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Protection conferred by an H5 DNA vaccine against highly pathogenic avian influenza in chickens: The effect of vaccination schedules

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ABSTRACT

H5 highly pathogenic avian influenza (HPAI) viruses of the Asian lineage (A/goose/Guangdong/1/96) belonging to clade 2.3.4.4 have spread worldwide through wild bird migration in two major waves: in 2014/2015 (clade 2.3.4.4c), and since 2016 up to now (clade 2.3.4.4b). Due to the increasing risk of these H5 HPAI viruses to establish and persist in the wild bird population, implementing vaccination in certain sensitive areas could be a complementary measure to the disease control strategies already applied.

In this study, the efficacy of a novel DNA vaccine, encoding a H5 gene (A/gyrfalcon/Washington/41088-6/2014 strain) of clade 2.3.4.4c was evaluated in specific pathogen-free (SPF) white leghorn chickens against a homologous and heterologous H5 HPAI viruses. A single vaccination at 2 weeks of age (1 dose), and a vaccination at 2 weeks of age, boosted at 4 weeks (2 doses), with or without adjuvant were characterized. The groups that received 1 dose with or without adjuvant as well as 2 doses with adjuvant demonstrated full clinical protection and a significant or complete reduction of viral shedding against homologous challenge at 6 and 25 weeks of age. The heterologous clade 2.3.4.4b challenge of 6-week-old chickens vaccinated with 2 doses with or without adjuvant showed similar results, indicating good cross-protection induced by the DNA vaccine. Long lasting humoral immunity was observed in vaccinated chickens up to 18 or 25 weeks of age, depending on the vaccination schedule. The analysis of viral transmission after homologous challenge showed that sentinels vaccinated with 2 doses with adjuvant were fully protected against mortality with no excretion detected. This study of H5 DNA vaccine efficacy confirmed the important role that this type of so-called third-generation vaccine could play in the fight against H5 HPAI viruses.

1. Introduction

Since 2014, H5 highly pathogenic avian influenza viruses (HPAIV) of clade 2.3.4.4 derived from the Asian lineage (A/goose/Guangdong/1/96) have geographically extended, causing outbreaks in poultry at an unprecedented worldwide scale [1,2]. Those viruses were spread by wild bird migration in two major waves: in 2014/2015 (clade 2.3.4.4c whose nomenclature has been revised in 2020 by WHO [3,4]) and since 2016 up to now (clade 2.3.4.4b) [1–3]. Clade-b viruses are particularly virulent in waterfowl, raptor and colony-breeding seabird species, but both sub-clades are highly pathogenic in chickens, causing significant economic losses in the poultry industry [1–3]. Due to the increasing risk of these H5 HPAI viruses establishing and persisting in the wild bird population, additional protective measures must be implemented [3,5]. Therefore, vaccination could be an effective additional management tool to first-line defense strategies such as biosecurity, rapid diagnosis, and surveillance [1,5]. According to the European Council and the WOAH, vaccination may be recommended under specific conditions such as emergency vaccination or longer-term preventive vaccination in areas at high risk of disease emergence from wildlife [6–8]. To date, licensed vaccines against influenza virus infections in poultry include inactivated whole viruses and recombinant viral vectors [9,10]. For biosafety and production reasons, the inactivated vaccines are viruses that have been engineered via reverse genetics [6,9,11,12]. Combined with mineral oil emulsion, they mainly induce humoral responses, generally against haemagglutinin (HA) which is one of the major antigenic glycoproteins of the viral lipid bilayer, and little or no cellular responses [6,12–14]. The conferred protection being directly dependent on the antigenic match with the HA of circulating strains, regular updates of inactivated vaccines are required [6,11,12]. Recombinant viral

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vector vaccines are live replicating viruses (e.g. fowlpox virus (FPV), Newcastle disease virus (NDV) and turkey herpesvirus (HVT)) that express an avian influenza gene, such as HA [6,9–12]. They offer several advantages over inactivated whole virus vaccines, including their compatibility with the DIVA strategy (Differentiating Infected from Vaccinated Animals) and their ability to induce humoral as well as cell-mediated immune responses, which actively participates in conferring cross-protection against both drifted and heterologous strains of avian influenza virus (AIV) [10–12,15]. Nevertheless, DNA vaccines, considered as a third-generation vaccination approach, present several advantages making them an interesting alternative to conventional vaccines [11,16,17]. These vaccines are composed of a bacterial plasmid that encodes an antigen of interest [16,18]. They are easy, rapid, and inexpensive to produce, and their stability facilitates their conservation and does not require cold chain infrastructure and finally they also allow the DIVA strategy [14,17,19,20]. They induce cellular and humoral responses by directly transfecting antigen presenting cells (APCs) or myocytes. The transfected myocytes then release antigens in the form of proteins or peptide chains, which, in turn, activate B cells. APCs, however, can be activated either by direct transfection of the DNA vaccine or by endocytosis of antigens released from apoptotic or not, transfected myocytes. The activated APCs then migrate to stimulate $CD8^+$, and $CD4^+$ T cells, through the presentation of antigenic peptides by the major histocompatibility complexes MHC I and MHC II, respectively [14,21].

Many DNA vaccine candidates against highly pathogenic avian influenza strains H5 and H7 have shown their efficacy in inducing full or near complete clinical protection and completely or strongly reducing viral shedding [11,13,22–24]. Numerous studies have focused on the optimization, formulation, and delivery of avian influenza DNA vaccines to improve these plasmids and their immunogenicity, leading to the first conditional approval by the USDA of a DNA vaccine against highly pathogenic H5 avian influenza in chickens in 2017 [17,22,25–32].

In this study, the efficacy of a novel DNA vaccine encoding the H5 gene of clade 2.3.4.4c was evaluated in specific pathogen-free (SPF) white leghorn chickens. Different vaccination schedules followed by challenge with homologous H5 HPAI virus were performed. In addition, the duration of humoral responses up to 25 weeks and the protection following a late challenge were investigated as well as the cross-protection induced after a heterologous challenge. Finally, this study also addressed the potential transmission of the challenge virus to sentinels and the application of the DIVA strategy following H5 DNA vaccination.

2. Materials and methods

2.1. Chickens

SPF White Leghorn chickens were hatched from embryonated eggs provided by Lohmann VALO BioMedia (Cuxhaven, Germany). After hatching, all birds were housed in biosafety level 3 isolators where food and water were accessible *ad libitum* throughout the duration of the experiments. All bird experiments were conducted under the authorization and supervision of the Biosafety and Bioethics Committees at Sciensano (Brussels, Belgium; bioethics authorization no. 20190131-01 & 20190131-02) following national and European regulations.

2.2. Vaccines

AIV bulk DNA (lot#DEC-010 for all experiments) has been provided by Huvepharma with sterile diluent (exp1: lot#051210518, exp2: lot#593-84 and exp3 & 4: lot#051-130919) and sterile ENABL adjuvant (exp1 & 2: lot#105023 and exp3 & 4: lot#105035). The AIV DNA encodes the AI HA gene adapted for plasmid construct from the A/gyrfalcon/Washington/41088-6/2014 strain (clade 2.3.4.4c). The H5 DNA vaccine was prepared sterilely at a concentration of 86 µg/dose with or without adjuvant on the day of vaccination according to the protocol provided by Huvepharma and on the basis of the different vaccination schedules to be studied. The VaxLiant ENABL adjuvant, designed to ensure stable release of a DNA vaccine, was used and represented 10 % of the total volume of vaccine prepared.

