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Effectuality of Three Inactivated H5 Vaccines of Different Clades Against Newly Emerged Egyptian HPAI H5N1/2022 and H5N8/2019belonging to Clade 2.3.4.4b and Immunopathology of Both Viruses on Some Immune Organs

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ABSTRACT

The H5Nx viruses belonging to clade 2.3.4.4b has become enormous intimidation globally. Outbreaks resulting from this clade have been reported in various countries resulting in an exceptional numeral bird mortalities. This work aimed to assess 3 H5 vaccines of different clades (2.3.2 - 2.3.4 -2.3.4.4b) against newly emerged Egyptian clade 2.3.4.4bHPAI H5N1/2022 and H5N8/2019 viruses in commercial broiler chickens. Clade 2.3.2 vaccine failed to protect clinically against both challenge viruses while, clade 2.3.4 (Re-5) showed 87 % and 40% protection against H5N8 and H5N1, respectively. Clade 2.3.4.4b showed 60% protection against H5N8 compared to 93% protection against H5N1.Obtained results demonstrated that clade 2.3.4.4b H5N8 vaccine antigenically matched effectively with the newly emerged clade 2.3.4.4b H5N1 virus& could afford high level of clinical protection. Histopathological lesions were recorded in different collected organs including cerebrum, trachea, lung, spleen, thymus and bursa of Fabricius. The lymphoid tissues displayed moderate to severe lesion scores including congestion, necrosis and depletion 0f lymphocytes. Thus, clade 2.3.4.4b viruses could have an immunosuppressive effect. Continuous evaluation of the validated AI H5 vaccines against newly emerging AI strains is substaitional to deal with that intimidating remark. Also, the vaccinal seed strains should updated regularly in order to obtain utmost protection levels and assure presence of antigenic matching among prevailing strain and vaccinal strains.

Key words: Clade 2.3.4.4b, Egyptian, H5Nx, HPAl, Immunopathology and Vaccine evaluation.

INTRODUCTION

Current situation of AI has largely evolved worldwide & HPAI cases persisted to be reported within both domestic birds, wild birds, mammals & humans (EFSA, 2023). Wild migratory birds are the cause of enormous dispersal of H5 AI viruses which give rise multiple intercontinental waves of AI outbreaks (Shi et al., 2023). Several countries Including Europe, Asia, Africa and North America have been reported clade 2.3.4.4b H5Nx outbreaks since 2020 which lead to an exceptional mortalities (Sagong et al., 2021 & Nagy et al., 2022).The HPAI epidemic observed in 2021/2022 was yet the most extensive epidemic recognized in Europe. That epidemic involved 6684 HPAI virus detections at 37 European countries

(EFSA, 2023). Based on recent studies, it is claimed that clade 2.3.4.4b H5N1 has gained dominance over clade 2.3.4.4b H5N8 viruses.H5N1 viruses possess increased prevalence and huge spread between wild birds and consequently increased transmission to domestic poultry in many countries (Sagong et al., 2022).

Egypt firstly reported isolation of HPAI H5Nl in December 2005 from wild birds (Saad et al., 2007). Then the virus became endemic at 2008 (Abdelwhab & Hafez, 201l). H5 strain that was detected in Egypt at the start belonged to clade 2.2 which evolved in China & then, diversified into 3 subclades (2.2.l.2, 2.2.l.l (a) & 2.2.2.l) (Abdelwahab et al., 2016). Clade 2.2.1.2 dominated at 2014 causing upsurge of AI outbreaks among poultry species at late 2014 (Arafa et al., 2015). In 2010, LPAI H9N2 outbreak was also reported in Egypt for the first time (Monne et al., 2013). Moreover, clade 2.3.4.4bHPAI H5N8 virus was introduced in 2016 through migratory wild birds and became endemic since then. Afterwards multiple cases of infection was reported (Selim et al., 2017& Salaheldin et al., 2018).Within 2019, novel HPAI H5N2 of clade 2.3.4.4b was found in commercial chicken & duck farms in Egypt. This H5N2 virus evolved through genetic reassortment between Egyptian HPAI H5N8 & LPAI H9N2 subtypes (Hagag et al., 2020 & Hassan et al., 2020). Thereafter, according to epidemiological data, clade 2.3.4.4b HPAI H5N8 replaced clade 2.2.1.2 H5N1 viruses that subsequently disappeared. Since then, the most considerably reported H5 subtype was clade 2.3.4.4b H5N8 in Egypt (Amer et al., 2021 & Tarek et al., 2021).Recently, isolation & fully characterization of clade 2.3.4.4b HPAI H5N5 virus was declared. H5N5 virus was isolated from purple heron that was apparently healthy during December 20l6 (Kandeil et al., 2023). The Egyptian HPAI H5N5 is genotypically similar to H5N5 that was detected in Russia &other European countries. H5N5 was proved to be antigenically distinct from H5N8despite it belong to the same clade 2.3.4.4b (Kandeil et al., 2023). Moreover, clade 2.3.4.4b HPAI H5N1 was detected at wild migratory birds in Egypt. That H5N1 clade 2.3.4.4b shared genetic characteristics with H5N1 strains that were reported in Europe, North America, Asia, & Africa at 2021/2022 (Mosaad et al., 2023). Afterwards, during an active surveillance, H5N1 clade 2.3.4.4b was isolated from 1 wild pintail duck and 3 domestic Pekin ducks that were present in live bird markets in early 2022 (El-Sheshny et al., 2022).

The current situation of AI disease in Egypt is very complicated and Egypt relies mainly on vaccination for AI controlling. Vaccination is thought to be of assistance in AI prevention and control. Although Egypt has used AI H5 vaccines for many years, AI viruses underwent many antigenic drifts. This was a consequence of immune pressure and subsequent vaccination failure (Kayali et al., 2016). Several commercially AI vaccines are available & widely applied. However, the extensive variations of HA gene together with variation in antigenicity within the same subtype, will lead to vaccination failure against those newly emerging H5Nx strains (Kandeil et al., 2018).Subsequently routine evaluation of the most commonly used commercial AI vaccines against the newly emerging H5Nx strains is urgently needed to establish the most appropriate and effective vaccination strategy.

Aim of work: experimental design was established in order to to assess the efficacy of 3 different commercial inactivated AI vaccines of different clades (clade 2.3.2 – (Re-5) clade 2.3.4 - clade 2.3.4.4b) in commercial broiler chickens against newly emerged clade 2.3.4.4b HPAI H5N1/2022& H5N8/2019. Assessment included recording of mortality rates, serum antibody titers and

the histopathological changes in six organs including spleen, bursa, thymus, cerebrum, trachea, and lung.

MATERIALS AND METHODS

Ethical consent:

All experiment procedures were conducted according to the guidelines of Laboratory animal use. Also, it was legally approved by the Committee of Ethics of Animal Experiments at the Animal Health Research Institute, Egypt. Experimental infection was done at isolators (BSL-3) animal biosafety level 3.Every attempts was carried out to reduce the birds' suffering.

Challenge Virus:

Formerly identified and characterized virus by the Reference Lab. For Veterinary Quality Control on Poultry Production clade 2.3.4.4b HPAI (H5N1) (A/Ibis/Egypt/RLQP/229S/2022); GenBank accession number OP491851 & clade 2.3.4.4b HPAI (H5N8) (A/duck/Egypt/SMG4/2019) GenBank accession number MN658766 were used for experimental infection study. Challenge procedures were conducted on day-31 via intranasal route using 10^6 EID_{50} /0.l m1 of the challenge viruses (El-Moeid *et al*., 2021).

Vaccines:

Three inactivated H5 commercially available AI vaccines of different clades (clade 2.3.2 – (Re-5) clade 2.3.4 - clade 2.3.4.4b) (Table-1) were used in current study to assess their efficacy in vaccinated challenged chickens. The vaccination procedures were conducted as per the manufacturers' recommendations.

