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Sero-prevalence of Avian Influenza Type A in Khartoum North and the Effect of Different Treatments on Sera Using the Indirect ELISA

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ABSTRACT

A serological survey was carried out to investigate the presence of avian influenza type A antibodies in Khartoum North. A total of 210 serum samples were collected from different layer chicken farms in Khartoum North in the last third of 2010. The sera were examined for avian influenza type A antibodies using indirect ELISA. The results showed that (3.3%) of the ser were positive to influenza type A antibodies. The second parts of the study was designed to know whether temperature, freezing/ thawing cycle will have any impact on avian influenza antibodies when tested using indirect ELISA. Tested sera were incubated at different temperatures (37^{o}C, and 45^{o}C for overnight) or treated with three cycles of freezing and thawing or kept in refrigerator at 4^{o}C for 15 days. The results showed that certain temperature, 3^{o}C for overnight and two weeks refrigerating at 4^{o}C had no effect on the testing results of ELISA. In addition, testing results for serum samples which had been incubated at 45^{o}C overnight and freezing and thawing for three cycles showed that two negative sera in each group changed to false positive. Therefore, it is recommended to store serum samples at appropriate temperature 4^{o}C for up to 14 days and -20^{o}C for more than two weeks to avoid the appearance of false results when tested, also monitoring the disease situation in the country through regular surveillance.

INTRODUCTION

Influenza originally referred to epidemics of acute, rapidly spreading catarrhal fevers of humans caused by viruses in the family *Orthomyxoviridae*, which are recognized as the causes of significant numbers of natural infections and diseases, usually of the upper respiratory tract, in humans, horses, domestic pigs and various bird species and sporadic cases of natural occurring diseases in mink and a variety of marine mammals(**Swayne and Halvorson, 2003**).

These viruses are further classified on the base of antigenic properties of the nucleoprotein and matrix proteins, as influenza A, B or C (**Clark and Hall, 2006**), only type A influenza viruses are known to cause natural infection in birds (**CIDRAP, 2011**), the type A is the most important of the three it is the pathogen responsible for epidemics and pandemics, since it is antigenic structure changes within a broader range due to recombination (Less frequent) (**Kayser *et al.*, 2005**).

There are many different subtypes of type A influenza viruses, these subtypes differ because of changes in certain proteins on the surface of the influenza A virus (heamagglutinin (HA) and

neuramindase (NA) proteins). There are 16 known HA subtypes and 9 known NA subtypes of influenza A viruses. Many different combination of HA and NA proteins are possible. Each combination represents different subtypes. All known subtypes of influenza A viruses can be found in bird (**CDC, 2010**).

Avian influenza subtypes in domestic chickens and turkeys are classified according to disease severity, with two recognized forms: highly pathogenic avian influenza (HPAI), also known as fowl plague, and Low Pathogenic Avian Influenza (LPAI). Avian influenza viruses that cause HPAI are highly virulent, and mortality rates in infected flocks often approach 100%. LPAI viruses are generally of lower virulence, but these viruses can sever as progenitors to HPAI viruses (**CIDRAR, 2011**). Low Pathogenic forms of AIV (LPAIV) are most common in wild birds, in which they do not appear to cause disease. Although egg production may be affected, the disease do not considered severe. There is little mortality and from a trade and production viewpoint, most Low- pathogenic forms of AI are not considered reportable diseases by the Organization International Epizootics (OIE). By contrast (HPAIV) forms rarely occur in wild birds and are considered primarily a problem of the industry (**Clark and Hall, 2006**).Due to excessive economically losses to the poultry industry, HPAI receive immense attention in the veterinary world and is globally treated as a disease immediately notifiable on suspicion to the authorities. Outbreaks of influenza have been recognized in domestic poultry (chickens and turkeys) for many years, and it can affect several species of food producing birds (chickens, turkeys, quails, guinea fowl, ect), as pet birds and wild birds with some subtypes resulting in high mortality rates, The virus has also been isolated from mammalian species including humans, rats and mice, weasels and ferrets, pigs, cats, tiger and dogs (**OIE, 2010**). In aquatic birds, influenza viruses replicate preferentially in the cells lining of the intestinal tract and are excreted in high concentrations in the faces, waterfowl transmit influenza viruses by the fecal- oral route through contaminated water (WHO, 2002). Avian influenza has received increasing attention over the years, not only because it represents a serious threat to the welfare of wild bird populations, but because it affects agriculture (i.e. poultry production and trade) and human health. Avian influenza viruses have a worldwide distribution with reports of isolations from Africa. Asia, Australia, Europe and north and South America (**Clark and Hall, 2006**).Countries may be under threat of introduction of avian influenza through exposure of poultry to wild birds, especially waterfowl. They may also be at risk from introduction of infected or contaminated poultry, poultry products or fomites. The control of AI in poultry is an objective that the veterinary community must achieve in order to manage the pandemic potential of the virus, to preserve profits of poultry industry and to guarantee food security to developing countries. Biosecurity must always be improved in the face of an AI threat and it may be used in conjunction with vaccination under certain circumstances (**Capua and Marangon, 2007**). In Sudan reports of the present of AI antibodies had been continuously observed since the year 1923 and also by the study performed by **Manal (2000)** who detected both antibodies and the virus. For the diagnosis, different serological tests were used such as agar gel diffusion test, heamagglutination and ELISA. Several ELISAs have been developed and evaluated by different laboratories; ELISA can detect antibodies against the nucleoprotein (NA). The occurrence of these AI antibodies means that the virus is still circulating; hence this study had been design to fulfill the following objectives:

