

Appraisal of Three Commercial Inactivated Avian Influenza Vaccines Efficacy Against Challenge with Egyptian Duck-Origin Clade 2.3.4.4b H5N8 in Commercial Broiler Chickens

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ABSTRACT:

The highly pathogenic avian influenza (HPAI) H5N8 virus belonging to clade 2.3.4.4b was detected in Egypt during 2016, which is one of the few countries depends on vaccination as a main control strategy. This study was conducted to assess the efficacy of three different commercial inactivated AI vaccines of different clades (clade 1, clade 2.3.4.4b and (Re-6 & Re-8)) in commercial broiler chickens against HPAI H5N8 clade 2.3.4.4b. The clinical protection percent was 67%, 80% and 87% in G1 (clade 1), G2 (clade 2.3.4.4b), and G3 (Re-6 & Re-8), respectively. The lowest mean antibody titer was recorded in G1 which exhibited unsatisfactory level of immune response. Also, Antisera raised against clade 1 vaccine showed minimal reactivity with clade 2.3.4.4b and Re-6&Re-8 antigens suggesting poor cross-protection. This was reflected on the antigenic relatedness (R-value) which was low (11.3%) indicating major antigenic difference. Samples were collected for examination of histopathological changes from thymus, trachea, spleen, bursa of Fabricius, lung, and cerebrum. Only mild cerebrum lesions were found in all challenged groups indicating reduced neurotropism. In lymphoid tissues, moderate to severe lesion score were detected. This impairment effect on some bird immune organs highlight the immunosuppressive effect of the virus, which can appear in the vaccinated exposed birds to HPAI virus. It is recommended to update the vaccine seed strains to be closely related to the circulating field strains to obtain better protection levels. Also, continuous evaluation of the validated AI vaccines against recent field strains is substantial.

Key words: Clade 2.3.4.4b, Histopathology, H5N8, HPAIV and Vaccine efficacy.

INTRODUCTION:

Highly pathogenic avian influenza (HPAI) viruses of the H5 subtype be regarded as a serious concern for both poultry and human health. Since the emergence of HPAI H5N1 (A/goose/Guangdong/1/1996) virus in China, descendants of this strain remain to

disseminate between avian species. Also, their HA has evolved into ten different phylogenetic clades (Smith and Donis, 2015). Viruses belonging to clade 2.3.4.4 are currently of special interest regarding their global spread. Clade 2.3.4.4 was divided into 8 subclades (2.3.4.4a to

2.3.4.4h) according to the current nomenclature (Smith and Donis, 2015) and consists of 7 different subtypes H5N1, H5N2, H5N3, H5N4, H5N5, H5N6, and H5N8 (Verhagen *et al.*, 2021).

In 2010, HPAI H5N8 virus of clade 2.3.4.4 was first detected in domestic ducks in eastern China. During 2014, H5N8 virus had caused multiple outbreaks in South Korea, Japan, China, North America, and Europe (Lee *et al.*, 2014). The worldwide spread of HPAI H5N8 virus strains has been linked to the overlapping flyways of migratory birds (Lee *et al.*, 2015). In Egypt HPAI H5N1 clade 2.2 virus has been endemic in poultry populations since 2006 (Abdelwhab *et al.*, 2016). Also, H9N2 AIV was isolated from the Egyptian poultry populations as early as December 2010 (Monne *et al.*, 2013). In December 2016, HPAI H5N8 clade 2.3.4.4b virus was first declared in Egypt via migratory birds (common coot, *Fulica atra*) in Damietta governorate (Selim *et al.*, 2017). Since then, several cases of H5N8 have been recorded among domestic poultry in live bird markets, backyard birds, and commercial farms in many governorates in Egypt (OIE, 2017). Although all Egyptian H5N8 isolates belong to the same clade (2.3.4.4b), several independent introductions of the virus have been detected since 2017 suggesting the widespread and continuous circulating in both commercial and backyard sectors in Egypt (Yehia *et al.*, 2018 & Tarek *et al.*, 2021). During 2019, novel HPAI H5N2 viruses were found in commercial chicken and duck farms in Egypt, as a result of genetic reassortment between HPAI H5N8 and LPAI H9N2 subtypes circulating in Egypt (Hageg *et al.*, 2019 & Hassan *et al.*, 2020). Moreover, new strains of HPAI H5N8 have been detected yearly and have caused economic losses in the poultry sector (Kandeil *et al.*, 2018). The Egyptian HPAI H5N8 viruses isolated in Egypt in 2019

were found to be phylogenetically related to HPAI H5N8 viruses reported in the second half of 2020 in Europe (Beerens *et al.*, 2020, Tarek *et al.*, 2021 & Lewis *et al.*, 2021). During 2021, the HPAI H5N8 virus was reported for the first time in humans who have a history of contact with infected poultry in Russia (WHO, 2021). Moreover, HPAI (H5N1) and (H5N5) belonging to clade 2.3.4.4b has been detected recently in Egypt at migratory birds (Mosaad *et al.*, 2023 & Kandeil *et al.*, 2023) which may lead to further complication of AI disease situation in Egypt.

The controlling of AI infections in Egypt depends mainly on vaccination as a routine control strategy to minimize losses in poultry production by limiting the spread of infection. Many factors could influence the efficacy of poultry vaccine. One of the critical factors is the genetic and antigenic matching between the circulating viruses and commercial vaccine seed strains (Wong and Webby, 2013). The variation between the HA of the AI viruses concurrently with antigenic variation leads to failure of the routine vaccination strategy against the newly emerging H5N8 strains and consequently mortalities (Kandeil *et al.*, 2018). Many studies have been designed to evaluate commercially available vaccines against newly emerged HPAI, and the obtained results were variable (Kandeil *et al.*, 2018, Hamouda *et al.*, 2019, Ali *et al.*, 2019, Nassif *et al.*, 2020 & El-Moeid *et al.*, 2021).

Aim of work: An experiment was designed to assess the efficacy of three different commercial inactivated AI vaccines of different clades (clade 1, clade 2.3.4.4b and (Re-6 & Re-8)) in commercial broiler chickens against HPAI H5N8 clade 2.3.4.4b. Assessment included recording of mortality rates, serum antibody titers, and the histopathological changes in six organs

including spleen, bursa of Fabricius, thymus, cerebrum, trachea, and lung.

MATERIALS AND METHODS:

Ethical consent:

All experimental procedures were conducted according to the guidelines of laboratory animal use and legally approved by the Committee of Ethics of Animal Experiments at the Animal Health Research Institute, Egypt. Experimental infection was performed in isolators at animal biosafety level 3 (BSL-3) and every effort was performed to minimize the birds' suffering.

Experimental chicks:

One-day-old commercial broiler chicks (n=150) were purchased from a commercial hatchery that had maternal antibodies against H5 AI acquired from their parents. Chicks were kept in isolator with food and water supplied adlibitum.

Commercial vaccines:

Three different commercially available inactivated avian influenza vaccines of different clades (Table-1) were used in current study to assess their efficacy in vaccinated challenged chickens. Chickens were vaccinated with 0.5 ml of the vaccine on day-10 via the subcutaneous route as per the manufacturers' recommendations.

Table (1): List of commercially available inactivated vaccines used in the experiment and their vaccinal seed strains:

Group	vaccine	clade	Vaccinal seed strain	Manufacturing country
G1	H5N3	1	A/chicken/Vietnam/C58/2004	USA
G2	H5N8 local	2.3.4.4b	Rg A/Chicken/ME-2018/H5N8	Egypt
G3	(Re-6 & Re-8) H5N1 imported	2.3.4.4g & 2.3.2.1c	Re-6(A/duck/Guangdong/s132 2110 Clade 2.3.2.1c) Re-8(A/Chicken/Guizhou/4/2013 clade 2.3.4.4g)	China
G4	Non-vaccinated Challenged (control positive)			
G5	Non-vaccinated Non-Challenged (control negative)			

Challenge Virus:

Previously identified and characterized virus by the Reference Lab. For Veterinary Quality Control on Poultry Production, Animal Health Research Institute, Dokki, Giza Egypt HPAI (H5N8) virus clade 2.3.4.4b (A/duck/Egypt/SMG-4/2019(H5N8)); GenBank accession No. MN658766 was used in the current study. The virus was previously isolated from non-vaccinated Muscovy duck farm in January 2019 in Port Said governorate with mortality

rate amounting to 23%. The virus revealed an intravenous pathogenicity index (IVPI) of 2.41 (Tarek *et al.*, 2021). Challenge procedures were conducted on day 31 via the intranasal route using 10⁶ EID₅₀ /0.1 ml of the challenge virus (El-Moeid *et al.*, 2021).

