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Study on the Infection of Zoo Birds by Highly Pathogenic H5N1 Avian Influenza Virus

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From February to June 2006 an epidemic of highly pathogenic avian influenza (HPAI) virus of subtype H5N1 affected all commercial poultry sectors as well as rural and backyard level in Egypt. This outbreak also was extended to include the zoo birds in Giza zoo. This study records the isolation and characterization of H5N1 virus from different species of the zoo birds and also studies the immune states of the vaccinated birds after application of H5N1 vaccine for the first time one month after the introduction of early outbreaks of 2006. Viral diagnosis was based on direct detection of viral RNA by real time PCR. Two positive cases from turkey and Grocer duck were processed for viral isolation and characterization. The level of antibodies was detected in vaccinated birds by HI test and the results were discussed to evaluate the role of vaccination in controlling the disease in these valuable zoo birds.

Key words: Avian influenza H5N1, Zoo birds, Egypt, real time PCR, HI test.

INTRODUCTION

Avian influenza virus (AIV) is type A orthomyxovirus which is found worldwide in a wide variety of wild and domestic birds **(Easterday *et al.*, 1997; Alexander 1982).** In domestic birds, AIV causes a range of clinical signs, depending on the virulence of the isolate, species and secondary infections. In free-living wild birds the infections are more common but clinical signs are rarely seen **(Slemons *et al.*, 1974; Stallknecht and Shane, 1988; Astorga *et al.*, 1994).**

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AIVs are most frequently isolated from migratory waterfowl; although these birds rarely show any clinical signs of diseases **(Stallknecht and Shane 1988).** Wild birds can serve as a silent reservoir for avian influenza viruses and these viruses routinely transmitted from this reservoir to poultry in many areas allover the world. If infected wild birds come into contact with, or contaminate an area populated by, commercial/ domestic or zoo birds the virus may transmit to these birds. Since zoo birds are reared outdoors, they are potentially exposed to AIV from wild birds sharing their habitat and penning area. The virus is labile in warm conditions, but can survive for months in a cold environment. **(Panigrahy *et al.*, 2002).**

In 1996, an H5N1 HPAIV (Asian subtype) was detected in geese in Southern China. Since then this virus established endemic infections in poultry, mainly ducks and geese, in many Southeast Asian countries **(Li *et al.*, 2004; Smith *et al.*, 2006).**

The Jakarta post reported on September 19, 2005 that the Ragunan Zoo in south Jakarta has been closed down for 21 days after multiple bird species were found to be infected with bird flu included eagles, herons, peacocks, mynahs, pigmy chickens, and wild ducks **(Lucey 2005).** Also, in Thailand two tigers and two leopards in a zoo died after experiencing high fever and respiratory distress; H5N1 infection was later confirmed as the cause of the illness. **(Keawcharoen *et al.*, 2004).**

Vaccination of zoo birds has taken place in many countries like Netherlands, Switzerland, Portugal, Belgium and France **(EAZA 2006).** Preparations to vaccinate were ongoing in Sweden, Denmark, Hungary and UK. Spain vaccinated zoo birds in August 2006. After vaccination, the first signs of immunity in vaccinated birds can be seen at 2 weeks and protection increases until maximum immunity at 5 weeks post vaccination. In most species a second dose of vaccine is advised at variable times either at 4 weeks or 6–10 weeks **(Philippa *et al.*, 2005).** Subsequent to these first two doses, regular boosters are likely to be required to maintain immunity. It is unsure how long immunity lasts for but the manufacturer suggests 6–12 months. **Oh *et al.*, (2005)** found that titers had decreased at 7–8 months. Whether zoo birds will need continual boosters is unknown and depends on the nature of the risk.

HPAI of subtype H5N1 was reported in Egypt in 17 February 2006 (**Aly *et al.*, 2006**). On February 19, Veterinary authorities in Egypt decided to close Giza zoo as a precautionary control measure after some dead birds tested positive for the H5N1 virus. The decision covers also other zoos in the various governorates. This closure lasted for about two months, during this period biosecurity, disinfection and quarantine measures has been adopted and also vaccination of all zoo birds was done by using commercial licensed vaccine available in that time (H5N1 Chinese origin). The inactivated, oil adjuvanted H5N1 vaccine induced at least 6 log2 haemagglutination inhibition (HI) units according to potency test was used to vaccinate the zoo birds after emergence of early 2006 outbreaks.

