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Efficacy of the recently registered inactivated Avian Influenza H5 (Re-13 & Re-14) vaccine in commercial broiler chickens compared to that of Re-5 & Re-6+Re-8 AI vaccines against the currently circulating HPAI H5N8 strain in Egypt

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ABSTRACT

Vaccination strategy against highly pathogenic Avian Influenza viruses (HPAI), with application of other measures was succeeded in limitation of the negative impact of that serious disease on the poultry industry in Egypt. The main factor determining the efficacy of an Avian Influenza vaccine is the vaccine composition matching the HPAI field strains. The prevalent HPAI field strains Nowadays in Egypt are related to the clade 2.3.4.4b, So the present study was designed to evaluate the efficacy of recently registered AI vaccine that composed of two inactivated RGAI strains which designated as Re-13 & Re-14 and related to the clade 2.3.4.4b in commercial broiler chickens. The obtained results were compared against that of two Re-6 + Re-8 and Re-5 AI vaccines, the results proved significantly higher efficacy, HI titer and reduction in both titers and number of shedders for the Re-13 & Re-14 vaccine.

Keywords: Highly Pathogenic Avian Influenza, AI vaccine, Egypt, broiler, efficacy.

1. Introduction

Avian influenza viruses (AIV) are a highly contagious avian disease, may cause serious economic losses in poultry industry worldwide and poses a potential threat to public health. (Neumann, 2015). In Egypt, the first introduction of HPAIV dates back to 2006. The causative virus of subtype H5N1 belonged to clade 2.2.1 of the goose/Guangdong lineage (gs/GD) of Chinese origin (Aly *et al*., 2008; Peyre *et al*., 2009). Since the emergence of this HPAI virus H5N1, descendants of this strain continue to spread among avian species and their hemagglutinin (HA) has evolved into multiple distinct phylogenetic clades, subclades and lineages (Smith *et al*., 2015). Three zoonotic AIVs have been detected in poultry in Egypt including H5N1 (2005–2020), H9N2 (since 2013) and recently H5N8 clade 2.3.4.4b (since 2016) (Kim *et al*., 2018; Hassan *et al*., 2021). The HPAI H5N8 virus was originally detected in migratory bird (common coots-Fulica atra) in late 2016 (Selim *et al.,* 2017). The original virus was phylogenetically closely related to other H5N8 viruses of clade 2.3.4.4b detected in Russia in 2016 (Yehia *et al*., 2018). Thereafter, the virus spread in a very short time among domestic poultry populations in different governorates in Egypt, posing a great threat to the poultry industry (Hassan *et al.,* 2020; Yehia *et al.,* 2020). And the epidemiologic data suggested that the HPAI H5N8 virus (clade 2.3.4.4b) has replaced the Egyptian H5N1 virus (clade 2.2.1.2), becoming the most commonly detected H5 subtype in Egyptian poultry sectors (Amer *et al.,* 2021).

To deal with this global threat caused by the Avian Influenza viruses, , The mass vaccination of poultry is highly useful to protect poultry from AIV and prevent spillover to other mammals including humans in addition to the biosecurity measures and culling strategy (Chen, 2009). Several AIV vaccines have been developed including inactivated whole virus vaccines and recombinant virus vector vaccines (Li *et al.,* 2014). The inactivated whole virus vaccine was first developed in the 1940s for the control and prevention of human influenza (Tang *et al.,* 2009). Over the past 30 years, it has also been the major type of vaccine used to control AIVs in poultry (Swayne, 2009). The evolution over time of the vaccinal strains is quite indicative of the need for regular reformulation. In 2004, killed heterologous H5N2

