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Research Article

Experimental study of the efficacy of three inactivated H9N2 influenza vaccines in broiler flocks

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Abstract

This study was carried out to evaluate the effect of different Low Pathogenic Avian Influenza (LPAI) H9N2 inactivated vaccines containing different virus strains (vaccine A, vaccine B, and vaccine C) on the productivity and immunity of 10 days-old broiler chickens. Two hundred 1 day-old Cobb broiler chicks were divided into eight groups, 25 chicks in each group. Six groups were vaccinated with Vaccine A, Vaccine B, and Vaccine C (2 groups/each vaccine) at 10 day-old. Chickens of groups 7 and 8 were kept as control groups. One group from each vaccine was challenged at 28 days old with 10^6 EID_{50}/0.2ml of A/chicken/Morocco/SF1/2016 (H9N2) virus via the oculonasal route. The remaining groups were kept unchallenged to evaluate the immune response. Chicks were sampled each week individually until 42 days old for zootechnical traits and serological evaluation. Two necropsies were done at 5 and 10 days post-challenge (DPC). Lungs and tracheas were collected for histopathology, and the virus shedding was monitored at 5, 7, 9, and 11 DPC by real-time RT-PCR. Results indicated that vaccine B provides significantly better growth performances ($P<0.05$), final body weight gain (2689.6±73.2 g), and feed conversion ratio (2.10±0.06) when compared to other vaccinated groups. During the challenge (28^{th}-35^{th} days), vaccine B showed a significant increase in antibody titer (26180±1129.1) than other vaccines. In contrast, the vaccine C group had a similar immune response to that of the control group. Both vaccines A and B were able to stop virus shedding by 11 DPC with higher mean Cq values. However, the vaccine C group continued to shed the virus until 11 DPC. Pathological examination of challenged birds revealed lesions predominantly in the respiratory tract. At 5 DPC, fibrinous sinusitis, tracheitis with fibrin plug, pneumonia, and fibrinous airsacculitis were observed. By 10 DPC, the fibrinous inflammation inceased, and only congestion in the trachea, lungs, and sinuses with thickening of air sacs were observed. Histopathology revealed lymphoplasmacytic tracheitis and congestive pneumonia. Scoring of lesions generally revealed more severe lesions at 5 DPC. Statistical analysis of both macroscopic and microscopic scores showed no significant differences between groups in both necropsies. In conclusion, vaccine B has significantly better seroconversion, better growth performance parameters, and a relatively early stop of viral shedding compared to other vaccines.

Keywords: LPAI H9N2, Broiler Chickens, Vaccine, ELISA, HI, PCR, Challenge, Pathology

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Introduction

Low pathogenic avian influenza H9N2 (LPAI H9N2) infection was reported for the first time in Morocco in 2016. The infection caused economic losses for the national poultry sector with 2-10% mortality rates in broiler chickens and 2-15% in laying hens and breeders, besides reduced performance in broilers and drop in egg production up to 80% in layers and breeders (El-Houadfi et al., 2016). The clinical signs included severe respiratory symptoms such as sneezing, coughing, rales, and respiratory distress. The lesions included congested or hemorrhagic tracheae with fibrinous exudates, fibrinous casts in bronchi, hemorrhagic lungs, pneumonia with fibrinous exudates, and airsacculitis (El-Houadfi et al., 2017).

Phylogenetic analysis of the LPAI H9N2 viruses isolated in the 2016 outbreaks showed that they belong to the G1 lineage and closely related to the viruses iso-

lated in the Middle East (El-Houadfi et al., 2016, 2017; Boumart et al., 2019; Essalah-Bennani et al., 2020). To control LPAI H9N2 infection, several measures were quickly implemented, including the immunization of all types of poultry production, along with the application of strict biosecurity measures.

After four years, applying these measures helped reduce the economic losses caused by the H9N2 LPAI virus, but it has not completely eradicated the disease (Essalah-Bennani et al., 2020). The antigenic drift of the LPAI H9N2, mainly due to the vaccination pressure, leads to vaccination failures (Park et al., 2011; Lee et al., 2016); hence, regular updating of the vaccine strain is recommended. The endemicity of the LPAI H9N2 virus in Morocco has raised several questions about the availability of vaccines that protect against the virus in broilers (Essalah-Bennani et al., 2021).

Therefore, evaluating different LPAI H9N2 vaccines used in Morocco is essential to provide an effective vaccination strategy. This study aimed to assess the efficacy and evaluate the immune response of three commercial H9N2 vaccines used in Morocco for broiler flocks against a Moroccan strain of the H9N2 (A/chicken/Morocco/SF1/2016 (Access no LT598532) (El-Houadfi et al., 2016).

Materials and Methods

LPAI H9N2 vaccines and viruses

The study was conducted at the Avian Pathology unit of Hassan II Agronomic and Veterinary Institute to evaluate the protective efficacy of three commercial inactivated oil emulsion H9N2 LPAI virus vaccines. Vaccine A has been produced on embryonated hen's eggs from the strain H9N2-D1991/2 isolated in the Middle East and inducing 256 HI titer. Vaccine B has been produced on cell culture from the strain H9N2-D3398 isolated in Morocco during H9N2 outbreak and inducing 256 HI titer/dose. Vaccine C has been produced on embryonated hen's eggs from the strain A/chicken/Morocco/ SF1/2016) containing a min titer of 10^7 EID_{50}). The challenge LPAI H9N2 A/chicken/Morocco/SF1/2016 (Accession no. LT598532) virus was kindly provided by the laboratory of the Division of Pharmacy and Veterinary Inputs.

Birds and diets

A total of 200 unvaccinated commercial one day-old chicks were used. The chicks were from the same hatch of breeders flock. Birds were assigned to 8 groups of 25 birds/group (Table 1). The birds were reared in separate rooms and had *ad libitum* access to water and a starter diet from day 1 to 21 day of age, grower diet from day 21 to 42 days of age.