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

Vaccine trade name	clade	subtype	Vaccinal strain	Manufacturing country	Manufacturer
B.E.S.T	2.3.2	H5N1	A/DK/China/E319-2/03		Boehringer Ingelheim
Re-5	2.3.4	H5N1	A/duck/Anhui/1/2006	china	Zhaoqing Dahuanong Biology Medicine
Vallyvac	2.3.4.4 b	H5N8	A/chicken/Egypt/S18182C/2020	Egypt	Egyptian company for biological and pharmaceutical industries

Chickens:

One-day-old commercial broiler chicks (n=135) that possessed maternal derived antibodies (MDA) against H5 HPAI gained from their parents were obtained from a commercial hatchery & kept in isolators with food and water supplied adlibitum.

Experimental design:

A total of one hundred and thirty five 1-day-old chicks were divided into 9 groups (n=15). Chickens in groupsG1, G2&G3received 0.5 ml/bird of clade 2.3.2, clade 2.3.4 (Re-5)& clade 2.3.4.4b inactivated vaccines on day-l0 of age, respectively, and challenged on day-31 with HPAI (H5N8/2019) clade 2.3.4.4b virus.G5, G6 &G7 received 0.5 ml/bird of clade 2.3.2, clade 2.3.4(Re-5) & clade 2.3.4.4b inactivated vaccines on day-10, respectively, and challenged on day-3l with HPAI

(H5N1/2022) virus. G4, G8 & G9 were kept non-vaccinated as control. G4 was challenged on day-31 with HPAI (H5N8/2019). While, G8 was challenged on day-31 with HPAI (H5N1/2022). G9 was kept non-vaccinated non-challenged as negative control (Figure-1). Clinical signs, PM lesions and mortalities were monitored for 12 dpc. Blood samples were collected for HI tests. Also, organ samples (spleen, bursa, thymus, cerebrum, trachea and lung) were gathered for histopathological examination.

Serology and antibody response:

The HI test was performed by using 3 different vaccinal antigens, supplied by local agencies, (Table-1) to obtain antibody response of the vaccines in the serum samples compiled on days 1, 10, 17, 23, 30 & 42. The HI titer is considered positive if there is inhibition of serum dilution equal to 1/8 (2^3 or 3 $\log_2$) or more against 8 HAU of antigens. The mean antibody titers were expressed on a $\log_2$ scales. The HI test was done in accordance with OIE protocol (OIE, 2018).

Determination of antigenic relatedness (R-Value):

The antigenic relatedness among vaccinal antigens of different clades were showed as (R-value) using the results of cross HI test. This was established on Archetti and Horsfall formula ($r = \sqrt{r1 x\, r2}$) (Archetti and Horsfall 1950). Where r1 obtained by dividing heterologous titer obtained from virus 2 by homologous titer obtained from virus 1.Also, r2 obtained by dividing heterologous titer obtained from virus 1 by homologous titer obtained from virus 2. The resulted R values get between 0 (if antigens were antigenically distinct) to 100% (if antigens were identical antigenically).Obtained R values were showed as percentage relatedness. Obtained percentage were interpreted according to Brooksby, (1967). Whereas if the R value is ranged from 0 to 10%, this indicate presence of serotype difference. If the R value is ranged from 11 to 32%, this indicates presence of major difference between the two subtypes. While if the R value is ranged from 33 to 70%, this indicates presence ofminor difference between the two subtypes. R values which are greater than 70% indicates presence of no or little differences between the two subtypes.

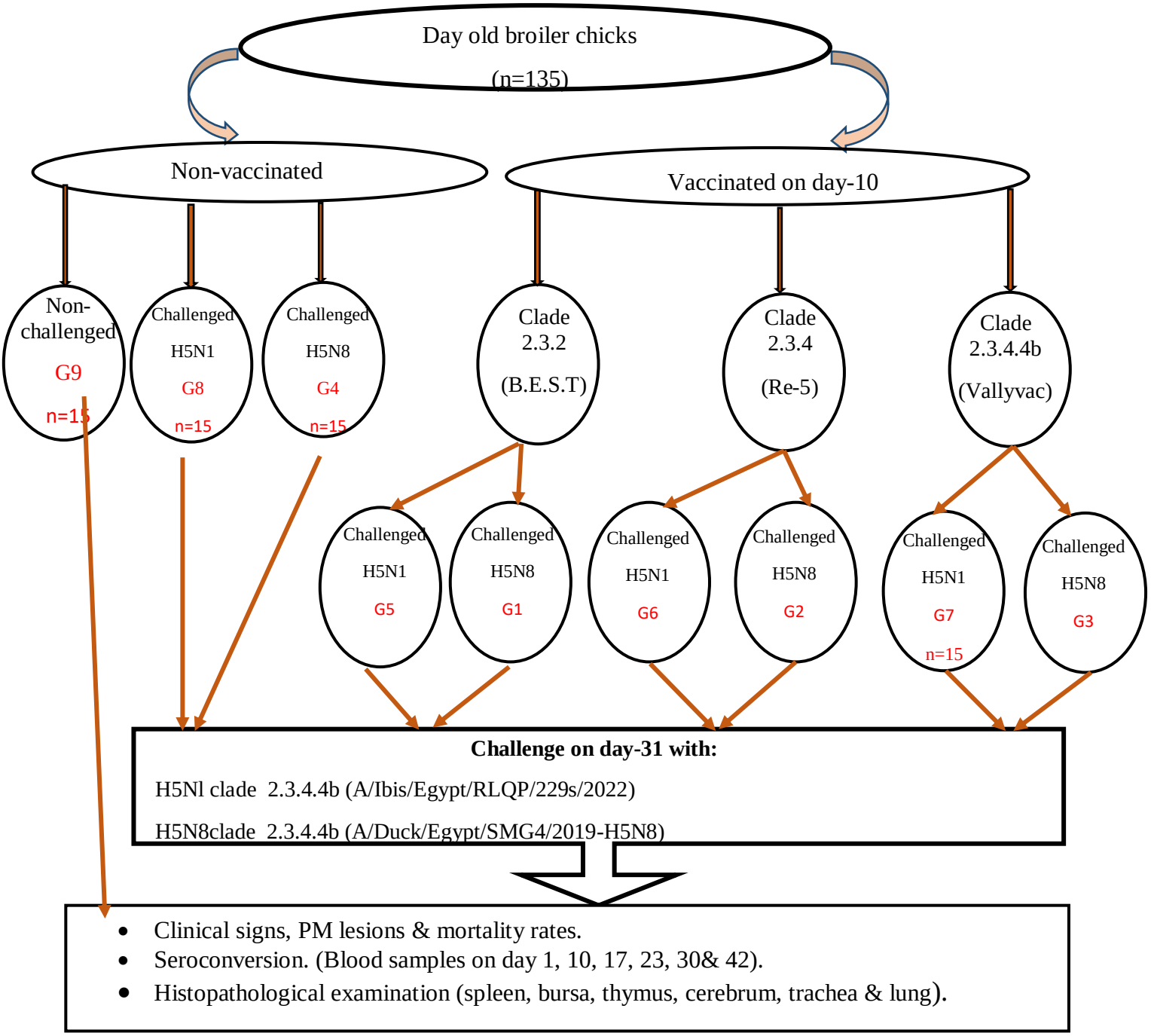
Histopathological examination:

Samples were collected from 6 organs thymus, trachea, spleen, bursa, lung, and cerebrum from all groups. The examined sample tissues were preserved in 10% buffered formalin. Dehydration of these samples was carried out by using ascending grades of alcohol. For cleaning, tissues placed in Xylol. 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 five micron thickness and prepared for staining by H&E and examined by light microscope (Bancroft *et al*., 2019).

Examination and scoring of random optical fields (n= 5) and calculation of mean lesion score (MLS) were done. The severity of histopathological changes was then scored. The score (0) = apparently normal; (1) = mild lesions;(2) = moderate lesions and (3) = sever lesions. The mean severity index (MSI) is obtained by the sum of MLS of examined organs per group divided by total number of examined organs as formerly described (Gibson *et al*., 2013 & Sultan *et al*., 2019).

