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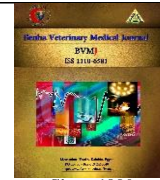

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Comparison of the efficacy of bivalent inactivated H5-Re13 and H5-Re14 vaccine with inactivated H5N2 vaccine against highly pathogenic avian influenza H5N1 virus bearing clade 2.3.4.4b

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ABSTRACT

Avian influenza, particularly the highly pathogenic H5N1 virus, continues to pose a persistent threat to poultry populations worldwide. In Egypt, the outbreaks of H5N1 have a significant economic and public health impact. To deal with this ongoing issue, vaccination remains a cornerstone strategy in mitigating the impact of avian influenza in broiler chicken populations. This study aims to evaluate selected commercially inactivated H5 vaccines in broiler chickens in Egypt, focusing on their effectiveness against the currently circulating highly pathogenic H5N1 strain clade 2.3.4.4b. Inactivated reassortant avian influenza virus vaccine (Re-13 & Re-14 strains) and inactivated avian influenza H5N2 vaccine, represented by G1 and G2 respectively were studied and by assessing their protective efficacy and immunological responses elicited by these vaccines. The mean HI titers (log2±SD) against the heterologous inactivated HPAI H5N1 antigen at 31 day old (DO) was 6.6 ± 0.52 and 3 ± 0.53 with mean shedding reduction 4 and 1.5 (log10) for G1 and G2, respectively. Moreover, the protection percentage after challenge infection with HPAI H5N1 clade 2.3.4.4b was 100% and 55 % for G1 and G2, respectively. Our results indicate that the reassortant avian influenza virus vaccine (Re 13 & Re 14 strains) was effective because the seed viruses in this vaccine are genetically close to the H5N1 virus clade 2.3.4.4b currently circulating in Egypt.

1. INTRODUCTION

The H5 subtype of highly pathogenic avian influenza (HPAI) viruses is thought to pose a serious risk to human and poultry health. Since the HPAI H5N1 (A/goose/Guangdong/1/1996) virus first appeared in China, its offspring viruses have persisted in spreading across other bird species. Furthermore, ten different evolutionary clades have emerged from their HA (Smith and Donis, 2015). Viruses from clade 2.3.4.4 are currently drawing significant attention due to their widespread distribution across the globe. Clade 2.3.4.4 has been categorized into eight subclades (2.3.4.4a to 2.3.4.4h) based on the latest naming conventions (Smith and Donis, 2015) and comprises the following seven subtypes: H5N1, H5N2, H5N3, H5N4, H5N5, H5N6, and H5N8. (Verhagen, et al., 2021). Since 2006, Egyptian poultry populations have been infected with the HPAI H5N1 clade 2.2 virus (Abdelwahab et al., 2016). The HPAI H5N8 clade 2.3.4.4b virus was first discovered in Egypt in December 2016 in the Damietta governorate in migrating birds, particularly the common coot (*Fulica atra*). (Selim et al., 2017) Since then, numerous cases of H5N8 have been reported in domestic poultry in a number of Egyptian governorates' commercial farms, backyard flocks, and live bird marketplaces (OIE, 2017). Even though all of the H5N8 samples from Egypt are members of the same clade (2.3.4.4b), since 2017 there have been several separate reports of the virus's arrival. This suggests that the virus is continuously spreading throughout Egypt's backyard and commercial poultry industries. (Yehia et al., 2018; Tarek et al., 2021). In 2019, new high-pathogenic avian influenza (HPAI) H5N2 viruses were identified in commercial chicken and duck farms in Egypt. This emergence was due to genetic reassortment between the circulating HPAI H5N8 and low-pathogenic avian influenza (LPAI) H9N2 subtypes in the region (Hagag et al., 2019; Hassan et al., 2020). Additionally, new variants of HPAI H5N8 are identified each year, leading to financial losses in the poultry industry (Kandeil et al., 2018). The HPAI H5N8 viruses isolated in Egypt in 2019 showed a phylogenetic connection to HPAI H5N8 viruses that were documented in Europe during the latter part of 2020 (Lewis et al., 2021; Tarek et al., 2021;). Highly pathogenic avian influenza (HPAI) strains H5N1, which belong to clade 2.3.4.4b, were recently discovered in domestic ducks and wild birds from live bird markets in Egypt during the winter of 2021–2022. According to the genomic investigation of those viruses, they shared genetic similarities with the H5 HPAI that was circulating in the Middle East, Africa, and Europe (El-Shesheny et al., 2023; Mosaad et al., 2023; Kandeil, et al., 2023). This development could further complicate the avian influenza disease scenario in the country.

