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Article:

Molecular and histopathological studies on commercial vaccines against H9 avian influenza in broilers

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Abstract

Avian influenza virus is one of the major threats that has been affecting the poultry industry in the Middle East region for decades. Many trials were made to eradicate the disease, but it doesn't work. Nowadays, there are many commercial vaccines that are either imported or prepared from circulating isolates from H9N2 in Egypt and Middle Eastern countries. This study focused on understanding the effectiveness of five commercial vaccines for their ability to give protection. Six groups of one day-old broiler chicks (n=15) were placed under normal conditions in separate pens. Each group was immunized with the specific vaccine at 4th day of age and then they were challenged with H9N2 virus at 14 days old. After challenge, respiratory signs, swelling of head, reduction in the feed consumption rate, decrease in the average body weight and general depression were observed. Postmortem findings of the euthanized birds revealed congestion of lung, tracheitis, swelling of kidneys and inflammation of bursa of fabricius. On histopathological examination, bursa of Fabricius shows different lesions of interfollicular edema, lymphocytic depletion, necrosis and sloughing of lymphoid cells which illustrates the immunosuppressive effect of the virus. The unvaccinated challenged group sheds the highest titer (mean $10^{4.7}$ EID_{50}/ml) while the remaining vaccinated groups sheds lowest titer (mean 10^{1} - 10^{4} EID_{50}/ml). The vaccinated birds show less signs of disease, less degree of pathological lesions and lower viral shedding titer. This explains the importance of using H9N2 different inactivated vaccines in poultry farms for controlling the spread of the disease.

Keywords: Avian influenza, Broiler, Vaccine, Challenge, H9N2.

Introduction

The poultry industry is considered one of the most important animal industries in Egypt and worldwide. It has been suffering from several pathogens such as the avian influenza virus, Newcastle disease virus, Infectious bronchitis virus, Infectious bursal disease virus, *E. coli*, Salmonella, Pasteurella, Mycoplasma and coccidia. All these pathogens have caused severe economic losses during recent decades **[1-10]**. Avian influenza virus (AIV) is a segmented single stranded negative sense RNA virus and is belonging to the Orthomyxoviridae family **[11, 12]**.

Up to today, 18 H subtypes have been identified (H1 to H18) and 11 N subtypes (N1 to N11) **[13]**. Avian influenza viruses are divided into milder low pathogenic and highly

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pathogenic avian influenza viruses. LPAI viruses frequently go unreported in poultry farms, while HPAI have the ability to cause mortalities up to 100% in birds **[14]**. If infection with the LPAI virus is combined with other pathogens, it may result in high mortality and high morbidity rates in chickens **[15, 16]**.

In Egypt, the virus is responsible for major economic losses since it was first reported in 2010 until now **[17]**. It is reported that humans were exposed occupationally to the H9N2 virus in Egypt **[18]**. The first case of human H9N2 was recorded in January 2015 from Egypt **[19]**.

In the last decade, RNA of the avian influenza virus can be demonstrated by real time RT-PCR (RRT-PCR) in clinical and laboratory samples **[20]**. To obtain effective control of H9N2 in poultry farms, immunization programs must be included in mind **[21]**. There are several experimental studies that indicate that inactivated avian influenza vaccines can stimulate antibody responses, which helps to decrease mortality rates and protect the infected birds from egg production decline **[22]**.

For evaluating the efficiency of the AI vaccine, we must observe the shedding of the virus in swabs from infected birds. An effective vaccine reduces viral shedding and prevents clinical disease **[23]**. The aim of this study is to investigate the effectiveness of different commercial vaccines against LPAI H9 and evaluate the value of applying the H9N2 vaccine at 4 days of life on the protection against the disease under standard laboratory conditions.

Materials and Methods

Ethical approval

Animal studies were approved by the veterinary medical research ethics committee, Faculty of Veterinary Medicine, Sohag University, Sohag, Egypt, according to the OIE standards for use of animals in research with approval number (Soh.un.vet/00061R). Every effort was made to minimize animal suffering.