Statistical analysis:

The data had been analyzed through the t-test or ANOVA if necessary, and then application of Duncan's new multiple range test to determine presence of significance differences among individual treatments and comparable controls (Steel and Torrie., 1960)



Clinical signs, PM gross lesions:

Ensuing challenge with both HPAI viruses (H5N1/2022& H5N8/2019) clade 2.3.4.4b, the main observed clinical signs in both HPAIV infections included moderate apathy and prostration. Subcutaneous edema, cyanosis of the comb and wattles were observed too. Also depression, loss of appetite, ocular & nasal discharges and gasping were common observation in different challenged groups. Clinical signs appeared to be more sever in clade 2.3.4.4b H5N1/2021 challenged groups especially for non-vaccinated G8 (H5N1 positive control).

PM examination of the dead birds challenged with both viruses (H5N1/2022& H5N8/2019) revealed similar lesions including, multifocal haemorrhages in

proventriculus, gizzard, pancreas and bursa of Fabricius. Hemorrhages of the legs, subcutaneous edema & lung consolidation. congestion in cerebrum and diffuse congestion of all internal organs were observed. Also, congested & edematous lungs together with enlarged mottled spleen were presented in some birds (Figure 2).There wasn't any clinical signs or pm gross lesions observations in negative control group G9.

Mortalities and protection %:

The mortalities started from 2 dpc and continued till 10 dpc as shown in Table-2.G1 and G5 vaccinated with clade 2.3.2 showed 60 %& 73 % mortality following challenge with H5N8/2019& H5N1/2022, respectively. While, G2 & G6 vaccinated with Re-5 clade 2.3.4 showed 13 % & 60 % mortality following challenge withH5N8/2019 and H5N1/2022, respectively. G3 & G7vaccinated with clade 2.3.4.4b showed 40 % and 7 % mortality following challenge with HPAI H5N8/2019 and H5N1/2022 clade 2.3.4.4b, respectively. Negative control group G9 showed 0 % mortality. Positive control groups (G4, G8) showed 100% mortality and the mortality rang per day was (2-6) dpc when challenged with clade 2.3.4.4b HPAI H5N8/2019&only (2-3) dpc when challenged with clade 2.3.4.4b HPAI H5N1/2022with 0% protection against both challenge viruses.

The highest clinical protection percent (87%) against H5N8/2019 challenge was recorded in G2 (Re-5 clade 2.3.4 vaccine) and much lower protection 40% & 60% was recorded in G1 (2.3.2 vaccine) & G3 (2.3.4.4b vaccine), respectively.

The highest protection percent (93%) against challenge with clade H5N1/2022 was recorded in G7 (2.3.4.4b vaccine) and very low protection 27% and 40% was recorded in G5 (2.3.2 vaccine) & G6 (Re-5 clade 2.3.4 vaccine), respectively.

Table 2: Mortality and protection %:

Group (n=15)	Vaccination on day 10	Challenge on day 31	Mortality/dpc (days post challenge)												Total mortality (%)	Mortality range /day	Protection (%)
			1	2	3	4	5	6	7	8	9	10	11	12			
G1	Clade 2.3.2	A/Duck/Egypt/SMG4/2019(H5N8) H5N8 clade 2.3.4.4b	-	1	-	-	2	3	1	1	1	-	-	-	9/15 (60%)	2-9	40 %
G2	Re-5 Clade 2.3.4		-	-	-	-	1	-	-	-	-	1	-	-	2/15 (13%)	5-10	87 %
G3	Clade 2.3.4.4b		-	1	1	-	-	2	1	1	-	-	-	-	6/15 (40%)	2-8	60 %
G4	H5N8 positive control		-	2	4	5	2	2	-	-	-	-	-	-	15/15 (100%)	2-6	0 %
G5	Clade 2.3.2	(A/Ibis/Egypt/RLQP/229s/2022) H5N1 clade 2.3.4.4b	-	-	3	3	2	1	1	1	-	-	-	-	11/15 (73%)	3-8	27 %
G6	Re-5 Clade 2.3.4		-	1	1	-	2	2	2	-	1	-	-	-	9/15 (60%)	2-9	40 %
G7	Clade 2.3.4.4b		-	-	-	1	-	-	-	-	-	-	-	-	1/15 (7%)	3-3	93 %
G8	H5N1 positive control		-	10	5	-	-	-	-	-	-	-	-	-	15/15 (100%)	2-3	0 %
G9	Negative control		-	-	-	-	-	-	-	-	-	-	-	-	0/15 (0%)	0	100 %