Experimental design:

A total of one hundred and fifty 1-day-old chicks were divided into five groups and were placed in negative-pressure-based BSL-3 isolators. Chickens in group 1 (G1 no=30) received 0.5 ml/bird of

monovalent H5N3 clade 1 vaccine subcutaneously on day 10. Chickens in group 2 (G2 no=30) and group 3 (G3 no=30) received 0.5 ml/bird of clade 2.3.4.4b local and (Re-6 & Re-8) imported vaccines, respectively, on day 10. Groups 4 and 5 were kept non-vaccinated as control. Groups (G1, G2, G3 and G4) were challenged on day 31 via intranasal route with H5N8 clade 2.3.4.4b (A/duck/Egypt/SMG-4/2019). While, G5 was kept non-vaccinated non-challenged as negative control (Figure-1). Clinical signs, gross pathological lesions and mortalities were monitored for 12 dpc in different groups.

Serology and antibody response:

The HI test was performed by using 3 different vaccinal antigens, supplied by local agencies (Table-1) to determine antibody response of the vaccines in serum samples collected from wing vein on days 1, 10, 17, 23, 31, 37 and 43. The HI titer was considered positive if there was inhibition of serum dilution 1/8 (2^3 or $3 \log_2$ when expressed as the reciprocal) or more against 8 HAU of antigens. The mean antibody titers were expressed on a \log_2 scale. The HI assay was carried out according to the OIE protocol (OIE, 2018).

Determination of antigenic relatedness:

The antigenic relatedness among vaccinal seed strains of different clades was expressed as an R-value based on the Archetti and Horsfall formula; $r = \frac{\sqrt{r_1 \times r_2}}{2}$ (Archetti and Horsfall 1950), using the cross HI results in serum samples collected after challenge at day 43. Where the ratio r_1 obtained by dividing the heterologous titer obtained with the virus 2 by the homologous titer obtained with the virus 1, and the ratio r_2 obtained by dividing the heterologous titer obtained with the virus 1 by the homologous titer with the virus 2. The R values ranged from 0 for isolates that are antigenically unrelated to

100% for isolates that are identical. The resulting R values were expressed as percentage relatedness and the interpretation of the results was done according to Brooks (1967) as follows: R value between 0:10% = serotype difference, R value between 11:32% = major subtype differences, R value between 33:70% = minor subtype differences and R value greater than 70% = a little or no differences.

Histopathological examination:

Samples were collected from 6 organs: thymus, trachea, spleen, bursa of Fabricius, lung, and cerebrum from dead birds from all challenged groups. Examined tissues were preserved in 10% buffered formalin. Dehydration of these samples was carried out by using ascending grades of alcohol. For cleaning, tissues were placed in Xylo. Impregnation was conducted by transferring the specimens in three changes of methyl paraffin wax. Finally, samples were block in hard paraffin cut into sections of 5-micron thickness and prepared for staining by H&E and examined by light microscope (Bancroft and Layton, 2019). Five random optical fields were examined and scored and then the mean lesion score of the five fields was calculated. The severity of histopathological changes was scored from 0 = apparently normal, 1 = mild lesions, 2 = moderate lesions and 3 = severe lesions.

The formula of the mean severity index (MSI) is given by the function: sum of mean lesion scores of six examined organs of five chickens per group divided by total number of examined organs as previously described (Sultan *et al.*, 2019 & Gibson *et al.*, 2013).

Statistical analysis:

Whenever necessary, the data were analyzed using the t-test or by ANOVA followed by application of Duncan's new multiple range test for determination of the significance of differences between

individual treatments and corresponding controls (Steel and Torrie., 1960).

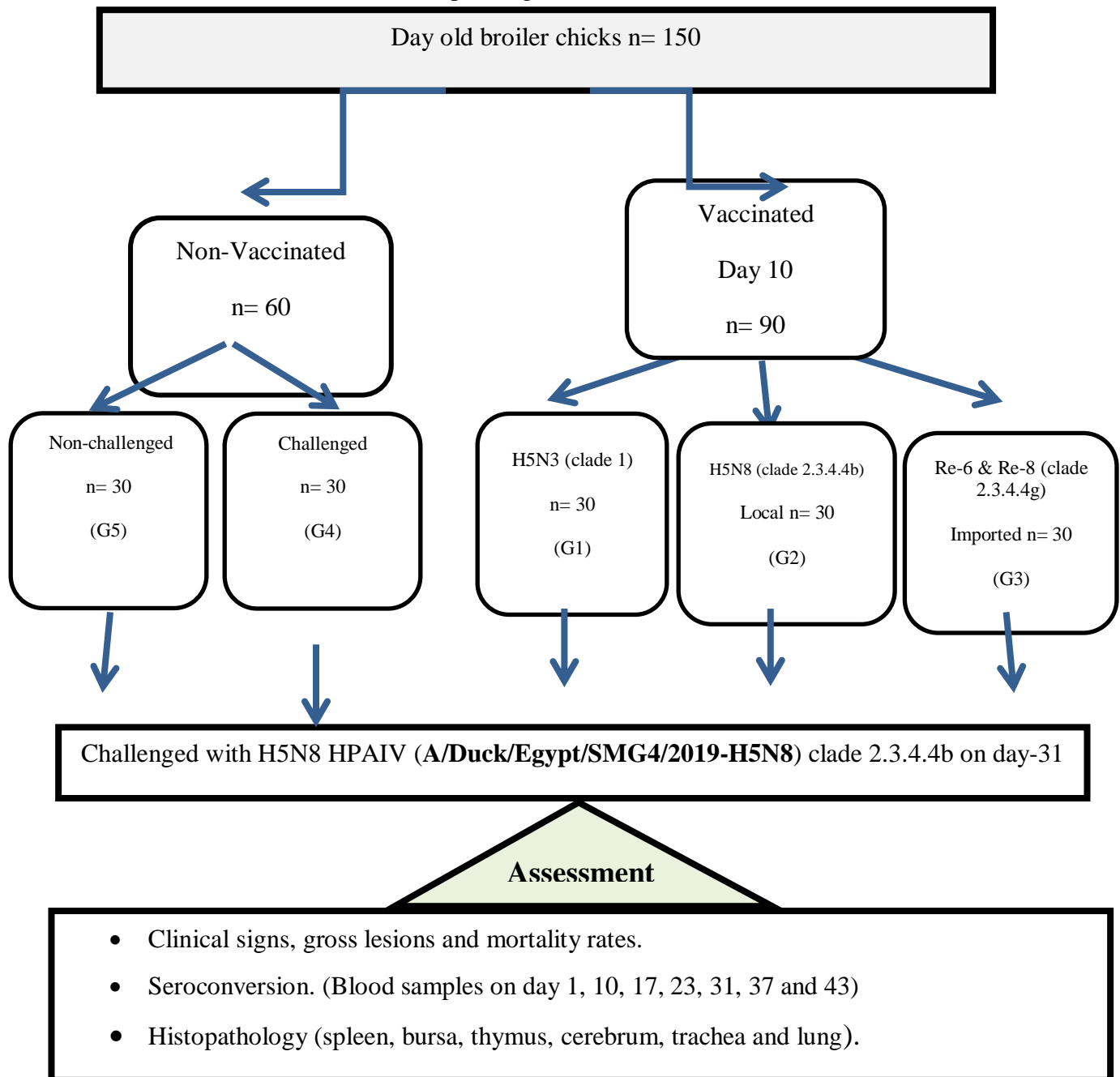


Figure-1: Experimental design for evaluation of the efficacy of three commercial inactivated vaccines of different clades against HPAIV H5N8 clade 2.3.4.4b in commercial broiler chickens.

RESULTS

Clinical signs, postmortem gross lesions, and mortalities:

Depression was found in all challenged groups with ruffled feathers and decrease in food intake from 1st dpc. Signs were more severe with Abnormal respiratory sounds, facial swelling, cyanosis, and whitish diarrhea with 30/30 (100 %) mortality in G4 (non-vaccinated challenged). From all vaccinated challenged groups, G1 was the most clinically affected group by the challenge with 10/30 (33%) mortality. Other groups G2 and G3 showed 6/30 (20%) and 4/30 (13%) mortality, respectively, as shown in Table-2.

