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Efficacy of a plant-produced clade 2.3.4.4 H5 influenza virus-like particle vaccine in layer hens

Celia Abolnik[a,*], Martha M. O'Kennedy[a,b], Mark-Anthony Murphy[a], Daniel B.R. Wandrag[a]

[a] Department of Production Animal Studies, Faculty of Veterinary Science, University of Pretoria, Onderstepoort, Pretoria, South Africa
[b] Next Generation Heath Cluster, Council for Scientific and Industrial Research (CSIR), Brummeria, Pretoria, South Africa

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ABSTRACT

Outbreaks caused by Goose/Guangdong H5 highly pathogenic avian influenza (HPAI)-lineage viruses continue to occur in unprecedented numbers throughout Eurasia, the Middle East and Africa, causing billions of dollars in economic losses and the deaths or destruction of hundreds of millions of poultry, and pose a zoonotic threat. Here, a recombinant virus-like particle (VLP) displaying the hemagglutinin protein of a clade 2.3.4.4b H5N8 HPAI strain was produced in tobacco plants (*Nicotiana benthamiana*) and its immunogenicity with four commercial adjuvants was compared in layer hens. After two immunizations with 250 hemagglutinating unit doses, hens that received intramuscular injections of H5 VLPs formulated with Emulsigen D, Emulsigen P or Montanide ISA 71VG seroconverted with hemagglutination inhibition geometric mean titres (GMTs) of 7.3 $\log_2$ (± 1.17), 8 $\log_2$ (± 1.08) and 7.9 $\log_2$ (±1.07), respectively, but the GMT of hens inoculated by eye drop with VLPs plus Carbigen only reached 2.05 $\log_2$ (± 1.64). The H5 VLP plus Emulsigen-P vaccinated hens and a sham-vaccinated group were then challenged with a high dose of the homologous H5N8 HPAI virus. Vaccinated hens were completely protected and showed no clinical signs, whereas the sham-vaccinated birds all died within 3–4 days. The average oropharyngeal shedding in vaccinated hens was reduced by 3,487-fold and 472-fold on days 2 and 3 post challenge, respectively, whereas average cloacal shedding was reduced by 2,360,098-fold and 15,608-fold on days 2 and 3, respectively, compared to the sham-vaccinated controls. No virus was detected in the vaccinated hens after day 8 post challenge, and the plant-produced H5 VLP vaccine completely prevented H5N8 HPAI virus transmission to eggs. This highly efficacious, safe and non-toxic plant-produced H5 VLP vaccine with DIVA (differentiation of infected from vaccinated animals) capability could be rapidly produced with a yield of at least 85,000 doses per Kg of plant leaf material.

1. Introduction

The goose/Guangdong (Gs/GD) lineage of highly pathogenic avian influenza (HPAI) H5-subtype viruses emerged in poultry in southern China in 1996, subsequently diversified into multiple phylogenetically and antigenically distinct clades, and repeatedly reassorted with low pathogenicity avian influenza viruses circulating in the wild bird reservoir in a continuing evolutionary process (Lee et al., 2021; Bertran et al., 2021). Gs/GD clade 2.3.4.4 H5 HPAI viruses were endemic in China since 2008 (Tatár-Kis et al., 2019), but in 2014 the virus jumped to migrant palearctic waterfowl populations, spreading within months from Southeast Asia to Siberia to the Middle East, Europe, Africa and North America (Lee et al., 2021; Abolnik et al., 2019). Unprecedented outbreaks have continued throughout Eurasia, the Middle East and Africa, causing billions of dollars in economic losses and the deaths or destruction of hundreds of millions of poultry and endangered bird species too (Abolnik et al., 2019; Pénzes et al., 2019). Spillover of Gs/GD H5 HPAIV to humans have a diagnosed case fatality rate of 53%, but thus far the viruses lack properties required for sustained human-to-human transmissibility (Ross et al., 2019).

Most countries opt to control sporadic epidemics of HPAI in poultry through surveillance, strict biosecurity, rapid diagnosis and the slaughter of infected and at-risk flocks to prevent the lateral spread of the virus (Kapczynski et al., 2017). However, since Gs/GD H5 lineages became endemic in poultry in China, South Korea, Indonesia, Vietnam, Egypt, and Bangladesh, these nations have applied vaccination to reduce transmission and minimise economic losses from HPAI infection (Sawitri Siregar et al., 2007; Kwon et al., 2021). The vast majority of vaccines used globally to combat H5 HPAI are prepared from chemically inactivated whole viruses propagated in eggs or cell cultures in high biocontainment facilities (Swayne, 2009). The hemagglutinin (HA) protein is the major antigen that induces virus-neutralizing antibodies, but substan-

* Corresponding author: Dr. Celia Abolnik, University of Pretoria Faculty of Veterinary Science, Private Bag X04, Onderstepoort, 0110, South Africa.
E-mail address: celia.abolnik@up.ac.za (C. Abolnik).

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tial antigenic variation already exists within the H5 HA subtype and the viruses are continuously evolving which reduces vaccine efficacy and requires that vaccines be constantly updated, a process that can take up to 6 months (Kapczynski et al., 2017; Abolnik et al., 2019; Swayne et al., 2011; World Organization for Animal Health (WOAH) 2019).