2.3. Challenge strains

The H5N8 clade 2.3.4.4c HPAIV (A/Turkey/Germany-MV/AR2472/2014) was provided by the Friedrich Loeffler Institute (Riems, Germany) and was considered as homologous to the vaccine on the basis of its distribution in the same clade and subclade 2.3.4.4c. The H5N8 clade 2.3.4.4b HPAIV (A/Brahma_Chicken/Belgium/6153/June-2017) was collected in a Belgian farm and was considered as heterologous to the vaccine. The virus stock was amplified in 9-day-old embryonated SPF eggs. After incubation for 3–5 days at 37 °C, allantoic liquids were harvested and stored at −80 °C. Viral titers expressed as 50 % embryo infectious dose (EID_{50}) per mL of virus were then determined by titration of allantoic fluid based on Reed and Muench calculations [33].

2.4. Experimental design

Four animal experiments applying different vaccination schedules to characterize the protection conferred against HPAI challenge strains were carried out (Table 1). For each experiment, a mixed-sex group of birds was used. For challenge experiment n°1, n°2, n°3.1 and n°4, 10 chickens were left unvaccinated and were used as negative control group. For experiment n°3.2, 3 unvaccinated chickens a week younger than the vaccinated chickens were used as negative control for the challenge. For all experiment, blood was sampled the day before challenge (−1 day post-infection (dpi)) and at 14 dpi for measurement of humoral immunity. The sera were stored at −20 °C until further analysis. For all vaccinations and blood sampling, 25G x 5/8″ − 0.5 x 16 mm Orange needles were used. Mortality was monitored daily for two weeks post-challenge. Tracheal (TRS) and cloacal swabs (CLS) were collected at 2, 5 and 9 dpi for individual follow-up of viral excretion and stored immediately at −80 °C in brain–heart infusion (BHI) medium with antibiotics until further analysis. Surviving birds were sacrificed at 14 dpi according to national animal welfare regulations.

Animal experiment n°1 (EXP1):

Two week-old chickens were vaccinated by intramuscular (im) route with one dose of H5 DNA vaccine with adjuvant (four 50 µl injections in the breast muscles), and were boosted the same way at the age of 4 weeks. At 6 weeks of age, 10 birds/group were challenged by oculo-nasal inoculation with 100 µl (50 µl in eye + 50 µl in nostril) of inoculum containing 10^6 EID_{50} of homologous H5N8 clade 2.3.4.4c HPAIV strain.

Animal experiment n°2 (EXP2):

The first vaccination group was similar to those in EXP1 with the addition of a second vaccination schedule: two week-old chickens were vaccinated by intramuscular route with one dose of H5 DNA vaccine without adjuvant (four 50 µl injections in the breast muscles), and were boosted the same way at the age of 4 weeks. At 6 weeks of age, 10 birds/group were challenged in the same way as in EXP1 with an inoculum containing 10^6 EID_{50} of heterologous H5N8 clade 2.3.4.4b HPAIV strain

Animal experiment n°3 (EXP3):

The first vaccinated group is identical to the one presented in EXP1 and for the vaccination schedule of the next two groups, two week-old chickens were vaccinated only once by intramuscular route (four 50 µl injections in the breast muscles) with one dose of H5 DNA vaccine with or without adjuvant. At 6 weeks of age the EXP3 was divided into two parts: one concerned the challenge at 6 weeks of age and the other the long-term serology monitoring followed by a challenge at 25 weeks of age.

First challenge (EXP3.1):

At 6 weeks of age, 10 birds/group were challenged with the

Table 1
Summary of the experimental design.

Experiment	Vaccination (Group/timing/route)	Nb. of SPF chickens	Challenge		Samplings	Sentinels
			Strain	**Age (wks of age)**		
1	G1: Unvaccinated G2: Vaccine with adjuvant / 2–4 wks / im	10 10	Homologous Clade 2.3.4.4c	6	Blood: −1 & 14 DPI Swabs: 2–5-9 DPI	No
2	G1: Unvaccinated G2: Vaccine with adjuvant / 2–4 wks / im G3: Vaccine without adjuvant / 2–4 wks / im	10 10 10	Heterologous Clade 2.3.4.4b	6	Blood: −1 & 14 DPI Swabs: 2–5-9 DPI	No
3.1	G1: Unvaccinated G2: Vaccine with adjuvant / 2–4 wks / im* G3: Vaccine with adjuvant / 2 wks / im G4: Vaccine without adjuvant / 2 wks / im	10 10 10 10	Homologous Clade 2.3.4.4c	6	Blood: −1 & 14 DPI Swabs: 2–5-9 DPI	No
3.2	G1: Unvaccinated G2: Vaccine with adjuvant / 2–4 wks / im G3: Vaccine with adjuvant / 2 wks / im G4: Vaccine without adjuvant / 2 wks / im	3 6–5(i) 3 3		25	Blood: From 3 to 24 wks of age; −1 & 14 DPI Swabs: 2–5-9 DPI	
4	G1: Unvaccinated G2: Vaccine with adjuvant / 2–4 wks / im G3: Vaccine with adjuvant / 1-day-old / im G4: Vaccine with adjuvant / 1-day-old / sc	10 10 10 10	Homologous Clade 2.3.4.4c	6	Blood: −1 & 14 DPI Swabs: 2–5-9 DPI	Yes 5 vaccinated sentinels added to G2 at 1 DPI

* G2 not challenged in experiment 3.1 (Details in section 2.4).
(i) The number of SPF chickens was 6 from 3 to 24 weeks of age and 5 at 25 weeks of age due to nonspecific mortality of one chicken.

homologous H5 HPAIV strain, except for the group that received the vaccine with adjuvant at 2 and 4 weeks of age. Since the vaccination and challenge conditions of this group were similar to those carried out in EXP1, the challenge of the group immunized with the vaccine with adjuvant at 2 and 4 weeks of age was not repeated in EXP3.1.

Monitoring of long-lasting serology and challenge (EXP3.2):

At 6 weeks of age, chickens not used for the first challenge were placed together in another isolator for long-term serology monitoring. Blood samples were taken regularly up to the age of challenge for measurement of humoral immunity. At 25 weeks of age, the chickens of the different groups (3 chickens for 2 groups with only one vaccination and 5 chickens for group with prime-boost) were separated in different isolators and challenged with the homologous H5 HPAIV strain (See EXP1 conditions).

Animal experiment n°4 (EXP 4):

This experiment included three vaccination groups, the first of which is identical to the one presented in EXP1. The second vaccinated group included 1-day-old chickens immunized with one dose of vaccine with adjuvant by intramuscular route (200 µl/chicken, 100 µl in right thigh and 100 µl in left thigh). The third vaccinated group was immunized with one dose of vaccine with adjuvant at 1-day-old by subcutaneous (sc) route (200 µl/chicken, 2× 100 µl in the neck). At 6 weeks of age, 10 birds/group were challenged with the homologous H5 HPAIV strain (See EXP1 conditions) and the day after the challenge, 5 sentinels vaccinated with adjuvant at 2 and 4 weeks of age were added to the challenged group having received the same vaccination schedule to study a potential transmission of the virus.