The protection percent following challenge with HPAI H5N8 clade 2.3.4.4b was 67%, 80% and 87% in G1, G2 & G3, respectively, as shown at (Figure-2 and table-2)

The postmortem gross lesions findings ranged from severe petechial hemorrhages, congestion in trachea, lung, and visceral organs, multifocal petechiae and necrotic areas in pancreas, multifocal petechiae in proventriculus and in the proventriculus-gizzard junction, Congestion in cerebrum, hemorrhages of variable intensity on legs, multifocal petechial hemorrhages in bursa of Fabricius (Figure-3). No clinical signs or macroscopic lesions were observed in negative control group G5.

Table-2: Mortality in different groups challenged on day-31 with HPAI H5N8 virus clade 2.3.4.4b:

Group	Vaccination on day 10	Mortality/dpc												Total mortality (%)	Mortality range /day	Protection %
		1	2	3	4	5	6	7	8	9	10	11	12			
G1	H5N3 ^a	-	1	-	1	3	1	-	2	2	-	-	-	10/30 (33%)	2-9	67%
G2	H5N8 local ^b	-	1	-	-	1	-	-	2	1	-	1	-	6/30 (20%)	2-11	80%
G3	Re-6 & Re-8 ^c	-	-	-	-	-	1	-	1		1		1	4/30 (13%)	6-12	87%
G4	Non-vaccinated challenged	-	11	13	-	3	1	1	1	-	-	-	-	30/30 (100%)	2-8	0%
G5	Non-vaccinated non-challenged	-	-	-	-	-	-	-	-	-	-	-	-	0/30 (0%)	0	100%

a: Inactivated Avian Influenza vaccine H5N3 subtype clade1 (A/chicken/Vietnam/C58/2004).

b: Inactivated local Avian Influenza vaccine H5N8 clade 2.3.4.4b (Rg A/Chicken/ME-2018/H5N8).

c: Inactivated imported Avian Influenza vaccine Re-6 & Re-8

Re-6(A/duck/Guangdong/s1322110 Clade 2.3.2.1c)

Re-8(A/Chicken/Guizhou/4/2013 clade 2.3.4.4g)

dpc: days post challenge

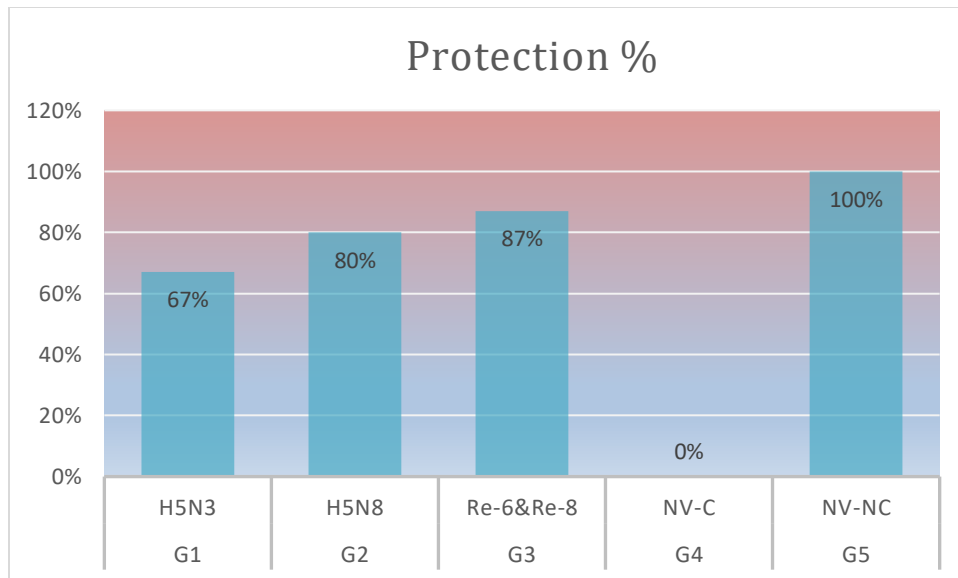


Figure 2: Protection percent in different groups following challenge.

G1: Vaccinated with Inactivated Avian Influenza vaccine H5N3 subtype clade1 (A/chicken/Vietnam/C58/2004).

G2: Vaccinated with Inactivated local Avian Influenza vaccine H5N8 clade 2.3.4.4b (Rg A/Chicken/ME-2018/H5N8).

G3: Vaccinated with Inactivated imported Avian Influenza vaccine Re-6 & Re-8
Re-6(A/duck/Guangdong/s1322110 Clade 2.3.2.1c), Re-8(A/Chicken/Guizhou/4/2013 clade 2.3.4.4g).

G4: non-vaccinated challenged (control positive).

G5: Non-vaccinated, non-challenged (control negative).

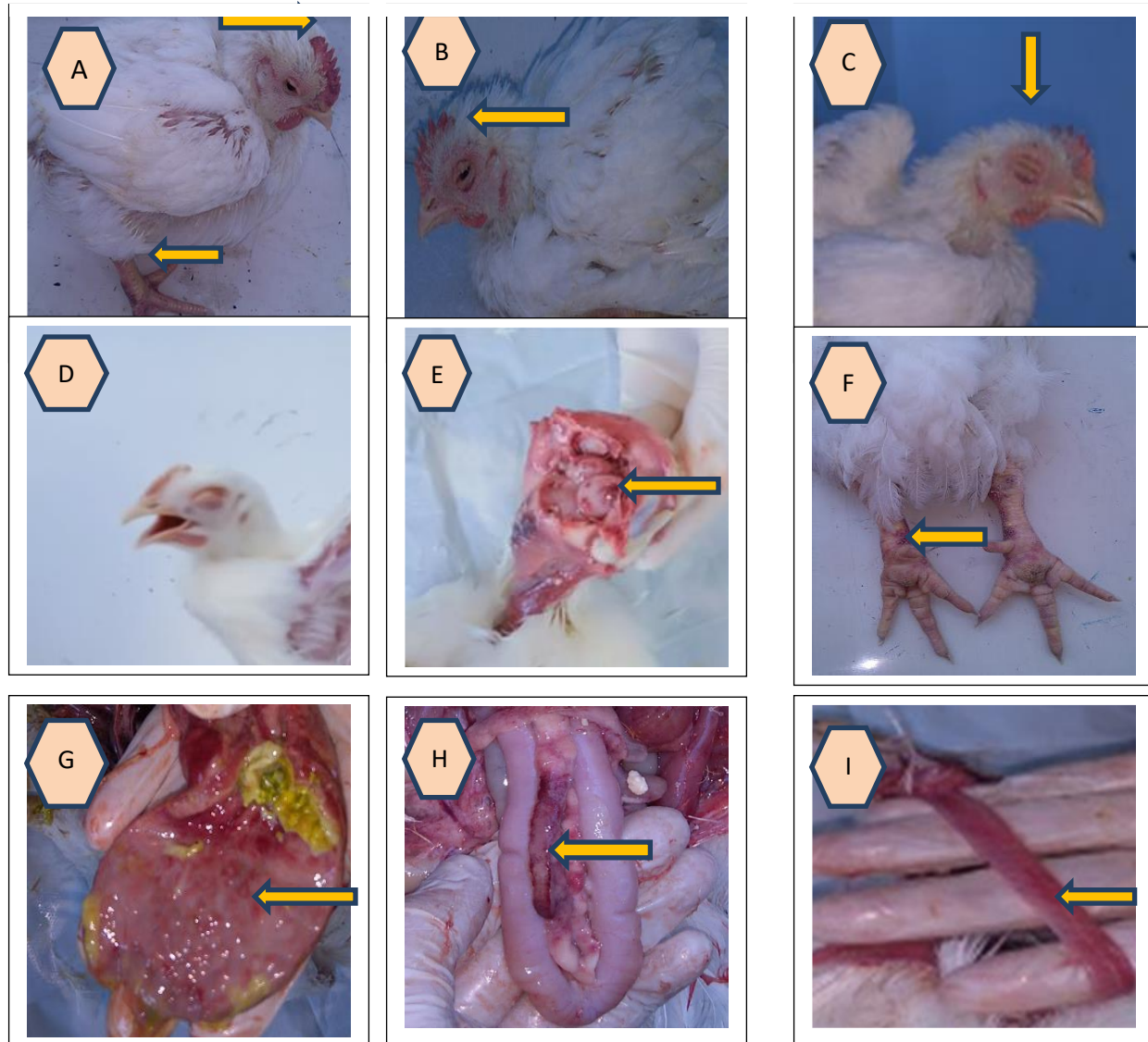


Figure-3: Signs and post mortum gross lesions following challenge with HPAI H5N8 virus clade 2.3.4.4b:

A: bird showing signs of facial swelling, cyanosis of comb and hemorrhage on leg, B and C: birds showing inflammation and congestion of eyes, D: respiratory distress; E: Brain of dead chicken showed congestion and hemorrhagic areas, F: hemorrhages on the legs, G: hemorrhages on proventriculus, H: multifocal petechiae and necrotic areas in pancreas, I: sever congestion in trachea.

