BENHA UNIVERSITY
FACULTY OF VETERINARY MEDICINE

BENHA VETERINARY MEDICAL JOURNAL

STUDY ON EXPERIMENTALLY INFECTED OSTRICHES WITH AVIAN INFLUENZA A VIRUS (H5N1)

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A B S T R A C T

The pathogenicity of avian Influenza of H5N1 subtype was studied in ostriches. Two groups of free and susceptible ostriches; two months old; were used to test the pathogenicity of avian influenza A, H5N1 subtype on ostriches. Group I (n=4) inoculated intranasally (IN) with 0.2 ml of HPAI H5N1 strain of 10^6 ELD50/0.1ml. Group II (n=2) was kept as control. In group I; observed clinical signs were in-appetence, loss of body weight, respiratory distress and nervous signs, while post-mortem lesions were congestion and hemorrhages predominated in respiratory, digestive and immune systems. In conclusion, H5N1 subtype of AIV caused severe form of the disease in ostriches. The clinical signs and post-mortem findings were specific for the diagnosis of the disease. To the best of our knowledge experimental reproduction of HPAI (H5N1) in ostrich seems to be first report in Egypt.

KEY WORDS: Avian Influenza, H5N1, Ostrich, Pathology

(BVMJ-24 [1]: 19-26, 2013**)**

1. I N T R O D U C T I ON

The ostrich industry has been a viable commercial agricultural industry for over 100 years in South Africa. Currently, this industry is expanding rapidly in many countries all over the world. In Egypt ostrich farming is one of new agricultural industry providing high investments and its future success depends on its high quality and disease free products. Avian influenza (AI) belongs to Genus Influenza virus type A of the family Orthomyxoviridea [7]. It is a contagious viral infection of many avian species including poultry, wild and exotic birds, shore birds, and migratory water fowl. Avian influenza viruses are distributed worldwide [8, 12, 19] and is a well-known threat to the Ratite industry causing production losses and trade restrictions [6]. Several outbreaks caused by (HPAI) subtypes had been reported in ostrich's farms; in South Africa H7N1 subtype (1991-1992) and H5N9 subtype (1994) were isolated during the outbreaks of the disease in ostriches [1, 2]. H5N2 subtype was isolated in ostriches during an outbreak of avian influenza in South Africa in 2004 and thousands of ostriches were culled-off to contain the diseases [5, 16]. In Saudi Arabia, 14 separate outbreaks of H5N1 AI occurred between 2003 and 2007; affected birds included falcons, peacocks, turkeys, parrots and ostriches. In one outbreak about 13,500 ostriches beside 4 million poultry were culled-off to contain the disease [18]. Therefore, the objective of this study was to describe the clinical, gross, histopathological findings and viral shedding observed in the ostriches experimentally infected by H5N1 of the AI virus.

2. MATERIALS AND METHODS

2.1. *Birds:*

Ostrich chicks of one-day-old (*Struthio Camelus*) were purchased from a commercial ostrich hatchery located in the Elasher city, Cairo, Egypt. The ostrich were reared in clean disinfected isolated room on floor and fed on ostrich ration till two months of age. Blood samples were collected for sera one week before the experiment where they were negative for HI-antibodies for AIV.

2.2. *Virus:*
The HPAI virus strain A/Ostrich/ Ismailia/ Egypt/ 2011 H5N1 (clade 2.2.1) which isolated and identified in our study in National Laboratory for Veterinary Quality Control on Poultry Production (NLQP) was used as challenge strain for inoculation. The virus titer was 10^6 ELD50/0.1ml

2.3. *Experimental protocol:*
Four two-Months-old ostrich chicks (Group I) were inoculated intranasally (IN) with 0.2ml of HPAI H5N1 strain of 10^6 ELD_{50}/0.1ml. The infected birds were housed in isolated units in the Central Laboratory for Veterinary Quality Control on Poultry Production (CLQP), Animal Health Research Institute, Dokki, Giza. Other two ostriches were left away from group I as control (Group II). Birds were identified by colored tabs in their legs into bird A, B, C and D. Birds were kept under daily observation till the end of experiment (14 days).