Multiple studies demonstrated that most efficacious H5 HPAI vaccines, i.e., those that prevent clinical disease and mortality as well as significantly reduce viral shedding from the upper respiratory and gastrointestinal tracts, are those that are antigenically closely matched to the field outbreak strain (Bertran et al., 2021; Kapczynski et al., 2017; Ross et al., 2019; Kandeil et al., 2018; Kwon et al., 2021; Swayne et al., 1999; Choi et al., 2013; Cavalcanti et al., 2017; Pua et al., 2017; Palya et al., 2018; Phan et al., 2020). Therefore, there is an absolute need to develop future vaccines using genetic technologies that enable them to be rapidly updated (Kapczynski et al., 2017). Virus-like particles (VLPs) are self-assembling macromolecules that mimic the geometry of an actual virus yet lack viral genetic material, rendering them completely non-infectious (D'Aoust et al., 2010 Jun; 8). VLP-based vaccines induce broader immune responses than standard split and protein subunit vaccines by directly stimulating dendritic cells, and consequently potent T-cell-mediated immune responses (Won et al., 2018). VLP vaccines displaying recombinant H5 HA proteins have demonstrated high efficacy in *in vivo* studies in poultry and other animals, but all prior H5 VLP vaccines developed for and tested in poultry have been produced in baculovirus-mediated insect or mammalian cell culture expression systems (Tatár-Kis et al., 2019; Pénzes et al., 2019; Wu et al., 2017; Kang et al., 2021; Choi et al., 2013; Pushko et al., 2017; Huang et al., 2021).

Gs/GD H5 VLP vaccines for humans were developed in tobacco plants (*Nicotiana benthamiana*) in readiness for a future human pandemic (Rybicki, 2014; D'Aoust et al., 2008; Landry et al., 2010), and were demonstrated to elicit protective humoral responses in mice, ferrets and humans (D'Aoust et al., 2008; Landry et al., 2010). Biopharming in tobacco plants is a potentially highly efficient, scalable and cost-efficient system for producing VLP vaccines for medical and veterinary use (Rybicki, 2014; D'Aoust et al., 2008; Landry et al., 2010; Smith et al., 2020; Peyret et al., 2021 Jul 13; Abolnik et al., 2022), but up until now, no plant produced H5 VLP vaccine has been specifically developed for and tested in poultry, the reservoir from which any pandemic human strain is predicted to emerge, and the most important animal protein source globally. In this study we used the *Agrobacterium tumefaciens*-mediated transient expression system in *N. benthamiana* to produce a clade 2.3.4.4b H5 VLP. Our prior study demonstrated that the use of an adjuvant with plant-produced VLP vaccines in chickens is essential (Abolnik et al., 2022), therefore we compared the immunogenicity of the H5 VLP formulated with four different adjuvants in layer hens. A challenge study with a clade 2.3.4.4b H5N8 HPAI virus evaluated the plant-produced VLP vaccine's efficacy in preventing disease and death, reducing viral shedding and vertical transmission in the eggs.

2. Materials and methods

2.1. Synthetic gene design and plant expression vector construction

The H5 VLP vaccine design was based on A/Speckled pigeon/South Africa/08–004B/2017 (H5N8), a clade 2.3.4.4b strain isolated during the 2017 HPAI epidemic (Abolnik et al., 2019). The full length hemagglutinin (HA) gene (accession number MH165604) was codon-optimized and synthesized by Bio Basic Inc., Toronto, Canada, to include an upstream *Mus musculus* monoclonal antibody heavy chain variable region (O'Hara et al., 2012), signal peptide, 6 x poly-histidine, and was flanked by Age*I* and Xho*I* restriction enzyme sites at the 5′- and 3′ ends, respectively. Methods for cloning of the synthetic gene into the pEAQ-HT plant expression vector, transformation into competent bacterial cells and verification of antibiotic-resistant clones were as previously described (Smith et al., 2020). The sequence-verified recombinant pEAQ-HT+H5 plasmid was transformed into *A. tumefaciens* AGL-1 competent cells (ATCC® BAA-101™) by electroporation, and selected antibiotic-resistant AGL-1 clones were verified by colony PCR (Smith et al., 2020).

2.2. VLP extraction, purification and confirmation of expression

The methods for the co-expression of pEAQ-HT+H5 with pEAQ-HT+M2 were as described previously (Smith et al., 2020; Abolnik et al., 2022), except that 6-week old wild type *N. benthamiana* plants were used. Sucrose density gradient centrifugation of the clarified leaf extract was also performed as previously described (Abolnik et al., 2022). 0.5 ml fractions were collected and analysed by hemagglutination assay with chicken red blood cells (World Organization for Animal Health (WOAH) 2019), 4–12% Bis-Tris Bolt™ (Life Technologies) sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and Western blot with H5N8 antiserum (Deltamune, South Africa) and goat anti-chicken IgY horseradish peroxidase conjugated antibody (Novex Life Technologies, ThermoFisher Scientific) used at 1: 1000 and 1:15,000 dilutions, respectively. Fractions with titres ≥ 1:64 hemagglutinating units (HAU) were pooled and dialysed twice in 2 L PBS in 3–12 ml Slide-A-Lyzer® Dialysis 3500 MWCO cassettes (Thermo Scientific, Waltham, USA). Tobacco leaf sections and the partially purified leaf extracts were examined by negative stain transmission electron microscopy (TEM) at the University of Pretoria (UP) Faculty of Veterinary Science's Electron Microscopy Unit.