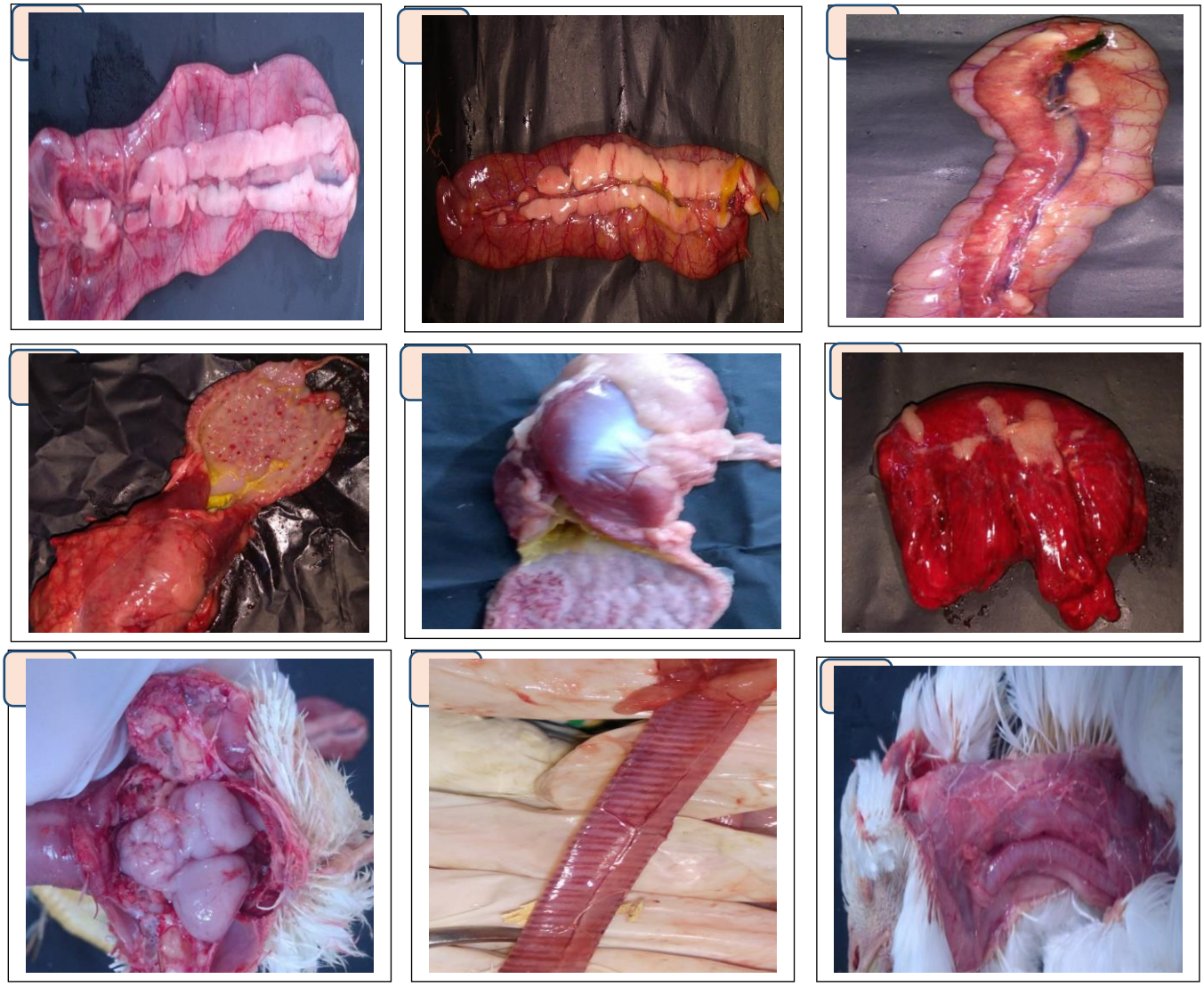
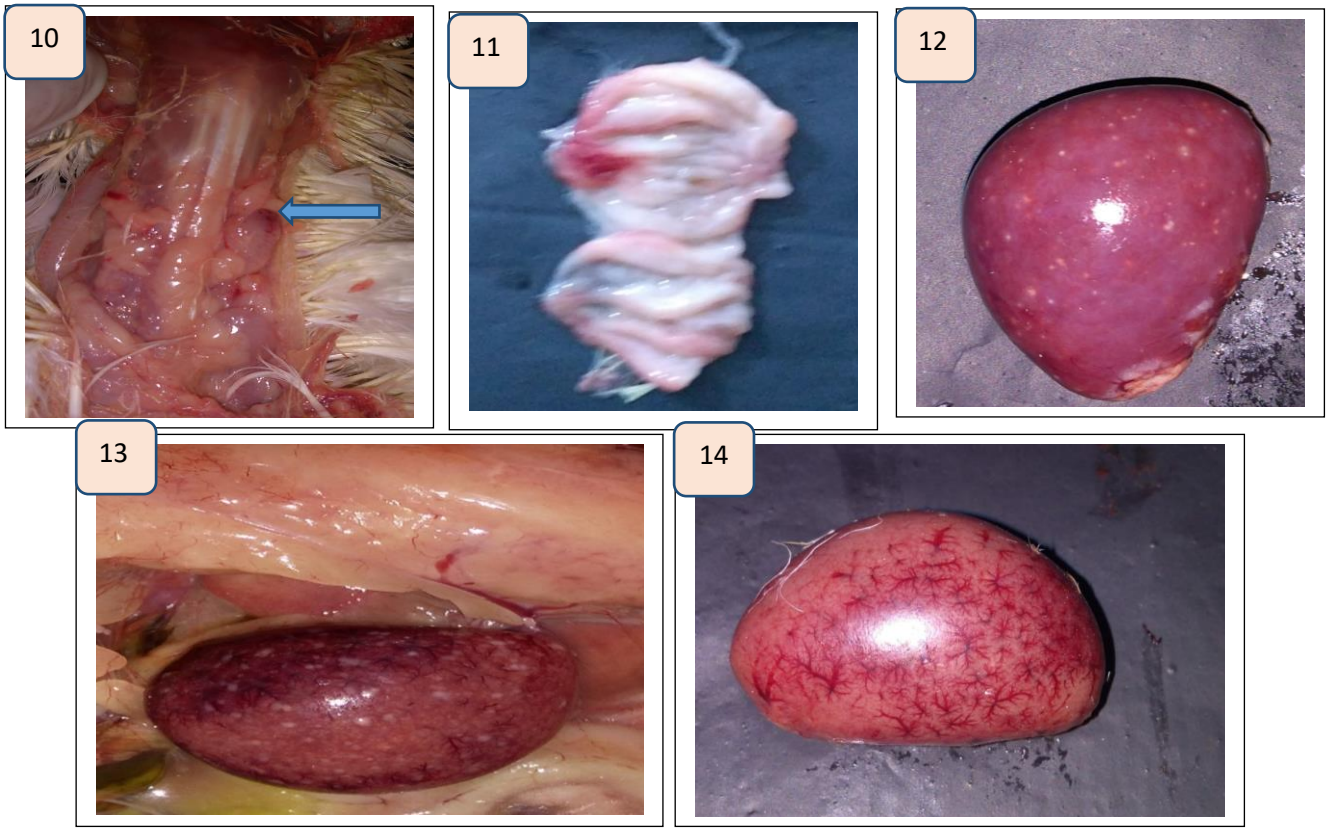


Figure 2: PM gross lesions following challenge on day-31 with clade 2.3.4.4b HPAIV H5N1/2022 and H5N8/2019.

1, 2 & 3: multifocal petechiae and necrotic areas in pancreas with sever congestion, 4 & 5: hemorrhages on proventriculus, 6: sever congestion in lung, 7: Brain of dead chicken showed congestion &hemorrhagic areas, 8 &9: sever congestion in trachea.

Continue, PM lesions:



10: congestion and hemorrhage in thymus, 11: hemorrhage in bursa and 12, 13 &14: congestion, hemorrhage and necrotic area in spleen.

Serum antibody response:

Serological responses after vaccination using three different antigens belonging to clade (clade 2.3.2, clade 2.3.4 (Re-5) & clade 2.3.4.4b) are shown at Tables 3, 4, 5 & 6, Figure 3

The mean HI titer of maternal derived antibodies (MDA) was not detectable in G9 (control negative) after 17th day.

The H5N1Clade 2.3.2 antigen showed very low reactivity with serum samples collected from different groups before or after challenge.

The mean HI titers at day 30 before challenge were 0.6, 0.4, & 0.3 log_2in G1 (2.3.2 vaccine) when tested using clade 2.3.2, 2.3.4 & 2.3.4.4b antigens, respectively.

The mean HI titers at day 30 before challenge were 0.4, 4.4, &4.0log_2 in G2 (2.3.4 vaccine) when tested using clade 2.3.2, 2.3.4 & 2.3.4.4b antigens, respectively.

The mean HI titers at day 30 before challenge were 0.3, 3.9, & 4.7 log_2 in G3 (2.3.4.4b vaccine) when tested using clade 2.3.2, 2.3.4 & 2.3.4.4b antigens, respectively.

At day 42, G1& G5 (2.3.2 vaccine) didn't react with 2.3.4 or 2.3.4.4b antigens. The mean HI titers using clade 2.3.4 antigen were 4.9, 4.3, 4.6&4.2 log_2 on G2, G3, G6&G7, respectively. The mean HI titers using clade 2.3.4.4b antigen were 4.6, 5.0, 4.3&5.2 log_2 on G2, G3, G6&G7, respectively (Table 6).

Table 3: Mean HI titer using clade 2.3.2 antigen (A/DK/China/E319-2/03) before challenge:

group	Type of Vaccine 0n day 10	Mean HI titre Log_2				
		Days /age				
		1	10	17	23	30
G1	Clade 2.3.2	1.1	0.7	0.5	0.5	0.6
G2	Clade 2.3.4	1.1	0.7	0.5	0.4	0.4
G3	Clade 2.3.4.4b	1.1	0.7	0.3	0.4	0.3
G9	Negative control	1.1	0.7	0	0	0

Table 4: Mean HI titer using Re-5 clade 2.3.4 antigen (A/duck/Anhui/1/2006) before challenge:

group	Type of Vaccine 0n day 10	Mean HI titre Log_2				
		Days /age				
		1	10	17	23	30
G1	Clade 2.3.2	1.3	0.9	0.6	0.5	0.4
G2	Clade 2.3.4	1.3	0.9	2.5	3.3	4.4
G3	Clade 2.3.4.4b	1.3	0.9	2	2.8	3.9
G9	Negative control	1.3	0.9	0.7	0	0

Table 5: Mean HI titer using clade 2.3.4.4b antigen (A/chicken/Egypt/S18182C/2020) before challenge:

group	**Type of Vaccine 0n day 10**	**Mean HI titre Log_2**				
		Days /age				
		1	**10**	**17**	**23**	**30**
G1	Clade 2.3.2	1.4	1.1	0.4	0.5	0.3
G2	Clade 2.3.4	1.4	1.1	2.3	3.0	4.0
G3	Clade 2.3.4.4b	1.4	1.1	2.6	3.8	4.7
G9	Negative control	1.4	1.1	0.8	0	0

