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EVALUATION OF PREDOMINANT COMMERCIAL VACCINES IN PROTECTION FROM AVIAN INFLUENZA DISEASE H5N1 IN BROILER CHICKENS

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ABSTRACT
With regard of economic importance of H5N1 avian influenza disease, some vaccines were evaluated against avian influenza experimental infection. 5 groups of one day old chick each group 30 chicks, group 1 non vaccinated non challenged; group 2 vaccinated with Vectormune then challenged; group 3 vaccinated with inactivated H5N2 then challenged, group 4 vaccinated with Inactivated H5N1 then challenged and group 5 non vaccinated challenged. serum samples were collected weekly and the HI titers were determined. The highest HI titers and protection percent was recorded in group vaccinated with Vectormune followed by group vaccinated with inactivated H5N1 then group vaccinated with inactivated H5N2. typical clinical signs and post mortem lesions of highly pathogenic avian influenza virus were observed in groups 3, 4 and 5 but Vectormune vaccinated group showed certain mortalities without obvious clinical signs. Tracheal shedding were detected by RT-PCR, Vectormune vaccinated group recorded the lowest shedding followed by H5N2 then H5N1 vaccinated group.

INTRODUCTION
Avian influenza (AI) is a highly contagious disease of poultry widely distributed all over the world. In Egypt, in February 2006, sever outbreaks of HPAI, H5N1; have emerged in several governorates and were associated with drastic mortality up to 100% in infected chickens (**Aly et al., 2006**). Economic importance of HPAI outbreaks associated with depopulation, disposal, high morbidity, high mortality, quarantine, surveillance, indemnities paid for elimination of marketing birds and the loss of foreign export markets (**Swayne and Halverson, 2003**). Egypt has been most severely affected by continuous outbreaks, resulting in severe losses in the poultry industry and efforts to control highly pathogenic H5N1 avian influenza virus in poultry and humans have failed despite increased biosecurity, quarantine and vaccination even three doses of vaccine (inactivated oil- whole- virus emulsion H5N1 vaccines from China and Europe) at poultry farms. The ongoing circulation of HP H5N1 AI in Egypt has caused >100 human infection and remains an unresolved threat to veterinary and public health (**Kim et al., 2010b**).

MATERIAL AND METHODS
Experimental birds
One hundred and fifty one day old commercial chicks, Avian breed were reared under good hygienic conditions and fed on a starter ration up to 3 weeks old then grower ration from 3 weeks old to the end of the experiment.

Vaccines
Vectormune AI® HVT AIV: vector vaccine contains a genetically engineered marek's disease vaccine THV expressing AI H5N1 key protective antigen (H- antigen) in frozen cell associated form preserved in liquid nitrogen.

- Other vaccines: (Intervet International B. V, Boxmeer – Holland).
- H5N1(Re-5 Strain) Merial®. - H5N2 CEVAC FLUKEM Ceva®.
- ND Clone 30 and IB MA5. - Gumboro D78.
- ND+IB inactivated oil adjuvant. - ND Clone 30.

Drugs:- tylosine - colistine – doxycycline - Amprolium.

Challenged virus: H5N1 strain obtained from Reference Laboratory for Veterinary Quality Control on Poultry Production, Agriculture Research Center, Egypt. It was tested by RRT-PCR to make sure it was negative for other Common avian pathogens, H9N2 AIV, Newcastle disease virus and Infectious bronchitis virus and this virus was titrated in SPF ECE to obtain the Allantoic fluid in a titer of 106 Embryo Infective dose (EID50)/mL which calculated according to **Reed and Muench, 1938.**

Experimental design: (Table 1) A total number of 150 one day old chicks, Avian breed used to evaluate three different commercial available vaccines experimentally, divided into 5 groups each group 30 chicks, group1 non vaccinated non challenged; group 2 vaccinated with

Vectormune AI® vaccine at 1 day old then challenged; group 3 vaccinated with inactivated H5N2 vaccine 0.5 ml s/c at 7 days old then challenged, group 4 vaccinated with Inactivated H5N1 vaccine 0.5 ml s/c at 7 day old then challenged and group 5 non vaccinated challenged. Challenge at 28 days old through naso-ocular route with 106/ EID 50, chickens kept under daily observation for signs, morbidity and mortality till the end of experiment.

Serum samples: were collected at one day old then weekly till 35 days of age and HI test carried according to OIE, 2012.

