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Efficacy Of Commercial Inactivated Avian Influenza Vaccines H5N1, H5N1 Re-6 And Re-8, H5N1 Re-6, Re-7 And Re-8, H5N2, H5N3 And H5N8 Against Current Circulating Field Strains In Egypt.

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Abstract

Avian influenza virus (AIV) represents a continuous major threat to all poultry sectors all over the world. Though routine and massive vaccination against different strains of influenza viruses however, sporadic and sudden epidemics in vaccinated and unvaccinated poultry flocks are still occurred. Continuous monitoring of used vaccines in field is necessary to ensure their efficacy against predominant influenza virus strains and updating vaccination protocols. In the present study, nine different batches of inactivated avian influenza vaccines were arbitrarily chosen throughout daily work at central laboratory for evaluation of veterinary biologics in 2023. Quality control of nine batches was carried out based on the Egyptian standard regulations for evaluation of veterinary Biologics. Completion of inactivation test of nine inactivated avian influenza vaccine batches showed negative haemaagglutination test against chicken erythrocytes. Based on manufacturer's instructions, immunization of one week old SPF chicks using batches of vaccines demonstrated no mortalities or clinical symptoms. Serological immune response of tested inactivated vaccines batches using haemagglutination inhibition test showed variable antibody titer (between 3.5 and 7.9 $log_{2)}$ after collection of serum samples from immunized chicks on 3rd and 4th weeks post vaccination. Testing of nine inactivated vaccine batches for challenge experiment on 4th week post immunization using current circulating influenza virus revealed variablelevel of protection (50% to 80%). Virus shedding of vaccinated challenged chicks in SPF eggs on 3rd, 5th, 7th and 10th days post challenge illustrated substantial quantity of virus in oropharengeal and cloacal swabs. This paper demonstratedcorroboration of some inactivated avian influenza vaccine batches in vaccination regimes and invalidation of other batches for use in poultry populations.

Keywords: *avian influenza, inactivated vaccines and quality control.*

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I. Introduction

Avian influenza virus is highly contagious, notifiable and devastating respiratory disease causing high economic losses with potential zoonotic impact **(Swayne and Sims, 2020)**. Influenzain birds is caused by virus infection of the *orthomyxoviridae*family in the genus *Alphainfluenza*virus (influenza A virus or influenza virus A)**(International Committee on Taxonomy of Viruses (ICTV), 2019)**.The viral nucleic acid issingle stranded RNA of negative sense measures about 13 kb including eight gene segments encoding ten proteins which are (polymerase basic PB1and PB2, polymerase acid PA, hemagglutinin HA, nucleoprotein NP, neuraminidase NA, matrix M1 and M2, and non-structural proteins NS1 and NS2). The surface glycoprotein HA is responsible for humeral immune response and host cell attachment whereas NP protein plays role in spreading virons progeny by detach sialic acid receptors from glycoproteins **(Palese*et al.*,2007)**. Based on antigenic characters of HN there are sixteen H subtypes and nine N subtypes**(Alexander, 2007)** with supposed novel recognized subtypes (H17, H18) from bats in Guatemala**(Swayne*et al.*, 2020)**. According to severity of disease, avian influenza virus is classified into highly pathogenic (HPAIV) and low pathogenic (LPAIV). The HPAIV strains (subtypes H5 and H7) have mainly been recovered from gallinaceous birds and associated with sever morbidity and mortality. In spite of HPAIV infrequently infect domestic wild birds or water fowl the Eurasian-African H5N1 HPAIV strains have developed in the past years and acquire ability to induce disease in wild birds and domestic ducks **(Chen *et al.*, 2020)**. LPAIV strains induce asymptomatic disease in aquatic wild birds however, when hosted