This study seeks to provide valuable insights into the performance of some vaccination strategies in the face of evolving avian influenza challenges by focusing on their effectiveness against the currently circulating highly pathogenic H5N1 strains clade 2.3.4.4b and the immunological responses elicited by these vaccines.

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2. MATERIAL AND METHODS

Ethical Approval
The study followed the requirements of the animal welfare committee, and the protocols were authorized by the Research Ethics Committee, Faculty of Veterinary Medicine at Benha University (Approval number BUFVTM 06-01-23).

2.1. Broiler chickens
A total of 120-day-old (DO) broiler chickens of the Ross breed were kindly provided from El Wadi Company for poultry production. All chickens were reared under proper hygienic conditions, throughout the trial. chickens were housed in Biosafety level 3 (BSL3) chicken isolators and given drinking water and feed *ad libitum*.

2.2. Challenge virus and antigen
The HPAI H5N1 clade 2.3.4.4b challenge virus strain with accession number OQ933425, which was kindly provided by the National Laboratory for Veterinary Quality Control on Poultry Production, Animal Health Research Institute, Giza, Egypt, was used in the challenge experiment. The allantoic fluid was harvested from SPF embryonated chicken eggs in order to propagate this virus. Once the virus's purity was established, it was titrated in SPF embryonated chicken eggs. The final concentration of 10^7 median egg infectious dose (EID_{50}) per milliliter was attained by diluting the viral challenge inoculum in PBS. The virus utilized in the current study had become inactive for use as an HI antigen after being treated with 0.05% beta-propiolactone for two hours at 37 ℃.

2.3. Vaccines
2.3.1. Reassortant Avian Influenza Virus Vaccine (Re 13 & Re 14 strains) ® is oil adjuvant commercial inactivated reassortant AI vaccine prepared from (H5N6 subtype, Re-13 strain (A/duck/Fujian/S1424/2020 clade 2.3.4.4h) and H5N8 subtype, Re-14 strain (A/whooper swan/Shanxi/4-1/2020).

2.3.2. Inactivated Avian Influenza H5N2 Vaccine is oil adjuvant inactivated reassortant avian influenza vaccine prepared from H5N2 subtype, LP strain (A/chicken/Hidalgo/28159-232/1994).

2.4. Vaccination and challenge protocol.
One hundred and twenty DO broiler chickens were allocated into four groups (from G1 to G4) of 30 chickens each. At 10th day of age, the chickens in G1and, G2 vaccinated with inactivated reassortant avian influenza (Re-13 & Re-14 strains) vaccine and Inactivated avian influenza (H5N2) vaccine, respectively. Every vaccine was administered subcutaneously at the base of the neck (0.5 ml per fowl). The chickens in G4 were regarded as a non-vaccinated, non-challenged group (control negative group), while the chickens in G3 were a challenged, non-vaccinated control group (control positive group). At 31st day of age, the challenge test was conducted on 20 chickens from each vaccinated group (G1 and G2) as well as from the control positive group (G3) using the HPAI H5N1 virus clade 2.3.4.4b. Each challenged chicken was inoculated intranasal (IN) with 100 µl of 10^6 EID_{50}/chicken, (equivalent to 100 CLD_{50}). All hens were observed and monitored daily for 10 days post-challenge (dpc) To report clinical symptoms, record mortalities, and identify viral shedding titer. The entire experiment will be carried out inside BSL3 chicken isolators as shown in Table (1).