2.5. Viral excretion

Quantification of the H5 HPAIV challenge strain in the tracheal and cloacal swabs has been conducted by quantitative real-time reverse transcription–polymerase chain reaction (qRRT-PCR) targeting the AIV matrix (M) gene according to standard procedure, as previously described [34]. Briefly, RNA was extracted by the use of the Magmax™ AI/ND 96 viral RNA kit (Ambion-Applied Biosystems, Lennik, Belgium) adapted for semi-automatic extraction using a Kingfisher magnetic particle processor (Fisher Scientific, Erembodegem-Aalst, Belgium). The AgPath-ID™ One-Step kit (Life Technologies, Merelbeke, Belgium) was used for amplification from purified RNA used as template on a LightCycler® 480 system (Roche Diagnostics, Machelen, Belgium). The run was divided into 1 reverse transcription cycle at 50 °C for 30 min, 1 denaturation cycle at 95 °C for 10 min and 50 PCR cycles (15 s at 95 °C, 34 s at 54 °C and 10 s at 72 °C) of amplification. Relative quantification was done towards a standard curve based on synthetic RNA (M1 part of the matrix gene) extracted from a dilution of an egg-titrated H5N1 high pathogenic (HP) influenza virus stock ("A/Swan/Hungary/4571/2006" strain). The Cp-cutoff value was determined at 40 during validation with a 99 % detection limit of $10^{2.7}$ viral RNA copies/ml swabs. Undetected or negative values for viral excretion were given a value of 10 (=1log10) viral RNA copies/ml swabs for statistical analysis, corresponding to a value well below the 95 % detection limit of the experimental method. Results were expressed as the number of viral RNA copies/milliliter of swabs (log10).

2.6. Measurement of humoral immune response

The haemagglutination inhibition (HI) tests were conducted according to standard procedures [12] with an adapted volume of 15 µl serum in 45 µl PBS at baseline. Two antigens were tested to quantify reactive antibodies in the sera towards the H5N8 clade 2.3.4.4c German strain (A/Turkey/Germany-MV/AR2472/2014) and the H5N8 clade 2.3.4.4b Belgian strain (A/Brahma_Chicken/Belgium/6153/June-2017). HI antibody titers were expressed as log2 and titers $> 2^3$ were considered positive.

H5 and NP antibody titers were determined using the commercial ID Screen® Influenza H5 antibody competition (FLUACH5) and ID Screen® Influenza A antibody competition (FLUACA) ELISAS from IDVet (Innovative Diagnostics,Grabels, France), respectively. The inhibition results are expressed as [100 - % inhibition] and negative values were given a value of 0.00 for calculating mean of Inhibition ± standard deviation and for graphical representation.

2.7. Statistical analysis

Statistical analyses were performed using GraphPad Prism9 software. Differences were considered significant at P-value < 0.05. The nonparametric Kruskal-Wallis test and Dunn's multiple comparisons test were used on the raw data to compare > 2 groups containing independent samples at each time point. The nonparametric Mann–Whitney test

was used on the raw data to compare 2 groups containing independent samples at each time point. These tests were chosen because the raw data didn't show a normal distribution or homogeneity of variance, and fewer than 30 animals per group and per time point were used. Nonparametric Wilcoxon test was used to compare the serological titers of paired data in each group at −1 dpi and 14 dpi.

3. Results

3.1. Clinical protection against 2 challenge strains after different vaccination schedules

All unvaccinated birds died between 2 and 5 dpi after the homologous challenge performed at 6 and at 25 weeks and between 3 and 6 dpi following the heterologous challenge performed at 6 weeks. After the homologous challenge at 6 weeks, the 1-day-old single vaccination schedule didn't provide full clinical protection as part of these birds died, regardless of the route of inoculation. These groups were therefore excluded from further virological and serological analysis. All the other vaccination schedules conferred complete protection without mortality (Table 2). Hence, vaccination at 2 weeks of age and prime-boost vaccination at 2 and 4 weeks of age, with or without adjuvant, were chosen to further investigate viral shedding after the challenges and the pre- and post-challenge humoral immune responses.

3.2. Viral shedding after different vaccination schedules and challenges

All excretion results are described in Table 3. At 2 dpi, all unvaccinated birds demonstrated tracheal excretion after homologous and heterologous challenges at 6 weeks of age. Similar results were observed for cloacal excretion, except after the heterologous challenge (EXP2) where the challenge strain was detected in 4 out of 10 chickens. The only surviving chicken at 5 dpi was after the heterologous challenge and showed tracheal and cloacal shedding but didn't survive until the next timing.

In experiment involving vaccination at 2 weeks of age with adjuvant followed by a homologous challenge at 6 weeks of age, a significant reduction in tracheal excretion was observed, with only 20 % and 30 % of birds excreting at 2 dpi and 5 dpi, respectively while at 9 dpi, no tracheal shedding was detected. No cloacal excretion was detected, regardless of the time point. At 2 dpi, the single vaccination performed without adjuvant induced a significant reduction in tracheal shedding, with excretion observed in 30 % of the birds, and complete reduction in cloacal shedding. All the remaining tracheal and cloacal swabs collected at 5 and 9 dpi were negative.

Following the homologous challenge at 25 weeks, only one on three unvaccinated chickens was alive at 2 dpi, and demonstrated high viral titers in both tracheal and cloacal swabs. The 2 groups vaccinated once exhibited tracheal shedding at 2 and 5 dpi, while no cloacal excretion was observed at these time points. No excretion was detected in both vaccinated groups at 9 dpi.

In experiment involving a vaccination at 2 weeks of age and a boost at 4 weeks, administrated with adjuvant, no shedding was detected in vaccinated chickens after the homologous challenge at 6 weeks. Birds subjected to the same vaccination schedule but exposed to heterologous strains at 6 weeks of age showed no shedding by either route and at the different time points except at 2 dpi, where tracheal shedding was detected in 6 individuals but was significantly reduced. When the challenge was performed at 25 weeks, tracheal shedding was detected at 2 and 5 dpi in 60 % of chickens but remained negative at 9 dpi. Only 1 chicken demonstrated cloacal shedding at 2 dpi. Finally, when birds were vaccinated at 2 weeks of age and boosted at 4 weeks without adjuvant, a complete protection against tracheal and cloacal shedding was observed after a heterologous challenged at 6 weeks.