into domesticated birds infection may be mild or yield sever clinical signs with damage to gastrointestinal system, respiratory and reproductive tract **(Hormane*t al.*, 2018)**. Moreover, the danger of LPAIV to be highly pathogenic by acquiring spontaneous mutations causing zoonotic sporadic infection that supports human to human transmission **(Cox *et al.*, 2017)**. In Egypt the first record of HPAIV H5N1 was in December 2005 in Damietta government during sampling of migratory birds. Molecular analysis of this virus revealed close relationship to the A/bar-headed goose/Qinghai/65/2005 clade 2.2 and this virus clade was re-isolated in 2006**(EMPRES, 2019)**. Rapid genetically and antigenically evolution of H5N1 resulting in categorization of clade 2.2 into subclades 2.2.1 and 2.2.1.1(emerged in late 2007 and remained till 2011due to ineffective vaccination of H5 vaccines in poultry farms). The strains 2.2.1.1 were seldom observed since 2012 however, the 2.2.1 strains sustained to evolve to form novel clade 2.2.1.2 due to gradual accumulation of mutations in HA gene and was dominant clusterbetween 2009 and 2014 in poultry populations regardless theirvaccination status**(Kayali*et al.*, 2016)**. In 2020 various genotypes of H5N1 strain within 2.3.4.4b clade were arose in wild birds and were observed in several countries in Asia, North America, Europe and Africa **(Sagong*et al.*, 2022)**. Now, the circulating clade 2.3.4.4b of H5N1 is attributed to global breaks of avian influenza and has been stated in domestic poultry, wild birds and mammls indicating danger of virus spread to new hosts **(Cui *et al.*, 2022)**. The newly identified clade 2.3.4.4b was reported in Egypt **(Mosaad*et al.*, 2023)**. In Egypt, unique-emerged H5N8 virus was recovered in year 2016 from wild birds. Phylogenetic tree demonstrated that Egyptian H5N8 was closely related toreassortant H5N8 viruses of clade 2.3.4.4b recovered from various Eurasian nations**(Kandeil*et al.*, 2017)**. Genetic analysis of H5N8 strain illustrated that six various genotypes are circulating in Egypt **(Moatasim*et al., 2019*)**. The H5N8 virus recorded in 2020 in Europe and phylogenetically was related to Egyptian strain isolated in 2019 **(Beerens*et al.*, 2020)**. LPAIV H9N2 viruses aredistributed globally causing extensive losses especially when other pathogens involved in infection. These viruses are classified into Eurasian and North American linages that prevalent in poultry flocks and wild birds **(weste*r et al.*, 1992)**. The Eurasian linage comprise six distinct genetically clades G1-like, Y280-like, G9-like, BJ94-like, Y439-like and Korean-like clades**(Tosh *et al.*, 2008)**. The G1 like clade is categorized into A, B, C and D subclades(**Fusaro*et al.*, 2011)**.Groups Aand B are widely circulating in the Middle Eastern countries and have been identified since 1999**(Bashashati*et al.*, 2013)**. G1-like H9N2 has been shared in developing reassortant by providing six internal genes to elicit multiple new genotypes of AIV such as H5N1 (**Iqbal *et al.*, 2009)** H7N9 **(Zhu *et al.*, 2016)**. In Egypt, the H9N2 were primary stated in 2011 from quail and the virus was closely related to Israeli circulating from 2006 to 2008 **(El-Zoghby*et al.*, 2012)** and was classified as Asian G1-like (A/Quail/Hong Kong/G1/97)**(Monne*et al., 2013*)**.Sequence analysis of Egyptian H9N2 viruses based on HA gene clustered viruses within B group **(Kandeil*et al.*, 2014)**. New variant H9N2 (Egy/G1var) cluster was detected in quails from 2011 to 2015 **(Adel *et al.*, 2017)**. Routine and massive vaccination against AIV is the primary approach for controlling the infection in poultry sectors but, wide range diversity of HA of AIV with antigenic variations in the same subtype leading to failure vaccination policies causing the emergence of new strains and subsequent mortalities and losses in vaccinated flocks **(Kapczynski*et al.*, 2017)**. Inactivated or killed AI vaccines were used over thirty years to combat infection in poultry sectors**(Swayne, 2009)**.Killed H5N1 Re-7 vaccine was produced in 2014 besides various inactivated vaccines were developed usingH5N1 Re-1/Re-4, Re-4/Re-5 and Re-4/Re-6 viral strains **(Guyonnet and Peters, 2020)**. Killed vaccine H5-Re11 was prepared fromA/duck/Guizhou/ S4184/2017(H5N6) field strain comprising clade 2.3.4.4h with NA and HA genes and protects against H5 viruses also, inactivated H5 Re-12 vaccine was constructed fromA/chicken/ Liaoning/SD007/2017(H5N1) field strain containing clade 2.3.2.1d HA gene **(Zeng *et al.*, 2020)**. Killed vaccine H7-Re3 from A/chicken/Inner Mongolia/ SD010/2019 (H7N9) was prepared and prevented AIV H7N9 strain in 2019. In 2020, killed trivalent H5-Re11, H5-Re12 and H7-Re3 have been developed to control H7N9 and H5 infections in poultry flocks **(MARA, 2020)**. Lately, novel HA and NA genes trivalent inactivated vaccine (H5-Re13, H5-Re14, and H7- Re4) induced strong immune response and adequate protection against concurrent field strains H5N6 virus, H5N8 virus, and H7N9 virus, correspondingly **(Zeng *et al.*, 2020)**. This paper was established to assess the effectiveness of commercial imported AIV killed vaccines in the protection against recent field virusin Egypt.