Table6: Mean HI titer following challenge at day 42 using three antigens:

Group	Type of vaccine	Challenge virus on day 31	Mean HI titre Log_2 at day 42		
			Clade 2.3.2	clade 2.3.4	clade 2.3.4.4b
G1	Clade 2.3.2	H5N8 clade 2.3.4.4b A/Duck/Egypt/SMG4/2019	0.3	0	0
G2	Clade 2.3.4		0	4.9	4.6
G3	Clade 2.3.4.4b		0	4.3	5
G5	Clade 2.3.2	H5N1 Clade 2.3.4.4b A/Ibis/Egypt/RLQP/229S/2022	0.2	0	0
G6	Clade 2.3.4		0	4.6	4.3
G7	Clade 2.3.4.4b		0	4.2	5.2

Figure 3: Serological response of broiler chickens following vaccination with inactivated H5AI vaccine clade 2.3.2 (G1), clade 2.3.4 (G2) and clade 2.3.4.4b (G3)

Antigenic relatedness:

The R-values% between different clades of the 3 vaccinal antigens was calculated using the cross HI results (table 7) at day 30 before challenge. Very low antigenic relatedness was detected between clade 2.3.2 and both clades 2.3.4 & 2.3.4.4b. Clade 2.3.2 R-value % was 7 & 4 with clade 2.3.4 & 2.3.4.4b, respectively. While the antigenic relatedness % between clade 2.3.4 & clade 2.3.4.4b was 79 % as shown at Table 8.

Table 7: Cross HI test within three commercial H5 inactivated AI vaccines of different clades:

Antigen	antisera HI titer means (Log_{-2}) at day 30		
	clade 2.3.2	clade 2.3.4	Clade 2.3.4.4b
clade 2.3.2	**0.6**	0.4	0.3
clade 2.3.4	0.4	**4.4**	4.0
Clade 2.3.4.4b	0.3	3.9	**4.7**

Table-8: Antigenic relatedness (R-values %) of 3 commercial H5 inactivated AI vaccines of different clades and their interpretation:

Item	clade 2.3.4		Clade 2.3.4.4b	
	R-value %	**Interpretation**	**R-value %**	**Interpretation**
clade 2.3.2[a]	**7 %**	Serotype difference	**4 %**	Serotype difference
clade 2.3.4[b]	-	-	**79 %**	Little subtype difference
Clade 2.3.4.4b[c]	-	-	-	-

Histopathologic examination:

The mean severity index (MSI) for clade 2.3.2 vaccinated groups (G1, G5) was 2 following challenge with both HPAI H5N8 (G1) and H5N1 (G5) viruses belonging to clade 2.3.4.4b. While, clade 2.3.4 (Re-5) vaccinated groups (G2, G6) showed 1.6 & 1.9 MSI following challenge with HPAI H5N8 (G2) and H5N1 (G6) viruses, respectively. Clade 2.3.4.4b vaccinated groups (G3, G7) showed 2&1.3 MSI following challenge with HPAI H5N8 (G3) & H5N1 (G7) viruses, respectively. Positive control groups (G4, G8) showed 2 and 2.1 MSI, respectively (Table 9).

The cerebrum in Groups (G1, G2, G3, and G4) challenged with H5N8 showed perivascular cuff, perineural edema, gliosis & mononuclear cells aggregation (figure 4), while cerebrum in Groups (G5, G6, G7, and G8) challenged with H5N1 showed perivascular cuff (figure 5).

The trachea in H5N8 challenged (G1, G2, G3, and G4) &H5N1 challenged Groups (G5, G6, G7, and G8) showed similar histopathological lesions including edema and thickening of mucosa with congestion, hyperplasia of lining epithelium, mononuclear cell infiltration and edema in lamina propriaat (figure 4& figure 5).

The lungin Groups (G1, G2, G3, and G4) H5N8 challenged showed fibrinopurulent materials in bronchi lumen, thickening in wall of blood vessels with thrombus formation, congestion in blood vessels and edema of parabronchi (figure 4), while Groups (G5, G6, G7, and G8) H5N1challenged showed focal interstitial granulocytes infiltration, congested blood vessels with thickening in its wall, perivascular edema and interstitial edema (figure 5).

The bursa in Groups (G1, G2, G3, and G4) H5N1 challenged showed sever depletion and necrosis of lymphocytes, depletion and atrophy of follicles, multiple cyst formation and interfollicular edema (figure 4). The bursa in groups (G5, G6, G7, and G8) H5N1 challenged showed depletion of lymphocytes with degeneration, interfollicular edema and subepithelial RBCs, hyperplasia of lining epithelium, microcysts and follicular cyst formation (figure 5).

The spleen in Groups (G1, G2, G3, and G4) H5N1 challenged showed multifocal coagulative necrosis of spleenocytes & multifocal depletion of spleenocytes (figure 4). The spleen in groups (G5, G6, G7, and G8) H5N1 challenged showed congestion in blood vessels, degeneration, coagulative necrosis and depletion of lymphocytes (figure 5).

The thymus in H5N8 challenged Groups (G1, G2, G3, and G4) &H5N1 challenged Groups (G5, G6, G7, and G8) showed similar histopathological lesions including congested blood vessels, depletion and necrosis of thymocytes (figure 4& figure 5). No histopathological lesion were found in negative control group (G9) and all examined organs showed normal structures.

Table 9: Histopathologic Mean lesion Scores (MLS) & Mean severity index (MSI):

Group	Type of vaccine	Challenge virus on day 31	Mean Lesion score (MLS)						MSI^B± SD
			Spleen	Thymus	Bursa	Trachea	Cerebrum	Lung	
G 1	Clade 2.3.2	H5N8 clade 2.3.4.4b A/Duck/Egypt/SMG4/2019(H5N8)	1.8	1	2.5	1.5	2.3	2.8	2.0±0.67[a]
G 2	Clade 2.3.4		1	1	3	1	2	1.3	1.6±0.81[b]
G 3	Clade 2.3.4.4b		1	3	2.3	1	2.8	2	2.0±0.86[a]
G 4	H5N8 Positive control		2.8	2.8	2.3	1.5	1.8	2	2.2±0.53[a]
G 5	Clade 2.3.2	H5N1 Clade 2.3.4.4b A/Ibis/Egypt/RLQP/229S /2022	1	2	2	2	2.8	2	2.0±0.75[a]
G 6	Clade 2.3.4		2.3	1.8	2	1	2	2	1.9±0.44[b]
G 7	Clade 2.3.4.4b		1.3	1.3	2	1	1	1.3	1.3±37[b]
G 8	H5N1 positive control		2.3	2.3	2.8	1.8	1.3	2	2.1±0.51[a]
G 9	Negative control		0	0	0	0	0	0	0[c]

Different lower case letters represent the presence of significant differences(*P≤0.05)* between the mean scores±SD within the same time interval**.**

0= apparently normal, **1**= mild lesion, **2**=moderate lesion,**3**= severe lesion