2.3. Preparation of vaccines

Trehalose dihydrate (15% w/v) (Sigma-Aldrich) was added to the dialyzed VLPs as a stabilizer (Lynch et al., 2012) prior to final quantification by hemagglutination assay and total protein concentration determination using a BCA Protein Assay Kit (Pierce). VLP vaccine batch sterility was confirmed through aerobic and anaerobic cultivation at the UP-Bacteriology Section. Doses of 250 HAU, the optimal dose for plant produced IAV VLPs in chickens (Abolnik et al., 2022), were diluted in sterile PBS or OculoNasal diluent (MSD Africa, South Africa) and mixed with one of the following four adjuvants for a total dose volume of 0.25 ml (Table 1). Montanide™ ISA 71VG (Seppic, France) is a water-in-mineral-oil adjuvant with a mannide oleate based surfactant, recommended for use in poultry at ratios of 50 to 70% v/v (Arous et al., 2013). H5 VLPs were mixed with 50% v/v Montanide ISA 71, and vortexed vigorously prior to administration. Emulsigen-P, Emulsigen-D and Carbigen adjuvants (MVP Techologies, Omaha, USA) were supplied by Phibro Animal Health (USA). Emulsigen-P is an emulsified oil-in-water dual adjuvant containing a unique polymer immunostimulant that has been demonstrated to stimulate T-cell responses, including interferon gamma and interleukin 12. Emulsigen-D is also an oil-in-water adjuvant with a T-cell immunostimulant. Emulsigen-P and -D are recommended for use at concentrations of 10 to 20% v/v, with only mild mixing required, and since they contain less oil than conventional water-in-oil or water-in-oil-water adjuvants, are easier to pass through a 25 guage needle and thus are less likely to cause adverse injection site reactions than conventional adjuvants. Carbigen is a carbomer-based adjuvant suspension containing a proprietary emulsified components. For injectables, Carbigen is recommened at 20- 40% v/v, but its also indicated for intranasal application by spray or immersion to produce IgA and IgG responses. Here, we administered the Carbigen-adjuvanted H5 VLPs to the mucosal surfaces by intra-ocular vaccination of chickens at 40% v/v. The carbigen-vaccinated H5 VLP vaccine was prepared according to the manufacturer's recommended procedure (accessible at https://mvpadjuvants.com/technical-bulletins/). The plant-produced H5 VLP vaccines were prepared on the day of the first immunization and stored at 4 °C until the booster.

Table 1
Study groups and H5 vaccine formulation.

No. hens	Adjuvant	Adjuvant v/v	H5 VLP	Volume and route
20	Montanide ISA 71VG	50%	250 HAU[a]	250 μl, intramuscular
20	Emulsigen-D	20%	250 HAU[a]	250 μl, intramuscular
20	Emusigen-P	20%	250 HAU[a]	250 μl, intramuscular
20	Carbigen	40%	250 HAU[b]	50 μl, intraocular
10	None (Sham)	None	None	250 μl[a], intramuscular

[a] diluted in PBS;
[b] diluted in Oculo-Nasal diluent

2.4. Challenge virus

A prior study determined that 10^6 egg infectious dose 50's (EID_{50}) of isolate A/Speckled pigeon/South Africa/08–004B/2017 (H5N8) administered by the intra-ocular or intra-nasal route to specific pathogen free (SPF) chickens produced 100% mortality within 3 to 4 days (Abolnik et al., 2019). A second passage stock was propagated in 9-to-11-day old SPF embryonated chicken eggs (ECEs) and the EID_{50} titres were determined according to the Reed and Muench method (Reed and Muench, 1938). The challenge dose of 10^6 EID_{50} per 50 μl (one drop of 25 μl in each eye) was diluted in OculoNasal diluent.

2.5. Experimental design

The White Leghorn layer hens aged 33 weeks used in this investigation were sourced from a prior study that tested various doses of a plant produced H6 VLP vaccine, but the birds were not challenged with any live IAV (Abolnik et al., 2022). There is no antigenic cross-reaction between IAV HA subtypes (World Organization for Animal Health (WOAH) 2019), therefore no immunological interference between H5 and H6 VLP vaccines was anticipated. The hens had also been vaccinated against Newcastle disease and Fowlpox (Abolnik et al., 2022). Ninety hens, identified individually by numbered wing tags, were randomly selected for enrolment in the H5 VLP vaccine trial. Housing and care conditions in a broiler house at UP's Faculty of Veterinary Science campus were as described previously (Abolnik et al., 2022). The hens were randomly divided into five groups and vaccinated as per Table 1. Three weeks later, the second immunization (booster) was administered. Blood was collected from the wing veins prior to the first vaccination, prior to the second vaccination, and three weeks after the second vaccination from all 90 hens. Birds were observed daily for signs of adverse vaccine reactions. For the challenge experiment, all ten hens from the sham-vaccinated group, plus the twenty birds from the vaccine group with the best H5-specific serological responses (results of the present study) were transferred into the UP Veterinary Faculty's negative pressure Poultry Biosafety Level 3 facility. The thirty hens were co-housed on sawdust on the floor, with perches and nesting boxes provided in an isolation room of 4.3 m^2 and *ad libitum* access to water and commercial layer feed. The isolation room had natural light and the ambient temperature was maintained at 24 °C. The hens were observed twice daily after challenge, with any clinical signs recorded in the early mornings, with deaths counted as the previous night. Nylon FLOQswabs® (Copan Diagnostics, Italy) were used to collected individual oropharyngeal and cloacal swabs daily that were stored in 2 ml of viral transport medium (VTM; brain–heart broth, 0.1 mg/mL doxycycline, 0.1 mg/mL enrofloxacin, 1 mg/mL penicillin–streptomycin and 10% v/v glycerol) at 4 °C until testing. Eggs were collected daily and stored at 4 °C. A sterile rayon-tipped swab (Copan Diagnostics, Italy) moistened in sterile distilled water was used to rub the surface of the egg and placed into 1 ml of VTM in a sterile 1.5 ml Eppendorf tube. The surface of the egg was disinfected with 70 % ethanol before making a hole in the shell to aseptically collect samples of the albumen and yolk into separate sterile tubes. Blood was collected from the survivors at day 10 post challenge, before the birds were humanely euthanized by cervical dislocation. All animal procedures were reviewed and approved by the UP and CSIR Research and Animal Ethics Committees.

2.6. Antibody detection

Influenza nucleoprotein (NP) specific antibodies were detected by competition ELISA (cELISA) with an Influenza A Antibody Test kit (IDEXX, South Africa) according to manufacturer's instructions. Sample to negative (S/N) ratios < 0.50 were considered as positive. Hemagglutination inhibition (HI) tests on egg yolks diluted 1:1 in PBS and sera were performed at the UP Department of Veterinary Tropical Diseases, according to the WOAH-recommended method (World Organization for Animal Health (WOAH) 2019). HI titres were expressed as $\log_2$ of the reciprocal of the highest dilution causing complete inhibition of 4 hemagglutinin units of antigen with 4 $\log_2$ considered as positive.