II. Material and methods

Ethical approval

This research was approved by Ethical Committee for medical research at the National researchcenter in accord with local outlines and procedures. All methods in this study were implemented according to Animal Research Reporting of In Vivo Experiments (**ARRIVE 2.0 guidelines**) **(Percie du Sert N et al., 2020)**.

Experimental design

1- Vaccine batches

- Nine imported commercial inactivated AIV vaccine batches were haphazardlychosenthrougheveryday routine work at central laboratory for Evaluation of Veterinary Biologics. These vaccine batches were presented in laboratoryat the first time in 2023for quality control forregistration and before validation. The vaccines batches were produced by nine different companies and their keys were supplied with theauthors. The tested vaccines were coded from 1 to 9**(table 1).**

Table no 1: demonstrate codesnumbers, designation of different tested vaccineandyear of entry to the lab.

Vaccine code number	Designation of tested inactivated avian influenza vaccinal strains	Year of entry to lab.
1	H5N1 (A/chicken/Egypt/M2583D/2010) (H5N1)	2023
2	H5N1 Re-6 (A/duck/Guangdong/S1322/2006) (H5N1)	2023
3	H5N1 Re-8 (A/chicken/ Egypt/18-H/2008) (H5N1 Re-8)	2023
4	H5N1 Re-6 (A/duck/Guangdong/s1322110) (H5N1 Re-6)	2023
5	H5N1 (Re-7 RG A/duck/Anhui/1/2006)(H5N1 Re-7)	2023
6	H5N1 (Re-8 A/Chicken/Guizhou/4/13)(H5N1 Re-8)	2023
7	H5N2 (A/chicken/Mexico/232/1994/CPA)(H5N2)	2023
8	H5N3 (A/chicken/Vietnam/C58/2004)(H5N3)	2023
9	H5N8 (RGA/green-winged tail/Egypt/877/2016)(H5N8)	2023

2- SPF (specific pathogen free) eggs

- Fertile embryonated chicken SPF eggs at age of 9-11 day were obtained from national project for production of SPF eggs (Koum-OshiemEl-Fayoum, Egypt). The eggs were used for completion of inactivation test of AIV inactivated tested vaccines and virus shedding quantitation.

3- Specific pathogen free chicks

- A total of two hundred and seventy (270) SPF of one week old chicks were obtained from SPF Egg Production Farm, KoumOsheim, El-Fayoum, Egypt. SPF chicks were used for challenge experiment and immunization using different inactivated avian influenza vaccines.

