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SEROPREVALENCE OF AVIAN INFLUENZA IN LAYERS OF HEAVILY POPULATED AREAS IN TOBA TEK SINGH AND ADJOINING LOCALITIES

M. Numan and M. Siddique

Department of Veterinary Microbiology, University of Agriculture, Faisalabad, 38040, Pakistan

ABSTRACT

A survey based on collection of serum samples for the determination of prevalence of avian influenza (AI) in non-vaccinated layer flocks was carried out in an area of Punjab having complaints similar to those represented by AI. Paired serum sampling was done from 14 non-vaccinated flocks against any subtype of AI virus (AIV). A total of 204 serum samples were collected (148 soon after exhibiting signs and symptoms and 56 after three weeks interval from positive farms for AIV antibodies) from 14 commercial layer farms. Haemagglutination Inhibition (HI) test was performed for the determination of serum antibodies against AIV. The results showed that out of 14 sampled farms, three farms had antibodies against only H7 subtype, two farms had antibodies against H9 and two farms showed antibodies against both subtypes, H7 and H9, while remaining 7 farms were negative for antibodies to AI. Furthermore, out of these positive farms only one farm was found seroconverted for H7 subtype.

Key words: Avian influenza, haemagglutination inhibition, seroconversion.

INTRODUCTION

Avian influenza (AI) is a contagious viral disease and is world wide in distribution. It affects the chickens of all ages with variable morbidity and mortality. With the highly pathogenic AI viruses, morbidity and mortality rates are very high (50-89%) and can reach 100% in some flocks (Capua *et al.*, 2000). AI viruses are classified in the family Orthomyxoviridae, genus Influenza virus A. The surface of AIV is covered by two types of glycoprotein projections, rod-shaped trimers of haemagglutinin (HA) and mushroom-shaped tetramers of neuraminidase (Cox *et al*., 2000). The HA is the major antigen that elicits antibodies which protect against death and clinical signs (Brugh and Stone, 1987).

Investment in poultry sector in Pakistan is about one billion US dollars. Every family in rural areas and every 5th family in urban areas are associated with poultry production activities in one way or the other (Sadiq, 2004). However, this industry is facing various managemental problems along with infectious diseases including AI. This disease of highly pathogenic type was first reported in Pakistan in 1995. The disease was caused by subtype H7N3 and caused high mortality among the affected flocks especially in the broiler breeder rearing areas of the country (Naeem and Hussain, 1995). Another influenza outbreak in northern areas of Pakistan was reported in 1999, which resulted in 10-20% mortality with decrease in 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 view of this situation, a survey was carried out with the objectives of determining prevalence of AI in commercial layer flocks in some areas of central Punjab heavily populated with layers and to see whether still disease is present in commercial layers or has been overpowered by the mass vaccination programmes.

MATERIALS AND METHODS

Collection of samples

The study was carried out in heavily populated areas of Punjab (including Toba Tek Singh, Kamalia, Pir Mahal, Sammundri and Rajana), Pakistan, from December 2004 to February 2005. A total of 204 blood samples (148 soon after manifestation of signs of AI and 56 after three weeks) were collected from 14 different commercial layer flocks having a history of non-vaccination against any AIV subtype. These blood samples were allowed to clot, sera were separated and frozen at -20°C till further use for HI test.

Haemagglutination and Haemagglutination Inhibition Tests

The serum samples were used to determine the antibodies against AIV, using the Haemagglutination (HA) and Haemagglutination Inhibition (HI) methods described earlier (Olsen *et al*., 2003). The antigens used

were AIV subtypes H7N3 and H9N2 taken from National Agricultural Research Center (NARC), Islamabad, Pakistan.

Washing and preparation of RBCs suspension

A total of 5 ml of chicken blood was collected aseptically in a disposable syringe containing 1 ml of sodium citrate (4% solution) as an anticoagulant. The blood was centrifuged at 1500 rpm for 15 minutes. The plasma and buffy coat was pipetted off. After adding phosphate buffer saline (PBS), the above procedure was performed for a total of three washings or until supernatant was clear. A 0.5 ml of washed RBCs was taken and 1.5 ml of PBS was added to make 25% RBCs stock solution and stored at 4°C. Then a 40 µl volume of 25% stock solution was taken and to it 1.96 ml of PBS was added to make a total volume of 2 ml. Suspension was mixed gently before use and stored at 4°C. Suspension was discarded when haemolysis occurred.