Vaccines

Different types of inactivated vaccines were used in this study to estimate the efficiency of each of them against avian influenza infection. Vaccine I (Nobilis H9N2 + ND P.) it is composed of inactivated avian influenza virus type A, subtype H9N2, strain A/CK/UAE/415/99 and inactivated Newcastle disease virus (strain clone 30) and manufactured by Merck Sharp & Dohme Animal Health, S.L. SA Salamanca – Spain. The vaccine was administered subcutaneously (under the skin of the neck) at a dose of 0.25 ml/bird.

Vaccine II (MEFLUVAC ™ H9 + ND7) is manufactured by MEVAC (made in Egypt) and was administered subcutaneously at a dose of 0.5 ml/bird.

Vaccine III (CEVAC ® NEW FLU H9 K) is Inactivated oil emulsion AIV H9N2 and Lasota strain vaccine (Ceva-Phylaxia Veterinary Biologicals Co. Ltd. 1107 Budapest – Hungary) and was administered subcutaneously at a dose of 0.2 ml/bird.

Vaccine IV (Gallimune Flu H9 M.E.) is produced by Merial incorporation, Lyon, France. It is composed of H9N2 (A/chicken/Iran/Av1221/1998) and Ulster 2C strain vaccine and was administered subcutaneously at a dose of 0.2 ml/bird.

Vaccine V (ValleyVac H9 – ND^{G7}) is manufactured by the Egyptian Company for Biological & Pharmaceutical Industries 101 extension of the sixth industrial zone-6th of October City, Egypt. The vaccine was administered subcutaneously at a dose of 0.5 ml/bird. All of these vaccines were applied to 4 days old chicks.

Other vaccines were given against infectious bursal disease (IBD), AIV-H5, Newcastle disease (ND) and infectious bronchitis (IB) viruses by eye drop route as shown in **Table 1**.

Experiment

Ninety, 1-day old broiler chicks were brought from a local hatchery in Egypt. The birds were supplied with feed and water and libitum and were reared under standard housing conditions resembling that of the farm. The birds were randomly divided into six separated groups on 4th day of age (15 birds/group) as the following: vaccine I (Nobilis H9N2 + ND P.), vaccine II (MEFLUVAC™ H9 + ND7), vaccine III (CEVAC® NEW FLU H9 K), vaccine IV (Gallimune Flu H9 M.E.) and vaccine V (ValleyVac H9 – ND^{G7}). Finally, the 6th group was unvaccinated challenged one (control positive) as illustrated in **Table 1**. On the 14th day of age, birds were challenged with H9 virus by intraocular route and observed daily for 7 days and all observations were recorded.

Challenge strain

A reference LPAI (H9N2) strain with accession number of ok148893 was used as the challenge virus. The infective dose was adjusted to 10^6 embryo infective dose 50 (EID_{50}) / ml and the birds were challenged via the ocular route.

Clinical signs and pm examination

All the designed groups of birds were observed daily for signs of disease and mortality for 7 days post challenge. Dead birds were necropsied to see the postmortem changes in the different internal organs of the bird and data was recorded separately for each group.

Histopathology

In a human manner, three chickens from each group were euthanized and exposed to necropsy at 7th day post challenge. Bursa was collected for histopathological study.

specimens from each group were fixed at 10% neutral buffered formalin and were dehydrated in a graded alcohol series, cleared with xylene, embedded in paraffin wax, sectioned at 4-5 um thickness and stained with hematoxylin and eosin for histopathological examination by light microscopy **[24]**. Stained tissue sections were examined by light microscopy (Olympus, Japan) and photographed using a digital camera (Olympus, Japan).