4- Avian influenza virus strain

- The local avian influenza currently circulating in Egypt designated as **(A/ibis/Egypt/RLQP-229S/2022-H5N1)** (accession number OP491851) was used as positive control virus for completion of inactivation test and the same virus was used in challenge experimental containing 106 egg infective dose fifty/ml **(Mosaad*et al.*, 2023).**

Quality control of commercial inactivated avian influenza vaccine batches

- All steps of quality control of killed avian influenza vaccine batches were carried out in consistent with**OIE, (2021)** and **Egyptian standard regulation for veterinary Biologics, (2017).**

1- Completion of inactivation test of tested vaccines:-

- Random vaccine was chosen from every provided batch for completion of inactivation test. Concisely, 0.2 ml of inactivated avian influenza vaccine was inoculated into five 9-11 day old SPF eggs at least for three passages via allantoic route. Five SPF eggs were inoculated with the same dose and route of currentcirculating field strain of avian influenza virus designated as (A/ibis/Egypt/RLQP-229S/2022-H5N1) (accession number OP491851) and used as positive control. Another five SPF eggs were none inoculated and kept as negative control group. The eggs were incubated at 37°C with 40-60% humidity for seven days and candled daily. Embryos dying during first 24 hours are considered nonspecific. Rapid slide HA test was used for detection of allantoic fluid collected from inoculated eggs to ensure from completion of inactivation process.

2-Vaccination of SPF chicks with inactivated avian influenza vaccines

-TenSPF chicks of one week old were immunized with the recommended dose of inactivated avian influenza vaccine via S/C route based on the instructions of the manufacturer.

3- Immunogenicity of avian influenza vaccines

- Collection of serum samples was carried out from five immunized chicks on 3rd week and 4th weekpost immunization using standard four HAU of the antigen to detect titer of antibody for avian influenza virus by HI test based on the geometric mean (G.M) usingreference antisera provided from GD- Holland Company. Serum samples were preserved at -20°C till use.

4- Experimental challenge of inactivated avian influenza vaccines (protection %)

- The vaccinated chicks challenged with the current circulating avian influenza field strain designated as (A/ibis/Egypt/RLQP-229S/2022-H5N1) (accession number OP491851) on 4th week post vaccination with a dose of 0.1mlcontaining 10^6 egg infective dose fifty/ml throughintramuscular route.

-Ten none vaccinatedone week old SPF chicks were inoculated with the same dose and route of above mentioned avian influenza virus and were kept as positive control group.

-Another none inoculated ten SPF one week old chicks were kept as negative control.

- Each group was kept in a separate isolator and observed for ten days to notice any signs that may arise.

- Validation of challenge experiment is achieved when none vaccinated challenged chicks (positive control group) showing mortalities not less than 90% within four days after challenge **(OIE, 2015)**

5- Virus shedding of inactivated avian influenza vaccines

- shedding of virus was determined by collection of oropharyengeal swabs from vaccinated challenged chicks on 3rd, 5th, 7th and 10th days post challenge. Swabs were transferred in 1mL phosphate buffered saline containing 1000µg streptomycin and 1000 IU penicillin followed by centrifugation at 3000 rpm for 30 minutes. Then, the supernatant is transferred to new vial and kept at -80°C untiluse. Also, oropharyengeal and cloacalswabs were collected from none vaccinated challenged group on 3rd day post inoculation and from negative control group on the same days with the same preparation guidelines.

-The quantitation of virus was implemented by inoculation of collected supernatant after preparation of oropharyengeal and clocal swabs in 9-11 days SPF old eggs via allantoic cavity and titration of virus was calculated according **to Reed and Muench, (1938).**

- All above mentioned steps for quality control were implemented for all nine commercial imported inactivated avian influenza vaccines used in this study.