Experiment design

Vaccines against H9N2 LPAI were administered by subcutaneous route on day 10 of age using the manufacturer's recommended doses. Groups 1, 3, 5, and 7 were challenged on the 28^{th} day of age via the intranasal and ocular route using 0.2 ml per bird containing 10^6 EID_{50} of the LPAI H9N2 challenge virus (A/chicken/Morocco/SF1/2016 (Accession no. LT598532). Groups 2, 4, 6, and 8 were transported to an isolated building and kept as the unchallenged vaccinated and negative control groups.

Studied traits

Key performance indicators

The birds in all groups were individually weighed to determine the body weight, and the body weight gain (BWG) was calculated at days 1 to 28, 28 to 35, and 1 to 42 intervals. The bird's feed intake and the feed conversion ratio (FCR) were determined at the same intervals.

Mortality, symptoms, and lesions

Mortality was recorded daily, and the mortality rates (%) were calculated for each group. The different group's chickens were observed daily from 1 DPC by the same observer for the clinical symptoms. Two necropsies were performed on the 5^{th} and 10^{th} DPC five birds/group randomly selected to compare the different group's lesions. Samples of lungs and trachea were fixed in 10% neutral buffered formalin, embedded in paraffin, and stained using the standard hematoxylin–Eosin (HE) stain on 5 µm sections (Suvarna et al., 2018).

Pathology and lesion scoring

Gross lesions were evaluated using a custom-designed scoring system to facilitate the comparison between the groups. The score of lesions varies from 0 to 3 depending on the severity (mild, intermediate, severe) and the lesion's extension (focal, multifocal, extensive). Histopathologic lesions of the trachea were evaluated as described previously (Bidoudan et al., 2021). A scoring system was also developed for the lungs to evaluate assess and compare lesions between groups. For this purpose, lesions were identified and scored for their severity and extent in the lung tissue.

Lesions identified as follows: proliferation of epithelial cells, mononuclear cell infiltrates, primarily lymphocytes and histiocytes, bronchus-associated lymphoid tissue (BALT) reaction, blood vessels hyperemia, edema, and hemorrhage. Each lesion varied on a scale from 0 to 3, with 0 corresponding to the absence of lesions, 1: mild/ focal histological changes, 2: moderate/ multifocal lesions, 3: severe/ extensive changes. Scores were attributed using light microscopy 10x objective (40x objective was used to confirm alterations). The macroscopic and histological scorings of the trachea and lung were carried in a single blinding setting in which groups were given codes to avoid biased evaluation (Day and Altman, 2000).

Serology

Blood samples were collected from 10 to 20 randomly selected chickens from each group on days 1, 7, 14, 21, 28, 35, and 42 of age to assess anti-H9N2 antibodies using both Enzyme-Linked Immunosorbent Assay (ELISA) and hemagglutination inhibition (HI) tests. The commercially available ID Screen® Influenza H9 Indirect ELISA kit (IDvet, Grabels, France) was used according to the protocol suggested by the manufacturer. The results were read using an ELISA reader,

Table 1: Vaccines and vaccination program for the experimental groups

Groups	Number of chicks	Vaccines[1]	Dose/bird	Challenge at 28^{th} day[2]
1	25	A	0.2 ml	Yes
2	25			No
3	25	B	0.2 ml	Yes
4	25			No
5	25	C	0.2 ml	Yes
6	25			No
7	25	Challenge control	-	Yes
8	25	Negative control	-	No

[1]Vaccine A= produced on embryonated chicken eggs from the strain H9N2-D1991/2, Vaccine B= produced on cell culture from the strain H9N2-D3398, Vaccine C= produced on embryonated hen's eggs from the strain A/chicken/Morocco/ SF1/2016)
[2]Challenge was done using LPAI H9N2 A/chicken/Morocco/SF1/2016 (Accession no. LT598532).

and the data were analyzed using ID SOFT™ software provided by IDvet. The HI test was conducted using the procedure (constant antigen and varying serum dilutions according to the OIE manual (OIE, 2019).

Virus shedding

To monitor the challenge virus shedding, tracheal swabs were collected at 3, 5, 7, 9, and 11 DPC. Swabs were also collected from unchallenged groups at 3 and 5 DPC to confirm the absence of LPAI H9N2 infection. The H9N2 challenge virus titers were determined by real-time RT-PCR (rRT-PCR). Viral RNA extraction was performed using ID Gene® Spin Universal Extraction Kit (IDvet, Grabels, France) according to the manufacturer's instructions. The RT-PCR reactions were performed on Agilent cycler using ID Gene® Influenza A Duplex (IDvet, Grabels, France) according to the manufacturer's instructions. The reaction cycle quantification values (Cq) were recorded on the provided software. Samples with a (Cq) value less than 35 were determined to be positive.

Statistical Analysis

The data of growth performance, serology, and organ index were analyzed using IBM SPSS statistics version 26 by one-way ANOVA followed by post hoc Duncan's test. The data are presented as the mean ± standard error. A p-value <0.05 was considered statistically significant.

Results and Discussion

Performance of experimental chickens

There was no significant difference in the different vaccine groups in average body weight gain (BWG) and feed conversion ratios (FCR) from day 1 until the 35th day ($P \geqslant 0.05$). However, the overall BWG and FCR (from day 1 to 42^{nd} day), birds in the vaccine B vaccinated group had significantly higher BWG and FCR ($P<0.05$) (2689.6±73.2 and 2.1±0.06, respectively) compared to the vaccine C group (2409.2±78.4 and 2.3±0.08, respectively). Whereas the use of vaccine A resulted in an FCR of (2.3±0.08) with no significant difference ($P \geqslant 0.05$) than the challenge control group (Table 2). Few data is available in the literature about the effect of H9N2 LPAI vaccines on growth performances. However, El-Sayed et al. (2011) and Safaa et al. (2011) studied the impact of different avian influenza vaccines on productivity and immune response of maternally immune broiler chicks. Though vaccinated chicks showed relatively higher body weight than non-vaccinated ones, there was no marked effect of the vaccine on the productive parameters, which agrees with our results. Furthermore, the results of this study are in line with Gharaibeh (2008), who studied the pathogenicity of LPAI H9N2 in experimentally infected broilers and specific-pathogen-free (SPF) chicks and reported decreased BWG as the most impactful pathogenic effect of H9N2 infection.