2.7. Virus detection

Total nucleic acid was extracted from 200 μl of the swab fluids and a pooled sample of the alantoic fluid and yolk from each egg with IndiMag Pathogen Kits according to the recommended procedure in an IndiMag™ 48 instrument (Indical BioSciences). Influenza A virus matrix and NP gene targets were detected by RT-qPCR using VetMax™-Gold AIV Detection Kits (ThermoFisher Scientific) in a StepOnePlus™ instrument (Life Technologies, Thermo Fisher Scientific) according to the recommended procedure, with the modification that a half volume reaction mix was used with 4 μl of template RNA. Standard curves were produced from 10-fold serial dilutions of nucleic acid extracted from 200 μl allantoic fluid of the titrated H5N8 challenge virus. PCR cycle threshold (Ct) values < 40 were considered positive, and all RT-qPCR results were expressed as EID_{50} equivalents/ ml. The limit of detection of the VetMax™-Gold AIV RT-qPCR assay was 0.195 EID_{50} equivalents / ml.

2.8. Statistical analysis

Data expressed as the mean +/- standard deviation (SD) were analysed by one-way ANOVA followed by Tukey's multiple comparison test (HI data) or un-paired T-tests (RT-qPCR data) in GraphPad Prism v 9.1.2 software for Windows (La Jolla, CA, USA). A P value < 0.05 was considered significant.

3. Results

3.1. Expression, purification and quantification of H5 VLPs

A distinct protein band of ~ 65.5 kDA consistent with the H5 HA protein was visible on the SDS-PAGE gel (Fig. 1(a)) and confirmed by Western blot (Fig. 1(b)). Abundant IAV-like VLPs measuring approximately 80–120 nm were visible in the partially- purified leaf extract (Fig. 2(a)) and in the leaf sections in the proximity of the plant plasma membrane (Fig. 2 (b)), consistent with a previous report that influenza VLPs in plants bud from the plasma membrane (D'Aoust et al., 2008). The concentration of the partially purified H5 VLPs (post dialysis in PBS,

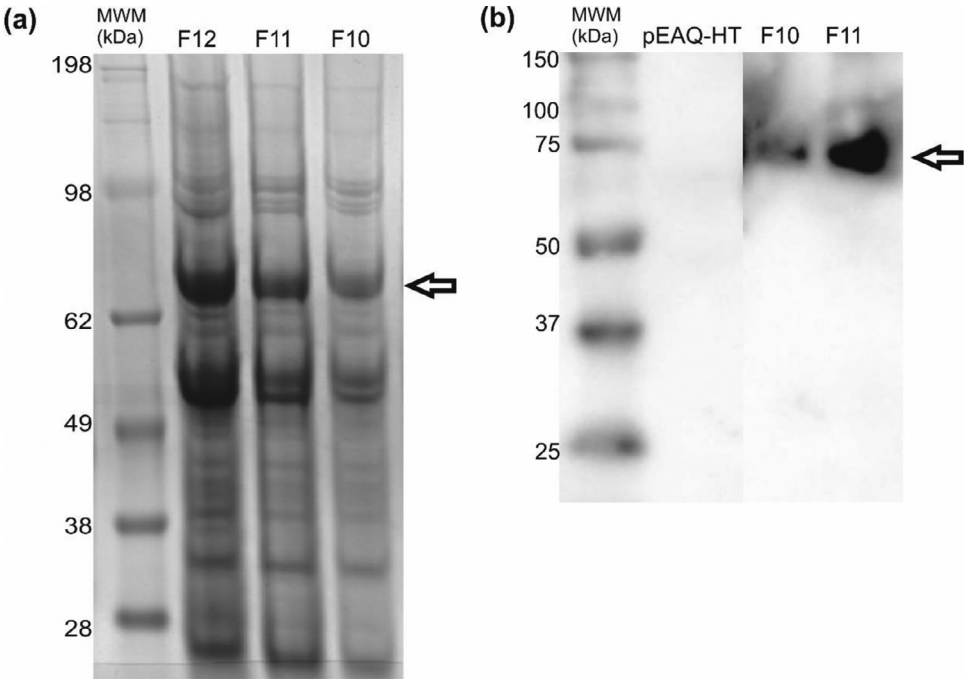

Fig. 1. *N.benthamina* expression of the 65 kDA H5 HA protein, indicated by the arrows, in fractions (F) 10, 11 or 12 in (a) SDS-PAGE and (b) Western blot analysis. The empty pEAQ-HT construct was agro-infiltrated as a negative control.

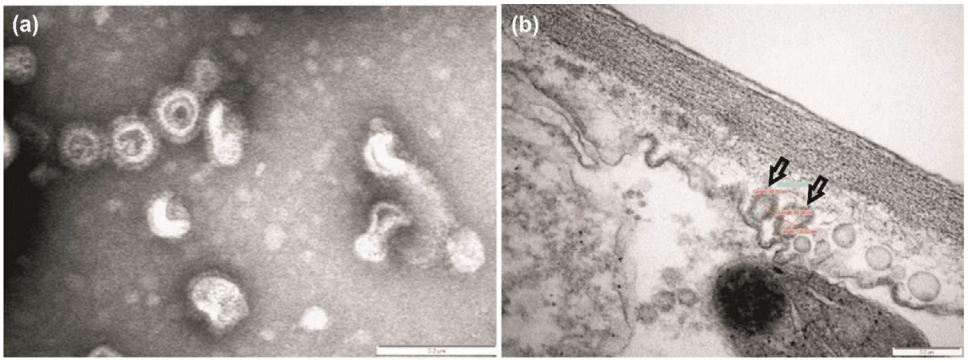

Fig. 2. Negative-strained transmission electron microscope images of H5 HA VLPs in **(a)** partially-purified plant leaf extracts and **(b)** as indicated by arrows in leaf sections of *N.benthamina*.

with 15% v/v trehalose) was 1:1024 HAUs (per 25 μl) and 581.87 μg/ml; therefore, a single 250 HAU dose (3.64 μg) was 6.25 μl. Conservatively estimated, 81,500×250 HAU doses were produced per Kg wild type *N. benthamiana* leaves, i.e. sufficient VLPs to prime-boost vaccinate 40,750 chickens.