2.4. *Samples*

2.4.1. *Blood samples:*
Serum samples were collected at 0, 3, 5, 7, 9, 12 and 14 days post inoculation from ostriches in the two groups. HI test were carried out according to OIE [18] using reference antigen (Antigen H5N1 Istituto Zooprofilattico delle Venezie OIE/FAO laboratory for AI Batch 2/08) supplied from local agency of (GD Lab., Holland). All sera collected from birds were tested by HI test 1 week before the experiment as well as immediately before the experiment; all sera detected no anti AIV (H5) antibodies

2.4.2. *Samples for re-isolation:*
The tracheal and cloacal swabs were collected from inoculated ostriches at 3, 5, 7, 9, 12 and 14 days post inoculation (PI). Tissue organs include lung, trachea, liver, heart, intestine, kidney, spleen; brain and thymus were collected for AIV re-isolation. The re-isolation were performed by inoculation of specific pathogen free (SPF) embryonated chicken egg (ECG) 9-11 days via allantoic route according to OIE [18].

3. RESULTS

3.1. *Clinical signs*
All birds in the experimentally infected group started to show clinical signs at four days post inoculation in form of in-appetence, loss of body weight and slight bleeding from the vent. Later on, the infected birds showed respiratory manifestation (nasal discharge and open mouth) at 6th day post inoculation, nervous signs (convulsion followed by paralysis) were shown shortly before death. The mortality percent recorded as 75% (3/4) (Table.1).

3.2. *Body weights*
The birds in group I showed loss in their mean body weights in comparable with that in group II as shown in table (2). The difference of mean body weights between birds in the two groups was 1.25 kg at day 3 PI and the gap of weight difference was gradual increased till reach 6.50 kg at day 14 PI.

3.3. *Postmortem findings*
The gross lesions in the three dead birds B,C and D in group I were highly sever in comparable with that observed in the sacrificed bird A, externally the gross lesions were in the form of facial edema, conjunctivitis, serous exudates in nostrils

and hemorrhages were seen in inner eye, legs and vent, fig. (1A). Internally, the most prominent lesion were seen as sever hemorrhages in subcutaneous tissue in face (fig. 2) at neck, in fat tissue and musculature. Severe hemorrhages on trachea with bloody exudates in its lumen (fig. 1B), pneumonia (fig. 1C); hemorrhages were extensively seen in coronary fat and the myocardium; in intestinal mucosa (fig. 1D), on liver surface, in pancreas, in the thymus gland, in bursa (fig. 1E) and in brain tissue.

Table 1 Mortality rate of two months old ostriches inoculated with influenza type A virus (A/Ostrich/Ismailia/Egypt/2011(H5N1)).

Group	N	Dose	No. of dead birds Days post inoculation														Dead birds (n)	Mortality rate (%)
			1	2	3	4	5	6	7	8	9	10	11	12	13	14		
I	4	200 µl of 10^6 ELD50/0.1ml	0	0	0	0	0	0	1	0	1	0	0	1	0	0	3	75%
II (control)	2	Not inoculated	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0%

Birds C, D and B dead at day 7, 9 and 12 post inoculations, respectively.

Table 2 Mean body weights of two months old ostriches inoculated with 10^6 ELD50 of influenza type A virus (A/Ostrich/Ismailia/Egypt/2011(H5N1)) in comparable with negative control.

Group	Mean body weight (kg) Days post inoculation						
	0	3	5	7	9	12	14
I	13.75	12.75	11.25	10.66	10.00	10.00	10.00
II (control)	13.50	14.00	14.50	15.00	15.50	16.00	16.50

0=day of inoculation

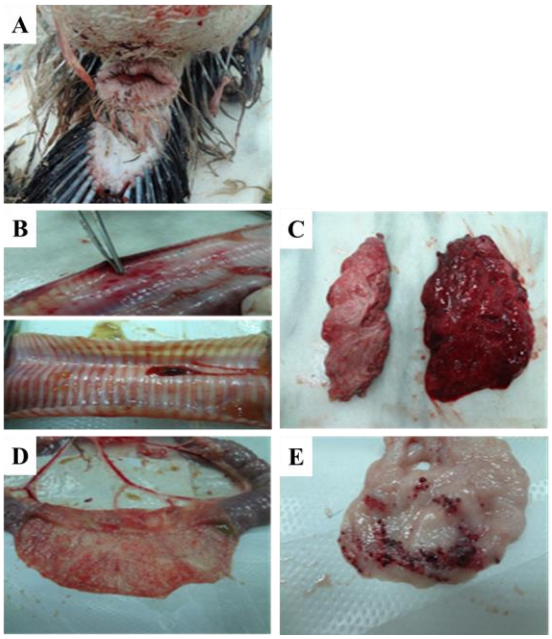

Fig. 1 Postmortem lesions in 72 days old ostrich experimentally infected with avian influenza virus. **A**: hemorrhage of the vent. **B**: hemorrhage in tracheal wall with bloody exudates. **C**: pneumonia in lung. **D**: hemorrhage of intestinal mucosa. E: hemorrhage of bursa.