2.5. Challenge validity
The challenge test will be considered valid when the control non-vaccinated challenged chickens show at least 90% mortality within 4 days post-challenge (OIE diagnostic manual, 2021).

2.6. Sampling
Ten Individual serum samples corresponding to ten blood samples during the immunization phase (at 1st, 10th, 17th, 24th and 31st DO) were collected from G1 and G2 as well as the control negative group (G4). These serum samples were used for the evaluation of AI vaccine potency through hemagglutination inhibition (HI) test. The wanning up of the maternally derived antibodies was examined in serum samples from (G4). Moreover, ten individual oropharyngeal swabs from each challenged group were collected onto dry swabs. These swabs were used for the evaluation of viral load. The sampling was done on the 3rd, 5th, 7th and 10th (dpc). The swabs were eluted by vortexing in 1 ml of PBS + 0.1% of an antibiotics stock solution (Penicillin, 100,000 units; Streptomycin, 100 mg / ml) and kept frozen at -80 ℃ till use.

2.7. Assessment of AI vaccine potency
The potency of AIV vaccines was assessed through monitoring post-vaccination responses against inactivated vaccines. This monitoring was conducted through hemagglutination inhibition test according to OIE diagnostic manual (2018). Each vaccine will be tested against heterologous challenge virus antigen by HI test. The antigen was adjusted to four hemagglutinating units. Data of HI testing will be analyzed based on HI mean (arythmatic) titer and standard deviation. A group of chickens that exhibit Sero-conversion ≥ 4 log2 HI titer will be considered positively seroconverted and protected against HPAI H5N1.

2.8. Challenge virus RNA quantification from oropharyngeal swabs
To evaluate the impact of vaccination on viral respiratory shedding, the challenge virus shedding from hens in the vaccinated and non-vaccinated challenged groups was measured using quantitative real-time reverse transcriptase PCR (qRT-PCR). Real-time RT-PCR was used to identify the extracted RNA using influenza matrix gene-specific primers and probes (Nagy et al., 2010). As others have done, a standard curve generated from 10-fold serial dilutions of the challenge material was used to infer the expected viral shedding concentration in the specimens from the Cq values (Lee & Suarez 2004). The results are shown as log10 copies per PCR reaction. A minimum of 2 log10 (100-fold) less virus should be shown to be shed from the respiratory tract in the challenged vaccinated chicken group as compared to the unvaccinated challenged chicken group (Maas, et al., 2009). It's seen as an essential requirement for vaccine effectiveness.
Mean shedding titer = sum of shedding titer / number of shedders birds. (10 from each group)

Table 1 An overview of the experimental design.

Group No.	Age of vaccination	Serology	No. of vaccinated chickens/group	Challenge	No. of challenged chickens/group
G1	10 D0	+	30	+	20
G2		+	30	+	20
G3	Not vaccinated		-	+	20
G4	Not vaccinated	+	-	-	0

G1: vaccinated with Re-13&Re-14 vaccine, G2: vaccinated with H5N2 vaccine, G3: non-vaccinated challenged group. G4: non-vaccinated non-challenged group.

2.9. Data Management and Analysis
Using the Statistical Package for Social Science (SPSS 27), the gathered data was updated, coded, tabulated, and loaded onto a personal computer. For each parameter, data was given, and appropriate analysis was conducted based on the type of data collected. For parametric numerical data, the descriptive statistics are mean, standard deviation (± SD), and range; for

non-parametric numerical data, they are median and interquartile range (IQR). The ANOVA test, which evaluates the statistical significance of the difference between more than two study group means, is one example of analytical statistics. The Post Hoc Test (used for comparisons of all possible pairs of group means), the Repeated Measure ANOVA test (used to evaluate the statistical significance of the difference between means measured more than twice for the same study group), and the Kruskal-Wallis test (used to evaluate the statistical significance of the difference between more than two study group ordinal variables). The P-value denotes the significance level ($P<0.05$: Significant (S) and $P>0.05$: Non-significant (NS).