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strain, A/Turkey/England/N-28/73 was used as viral strain in H5N1 vaccines Then in 2005, the viral strain H5N1 (A/Goose/Guangdong/96) Re-1, killed was developed. Between 2006 &2012, different vaccines were produced using killed H5N1 Re-1/Re-4, Re-4/Re-5 and Re-4/Re-6 viral strains. In 2014, H5N1 Re-7, killed was developed (Guyonnet and Peters, 2020). Later on, H5-Re11 contains the HA and NA genes from A/duck/Guizhou/ S4184/2017(H5N6) and was developed to protect against H5 viruses carrying the clade 2.3.4.4h HA gene; H5-Re12 contains the HA and NA genes of A/chicken/ Liaoning/SD007/2017(H5N1) and was developed to prevent infection with H5 viruses carrying the clade 2.3.2.1d HA gene. H5-Re11 and H5-Re12 have been used for vaccine production since December 2018 (Zeng *et al.,* 2020). In 2019, the generation of H7-Re3, which contains the HA and NA genes of the A/chicken/Inner Mongolia/ SD010/2019 (H7N9) virus was done for the prevention of H7N9 avian influenza. Also, an inactivated trivalent H5/H7 vaccine, produced by using H5-Re11, H5-Re12, and H7-Re3, had been widely used to prevent and control lethal H5 and H7N9 virus infections in poultry since July 2020 (MARA 2020). Recently, an updated trivalent vaccine (H5-Re13, H5-Re14, and H7-Re4, of which the HA and NA genes originated from the newly detected H5N6 virus, H5N8 virus, and H7N9 virus, respectively) has been developed and animal studies proved that the novel H5/H7 trivalent vaccine is immunogenic and could provide solid protection against viruses that are currently circulating in nature (Zeng *et al*., 2020).

The aim of our study to evaluate the protective efficacy of the recently registered inactivated Avian Influenza H5 (Re-13 & Re-14) vaccine in commercial broiler chickens compared to that of Re-5 & Re-6+Re-8 AI vaccines against the currently circulating HPAI H5N8 strain in Egypt.

2. Material and Method

2.1. Ethical approval

All methods in the study were performed according to relevant guidelines and regulations. All experiments were carried out according to ARRIVE 2.0 guidelines and were approved by the Institutional Animal Care and Use Committee (IACUC) in the Faculty of Veterinary Medicine, Cairo University (Code: VetCU01102020217)."

2.2. Experimental birds and housing

A total of 150 commercial broiler one day old (DO) chicks of cub breed, were kindly supplied from Cairo Company for poultry production, carrying significant level of maternally derived antibodies (MDA) to H5 AIV, representative of the common situation in Egypt. Chicks were hatched from breeders vaccinated, more than one time, against avian influenza with different inactivated H5 vaccines. All chicks were housed inside poultry BSL3 chicken isolators during the whole experiment period. All chicks were reared under proper Hygienic conditions, feed and water will be supplied adlibitum.

2.3. Vaccines

I. Reassortant Avian Influenza Virus (Subtype H5) Vaccine, Inactivated (H5N6 H5 – Stain Re13 + H5N8 H5 – Strain Re14)

It is oil adjuvant inactivated reassortant avian influenza vaccine prepared from (H5N6 H5 – Stain Re13 A/duch/Fujian/S1424/2020 clade 2.3.4.4h + H5N8 H5 – Strain Re14 A/whooper swan/Shanxi/4-1/2020 clade 2.3.4.4b). Manufacture date: 25/8/2022 and Expiry date: 24/8/2024.

II. Reassortant Avian Influenza Virus H5 Subtype Vac. Inact. (Re-5) strain:

It is oil adjuvant inactivated reassortant avian influenza vaccine prepared from (H5N1 subtype, Re-5(A/duck/Anhui/1/2006 clade 2.3.4) strain. Manufacture date: 30/1/2022 and Expiry date: 29/1/2024.

III. Reassortant Avian Influenza Virus H5 Subtype Vac. Inact. (Re-6 &Re-8 strains)

It is oil adjuvant inactivated reassortant avian influenza vaccine prepared from (H5N1subtype, Re-6(A/duck/Guangdong/s1322110 Clade 2.3.2.1) strain and Re-8(A/Chicken/Guizhou/4/13 clade 2.3.4.4g) strain). Manufacture date: 9/3/2022 and Expiry date: 8/3/2024.