Fig. 2 Postmortem lesions in 69 days old ostrich experimentally infected with avian influenza virus showed subcutaneous hemorrhages with coagulated blood at face region.

3.4. *Serology results*

Only one bird (A) from group I showed detectable specific antibodies appeared on day 7 post-infection and persisted until the termination of the experiment (14 days) while the other infected birds B, C and D were seronegative before death. HI titers were low in positive bird (A) and ranged

from 3 log2 (23) at (7^{th} and 9^{th} day PI) to 4 log2 (24) at (12^{th} and 14^{th} day PI) dpi. The birds in group II (control) showed no detectable anti-AIV (H5) antibodies through the experimental period (Table 3).

Viral re-isolation from tracheal and cloacal swabs

Successful virus recovery from tracheal and cloacal swabs was shown in table (4). Shedding of the virus differs per days post inoculation and the virus was shed until 12 day PI. At day 3 PI successful recovery of virus was from tracheal swab with percentage of 25% (1/4). Virus re-isolation was failed from cloacal swabs at the same day. The highest recovery percentage of 100% (4/4) was recorded from tracheal swabs at day 5 PI. In contrast, the cloacal swabs the highest percentage of 100% (3/3) was recorded at day 7 PI.

Viral re-isolation from examined organs

Successful virus re-isolation were attempted from different tested organs from group I, AIV was recovered from all tested organs except liver, intestine and heart with recovery percentage of 100 % (4/4) as shown in table (5). Most of SPF embryos died within 24 to 48 hours after inoculation and showed haemorrhage.

Table 3 Detection of avian influenza virus antibodies by haemagglutination inhibition assay (HI) in 2months old ostriches post-inoculation with influenza type A virus (A/Ostrich/Ismailia/Egypt/ 2011(H5N1)).

Groups	Birds	Serum examination by (HI test) using reference antisera Days post inoculation						
		0	3	5	7	9	12	14
I	A	-ve	-ve	-ve	Pos. 2^3	Pos.2^3	Pos. 2^4	Pos. 2^4
	B	-ve	-ve	-ve	-ve	-ve	Neg.*	_
	C	-ve	-ve	-ve	_	_	_	_
	D	-ve	-ve	-ve	Neg.	_	_	_
II	E	-ve	-ve	-ve	-ve	-ve	-ve	-ve
	F	-ve	-ve	-ve	-ve	-ve	-ve	-ve

Pos. =positive. - = dead bird. *= serum collected before death immediately

Table 4 virus re-isolation from tracheal and cloacal swabs from inoculated 2 months old ostriches with influenza type A virus (A/Ostrich/Ismailia/Egypt/2011(H5N1).

Days post inoculation	Group	n	Re-isolation results /Swab type: Tracheal			Cloacal		
			No. of positive	%*	Birds showed positive re-isolation.	No. of positive	%*	Birds showed positive re-isolation.
3	I	4	1	25%	C	0	0%	
	II	2	0	0%		0	0%	
5	I	4	4	100%	A, B, C & D	3	75%	A,C&D
	II	2	0	0%		0	0%	
7	I	3	2	66.7%	B & D	3	100%	A,B&D
	II	2	0	0%		0	0%	
9	I	2	0	0%		0	0%	
	II	2	0	0%		0	0%	
12	I	2	1	50%	B*	1	50%	B*
	II	2	0	0%		0	0%	
14	I	1	0	0%		0	0%	
	II	2	0	0%		0	0%	

%*=percent of positive sample /daily total number of bird. B*= Samples collected before death immediately.

Table 5 Virus re-isolation from different examined organs from inoculated 2 months old ostriches with influenza type A virus (A/Ostrich/Ismailia/Egypt/2011(H5N1))

Infected birds	Recovery of AIV from organs								
	Lung	trachea	Brain	intestine	liver	heart	Kidney	Spleen	thymus
A	+ve	+ve	+ve	-ve	-ve	-ve	+ve	+ve	+ve
B	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
C	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
D	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
Total*	4/4	4/4	4/4	3/4	3/4	3/4	4/4	4/4	4/4
%*	100%	100%	100%	75%	75%	75%	100%	100%	100%
HA titer	108	-108	107	-108	107	107	107	107	109

Total * = positive virus sample /total number of examined samples per organ. %*= percentage of positive virus sample / total no. of examined samples per organ.

