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Original Article

Sero-Virological studies on Newcastle Disease and Avian Influenza in Farmed Ostriches (*Struthio camelus*) in Saudi Arabia

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ABSTRACT

Two hundred and sixteen samples (80 Tracheal swabs and 136 blood serum) were collected from four commercial ostrich farms in the Kingdom of Saudi Arabia for isolation of Newcastle disease (ND) and Avian Influenza (AI) viruses or detection of their antigens and antibodies by RT-PCR and Enzyme Linked Immune Sorbant Assay (ELISA) respectively. Neither AI and ND viruses nor their antigens were isolated or detected in any of the collected swabs samples. Also, Antibodies to avian influenza virus were not detected in the collected serum samples. Antibodies to ND virus were detected in 0.0%, 80.0%, 74.1%, and 80.0% in serum samples from farms 1, 2, 3 and 4, respectively. Mean ND ELISA antibody titer were 0.0, 646.3, 1068.5 and 1440.5 in farms 1, 2, 3 and 4, respectively. Ostriches vaccinated with live ND vaccine by drinking water were negative for ND antibodies, while ostriches vaccinated with live ND vaccine by spray method and boosted by inactivated oil emulsion ND vaccine gave the highest immune response. Ostriches vaccinated with inactivated oil emulsion ND vaccine (4x chicken dose/ostrich) elicited effective immune response and transferred maternal antibodies to ostrich chicks at the rate of 85.7% and 66.7% with mean ELISA antibody titer of 1450.5 and 1350.15 at 7 and 21 day old, respectively.

Key words: Avian Influenza, ELISA, Newcastle disease, ostrich, RT-PCR, Saudi Arabia.

INTRODUCTION

Newcastle Disease (ND) and Avian Influenza (AI) are among the most economic important viral diseases threatening ostrich industry worldwide. Newcastle Disease Virus (NDV) is an enveloped virus; has a negative sense single strand RNA genome caused by Avian paramyxovirus type1 which is classified with the other avian paramyxoviruses in the genus Avulavirus, subfamily Paramyxovirinae, family Paramyxoviridae, order Monanegavirals (Lamb *et al*., 2005). Newcastle Disease was first described in commercial ostrich flock aged 5 to 9 months with a mortality rate of 28% in Israel (Samberg *et al*., 1989). Cadman *et al*., (1994) carried out a serological survey of nine ostrich farms in Zimbabwe and reported that 23% of the birds tested were positive for ND virus antibodies. The disease was described in ostrich chicks with facial edema and consistent nervous signs in the Republic of South Africa (RSA) (Huchzermeyer, 1996). Virulent NDV was isolated from ostriches dying while held in quarantine in Denmark (Jorgensen *et al*., 1998). Recently, NDV was first reported in the Iranian commercial ostrich farms by isolation from 4 brain samples of ostriches that died following nervous signs (Ghiamirad *et al*., 2010)**.**

Worldwide many subtypes of Influenza Viruses were recorded in ostriches. First isolation of AI in ostrich was reported in RSA from one month old ostrich showing 80% mortality; the subtype of the isolated virus was Low Pathogenic Avian Influenza (LPAI) H7N1 (Allwright *et al.*, 1993). Other LPAI (i.e., H9N2, H6N8 and H5N9) infection in ostriches were reported in RSA (Manvell *et al*., 1998, Abolnik *et al*., 2010). Highly Pathogenic Avian Influenza (HPAI) (*e.g*., H5N2 subtypes) was also reported in ostriches in USA (Panigrahy *et -al.*, 1995) and HPAI H7N1 in Italy (Capua *et al.*, 2000). First HPAI (H5N1) in commercial ostriches was reported in Saudi Arabia in 2007 (Lu *et al*., 2010) in an outbreak where 13460 ostriches were culled.

There is little information about infectious diseases in commercial ostrich farms in Saudi Arabia. Since ostrich (*Struthio camelus*) are kept in free range farms, they may come in contact with wild birds and become exposed to different pathogens such as NDV

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and avian influenza virus. The aim of the present study is to conduct a sero-virological investigation on the current situation of avian influenza and ND in commercial farmed ostriches in Saudi Arabia.

MATERIALS AND METHODS

Samples

One hundred and thirty-six blood serum and 80 tracheal swab samples were collected between September 2011 to November 2012 from four ostrich farms located in Central, North and West regions in Saudi Arabia (Table 1).

Enzyme-Linked Immunosorbent Assay (ELISA)

ELISA test was performed using IDEXX Flock Check (ND and AI viruses antibody test kits).

The test procedure was in accordance with IDEXX Flock Check Manual instructions.

Virus Isolation

Virus Isolation was done by inoculation of 0.2 ml swab supernatant into the allantoic cavity of 9-11 days old embryonated SPF eggs (Alexander and Senne., 2008, Allwright *et al*., 1993) and the eggs were candled daily for 4 days. All eggs with dead embryos were chilled while with surviving embryos were chilled on day 6. Harvested allantoic fluid was tested for Haemagglutination (HA) activity.