2.4. Virus and antigen

The HPAI H5N8 (A/Turkey/Egypt/A2/2021 (H5N8)) clade 2.3.4.4b virus was used as challenge virus with a dose: 100ul contain 100 CLD_{50}/bird which is equivalent to 6 (log 10) EID_{50}, in addition used as HI antigen after treatment with Binary ethylenimine (BEI).

2.5. Vaccination and challenge study

A total of 150 chicks were divided into 5 groups each of 30 chicks at 10th DO and the experimental groups were treated as illustrated in table (1). All vaccinated groups were injected with mentioned vaccines at the recommended dose according to the manufacturer instructions via S/C route. The challenge was conducted at 31st day old on twenty chickens from groups 1, 2 and 3 and ten chickens from group 4. Each challenged chicken was inoculated via intranasal route with 100ul contain 100 CLD_{50}/bird which is equivalent to 6 (log 10) EID_{50}.

All chickens were subjected to daily monitoring for clinical singes and mortalities for 10-day post challenge (DPC). Organs and swabs were collected from dead birds to ensure the cause of death.
The challenge test considered to be valid when the control non-vaccinated challenged chickens showing not less than 90% mortality within 4 days Post challenge (OIE, 2015).

2.6. Serology

Individual Serum samples were collected from 10 chickens in each group (1-4gp) at 1st, 10th, 17th, 24th and 31th day old. Serum sample from (gp4) were tested for waning up of the MDA. Haemagglutination Inhibition (HI) test was conducted on those samples according to OIE diagnostic manual (2018) using standard 4 HAU of the antigen. Serum samples were preserved at -20°C till time of testing. Each vaccine was tested against the heterologous challenge virus antigen. Data of HI testing were analyzed based on HI mean (arithmetic mean) titer.

Table 1: Experimental groups

Group No	Age of vaccination (DO)	Vaccine	Manufacture date	No of birds/group
1	10 DO	Reassortment Avian Influenza Virus H5 Subtype Vac. Inact. (Re-13 &Re-14)	25- 8 - 2022	30/group
2		Reassortment Avian Influenza Virus H5 Subtype Vac. Inact. (Re-5) strain	30 - 1 -2022	
3		(Reassortment Avian Influenza Virus H5 Subtype Vac. Inact. (Re-6 &Re-8 strains)	8- 3 -2022	
4	Control positive group	-	-	
5	Control negative group	-	-	

2.7. Virus shedding titer detection

Individual Oro pharyngeal swab samples were collected at 3rd, 5th, 7th and 10th day post challenge (DPC). Swab samples were re-suspended in 1ml PBS containing 1000 IU penicillin and 1000 ug Streptomycin by cutting the cotton part in cryo vial and doing vigorous vortexing. Supernatant were transferred to new vial for preservation at -80°C till time of testing.

Extraction was done using Qiagen RNA extraction kit the total sample volume of extraction was 200 ul. Real time rt -PCR kit was Invitrogen. The primers, probe and cycle conditions were according to Slomka *et al.* (2007).

The Shedding titer (PCR copies/ml) was conducted targeting the M gene and results of CT values were calculated against challenge virus standard curve.

Mean shedding titer= sum of shedding titers/number of shedder birds.

3. Results

The effectiveness of vaccination was evaluated on the basis of clinical protection (morbidity and mortality) and measurement of virus shedding after challenge. Immune response to vaccination was by evaluation the serological response (mean HI titer).

3.1. Antibody titer using HI test.

The recorded results of mean HI antibody titer (log2) tested using the prepared antigen from the heterologous HPAI H5N8 (A/Turkey/Egypt/A2/2021 (H5N8)) clade 2.3.4.4b virus of vaccinated as well as control group at 1st, 10th, 17th,24th and 31th DO were illustrated in table (2) and figs (1and 2) and they were as follow:

The mean log 2 HI antibody titer at 31st DO were 5.9, 4.3 and 5.1 for groups 1, 2,3 respectively. In addition, in control group (gp4) were 5.6, 3, 1.8, 1., 0 HIU and this mean that result of HI titer for waning up of the MDA in this group was no longer detectable at the age of 31st as shown in fig (2).