3.5. *Histopathological findings:*

The most prominent histopathological changes in birds of group I were seen as loss of tracheal epithelium with severe submucosal congestion (fig. 3A). Lung revealed severe congested blood vessels (fig. 3B) and hemorrhage in addition to thickening in the wall of blood vessels with interstitial fibrinous pneumonia (fig. 3C). Some alveoli are filled with granulocytes. Interstitial and intra-alveolar deposition of esinophilic material was seen (fig. 3D). Pancreas showed severe congestion and hemorrhage with pronounced vacuolar degeneration of the epithelium of pancreatic acini with necrosis (fig. 3E). Denudation of pancreatic acini was also seen (fig. 3F). Depletion of lymphocytes and necrosis are also observed and pronounced proliferations of sheathed capillaries were revealed. Thymus showed severe congested blood vessels (fig. 3G) with severe hemorrhage in addition to depletion of lymphocytes (fig. 3H). The intestine exhibited hyperplasia and sloughing of the lining epithelium. The lamina propria showed severe congestion with mononuclear cell infiltration (fig. 3I). Immune organs were severely affected, spleen revealed thickening of the splenic capsule with severe congestion and hemorrhage in addition to focal amyloidosis (fig. 3J).

4. DISCUSSION

Avian influenza infection in domestic poultry comes out within different clinical syndromes. The occurrence of the disease syndrome or the degree of severity of the disease depends on multiple factors, including the pathogenicity of the virus, care, feeding conditions, the host species, age of the host, route of infection, and existence of secondary bacterial infections [3, 19]. Besides, it was reported that the severity of the disease depended whether the infection was natural or experimental. Some AIV strains caused severe systemic infections and high mortality in natural conditions [4, 13, 14]. Many authors who studied the pathogenicity of HPAI (H5) in ostriches they found that, despite the high dose of infection, clinical signs were mild [6, 9]. In our study severe respiratory distress and nervous manifestations were the prominent signs induced post inoculation. Similar finding of respiratory signs were observed by Toffan et al. [21].

Although some of our post-mortem findings agreed with that observed by Clavijo et al. [6] but differ in their severities. On the other hand, our PM findings were disagreed with Manvell et al. [10] who found that, the experimentally infected 11 weeks-old ostriches (including sentinel birds) with highly pathogenic AIV were grossly normal except for localized pneumonic lesions. In comparison with previous experimental studies of AIV in ostriches [6, 9, 10], in our experimental work recorded high severity of clinical

signs and PM lesions as well as high mortality rate. These finding may be attributed to young age of ostriches and virulence of virus with respect of the other factors.