3. RESULTS

3.1. Protection against HPAI H5N1 challenge

The protection percentages of the vaccinated and non-vaccinated chickens after the challenge with HPAI H5N1 clade 2.3.4.4b virus were shown in Table (3). Non-vaccinated chickens challenged with the HPAI H5N1 virus had 100% mortality and showed typical clinical signs of Avian Influenza disease such as depression, pink eye, ruffled feathers, haemorrhage in the shanks, cyanosis of the comb and wattle, diarrhea, respiratory distress and nervous manifestations at 2nd and 3rd dpc. On the other hand, G1-vaccinated with Re-13 & Re-14 vaccine- showed 100% protection against the challenge, which is significantly higher than G2, vaccinated with inactivated H5N2 vaccine which showed 55% protection.

3.2. Serological responses

3.2.1. MDA profile

The mean MDA titer for all experimental chickens at one day old ranged from 5 to 5.1 log2 using H5N1/Ag for HI. The mean MDA titer constantly decreased until 17 DO in all vaccinated groups (G1 with Re-13 & Re-14 vaccine, G2 with inactivated H5N2 vaccine), and until 31 DO for G4 (unvaccinated control group) as shown in Table (2).

3.2.2. Antibody response to vaccination

The post-vaccination immune response to inactivated Re-13 & Re-14 and inactivated H5N2 vaccines in G1 and G2, respectively was determined using H5N1/Ag. At 24 and 31 DO, the weekly mean HI titters for G1 were (4.2 ± 0.42 log2 and 6.6 ± 0.52 log2) and for G 2 (2.8 ± 0.42 log2 and 3 ± 0.53 log2) as shown in Table (2). The weekly mean HI titters for G1 were significantly ($P < .05$) higher than G2 and G4 (un-vaccinated control group).

Table 2 Mean HI titers (log2±SD) against heterologous inactivated HPAI H5N1 challenge antigen

Age (day)	1DO	10DO	17DO	24DO	31DO
G1	5± 0.22	3.2 ± 0.42	2.5 ± 0.53	4.2±0.42	6.6±0.52
G2	5.1 ± 0.32	3.2 ± 0.42	2 ± 0.47	2.8±0.42	3±0.53
G4	5.1 ± 0.32	3.3± 0.32	1.7 ± 0.48	0.9±0.32	0.0±0.0

G1 vaccinated with Re-13&Re-14 vaccine, G2 vaccinated with H5N2 vaccine and G4 (non-vaccinated non-challenged group)

3.3. AIV shedding

All chickens in the control positive group (G3) shed challenge virus by oropharyngeal route with titers above 5 log10 EID_{50}/ml after challenge as shown in figure 1. The control positive group (G3) at 3 dpc had the highest viral shedding titer of 5.2 (log10) among all the challenged groups. In addition, the two immunized challenged groups shed significantly less challenge virus, with reductions of 4 and 1.5 (log10), respectively, when compared to the control positive group (G3). Moreover, there was a statistically significant difference between the groups that received vaccinations as the mean viral shedding titer was significantly lower in G1 immunized with Re-13&Re-14 vaccine than in G2 immunized with inactivated H5N2 vaccine at 3, 5, 7 & 10 dpc.

Table 3 Shedding titer variation between the non-vaccinated challenged and vaccinated challenged groups (log10 EID_{50}/ml) and protection percentages.

Group No.	Mean shedding Reduction	Protection %	Mortality Pattern									
			Days post challenge									
			1st	2nd	3rd	4th	5th	6th	7th	8th	9th	10th
G1	4	100%	0	0	0	0	0	0	0	0	0	0
G2	1.5	55%	0	0	0	0	1	3	2	2	1	0
G3	0	0%	0	6	9	5	0	0	0	0	0	0

G1 vaccinated with Re-13&Re-14 vaccine, G2 vaccinated with H5N2 vaccine and G3 (non-vaccinated challenged group).