Test procedure

A 2-fold serial dilution of the AIV-subtypes H7 and H9 was made in PBS (pH 7.2) in separate 96-well microtitration plates. Chicken RBCs were added to each well at 0.5% concentration. The plates were incubated for 30 minutes at 37°C and 4 HA units were calculated for both of the antigens separately. HI titer of each serum sample was also determined. For this purpose, 25 µl of the test sera were serially diluted in PBS (pH 7.2) using two 96-well microtitration plates. To these, 25 µl of 4 HA units of AIV subtypes H7 and H9 were added separately in each well. The plates were incubated for 30 minutes at 37°C. Then 50 µl of 0.5% chicken RBCs were added to each well and the plates were again incubated for 30 minutes at 37°C. The results were recorded when complete button formation was observed in the control well and subjected to geometric mean titer (GMT) analysis, as described by Brugh (1978).

RESULTS

A total of 204 serum samples were collected (148 soon after exhibiting signs and symptoms and 56 after three weeks) from 14 commercial layer farms with a history of non-vaccination against H7 and H9 subtypes of AIV. Out of these 14 sampled farms, three farms had antibodies against only H7 subtype, two farms showed antibodies against H9 subtype and two farms had antibodies against both of the subtypes; H7 and H9, while remaining 7 farms were negative for antibodies to AIV. In 2nd sampling, instead of serum samples from all 7 seropositive flocks, serum samples from 5 flocks could be collected, as two flocks had been sold. Sample's distribution on the basis of overall positive and negative percentages was 48.65 and 51.35%, respectively (Table 1).

In 1st sampling, in layers of 24 weeks age, the antibody titers ranged from log24 to log216 with GMT value of 7.5 (H7). Birds aged 28 weeks showed antibody titer in the range of log28-log216 with GMT value of 10.6 (H9). Antibody titers of 17 weeks old birds were found in the range of log216-log232 with GMT value of 26.0 (H7) and also log232-log264 with GMT value of 34.3 (H9). Age group of 21 weeks showed serum antibodies in the range of log216-log264 with 27.9 GMT value (H7). In 26 weeks age group, the antibody titers ranged from log28 to log216 with GMT value of 8.6 (H7). Another flock of age 26 weeks showed antibodies in the range of log28 to log216 with GMT value of 11.3 (H9). Birds aged 46 weeks showed antibody titer in the range of log232-log264 with GMT value of 34.3 (H7) and log232-log264 with GMT value of 36.8 (H9) (Table 2).

Table 1: Serum samples of commercial layers exhibiting positive or negative results to AIV using HI test

Flock No.	Age (weeks)	No. of samples	Antibodies to H7	Antibodies to H9	Positive samples for AIV antibodies	Negative samples for AIV antibodies	% positive samples for AIV antibodies	% negative samples for AIV antibodies
1	24	10	+	-	10	-	100	Zero
2	28	15	-	+	15	-	100	Zero
3	27	10	-	-	-	10	Zero	100
4	32	10	-	-	-	10	Zero	100
5	32	12	-	-	-	12	Zero	100
6	17	8	+	+	8	-	100	Zero
7	19	10	-	-	-	10	Zero	100
8	21	12	+	-	12	-	100	Zero
9	42	10	-	-	-	10	Zero	100
10	26	10	+	-	10	-	100	Zero
11	19	15	-	-	-	15	Zero	100
12	26	6	-	+	6	-	100	Zero
13	46	11	+	+	11	-	100	Zero
14	27	9	-	-	-	9	Zero	100
Total		148	5	4	72	76	48.65	51.35

Table 2: Distribution of layers on the basis of log_2 HI titers obtained against AIV subtypes (first sampling)