H9N2 virus shedding

A total of 18 tracheal swabs were collected (n=3/group) at 4 days post challenge (dpc). Swabs were immersed in viral transporting media (DMEM medium) and then Kept at -80ºC till being examined. The process of viral RNA extraction was carried out according to the instructions of the QIAamp Viral RNA Mini Kit (Qiagen) with catalogue NO. 52904. Quantitative real time RT-PCR (qRRT-PCR) was done according to QuantiTect probe RT-PCR with catalogue NO. 204443, using the designed primer pairs and probe targeting the H-gene of H9N2 virus as illustrated in **table 2**.

Table 1 illustrating Experimental design for evaluation of H9N2 inactivated commercial vaccines in broilers.

	Vaccine I	Vaccine II	Vaccine	Vaccine IV	Vaccine V	Unvaccinated challenged
Number of birds	15	15	15	15	15	15
H9N2 vaccine	+	+	+	+	+	-
ND vaccine	+	+	+	+	+	+
IB vaccine	+	+	+	+	+	+
H5N1 vaccine	+	+	+	+	+	+
IBD vaccine	+	+	+	+	+	+

Table 2 illustrating Oligonucleotide primers used for the amplification of the H9N2 H-gene.

Virus	Gene	Primer/ probe sequence 5'-3'	Ref
H9	H	**H9F** GGAAGAATTAATTATTATTGGTCGGTAC	**[25]**
		H9R GCCACCTTTTTCAGTCTGACATT	
		H9 Probe [FAM]AACCAGGCCAGACATTGCGAGTAAGATCC[TAMRA]	

Statistical analysis

The obtained data of virus shedding analyses were expressed as mean ± standard error of mean (SEM) using one way ANOVA, followed by Tukey's Honestly Significant Difference (Tukey's HSD) test as a post hoc test. Statistical significance was set at 0.05.

Results

Clinical and postmortem findings

Unvaccinated challenged birds show respiratory signs such as conjunctivitis, sneezing, nasal discharge, ocular discharge, swelling of the head, reduction in the feed consumption rate, decrease in the average body weight and general depression. In contrast, the other vaccinated birds show milder signs than unvaccinated challenged birds. The lowest signs were observed in vaccine I, vaccine V, vaccine II, and vaccine III groups, respectively.

The postmortem findings of the examined birds revealed congestion of lung, tracheitis, swelling of kidneys, inflammation of bursa of fabricius and splenomegaly with hemorrhages. Unvaccinated challenged birds show a severe degree while the other vaccinated ones show a lesser degree than non-vaccinated birds. The lowest P.m. lesions were observed in vaccine I and vaccine V groups. Mortality rate reached up to 20% in unvaccinated challenged birds, while they reached 0-13% in the different immunized groups. It reaches 0% in I, II and V vaccinated groups while it reaches 6.7 % in vaccine IV group and 13.3% in Vaccine 3 group.

Histopathological examination

The bursa of fabricius shows different lesions of interfollicular edema, lymphocytic depletion, necrosis and sloughing of lymphoid cells by examination under a light microscope. The degree of lesion varies from very mild in the vaccine I group to a highly severe degree in the unvaccinated challenged group as shown in **Figure 1** and **Table 3**. The bursa of fabricius section of the unvaccinated challenged group shows severe interfollicular edematous areas and depletion of lymphocytes. The bursa of fabricius section of birds vaccinated with vaccine (I) shows normal architecture while in birds vaccinated with vaccine (II) shows mild interfollicular edema and lymphocytic depletion. For the vaccine III group, it shows moderate lymphocytic depletion. vaccine IV group shows severe multiple areas of necrosis of lymphoid cells and sloughing in the bursal follicles. Finally, bursa of the vaccine V group shows a mild increase in the thickness of the septae of interfollicular tissue.