III. Results

1- Result of completion of inactivation test

- Inoculation of all tested nine commercial killed avian influenza vaccines in 9-11 day old SPF eggs with 0.2 ml throughallantoic cavity demonstrated no deaths the second day of inoculation.

- Candling of eggs inoculated with avian influenza field strain that used as positive control showed death of all five SPF eggs embryos the second day post inoculation.

- Testing of allantoic fluid harvested from positive control group of inoculated virus showed positive agglutination of 10% chicken erythrocytes by rapid slide HA test.

- No deaths were detected at the end of incubation period for negative control group.

2- Result of vaccination of tested vaccines in SPF chicks

- No clinical signs or mortalities were detected in one week old SPF chicks inoculated with all nine killed avian influenza tested vaccines afterten days observation period indicating the safety of all tested vaccines.

3- Result of immunogenicity of tested vaccines

- Collection of serum samples at interval times 3rd and 4thweeks post vaccination (WPV) from immunized chicks showed good immune response of all nine tested inactivated vaccines expect vaccine codes 3 and 4 based on geometric mean (G.M) calculated from five chicken sera using HI test and reference antisera as demonstrated in **table 2**

Table no 2: Summarize mean HI titers of nine tested vaccines on 3rd and 4th week post vaccination (WPV) against avian influenza virus.

Vaccine code number	Designation of tested inactivated avian influenza vaccinal strains.	3rd WPV	4th WPV
1	H5N1 A/chicken/Egypt/M2583D/2010 (H5N1)	6.2 log2	7.2 log2
2	H5N1 Re-6 (A/duck/Guangdong/S1322/2006 (H5N1)	6.4 log2	7.4 log2
3	H5N1 Re-8 (A/chicken/ Egypt/18-H/2008 (H5N1 Re-8)	4 log2	5.1 log2
4	H5N1 Re-6 A/duck/Guangdong/s1322110 (H5N1 Re-6)	3.5 log2	4.9 log2
5	H5N1 Re-7 RG A/duck/Anhui/1/2006(H5N1 Re-7)	6 log2	7.1 log2
6	H5N1 Re-8 A/Chicken/Guizhou/4/13(H5N1 Re-8)	5.6 log2	7 log2
7	H5N2 A/chicken/Mexico/232/1994/CPA(H5N2)	6.6 log2	7.7 log2
8	H5N3 A/chicken/Vietnam/C58/2004(H5N3)	6.9 log2	7.9 log2
9	H5N8 (RGA/green-winged tail/Egypt/877/2016)(H5N8)	6.7 log2	7.6 log2

4-Result of challenge experiment of inactivated avian influenza vaccines (Protection %)

- Challenge experiment of nine commercial inactivated avian influenza vaccines revealed variations in protection percentage of vaccinated challenged chicks against current circulating avian influenza field strain designated as (A/ibis/Egypt/RLQP-229S/2022-H5N1) (accession number OP491851) as shown in **table 3.**

- Based on protection percent of tested vaccines, vaccine codes 3 and 4 were not valid for registration at central laboratory for evaluation of veterinary Biologics due to protection percent less than 80% according toEgyptian standard regulation for veterinary Biologics that stated valid inactivated avian influenza vaccines only must have 80% protection percent and above against challenge with field strains. So vaccine batches number 3 and 4 are not approved for vaccination in poultry flocks.

- Positive control group developed characteristic signs on 2nd and 3rd days post challenge of avian influenza virus such as hemorrhage in shanks and feet, cyanosis in wattle and comb, nasal discharge and conjunctivitis.100 % mortality was attained on 4th day post challenge.

- No clinical signs or mortalities were developed on negative control group along ten days observation period

Table No3: demonstrates protection level of vaccinated challenged chicks achieved by different tested vaccines at 4thWPV against field circulating strain.