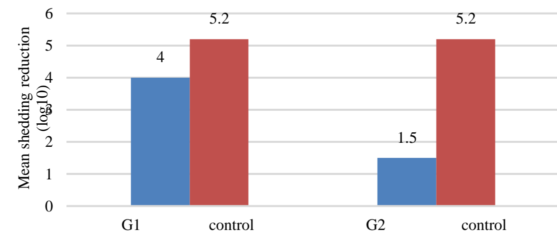

Fig 1 Reduction in the shedding titers of HPAI H5N1 in the vaccinated challenged groups compared with non-vaccinated challenged groups. G1 vaccinated with Re-13&Re-14 vaccine, G2 vaccinated with H5N2 vaccine and G3 (non-vaccinated challenged group)

4. DISCUSSION

In Egypt, managing AI outbreaks primarily depends on vaccination as a standard control method to reduce poultry production losses. Various factors may affect the effectiveness of poultry vaccines. Numerous studies have been conducted to assess the efficacy of commercially available vaccines against newly emerged highly pathogenic avian influenza (HPAI), yielding varying results (Nassif et al., 2020; El-Moeid et al., 2021).

In this study, we compared oil adjuvant inactivated reassortant avian influenza vaccine made from H5N2 subtype, LP strain, and the protective effectiveness of the new H5 bivalent inactivated vaccine, which consists of strains H5-Re13, which contains 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. The effectiveness was evaluated by the reduction in the shedding titers of HPAI H5N1 after the challenge against the currently circulating H5N1 HPAI strain in Egypt. Also, the immune response to vaccination was evaluated by (mean HI titer).

The maternally derived antibodies had a great effect on the level of HI antibodies at the 1st two weeks post-vaccination (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 the maternally derived antibodies may still interfere with vaccination to a lesser extent because they are present up to 3 weeks post-hatch.

Our results revealed that the highest HI antibody titre was determined in G1 (6.6 ± 0.52) at 31 DO while in G2 (3 ± 0.53) as shown in Table (2). In addition, the lowest reduction in the mean of virus shedding titre compared to G3 (non-vaccinated challenged group) was noticed in G2 (1.5 (log10)) with protection 55% while G1 showed the highest reduction in the mean of virus shedding titre 4 (log10) with protection 100% as shown in Table (3) and Figure (1). our results agreed with Ying et al. (2022) who noted that strains of Re-13 and Re-14 vaccine and the currently circulating avian influenza H5 viruses in Egypt had high antigenic and genetic relatedness between each other. In addition, the changes in the hemagglutinin (HA) of avian influenza (AI) viruses, along with antigenic shifts, hinder the effectiveness of standard vaccination approaches against the emerging H5Nx strains, leading to increased mortality rates (Kandeil et al., 2018).

Furthermore, Mo, et al. (2023) found that inactivated vaccines would give better protection when the vaccine antigen had a greater HA1 amino acid relatedness (≥95%) with the challenge virus. Also, The current findings were matched with those of Swayn et al. (2015) and Zhang et al. (2024), who mentioned that as the challenge and vaccine viruses are genetically and antigenically closely related, the range of protection offered by existing vaccines would be increased

The necessity for broadly protective influenza vaccinations is highlighted by the fact that the antigenic drift and shift of influenza viruses necessitate regular updating of vaccine strains. A crucial element in extending the range of protection offered by existing vaccinations is the genetic and antigenic similarity between the viruses in use and the seed strains used in commercial vaccines (Zhang, et al., 2024).