3.3. Serological response of different vaccination schedules

3.3.1. After homologous challenge

One day before the challenge at 6 weeks, the birds that received a single vaccination at 2 weeks with and without adjuvant, and the birds vaccinated at 2 and 4 weeks with adjuvant presented a median HI titer ranging between 5 and 6 log2 against a homologous antigen. At −1 dpi, the group vaccinated once at 2 weeks of age without adjuvant had a significantly lower HI titers than the group that received the vaccine combined to an adjuvant at 2 and 4 weeks. HI titers of both groups vaccinated at 2 weeks of age were significantly increased between before (-1dpi) and after (14dpi) challenge, suggesting a boost effect of the challenge strain. For the group vaccinated at 2 and 4 weeks with adjuvant, no significant difference in HI titers was observed between before and after challenge, and the post-challenge titers were

Table 2
Clinical protection against mortality of vaccinated and unvaccinated SPF chickens.

Vaccinated groups			Challenge		
			6 wks of age		25 wks of age
Adjuvant	Vaccination age	Injection route	Homologous strain	Heterologous strain	Homologous strain
With adjuvant	Once at 1-day-old	Intramuscular Thigh muscles	80 %[(i)] *(EXP4)*	N.D.	N.D.
		Subcutaneous Neck	20 % *(EXP4)*	N.D.	N.D.
	Once at 2 weeks	Intramuscular Breast muscles	100 % *(EXP 3.1)*	N.D.	100 % *(EXP 3.2)*
	Prime/Boost 2–4 weeks	Intramuscular Breast muscles	100 % *(EXP 1)*	100 % *(EXP 2)*	100 % *(EXP 3.2)*
Without adjuvant	Once at 2 weeks	Intramuscular Breast muscles	100 % *(EXP 3.1)*	N.D.	100 % *(EXP 3.2)*
	Prime/Boost 2–4 weeks		N.D.	100 % *(EXP 2)*	N.D.
Unvaccinated groups			0 % *(EXP1 – EXP3.1 – EXP4)*	0 % *(EXP2)*	0 % *(EXP 3.2)*

(i) Data represent survival rate 2 weeks after challenge by oculo-nasal route with H5N8 HPAIV strain.
N.D.: not determined.

Table 3
Viral shedding of H5 HPAI strains after challenge of vaccinated and unvaccinated SPF chickens. Data are determined by qRRT-PCR on 1 ml of swabs taken at specific time after challenge and the overall arithmetic mean ± standard deviation of viral excretion was calculated per group and per time point based on all birds (negative and positive shedders).

Vaccinated groups				Challenge					
				6 wks of age				25 wks of age	
Adjuvant	Vaccination age	Injection route	DPI	Homologous strain		Heterologous strain		Homologous strain	
				TRS	CLS	TRS	CLS	TRS	CLS
With adjuvant	Once at 2 weeks	Intra-muscular	2	2/10(i) 2.24 ± 1.50(ii)*	0/10 1.00 ± 0.00	N.D.		1/3 3.73 ± 2.88(iii)	0/3 1.00 ± 0.00
			5	3/10 2.02 ± 1.10(iii)	0/10 1.00 ± 0.00			1/3 2.29 ± 1.27(iii)	0/3 1.00 ± 0.00
			9	0/10 1.00 ± 0.00	0/10 1.00 ± 0.00			0/3 1.00 ± 0.00	0/3 1.00 ± 0.01
				(EXP 3.1)				*(EXP 3.2)*	
	Prime/Boost 2–4 weeks	Breast muscles	2	0/10 1.00 ± 0.00	0/10 1.00 ± 0.00	6/10 4.28 ± 3.03*	0/10 1.00 ± 0.00	3/5 3.25 ± 1.74(iii)	1/5 1.87 ± 1.96(iii)
			5	0/10 1.00 ± 0.00	0/10 1.00 ± 0.00	0/10 0.96 ± 0.13	0/10 1.00 ± 0.00	3/5 2.76 ± 1.37(iii)	0/5 1.36 ± 0.52
			9	0/10 0.97 ± 0.36	0/10 1.00 ± 0.00	0/10 1.00 ± 0.00	0/10 1.00 ± 0.00	0/5 1.00 ± 0.00	0/5 1.00 ± 0.00
				(EXP 1)		*(EXP 2)*		*(EXP 3.2)*	
Without adjuvant	Once at 2 weeks	Intra-muscular	2	3/10 2.00 ± 1.19*	0/10 1.00 ± 0.00	N.D.		3/3 4.47 ± 1.55(iii)	0/3 0.70 ± 0.52
			5	0/10 0.91 ± 0.27	0/10 1.00 ± 0.00			2/3 3.37 ± 2.19(iii)	0/3 1.00 ± 0.00
			9	0/10 1.00 ± 0.00	0/10 1.00 ± 0.00			0/3 0.89 ± 0.25	0/3 1.08 ± 0.14
				(EXP 3.1)				*(EXP 3.2)*	
	Prime/Boost 2–4 weeks	Breast muscles	2	N.D.		0/10 1.16 ± 0.50	0/10 1.00 ± 0.00	N.D.	
			5			0/10 1.00 ± 0.00	0/10 1.00 ± 0.00		
			9			0/10 1.00 ± 0.00	0/10 1.00 ± 0.00		
						(EXP 2)			
Unvaccinated groups			2	9/9 8.87 ± 0.46 *(EXP 1)* 7/7 8.11 ± 0.82 *(EXP 3.1)*	9/9 6.82 ± 0.80 7/7 6.46 ± 1.07	10/10 7.14 ± 0.87	4/10 3.20 ± 2.90	1/1 8.91	1/1 6.76
			5	S.M.	S.M.	1/1 5.89	1/1 5.21	S.M.	S.M.
			9	S.M.	S.M.	S.M.	S.M.	S.M.	S.M.
						(EXP 2)		*(EXP 3.2)*	

(i) Frequency (number of positive/total tested chicken) of virus detection in 1 ml of swabs.
(ii) Data represent mean ± standard deviation of concentration in viral RNA copies/ml (log10) at each time point for all chickens of each group.
(iii) Statistical analysis not possible due to complete or almost complete (only 1 surviving individual) specific mortality of unvaccinated individuals due to challenge.
* Viral excretion significantly lower (P-value < 0.05) than that of unvaccinated group in the corresponding experiment.
N.D.: not determined.
S.M.: not determined because of specific mortality due to challenge.

significantly lower than the ones after a simple vaccination (Fig. 1A).

Before the challenge at 25 weeks of age, the HI titers of the group vaccinated at 2 weeks with adjuvant is significantly higher than the same group without adjuvant, but no difference between these groups and the group vaccinated at 2 and 4 weeks could be demonstrated. No significant difference between the HI titers of vaccinated groups was observed after challenge. The group vaccinated at 2 weeks without adjuvant seems to show a boost effect but cannot be statistically reliable given the too small number of individuals per group (Fig. 1B).

The quantification of H5 antibodies by ELISA test supported the HI results. The groups vaccinated once at 2 weeks of age with or without adjuvant had significantly lower H5 antibody titers than the group vaccinated at 2 and 4 weeks with adjuvant but after challenge, no significant difference between the vaccinated groups was observed. Between before and after challenge, a significant increase in H5 antibody titers was demonstrated in both groups vaccinated once at 2 weeks (Fig. 2A). No significant difference was observed in H5 antibody titers of the vaccinated groups before and after the challenge at 25 weeks of age and all vaccinated birds were positive for H5 antibodies after the challenge (Annex 1).