General objectives:

1. Sero-survilance of avian influenza in Khartoum North city
2. To detect antibodies to avian influenza by using the Indirect ELISA
3. To evaluate the effect of temperature, freezing and thawing on the level of avian influenza antibodies in poultry serum using indirect ELISA test

MATERIAL AND METHODS

Study area

The study was conducted in Khartoum State during the period of August and December in 2010 in layer chickens from ten farms practicing an open system (Table 1).

Samples

Two hundred and ten of serum samples were collected from layer chickens at different ages.

Collection of blood samples for serological survey

Whole blood (2-3 ml) was taken from birds aseptically from wing and jugular vein in 5 ml syringe they were then left to clot at room temperature. Sera were separated from the clot and kept in an eppendorf tubes labeled and stored at – 20 ^{0}C until used.

Serum treatment strategy

Fifteen sera included a positive and negative serum were chosen from the 210 samples after testing them with ELISA, then each serum samples was further sub divided into 5 different ependorf tubes and labeled. Each fifteen samples represent a group.

Group No 1: of the fifteen selected samples was kept without any treatment and used as control group.

Temperature treatment

Group No 2 was incubated at 37^{0}C overnight, while group 3 was incubated in a water bath at 45^{0}C overnight.

Thawing and freezing strategy

Group 4 was thawed and frozen for three cycles and then tested.

Refrigerating strategy

Group 5 was kept in refrigerator for 15 days and then tested

Enzyme Linked Immunosorbent Assay (ELISA)

The AI ELISA Kit (Synbiotics Laboratories, Canada), Was used for detection of antibodies of avian influenza virus type A.

Calculations:

Using samples/ positive ratio (S/P ratio) which is calculated as following

$$S/P = \frac{\text{Mean of test sera – Mean of negative control}}{\text{Corrected positive control absorbance*}}$$

*(Mean of positive control- Mean of negative control)

Table 1: Number of serum samples collected

Farm Location	Type of Breed	Total Flock No	Chicken age/ week	Samples collected
Shambat A	Local (Zallot)*	500	12	30

Alhalfaya	Lohman	1000	12	10
Alkhojalab	Hiline	1200	24	20
Shambat B	Lohman	700	14	18
Om Deraiwa	Hi sex	1600	42	24
Aldrushab	Hiline	2000	52	24
Alfakihashim	Hiline	3500	132	24
Altibna	Hiline	4000	52	20
Hillat Koko	Hiline	550	91	20
Alwihda Market	Unknown	50	Above 104	20
Total Number	-	15100	-	210

Zollatis a local Sudanese breed of chicken with a pare neck

RESULT AND DISCUSSION

Field observation

Birds where serum samples were collected did not show any respiratory signs although there were some common and unspecific signs for many diseases like depression, ruffled feather and swelling of the face.

Detection of AI antibodies as tested by means of ELISA

The results showed that seven out of 210 sera were found positive to AIV antibodies with a prevalence percentage of 3.3 (Table 2).

Treated\sera

Untreated control sera

From the fifteen sera tested for AI antibodies using ELISA seven samples were found positive. The result was considered as a control for the rest of the trail (Table 3).

Sera treated at 37 0C

After an overnight incubation at 370C the same result as that of control was obtained, only 7 out of 17 sera tested using ELISA were found positive to AI antibodies.