Clinical symptoms and mortality rates in different groups

The H9N2 challenge in the experimental groups resulted in the death of 1 of 30 birds (3.33%) in the vaccine A and vaccine C groups and the challenge control group (Table 3). Clinical signs reached the peak at 4 DPC, which included non-respiratory symptoms such as lethargy which was noted during the first seven days post-challenge, and conjunctivitis, which lasted for three days in vaccine A, two days in vaccine B, and six days in vaccine C and control group. The latter could be attributed to a local reaction following the ocular challenge. Ladman et al. (2008) reported mild and transient conjunctivitis that lasted from 3 to 5 DPC following a viral challenge with LPAI H7N2 via the ocular route in broilers, SPF Leghorns, and turkeys. The authors also reported a more severe impact on the eye used for the challenge than the opposite side. Furthermore, another study by Kim et al. (2013) proposed conjunctivitis as a clinical sign based efficacy testing for AIV vaccines. The authors used the biocular installation of LPAI H9N2 to challenge SPF birds and reported a significant difference between vaccinated and non-vaccinated birds, which correlated with virus reisolation testing and virus titers. Our results confirm in part this model as conjunctivitis lasted longer in control challenged group compared to vaccinated birds except for birds of group C.

Diarrhea appeared during the peak of the symptoms from 4 to 6 DPC and lasted for two days in most

Table 2: The effect of different vaccines against H9N2 virus on the growth performance of vaccinated broilers

Parameters[1]	Experimental groups[2]			
	Vaccine A	**Vaccine B**	**Vaccine C**	**Control**
1 to 28 days-old (Before the H9N2 challenge)				
BWG (g)	1388.8±31.9^{a}	1348.6±31.9^{a}	1341.8±46.1^{a}	1368.2±35.6^{a}
FCR	1.7±0.47^{a}	1.7±0.40^{a}	1.7±0.62^{a}	1.7±0.44^{a}
28 to 35 days-old (Period of H9N2 challenge)				
BWG (g)	1811.2 ±57.6^{a}	1820.0±52.4^{a}	1840.6±27.5^{a}	1771±54.7^{a}
FCR	2.3±0.08^{a}	2.2±0.06^{a}	2.1±0.03^{a}	2.3±0.07^{a}
Overall the experiment period)				
BWG (g)	2532.8±82.4^{ab}	2689.6±73.2^{a}	2409.2±78.4^{b}	2596.1±74.3^{ab}
FCR	2.3±0.08^{ab}	2.1±0.06^{b}	2.3±0.08^{a}	2.2±0.07^{ab}

[1]Abbreviations: BWG (g); body weight gain (gram), FCR; feed conversion ratio.

[2]Data presented as the average ± standard error. Different superscript letters in the same row indicate a significant difference (P<0.05).

Table 3: Frequency and severity of gross pathological lesions at 5 and 10 days post-challenge (DPC)

Lesions	Experimental groups[1]			
	Vaccine A	**Vaccine B**	**Vaccine C**	**Control**
Mortality %	3.33	0	3.33	3.33
5 DPI				
Sinusitis	(5/5) 2±0.45^{c}	(5/5) 2.6±0.4^{ab}	(3/5) 1±0.45^{a}	(1/5) 0.2±0.2^{bc}
Fibrinous sinusitis	(1/5) 0.2±0.2^{a}	(0/5) 0^{a}	(0/5) 0^{a}	(0/5) 0^{a}
Tracheitis	(4/5) 2±0.55^{a}	(4/5) 1.8±0.49^{a}	(4/5) 1.2±0.37^{a}	(4/5) 1.6±0.51^{a}
Fibrinous plugs	(1/5) 0.2±0.2^{a}	(0/5) 0^{a}	(0/5) 0^{a}	(1/5) 0.2±0.2^{a}
Pneumonia	(1/5) 0.6±0.6^{a}	(3/5) 1.4±0.6^{a}	(3/5) 1.2±0.58^{a}	(3/5) 1±0.45^{a}
Thickening of air sacs	(1/5) 0.6±0.6^{a}	(2/5) 0^{a}	(3/5) 1±0.55^{a}	(4/5) 1.2±0.37^{a}
Fibrinous airsacculitis	(1/5) 0.6±0.6^{a}	(2/5) 0.4±0.24^{a}	(1/5) 0.6±0.6^{a}	(1/5) 0.4±0.4^{a}
Conjunctivitis	(3/5) 1.6±0.68^{a}	(3/5) 1.4±0.6^{a}	(2/5) 1±0.63^{a}	(0/5) 0^{a}
Splenomegaly	(1/5) 0.4±0.4^{a}	(1/5) 0.4±0.4^{a}	(1/5) 0.4±0.4^{a}	(0/5) 0^{a}
Spleen congestion	(2/5) 0.6±0.4^{ab}	(3/5) 1±0.45^{ab}	(4/5) 1.4±0.4^{ab}	(0/5) 0^{a}
10 DPI				
Sinusitis	(2/5) 0.8±0.58^{a}	(1/5) 0.2±0.2^{a}	(3/5) 0.6±0.24^{a}	(3/5) 0.8±0.37^{a}
Fibrinous sinusitis	(0/5) 0^{a}	(0/5) 0^{a}	(0/5) 0^{a}	(0/5) 0^{a}
Tracheitis	(4/5) 1±0.32^{a}	(3/5) 1.2±0.58^{a}	(3/5) 0.8±0.37^{a}	(3/5) 1±0.45^{a}
Fibrinous tracheitis	(0/5) 0^{a}	(0/5) 0^{a}	(0/5) 0^{a}	(0/5) 0^{a}
Pneumonia	(4/5) 1±0.32^{ab}	(4/5) 1.4±0.4^{a}	(3/5) 1±0.45^{ab}	(0/5) 0^{b}
Thickening of air sacs	(3/5) 1.2±0.49^{a}	(3/5) 0.8±0.37^{a}	(3/5) 1.6±0.68^{a}	(3/5) 1.4±0.68^{a}
Fibrinous airsacculitis	(0/5)0^{a}	(0/5) 0^{a}	(0/5) 0^{a}	(0/5) 0^{a}
Conjonctivitis	(0/5) 0^{b}	(1/5) 0.2±0.2^{ab}	(3/5) 0.8±0.37^{a}	(0/5) 0^{b}
Splenomegaly	(0/5) 0^{a}	(0/5) 0^{a}	(0/5) 0^{a}	(0/5) 0^{a}
Spleen congestion	(1/5) 0.4±0.4^{a}	(1/5) 0.2±0.2^{a}	(0/5) 0^{a}	(0/5) 0^{a}