This study was conducted to investigate the first introduction of H5N1 HPAIV in different bird species in Giza zoo and also studies the immune status of the vaccinated birds after application of H5N1 vaccine for the first time in 2006.

MATERIAL AND METHODS

I- Sample collection:

Serum samples (n=131) and cloacal swabs (n=175) were collected from 15 species of zoo birds and one specie from mammals. The sampled species included GALLIFORMES (Turkey, Peacock, Guinea fowl, Chicken); PASSERIFORMES (Sky sparrow, Crew), ANSERIFORMES (Grocer duck, Wild Duck, Geese); COLUMBIFORMES (Wild pigeon); CICONIIFORMES (Cattle Egret, Greater Flamingo, Little Egret); STRUTHIONIFORMES (Ostrich, Emus) and one specie from mammals, Feline (Black tiger).

1- 3 ml of whole blood were taken and allowed to clot, the serum samples were centrifuged at 2500 rpm/10 minutes to separate serum and kept at 4 °C until tested and then stored at -20 °C. Swabs were placed in viral transport medium and were immediately chilled in ice boxes till return to the laboratory.

II. Direct detection of AIV by real-time RT-PCR:

The molecular detection of the M gene of avian influenza from samples collected from each type of zoo birds was carried out by using Real time AIV RT-PCR (PG-Biotech; QIAGEN, Valencia,

California, USA) as described by the manufactures. RNA was extracted from pooled cloacal swabs by using virus RNA Extraction Kit (QIAGEN, Valencia, Calif,USA). Positive samples were further processed for virus isolation.

III. Virus isolation and Characterization:

The cloacal swab samples from 5 types of zoo birds were pooled and inoculated in 9-day-old SPF embryonated chicken eggs for up to 5-7 days at 37°C. The allantoic fluids were harvested and tested for HA activity as previously described by ***OIE Manual, (2005)***.

The isolated viruses were further identified and subtyped by using Real time RT-PCR for H5N1 (Roche, Mannheim, Germany) kits as described by the manufactures.

The nucleotide sequence of the HA gene was investigated in order to identify the pathogenicity of the isolated strains. One isolate from turkey was submitted to NAMRU-3 unit, Cairo, where the PCR product of HA gene was directly sequenced and complete characterization and typing of isolate was done.

IV. Serological monitoring by hemagglutination-inhibition (HI):

The birds were vaccinated three times with 8 weeks and 8 months intervals via the subcutaneous route. The vaccine dose administered was calculated according to body weight.

Serum samples were pretreated with 10% chicken RBCs to remove the nonspecific HA binding and tested for presence of antibodies against H5 in vaccinated zoo birds by HI test, where the HI titers were determined according to standard methods ***(OIE Manual., 2005)*** by using chicken erythrocytes and 4 hemagglutinating units of H5 antigen. In empirical bases, an HI titer more than 4 log2 suggested a positive antibody titer; an HI titer less than 4 log2 was considered negative.

RESULTS

I. Virological testing:

Testing of cloacal swabs for presence of AIV by real-time RT-PCR for the M gene was positive for 5 of 175 examined samples. (Table 1).

Four positive samples from the 5 and another negative sample of wild pigeon were subjected to virus

isolation. The HA activity has been demonstrated for 2 isolates from turkey and Grocer duck after the first passage, the allantoic fluid of inoculated SPF embryos were collected from died eggs (within 5 days). HA titer of the isolated viruses was 7 and 5 log2 respectively.

The 2 isolates were tested positive for presence of H5 and N1 genes by real-time RT-PCR.

One isolate from turkey was submitted to NAMRU-3 unit. Cairo, where the PCR products of HA gene were directly sequenced. The HA cleavage site were (PQGERRRKKRGLFGAIA) identified this isolate as a highly pathogenic avian influenza (HP AIV).