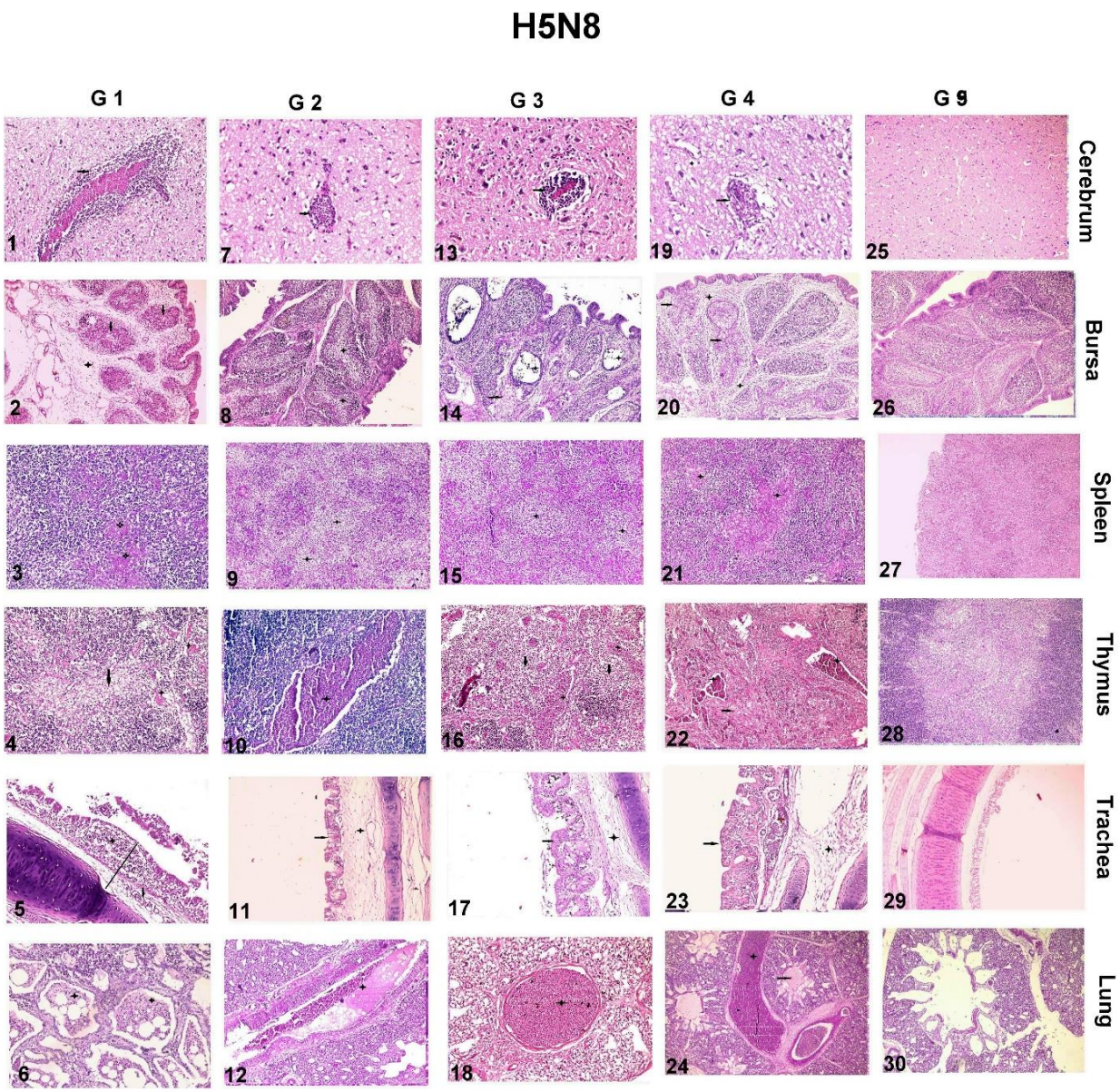


Figure 4: Histopathological lesions in six organs following challenge withHPAIclade 2.3.4.4b H5N8/2019:

G1 vaccinated with clade 2.3.2 vaccine:

1: **Cerebrum** of G1 showing perivascular cuff (arrow), lymphocytes aggregation in perivascular space,

2: **Bursa** of G1 showing severe depletion &necrosis of lymphocytes (arrow), with edema (star),

3: Spleen of G1 showing multifocal coagulative necrosis of spleenocytes (star),

4: **Thymus** of G1 showing congested blood vessels (star) & focal depletion of thymocytes (arrow),

5: **Trachea** of G1 showing focal thickening of mucosa (line), with edema, congestion (arrow) & monnuclear (star) cells infiltration&

6: **Lung** of G1 showing fibrinopurulent materials in tertiary bronchi lumen (star).

G2 vaccinated with clade 2.3.4 vaccine:

7: Cerebrum of G2 showing aggregation of mononuclear cells bordered the blood vessel (arrow),

8: Bursa of G2 showing depletion of lymphocytes in the medulla (star),

9: Spleen of G2 showing multifocal depletion of spleenocytes (star),
10: Thymus of G2 showing congested blood vessels (star),
11: Trachea of G2 showing mild hyperplasia of lining epithelium (arrow), with edema of lamina propria (star) &
12: Lung of G2 showing thickening of the wall of blood vessels with thrombus formation (star).
G3vaccinated with clade 2.3.4.4b vaccine:
13: Cerebrum of G3 showing perineural edema, gliosis & perivascular cuff formation (arrow),
14: Bursa of G3 showing depletion & atrophy of follicles, edema (arrow) in addition to formation of multiple cysts (star),
15: **Spleen** of G3 showing multifocal depletion of spleenocytes (star),
16: **Thymus** of G3 showing severe congested blood vessels in cortex & medulla (star) & depletion of thymocytes (arrow),
17: **Trachea** of G3 showing hyperplasia of lining epithelium (arrow), with activation of mucous glands & edema of lamina propria (star).
18: Lung of G3 showing congested blood vessels (star).
G4 control positive (non-vaccinated challenged):
19: **Cerebrum** of G4 showing perivascular cuff (star).
20: **Bursa** of G4 showing hyperplasia with depletion of lymphocytes (arrow) & cysts formation, in addition to interfollicular edema (star),
21: **Spleen** of G4 showing coagulative necrosis of spleenocytes (star),
22: **Thymus** of G4 showing congested blood vessels (star)with depletion & necrosis of thymocytes (arrow),
23: **Trachea** of G4 showing hyperplasia of lining epithelium (arrow), congestion & edema of lamina propria (star)**.**
24: **Lung** of G4 showing congested blood vessels (star)& edema of parabronchi (arrow).
G9 control negative (non-vaccinated non-challenged):
25: **Cerebrum** of negative control group showing apparently normal architectures,
26: **Bursa** of negative control group showing apparently normal architectures,
27: **Spleen** of negative control group showing apparently normal architectures,
28: **Thymus** of negative control group showing apparently normal architectures,
29: **Trachea** of negative control group showing apparently normal architectures,
30: **Lung** of negative control group showing apparently normal architectures.