Sera treated at 450C

Sera which were incubated at 450C overnight when tested using AIV ELISA showed that nine samples of the 15 tested sera were found positive (Table 3)

Thawing and freezing cycle

Sera which were freezed and thawed three times and then tested using AIV ELISA the result showed that nine out of 15 samples tested were found positive (Table 3)

Refrigerated sera

Sera\which were refrigerated for 15 days at 40C showed seven samples to be positive out of 15 samples tested for AI antibodies using ELISA (Table 3)

Table 2: Area and total number of chickens sera showed antibodies against AIV type A using ELISA

Area	Samples tested	No. positive	% of positive
Shambat A	30	1	3.3
Alhalfaya	10	0	0
Alkhojalab	20	0	0
Shambat B	18	0	0
Om Deraiwa	24	1	4.2
Aldrushab	24	1	4.2
Alfakihashim	24	0	0
Altibna	20	1	5
Hillat Koko	20	1	5
Alwihda Market	20	2	10
Total	210	7	3.3

Table 3: ELISA results of the different trials used for chicken sera positive to AI type A

Number	Control	37^0C	45^0C	Th	Refrigerated
1.	+	+	+	+	+
2.	+	+	+	+	+
3.	-	-	-	+	
4.	-	-	+	+	
5.	+	+	+	+	+
6.	-	-	-	-	-
7.	+	+	+	+	+
8.	+	+	+	+	+
9.	+	+	+	+	+
10.	-	-			
11.	-	-			
12.	-	-	+		
13.	-	-			
14.	-	-			
15.	+	+	+	+	+
Total	7	7	9	9	7

Th: Thawed and frozen for three cycles

Refrigerated for 15 days

Discussion

Avian influenza (AI) is a highly contagious viral disease affecting several species of birds as\well as pet and wild birds (OIE, 2011)some of the subtypes such as H5 and H7 are a notifiable disease and has a zoonotic nature so it can be a source of thread for both human and birds. Due to this avian influenza has an international interest and all possible precautions have been adopted to asses early detection and consequence possible control of the disease. This contribution was carried out to investigate the presence of AI type A antibodies in layer chickens in /Khartoum North at Khartoum State using the ELISA and also to evaluate the effect of those antibodies when stored in different

condition. It should be stated that this surveillance was conducted on the base of some uncharacteristic observed clinical signs and not as a results of an outbreak. The noticeable signs seen were common and summarized on signs such as depression, ruffled feather and swelling of the face. Manal (2000) mentioned approximately similar clinical signs in the chicken flock survived by her and she was\able to detect antibodies to AI type A and isolated the virus also same clinical signs had been observed by Hayfa (2008). Shaza (2008) detected antibodies to avian influenza type A from apparently health birds. For the explanation of these extremities a possibility of mixed infection of avian influenza and other pathogens and or circulation of low pathogenic avian influenza or another pathogen. Using the indirect ELISA seven sera from a total of 210 samples examined were found positive to AI type A with a prevalence rate of 3.3%.

A prevalence rate of 28.4% to AI type A antibodies was detected by Manal (2000), Sahar (2007) detected 56.3% positive results using AGID test and AGID is an accepted protocol in OIE manual for the detection of AI, it is well known that the ELISA is more sensitive but during 2007 chicken populations was infected and also the fact that vaccination was conducted during this period this may explain the high percentage of antibodies. Hayfa (2008) found 16% positive result after the outbreak period and 37% positive results before the outbreak period and Shaza (2008) found 11.9% positive result in layer chicken. The justification of this variant may be due to depopulation of all infected and suspected birds after the outbreak at 2006 and also all of these researches were done during the period of an outbreak. It could be as a result of the vaccination or to that the survived birds got a previous infection of low dose.

A appropriate storing and shipping conditions of specimen and even the reference reagent are the key point of accurate testing results for detection of infection. For viral infections, the detection of corresponding antibody responses is easy and reliable.

According to the results obtained in this study incubation of the sera at 37^{o}C overnight has no effect on the sensitivity of ELISA for detection of AI type A antibodies. However, Dundon *et al.,* (2007) who did not found a change in HI titer when incubating the sera at 37^{o}C for up to 21 days. On the other hand storage of sera at 45^{o}C overnight and conducting the ELISA our results showed that some of the negative sera changed to positive which was considered to be a false positive, this results indicated that heat inactivation of serum specimens before screening by ELISA can give false positive results because heat inactivation has got some effect on antibodies as has been stated by Jones (1927), as had been known that an antibody is amino acid which is heat liable, high temperatures may cause denaturation of proteins. And also like any lab test, ELISA can give false positive when there is a contamination issue. Therefore, it is important to ensure that the samples to be tested are at good quality and under good conditions. Also the tested sera which was stored at 4^{o}C for 15 days showed no change And it agreed with the results of Dundon *et al.,* (2007) who showed no change in the result when reference sera of avian influenza were kept at 4^{o}C for 21 days.

With regard to the treatment of serum by freezing and thawing three times our results showed that some of the sera change from negative to positive which was considered to be false positive whereas fast freezing with slow thawing resulted in more severe damage to proteins

(Cao *et al.,* 2003) which can give false positive results.

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