3.2. Comparison of immune responses with different adjuvants

The immunogenicity of the plant produced H5 VLPs formulated with four different commercial adjuvants after a single and double vaccination was evaluated. Three weeks after a single vaccination, hens that received intramuscular injections of H5 VLPs with Emulsigen D, Emulsigen P or Montanide ISA 71VG adjuvants seroconverted with HI geometric mean titres (GMTs) of 5.75 $\log_2$ (± 0.97), 6.35 $\log_2$ (± 1.04), and 6.0 $\log_2$ (± 2.0), respectively (Fig. 3), but the differences between these groups were not significant (Fig. 4). H5 VLPs with Carbigen adjuvant administered by eye drop only elicited a GMT of 1.1 $\log_2$ (± 1.21) after a single vaccination, that was not statistically higher than the sham-vaccinated control.

After the second vaccination, the GMTs for the vaccines injected intramuscularly increased by at least 2 logs to 7.3 $\log_2$ (± 1.17) for Emulsigen-D, 8 $\log_2$ (± 1.08) for Emulsigen P and 7.9 $\log_2$ (± 1.07) for Montanide ISA 71VG, but again, these HI differences were not significant. The Carbigen group's GMT increased to 2.05 $\log_2$ (± 1.64) after the second vaccination, which was significantly higher ($P < 0.05$) compared to the sham vaccinated controls, but still below the threshold for HI positivity. Since the Emulsigen-P group had a lower standard deviation after a single vaccination and slightly higher HI GMTs compared to the Montanide ISA 71-VG-adjuvanted group, the Emulsigen-P H5 vaccinated hens were considered to have the best H5-specific immune responses and were enroled in the challenge study. No adverse vaccine effects were observed in any of the hens after either a double or a single immunization.

3.3. Clinical signs following H5 challenge

No clinical signs were observed in the vaccinated hens over the 10-day duration of the challenge study, but 70% of the sham-vaccinated hens died before 3 days, and none survived to day 4. The first clinical signs in the sham-vaccinated birds were observed at day 2, and included depression, ruffled feathers, weakness, lethargy and diarrhoea. Later signs of conjunctivitis, cyanotic combs and nasal discharge were quickly followed by death. The increase in the GMTs of the vaccinated hens from 8 $\log_2$ pre-challenge to 9.65 $\log_2$ post challenge, and the appearance of NP-specific antibodies in 75 % of the hens by day 10 (Supplemental table 1), affirmed that these birds had been exposed to the challenge virus, even though they appeared clinically normal.

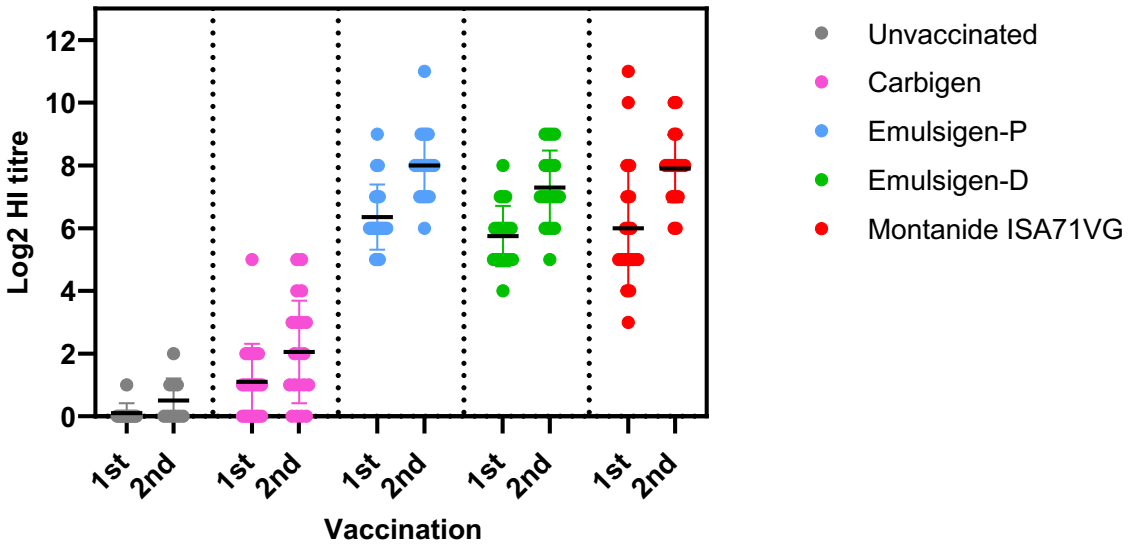

Fig. 3. H5-specific influenza antibody responses in hens immunized once or twice with 250 HAU doses of plant-produced H5 VLP vaccines formulated with four different adjuvants.