Finally, as expected, no NP antibodies were detected before challenge in the different groups. After the homologous challenge, the group vaccinated at 2 and 4 weeks of age remained NP negative, while NP antibodies were detected in the 2 groups vaccinated once at 2 weeks of age with and without adjuvant suggesting an effect of the challenge strain replication. However, there was no significant difference between these two groups (Fig. 3A). After the challenge at 25 weeks of age, all vaccinated chickens were positive for NP antibodies, with no significant

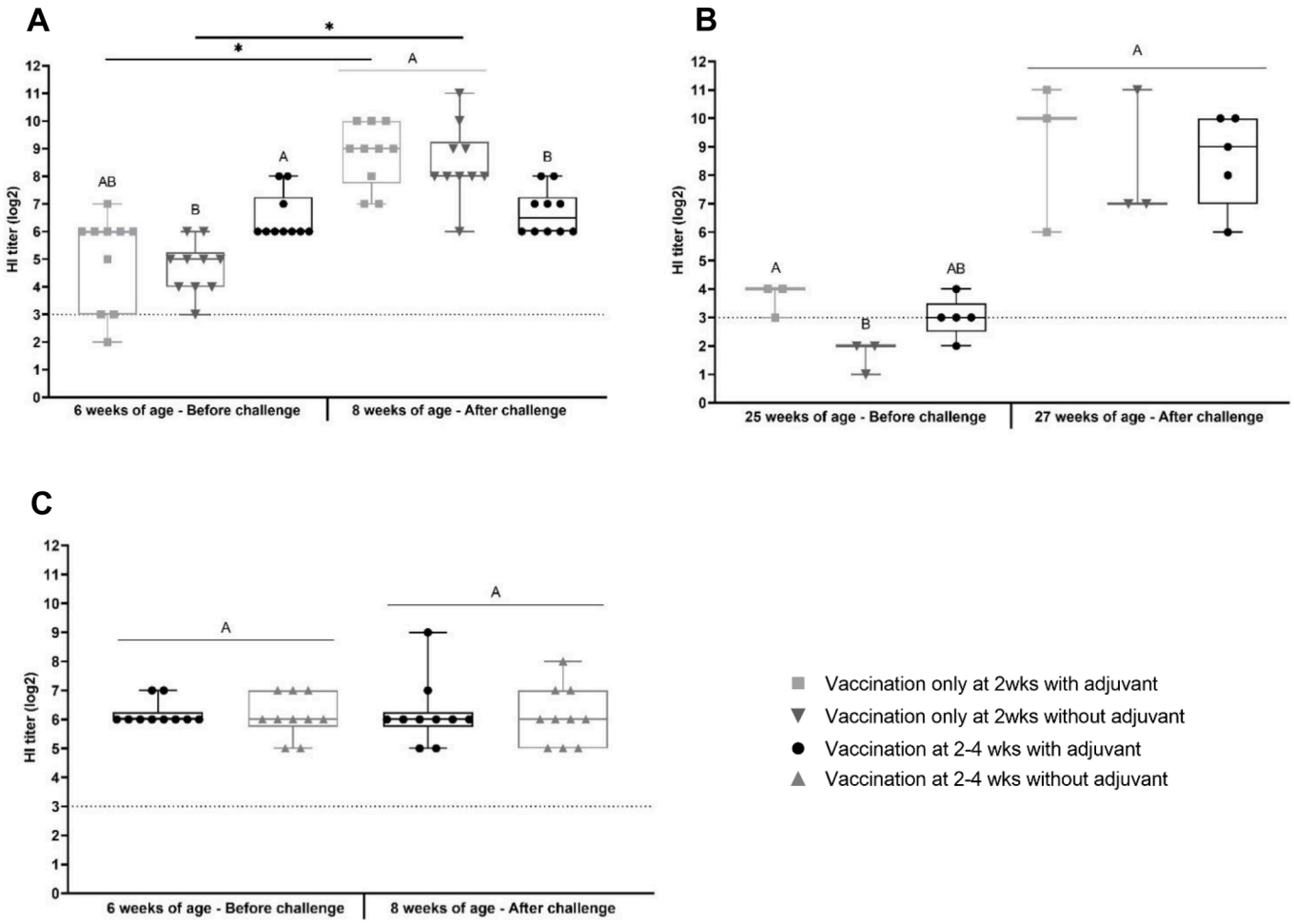

Fig. 1. H5-specific antibody titer measured by HI test for vaccinated SPF chickens before and after challenge. Homologous strain (A/Turkey/Germany-MV/AR2472/2014) H5N8 clade 2.3.4.4c was used for challenge and as antigen at 6-week-old (A) and at 25-week-old (B) and heterologous strain (A/Brahma_Chicken/Belgium/6153/June-2017) H5N8 clade 2.3.4.4b was used at 6-week-old for challenge and as antigen (C). Results are presented as boxplots with dots representing individual biological replicates. The HI titers correspond to the last dilution showing an inhibition of haemagglutination of 4 haemagglutination units of HA antigen. Different capital letters indicate a significant difference (P-value < 0.05) of HI titers between groups at a given time point (before or after the challenge), while (*) indicates a significant difference (P-value <0.05) between -1 dpi and 14 dpi times points for each group.

difference between the vaccinated groups (Annex 2).

3.3.2. *After heterologous challenge*

Before the challenge at 6 weeks of age, HI titers against the heterologous antigen remained similar between the two groups vaccinated twice and no difference between HI titers at −1dpi and 14 dpi was observed for the two groups, indicating the absence of measurable challenge-induced stimulation (Fig. 1C). The results of the detection of H5 antibodies by ELISA test support the HI data since no significant difference was observed between the double-vaccinated groups before and after the heterologous challenge and no increase in the level of H5 antibodies in both groups following the challenge (Fig. 2B). After the heterologous challenge, only one chicken in each double-vaccinated group presented a seroconversion for the NP antigen but no difference in NP antibody titers between −1dpi and 14 dpi could be demonstrated, suggesting the weak H5N8 HPAIV replication in both double-vaccinated groups (Fig. 3B).

3.4. *H5-specific antibody persistence*

Seroconversion measured by HI test using homologous HI antigen was detected in all vaccinated groups from 4 weeks of age and remained detectable up to 18 weeks. Between 19 and 25 weeks of age, seroconversion was detected in the group vaccinated at 2 and 4 weeks of age with adjuvant at all timings as well as in the single vaccinated group with adjuvant (Fig. 4). From 5 weeks until 24 weeks, the group vaccinated at 2 and 4 weeks of age with adjuvant was significantly higher than the group vaccinated once at 2 weeks without adjuvant, excepted at 21 and 22 weeks where no significant difference was observed between the different vaccinated groups.

3.5. *Protection, shedding and serology of vaccinated sentinels*

To evaluate the viral transmission between vaccinated birds, 5 sentinel birds having received a double-vaccination with adjuvant were co-housed at 1 dpi with 10 chickens similarly vaccinated and infected with H5N8 clade 2.3.4.4c HPAIV (A/Turkey/Germany-MV/AR2472/2014) strain. A full protection against mortality has been observed during the 14 days observation period with no excretion detected in the sentinels at the different timings studied.