Table 2: Mean HI antibody titers (Log 2) against the heterologous A/Turkey/Egypt/A2/2021 HPAI H5N8 antigen of broiler vaccinated as well as control groups

	Mean HI results				
D.O / **Group**	**1 D.O**	**10 D.O**	**17 D.O**	**24 D.O**	**31 D.O**
Group1	5.4 ±0.69	3 ±0.66	2.8±0.63	4.2 ±0.78	5.9 ±0.87*
Group2	5.4 ±0.69	2±0.66	2.4±0.84	3.3 ±0.67	4.3 ±0.67*
Group3	5.4 ±0.69	3 ±0.66	2.5±0.71	3.9 ±0.73	5.1 ±0.73*
Control	5.4 ±0.69	3 ±0.66	1.8 ±0.42	1 ±0.047	0

The arithmetic mean and ±standard deviation of HI titers are shown
*= statistically significant difference at P<0.

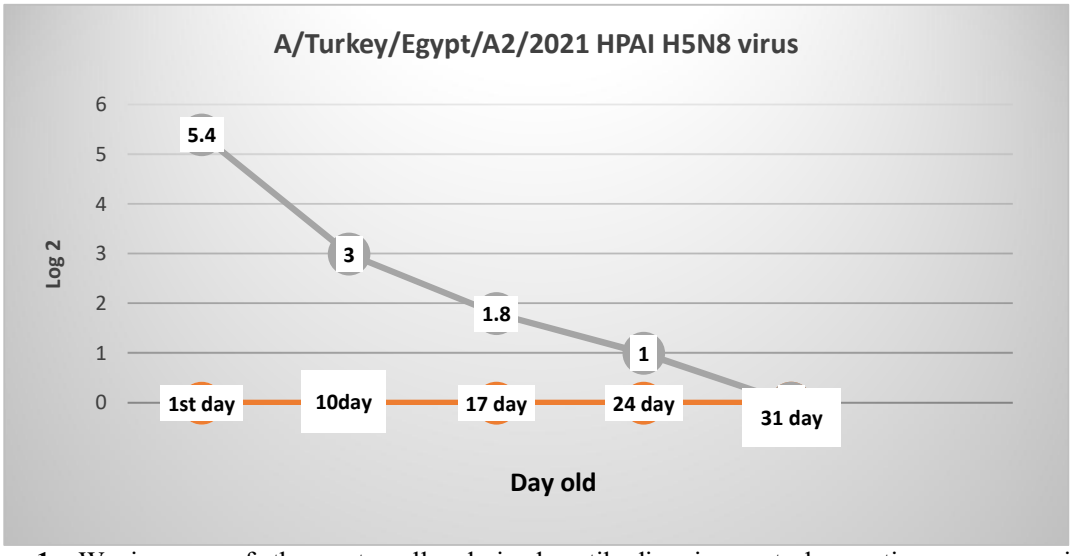

Fig. 1: Waning up of the maternally derived antibodies in control negative group against A/Turkey/Egypt/A2/2021 HPAI H5N8 antigen