[1]Data presented as the average± standard error. Different superscript letters in the same row indicate a significant difference (P<0.05).

groups. Other studies (unpublished data) carried out in our unit also noted (consistent observation) watery diarrhea following the H9N2 challenge with this strain in broilers. The Moroccan strain of the virus is likely enterotropic however, further investigations need to be carried out to confirm this hypothesis.

Our results are in accordance with other published data (Ebrahimi et al., 2011; Aslam et al., 2015; Zhang et al., 2020) that reported digestive tract impairment following a challenge with LPAI H9N2 in poultry. Furthermore, Aslam et al. (2015) reported virus detection in the intestine at 5 DPC using immunohistochemistry. Simultaneously, Zhang et al. (2020) reported diarrhea due to ileal and caecal damage of the mucosal layer following experimental infection with H9N2 in broilers.

On the other hand, Li et al. (2018) have proven that H9N2 affects the intestinal microbiota and the intestinal mucosal barrier by promoting the proliferation of endogenous *Enterobacteriaceae*, resulting in intestinal bacterial translocation and promoting secondary bacterial infection, destruction of tight junctions of intestinal epithelial cells, and upregulating the expression of proinflammatory cytokines namely IFN-γ, IL-22, IFN-α, and IL-17A in the intestinal epithelium that promotes the inflammatory response and intestinal injury. Respiratory distress, coughing, and sneezing were noted during the first days of the trial and lasted six days in vaccine A and vaccine B groups, five days in vaccine C and control groups, while rales began at 2 DPC and lasted for 11 DPC. These results align with previously published data (Nili and Asasi, 2002; Bano et al., 2003; Gharaibeh, 2008; Swayne and Pantin-Jackwood, 2008; Bidoudan et al., 2021).

Post-challenge gross pathology

Upon necropsy on 5 DPC, the most prominent lesions in birds were found in the respiratory tract (Table 4). Hypertrophy and congestion of the spleen were reported at 5 DPC and subsided at 10 DPC necropsy. The spleen reaction during the systemic and acute phase of the disease would explain splenic congestion and hypertrophy. Similar findings were reported by Qiang and Youxiang (2011); in fact, their study also proves the immunosuppressive effect of the LPAI H9N2 virus and sheds light on the impact of the virus on the lymphoid organs, including thymus, bursa of Fabricius, and spleen. They also reported lymphoid cell depletion and necrosis, reticular cell hyperplasia, congestion, and hemorrhage following H9N2 infection, which would explain the splenomegaly and congestion noted during our study. Arafat et al. (2018) also noted atrophy in the bursa of Fabricius and thymus and the dissemination of LPAI H9N2 infected lymphocytes throughout the body via blood and lymphatic circulation.

Organs of the respiratory system affected in the 5 DPC necropsy were the sinus and the trachea and, to a lesser extent, the air sacs and lungs (Table 3), which is consistent with previous descriptions of the pathology (Bano et al., 2003; Gharaibeh, 2008; Swayne and Pantin-Jackwood, 2008; Fletcher and Abdul-Azi, 2016). Overall, unexpectedly the challenged control group showed less severe lesions than all other groups except for the thickening of air sacs (Figure 1A). Respiratory lesions, including fibrinous airsaculitis and membrane thickening, were more severe in vaccine A, vaccine C, and the control group compared to vaccine B group. Contrarily to the study carried by Hassan et al. (2017), who found slight airsaculitis in all groups, even in non-vaccinated birds against H9N2 and challenged simultaneously by H9N2 and IBV viruses group. No visual differences between the groups in the lung congestions (Figure 1B). A fibrin plug in the trachea (Figure 1C) was noted in one bird of each of the challenged control and vaccine A groups (Nili and Asasi, 2002; Swayne and Pantin-Jackwood, 2008; El-Houadfi et al., 2016). Tracheitis (Figure 1D) was more prominent in the vaccine A group, followed by vaccine B, control, and vaccine C groups. Sinusitis (Figure 1E) was more severe in vaccine B, followed by vaccine A, vaccine C, and lastly, the control group. At the 10 DPC, a decrease in the severity of all the lesions was noted. This necropsy revealed that the control group and vaccine C groups showed more conjunctivitis, thickening of air sacs, and sinusitis. As for the congestion of lungs, vaccine B and C groups had the highest scores than vaccine A and the challenged control groups. Congestion in the trachea was noted in all groups, with a slight difference between vaccine A and C groups compared to vaccine B and control groups.

Trachea and lung histopathology

A lymphoplasmacytic tracheitis was noted in all birds of all challenged groups. The severity of the lesion varied between the groups and the timing of necropsy. The lesions tend to be more severe in the first necropsy at 5 DPC (Figure 2A, B, C, D). Furthermore, the vaccine C group and the controls showed the lowest tracheitis scores at 10 DPC (Figure 2E, F, G, H). While vaccine A and B groups had the lowest average at 5 DPC. Overall, scores of lesions between groups are not significantly different (Table 4).

The results of the gross and histopathologic lesions noted during this study are in accordance with previous reports of the pathological effect of LPAIV H9N2 infection. Damage of the respiratory organs was described; gross lesions include catarrhal or fibrinous sinusitis, congestive trachea with occasionally a fibrinous exudate that is the primary cause of death of affected birds due to asphyxia, congestion in the lungs, and thickened air sacs. Histologically, congestion and lymphoplasmacytic inflammation are described in respiratory organs. Tracheitis is characterized by complete loss of cilia and depletion of goblet cells with lymphoplasmacytic infiltrates in the submucosa, lymphocytic bronchitis associated with peribronchiolar lymphocytic pneumonia (Nili and Asasi, 2002; Bano et al., 2003; Degen et al., 2006; Gharaibeh, 2008; Swayne and Pantin-Jackwood, 2008; Mahana et al., 2019; Javan et al., 2020; Bidoudan et al., 2021). Non-respiratory organs included conjunctivitis (Ladman et al., 2008; Kim et al., 2013; Mahana et al., 2019).