Tracheal swabs: were collected at 2 and 5 dpi under complete a septic condition to detect the challenge virus shedding, each 3 birds represent one pooled sample collected in 2 ml transport medium (PBS 7.2 PH, penicillin 2000 unit, nystatin 1000 IU, streptomycin 2mg, gentamicin 50 µg/ml).

RRT-PCR Requirements (Real time-Reverse Transcription polymerase Chain Reaction), Master Mix kit (Reagents and buffers for extraction of viral RNA) a) Hot Start Taq DNA polymerase. b) Rt-PCR buffer (Tris-CL, KCL, (NH4)2SO4, 8µM MgCl2, dNTP mix (Contains dATP, dCTP, dGTP and dUTP of ultrapure quality). c) Fluorescent dye. d) QuantiTect RT mix (Contains an optimized mixture of the QIAGEN proteases). e) Rnase free water (PCR grade), **Primers** used for Real time Reverse Transcriptase-Polymerase Chain Reaction **a)** Primers and probe sequences for **AI type A** (matrix gene) according to (**Spackman et al., 2002**).
Sep1: 5- AGA TGA GTC TTC TTA CCG AGG TCG-3
Sep2: 5-TGA AAA AAC ATC TTC AAG TCT CTG3-
SEPRO: 5-{6-FAM}-TCA GGC CCC CTC AAA GCC GA {TAMRA} -3
b) - Primers and probe sequences for H5 gene detection as mentioned by **Lòndt et al. (2008)**. H5 LH1: 5-ACATATGACTACCCACARTATTCAG-3
H5 RH1: 5-AGACCAGCTAYCATGATTGC-3
H5 PRO: 5-{6-FAM}-TCAGGCCCCCTCAAAGCCGA {TAMRA}-3
- Sterile 0.5 ml micro centrifuge tube. - 10, 10 - 20 and 20-200 µl adjusted pipette (Biohit, Germany).
- Filter tips of different sizes. - Optical tubes (0.2 ml).
- Optical caps. - Real time PCR machine (Stratagene MX3005P).

RESULTS

Serological evaluation of different vaccines: serum samples were collected weekly up to 35 days of age and the HI titers were determined (Table 2). The highest HI titers was recorded in group 2 (vaccinated with Vectormune AI® vaccine) 7.4 log2 and 6.35 log2 followed by group 4 (vaccinated with inactivated H5N1 vaccine) 6.6 log 2 and 6 log 2 then group 3 (vaccinated with inactivated H5N2 vaccine) 6.6 log 2 and 5.6 log 2 at 35 days While control groups have the lowest titers 1.2 log 2 and 0.35 log 2 at 28 and 35 days respectively.

Clinical observations after challenge: All chickens observed daily after challenge to record mortalities, protection percent, signs and post mortem lesions. Data of mortality and protection percent are presented in (Table 3), the highest percent of protection was recorded in group 2 followed by group 4, 3 then 5 with 83.3%, 33.3%, 16.7% and 0% respectively. The challenged group 2 showed no clinical signs while the other groups 3, 4 and 5 explained typical clinical signs and post mortem lesions of highly pathogenic avian influenza virus, firstly clinical signs were coughing, sneezing, oedema in comb and wattle with cyanosis, Off food and depression, also conjunctivitis and haemorrhages on shanks were observed. Onset of mortality was at 5th, 4th, 4th, 3rd day post infection in group 2, 3, 4 and 5 respectively. Pm lesions in dead birds were congestion in lung and trachea and haemorrhage in visceral organs with necrosis of pancreas.

H5N1 HPAI virus shedding within different challenged groups: Tracheal shedding of H5N1 HPAI virus in birds of different groups were detected at 2 and 5 dpi by quantitative RRT-PCR. At 2 dpi, there was no tracheal shedding in group 2. While the other three groups shed the challenged virus as follow: group 3 shed virus by 2.82 X 101 EID50 and group 4 shed more little virus 2.87 X 101 EID50 but group 5 shed more virus 2.93 X 102 EID. Also, at 5 dpi, group 4 was the highest group shed virus by 6.1 X 102 EID50 followed by group 3 which shed 4.025 X 102 EID50, finally group 2 was the lowest group which shed 2.85 X 102 EID50 (table 4&5), (fig. 1).