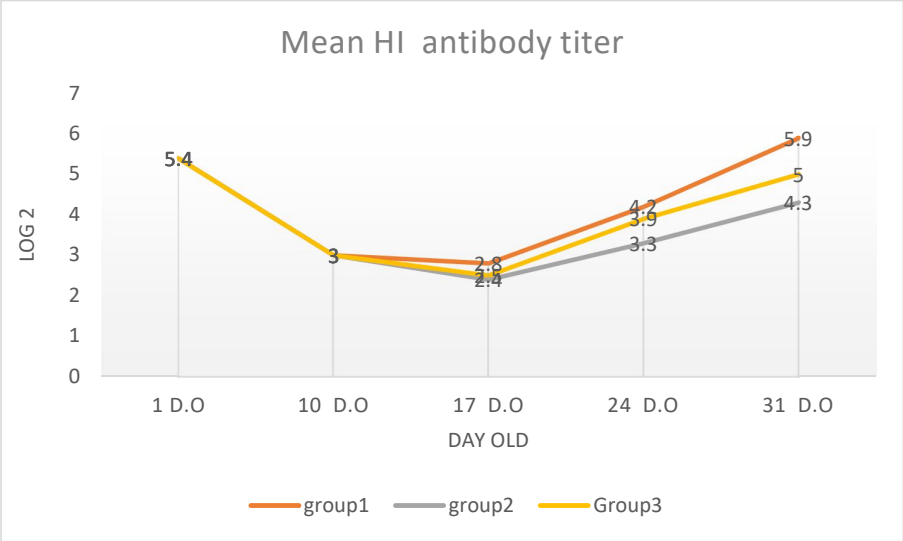

Fig. 2: Mean HI antibody titers (Log 2) of commercial broiler chickens vaccinated with different AI vaccines against the prepared heterologous A/Turkey/Egypt/A2/2021 HPAI H5N8 virus antigen the result indicates that group 1 which vaccinated with Reassortment Avian Influenza Virus H5 Subtype Vac. Inact. (Re-13 &Re-14) has the highest mean of the antibody titer followed by group 3 which vaccinated with (Reassortment Avian Influenza Virus H5 Subtype then group 2 which vaccinated with Reassortant H5N respectively

3.2. Protective efficacy of the newly reassortant H5 bivalent vaccine (Re-13 and Re- 14 strains) against the recently HPAI local H5N8 (A/Turkey/Egypt/A2/2021 (H5N8)) clade 2.3.4.4b virus

The results of daily observation post challenge at 31st DO of the experimental (1-3) groups, as well as control positive group, recording of the mortalities, protection percentage, mean virus shedding titer and difference of mean virus shedding titer between vaccinated groups and challenged non vaccinated group are illustrated figs (3) and tables (3,4 and 5).

- At 2nd, 3rd and4th DPC the frequently observed clinical signs in challenged non vaccinated group (gp4) were sever cyanosis of comb and wattle, ecchymosis on the shanks and feet, nervous signs represent in torticollis and tremors, severely respiratory signs including virulent coughing, sneezing, nasal discharge, gasping and dyspnea conjunctivitis mainly of frothy type associated with abundant lacrimation, the mortality rate was 100% at 4th DPC and this mortality pattern is characteristic for this challenge virus. The mean of virus shedding titer in 3rd DPC was 5.75 (log10).

- There are no noticed clinical signs observed in group 1 meanwhile there are minor noticed signs in 3 chickens in group 2 mainly were depression and ruffled feather and also this signs observed in 2 chickens in group 3. The mortality started from 7th DPC for group 1 and 6th DPC for both groups 2 and 3 with protection percentage 95%,85% and 90% for groups 1,2 and 3 respectively. The cumulative mean of virus shedding titer in 3rd, 5th ,7th and 10th DPC were2.85, 3.32 and 3 (log10) for the three groups respectively , while the reduction in mean of virus shedding titer of the three vaccinated groups compared to challenge non vaccinated group were 2.85, 2.37 and 2.7 log (10) for the groups 1,2 and 3 respectively.

- In the negative control group (gp5); There was no mortality during the whole observation period indicating that no AIV or other life-threatening infectious agents.