5. CONCLUSIONS

This study was aimed at assessing the efficacy of Re-13 & Re-14 strains. The oil adjuvant commercial inactivated reassortant AI vaccine prepared from (H5N6 subtype, Re-13 strain (A/duck/Fujian/S1424/2020 clade 2.3.4.4h) and H5N8 subtype, Re-14 strain (A/whooper swan/Shanxi/4-1/2020) with Inactivated Avian Influenza H5N2 reassortant avian influenza vaccine prepared from H5N2 subtype, LP strain. These vaccines exhibited varying levels of effectiveness, HI titer, and reduction in virus shedding titer; these variations could be attributed to differences in the percentages of nucleotide sequence identity between the challenge virus and the vaccine seeds. these results showed the importance of continuous evaluation of the validated AI vaccines against recent field strains to deal with a persistent threat to poultry populations in Egypt.

6. REFERENCES

1. Abdelwhab, E. M., Hassan, M. K., Abdel-Moneim, A. S., Naguib, M. M., Mostafa, A., Hussein, I. T. M., Arafa, A., Erfan, A. M., Kilany, W. H., Agour, M. G., El-Kanawati, Z., Hussein, H. A., Selim A. A., Kholousy, S., El-Naggar, H., El-Zoghby, E. F., Samy, A., Iqbal, M., Eid, A., Ibraheem, E. M., Pleschka, S., Veits, J., Nasef S. A., Beer, M. Mettenleiter, T. C., Grund, C., Ali, M. M., Harder, T. C. and Hafez, H. M..2016. Introduction and enzootic of A/H5N1 in Egypt: Virus evolution, pathogenicity and vaccine efficacy ten years on. Infect Genet Evol. 2016 Jun;40: 80-90. doi: 10.1016/j.meegid.2016.02.023.
2. El-Moeid, A. A., EL-Deeb, A. H., Elsaied, M. F., Soliman, R. A, EL-Safty M. M. and Hussein, H. A. .2021. Discrepancies in the efficacy of H5 inactivated avian influenza vaccines in specific-pathogen-free chickens against challenge with the Egyptian H5N8 clade 2.3.4.4 Group B virus isolated in 2018, *Veterinary World*, 14.8. 2131-2141.
3. El-Shesheny, R., Moatasim, Y., Mahmoud, S. H., Song, Y., El Taweel, A., Gomaa, M., Kamel, M. N., Sayes, M. E., Kandeil, A, Lam, T. T. Y., McKenzie, P. P., Webby, R. J., Kayali, G. and Ali, M. A. .2023. Highly Pathogenic Avian Influenza A.H5N1) Virus Clade 2.3.4.4b in Wild Birds and Live Bird Markets, Egypt. Pathogens. Dec 26;12.1.36. doi: 10.3390/pathogens12010036.
4. Hagag, N. M., Erfan A.M., El-Husseiny, M., M., Shalaby, A. G., Saif, M. A., Tawakol, M. M., Nour, A. A., Selim A. A., Arafa, A. S., Hassan, M. K., Hassan W. M., Fahmy H. A., Ibraheem, E., Attia, M., Abdelhakimy, A. M. M., Shahein, M. A. and Naguib, M. M. .2019. Isolation of a Novel Reassortant Highly Pathogenic Avian Influenza .H5N2) Virus in Egypt. Viruses. 2019 Jun 18;11.6.565. doi: 10.3390/v11060565.
5. Hassan, K.E.; King, J.; El-Kady, M.; Afifi, M.; Abozeid, H.H.; Pohlmann, A.; Beer, M. and Harder, T. .2020. Novel Reassortant Highly Pathogenic Avian Influenza A.H5N2) Virus in Broiler Chickens, Egypt. Emerg. Infect. Dis. 2020, 26, 129–133.
6. Kandeil, A., Sabir, J.S., Abdelaal, A., Mattar, E.H., El-Taweel, A.N., Sabir, M.J., Khalil, A.A., Webby, R., Kayali, G. and Ali, M.A. .2018. Efficacy of commercial vaccines against newly emerging avian influenza H5N8 virus in Egypt. Sci. Rep., 8.1. 1-6.
7. Kandeil, A., Kayed, A., Moatasim, Y., Aboulhoda, B. E., El Taweel, A. N., Kutkat, O. and Ali, M. A. .2023. Molecular identification and virological characteristics of highly pathogenic avian influenza A/H5N5 virus in wild birds in Egypt. Microbial Pathogenesis, 174, 105928.
8. Lee, C.W. and Suarez, D. L. .2004. Application of real-time RT-PCR for the quantitation and competitive replication study of H5 and H7 subtype avian influenza virus. J Virol Methods.; 119.2.151-8. doi: https://10.1016/j.jviromet.2004.03.014.