Table 4: Results of histopathological scoring of trachea and lungs

Organ/lesion		Experimental groups[1]			
		Vaccine A	**Vaccine B**	**Vaccine C**	**Control**
5 DPC					
Trachea		(5/5) 2.4±0.4^a	(5/5) 2.4±0.24^a	(4/5) 2.6±0. 26^a	(3/3) 3±0.0$^{a\,2}$
Lungs	Congestion	(5/5) 2.4±0.24^a	(5/5) 2±0.45^a	(5/5) 2±0.55^a	(4/4) 2.5±0.3^a
	Hemorrhage	(5/5) 2.4±0.24^a	(5/5) 2.6±0.24^a	(5/5) 2.2±0.37^a	(4/4) 2.25±0.25^a
	Edema	(5/5) 2.4±0.24^a	(3/5) 1.33±0.33^a	(5/5) 1.8±0.5^a	(4/4) 2±0.4 ^{a}
	Lymphoplasmacytic Infiltration	(5/5) 2.2±0.37^a	(5/5) 2.2±0.2^a	(5/5) 1.8±0.37^a	(4/4) 1.75±0.25^a
	Proliferation of epithelial cells	(5/5) 3±0.0^a	(5/5) 2.2±0.37^a	(5/5) 2.8±0.2^a	(4/4) 2.75±0.25^a
	Bronchitis	(2/5) 1.67±0.88^a	(5/5) 2.2±0.2^a	(4/5) 1.8±0.5^a	(4/4) 2.5±0.3^a
	Reactive BALT	(2/5) 1.2±0.73^a	(0/5) 0^a	(4/5) 1.4±0.5^a	(1/4) 0.25±0.25^a
10 DPC					
Trachea		(5/5) 0.8±0.84^a	(4/5) 0.75±0.48^a	(5/5) 0.4±0.24^a	(5/5) 0.4±0.55^a
Lungs	Congestion	(4/5) 1.4±0.4^a	(3/5) 1.25±0.5^a	(4/5) 1.8±0.5^a	(3/4) 0.75±0.25^a
	Hemorrhage	(5/5) 1.4±0.24^a	(3/5) 1.5±0.6^a	(5/5) 1.6±0.4^a	(4/4) 0.75±0.25^a
	Edema	(4/5) 1±0.31^a	(3/5) 1.67±0.33^a	(5/5) 2±0.31^a	(4/4) 1.25±0.25^a
	Lymphoplasmocytic Infiltration	(4/5) 1.2±0.37^{ab}	(3/5) 1.25±0.48^{ab}	(5/5) 2.2±0.37^a	(2/4) 0.5±0.29^b
	Proliferation of epithelial cells	(4/5) 1.6±0.5^a	(4/5) 2.25±0.48^a	(5/5) 2.2±0.2^a	(4/4) 2±0.4^a
	Bronchitis	(1/5) 0.2±0.2^a	(1/5) 0.5±0.5^a	(3/5) 1±0.45^a	(2/4) 0.75±0.48^a
	Reactive BALT	(4/5) 1.2±0.37^a	(3/5) 1.25±0.48^a	(3/5) 1.4±0.68^a	(0/4) 0^a

[1]Data presented as the average± standard error. Different superscript letters in the same row indicate a significant difference (P<0.05). [2]Few specimens were lost during the processing of tissues.

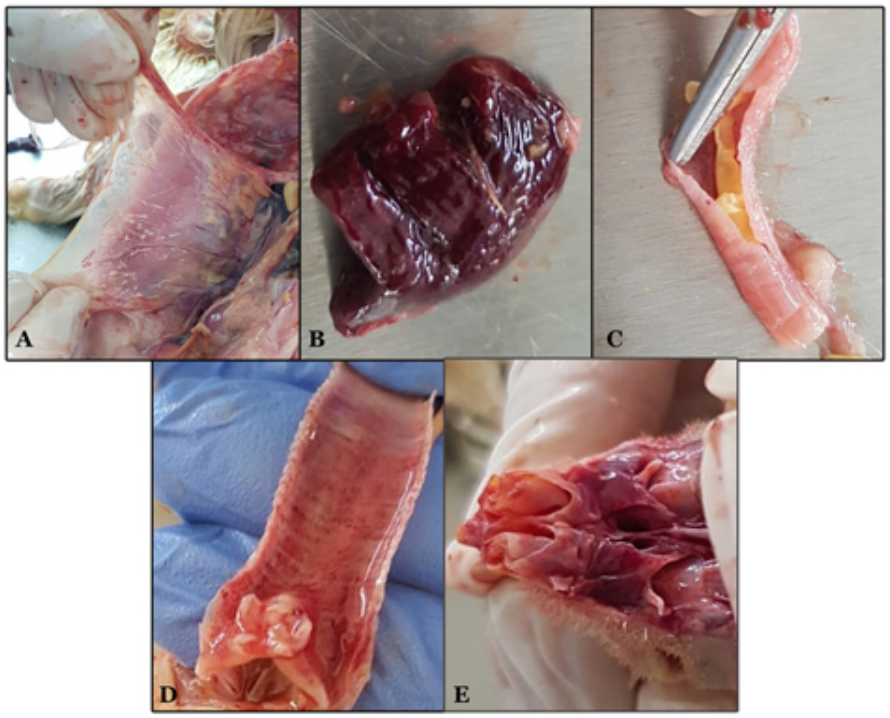

Figure 1: Gross pathological lesions at 5 DPC noted in experimentally infected broilers with LPAI H9N2. **(A)** score 2 thickening of air sacs in the vaccine A group, **(B)** score 3 congestive lungs in the vaccine C group, **(C)** score 3 fibrin plug in the lumen of the trachea in the challenged control group, **(D)** score 2 congestion in the trachea in the vaccine B group, **(E)** score 2 congestive sinusitis in the vaccine b group.