DISCUSSION

In Egypt, HPAI H5N1 not only considered as an endemic infection in poultry farms but also the infections of vaccinated poultry may represent a potential challenge for the development of an effective vaccines, therefore, Rigorous evaluation of the potency of vaccines against Egyptian strains of H5N1 is essential to effectively control the disease in poultry. **So that this study was planned for:** Evaluation of three commercial available vaccines against HPAIV H5N1 (two oil emulsion inactivated vaccines and one vectored vaccine), determination of the role of these vaccines in morbidity and mortality reduction, measurement of the immunity levels and determination of the viral shedding.

It's clear that Vectormune vaccinated group has the highest HI titers 7.4 log2 at 28 days directly before challenge achieving the highest protection percent 83.3 % without virus shedding detected at 2 dpi while at 5 dpi was less virus shedding, 2.85 X 10^2 EID50.

There was a correlation between HI antibody titer, protection against disease and virus shedding at HI titer of 4 to 6 log 2 (**Kumar et al., 2007**) but such relations are only valid if there is a close antigenic match between the vaccine and the challenge viruses (**Abdelwhab et al., 2012**).

Also, **Rauw et al. (2012)** found the protection percent was 90% and 70% in chickens vaccinated with rHVT-H5 then challenged with highly diverse clade 2.2.1 2007 and 2008 Egyptian strain respectively. On the other hand, the reduction of challenge virus shedding was significantly higher in the chickens vaccinated with rHVT-H5 alone when compared to those vaccinated with inactivated vaccines. **Soejoedono (2012)** recorded clinical protection of 80% in chickens were vaccinated at one day old with rHVT-H5 alone when challenged with Indonesian HPAI H5N1 field isolate, while the protection percent was 95% in the second trial. While, **Kapczynskia et al. (2015)** found that all SPF broiler chicks vaccinated with rHVT-Hu4999 at one day old induced average HI titers of 2^5–2^6 prior to challenge achieving the protection percent of 80% with significantly decreased virus tracheal shedding at 2 dpi when compared to the challenged control intranasally with HPAI H5N1 clade 2.1.3 virus at 10^6 EID50 (all birds died within 2 days) and H5N2 vaccinated group at 10 days of age (Only one bird was survived, lower HI titers of $2^{1.7}$).

On the other hand, H5N2 and H5N1 vaccinated groups have less HI titers 6.6 log2 of each with less protection percent 16.7% and 33.3% respectively. The challenge virus shedding was as follow, group 3 shed virus by 2.82 X 10^1 EID50 in 25% of tested swabs and group 4 shed more little virus 2.87 X 10^1 EID50 in 20% of tested swabs at 2 dpi but at 5 dpi group 4 (H5N1 vaccinated) was the highest group shed virus by 6.11 X 10^2 EID50 followed by group 3 (H5N2 vaccinated) shed 4.025 X 10^2 EID50 in 33.3% of tested swabs of each. The failure of these used vaccines to protect chickens from HPAIV H5N1 or to decrease its shedding may be due to:

Firstly although vaccines such as oil adjuvant whole inactivated AI virus antigens have been used in numerous countries as an aid in control efficacy are not optimum unless there is antigenic match between the vaccine and field strain achieved **(Swayne and Kapczynski, 2008)**.

As in Egypt, control program for HPAIVs is dependent on the use of imported vaccines as the commercial vaccines available currently for H5 AIVs include two oil-emulsion formulations: one derived from killed low-pathogenic H5N2 virus A/chicken/Mexico/232/94/CPA and the other from a reassortant H5N1 virus from China. A major problem with using these vaccines was that they were not evaluated for their protective efficacy against a challenge infection with Egyptian isolates before their introduction to the market and this is the probably reason for the disappointing results of protective efficacy for both vaccines and there are concerns that these vaccines may not be completely effective practically and may not prevent virus shedding and spread (**Bahgat et al, 2009**). Antigenic match between the vaccine strain and the circulating field virus is one of the most decisive factors in determining the H5 vaccine efficacy to prevent the replication and transmission of H5N1 virus (**Kim et al., 2010**) and the Egyptian H5N1 HPAI viruses continue to mutate and rapidly evolve over time (**Arafa, et al., 2012a**).

Also we can say extensive vaccination with inactivated vaccines used in Egypt has been suspected to induce the emergence of vaccine escape mutants as in Mexico (**Lee et al., 2004**). In addition to viruses isolated from vaccinated poultry showed stepwise acquisition of mutations in the immunogenic epitopes of the HA resulting in divergence at increased evolution rate to escape from the immunity induced by the employed vaccine strains. This immune escape resulted in severe outbreaks in poultry farms despite continuing vaccination as reported by **Arafa et al. (2012b)**.