Seroconversion was detected at 6 and 8 weeks of age by the HI test in vaccinated sentinels. Before to contact with challenged individuals, 100 % of the sentinels showed a positive HI titer, while 80 % seroconversion was observed after contact, suggesting that the challenge had little or no

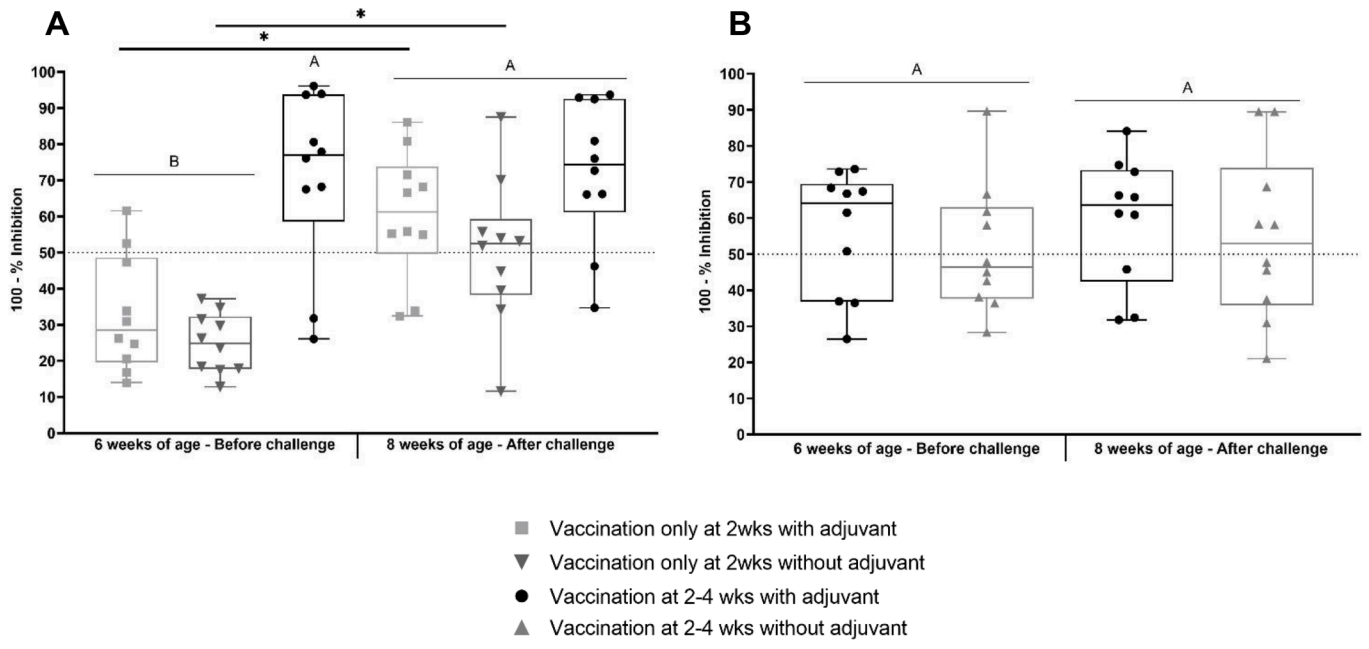

Fig. 2. H5-specific antibody titer measured by competitive ELISA (FLUACH5) for vaccinated SPF chickens before and after challenge at 6-week-old. The homologous strain (A/Turkey/Germany-MV/AR2472/2014) H5N8 clade 2.3.4.4c (A) and heterologous strain (A/Brahma_Chicken/Belgium/6153/June-2017) H5N8 clade 2.3.4.4b (B) were used for challenge. Results are presented as boxplots with dots representing individual biological replicates. The commercial ELISA was performed following the manufacturers recommendations and the sample was positive if [100 - % Inhibition] > 50 %. Different capital letters indicate a significant difference (P-value < 0.05) of Inhibition between groups at a given time point (before or after the challenge), while (*) indicates a significant difference (P-value <0.05) between -1 dpi and 14 dpi times points for each group.

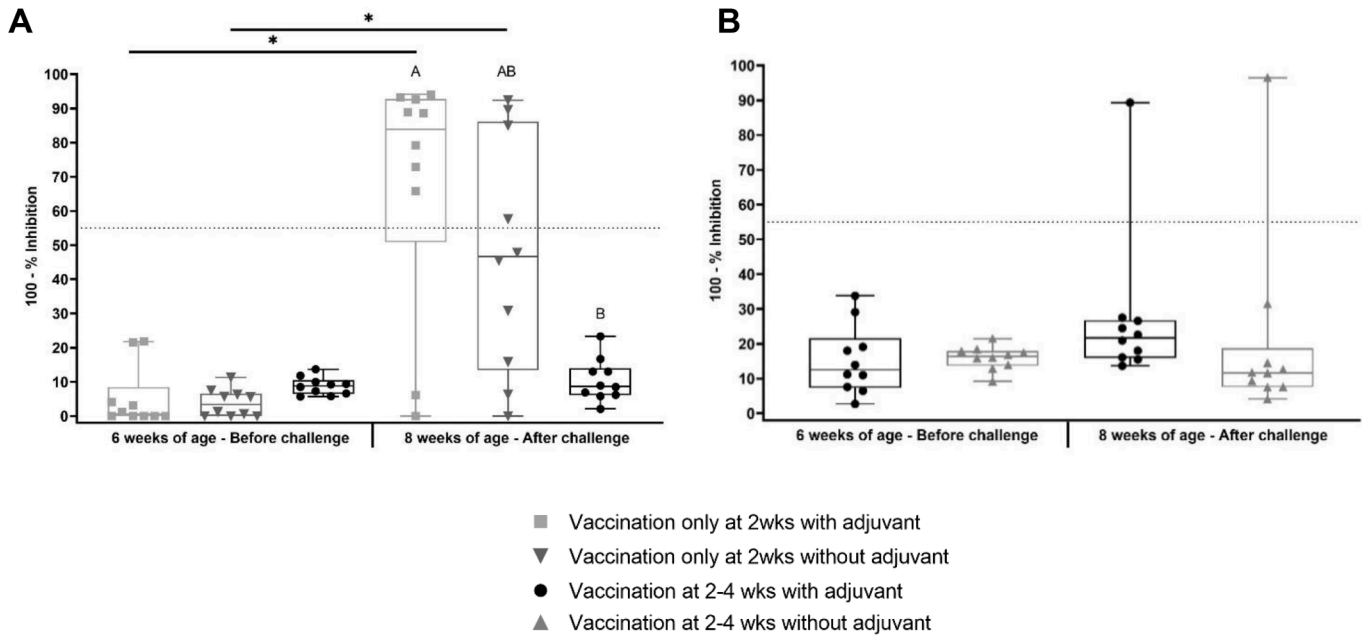

Fig. 3. NP-specific antibody titer measured by competitive ELISA (FLUACA) for vaccinated SPF chickens before and after challenge at 6-week-old. The homologous strain (A/Turkey/Germany-MV/AR2472/2014) H5N8 clade 2.3.4.4c (A) and heterologous strain (A/Brahma_Chicken/Belgium/6153/June-2017) H5N8 clade 2.3.4.4b (B) were used for challenge. Results are presented as boxplots with dots representing individual biological replicates. The commercial ELISA was performed following the manufacturers recommendations and the sample was positive if [100 - % Inhibition] > 55 %. Different capital letters indicate a significant difference (P-value < 0.05) of Inhibition between groups at a given time point (before or after the challenge), while (*) indicates a significant difference (P-value <0.05) between -1 dpi and 14 dpi times points for each group.

effect. However, H5 antibody detection demonstrated that 40 % of vaccinated sentinels were positive before contact, and 60 % after contact. No NP antibodies were detected in sentinels post-contact, suggesting the absence of transmission and viral replication (Table 4).