Serum antibody response to vaccination:

Serological responses after vaccination using different antigens are shown at Fig. 4. The mean HI titer of maternal derived antibodies (MDA) was not detectable in non-vaccinated group on day 23. The mean HI titers using the homologous antigen of AI H5N3 were 1, 1.4, 2 and 2.6 \log_2 on day 23, 31, 37 and 43, respectively. Positive antibody response 3.4 and 2.8 \log_2 on day 17 and 23, respectively, using the homologous antigen of AI H5N8 2.3.4.4b was detected. The mean HI titer \log_2 increased to 4.8, 6.2 and 6 \log_2 on day 30, 37 and 43, respectively. The mean HI titers using the homologous antigen of AI Re-6 & Re-8 were 0.6, 4, 5.2, 6 and 5.6 \log_2 on day 17, 23, 30, 37 and 43, respectively.

Antigenic relatedness:

Antigenic relatedness (R-values) % between different clades of the vaccinal seed strains was calculated based on the Archetti and Horsfall formula (1950) using the cross HI results shown in (Table-3, Figure-5) and the interpretation of the results was done according to Brooksby, 1967. The R-values results are shown in (Table-4).

The antigenic relatedness between H5N3 clade 1 and H5N8 clade 2.3.4.4b was low with R value 11.3% indicating major antigenic difference between the two subtypes. A minor subtype difference in antigenicity was detected between Re-6 & Re-8 with H5N3 clade 1 and H5N8 clade 2.3.4.4b with R value 49.7 & 59.2, respectively.

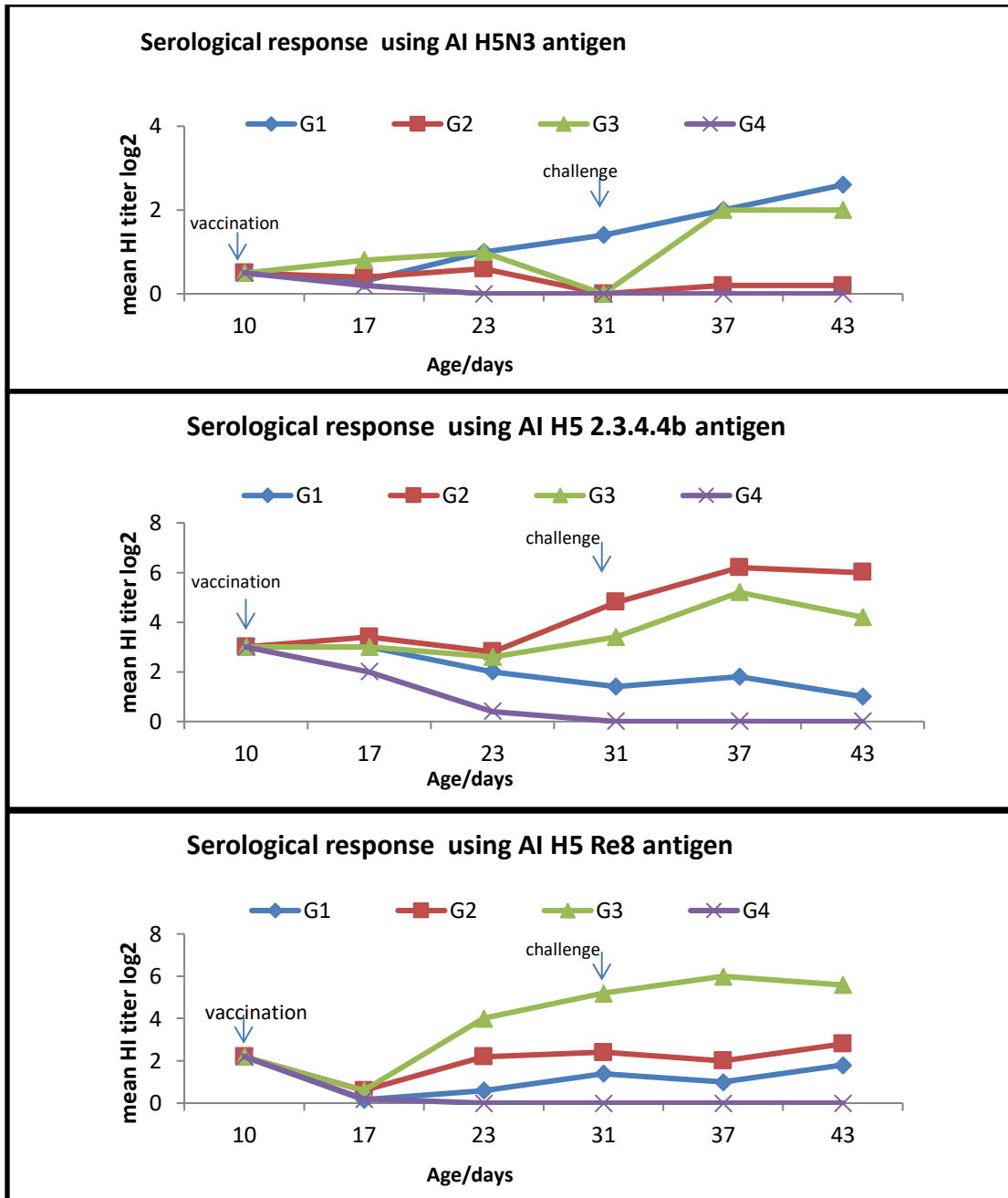


Figure-4: Serological response of broiler chickens after vaccination with inactivated oil emulsion avian influenza vaccine H5N3 clade 1 (G1), H5N8 clade 2.3.4.4b local (G2) and Re-6 & Re-8 (G3) H5N1 imported and challenged with A/Duck/Egypt/SMG4/2019-H5N8 clade 2.3.4.4b using AI H5N3, AI H5 2.3.4.4b, and AI H5 Re-6 & Re-8 antigens.

Table-3: Cross haemagglutination inhibition (HI) test between three commercial inactivated Avian Influenza vaccines of different clades:

Antigen	Antisera HI titer means (Log ₂)		
	clade 1 ^d	clade 2.3.4.4b ^e	Re-6 & Re-8 ^f
clade 1 ^a	2.6	0.2	2.0
clade 2.3.4.4b ^b	1.0	6.0	4.2
Re-6 & Re-8 ^c	1.8	2.8	5.6

a: A/chicken/Vietnam/C58/04/H5N3 (Inactivated Avian Influenza vaccine H5N3 subtype)

b: Rg A/Chicken/ME-2018/H5N8 (Inactivated local Avian Influenza vaccine H5N8)

c: Re-6(A/duck/Guangdong/s1322110 Clade 2.3.2.1c), Re-8(A/Chicken/Guizhou/4/2013 clade 2.3.4.4g)

d: antiserum against A/chicken/Vietnam/C58/04/H5N3

e: antiserum against Rg A/Chicken/ME-2018/H5N8

f: antiserum against Re-6 & Re-8 strain.

*The homologous titers are shown in bold.