Vaccine code number	Designation of tested inactivated avian influenza vaccinal strains	Protection percent
1	H5N1 (A/chicken/Egypt/M2583D/2010) (H5N1)	80%
2	H5N1 Re-6 (A/duck/Guangdong/S1322/2006) (H5N1)	80%
3	H5N1 Re-8 (A/chicken/ Egypt/18-H/2008 (H5N1 Re-8)	60%
4	H5N1 Re-6 (A/duck/Guangdong/s1322110 (H5N1 Re-6)	50%
5	H5N1 Re-7 RG A/duck/Anhui/1/2006(H5N1 Re-7)	80%
6	H5N1 Re-8 A/Chicken/Guizhou/4/13(H5N1 Re-8)	80%
7	H5N2 A/chicken/Mexico/232/1994/CPA(H5N2)	90%
8	H5N3 A/chicken/Vietnam/C58/2004(H5N3)	90%
9	H5N8 (RGA/green-winged tail/Egypt/877/2016)(H5N8)	80%

5-Result of viral shedding of inactivated avian influenza vaccines

- Quantitation of virus shedding from oropharyngeal and cloacalswabs collected from vaccinated challenged chicks on 3rd, 5th, 7th and 10th days post challenge (DPC) inoculated through allantoic cavity in 9-11 day old SPF eggs of tested vaccines revealed different amount of virus load. **(Tables 4 and 4)**.

- Quantitation of virus shedding from oropharyngeal and cloacal swabs collected from positive control group on 3rd day post challenge showed that the mean virus load titer was 6(log10) and 5 (log10) respectively. **(Tables 4 and 5).**

-No virus shedding was detected of cloacal and oropharyngeal swabs collected on 3rd, 5th, 7th and 10th days and inoculated through allantoic cavity in 9-11 day old SPF eggs of negative control group.

Table No 4:Mean reduction titer of viral shedding of tested inactivated avian influenza vaccines collected from oropharyngeal swabs of vaccinated challenged chicks.

Vaccine code number	Mean reduction titer of viral shedding (log10) Vacc.= vaccinated, DPC= day post challenge N/A = not assed due to death of birds at these days							
	3rd DPC		5th DPC		7th DPC		10th	
1	Vacc.	control	Vacc.	control	Vacc.	control	Vacc.	control
	3.8	6	3.7	N/A	2.8	N/A	2.7	N/A
2	3.6	6	2.8	N/A	2.9	N/A	2.8	N/A
3	5	6	3.6	N/A	2.8	N/A	2.9	N/A
4	5.7	6	3.4	N/A	2.7	N/A	2.5	N/A
5	3.8	6	2.8	N/A	2.2	N/A	1.9	N/A
6	3.6	6	2.7	N/A	2.3	N/A	1.8	N/A
7	3.8	6	2.5	N/A	1.7	N/A	2.2	N/A
8	3.4	6	2.8	N/A	1.6	N/A	1.8	N/A
9	3.3	6	2.9	N/A	1.8	N/A	2	N/A

Table No 5: Mean reduction titer of viral shedding of tested inactivated avian influenza vaccines collected from clocal swabs of vaccinatedchallenged chicks.

Vaccine code number	Mean reduction titer of viral shedding (log10) Vacc.= vaccinated, DPC= day post challenge N/A = not assed due to death of birds at these days.							
	3rd DPC		5th DPC		7th DPC		10th	
1	Vacc.	Control	Vacc.	Control	Vacc.	Control	Vacc.	Control
	3	5	2.9	N/A	2	N/A	1.9	N/A
2	2.8	5	3	N/A	2.1	N/A	2	N/A
3	4.2	5	2.8	N/A	2	N/A	2.1	N/A
4	4.9	5	2.6	N/A	1.9	N/A	1.7	N/A
5	3	5	2	N/A	1.4	N/A	1.1	N/A
6	2.8	5	1.9	N/A	1.5	N/A	1	N/A
7	2.9	5	1.7	N/A	0.9	N/A	1.4	N/A
8	2.6	5	2	N/A	0.8	N/A	1	N/A
9	2.5	5	2.1	N/A	1	N/A	1.2	N/A

