. After four days, the eggs were chilled over night. Under aseptic conditions, the allantoic fluid (AF) was collected. The embryos were also examined for lesions. The AF from each egg was tested for haemagglutinating activity as previously described (Naeem *et al.*, 1999). The negative samples were repassaged in fresh embryonated eggs.

Haemagglutination and Haemagglutination inhibition (HI) tests: In order to monitor the antibody titres against AI viruses in wild birds, HA and HI tests were performed according to the protocols described earlier (Olsen *et al.*, 2003). Briefly, 2- fold dilution of the AF was made in PBS (pH 7.2) in a 96-well micro titration plate. Chicken RBC's were added to each well at 0.5% concentration. The plates were incubated for 30 mins at 37°C before recording the haemagglutinating activity. Haemagglutination inhibition (HI) titre of each serum sample was also determined. Briefly, 25 µl of the test sera were serially diluted in PBS (pH7.2) using a 96-well titration plate. To this 25 µl of the known serotype of the AIV (4HA) was added in each well. The plates were incubated for 30mins at 37°C. Now 50ml of 0.5% of the chicken RBC's were added to each well and the plates were again incubated for 30 mins. at 37°C. The results were recorded and subjected to geometric mean titres (GMT) analysis.

Virus Neutralization (VN) test: The constant-serum, diluted-virus method was used for the identification of the isolated serotypes using the method as previously described by Beard, (1980).

Results

Out of 84 serum samples collected from the wild birds, 12 samples representing every bird type showed HI antibody titres against H9N2 AIV serotype. Table 1 shows the results of HI against AIV among different species of wild birds. As shown, the eagles had the highest GMT value of 16. All other birds had a GMT value within a range of 1 - 4 (myna 1, crows 3, ducks 2.3, common goose 3.5, waterfowls 4 and the doves 2). All the samples tested for the presence of antibodies against H7N3 were found negative.

Table 2: Distribution of avian Influenza virus isolation from tissues of seropositive samples

Bird Type	Virus Detection at Passage (P) Level		
	P1	P2	P3
Fowl #1	-	+	++
Eagle# 1	-	-	+
Crow#1	-	+	++
Crow#2	-	-	+
Crow#3	-	+	++
Crow#4	-	-	+
Duck#1	-	-	-
Goose#1	-	-	-
Goose#2	-	+	++
Goose#3	-	-	-
Goose#4	-	-	+

- = Negative + = Positive. NOTE: All the samples tested for the presence of H7N3 serotype was found negative.

Table 2 shows HA activity of the isolates recovered from the tissue samples of wild birds. All the samples were processed and passaged twice in eggs. Out of 84 samples, 8 samples showed haemagglutinating activity. All samples were further tested through Agar Gel Precipitation Test (AGPT), using AIV polyvalent antisera and 4 samples were found positive on AGPT (data not shown).

Fig. 1 shows the HA activity of isolates recovered from the cloacal swabs and tissues of these wild birds. The crows showed the highest activity i.e.128, 32 and 2, while fowls showed the HA activity of 32, 8, and 4; ducks showed the HA activity of 2, 2, and 2; eagles showed the HA activity of 4, 2, and 2; while the goose showed the HA activity of 32, 4, and 2.

Table 3 shows the results of virus neutralization test (VNT) performed for the serotype identification of AIV. Eight isolates were positively identified as H9N2 serotypes using the reference antisera. No positive isolates of H7N3 were found.

Fig. 2 depicts the distribution of AIV positive cases among various species of wild birds, tested by HI. It was seen that 50% eagles, 17% crows, 10% ducks, 30% goose and 33% fowls were the carriers of AIV (H9N2 serotypes). It was further seen that all the samples tested for the presence of antibodies against H7N3 were found negative.

Discussion

There have been a number of AIV out-breaks in poultry during the past decade in different parts of the world. A recent outbreak involving H7N3 and H9N2 occurred in Pakistan from November 2003 to May 2004. The study under report was carried out to analyze the role of wild birds in the spread of avian influenza viruses in this outbreak.