9. Lewis, N. S., Banyard, A. C., Whittard, E., Karibayev, T., Al Kafagi, T., Chvala, I., Byrne, A., Meruyert Akberovna, S., King, J., Harder, T., Grund, C., Essen, S., Reid, S. M., Brouwer, A., Zinyakov, N. G., Tegzhanov, A., Irza, V., Pohlmann, A., Beer, M., Fouchier, R. A. M., Akhmetzhan Akievich, S., Brown, I. H. .2021. Emergence and spread of novel H5N8, H5N5 and H5N1 clade 2.3.4.4 highly pathogenic avian influenza in 2020. Emerg Microbes Infect. Dec;10.1.148-151. doi: 10.1080/22221751.2021.1872355.
10. Maas, R., Tacken, M., van, Zoelen, D. and Oei H .2009. Dose response effects of avian influenza .H7N7) vaccination of chickens: serology, clinical protection and reduction of virus excretion. Vaccine. 2009; 27.27. 3592-7. doi: https://10.1016/j.vaccine.2009.03.066.
11. Mo, J., Spackman, E. and Swayne, D. E. .2023. Prediction of highly pathogenic avian influenza vaccine efficacy in chickens by comparison of in vitro and in vivo data: A meta-analysis and systematic review. Vaccine. 31; 41 .38. 5507-5517.
12. Mosaad, Z., Elhusseiny, M. H., Zanaty, A., Fathy, M. M., Hagag, N. M., Mady, W. H. and Naguib, M. M. .2023. Emergence of Highly Pathogenic Avian Influenza A Virus .H5N1) of Clade 2.3. 4.4 b in Egypt, 2021–2022. Pathogens, 2023, 12.1, 90.
13. Nagy, A., Vostinakova, V., Pirchanova, Z., Cernikova, L., Dirbakova, Z., Mojzis, M., Jirincova, H., Havlickova, M., Dan, A., Ursu, K., Vilcek, S. and Hornickova, J. .2010. Development and evaluation of a one-step real-time RT-PCR assay for universal detection of influenza A viruses from avian and mammal species. Arch Virol. 2010 May;155.5.665-73. doi: 10.1007/s00705-010-0636-x.
14. Nassif, S., Zaki, F., Mourad, A., Fouad, E., Saad, A., Setta, A. and Palya, V. .2020. Herpesvirus of turkey-vectored avian influenza vaccine offers cross-protection against antigenically drifted H5Nx highly pathogenic avian influenza virus strains. Avian Pathology, 49.6, 547-556.
15. OIE .2017. Follow Up Report 3. Highly pathogenic influenza A viruses. terrestrial poultry, Egypt. http://www.oie.int/ wahis_2/public/ wahid.php / Reviewreport/ Review report id:23232.
16. OIE Terrestrial Manual. .2018. Avian Influenza. Infection with Avian Influenza Viruses. OIE Terrestrial Manual, Paris, France. p821-843.
17. OIE Terrestrial Manual. .2021. Avian Influenza .Infection with Avian Influenza Viruses. OIE Terrestrial Manual, Paris, France. p821-843.
18. Selim, A. A., Erfan, A. M., Hagag, N., Zanaty, A., Samir, A. H., Samy, M., Abdelhalim, A., Arafa, A. A., Soliman, M. A., Shaheen, M., Ibraheem, E. M., Mahrous, I., Hassan, M. K. and Naguib, M, M .2017. Highly Pathogenic Avian Influenza Virus .H5N8) Clade 2.3.4.4 Infection in Migratory Birds, Egypt. Emerg Infect Dis. 2017 Jun;23.6.1048-1051. doi: 10.3201/eid2306.162056.
19. Smith, G.J. and R.O. Donis .2015. Nomenclature updates resulting from the evolution of avian influenza A .H5) virus clades 2.1.3.2a, 2.2.1, and 2.3.4 during 2013-2014. Influenza and Other Respiratory Viruses, 9, 271–276.
20. Swayne, D. E., Suarez, D. L., Spackman, E., Jadhao, S., Dauphin, G., Kim-Torchetti, M., McGrane, J., Weaver, J., Daniels, P., Wong, F., Selleck, P., Wiyono, A., Indriani, R., Yupiana, Y., Sawitri Siregar, E., Prajitno, T., Smith, D. and Fouchier, R. .2015. Antibody titer has positive predictive value for vaccine protection against challenge with natural antigenic-drift variants of H5N1 high-pathogenicity avian influenza