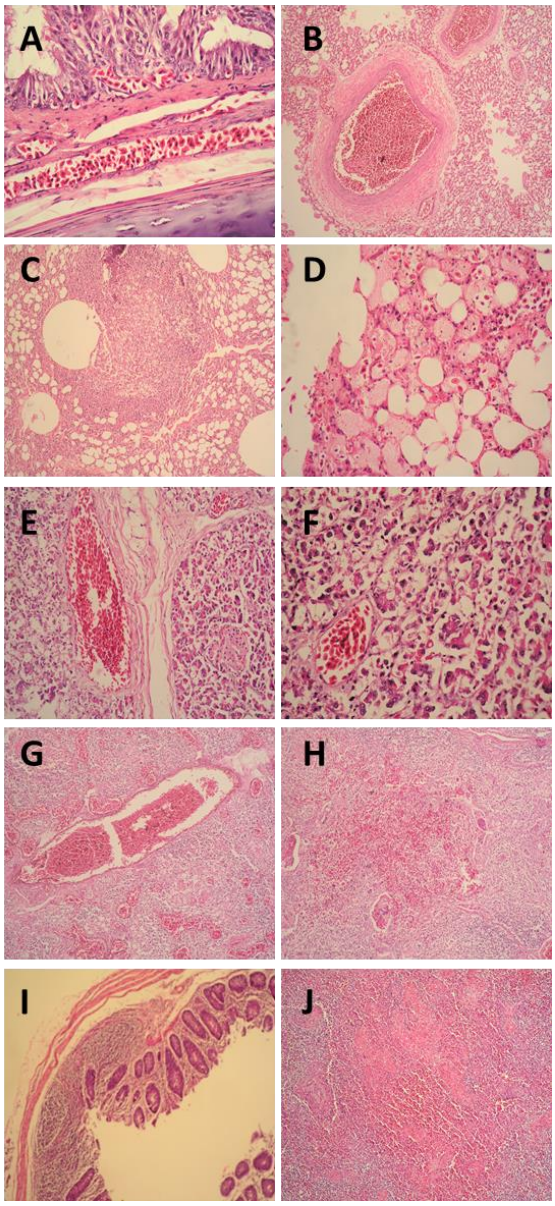

Fig. 3 Histopathological changes in ostrich experimentally infected with avian influenza virus stained with H&E. **A**: Trachea showed submucosal congested blood vessels (×40). **B**: Lung showed severe congestion of blood vessels and perivascular connective tissue proliferation (×10). **C**: Lung showed interstitial fibrinous pneumonia (×10). **D**: Lung showed esinophilic material filled the alveoli (×40). **E**: Pancreas showed severe congested blood vessels with degeneration of the acini (×20). **F**: Pancreas showed denuded and degenerated acini (×40). **G**: Thymus showed severe congested blood vessels (×20) with depletion of lymphocyte. **H**: Thymus showed depletion of lymphocytes and hemorrhage (×10). **I**: Intestine showed mononuclear cells infiltration in lamina propria (×10). **J**: Spleen showed hemorrhage and focal amyloidosis (×10).

Our serological findings share agreement with former results [9] recorded exceptionally low detectable antibody titers by HI test in ostriches challenged by IN route (2-4 log2), also our serological results agreed with former report [21] recorded that, detectable specific antibodies appeared on day 7 post-infection and persisted until the termination of the experiment. On the other hand, our serological results disagreed with Easterday et al. [10] who recorded no significant levels of antibody (HI titers of $\leq 2^1$) were detected in experimentally infected ostriches with AIV (H7).

In this study AIV was most efficiently isolated from tracheal swabs (table 4). The shedding pattern was compatible with the pathogenesis of the AI in that the virus replicates first in the upper respiratory tract and later becomes systemic affecting other organs including gastrointestinal tract. Therefore, cloacal shedding will come a later. A similar shedding pattern was obtained in ostrich using the AIV strain A /Emu /Texas / 39924 /93(H5N2) by Clavjio et al. [6]. Our results indicate that tracheal swabs are the sample of choice for detection of AIV infection in ostrich. The last detection of the viral shedding was at day 12 PI from both tracheal and cloacal swabs. Similar finding was obtained by Manvell et al. [9] in their experimental study of AIV in ostrich while the length of shedding period was shorter (12dpi) than that reported by Manvell et al. [10] who reported that, Virus was shed until day 14 PI in the ostriches group infected with A / Ostrich / Italy / 1038 / 2000.

Our viral re-isolation results from organs (table 5) were higher than that recorded by Clavijo et al. [6]. Our findings indicated that inoculation of ostriches with HPAI virus by a route (IN) that stimulates the natural route of exposure resulted in systemic viral replication.

Histopathological findings were one of the important aspects in our experimental

work to localize the pathological changes in different tissues associated with AIV (H5) infection in ostriches. In fact the frequency and severity of the lesions in each organ were probably related to tissue tropism, host species and how long the ostriches had survived before deaths. In the present study the histopthological findings of respiratory tract were the same like that recorded by Mehrabanpour et al. [14] in 6 weeks old experimentally turkeys infected with AIV. The prominent pathological lesions observed in the intestine and pancreas were similar to PM findings recorded by Manvell et al. [9] and Saif [17]. Sever congestion; hemorrhages and depletion of lymphocytes were commonly pictures seen in immune system organs especially in thymus gland. Lymphocyte depletion may have important repercussions for birds that survive from infection with the AIV H5N1 virus in that immunosuppression and increased susceptibility to other potential pathogen would result [22].

5. CONCLUSION

In conclusion, in this study, it was determined that the H5N1 sample of AIV resulted in sever disease in the ostriches, and that the clinical and pathological findings were specific for the diagnosis. And that in addition to clinical and pathological findings, other laboratory techniques would be useful for the diagnosis of the disease.

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