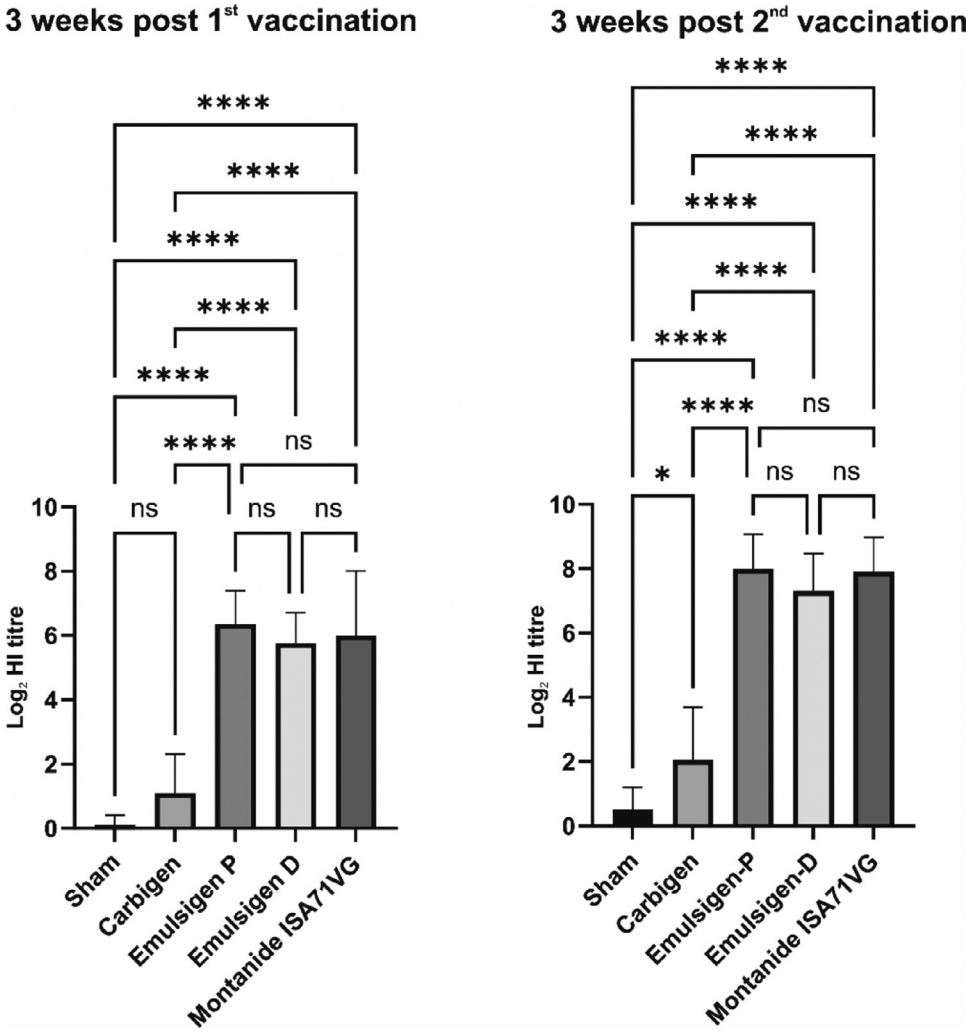

Fig. 4. Comparison of hens immunized once or twice with 250 HAU doses of plant-produced H5 VLPs formulated with four different adjuvants. Statistical significance between groups is indicated as: *p <0.05; **p <0.01; ***p<0.001; ****p<0.0001; ns- not significant.

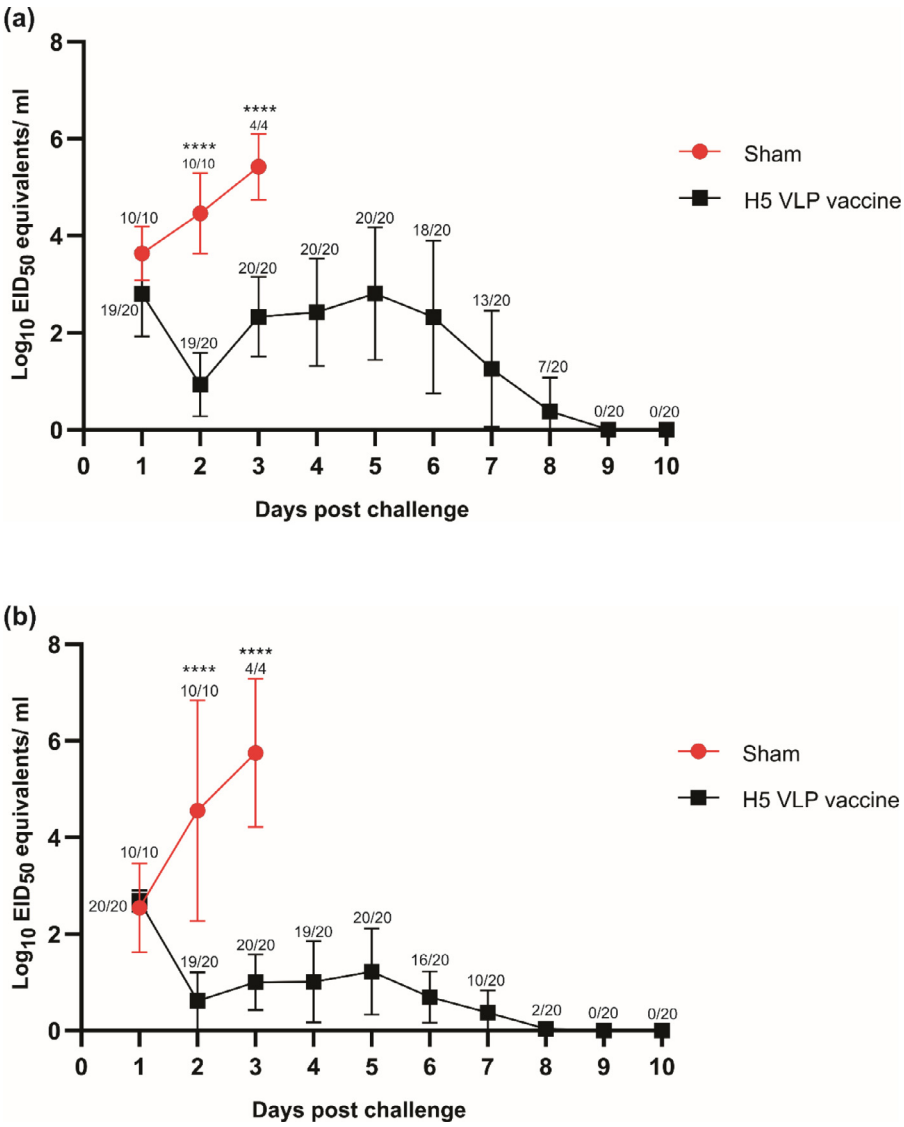

Fig. 5. Virus shedding as detected by RT-qPCR in **(a)** oropharyngeal and **(b)** cloacal swabs of hens challenged with H5N8 HPAIV. ****p<0.0001.