H9N2 shedding

The unvaccinated challenged birds shed high titers of virus in the trachea (mean $10^{4.7}$ EID_{50}/ml) and the same with the vaccine IV group shows high titers of virus (mean $10^{4.7}$ EID_{50}/ml). For the vaccine V group, birds shed also high titers of virus (mean 10^{4} EID_{50}/ml). On the other hand, the vaccine I group sheds the lowest titers of virus in the trachea (mean 10^{1} EID_{50}/ml). The remaining other groups vaccine II and vaccine III, range in between the previous groups (mean $10^{2.7}$ EID_{50}/ml). The results of virus shedding were indicated as mean ± standard error of mean (SEM) as shown in **Figure 2** and **Table 4**.

Table 3 showing degree of pathologic lesion in bursa of fabricius in broilers

Groups	Very mild	mild	moderate	severe	Highly severe
Vaccine I	+	-	-	-	-
Vaccine II	-	+	-	-	-
Vaccine III	-	-	+	-	-
Vaccine IV	-	-	-	-	+
Vaccine V	-	-	+		-
unvaccinated challenged (control positive)	-	-	-	-	+

Table 4 showing Statistical analysis of virus shedding in tracheal swabs at 4 days post challenge by H9N2 virus at 14 days of age.

Groups	Challenged with H9 at 14 days of age
Vaccine I	7.3517 ± 0.10533 [d]
Vaccine II	9.5614 ± 0.34496 [bc]
Vaccine III	9.1945 ± 0.27865 [c]
Vaccine IV	10.1083 ± 0.36078 [a]
Vaccine V	10.0416 ± 0.27297 [ab]
unvaccinated challenged (control positive)	10.1162 ± 0.42472 [a]

Data represented as mean ± SEM. [Abc] Means within the same column carrying different superscripts are significantly different at $P < 0.05$ based on Tukey's Honestly Significant Difference (Tukey's HSD) test. [a] represents the highest result of shedding of H9N2 virus. [d] represents the lowest result of shedding of H9N2 virus. The rest of the letters represent a result in between the highest result ([a]) and the lowest result ([b]).