4. Discussion

Since the fall of 2021, Europe has experienced the most devastating outbreaks of HPAI viruses which severely affected both poultry and captive birds, as well as wild birds [3]. The loss of seasonality and

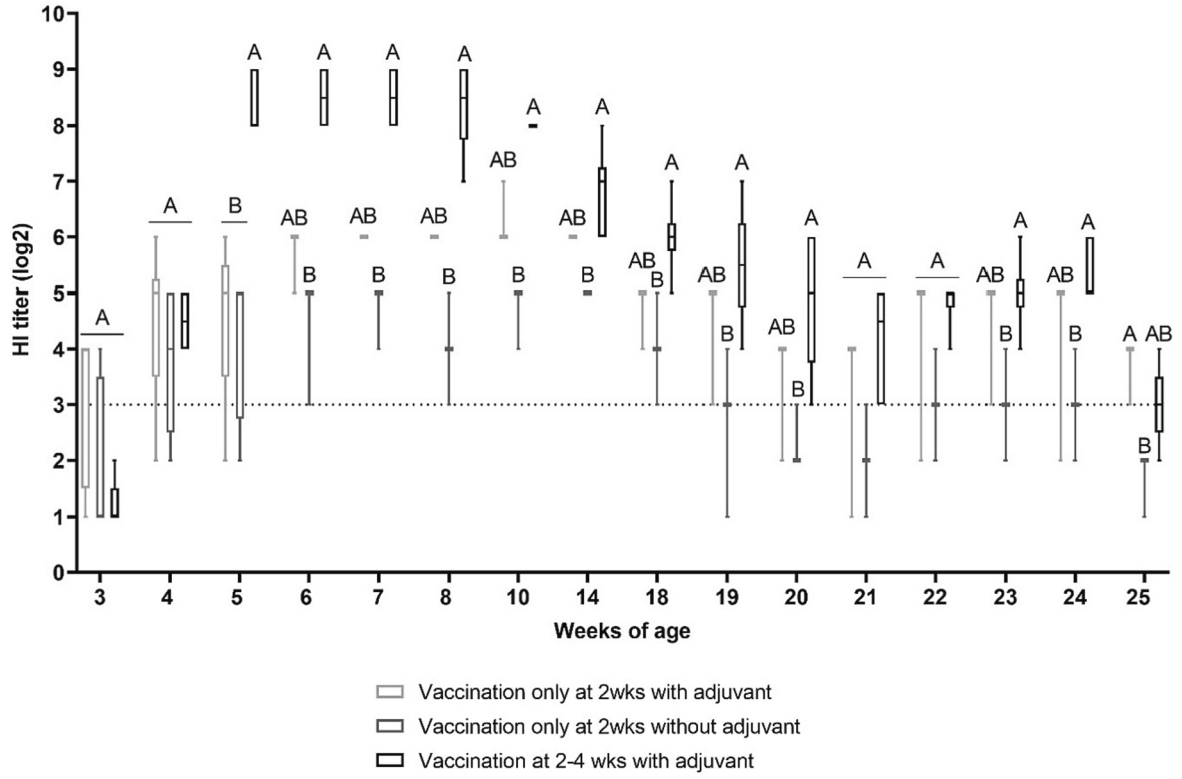

Fig. 4. H5-specific antibody persistence after different vaccination schedules. Haemagglutination inhibition antibody titers were monitored from 3 to 25 weeks of age for vaccinated chickens with H5N8 clade 2.3.4.4c HPAIV (A/Turkey/Germany-MV/AR2472/2014) as antigen and results are presented as boxplots. The HI titers correspond to the last dilution showing an inhibition of haemagglutination of 4 haemagglutination units of HA antigen. Different capital letters indicate a significant difference (P-value < 0.05) of HI titers between groups at a given time point.

endemization of H5 clade 2.3.4.4b HPAI viruses observed in the European wild bird population increased the pressure on poultry farms as never before. In this context, the implementation of vaccination in certain susceptible areas is increasingly considered as a complementary measure to existing disease control strategies. The limitations of currently used inactivated vaccines against HPAIV due to among others the rapid antigenic evolution of current HPAI viruses underline the need to develop new generation vaccines that can be easily updated to protect against circulating strains as well as a wide spectrum of different H5HP clades or subclades circulating. The objectives of the present study were to characterize the protection and cross-protection conferred by a new H5 DNA vaccine candidate, the duration of induced immunity, and to determine the optimal conditions of application.

The H5 DNA vaccine was able to induce complete protection against mortality following a homologous challenge with a clade 2.3.4.4c HPAIV at 6 weeks of age in birds vaccinated and boosted at 2 and 4 weeks of age. The complete clinical protection was also demonstrated after a single dose of vaccine at 2 weeks with and without adjuvant. These results are consistent with previous studies that showed full or high level of clinical protection in animals immunized with a single dose of H5 DNA or after a prime-boost vaccination against HPAI challenge strains closely related to the H5 vaccine [13,22,30,35]. The H5 HA sequences of the vaccine (A/gyrfalcon/Washington/41088–6/2014) and the clade 2.3.4.4c HPAI challenge strain (A/Turkey/Germany-MV/AR2472/2014) used in the present study, shared 98,35 % homology. As hatchery vaccination has become a common practice in the poultry industry to control economically important poultry diseases [36,37], the protection conferred by a single intramuscular or subcutaneous vaccination at one day of age was also evaluated. Complete clinical protection could not be achieved against a challenge with a homologous strain at 6 weeks. This is consistent with previous results reported after DNA vaccination against infectious bursal disease virus of 1-day-old SPF and broiler chickens however carrying maternal antibodies [38,39]. The poor protection conferred by 1-day-old vaccination could be explained by the immaturity of the adaptive immune system at that age, impairing the development of the immune responses initiated by DNA vaccines [36,40,41].