viruses from Indonesia. J Virol. 2015 Apr;89.7.3746-62. doi: 10.1128/JVI.00025-15. Epub 2015 Jan 21.

21. Tarek, M.; Naguib, M.M.; Arafa, A.-S.; Tantawy, L.A.; Selim, K.M.; Talaat, S. and Sultan, H.A. .2021. Epidemiology, Genetic Characterization, and Pathogenesis of Avian Influenza H5N8 Viruses Circulating in Northern and Southern Parts of Egypt, 2017–2019. Animals 2021, 11, 2208. https://doi.org/ 10.3390/ani11082208.
22. Verhagen, J. H., Fouchier, R. A. M. and Lewis, N. .2021. Highly pathogenic avian influenza viruses at the wild domestic bird interface in Europe: Future directions for research and surveillance. Viruses 13:212.
23. Vriese, J.D., M. Steensels, V. Palya, Y. Gardin, K. Moore Dorsey, B. Lambrecht, S. Van Borm and Berg. T.V.D. .2010. Passive Protection Afforded by Maternally-Derived Antibodies in Chickens and the Antibodies' Interference with the Protection Elicited by Avian Influenza-Inactivated Vaccines in Progeny. Avian Diseases, 54, 246-252.
24. Yehia, N.; M.M. Naguib; R. Li; N. Hagag; M. El-Husseiny; Z. Mosaad; A. Nour; N. Rabea; W.M. Hasan; M.K. Hassan; T. Harder and Arafa A.A. .2018. Multiple introductions of reassorted highly pathogenic avian influenza viruses .H5N8) clade 2.3.4.4b causing outbreaks in wild birds and poultry in Egypt. Infection, Genetics and Evolution, 58; 56–65.
25. Ying, Z.X., H.X. Wen, M. Fei, M. Qi, W. Yan, B. Hong-mei, L. Yan-jing, D. Guo-hua, S. Jian-zhong, L. Yan-bing, T. Guo-bin and Hua-lan, C. .2022. Protective efficacy of an H5/H7 trivalent inactivated vaccine .H5-Re13, H5-Re14, and H7-Re4 strains) in chickens, ducks, and geese against newly detected H5N1, H5N6, H5N8, and H7N9 viruses. Journal of Integrative Agriculture, 21.7. 2086–2094.
26. Zhang, Y., Cui, P., Shi, J., Zeng, X., Jiang, Y., Chen, Y, Zhang, J., Wang, C., Wang, Y., Tian, G., Chen, H., Kong, H. and Deng, G .2024. A broad-spectrum vaccine candidate against H5 viruses bearing different sub-clade 2.3.4.4 HA genes. NPJ Vaccines. 19;9.1.152. doi: 10.1038/s41541-024-00947-4.