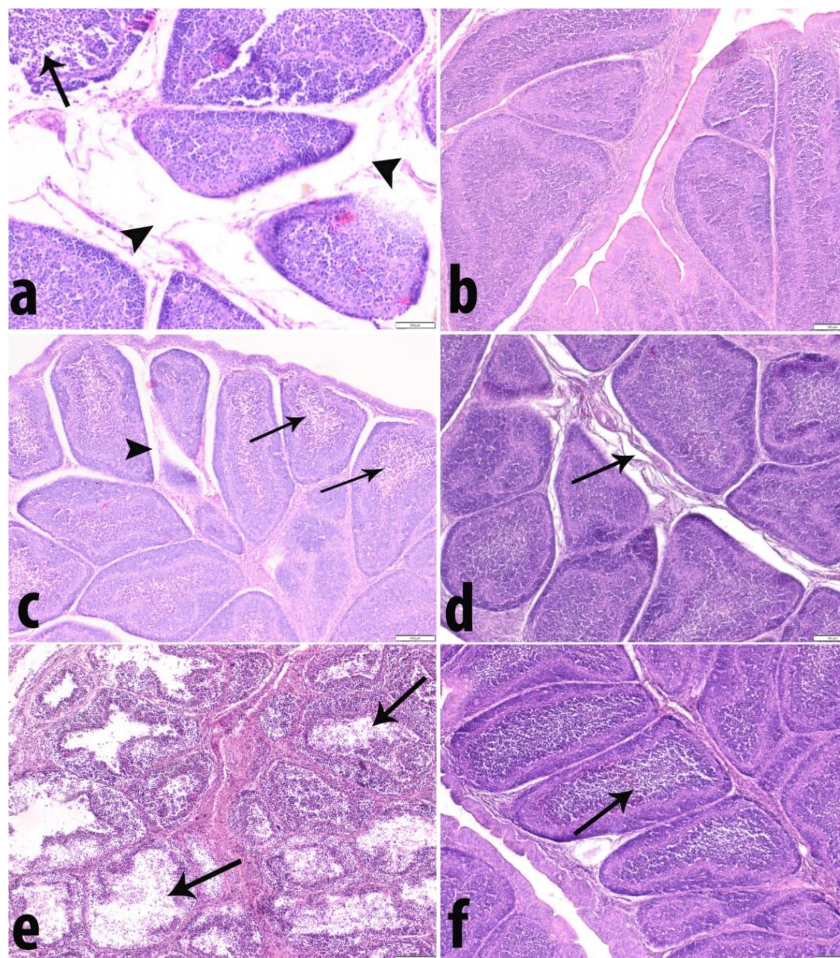

Figure 1 ***a)*** *bursa of unvaccinated challenged (control positive) group showing severe interfollicular edematous areas (arrow heads), and lymphocytic depletion (arrow) (HE, Bar = 100 µm).* ***b)*** *bursa from vaccine I group showing normal architecture (HE, Bar = 100 µm).* ***C)*** *bursa from vaccine II group showing mild interfollicular edema (arrow head) and lymphocytic depletion (arrow) (HE, Bar = 100 µm).* ***d)*** *bursa from vaccine V group showing mild an increase in the thickness of septae of interfollicular tissue (arrow) (HE, Bar = 100 µm).* ***e)*** *bursa from vaccine IV group showing severe multiple area of necrosis of lymphoid cells and sloughing in the bursal follicles (arrow) (HE, Bar = 100 µm).* ***f)*** *bursa from vaccine III showing moderate lymphocytic depletion (arrow) (HE, Bar = 100 µm).*

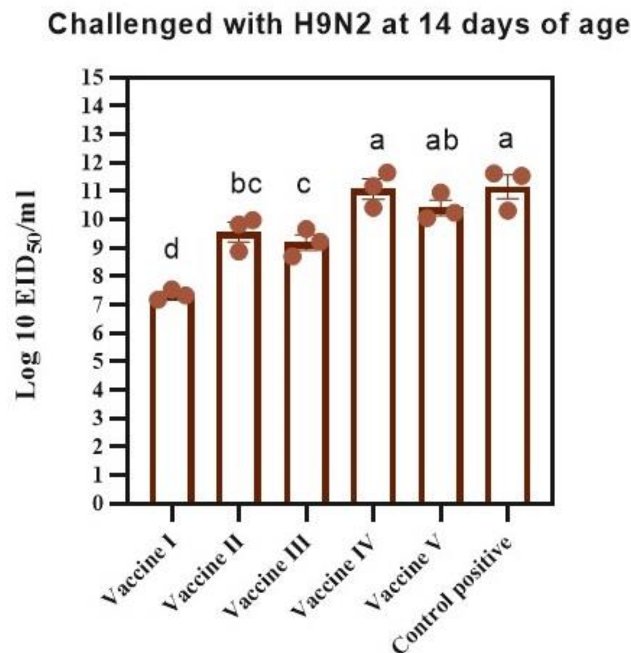

Figure 2 H9N2 shedding in birds vaccinated with different commercial inactivated H9N2 vaccines and unvaccinated challenged (control positive)birds in broiler chickens and challenged with LPAI H9N2 at 14 days old. Scatter plots of tracheal shedding from vaccinated and unvaccinated challenged (control positive) birds at 4 dpc with Avian influenza virus H9N2 G1 lineage (A/chicken/Egypt/FAO-S33/2021(H9N2)) with an accession number of (ok148893) at 14 days old. Shedding titres are expressed as log10 with error bars included. The data were graphed using GraphPad Prism version 8 and the statistical analysis was performed using ANOVA. Different letters indicate a significant difference between groups at the same time-point

Discussion

In the Middle East, the LPAI H9N2 virus has been reported for several years and it causes great economic losses in poultry farms if it is combined with other pathogens. In this study, vaccine efficacy was evaluated through observation of clinical signs every day, recording shedding of the virus in tracheal swabs and histopathological findings under a light microscope.