Age (weeks)	Positive samples for AIV H7 antibodies	Positive samples for AIV H9 antibodies	Antibody titers using HI test											
			1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	GMT	
													H7	H9
24	10	-	-	2	7	1	-	-	-	-	-	-	7.5	-
28	-	15	-	-	8	7	-	-	-	-	-	-	-	10.6
17	8	-	-	-	-	2	6	-	-	-	-	-	26.0	-
	-	8	-	-	-	-	7	1	-	-	-	-	-	34.3
21	12	-	-	-	-	4	6	2	-	-	-	-	27.9	-
26	10	-	-	-	9	1	-	-	-	-	-	-	8.6	-
26	-	6	-	-	3	3	-	-	-	-	-	-	-	11.3
46	11	-	-	-	-	-	10	1	-	-	-	-	34.3	-
	-	11	-	-	-	-	9	2	-	-	-	-	-	36.8

Table 3: Distribution of layers on the basis of log_2 HI titers obtained against AIV subtypes (sampling done three weeks interval)

Age (weeks)	Positive samples for AIV H7 antibodies	Positive samples for AIV H9 antibodies	Antibody titers using HI test											
			1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	GMT	
													H7	H9
24	10	-	3	4	3	-	-	-	-	-	-	-	8.0	-
28	15	-	-	2	8	5	-	-	-	-	-	-	-	9.2
17	8	-	-	-	-	3	5	-	-	-	-	-	24.3	-
	-	8	-	-	-	-	6	2	-	-	-	-	-	36.8
21	12	-	-	-	-	-	-	-	10	2	-	-	147.0	-
46	11	-	-	-	-	1	8	2	-	-	-	-	34.3	-
	-	11	-	-	-	-	8	3	-	-	-	-	-	39.4

In 2nd sampling (sampling done after three weeks interval), in layers of 24 weeks age, the antibody titers ranged from log22 to log28 with GMT value of 8.0 (H7). Birds of age 28 weeks showed antibody titer in the range of log24-log216 with GMT value of 9.2 (H9). Antibody titers of 17 weeks old birds were found in the range of log216 to log232 with GMT value of 24.3 (H7) and also log232 to log264 with GMT value of 36.8 (H9). Birds aged 21 weeks showed serum antibodies in range of log2128 to log2256 with 147.0 GMT value (H7). Birds aged 46 weeks had antibody titer in range of log216 to log264 with GMT value of 34.3 (H7) and log232-log264 with GMT value of 39.4 (H9) (Table 3).

DISCUSSION

Paired serum samples collected soon after manifestation of signs of AI and three weeks later were subjected to HI test to see the difference in the titres of antibodies against AIV in both of the samplings. Only samples of one and only farm of age 21 weeks showed more than four fold increase (27.9 to 147.0 GMT) in the antibodies against H7 as compared to the first serum sampling. Furthermore, this seroconverted flock had a history of about 3,000 deaths of the birds out of 30,000 flock size within 36 hours, while egg production was reduced to 51%. Other seropositive flocks in the first sampling did not show such pattern. These results suggest that seroconversion had taken place for AIV subtype H7 and birds of these farms were suffering from infection by AI. Naeem *et al.* (2003) also observed similar pattern of seroconversion for H9N2. Other serum samples which showed positive results by HI test in 1st sampling but did not show seroconversion in their 2nd sampling seem to have been exposed to respective AIV subtype in the past but remained un-noticed by the farmer. Birds of these farms might have suffered from AI infection in the past and recovered with the passage of time, and thus showed antibodies against respective AIV subtypes.

It's obvious that this AIV is spreading among humans, wild birds and poultry and may cause new outbreaks after its mutation during interspecies transfer and replication. Poorly controlled movement of birds and lack of biosecurity caused AI to become endemic in poultry populations, especially in Europe and few areas of Asia (Swayne and Halvorson, 2003). Presence of different subtypes of AIV in the field indicates that there is always a likelihood of generating new virus by gene reassortment between subtypes pathogenic in birds and mammals. Thus, there is a constant need to carry out a coordinated surveillance for influenza viruses both in birds and humans in the country and farmers should be advised to immunize their birds regularly

with inactivated vaccines against AIV-subtype H7 as well as against H9. In this way chances of emergence of new strains may be reduced to its minimum in the area.

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