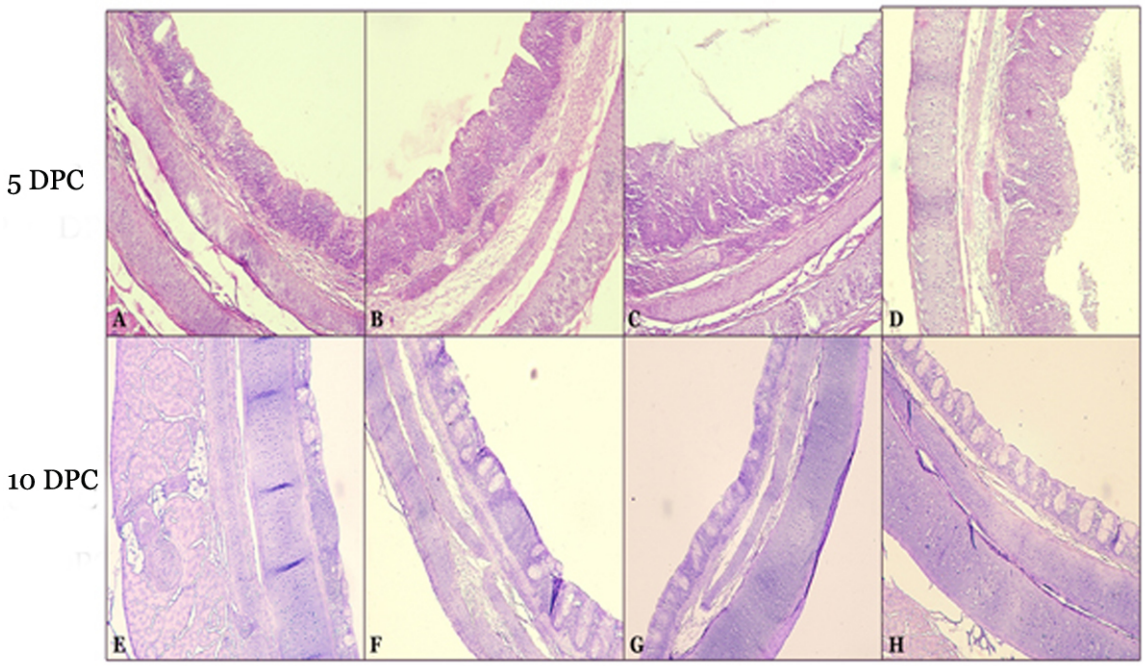

Figure 2: Lymphoplasmocytic tracheitis in challenged birds at 5 DPC (**(A)** Vaccine A- grade 2, **(B)** vaccine B-grade 2, **(C)** vaccine C– grade 2, **(D)** challenged control- grade 3) illustrating complete loss of cilia and goblet cells, lymphocytic infiltration, and hyperplasia in grade 3 tracheitis in the challenged control group. Less sever lesions in grade 2 with loss of cilia, proliferation of epithelial cells, and infiltration of the submusucosa and reduction of the number of goblet cells. At 10 DPC **(E)** vaccine A- grade 1, **(F)** vaccine B- grade 1 **(G)** vaccine C- grade 0, **(H)** challenged control- grade 0) illustrating recovery of the structure of the trachea (grade 0 in g and h and grade 1 with mild infiltration in e and f) (x100-H&E).

Serology

The average level of maternally derived antibodies specific for LPAI H9N2 was 26585.8±350.19, which decreased over time in the non-vaccinated control groups to reach values of (5135.35±745.95) by the 28th day (challenge day). There were no significant differences between ELISA antibody titers of all groups on the challenge day (P⩾0.05) (Figure 3). However, by the 35th day, the three vaccinated groups showed an increase in the ELISA antibody titers. Meanwhile, the vaccine B and vaccine A groups antibody titers were significantly (P<0.05) higher than both vaccine C and the control groups (26180±1129.1, 21774.45±1407.1, 14674.65±1823.8, and 12691.6±1041.5, respectively). However, on the 42nd day, the vaccine B and C groups showed significantly (P<0.05) the highest ELISA antibody titers than vaccine A and control groups (Figure 4A). In the unchallenged groups, at the 35th day, both vaccine A and B groups showed a significant increase in ELISA antibody titers (P<0.05) (12024.7±1575.8 and 7865±2964.1, respectively) compared to vaccine C and control groups (Figure 4B).

The vaccinated birds showed elevated HI antibody titers by the 5th week of age (Figure 5). HI antibody titers were significantly increased in vaccine A and vaccine B groups on the 35th day (2.9 and 3 Log_2, respectively). All tested vaccines developed a protective immune response. Vaccine A and in particular, vaccine B were potent in increasing antibody titers in the challenged and unchallenged groups. Despite the low homology level between vaccine A strain (Middle East isolate) and challenge virus (Moroccan isolate), the vaccine induced a high immune response, especially in the challenged groups compared to vaccine C group containing a homologous strain. This could be presumably due to the high quality of the vaccine composition and the used adjuvant effect. Previous studies evidenced that vaccine formulation can interfere with the inactivated water-in-oil vaccine performance (Kilany et al., 2016b; Colvero et al., 2017). The lower antibody titers detected in vaccine C group compared to the vaccine B group could be explained by the lower dose of antigen in vaccine C where previous studies have shown that the immune response is positively correlated with the antigen level of the vaccine (Sasaki et al., 2009; Kilany et al., 2016a).