PCR

- RNA extraction and first-strand cDNA

Viral RNA was extracted from tracheal swabs using QIAamp® Viral RNA Mini Kit (50) Isolation kit QIAGEN®GmbH). Then Reverse transcription to the extracted RNA using Transcriptor® First Strand cDNA Synthesis Kit (Roche® Version 6.0) was performed according to Manufacturer´ instructions.

- Realtime-RTPCR for detection of AIV:

Primers; LightMix® KIT Inf A M2 primer (Cat. No. 40-0234-16) labeled with flourescein probe containing internal positive control (IPC) and positive control for AIV M2 gene (synthetic oligonucleotides) (TIB MOLBIOL Syntheselabor GmbH).

- A LightCycler® FastStart DNA Master plus HybProbe KIT (Roche) and Light Cycler® 2.0 device were utilized for real-time PCR using 5 µℓ of the cDNA in a total volume of 20 µℓ. The temperature profile was used as follows: 10 min at 95 °C, and 50 cycles of 5s at 95 °C, 5s at 62 °C, and 15s at 72 °C. Fluoro phore-specific emission data were collected during the annealing step. Crossing point (Cp) values were calculated with LightCycler 3.5 software using the second derivative maximum method.

- One Step RealTime RT-PCR for NDV detection: Primers;

APMV-1 - F M + 4100 5_-AGTGATGTGCTCGGACCTTC-3_; APMV-1-R M-4220- 5_-CCTGAGGAGAGGCATTTGCTA-3_ and APMV-1 Probe- 4169 5_FAM]TTCTCTAGCAGTGGGACAGCCTGC[TAMRA]3(Biosearchtech.,INC.Novato,CA) For the RRT-PCR, the Qiagen® one-step RT-PCR kit and LightCycler® 2.0 device, 20ul reaction volumes were used. Extensive optimization was performed on all three primer-probe sets on the following parameters: annealing temperature, MgCl2 concentration, primer concentration, probe concentration, and primer ratios. The assays for M gene were developed with the following amounts per reaction; 5ul of the extracted RNA template, 0.5 ul of kit-supplied enzyme mix (including Hot Start Taq polymerase and RT), 4ul of kit-supplied buffer (5x),10 pmol of the reverse primer, 10pmol of the forward primer, 6 pmol of probe, 0.8 ul of kit-supplied deoxynucleoside triphosphates (final concentration: 320 mM each), 1ul of 25 mM MgCl2 (combined with MgCl2 in kit-supplied buffer, final concentration 3.70 mM) and 13 U of RNase inhibitor (Promega, Madison, Wis.). For each primer set, the RT step was 30 min at 50°C, followed by 15 min at 95°C. The cycling conditions for the APMV-1 matrix primers consisted of 40 cycles of 10 s of denaturation at 94°C, 30 s of annealing at 52°C, and extension at 72°C for 10s (Wise *et al*., 2004).

Table 1. serum and tracheal swabs samples from ostrich farms

Farm No.	Sample				
	Serum				Swabs
	7 days	21 days	6 month	Breeders	Tracheal
1	NA[1]	NA	NA	20	20
2	10	10	10	NA	20
3	14	12	10	20	20
4	NA	NA	10	20	20

[1]NA: Not Available

RESULTS AND DISCUSSION

Ostrich farming is considered a new industry compared with the highly advanced and modernized poultry industry established in Saudi Arabia many years ago.

Saudi Arabia is considered NDV endemic country in its commercial poultry sector. Moreover HPAI (H5N1) was previously reported in poultry and ostrich farms in Saudi Arabia in 2007. The present study aimed to evaluate the current situation of both diseases in commercial ostrich farms in Saudi Arabia.

The obtained results revealed that, Neither AI and ND viruses nor their antigens were isolated in embryonated chicken eggs or detected by RT-PCR in any of the 80 collected swab samples.

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As previously reported, there is a conflict on the suitable serological test for detection of antibodies against avian influenza in ostrich sera. Williams *et al.* (1997) reported that ostrich sera are highly cross reactive when tested with Haemagglutination Inhibition test (HI) for the presence of non specific inhibitors of haemagglutinin resulting in a high number of false positive results. The study by Toffan *et al.* (2010) that evaluated the best serological test for detection of antibodies against avian influenza in ostrich sera concluded that validated ELISA is considered suitable test for screening purpose and should be preferred to Agar Gel Immune Diffusion test (AGID).

In the present study, ELISA seemed to be the best screening method to detect avian influenza and ND antibodies in ostrich sera. The obtained results showed that all tested sera were negative for avian influenza antibodies, which revealed that ostrich farms in Saudi Arabia are considered free from avian influenza.