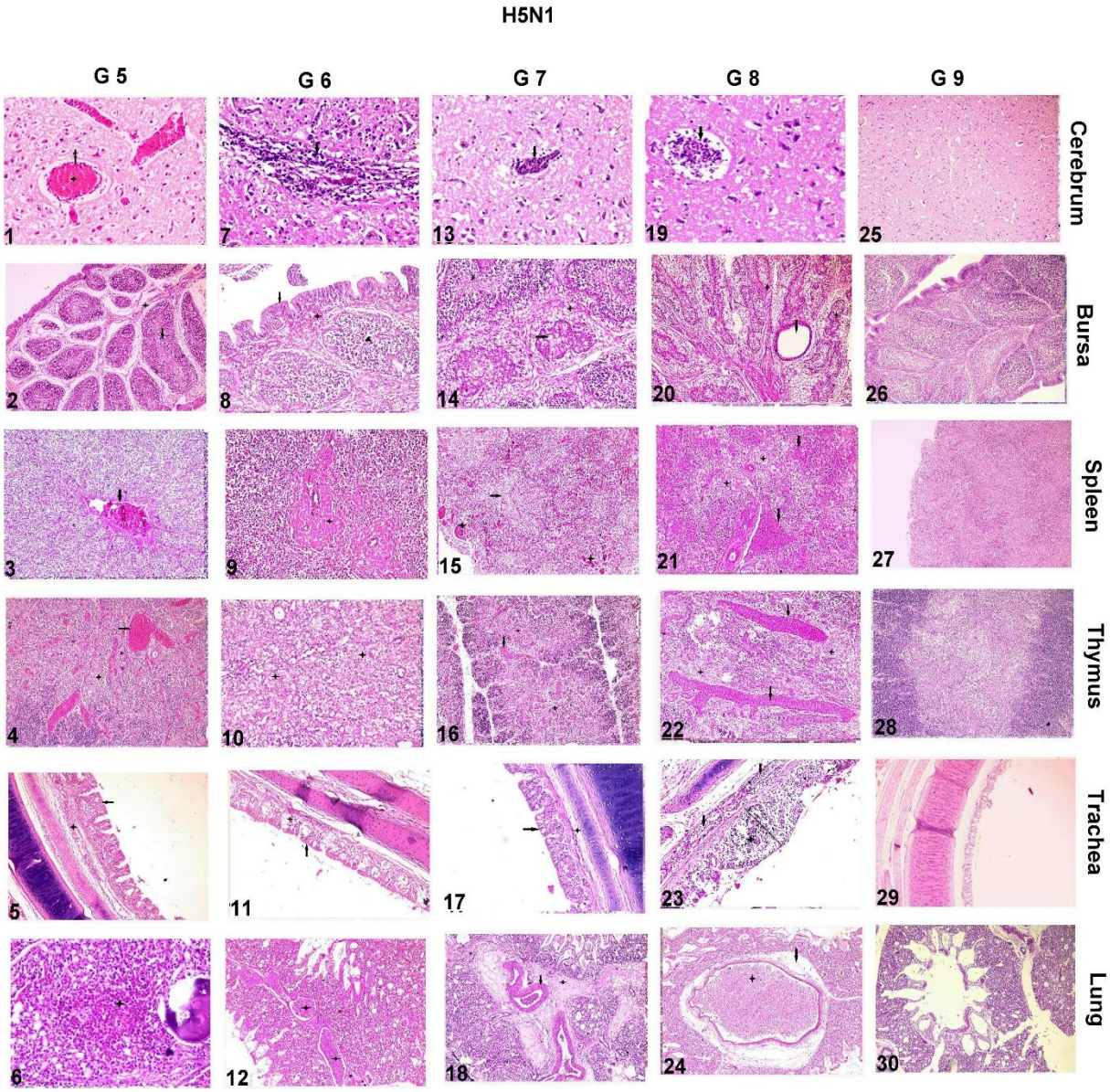


Figure 5: Histopathological lesions in six organs following challenge withHPAI clade 2.3.4.4b H5N1/2022:

G5vaccinated with clade 2.3.2 vaccine:

1: Cerebrum of G5 showing perivascular cuff (star),

2: **Bursa** of G5 showing depletion of lymphocytes (arrow) with interfollicular edema (star),

3: **Spleen** of G5 showing congested blood vessels (arrow),

4: **Thymus** of G5 showing congested blood vessels (arrow) & depletion of lymphocytes (star),

5: **Trachea** of G5 showing hyperplasia of lining epithelium (arrow), with edema and inflammatory cells infiltration (star) in lamina propria,

6: **Lung** of G5 showing focal interstitial granulocytes infiltration (star).

G6vaccinated with clade 2.3.4 vaccine:

7: Cerebrum of G6 showing perivascular cuff (arrow),

8: Bursa of G6 showing hyperplasia of lining epithelium (arrow), depletion of lymphocytes (triangle) & interfollicular edema and subepithelial RBcs (star),

9: **Spleen** of G6 showing coagulative necrosis (star),
10:Thymus of G6 showing depletion & degeneration of thymocytes (star),
11: Trachea of G 6 showing hyperplasia of lining epithelium (arrow) & activation of mucous glands (star),
12: Lung of G 6 showing congested blood vessels (star).
G7vaccinated with clade 2.3.4.4b vaccine
13: **Cerebrum** of G7 showing perivascular cuff (arrow),
14: Bursa of G7 showing depletion of lymphocytes, with microcysts formation (arrow) & interfollicular edema (star),
15: **Spleen** of G7 showing depletion of spleenocytes (arrow) & congested blood vessels (star),
16: **Thymus** of G7 showing depletion of thymocytes (star) & congested blood vessels (arrow),
17: **Trachea** of G7 showing depletion hyperplasia of lining epithelium (arrow) & edema of lamina propria (star)
18: **Lung** of G7 showing congested blood vessels with thickening of its wall (arrow) & interstitial edema (star).
G8control positive non-vaccinated challenged:
19: Cerebrum of G8 showing perivascular cuff (arrow),
20: **Bursa** of G8 showing depletion of lymphocytes with degeneration (star) & follicular cyst formation (arrow),
21: **Splee**n of G8 showing depletion of lymphocytes (star) with degeneration & necrosis (arrow),
22: **Thymus** of G8 showing congested blood vessels (arrow) & depletion of thymocytes (star),
23: **Trachea** of G8 showing thickening of mucosa (line), & mononuclear cells infiltration (star), edema & congested blood vessels (arrow),
24: **Lung** of G8 showing congested blood vessels (star) & perivascular edema (arrow).
G9control negative non-vaccinated non-challenged:
25: Cerebrum of negative control group showing apparently normal architectures,
26: **Bursa** of negative control group showing apparently normal architectures,
27: **Spleen** of negative control group showing apparently normal architectures,
28: **Thymus** of negative control group showing apparently normal architectures,
29: Trachea of negative control group showing apparently normal architectures,
30: **Lung** of negative control group showing apparently normal architectures.

DISCUSSION

Succeeding detection of clade 2.3.4.4b HPAI during May 2016 at Lake Uvs-Nur, Russia (Lee et al., 2017) & Qinghai Lake, China (Li et al., 2017), this clade became widespread and moved to Europe & Africa. This resulted in one of the largest reported epizootics (Alarcon et al., 2018). Clade 2.3.4.4b H5N8viruses reassorted with other AI viruses during their circulation in nature. This resulted in generation of several other H5 subtypes which belong to this clade (2.3.4.4b). This other subtypes including H5Nl, H5N2, H5N3, H5N4, H5N5 & H5N6 Viruses (Lewis *et al.*, 202l; Engelsma *et al.*, 2022; cue *et al.*, 2022 & Shi *et al.*, 2023). From all this subtupes, the novel H5Nl clade 2.3.4.4b virus that was firstly detected at Netherlands (Lewis et al., 2021)is considered to be the most critical descendant of H5N8 virus. The H5Nl virus have started the 4th large scale, intercontinental Spread causing increasing reports of mass mortality within wild birds & poultry (cue *et al.*, 2022 & Shi *et al.*, 2023).Based on recent studies, it is claimed that clade 2.3.4.4b H5Nl became dominant over 2.3.4.4b H5N8, with high prevalence & spread among wild birds & further transmission into domestic poultry at several countries (Sagong *et al.*, 2022 & Xie *et al.*, 2022).

Several introductions of clade 2.3.4.4b viruses have been reported in Egypt. The HPAI H5N8

virus clade 2.3.4.4b was introduced during 2016 through migratory birds & have become endemic. Then, several cases have been reported within local poultry (Selim *et al.*, 2017& Salaheldin *et al.*, 2018). Thereafter, according to the epidemiological data, clade 2.3.4.4b HPAI H5N8 replaced clade 2.2.l.2 H5Nl.Then, clade 2.3.4.4b H5N1turn into the most frequently reported H5 subtype in Egyp (Amer et al., 2021 & Tarek et al., 2021).Additionally, isolation & fully characterization ofclade 2.3.4.4b HPAI H5N5 virus was detected within a healthy purple heron during December 2016. (Kandeil *et al.*, 2023). Moreover during 2022, clade 2.3.4.4b HPAI H5Nl subtype was isolated from wild birds recording its introduction into Egypt. Egyptian clade 2.3.4.4b H5N1 shared genetic characteristics with HPAI H5Nl strains that were reported within Europe, North America, Asia& Africa during 2021/2022 (Mosaad et al., 2023). Afterwards, during an active surveillance, H5N1 of clade 2.3.4.4b was isolated from 1 wild pintail duck &3 domestic Pekin ducks from live bird markets at 2022 (El-Sheshny et al., 2022).