Virus shedding

Virus shedding was monitored by quantifying the viral titers in oropharyngeal swabs collected at 3, 5, 7, 9, and 11 DPC. The vaccinated groups had a more extended shedding virus period than the control group, where the virus shedding stopped at the 9 DPC (Table 5). Virus shedding peaked at the 5th DPC in vaccine A and vaccine B groups until the 7th DPC in vaccine C and control groups. By the 9th DPC, virus excretion has been insignificantly (P⩾0.05) reduced in vaccine A and B groups compared to vaccine C groups. Based on the Cq values, vaccines A and B have rapidly reduced the virus shedding compared to the vaccine C group, which showed the highest viral shedding until 11 DPC. This

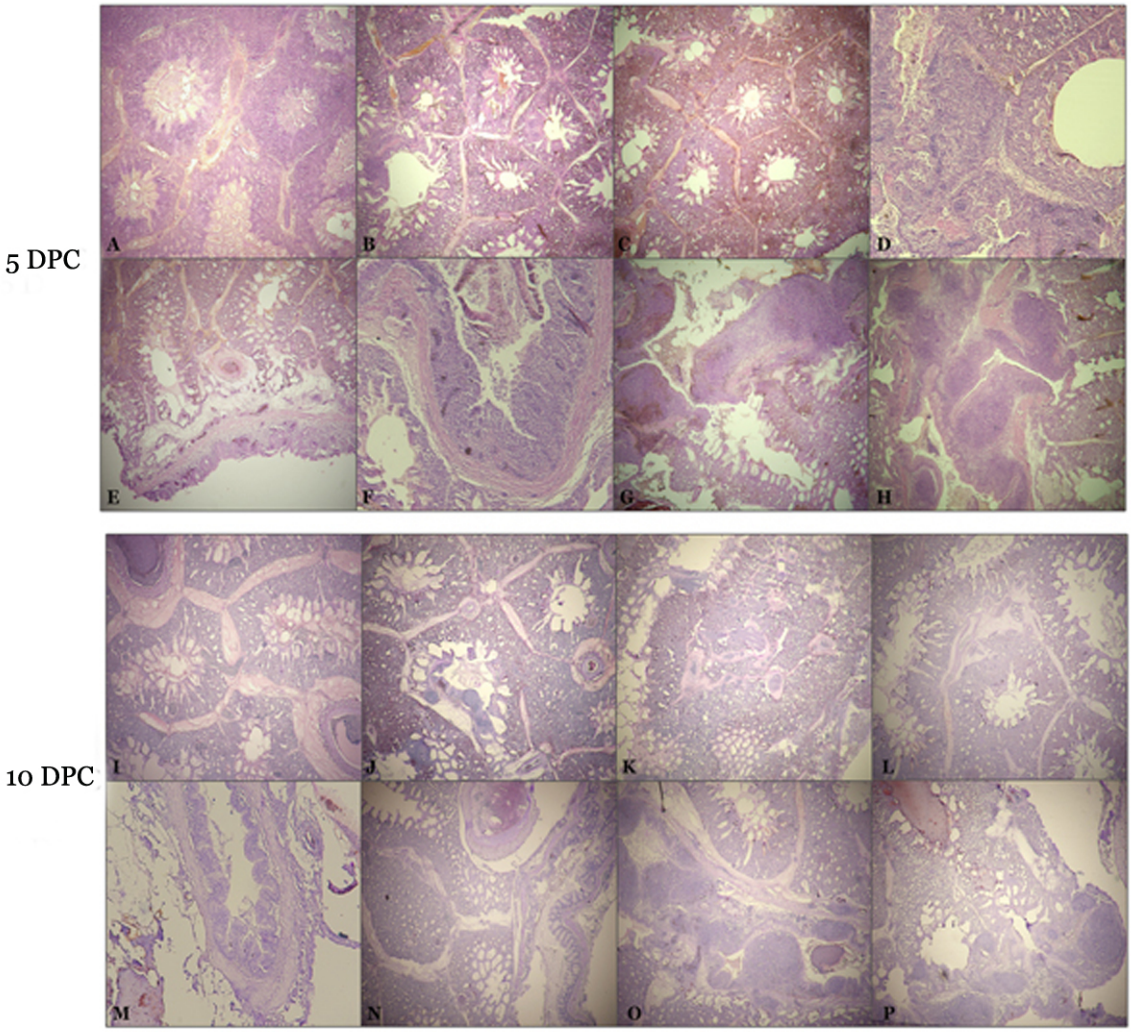

Figure 3: Histopathological features of pneumonia noted in challenged birds at 5 DPC (**A** and **E** = vaccine A, **B** and **F** = vaccine B, **C** and **G** = vaccine C, **D** and **H** = challenged control). Characterized by edema, congestion, and thickening of parabronchial walls due to proliferated epithelial cells and a severe bronchitis (**E, F, G, H**). At 10 DPC (**I** and **M** = vaccine A, **J** and **N** = vaccine B, **K** and **O** = vaccine C, **L** and **P** = challenged control) reduction of the severity of pathological lesions with persistence of mild edema and grade 1 bronchitis in group C and challenged control, (**O, P**) bronchi are normal in group A and B (**M, N**) (x40 - HE).

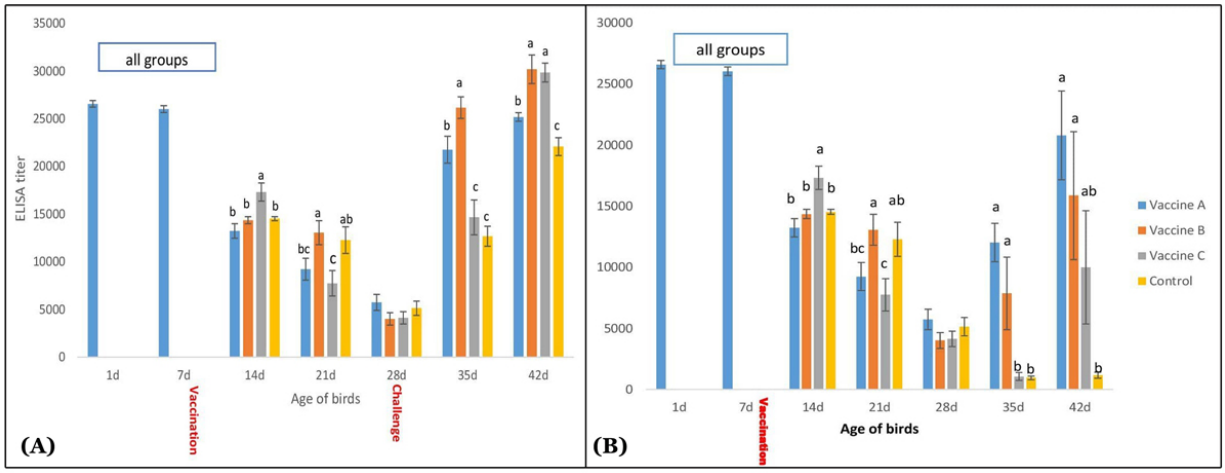

Figure 4: Geometric mean ELISA antibody titers of LPAI H9N2 in the the vaccinated and challenged **(A)** and vaccinated unchallenged **(B)** experimental groups using ID Screen® Influenza H9 Indirect test kit. Different letters at each observation time indicate significant differences ($P<0.05$).