Table 3: Virus Neutralization Test for serotype identification of avian influenza isolates

Isolate designation	Virus neutralization activity against	
	H9N2	H7N3
Fowl#1	+	-
Fowl#2	-	-
Dove#1-7	-	-
Common Myna# 1-23	-	-
Mallard Duck#1	-	-
Crow#1	+	-
Crow#2	+	-
Crow#3	+	-
Crow#4	+	-
Goose#1	-	-
Goose#2	+	-
Goose#3	+	-
Goose#4	+	-

For this purpose mallard ducks, geese, crows, common myna, Indian fowl, doves and eagles were examined. Blood samples, cloacal swabs and the tissue samples, where available, were collected. The antibody titres against AIV sub types H9N2 and H7N3 were tested by HI. 10 % of the mallard ducks showed the high antibody titres against influenza virus sub type H9N2, which migrated in summer to Lake Kalar Kahar (Chakwal). No titres were found against H7N3 in these birds. Presence of high titres of HA antibody supported the idea that mallard ducks may be the natural carrier of AIV. Similarly the titres against AIV were also checked in other wild birds. The percentage of seropositive birds against H9 antibodies was 30% geese, 17% crows, 50% eagles and 33% Indian fowls.

This shows that the wild birds are the natural carriers of AIV serotype H9N2. The titre range (GMT) in mallard ducks was 2-8, in geese 2-256, in crows 2-64, in eagles 2-128 and Indian fowls 8-32. The high antibody titres against subtype H9N2, is the indication for the presence of AIV (H9) in these birds persistently.

High antibody titres against H9N2 were found in 17% of these samples. This shows the role of crows in the spread of avian influenza virus in poultry population. As the crows eat poultry waste and are carnivorous animal, this might be the reason of transfer of virus to crows. On the other hand common myna showed no titres against the influenza virus and seems not to be participating in influenza spread.

The doves were also examined in this study. It was found that there was no antibody titre against AIV (H9 and H7) in these birds. Moreover no virus was isolated from the tissues of these doves. As doves are not involved in flesh eating, they remained free from virus exposure. It further indicates that the water reservoirs in the vicinity from where sampling was done are free from

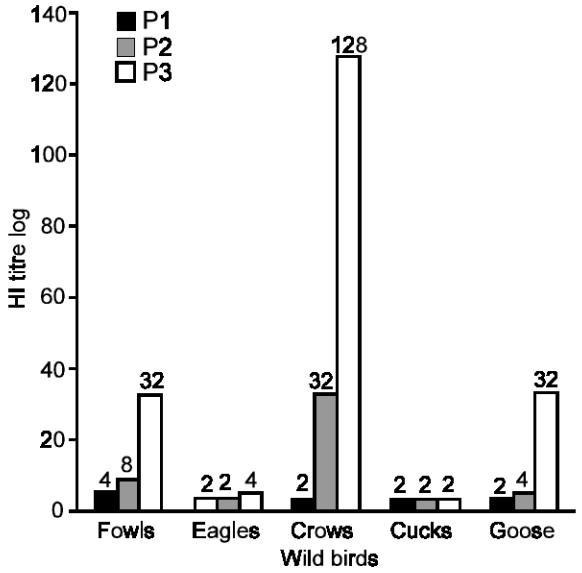

Fig. 1: Haemagglutinaion (HA) titres of AIV isolates recovered from cloacal swabs and tissues

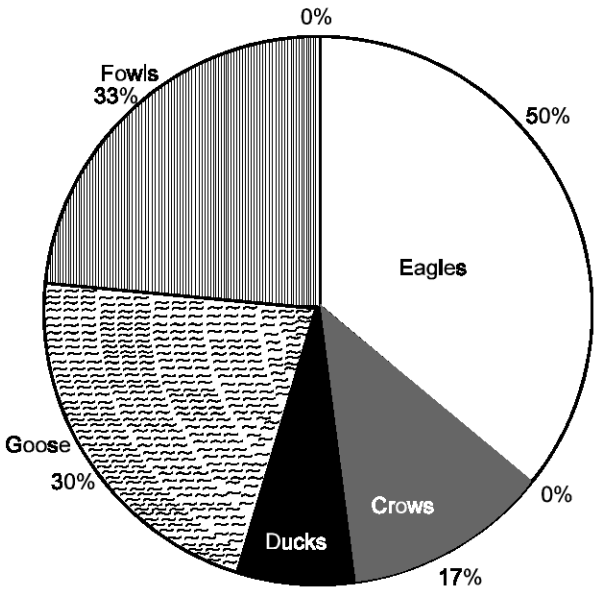

Fig 2: Distribution of aiv positive cases among different species of wild birds as tested by HI

AIV as there is great possibility of sharing of water reservoir among all types of birds which could have transmitted the virus to myna and doves as well.