The Egyptian H5N1 HPAI virus from 2008 exhibited a low cross reactivity in HI tests against the Mexican vaccine seed strain (H5N2) commonly used in Egypt, suggesting that significant antigenic drift occurred (**Cattoli et al., 2011**).

These findings are matched with **El-Zoghby et al. (2013)** who isolated a HPAIV H5N1 from twenty weeks old layers chickens that vaccinated with a homologous H5N1 vaccine at 1, 7 and 16 weeks old and suffered from an outbreak of H5N1 virus with 27% mortality. Examined serum samples showed antibody titer in HI test (Log2 3.2± 4.2). Conspicuous mutations in the HA and NA genes including a deletion within the receptor binding domain in the HA globular head region were observed.

The second reason of this failure may due to high maternal derived immunity (MDA) due to extensive vaccination in breeders. **Kim et al. (2010)** mentioned that in Egypt, efforts to control highly pathogenic H5N1 avian influenza virus in poultry and humans have failed despite increased biosecurity, quarantine and vaccination at poultry farms which may be due to the passive transfer of maternal H5N1 antibodies to chicks which inhibit their immune response to vaccination.

After several rounds of routine vaccination of the breeders, high levels of MDAs are transferred to the progeny. MDAs may still interfere with vaccination to a lesser extent because they are present up to 3wk post hatch. Therefore, in areas with high infection pressure, when possible, two vaccinations are recommended for optimal protection. Also, it might be advisable to take into account day-old AI MDA titers when one is determining the optimal age of vaccination (**De Vriese, et al., 2010**). MDAs interfere with successful vaccination of young chicks because of their capacity to neutralize the vaccine virus and increase the clearance of the vaccine antigens, thereby preventing optimal exposure to the immune system (**Poetri et al., 2011**).

The rHVT-H5 vaccine appeared very protective for commercial chickens when used in one day-old chickens

possessing MDA as these experiment confirmed that the rHVT-H5 vaccine applied alone could provide high level of clinical protection against divergent HPAI H5N1 field isolates with reduction in the excretion of challenge virus and could therefore be recommended in areas with high infection pressure for optimal protection.

So in endemic countries like Egypt, rigorous control measures including biosecurity, culling of infected birds and constant update of vaccine virus strains are highly required to prevent circulation of HPAIV H5N1 between commercial poultry.

Table 1: Experimental design.

Group No.	Vaccination program
1	Non Vaccinated non infected
2	Vaccinated with Vectormune AI vaccine 0.2 ml S/C at 1 day old then challenged
3	Vaccinated with inactivated H5N2 vaccine 0.5 ml S/C at 7 days old then challenged
4	Vaccinated with inactivated H5N1 vaccine 0.5 ml s/c at 7 days old then challenged
5	Non vaccinated challenged

Table 2: HI geometric means titers of H5N1 AI virus vaccines in broiler chickens.

Group no.	Vaccination program	Geometric means HI titer/day					
		1 st	7th	14th	21th	28th	35th
1	Negative control non vaccinated non Challenged	4.8	3.2	2.4	2	1.2	0.35
2	Vectormune AI® vaccine 0.2 ml s/c at 1 day old.		4.2	4.7	5.2	7.4	6.35
3	Inactivated H5N2 vaccine 0.5 ml at 7 days old.		3.2	3.1	3.2	6.6	5.6
4	Inactivated H5N1 vaccine 0.5 ml at 7 days old.			2.6	2.7	6.6	6
5	Positive control non vaccinated challenged with AI virus			2.35	1.85	1.1	- (died)

***Broiler chickens were challenged at 28 days of age with H5N1 AIV at a dose of 0.1 ml 10^6 EID50 intra naso-ocular.**

Table 3: Mortality and protection percent in challenged groups.

Group no.	Mortality/day								total	Mortality %	Protection %
	1st	2Nd	3rd	4th	5th	6th	7th	8th			
2	-	-	-	-	1	2	2	No mortalities	5/30	16.7%	83.3%
3	-	-	-	7	6	8	4		25/30	83.3%	16.7%
4	-	-	-	8	5	3	4		20/30	66.7%	33.3%
5	-	-	17	13	-	-	-		30/30	100%	0%

Table 4: Tracheal shedding detected by quantitative RRT-PCR within Different challenged groups.