In addition to inducing clinical protection, effective vaccination should prevent shedding to reduce the risk of transmission. Viral shedding was therefore assessed for the vaccination schedules that induced full clinical protection. The results showed that a boost at 4 weeks was required to fully prevent both tracheal and cloacal excretion after a homologous challenge at 6 weeks, since viral excretion, although reduced, could be still detected after a single vaccination at 2 weeks confirming previous studies [22,30]. Moreover, serological results showed no increase in HI and H5 antibody titers following the challenge of the group boosted and the absence of NP antibodies detection, while the latter were detected in the challenged group vaccinated with a single dose. As the viral replication was not completely blocked after a single dose of vaccine at 2 weeks (with and without adjuvant), a higher amount of viral antigen will be available to stimulate the immune system. This could consequently explain the significant increase observed in serological responses to H5 and NP antigens. The HI titers ranging between 6 and 8 log2 in the boosted group before the challenge was furthermore consistent with the complete clinical protection observed after the challenge. This indicates that vaccination, especially the booster, seems to prevent systemic infection and thus confirms the excretion results. Regarding the transmission study performed after a homologous challenge at 6 weeks, although that 20 % of the challenged boosted group showed positive NP serology post-challenge, this didn't induce transmission in view of the absence of viral shedding and NP antibody detection in the vaccinated sentinel birds. This suggests that the application of DNA vaccination could not promote silent circulation of H5 HPAIV in vaccinated flocks if there is a good match between the H5

Table 4
Serological responses (HI and ELISA tests) of vaccinated challenged SPF chickens and vaccinated sentinels. For HI test, data represent mean ± standard deviation of HI titer (log2), which corresponds to the last dilution showing an inhibition of haemagglutination of 4 haemagglutination units of HA antigen. For ELISA tests, data represent mean of Inhibition ± standard deviation determined at specified time of age. The commercial ELISA was performed following the manufacturers recommendations and the sample was positive if [100 - % Inhibition] > 50 % and 55 % for competitive ELISA-H5 and ELISA-NP, respectively.

Tests	Groups	Challenge at 6 wks of age + added sentinels at 1dpi	
		Titer at − 1dpi	Titer at 14 dpi
HI test using H5N8 clade 2.3.4.4c (A/Turkey/Germany-MV/AR2472/2014) as antigen	Vaccination at 2–4 wks with adjuvant Intramuscular route	10/10[(i)] 8.7 ± 1.4[(ii)]	10/10 5.9 ± 1.2
	Sentinels	5/5 8.2 ± 0.8	4/5 4.8 ± 1.1
Competitive ELISA-H5	Vaccination at 2–4 wks with adjuvant Intramuscular route	8/10 55.4 ± 14.2	5/10 52.9 ± 19.4
	Sentinels	2/5 42.8 ± 21.0	3/5 46.2 ± 17.2
Competitive ELISA-NP	Vaccination at 2–4 wks with adjuvant Intramuscular route	0/10 10.6 ± 7.0	2/10 17.8 ± 25.8
	Sentinels	0/5 10.2 ± 6.4	0/5 2.5 ± 5.6

(i) Frequency (number positive/total tested chickens).
(ii) Data represent mean ± standard deviation.

vaccine antigen and the antigens circulating in the field. Nevertheless, given the low seroconversion rate for post-challenge NP antibodies, the implementation of DNA vaccination should be combined with the optimization of currently used NP ELISA and the development of new DIVA strategies to ensure efficient serological surveillance [42].

To assess the cross-protection induced by the H5 DNA vaccine, its efficacy was evaluated against the dominant subclade 2.3.4.4b circulating today, which emerged in 2016 as a result of the continued evolution and diversification of the A/Goose/Guangdong/1/1996 lineage observed over the past decade. The results indicated that prime-boost vaccination with and without adjuvant induced complete clinical protection, and nearly complete reduction of shedding against a heterologous challenge strain at 6 weeks of age. Furthermore, the heterologous challenge of the groups boosted at 4 weeks didn't increase the HI and H5 antibody titers 14 days post challenge. Before to challenge, HI antibody titers were positive against the heterologous strain (A/Brahma_Chicken/Belgium/6153/June-2017) for both adjuvanted and non-adjuvanted boosted groups as observed against the homologous strain at 6 weeks. These results suggest that H5 DNA vaccination could offer protection against a broad spectrum of H5 HPAI strains. However, the limited viral excretion observed after the heterologous challenge in the adjuvanted group, along with the seroconversion to NP antibodies in one individual in each group indicate an incomplete cross-protection conferred by the DNA vaccine. Further studies should therefore be carried out to examine the protection induced against more antigenically distant strains such as clade 2.3.2.1 HPAIV currently circulating in Asia [43].

One of the key requirements for successful vaccination with new generation vaccines is the induction of long-term immunity, especially in long-lived poultry such as breeders and layers. While H5 seroconversion was demonstrated in all vaccinated chickens between 4 and 18 weeks of age, the chickens immunized at 2 weeks without adjuvant became seronegative between 19 and 21 weeks of age. Although the use of the adjuvant did not have an effect on the excretion after a challenge at 6 weeks of age, these results suggest that the adjuvant may have a role in maintaining the vaccine-specific humoral response over time when a single dose is administered at 2 weeks. On the other hand, a reduced H5 antibody response in all vaccinated birds was observed from 14 weeks of age. This may be related to the beginning of the laying period, as it has been shown that cell-mediated immune responses can be significantly reduced in 16 to 20-week-old laying chickens before increasing gradually from 24 weeks of age [44,45]. It has been established that an efficient B-cell activation requires interaction with TH2-cells via cell-mediated immunity [46,47], so a reduced cellular response during the laying period could alter B-cell activation, and thus antibody production. Unexpectedly, the serological titers measured at 25 weeks of age dropped compared to the values obtained at 24 weeks of age for all groups. Nevertheless, complete protection against mortality was observed after a homologous challenge at 25 weeks, independent of the use of an adjuvant and the vaccination schedule applied. It can therefore be hypothesized that the clinical protection was dependent, at least partially, on vaccine-induced cell-mediated immune responses, which might be complementary to the limited humoral immunity measured at that age. Nevertheless, although the overall excretion was reduced in all vaccinated groups compared to the unvaccinated group, the shedding was not completely prevented by any of the vaccination schedules tested. This could likely be explained by the lower HI and H5 antibody titers observed in all vaccinated group before to challenge. Furthermore, the detection of NP antibodies in all vaccinated and challenged groups confirmed a reduced protection of 25-week-old chickens, indicating that vaccine-induced immunity is not maintained over time and may require additional booster. Additional studies on larger cohorts will be needed to confirm these results.

In this study, the H5 DNA vaccine was shown to provide cross-clade protection by preventing mortality as well as reducing the viral replication in the respiratory and intestinal tracts and inducing a long-lasting antibody response under certain controlled conditions. Although these results are encouraging, further studies are needed to collect field data but also to fully understand the immune mechanisms triggered by these new vaccine technologies and determine the correlates with protection. Finally, it is important to highlight that the use of AI vaccines in the field requires adequate surveillance to prevent silent circulation in vaccinated flocks which, combined with the evolution of influenza strains through antigenic drift, could lead to the emergence of variant viruses. The development of adequate and sufficiently specific methods to differentiate vaccinated and infected birds is therefore of critical importance to ensure optimal surveillance and control of the disease.

CRediT authorship contribution statement

Julie Valentin: Methodology, Investigation, Writing – original draft, Writing – review & editing. **Fiona Ingrao:** Writing – review & editing. **Fabienne Rauw:** Conceptualization, Methodology, Supervision, Project administration. **Bénédicte Lambrecht:** Conceptualization, Methodology, Writing – review & editing, Supervision, Project administration,

Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary material

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