The AIV serotype H9N2 was also detected from the cloacal swabs of the birds examined above. Here it was found to be 50 % (2/4) in geese, 50% (½) in fowls, 50% (½) in eagles and 75% (3/4) in crows. The AIV subtype H9N2 was recovered from the cloacal swabs of these birds by *in ovo* inoculation. The propagation of influenza virus in embryonated eggs confirms the previous knowledge about the reliability of this system. It was also found that most of the positive isolates in this study were detected after 2nd passage through the embryonated eggs.

Role of wild birds in maintaining and spreading the avian influenza virus has also been reported by some other workers. Lee *et al.* (2000) reported the isolation of an H9N2 influenza virus (A/duck/North Carolina/91347/01) (Dk/Nc) from wild ducks in the United States. Genetic analysis showed that this duck virus had the same human /classical swine/avian reassortment genotype as the H9N2 viruses that have been isolated from pigs and turkeys in the US since 1999. In another study, serum samples from 163 slaughter age ostriches in Ohio and Indiana were tested for antibodies to avian influenza virus. Some ostrich had antibodies to AIV H5N9. This is the first report of antibodies to avian influenza in ostriches in the United States. In another report AIV serotype H7N3 was recovered from a falcon in Syria in 1995. Upon the molecular analysis of these isolates, the sequencing data revealed its homology to the isolates from Pakistan in 1995. It was eventually found that this falcon was brought from Pakistan (Banks *et al.*, 2000).

Such studies of wild bird monitoring have been helpful in predicting the new serotypes of AIV in an area. Such intensive surveillance study of aquatic birds especially in Siberia has earlier provided information on the future pandemics of influenza viruses and for vaccine preparation (Okazaki *et al.*, 2000). Significance of such studies is also reflected from many previous studies indicating the inter species transmission of influenza A viruses circulating in the wild aquatic birds occasionally results in influenza out breaks in mammals, including humans (Matrosovich *et al.*, 2000). This happens due to genetic reassortment among different types of AIV infecting wild birds. The role of AIV serotype H9N2 in causing infection in humans in Hong Kong and China (Peires *et al.*,1999) also signifies the prevalence of H9N2 serotype in poultry. In this part of the world, it is highly likely that this virus is spreading among humans, wild birds and poultry and may result in causing new outbreaks after its mutation during interspecies transfer and replication. Therefore the wild birds can be considered as major reservoirs of H9N2 in this area.

In the above scenario, the detection of AIV H9N2 in wild birds in this country poses a continuous threat for the emergence of more pathogenic strains of influenza virus in poultry, along with the possibility of further spread of these viruses to other parts of the Pakistan. The persistence of this AIV serotype from the wild birds, in this part of the world signifies the existence of virus as a possible candidate for future out-breaks in poultry in Asia. Based on the significance of wild birds as the carrier of AIVs, it is recommended to keep an eye on the migratory patterns of the wild birds in a region. As the

global climate is changing rapidly, the temperature of the earth is increasing and the glaciers are melting, the patterns of the migration of these birds are fluctuating as well. So it is necessary to watch the patterns of migration and seromonitor these birds on regular basis. It will help to implement the proper precautionary measures in Asia and Sub-continent to control the frequent relapse of AIV in poultry population in Pakistan.

Serological analysis of various species also indicates the activity of this subtype in several types of wild birds. It would, therefore, be appropriate to seromonitor the wild birds on regular basis to evaluate the rapidly changing status of this virus. It is further suggested that improvement in biosecurity at the farms should be given top priority to stop the spread of AIV serotypes through contacts between wild birds and commercial poultry.

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