Table 1. Virological examination for detection and isolation of AIV from Zoobirds:

Bird type (Common Name)	Date of collection	No. of tested birds	RT-PCR[1] (M)	AI isolation	
				HA[2]	RT-PCR[3] (H5N1)
Turkey	18/2/2006	18	+	+	+
Peacock (1)	20/2/2006	6	+	Nd[4]	
Sky sparrow	20/2/2006	6	-	Nd	
Grocer duck	20/2/2006	5	+	+	+
Wild pigeon	20/2/2006	4	-	-	Nd
Greater Flamingo	20/2/2006	3	+	-	Nd
Crow	22/2/2006	1	+	-	Nd
Cattle Egret	5/3/2006	3	-	Nd	
Emus	6/3/2006	2	-	Nd	
European Chicken	6/3/2006	22	-	Nd	
Wild Duck	6/3/2006	12	-	Nd	
Black tiger*	7/3/2006	8	-	Nd	
Ostrich	12/3/2006	3	-	Nd	
Geese	12/4/2006	22	-	Nd	
duck	12/4/2006	18	-	Nd	
Peacock (2)	12/4/2006	12	-	Nd	
Guinea fowl	19/4/2006	30	-	Nd	
Total		175	5/17	2	2

[1]RT-PCR (M) = real-time PCR for M gene; [2]HA = Hemagglutination test; [3]RT-PCR (H5N1) = real-time PCR for H5 and N1 genes; [4]Nd = Not done; * Black tiger= one specie from mammals (Feline).

II. Serological testing:

Serum samples were tested for monitoring of AI antibodies against HA protein after each vaccination of avian influenza by using HI test and the results after first vaccination were positive for 8 types of birds examined and the HI titer was more than 4 log2. Only one type (Little Egret) vaccinated in the same date give low antibody response (less than 4 log2) among 9 types of zoo birds examined. The positive titer was also noticed after the second vaccination for 7 types of birds examined, and for 4 types after third vaccination. (Table 2).

DISCUSSION

During the first incursion of HPAI of H5N1 virus in Egypt, in 18 February 2006, turkey in Giza zoo exhibited symptoms suspected to be avian influenza disease as severe congestion in snout, sudden death. Some birds were submitted to the laboratory for laboratory investigations, and this was followed by submission of other samples from the zoo from other different bird species two days after.

The results of virus isolation, identification and molecular characterization using Real-time PCR as well as genetic analysis of HA cleavage site confirmed that the observed clinical signs, lesions and mortalities were due to HPAI of subtype H5N1.

There were differences in susceptibility between birds species observed where the GALLIFORMES especially turkey and peacock were more affected; ANSERIFORMES (Grocer duck, Wild Duck, Geese), PASSERIFORMES, and CICONIIFO-RMES were less affected while COLUMBIFORMES, STRUTH-IONIFORMES and the Black tigers were not affected.

The effect of H5N1 infection on birds varies greatly between species. During the H5N1 outbreak in Hongkong 2003, Flamingos and several other bird species appeared to be susceptible, resulting in a high mortality rate. None of the caged passerines were infected, although this may have been owing to the enhanced biosecurity rather than to any innate species resistance **(Ellis *et al.*, 2004).** Some passerine species are not likely to represent a significant reservoir of AI viruses as they rarely get infected or, like psittacines, die **(Perkins and Swayne, 2003).**

Waterfowls and free flying wild birds are the important natural reservoir of avian influenza (AI) viruses. Wild waterfowl provide a

reservoir of most HA subtypes **(Stallknecht 1997).** Wild waterfowl are usually asymptomatic for AIV infection, may excrete virus in the feces for long periods, may be infected with more than one subtype, and often do not develop a detectable antibody response. Zoo birds may acquire AIV infection as they come in contact with their wild counterpart. Thus the waterfowl and any creature sharing that waterfowl environment (such as free-flying birds, small mammals, and man) may spread the disease.

Differences in responses between and within taxonomic orders were seen as previously reported ***(Oh et al., 2005).*** **Heckert *et al.*, (1999)** suggested that emus are similar to wild waterfowl in their susceptibility and response to AIV infection.