IV. Discussion

Vaccination is an effective platform for avian influenza control and prevention. Although hard efforts for virus eradication and financial costs for different vaccination policies in Egypt since the emergence of highly pathogenic avian influenza H5N1, low pathogenicH9N2 besides newly emerged H5N8, the virus became endemic in poultry flocks and acquiring public health importance**(ElMasry*et al.*, 2017).** Additionally, biosecurity and biosafety strategy, poultry movement restrictions between localities and infected birds culling to avoid virus transmission should be taken into account for combating AIV **(Kayali*et al.*, 2016).**Inactivated emulsified oil based vaccines are common use in Egypt and at least twenty four available commercial inactivated AI vaccines are applied and licensed for controlling infection in Egypt. These vaccines contain wide range of antigen ranging from traditional old H5 linage strains to various goose / Guangdong (Gs/GD) linages strains. **(Kayali*et al.*, 2013).**Some of vaccines tested under laboratory circumstances were immunogenic and confer protection against challenge with highly pathogenic avian influenza H5N1**(Kim *et al.*, 2010)**Nevertheless, H5N2 and H5N1 prepared inactivated avian influenza vaccines failed to produce sufficient immunity after field application in Egypt**(Kandeil*et al.*, 2016)**this is might be account for genetic diversity between recent circulating field strains and available traditional vaccines has induced inadequate protection and further subsequently result in mutants escaping as observed in the emergence of variant antigenic drift clade 2.2.1.1 in poultry sectors**(Swayne *et al.*, 2012)**.Therefore, efficacy reassessment and modification of currently used killed avian influenza vaccines is extremely necessary to keep the most effective immunization policy in addition to checking field strains to detect newly merging variants**(Swayne *et al.*, 2014)**. In this respect the current work was designed to evaluate the efficacy of commercial imported inactivated avian influenza vaccines.Nine vaccines were randomly chosen from nine batches throughout everyday work at Central Laboratory for Evaluation of Veterinary Biologics (CLEVB) in 2023; these vaccines were coded from one to nine and were tested for quality control before registration and approval.

These newly presented inactivated vaccines were tested in their efficacy against currently circulating avian influenza field strain designated as (A/ibis/Egypt/RLQP-229S/2022-H5N1) (accession number OP491851)**(Mosaad*et al.*, 2023).** Based on Egyptian evaluation protocols applied in CLEVB, **(OIE, 2021)** the nine killed avian influenza vaccines was tested for completion of inactivation process in SPF eggsfor at least three passages to assure the efficiency of inactivation process and absence of heamagglutinaitng virus activity during vaccine production.

Since using phylogenetically distinct inactivated AI/H5 seed vaccines in 2006 serological response was variable against Egyptian H5N1 strains **(Spackman*et al.*, 2014).** In this study, the newly inactivated vaccines induced considerable antibody titers **(Table-1)** on 3rd and 4th week post vaccination using HI test against field strain used in this study. The mean HI antibody titer ≥7 log obtained in serum samples after 4th week vaccination is required for vaccine validation**(OIE, 2021).**All tested vaccines induced inadequate antibodies on 3rd WPV however; serological response was peaked on 4th WPV as reported by **Ali *et al.*, (2017).** Vaccine codes 3 (H5N1 Re 8) and 4 (H5N1 Re-6) produced insufficient serological response and this might be due to genetic divergence reduced reactivity between these vaccines and current circulating avian influenza virus strain **(Kandil*et al.*, 2018).** Furthermore, differences in antigenic loci in relation to challenge virus implicated in this poor immune response.**(Ibrahim *et al.*, 2015)**. Also, the closer HA gene sequence identity between the circulating field virus and vaccinal strain, the higher protection and immunity accompanied with replication reduction in respiratory tract of the challenged virus**(Swayne *et al.*, 2000)**.Conversely, trivalent vaccine masterseeds H5-Re13, H5-Re14, and H7-Re4 harboring NA and HA genes of recently identified H5N6 virus, H5N8 virus, and H7N9 virus, respectively induced high titer of antibody with adequate protection against H5N1,H5N6 H5N8, and H7N9 viruses under field conditions in poultry flocks**(Ying *et al.*, 2022)**.The Relationship between immune response and protection against mortality and is indicator for vaccine efficacy. Also,the protection from mortality relies on serological potency**(OIE, 2021)**. When close antigenic and genetic relationship between the challenge and vaccine viruses, the predicted serological titer induce better protection**(Kumar *et al.*, 2007).**