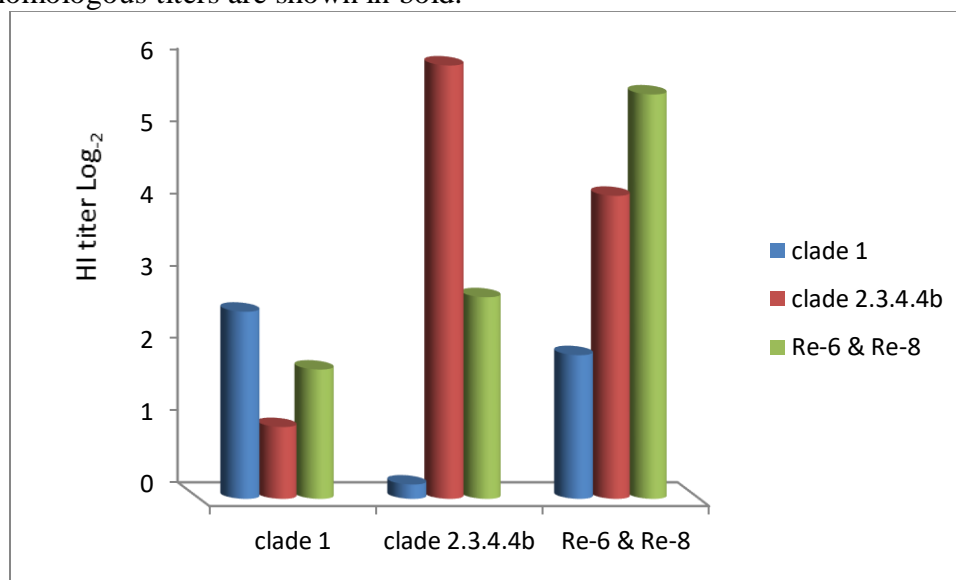


Figure-5: Cross haemagglutination inhibition (HI) test between three commercial inactivated Avian Influenza vaccines of different clades after challenge with HPAIV H5N8 clade 2.3.4.4b: **clade 1:** A/chicken/Vietnam/C58/04/H5N3 (H5N3 subtype), clade 2.3.4.4b : Rg A/Chicken/ME-2018/H5N8 (H5N8 subtype), Re-6 & Re-8 : Re-6(A/duck/Guangdong/s1322110 Clade 2.3.2.1c), Re-8(A/Chicken/Guizhou/4/2013 clade 2.3.4.4g)

Table-4: Antigenic relatedness (R-values %) of three commercial inactivated Avian Influenza vaccines of different clades and their interpretation:

Item	clade 2.3.4.4b		Re-6 & Re-8	
	R-value %	Intpr.	R-value %	Intpr.
clade 1 ^a	11.3	MjSD	49.7	MSD
clade 2.3.4.4b ^b	-	-	59.2	MSD
Re-6 & Re-8 ^c	-	-	-	-

a: Inactivated Avian Influenza vaccine H5N3 (A/chicken/Vietnam/C58/2004).

b: Inactivated local Avian Influenza vaccine H5N8 (Rg A/Chicken/ME-2018/H5N8).

Re-6 & Re-8: Inactivated imported Avian Influenza vaccine Re-6(A/duck/Guangdong/s1322110 Clade 2.3.2.1c) Re-8(A/Chicken/Guizhou/4/2013 clade 2.3.4.4g)

R-value: Relatedness value.

Intpr.: Interpretation

MSD: Minor subtype difference.

MjSD: Major subtype difference

Histopathologic examination:

Samples were collected from thymus, trachea, spleen, bursa, lung, and cerebrum from dead or severely-affected chickens. Mild to moderate lesions were observed in G1, G2, and G3 with MSI 1.5, 1.7, and 2.0, respectively. While moderate lesions of thymus & trachea and severe lesions of spleen, bursa & lung were observed with MSI 2.3 in G4. Only mild cerebrum lesions were found in all challenged groups. All examined organs were apparently normal in negative control birds as shown in (Table-5).

The predominant microscopic lesions in the tissues were congested blood vessels, areas of necrosis and hemorrhages with mixed inflammatory infiltrate. In lymphoid tissues, including spleen, thymus and bursa of Fabricius, congested blood vessels and multifocal areas of necrosis of variable intensity

with depletion of lymphocytes were present. The bursa also showed hyperplasia in lining epithelium, epithelization and interfollicular connective tissue (Fig. 6, 7 & 8).

The trachea showed hyperplasia of lining epithelium, with edema, mononuclear cells infiltration and congestion in mucosa, submucosa and muscular layer as shown at (Fig. 9). In the cerebrum, congested blood vessels with perivascular edema and perivascular cuff were observed. Also, mononuclear cells infiltration and focal gliosis were observed as shown at (Fig. 10).

The lung showed severe congested blood vessels with perivascular edema and thickening of the wall of blood vessels associated with proteinaceous fluid and inflammatory cells infiltration in the lumen of parabronchi as shown at (Fig. 11).

Table-5: Histopathologic lesion Scores in vaccinated and non-vaccinated groups challenged with HPAI H5N8 virus clade 2.3.4.4b:

G/organ	Mean Lesion score						MSI± SD
	Thymus	Trachea	Spleen	Bursa	Lung	Cerebrum	
G 1	2	1	2	1	2	1	1.5±0.54^a
G 2	1	2	2	2	2	1	1.7±0.51 ^a
G 3	3	2	2	2	2	1	2.0±0.63 ^b
G 4	2	2	3	3	3	1	2.3±0.81 ^b
G 5	0	0	0	0	0	0	0 ^c

Different lowercase letters indicate the presence of significant differences ($P \leq 0.05$) between the mean scores \pm SD within the same time interval.

0: apparently normal, **1:** mild lesion, **2:** moderate lesion, **3:** severe lesion, **MSI:** Mean Severity Index.

G1: vaccinated with Inactivated Avian Influenza vaccine H5N3 subtype clade1

G2: vaccinated with inactivated local Avian Influenza vaccine H5N8 clade 2.3.4.4b

G3: vaccinated with inactivated imported Avian Influenza vaccine, Re-6 + Re-8 strain

G4: Non-vaccinated challenged (control positive)

G5: Non-vaccinated non-challenged (control negative)

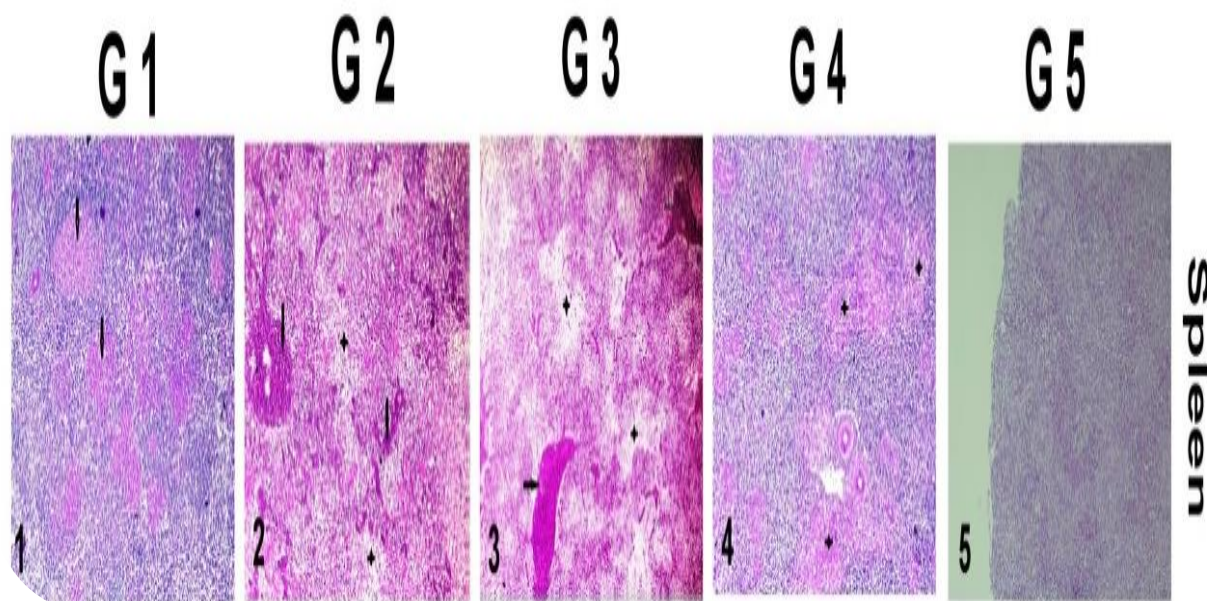


Figure-6: Histopathological lesions in spleen of different groups after challenge with HPAI H5N8 virus clade 2.3.4.4b:

1: Spleen of G1 at 2 dPC showing multifocal necrosis (arrow) H&E X100, **2:** Spleen of G2 at 2 dPC showing congested blood vessels (arrow), depletion and necrosis of lymphocytes (star) H&E X100, **3:** Spleen of G3 at 8 dPC showing severe congested blood vessels (arrow) and depletion of lymphocytes (star) H&E X50, **4:** Spleen of G4 at 3 dPC showing severe multifocal degeneration and necrosis of splenocytes (star) H&E X100 and **5:** Spleen of G5 at 21 dPC showing apparently normal structures (H&E X100).