3.4. Viral shedding from the respiratory and gastrointestinal tracts

Efficacy of the plant-produced H5 VLP vaccine was assessed by its ability to prevent mortality and clinical signs following challenge as well as the reduction in viral shedding, compared to a sham-vaccinated control group. Oropharyngeal and cloacal swabs were collected daily from each hen and tested by RT-qPCR to determine the relative magnitude and duration of virus shedding. Virus detected on day 1 in the oropharyngeal swabs (Fig 5(a); Supplemental table 2(a)) or cloacal swabs (Fig 5(b); Supplemental table 2(b)) was assumed to be residual challenge inoculum in the respiratory and alimentary tracts. On day 2, a significant reduction ($P < 0.0001$) in viral shedding was evident in the vaccinated birds compared to the sham-vaccinated group, with $10^{1.49}$ vs $10^{5.03}$ EID_{50} eqivalents/ ml in the oroharynx and $10^{1.22}$ vs $10^{7.6}$ EID_{50} eqivalents/ ml in the cloaca, respectively. All sham-vaccinated chickens had died by the end of day 3, but average peak titres of $10^{5.65}$ EID_{50} eqivalents/ ml were detected in the orpoharynx and $10^{6.74}$ EID_{50} eqivalents/ ml from the cloaca, compared with significantly lower titres ($P < 0.0001$) of $10^{2.97}$ and $10^{1.52}$ EID_{50} eqivalents/ ml for vaccinated hens, respectively (the logarythmic means are represented on the graphs wherease the average titers are provided in the supplemental data).

In the vaccinated hens, viral shedding peaked on day 5 in both the oropharynx and cloaca with average titres of $10^{4.19}$ and $10^{2.51}$ EID_{50} eqivalents/ ml, respectively. On day 6 the proportion of shedding hens and the average viral titres started to decline, with an average titre of $10^{3.83}$ EID_{50} eqivalents/ ml from the oropharynx and $10^{0.96}$ EID_{50} eqivalents/ ml from the cloaca, and by day 8 only 35% of hens were still shedding detectable average titres of $10^{1.13}$ EID_{50} eqivalents/ ml virus from the respiratory tract, and 10% of birds shed virus from the gastrointestinal tracts with an average titre of $10^{0.28}$ EID_{50} eqivalents/ ml, but no virus was detected from either source from day 9 onwards.

3.5. Virus detection in eggs

To determine whether IAV was vertically transmitted, the internal contents of eggs (as well as the external surfaces) that were laid at the peaks of viral shedding were tested, i.e., days 2 and 3 when all vaccinated and some non-vaccinated hens were alive, and at day 5 when only vaccinated hens still survived. None of the eggs collected were abnormal in appearance. No virus was detected in the internal contents of eggs from day 2 (n = 16), day 3 (n = 14) or day 5 (n = 16) but HPAIV was detected on the shells of 31.3%, 64.3% and 87.5%, respectively, at titres ranging from 1.17 to 3.83 $\log_{10}$ EID_{50} equivalents/ ml (Table 2). The daily increase in the proportion of surface-contaminated eggs was consistent with accumulation of the virus in the environment. H5-specific antibody HIs titres > 6 $\log_2$ were detected in all eggs on days 2 and 3,

Table 2
H5N8 HPAI virus in egg contents and on the shell exterior as detected by RT-qPCR, plus H5-specific antibody detection in the yolks.

	Egg. No.	Contents Log_{10} EID_{50} equivalents/ ml (Ct)	Surface Log_{10} EID_{50} equivalents/ ml (Ct)	Yolk Log_2 HI titre
Day 2 post challenge	1	Not detected	**1.58 (37.83)**	8
	2	Not detected	Not detected	7
	3	Not detected	Not detected	7
	4	Not detected	Not detected	7
	5	Not detected	**1.98 (36.41)**	8
	6	Not detected	**1.34 (38.70)**	8
	7	Not detected	Not detected	8
	8	Not detected	Not detected	7
	9	Not detected	Not detected	7
	10	Not detected	Not detected	7
	11	Not detected	**1.51 (38.09)**	7
	12	Not detected	**2.06 (36.15)**	7
	13	Not detected	Not detected	7
	14	Not detected	Not detected	8
	15	Not detected	Not detected	7
	16	Not detected	Not detected	7
Day 3 post challenge	1	Not detected	Not detected	7
	2	Not detected	**3.28 (31.83)**	8
	3	Not detected	**1.99 (36.38)**	8
	4	Not detected	**1.61 (37.72)**	7
	5	Not detected	**2.05 (36.19)**	7
	6	Not detected	Not detected	7
	7	Not detected	**2.51 (34.54)**	7
	8	Not detected	**2.55 (34.41)**	8
	9	Not detected	**1.17 (39.30)**	6
	10	Not detected	Not detected	6
	11	Not detected	**1.94 (36.56)**	8
	12	Not detected	**3.35 (31.59)**	8
	13	Not detected	Not detected	9
	14	Not detected	Not detected	10
Day 5 post challenge	1	Not detected	**1.93 (36.59)**	Not tested
	2	Not detected	**1.99 (36.39)**	Not tested
	3	Not detected	**3.83 (29.90)**	Not tested
	4	Not detected	**2.00 (36.34)**	Not tested
	5	Not detected	**3.14 (32.31)**	Not tested
	6	Not detected	**2.75 (33.72)**	Not tested
	7	Not detected	**1.92 (36.64)**	Not tested
	8	Not detected	Not detected	Not tested
	9	Not detected	**1.92 (36.62)**	Not tested
	10	Not detected	**2.16 (35.78)**	Not tested
	11	Not detected	**1.81 (37.01)**	Not tested
	12	Not detected	**2.05 (36.19)**	Not tested
	13	Not detected	**2.18 (35.73)**	Not tested
	14	Not detected	Not detected	Not tested
	15	Not detected	**1.99 (36.39)**	Not tested
	16	Not detected	**1.72 (37.35)**	Not tested

indicating that all the eggs tested were laid by vaccinated hens, because HA-specific antibodies detectable by HI in response to a 10^6 EID_{50} challenge only develop in chickens after day 5 post challenge (Dundon et al., 2007).