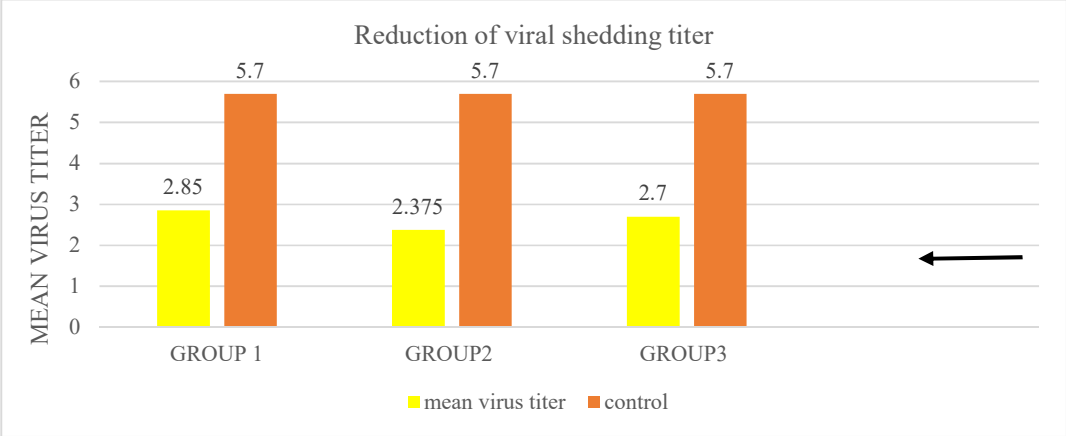

Fig. 3: Reduction in mean of virus shedding titer of vaccinated groups comparing to challenged non vaccinated control group

Table 3: - Protection percentage of Broiler chickens vaccinated with different AI vaccines against A/Turkey/Egypt/A2/2021 HPAI H5N8 virus

Group	No. of challenged birds	No. of dead birds	Protection %
Group1	20	1 (AT 7th DPC)	95%
Group2	20	3 (1 AT 6th & 2 at 7th DPC)	85%
Group3	20	2 (1 at 6th & 2 at 8th DPC)	90%
Group4 NVC	20	20(14 at 3rd & 6 at 4th DPC)	0%
Group5 NVNC	20	0	0

***NVC:** Non vaccinated challenged group * **NVNC:** Non vaccinated non challenged group

Table 4: Cumulative results of virus shedding titer after challenge with A/Turkey/Egypt/A2/2021 HPAI H5N8 virus at 3rd, 5th, 7th, 10th in all groups

Group number	3 DPC	5 DPC	7 DPC	10 DPC	**Cumulative mean	***Reduction in mean titer LOG(10) comparing to positive control group	Protection %
Group 1	3.7 ±0.21*	3.1 ±0.29	2.5 ±0.34	2.1 ±0.68	2.85	2.85	95%
Group 2	3.9 ±0.22*	3.6 ±0.31	3.1 ±0.29	2.7 ±0.44	3.325	2.375	85%
Group 3	3.8 ±0.27	3.2 ±0.24	2.7 ±0.29	2.4 ±0.35	3	2.7	90%
Group 4 (control+)	5.7 ±0.22	-	-	-			
Group 5 (control-)	-	-	-	-	-	-	

* Data represent arithmetic mean ±standard deviation of H5 gene copies in ml of swabs (Arithmetic mean shedding titer = sum of shedding titer (log10 HPAI H5N1 virus titer) /number of shedders. (10from each group)
** Cumulative mean= cumulative mean shedding titer of four days.
*** Reduction in mean of virus shedding titer between vaccinated groups and challenged non vaccinated control group which should be at a minimum of (10^2) 2 logs (100 fold) less virus in vaccinated compared to non-vaccinated chickens (Suarez *et al*., 2006)

Table 5: Summary of data record after challenge with A/Turkey/Egypt/A2/2021 (H5N8) HPAI virus in all groups

Group	No. of challenged birds	Total mortalities	Protection %	Mean HI titer at 31st DO (log2)	Reduction in mean titer log (10) comparing to positive control group
Group1	20	1	95	5.9 ±0.87	2.85
Group2	20	3	85	4.3 ±0.67	2.375
Group3	20	2	90	5.1 ±0.73	2.7
Group4	20	20	0.0	0	0
Group5	0	0	-	0	-

4. Discussion

Vaccination strategy against highly pathogenic avian influenza viruses (HPAI) with the applications of other measures such as education of workers, biosecurity measures using a good quality diagnostics. Active surveillance were succeeded in the limitation of the negative impact of that serious disease on the poultry industry in Egypt.