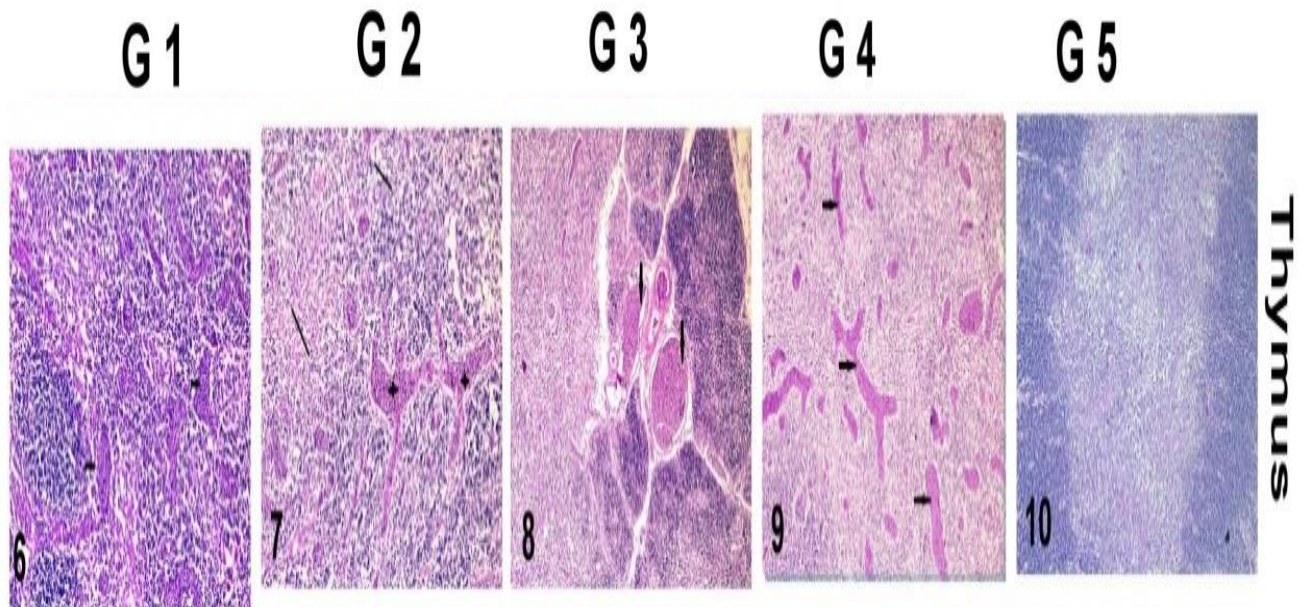


Figure-7: Histopathological lesions in thymus of different groups after challenge with HPAI H5N8 virus clade 2.3.4.4b:

6: Thymus of G1 at 2 dPC showing congested blood vessels (arrow) H&E X200, **7:** Thymus of G2 at 5 dPC showing congested blood vessels (star) and depletion of lymphocytes (line) H&E X200, **8:** Thymus of G3 at 6 dPC showing congested blood vessels (arrow) H&E X100, **9:** Thymus of G4 at 3 dPC showing severe congested blood vessels (arrow) H&E X100 and **10:** Thymus of G5 at 21 dPC showing apparently normal structures (H&E X100).

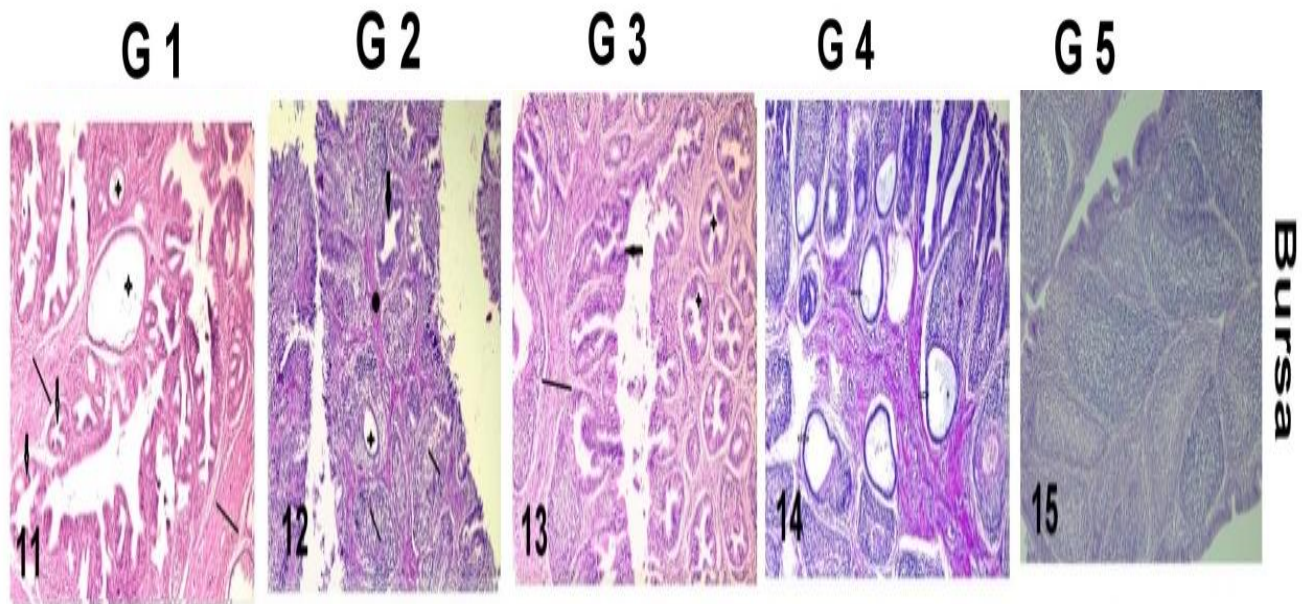


Figure-8: Histopathological lesions in bursa of different groups after challenge with HPAI H5N8 virus clade 2.3.4.4b:

11: Bursa of G1 at 9 dPC showing severe epithelization (arrow) and cysts formation (star) with interfollicular connective tissue formation (line) H&E X100, **12:** Bursa of G2 at 5 dPC showing depletion of lymphocytes (line), epithelization (arrow) ,cyst (star) and interfollicular connective tissue proliferation (circle) H&E X100, **13:** Bursa of G3 at 6 dPC showing hyperplasia of lining epithelium (arrow), epithelization (star) and interfollicular connective tissue (line) H&E X200, **14:** Bursa of G4 at 3 dPC showing several cysts formation (star) H&E X100 and **15:** Bursa of G5 at 21 dPC showing apparently normal structures (H&E X100).

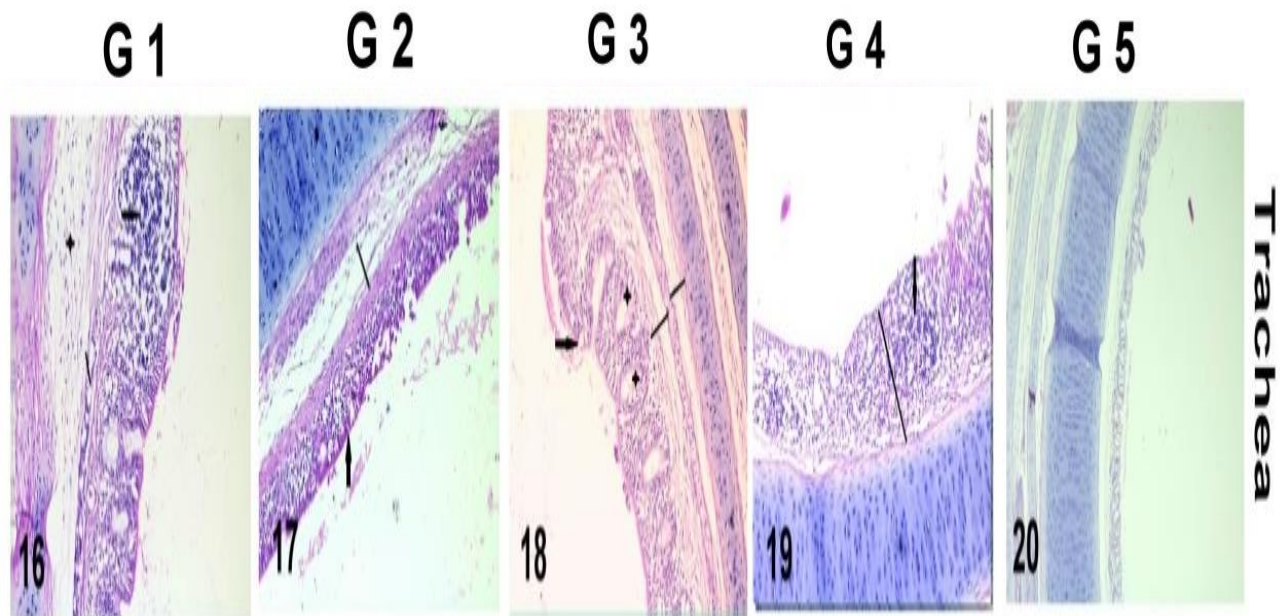


Figure-9: Histopathological lesions in trachea of different groups after challenge with HPAI H5N8 virus clade 2.3.4.4b:

16:Trachea of G1 at 2 dPC showing edema (star), congested blood vessels (line) and mononuclear cells infiltration in lamina propria (arrow) H&E X200, **17:**Trachea of G2 at 5 dPC showing mild thickening of mucosa (arrow) due to edema and mononuclear cells infiltration (line) H&E X200, **18:**Trachea of G3 at 8 dPC showing hyperplasia of lining epithelium (arrow), activation of mucous glands (star) and edema in lamina propria (line) H&E X200, **19:** Trachea of G4 at 5 dPC showing mild thickening in mucosa with edema (line) and mononuclear cells infiltration in lamina propria (arrow) H&E X200 and **20:** Trachea of G5 at 21 dPC showing apparently normal structures (H&E X100).