Group No.	Sample NO.	Result	CT	Conc. (EID50/ml) Acc. To $10^{7.5}$/ml Standard	GM Conc. (EID50/ml)
	2dpi samples				
2	T1	Negative	-	-	-
	T2	Negative	-	-	
	T3	Negative	-	-	
	T4	Negative	-	-	
	T5	Negative	-	-	
3	T9	Positive	29.02	2.82×10^1	$.282 \times 10^2$
	T10	Negative	-	-	
	T11	Negative	-	-	
	T12	Negative	-	-	
4	T13	Negative	-	-	$.287 \times 10^2$
	T14	Negative	-	-	
	T15	Positive	28.94	2.87×10^1	
	T16	Negative	-	-	
	T17	Negative	-	-	
5	T18	Positive	28.54	2.93×10^2	2.93×10^2
	T19	Negative	-	-	
	T20	Negative	-	-	
	5dpi samples				

2	T21	Negative	-	-	2.85×10^2
	T22	Positive	26.93	2.85×10^2	
	T23	Negative	-	-	
	T24	Negative	-	-	
3	T25	Negative	-	-	4.025×10^2
	T26	Positive	26.75	4.025×10^2	
	T27	Negative	-	-	
4	T28	Positive	23.6	6.11×10^2	6.11×10^2
	T29	Negative	-	-	
	T30	Negative	-	-	

Each sample = Pooling of 3 chicks**. CT** = Cycle threshold **GM** = Geometric mean **T:** Tracheal sample**.**

Table 5: Number of positive / total tracheal samples for detection of shedding of the challenge H5N1 HPAI virus in different groups.

Group No.	2dpi		5dpi	
	Tracheal sample		tracheal sample	
	Shedding Virus/Total sample	%	Shedding Virus/Total sample	%
2	0/5	0	1/4	25%
3	1/4	25%	1/3	33.3%
4	1/5	20%	1/3	33.3%
5	1/3	33.3%	- (died)	-

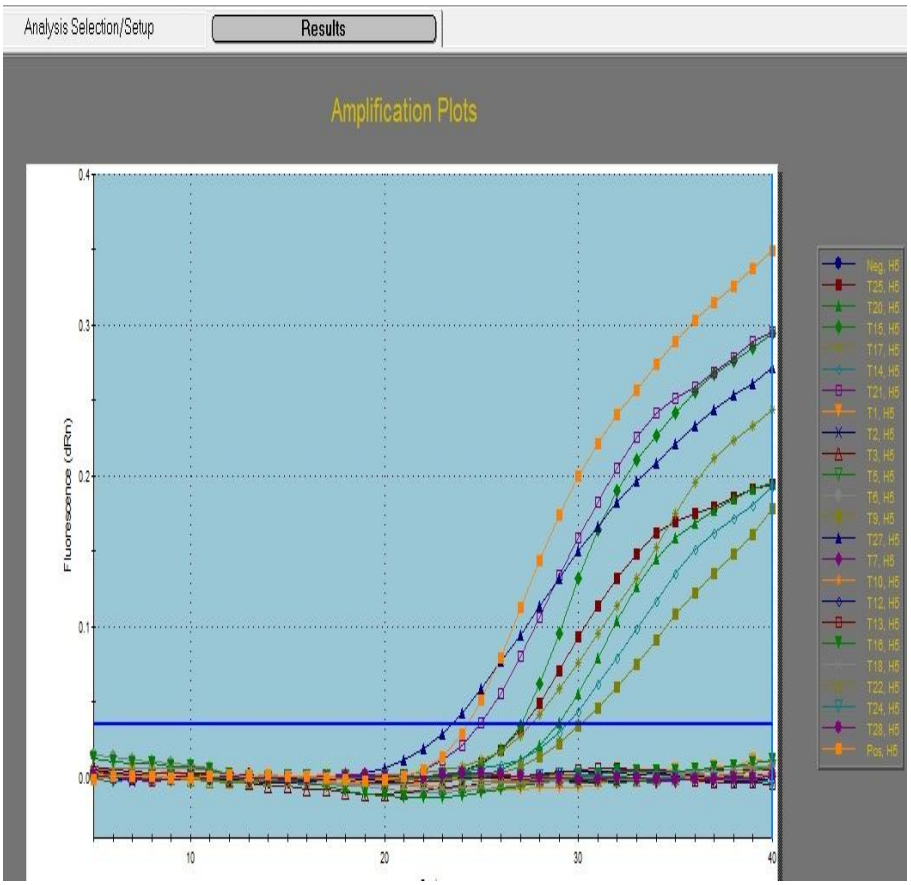

Fig. 1: Results of tracheal shedding of different vaccinated groups.

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