The examined broilers revealed clinical signs such as sneezing, nasal discharge, ocular discharge, conjunctivitis and swelling of periorbital tissues. Other studies revealed the same clinical signs were observed among H9N2 infected birds **[26-28]**. It is also reported by previous studies that respiratory signs were observed in SPF and commercial chickens vaccinated at day 1 and infected with the H9N2 virus alone **[29, 30]**. Respiratory signs in most of the challenged groups were attributed to trypsin like proteases which are responsible for the replication of the virus easily in the epithelial tracheal cells **[31]**. Other studies revealed that the only findings were depression and diarrhea **[32-34]**.

The average body weight in the unvaccinated challenged birds was lower than the vaccinated one. These results were attributed to the impairment of production of pancreatic enzymes, which affect digestion due to the effect of the H9N2 virus on the pancreatic tissues **[23]**.

Necropsy examination of birds revealed lesions such as congestion of the lungs, tracheitis, swelling of the kidneys, inflammation of the bursa of fabricius and splenomegaly. The same postmortem findings as congestion in the trachea and airsacculitis were reported by other studies **[30, 35]**.

Our study revealed a 20% mortality rate in the unvaccinated challenged birds while in other immunized birds it reaches 0-12%. Comparable results were obtained by one study which illustrated that 20% cumulative mortality in chickens challenged with the H9N2 virus **[36]**. Also in field conditions, LPAI H9N2 causes frequent outbreaks of disease with high mortality rates in different poultry production types worldwide **[37-40]**. In contrast, other studies said that there wasn't any mortality

observed and explained that due to the low pathogenic nature of the H9N2 virus **[32, 33, 41]**.

In the present study, the histologic changes in the bursa of fabricius were lymphocytic depletion, interfollicular edema, necrosis and sloughing of lymphoid cells. These pathological findings in the immune organs explain the immunosuppressive effect of the LPAI H9N2 virus on infected broilers. These results were similar to other studies in bursa of fabricius in which chickens were inoculated with 10^6 EID_{50} of H9N2 virus / bird by intranasal route **[32, 36, 42]**. Atrophy of bursa was also documented by other studies in broiler chickens challenged with H9N2 virus **[43]**. These variations could be attributed to if the experimental birds were SPF or commercial and to the strain of the challenged virus.

For evaluation of the efficacy of vaccines to stop or reduce the virus shedding, tracheal swabs were collected and tested. In this study, no vaccine was found to be able to stop the virus shedding. It was found that most of the used vaccines in this experiment reduced the shedding titer of H9N2 virus in tracheal swabs in comparison to the unvaccinated challenged but not total prevention of H9N2 virus shedding in tracheal swabs at 4 dpc. Other experiments using inactivated avian influenza vaccines have shown a reduction in virus shedding, but not total prevention of LPAI infection **[44]**.

Conclusion

Based on the presented study it could be concluded that immunized birds with inactivated H9N2 avian influenza virus vaccines are effective in reducing shedding of the virus and the degree of lesion than non-vaccinated birds and limit the spread of the H9N2 virus in poultry farms. Vaccination programs should be combined with other control measures, such as biosecurity and periodical surveillance to limit the opportunity of appearing of Avian influenza disease worldwide.

Authors' contribution

Hesham Asaad: Investigation, Methodology, Visualization, Data curation, Writing – review & editing. Ola Hassanin: Conceptualization, Data curation, Formal analysis, Investigation Methodology, Project administration, Supervision, Validation and Visualization. Mostafa saif-Edin: Conceptualization, Project administration and Supervision. Ragab S. Ibrahim: Visualization and Supervision. Moemen A. Mohamed: Visualization and Supervision. Mustafa Hamad: Methodology and resources. Tamer Mahmoud Abdullatif: Formal analysis, Methodology and Software. Mohamed Gamal: Examine slides under microscope and writing the result of pathology. Sary Kh. Abd-El Ghaffar: Review the result of pathology.

Conflict of interest

There is no conflict of interest.

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