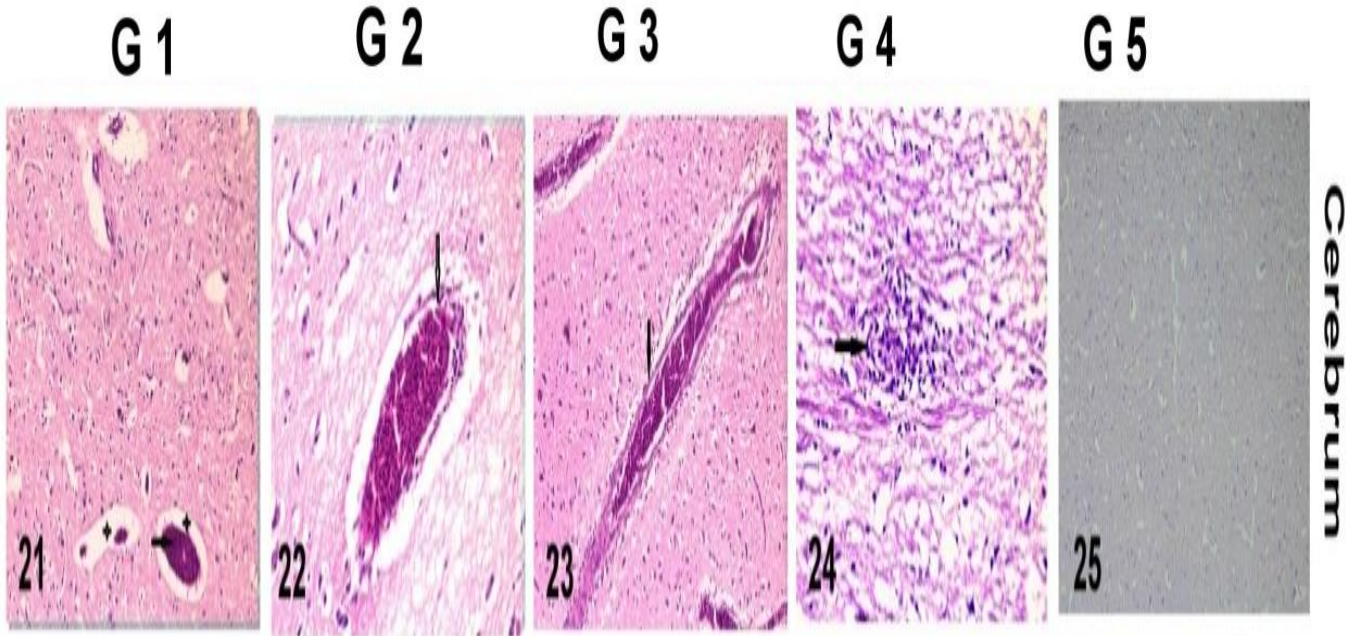


Figure-10: Histopathological lesions in cerebrum of different groups after challenge with HPAI H5N8 virus clade 2.3.4.4b:

21: Cerebrum of G1 at 2 dPC showing congested blood vessels (arrow) with perivascular edema (star) H&EX200, **22:** Cerebrum of G2 at 5 dPC showing perivascular cuff (arrow), few lymphocytes bordered the blood vessel H&E X200, **23:** Cerebrum of G3 at 6 dPC showing mononuclear cells infiltration in the Virchow-Robin space (arrow) H&E X200, **24:** Cerebrum of G4 at 2 dPC showing focal gliosis (arrow) H&E X400 and **25:** Cerebrum of G5 at 21 dPC showing apparently normal structures (H&E X100).

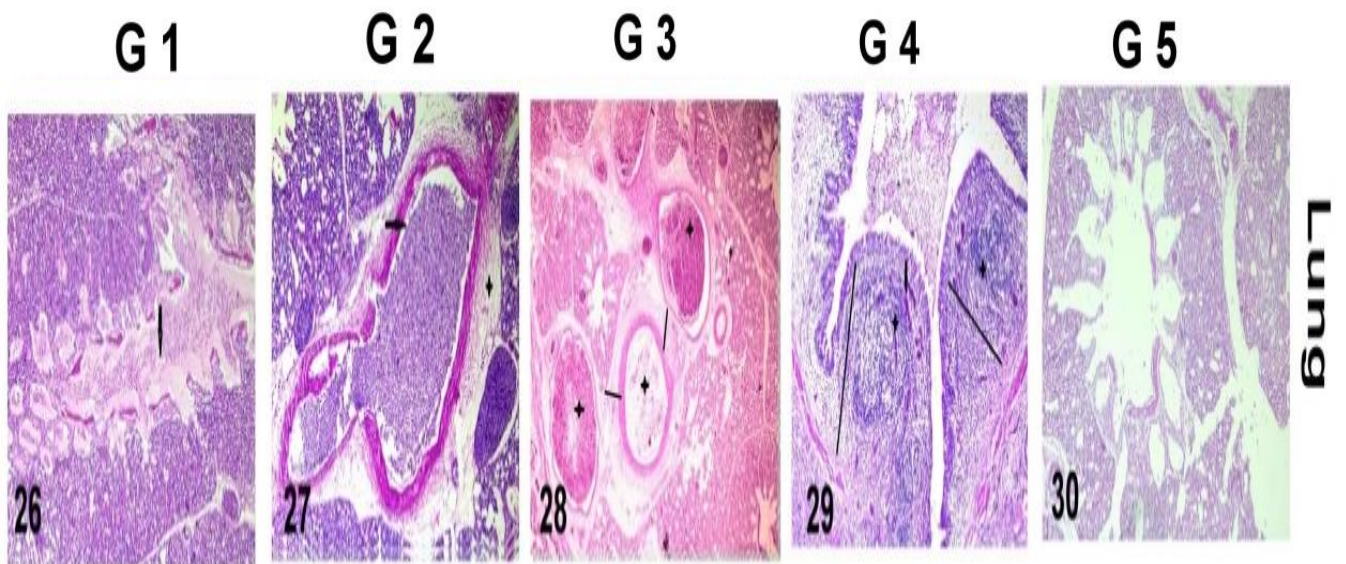


Figure-11: Histopathological lesions in lung of different groups after challenge with HPAI H5N8 virus clade 2.3.4.4b:

26: Lung of G1 at 5 dPC showing parabrachial proteinaceous fluid in the lumen (arrow) H&E X100, **27:** Lung of G2 at 2 dPC showing congested blood vessels and air

capillaries (arrow) with perivascular edema (star) H&E X100, **28**: Lung of G3 at 6 dPC showing severe congested blood vessels (star) with interstitial edema (line) H&E X50, **29**: Lung of G4 at 5 dPC showing thickening of mucosa of secondary bronchiole with edema (line), congestion (arrow) and mononuclear cells infiltration (star) H&E X200 and **30**: Lung of G5 at 21 dPC showing apparently normal structures (H&E X100).