Philippa *et al.*, (2005) described three orders that seemed to show a lower antibody response Pelecaniformes, Passeriformes and Columbiformes. Although the order of Galliformes showed favorable overall antibody responses, guinea-fowl, reacted with low titers. While **Bertelsena *et al.*, (2007)** reported a significant species variation in response; pelicans, ducks, geese and Guinea fowl showed very poor response to vaccination, while very high titers and seroconversion rates were seen in flamingos, ibis , Congo peafowl and amazon parrots.

Ducks have been documented with antibodies up to 10 months post-vaccination, and were protected from challenge infection at this time, but the longevity of antibodies in geese was much shorter **(Tian *et al.*, 2005).** In Singapore, a small sub-sample of vaccinated zoo birds showed persistence of serum antibodies when tested 6 months post H5 vaccination **(Oh *et al.*, 2005).** Re-vaccination 6–10 months post-vaccination may therefore be required to maintain protective titers among the large variety of avian species in zoos. **Redrobe (2007)** recommends further research into the longevity of serum antibody titers upon vaccination in different exotic species.

The data from this work revealed that vaccination with an inactivated vaccine is useful and necessary component of the preventive measures applied for avian influenza H5N1 control. Also, monitoring of the vaccinated zoo birds for AI viruses and serological monitoring should remain a priority as part of the surveillance program. Other measures for prevention of direct and indirect contact with

Table 2. Serological examination for monitoring of H5N1 antibodies in vaccinated Zoobirds:

Bird type	1st vaccination:13/3/2006			2nd vaccination: 17/5/2006			3rd vaccination: 14/1/2007		
	Date of testing	No. of birds	HI[1] Mean	Date of testing	No. of birds	HI[1] Mean	Date of testing	No. of birds	HI[1] Mean
Guinea fowl (1)	9/4/2006	9	5.9	17/6/2006	2	8.5	4/3/2007	3	5.7
Guinea fowl (2)		9	6.8		2	8		2	5.5
European Chicken		9	5.1		2	8.5		-	-
Pea fowl (1)		8	8		2	8.5		-	-
Pea fowl (2)		10	7.9		2	7		4	4.8
White pea fowl		11	7.7		4	8.25		4	6.5
Mallard duck		11	6.7		6	7.8		5	7.6
Greater Flamingo		9	6.1		-	-		-	-
White Stork		9	7.6		2	8		-	-
White pelican		9	7.4		2	9		-	-
Little Egret	15/4/2006	2	2.5		-	-		-	-
Total[2]		96			24			18	

[1]HI = Hemagglutination inhibition test; [2]Total number of birds tested.

should be applied; for example, keep zoo birds inside where possible, use appropriate sized mesh on aviaries and roof-meshed aviaries; bio-security measures must be in place; removal of manure and other waste products must be periodically; the movement of people, domestic animals and vehicles, etc., is subject to conditions and authorization by Zoo management. The key to the prevention and control of AI disease in zoos is good bio-security measures.

REFERENCES

Alexander. D.J., (1982). Isolation of influenza A viruses from birds in Great Britain during 1980 and 1981. Vet Rec. 1982 Oct 2; 111 (14):319-21.

Aly M.M, Hassan M.K, and Arafa A., (2006): Emergence of highly pathogenic H5N1 avian influenza virus in poultry in Egypt: First record of 2006 outbreaks. Journal of the Egyptian veterinary medical association, vol.66 (2): 263-276

Astorga, R.J., Leon, L., Cubero, M.J., Arenas, A., Maldonado, A., Toradas M.C. and Perea, A. (1994). Avian influenza in wild waterfowl and shorebirds in the Doñana National Park: serological survey using the enzyme-linked immunosorbent assay. Avian Pathol. 23(2). 339-344

Bertelsena, M. F., Klausenb, J., Holmb, E., Grondahla, C. and Jorgensen, P. H. (2007). Serological response to vaccination against avian influenza in zoo-birds using an inactivated H5N9 vaccine. Vaccine Volume 25, Issue 22, 4345-4349

Easterday, B. C., Hinshaw, V. S. and Halvorson, D. A., (1997): Influenza. In Diseases of poultry (10th edn). 583– 605. Calnek, B. W., Barnes, H. J., Beard, C. W., McDougald, L. R. & Saif, Y. M. (Eds). Ames, IA: Iowa State University Press.