Our study aimed to assess the efficacy of 3 commercial H5 inactivated AI vaccines (clade 2.3.2, clade 2.3.4(Re-5) & clade 2.3.4.4b) within commercial broiler chickens against Egyptian newly emerged HPAI H5N1/2022 and H5N8/2019 belonging to clade 2.3.4.4b challenge on day-31. Succeeding challenge with both viruses, characteristic clinical signs for HPAI infection were observed. Clinical presentation of the disease included subcutaneous edema, cyanosis of comb & wattles, depression, loss of appetite, ocular & nasal discharges and gasping. The PM lesions included multifocal haemorrhages in proventriculus, gizzard and pancreas, hemorrhages of variable intensity in the legs, subcutaneous edema, congested and edematous lung, congestion in cerebrum and diffuse congestion within internal organs. Also, multifocal petechias in bursa & enlarged mottled spleen were common observation. Former studies recorded similar results (Tarek *et al*., 2021, El-Moeid *et al*., 2021 & Rohaim *et al*., 2021).

Clinical signs appeared to be more sever in H5N1 clade 2.3.4.4b challenged groups especially for G8 (non-vaccinated) in which, the mortality range/day was 2-3 days &mortality percent up to 100 %. This might be resulted from increased virulence of the HPAI H5N1 clade 2.3.4.4b virus.Mosaad et al., (2023) previously stated that, the amino acid analyses of HA gene& other internal proteins of Egyptian HPAI H5Nl clade 2.3.4.4b virus (A/Ibis/Egypt/RLQP/229S/2022) revealed the presence of many substitutions related to virulence in birds.

Contrasting this results, a previous study demonstrated the silent infection behavior of some clade 2.3.4.4.b H5Nl outbreaks which occurred within broilers at Italy during 2021/2022 HPAI epidemic. It was found that, 12 live birds out of 60 were tested positive for HPAI H5Nl (20%) despite showed absence of notable clinical signs (Gobbo et al., 2022).

The highest protection percent (87%) against challenge with HPAI H5N8 clade 2.3.4.4b was recorded in G2 (Re-5 clade 2.3.4 vaccine) and much lower protection 40% and 60% was recorded in G1 (2.3.2 vaccine) and G3 (2.3.4.4b vaccine), respectively. Near results were obtained by Nasssif *et al*., 2022 who recorded 85 % protection against H5N8/2021 clade 2.3.4.4b isolate. Unsatisfactory level of protection against mortality (40%) following vaccination with clade 2.3.2 vaccine was recorded. This could be due to genetic and\or antigenic dissimilarity between clade 2.3.2 and clade 2.3.4.4b.The highest protection percent (93%) against challenge with HPAI H5N1 clade 2.3.4.4b was recorded in G7 (2.3.4.4b vaccine) and very low protection 27% and 40% was recorded in G5 (2.3.2 vaccine) and G6 (Re-5 clade 2.3.4 vaccine), respectively. Our obtained results demonstrated that clade 2.3.4.4b H5N8 vaccine antigenically matched effectively with the newly emerged clade

2.3.4.4b virus and could afford high level of clinical protection.

If appropriate matched pairs of HI antigen and challenge virus are used, HI titers are thought to be indicative of protective effectiveness (Swayne, 2000). The homologus vaccinal antigen has been used in HI test regularly for evaluation of vaccine efficacy (Hafez *et al*., 2010). It is claimed that titers > 4-$\log_2$couldindicate for clinical protection. Also titers >6-$\log_2$couldindicate for viral shedding reduction as formerly stated (Kumar *et al*., 2007).

The HI test was performed using 3 vaccinal antigens (clade 2.3.2, clade 2.3.4 & 2.3.4.4b) to determine antibody response of the vaccines in serum samples collected on days 1, 10, 17, 23, 30, & 42.Clade 2.3.2 antigen showed very low reactivity with serum samples collected from different groups before or after challenge. The mean HI titers at day 30 before challenge in G1 (2.3.2 vaccine) were 0.6, 0.4, & 0.3 $\log_2$ when tested using clade 2.3.2, 2.3.4 & 2.3.4.4b antigens, respectively. This might be due to antigenic variation between this clades. Also, it could be a cause of decreased protection % in G1 (40%)& G5 (27) vaccinated with clade 2.3.2 against both clade 2.3.4.4b H5N1/2022 & H5N8/2019 challenge viruses.

By calculating the R-values% between different clades of the 3 vaccinal antigens, very low antigenic relatedness was detected between clade 2.3.2 and both clades 2.3.4 (7 %) & 2.3.4.4b (4 %). This results indicate presence of serotype difference between clade 2.3.2 and other both clades used in this study. Also it might explain the poor cross reactivity between clade 2.3.2 and clades (2.3.4, 2.3.4.4b).While the antigenic relatedness % between clade 2.3.4 & clade 2.3.4.4b was 79 % indicating little differences within the two clades.

Histopathological changes were investigated in thymus, trachea, spleen, bursa, lung, and cerebrum. The trachea in all challenged groups showed mild lesion score & similar histopathological lesions including edema and thickening of mucosa with congestion, hyperplasia of lining epithelium, mononuclear cell infiltration and edema in lamina propria. The lung showed mild to moderate lesion score. H5N8 challenged groups (G1, G2, G3& G4) showed fibrinopurulent materials in bronchi lumen, thickening in wall of blood vessels with thrombus formation, congestion in blood vessels and edema of parabronchi, while H5N1challenged groups (G5, G6, G7& G8) showed focal interstitial granulocytes infiltration, congested blood vessels with thickening in its wall, perivascular edema & interstitial edema. The cerebrum in H5N8 challenged groups (G1, G2, G3& G4) showed perivascular cuff, perineural edema, gliosis & mononuclear cells aggregation, while H5N1challengedgroups (G5, G6, G7& G8) challenged with HPAI H5N1 clade 2.3.4.4b showed perivascular cuff. Similar results were recorded formerly by Rohaim *et al*., (2021). Also, Gobbo et al., (2022) pathobiology results showed that clade 2.3.4.4b 2021/2022 HPAI H5Nl infection involved different organs with a specific respiratory tissue tropism for lung, endothelium & brain.

The bursa H5N8 challenged groups (G1, G2, G3& G4) showed sever depletion & necrosis of lymphocytes, depletion and atrophy of follicles, multiple cyst formation and interfollicular edema.H5N1 challenged groups (G5, G6, G7& G8) showed depletion of lymphocytes with degeneration, interfollicular edema and subepithelial RBCs, hyperplasia of lining epithelium, microcysts and follicular cyst formation. The spleen of H5N8 challenged groups (G1, G2, G3 & G4) showed multifocal coagulative necrosis of spleenocytes and multifocal depletion of spleenocytes. While H5N1 challenged groups (G5, G6, G7& G8) showed congestion in blood vessels, degeneration, coagulative necrosis and depletion of lymphocytes. The thymus showed similar histopathological lesions in all groups including congested blood vessels, depletion and necrosis of thymocytes. Similar results were recorded by Sánchez-González *et al*., (2020), Gobbo et al., (2022) & Gaide et al.,

(2022).This impairment effect from both H5N1 & H5N8 (clade 2.3.4.4b) to some immune organs suggests the immunosuppressive effect of both viruses.

CONCLUSION

This work was aimed to assess the efficacy of 3 different vaccines of different clades (2.3.2, 2.3.4 (Re-5) & 2.3.4.4b) against HPAI H5N1/2022 and H5N8/2019 (clade 2.3.4.4b) virus. Obtained results demonstrated that clade 2.3.4.4b H5N8 vaccine antigenically matched effectively with the newly emerged clade 2.3.4.4b H5N1 virus& could afford high level of clinical protection.

The genetic & 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. 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. Finally, continuous evaluation of the validated AI vaccines against recent field strains is substantial. Also, updating the vaccine seed strains to be closely related to the circulating field strains is recommended to obtain better protection levels.

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