All farms included in the present study practiced vaccination against ND, but the vaccination regimen (Method and dose) varied between farms (Table 2).

Table 2. Vaccination programme applied in the four ostrich farms

Farm	Age	Type of vaccine	Dose	Method of administration
1	15 day old	Hitchner live	1Xchicken dose/ostrich	Drinking water
	3 month	Lasota live		
2	7 day old	Lasota live	1Xchicken dose/ostrich	Eye drop
	30 day old	Inactivated oil emulsion ND		Intramuscular
3	15 day old	Inactivated oil emulsion ND	1ml/ostrich	Intramuscular
	3month			
	Breeder	Inactivated oil emulsion (ND)	2ml/ostrich (4x chicken dose)	
4	1 day old	(ND+IB) Live	1Xchicken dose/ostrich	Spray
	21 day old	Inactivated oil emulsion ND	0.3ml/2kg	Intramuscular
	3 month		0.3ml/2kg	
	6 month	Inactivated oil emulsion (ND+IB)	2.5ml/10kg	
	breeder		8ml/1bird (16x chicken dose)	

[1]NA: Not Available; ND: Newcastle Disease; IB: Infectious Bronchitis

Table 3. Positive ND antibodies in serum samples from ostrich

Farm No.	Growing			Breeders	Mean positive %
	7days	21days	6 month		
1	NA[1]			0.0%	0.0
2	0.0%	80.0%	0.0%	NA	80.0
3	85.7%	66.7%	0.0%	70.0%	74.1
4	NA		0.0%	80.0%	80

[1]NA: Not Available

Table 4. ND HI titer at different age of the four ostrich farms

Farm		Age			
		7	21	6 Month	Breeder
1	Mean	NA[1]			0.0%
2	Mean	0.0%	646.3	0.0%	NA*
3	Mean	1452.2	1350.15	0.0%	1068.5
4	Mean	NA		0.0%	1440.5

[1]NA: Not Available

In the first farm, ostriches that were vaccinated with Hitchner and La Sota live vaccines (1x chicken dose/ostrich by drinking water) did not elicit antibodies in any of tested sera, This result in consistent with result obtained by (Sakai *et al*., 2006) who found that only 9 from 30 tested serum samples from ostrich immunized 3 times at 2 weeks intervals with live ND vaccine by drinking water tested positive.

In the second farm, young ostriches were primed at 7 day of age with (1x chicken dose/ostrich live La Sota vaccine by eye drop and boosted at 30 days old by Inactivated ND vaccine 1x chicken dose/ostrich by intramuscular route. In this regimen, ND antibodies were found in 0.0%, 80% and 80% of tested sera at 7, 21 days and 6 months of age, respectively (Table 3). This finding revealed that live la Sota vaccine elicit immune response when given by eye drop at a dose of 1x chicken dose/ostrich at 7 days of age. This finding is in disagreement with the results obtained by Blignaut *et al.* (2000) who reported that immunization by eye drop with a live La Sota vaccine in 5 weeks old ostriches did not elicit a humoral immune response.

In both third and fourth farms, we noticed that, Vaccination regimens for ND depends mainly on inactivated oil emulsion vaccines with gradual increase in vaccination doses/ostrich by age. In farm 3

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vaccination regimen started with double chicken dose/ostrich at 15 day old, then the dose increased to reach 4x chicken dose/ostrich for older birds. Whereas in farm 4, ND vaccination dose reached 16x chicken dose/breeder ostrich (8ml/ostrich).

The positive tested breeder sera from farms 3 and 4 were 70% and 80%, respectively (Table 3). The higher percentage of positive samples in farm 4 agreed with Maderios (1997) who recommended gradual increase in ND oil emulsion vaccine starting with 2x chicken doses at 2 weeks old and thereafter increasing gradually by age until reaching 10x chicken dose/ostrich at 12 months of age.

The mean ND ELISA antibody titer were 0.0%, 646.3,1068.5 and 1440.5 in farms 1, 2, 3 and 4, respectively (Table 4). This finding revealed that higher dose in farm 4 gave the highest antibody titer while vaccination with eye drop 1x chicken dose/ostrich gave the lowest titer.

Maternally derived antibodies (MDAbs) against ND were not detected in any of the tested sera collected from farm 2, whereas in farm 3 MDAbs were detected in 85.7% and 66.7% of the tested sera in 7 and 21 day old ostriches with mean ELISA titer of 1450.5 and 1350.15, respectively (Table 3). This finding revealed that the vaccination regimen using 4x chicken dose /ostrich is effective to induce maternally derived antibodies in ostrich chicks.

The present results revealed that ostrich farms in Saudi Arabia are considered free from avian influenza. Live ND Vaccines administered through drinking water failed to produce immune response, while vaccination by spray method and boosted by inactivated vaccine appeared to be effective to induce immune response with maternal derived antibodies in ostrich chicks. The antibody titer is correlated with the dose of inactivated vaccine.

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