In this study we compared the protective efficacy of the novel H5 bivalent inactivated vaccine which consist of strain H5-Re13 containing the hemagglutinin (HA) and neuraminidase (NA) genes of an H5N6 virus that bears the clade 2.3.4.4h HA gene and H5-Re14 which contains the HA and NA genes of an H5N8 virus that bears the clade 2.3.4.4b HA gene. With the two commercially available inactivated Avian Influenza vaccines Re-5 H5N1 subtype, which contain (Re-5 A/duck/Anhui/1/2006 clade 2.3.4) strain. And Re-6+Re-8 vaccine which contain (Re-6 A/duck/Guangdong/s1322110 Clade 2.3.2.1) strain and (Re-8 A/Chicken/Guizhou/4/13 clade 2.3.4.4g) strain and this effectiveness was evaluated on the basis of clinical protection (morbidity and mortality) and measurement of virus shedding after challenge against the currently circulating H5N8 HPAI strain in Egypt. Also, the immune response to vaccination was done by evaluation the serological response (mean HI titer).

The maternally derived antibodies (MDA) found to affect the level of PV HI antibodies at the 1st two weeks PV when compared to the control group which has no longer detectable mean HI titer at the age of 31st DO and these results were agreed with Vriese *et al*., (2010) who found that MDAs may still interfere with vaccination to a lesser extent because they are present up to 3 week post hatch.

And here in our study when evaluate immune response using HI test in vaccinated groups, the highest mean antibody titer was noticed in group 1 (5.9) log2 HIU which was vaccinated with H5-Re13 and Re- 14 vaccine then group 3 (5.1) log2 HIU then group 2 (4.3) log2 HIU at 31st DO as shown in table 2 and this result was because of the high antigenic and genetic relatedness between the strains of Re-13and Re-14 vaccine and the currently circulating avian influenza H5 field viruses now in Egypt and this results were in agreement with Ying *et al*. (2022).

The prevention of infection or the qualitative and/or quantitative reduction in virus replication in respiratory and digestive tracts are essential protective criteria that indirectly assess the role of the vaccine to limit field virus spread and are critical for control (Beard, 1992; Swayne, 2003 and Capua *et al*., 2004).

Our results reveal that the highest reduction in mean of virus shedding titer Log (10) compared to positive control group was noticed in group 1 (2.85) Log (10) then group 3 (2.7) Log (10) then group 2 (2.375) Log (10). Also, protection percentage was 95% in group 1 which vaccinated with inactivated Re-13&Re14 vaccine and 85%, 90% in group 2 and 3 which vaccinated with inactivated Re-5 strain and Re-6& Re-8 strain vaccines respectively our results indicate that hat the newly registered H5 vaccine can provide solid protection against the H5 viruses that are currently circulating in nature and that's because of the high similarity between this newly vaccinal strains and the currently circulating avian influenza H5 field viruses now in Egypt. And this results were in agreement with Ying *et al*. (2022) who found that the newly updated H5/H7 trivalent vaccine which is composed of train H5-Re13 contains the hemagglutinin (HA) and neuraminidase (NA) genes of an H5N6 virus that bears the clade 2.3.4.4h HA gene, H5-Re14 contains the HA and NA genes of an H5N8 virus that bears the clade 2.3.4.4b HA gene, and H7-Re4 contains the HA and NA genes of H7N9 virus detected in 2021 can

provide solid protection against the H5 and H7N9 viruses that are currently circulating in nature when he evaluated the protective efficacy of this novel H5/H7 trivalent inactivated vaccine in chickens, ducks, and geese

4. Conclusion

To have a successful AI vaccine, it is very crucial to update the seed virus strains of AI vaccines to match the field strains.

5. Recommendations

1- Continuous active surveillance for HPAI viruses.
2- Continuous follow up the HPAI epidemiological map worldwide with continuous supervision of the migratory birds pathways.
3- Continuous update of AI vaccine seed virus matching to the field strains.
4- Continuous evaluation of the AI vaccines batch by batch using the recent prevalent field HPAI virus strain.

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