EAZA (2006): Vaccination of zoo birds against avian influenza. Amsterdam: European Association of Zoos and Aquaria (EAZA). News at http://www.eaza.net/

Ellis, T. M., Bousfield, R. B., Bissett, L. A., Dyrting, K. C., Luk, G. S. M., Tsim, S. T., Sturm-Ramirez, K., Webster, R. G., Guan, YI and Malik Peiris, J. S. (2004): Investigation of outbreaks of highly pathogenic H5N1 avian influenza in waterfowl and wild birds in Hong Kong in late

2002. Avian Pathology 33: 492–505.

Heckert, R.A., McIsaac, M., Chan, M., & Zhou, E-M., (1999). Experimental infection of emus (Dromaiius novaehollandiae) with avian influenza viruses of varying virulence: clinical signs, virus shedding and serology. Avian Pathology, 28, 13-16

Keawcharoen,J., Oraveerakul, K., Kuiken, T., Fouchier, R. A., *et al.*, (2004): Avian influenza H5N1 in tigers and leopards. Emerging Infectious Diseases 10: 2189–2191

Li, K.S., Guan, Y., Wang, J., Smith, G.J., Xu, K.M., *et al.*, (2004). Genesis of a highly pathogenic and potentially pandemic H5N1 influenza virus in eastern Asia. Nature 430, 209–212.

Lucey, D. R., (2005): Bird Flu closes Jakarta Zoo, H5N1 human case(s) confirmed. Website for this "newsletter" posting http://www.bepast.org/ http://www.bepast.org/docs/washington%20newsletter/2005-09-20_newsletter_jakarta_zoo.htm

Oh, S., Martelli, P., Hock, OS., Luz, S., Furley, C., Chiek, EJ., et al., (2005). Field study on the use of inactivated H5N2 vaccine in avian species. Vet Rec.157 (10):299–300.

OIE Manual (2005): OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals. Chapter 2.7.12. Version adopted May 2005. http://www.oie.int/eng/normes/mmanual/A_00037.htm

Panigrahy, B., Senne, D. A. and Pedersen, J.C. (2002). Avian influenza virus subtypes inside and outside the live bird markets, 1993-2000: a spatial and temporal relationship. AVIAN DISEASES 46:298–307, 2002

Perkins, L. E. L. and Swayne, D. E. (2003): Varied pathogenicity of a Hong Kong origin H5N1 avian influenza virus in four passerine species and budgerigars. Veterinary Pathology 40: 14–24.

Philippa, J. D. W., Munster, V. J., Van Bolhuis, H., Bestebroer, T. M., Schaftenaar, W., Beyer, W. E. P., Fouchier, R.A.M., Kuiken, T. and Osterhaus, A. D. M. E., (2005): Highly pathogenic avian influenza (H7N7): vaccination of zoo birds and transmission to non-poultry species. Vaccine 23: 5743–5750.

Redrobe, S.P., (2007). Avian influenza H5N1: a review of the current situation and relevance to zoos. Int. Zoo Yb. 41: 96–109

Slemons, R. D., D. C. Johnson, J. S. Osborn, and F. Hayes. (1974). Type-A influenza viruses isolated from wild free-flying ducks in California. Avian Dis 18:119—124.

Smith, G.J.D., Fan, X.H., Wang, J., Li, K.S., Qin, K., et al., (2006). Emergence and predominance of an H5N1 influenza variant in China. Proc. Natl. Acad. Sci. U.S.A. 103, 16936–16941.

Stallknecht, D. E. (1997): Ecology and epidemiology of avian influenza viruses in wild bird populations: waterfowl, shorebirds, pelicans, cormorants, etc. In: Proc. 4th International Symposium on Avian Influenza, U.S. Animal Health Association, Athens, GA. pp. 61–69.

Stallknecht and Shane (1988): Host range of avian influenza virus in free-living birds. Veterinary Research Communications 12: 125–141

Tian G, Zhang S, Li Y, Bu Z, Liu P, Zhou J, et al. (2005): Protective efficacy in chickens, geese and ducks of an H5N1-inactivated vaccine developed by reverse genetics 6. Virology;341(1):153–62.