In the current study, immunized chicks were challenged with circulating avian influenza virus on the 4th week post vaccination and all tested vaccines achieved 80% protection from deaths and clinical signs as reported by**OIE, (2021)** for being registered and approved**(Table-3)** expect vaccines code 3 and 4. This might be genetic divergence and reduced reactivity between these vaccines and challenge virus**(OIE, 2018).**Moreover, adaptation and progress of reassortant avian influenza virus from former and current circulating strains present in Egyptian poultry populations can be attributed in reduction of protection**(Hagag*et al.,* 2019)**.Vaccine code 7 H5N2 elicits the highest protection level as cross protection may be expected within different influenza virus subtypes and high HA amino acid similarity. Likewise, commercial killed avian influenza H5N2 vaccine designated as A/CK/Mexico/ 232/94 induce protection against low dose of challenge virus A/CK/HK/86.3/02 (H5N1) and despite of high titers of HI antibodies; vaccinated challenged birds were not completely protected**(Liu *et al.,* 2003)**. In context with this study inactivated H5N2 strain based on H5 from H5N1egyptian virus and N2 from Egyptian H9N2 was developed and proved its efficacy and mortality decrease upon challenge with H5N1 and H9N2**(Kandeil*et al.,* 2016)**.Also,Vaccine code 8 H5N3 elicits the highest protection percent owing to cross reactivity within influenza subtypes. Similarly, H5N3vaccine was developed based on A/Puerto Rico/8/34 (PR8) backbone reassortant mutants at 140th loop and 190th helix haemagglutin. The newly reasserted vaccine elicits both cross neutralizing antibodies and cross protection against H5N1 virus infection (**Rajesh Kumar*et al.,* 2017)**.

Qualitative or quantitative of virus shedding and replication in gastrointestinal and respiratory tract are fundamental protective measure that plays a role of the vaccine to reduce spreading of virus and consequently for epidemics control **(Capua *et al.,* 2004)**. The vaccinated challenged chicks developed disseminating of virus on 3rd, 5th. 7th and 10th days after challenge of all tested killed avian influenza vaccines **(tables 4 and 5).**

Reduction of virus load from respiratory tract should decrease in vaccinated challenged group by (10^2) 2 logs (100 fold) than non-vaccinated challenged group or positive controlgroup for validation and registration of inactivated avian influenza vaccines**(Suarez *et al.,* 2006)**.The tested vaccines could not inhibit virus dissemination indicating the potential risk of virus transmission to susceptible birds besides the endemic virus status causing antigenic drift. Similarly, effective killed avian influenza virus vaccines at complete dose decrease virus dissemination from digestive and respiratory tract **(Swayne *et al.,* 2006)**. The shedding of challenge virus is associated with the antigenic similarity degree and content between HA gene of inactivated avian influenza vaccine and challenge **virus (Swayne *et al.,* 2000).** Therefore, the significant reduction in virus shedding upon using challenge virus as autogenously vaccine is attained **(Kapczynski*et al.,* 2017).**

V. Conclusion

Control of avian influenza depends mainly on vaccination besides biosafety and biosecurity approaches. Vaccinal strains should be modified based on epidemiological state and implementation of vaccine assessment regimes according to recent existing viruses to overcome virus escape mutants and remerging viruses. Also, construction of novel platform of vaccines is highly needed to augment the protective efficacy and effective immunization in field

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