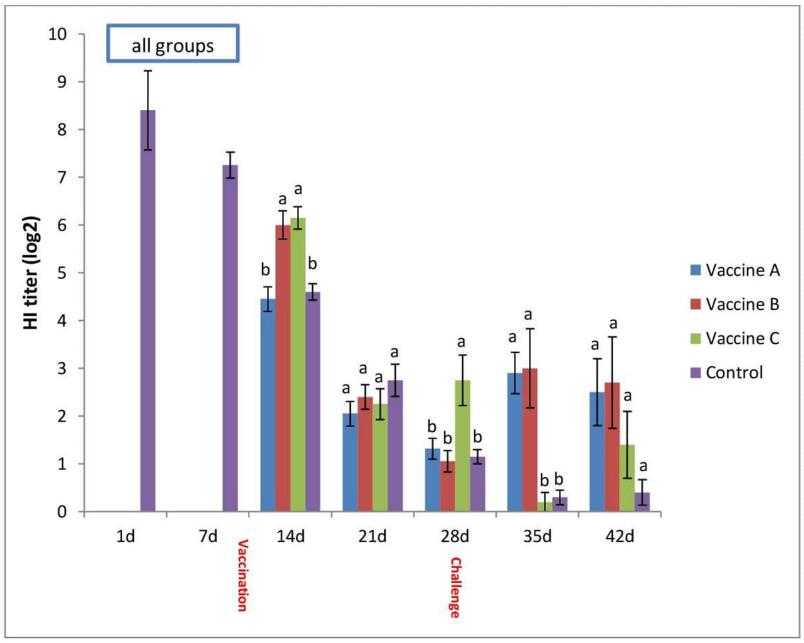

Figure 5: Mean HI titers (log_2) of H9N2 AIV antibodies in different groups throughout the unchallenged experimental period. Different letters at each observation time indicate to significant difference (P<0.05).

Table 5: Results of histopathological scoring of trachea and lungs

Days post-challenge	Experimental groups[1]			
	Vaccine A	Vaccine B	Vaccine C	Control
3	(3/5) 13.9±5.6^a	(4/5) 18.5±4.6^a	(3/5) 13.5±5.5^a	(3/5) 23±1^a
5	(5/5) 27.7±2.1^a	(5/5) 27.3±0.7^a	(5/5) 23.2±1^a	(5/5) 26.3±0.81^a
7	(4/5) 17.9±7.4^a	(2/5) 12.4±7.6^a	(4/5) 23.7±6^a	(3/5) 32.4±0.7^a
9	(1/5) 33.99	(1/5) 33.72	(3/5) 19.8±8.1^a	(0/5) 0
11	(0/5) 0	(0/5) 0	(1/5) 33.24^a	(0/5) 0

[1]Data presented are No of positive samples for H9N2 virus /total samples, and mean Cq value±SE. Different superscript letters in the same row indicate a significant difference (P<0.05).

data indicates that none of the three vaccines tested in our challenge study completely prevent virus shedding, but they can reduce the LPAI H9N2 viral shedding in broiler chicks. Previous studies have shown a prolonged influenza virus shedding in vaccinated birds (Humberd et al., 2007; Carranza-Flores et al., 2013).

The discordance between our study and previous reports is probably due to a technical issue during the inoculation. Birds tend to shake their heads after the challenge, thus reducing the challenge dose (Kim et al., 2013). Furthermore, it is well acknowledged that an effective AIV vaccine protects first against mortality and morbidity, then a significant reduction of shedding from the alimentary tract. However, virus shedding reduction from the respiratory tract is difficult to establish (Swayne and Pantin-Jackwood, 2008). Testing of the cloacal shedding could be of great interest in comparison to respiratory shedding. In addition, vaccines A and B have shown to be more effective. The oil-based LPAI H9N2 inactivated vaccines reduce but not eliminate challenge virus shedding and protect against morbidity and mortality in broilers (Vasfi Marandi et al., 2002; Tavakkoli et al., 2011).

Conclusions

The LPAI H9N2 G1- like genotype is the dominant genotype since 2016 in Morocco. The current study compared the efficacy of three different commercial inactivated vaccines under experimental conditions in broilers, including a homologous cell-culture-based vaccine against Moroccan LPAI H9N2. The vaccine B challenged birds showed no mortality, and the vaccine was able to protect birds against severe clinical signs with better post-challenge performance in terms of BWG and FCR. In terms of the challenge virus shedding, both vaccines A and B rapidly reduced the virus shedding compared to the vaccine C group and the challenged control. Although cell-culture-based vaccine C contains a homologous LPAI H9N2 strain, its protective efficacy was lower than the other two vac-

cines, probably due to the vaccine's lower antigen content.

Vaccination with H9N2 inactivated oil emulsion vaccines is a valuable tool to control LPAI H9N2 disease. Even though the vaccines cannot stop the infection, partial protection against LPAI H9N2 can be achieved concurrently with farm biosecurity. The antigenic match between the vaccine strain and circulating strains and the antigen content contributes to vaccination efficacy. Therefore, continuous surveillance of the efficacy against LPAI H9N2 vaccination and matching the vaccine strain to the field strain are valuable tools in controlling the disease, especially in endemic countries (Suarez et al., 2006; Lee and Song, 2013; Talat et al., 2020).

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Conflict of Interest.The authors declare no conflict of interest.

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