DISCUSSION:

During 2016, HPAI H5N8 clade 2.3.4.4b virus was first declared in Egypt via migratory birds (common coot and green winged teal) in Damietta governorate (Selim *et al.*, 2017 & Kandeil *et al.*, 2017). Since then, several cases of H5N8 have been recorded among domestic poultry in live bird markets, backyard flocks, and commercial farms in several governorates in Egypt as a result of multiple introductions (Salaheldin *et al.*, 2017, OIE, 2018, Yehia *et al.*, 2018, Kandeil *et al.*, 2019 & Yehia *et al.*, 2020). The prevalence of H5N1 has been gradually declined and is being replaced by H5N8 viruses (Tarek *et al.*, 2021). The controlling of AI infections in Egypt depends mainly on vaccination as the main control strategy. This work was designed to assess the efficacy of three different commercial inactivated AI vaccines of different clades (clade 1, clade 2.3.4.4b and Re-6 & Re-8) in commercial broiler chickens against challenge on day-31 with HPAI H5N8 clade 2.3.4.4b. Following HPAIV challenge, clinical signs which is characteristic for HPAI infection were observed. Clinical presentation of the disease included depression, abnormal respiratory sounds, facial swelling, oculonasal discharges, and cyanosis of comb & wattles, and hemorrhages shank. The postmortem gross lesions findings ranged from severe petechial hemorrhages, congestion in trachea, lung, and visceral organs, multifocal petechiae and necrotic areas in pancreas, multifocal petechiae in proventriculus

and in the proventriculus-gizzard junction, Congestion in cerebrum, hemorrhages of variable intensity on legs, multifocal petechial hemorrhages in bursa of Fabricius (Figure-3) as previously recorded by (Tarek *et al.*, 2021, Ibrahim *et al.*, 2021, El-Moeid *et al.*, 2021 & Rohaim *et al.*, 2021).

From all vaccinated challenged groups, G1 (vaccinated with clade 1) was most affected by challenge with 67% protection, and the clinical signs were more severe than other vaccinated challenged groups. This is probably due to difference in antigenicity between clade 1 and clade 2.3.4.4b AI viruses. Other groups G2 and G3 showed 80% and 87% protection, respectively, as shown in Table-2. G4 (positive control) and G5 (negative control) showed 0% and 100% protection, respectively. Any vaccine should conserve at least 80% protection for the vaccinated birds according to the OIE manual for vaccine assessment (OIE, 2018). Unsatisfactory level of protection against mortality (70%) following vaccination with H5N3 belonging to clade 1 was also obtained by Elsafty *et al.*, 2023 after challenge with HPAI H5N8 clade 2.3.4.4b 2020 isolate compared to 85 % protection after challenge with HPAI H5N8 clade 2.3.4.4b 2018 isolate. Also Hegazy *et al.*, 2021 previously recorded 70 % protection after vaccination with clade 1 in layer chickens challenged with H5N8 clade 2.3.4.4b 2018 isolate. In contrast to these results, El-Moeid *et al.*, 2021 showed the ability of H5N3 clade 1 vaccine to act efficiently against H5N8 clade 2.3.4.4b 2017 isolate with 100 %

protection when challenged two and three weeks post vaccination. While challenge with H5N8 2018 isolates revealed 0 % and 92 % protection when challenged two and three weeks post vaccination, respectively.

Protection percent following vaccination with Re-6 & Re-8 vaccine was the highest between the three vaccinated groups 86 %. Close results were obtained by Nassif *et al.*, 2022 who recorded 90 % protection against H5N8 2021 isolate.

HI titers are considered to be of predictive value concerning protective efficacy if suitable matching pairs of HI antigen and challenge virus are used (Swayne, 2009). Serological monitoring of H5 vaccinated flocks by the HI test using the homologous vaccine antigen is a routine laboratory procedure to evaluate vaccination efficacy of poultry in Egypt and elsewhere (Hafez *et al.*, 2010). Titers greater than 4-log_2 have been claimed to be an indicator for clinical protection and titers greater than 6-log_2 indicator for prevention of viral shedding as previously stated (Kumar *et al.*, 2007).

The HI test was performed using 3 vaccinal antigens (clade 1, clade 2.3.4.4b and Re-6 & Re-8) to determine antibody response of the vaccines in serum samples collected on days 1, 10, 17, 23, 31, 37 and 43. The lowest mean antibody titer was recorded in G1 vaccinated with H5N3 clade 1 vaccine which exhibited unsatisfactory levels of immune response as previously recorded by Elsafty *et al.*, 2023. That was reflected on the unsatisfactory level of clinical protection against mortality (67%). This could be on account of the presence of variation in antigenicity between the vaccinal seed strain and the H5N8 challenge virus. The highest mean antibody titer was noticed in G2

vaccinated with H5N8 clade 2.3.4.4b. This comes in agreement with Swayne *et al.* (2000) who stated that the greater the similarity of the HA gene sequence between the vaccination and field viruses, the better the protection afforded and the reduction in challenge virus reproduction in the respiratory tract.

Differences in HI titers between vaccinated birds in different groups may be due to differences in the antigens used in HI testing, as been confirmed by using the homologs and heterologous antigens in the current study. One of the critical factors influencing the efficacy of the vaccine is the genetic and antigenic matching between the circulating viruses and commercial vaccine seed strains (Wong and Webby, 2013). Our results goes in line with previous studies which have interpreted the poor seroconversion resulted from the genetic dissimilarity and the poor reactivity between the H5 commercial vaccines and the circulating H5N8 virus (Kandeil *et al.*, 2018, Ali *et al.*, 2019).

The antigenic relatedness between the three clades of vaccinal seed strains was calculated based on the Archetti and Horsfall formula (1950) using the cross HI results and the interpretation of the results was done according to Brooksby (1967) as shown in (table-4). Antisera raised against clade 1 vaccine showed minimal reactivity with clade 2.3.4.4b and clade 2.3.4.4g suggesting poor cross-protection. This was reflected on the R- value which was low (11.3%) indicating major antigenic difference. While, a minor subtype difference was detected between clade 2.3.4.4b and Re-6 & Re-8 with R-value 59.2 %.

It is essential to raise awareness of the gross pathological and histopathological features of HPAI viruses which will then help in the

disease investigation via the pathological indicators following infection. Several factors could impact clinico-pathological and scoring results between different species including Galliformes and Anseriformes (Gaide *et al.*, 2022). In chickens, infection resulted in severe and systemic disease, but clinical expression varied between different flocks (Gaide *et al.*, 2022)

In our study, samples were collected for examination of histopathological changes from thymus, trachea, spleen, bursa, lung, and cerebrum from dead or severely-affected chickens. Only mild cerebrum lesions were found in all challenged groups indicating reduced neurotropism of HPAI virus H5N8 belonging to clade 2.3.4.4b. Histopathological lesions included congestion, perivascular cuff, focal gliosis and mononuclear cell infiltration. This goes with previous study that recorded a reduced neurotropism based on the comparatively lower amounts of viral antigen and lesions detected in the brain of H5N8 inoculated chickens (Sánchez-González *et al.*, 2020).

The trachea showed thickening in mucosa, congested blood vessels, mononuclear cell infiltration. Histopathological lesions in the lung included congestion, perivascular & interstitial edema and mononuclear cell infiltration. Similar results were recorded previously by Rohaim *et al.* (2021).

In lymphoid tissues, including spleen, thymus and bursa of Fabricius, moderate to severe lesion score were detected. The spleen lesions included multifocal degeneration and necrosis, congestion and depletion of lymphocytes. Thymus also showed depletion of lymphocytes and congested blood vessels. Bursa showed depletion

of lymphocytes, epithelization, interfollicular connective tissue proliferation and several cyst formation.

Similar results were recorded by Sánchez-González *et al.*, (2020). Descriptions for the effect of AIV-H5N8 clade 2.3.4.4b on the bird immune organs (bursa, thymus, spleen) and the impairment effect on it highlight the immunosuppressive effect of the virus, which can appear in the vaccinated exposed birds to highly pathogenic avian influenza virus in the endemic countries which implemented vaccination strategies like in Egypt.

CONCLUSION

This study was aimed to assess the efficacy of three different vaccines of different clades against HPAI H5N8 clade 2.3.4.4b virus and the results obtained showed that genetic and antigenic variation between vaccinal seed strain and the challenge virus could reflect the clinical protection percent of the vaccine. Also, the immunopathological effects of the virus on some immune organs highlight the immunosuppressive effect of the virus, which can appear in the vaccinated exposed birds.

Finally, it is recommended to update the vaccine seed strains to be closely related to the circulating field strains to obtain better protection levels. Also, continuous evaluation of the validated AI vaccines against recent field strains is substantial to deal with the threat to the economy. Moreover, Continuous efforts to characterize the pathobiology and the pathological impact of the disease is necessary to update the presentation of HPAI Viruses to guide early diagnosis and control of the disease.

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