4. Discussion

Alarming year-on-year increases in the number of outbreaks of Gs/GD HPAI H5Nx in poultry and wild birds are being reported to the World Organisation for Animal Heath from the European Union, Russia, Asia and Africa (Adlhoch et al.). Clade 2.3.4.4b H5 viruses reached South Africa via the seasonal movements of wild waterfowl in 2017 (Abolnik et al., 2019), 2018 (Abolnik, 2020), and 2021 (World Organization for Animal Health (WOAH), 2021). To eradicate the infection in South Africa, 5.4 million chickens were culled in the H5N8 epidemics of 2017 and 2018 with total losses to farmers estimated at US$ 140 million (Abolnik et al., 2019), and thus far in the 2021 H5N1 epidemic > 100,000 poultry have died from the infection and an additional 1, 7 million healthy chickens have been pre-emptively culled to prevent spread (World Organization for Animal Health (WOAH), 2021). HPAI H5Nx strains continued to circulate in domestic and wild bird populations in Eurasia throughout the summer of 2021, including those in the migratory breeding and stopover sites (Adlhoch et al.), there is thus a high risk that novel Gs/GD H5 lineages will again be spread southwards in 2022. With more frequent introductions and outbreaks, continuous stamping out even in non-endemic countries becomes unsustainable and there has been increased demand for vaccination by poultry producers and animal welfare organizations (Kang et al., 2021).

Countries that use stamping out as a control measure for HPAI are reluctant to apply vaccination for various reasons. Firstly, vaccination is believed to suppress clinical signs without controlling transmission of challenge virus (Tatár-Kis et al., 2019; Suarez, 2005). These concerns are well-founded because substantial data from field and experimental infections demonstrate that, even when properly applied, whole inactivated virus or commercial live recombinant turkey herpesvirus (HVT) and recombinant fowlpox virus vaccines with 100% protection against clinical signs and mortality fail to significantly reduce viral shedding when the challenge virus and vaccine is heterologous (Kapczynski et al., 2017 Nov 1; Kandeil et al., 2018; El-Moeid et al., 2021; Cavalcanti et al., 2017). There is a significant correlation between the antigenic distance

and the amount of virus shed (Peeters et al., 2017), and the risk of "silent infections" can consequently be greatly minimised by using the homologous field virus HA in the vaccine. Recombinant VLP vaccines expressing H5 HA produced in insect or mammalian cell cultures, administered to chickens at doses ≥ 500 HAUs elicited high pre-challenge antibody titres ranging from 4.5 $\log_2$ to 9 $\log_2$ after single (Pénzes et al., 2019; Choi et al., 2013; Kapczynski et al., 2016; El-Husseiny et al., 2021) or double (Kang et al., 2021; Kapczynski et al., 2016; El-Husseiny et al., 2021) immunizations with 100% protection against morbidity and significant reductions in virus shedding from the respiratory and gastrointestinal tracts (Pénzes et al., 2019; Kang et al., 2021; Choi et al., 2013; Kapczynski et al., 2016; El-Husseiny et al., 2021). A drawback of these cell culture-based systems is that they may require additional chemical and purification treatments to inactivate and remove baculoviruses, host cell pathogens and DNA (Kapczynski et al., 2016). Plant-produced VLP vaccines on the other hand are completely safe and non-toxic in chickens and other species (D'Aoust et al., 2010; Rybicki, 2014; Smith et al., 2020; Peyret et al., 2021; Abolnik et al., 2022). When 250 HAU doses of our plant-produced H5 VLP were formulated with Emulsigen-P as an adjuvant, GMTs of 6.35 $\log_2$ and 8 $\log_2$ were obtained after single and double immunizations, respectively, which is comparable to the insect and mammalian cell-produced VLPs that were used at twice the dosage. Hens vaccinated twice before challenge with H5N8 HPAI were completely protected and showed no clincal signs, whereas the sham-vaccinated controls all died within 3–4 days. The plant-produced H5 VLP vaccine reduced the average oropharyngeal shedding by 3487-fold and 472-fold on days 2 and 3 post challenge, respectively, whereas average clocal shedding was reduced by 2360,098-fold and 15,608-fold on days 2 and 3, respectively, compared to the sham-vaccinated controls. VLPs and inactivated whole virus vaccines prevent systemic replication of the challenge virus, but they do not completely inhibit local virus replication (Choi et al., 2013). Although IAV is highly infectious, it typically takes many infectious viral particles (10 - 1000) to routinely infect a bird experimentally (Suarez, 2005), thus it's possible that the minimal levels of shedding detected by day 8 (≤ $10^{1.13}$ EID_{50} equivalents/ ml) would be below the threshold required to infect other chickens, although we did not use non-vaccinated contact birds to assess the infectivity here.

In both natural infections and experimental infections with non-vaccinated chickens, HPAI virus replication occurs systemically, and the virus has been isolated from multiple internal organs including the reproductive tract of hens, with the transmission of virus to eggs occurring at any point from ovulation but before the formation of the shell (Bertran et al., 2015, Uchida et al., 2016). HPAI virus-infected hens cease laying about 72 hrs post-infection unless they die sooner, and the final eggs laid are frequently thin-shelled, soft-shelled, or deformed with HPAI virus on the eggshell surface, in the yolk and albumen (Bertran et al., 2015, Uchida et al., 2016, Stephens et al., 2020). Peak viral quantities of 5.3 $\log_{10}$ and 4.4 $\log_{10}$ EID_{50}/ ml were reported in the albumen and egg yolk, respectively of some eggs laid by hens infected with Gs/GD H5 HPAI viruses (Bertran et al., 2015, Uchida et al., 2016). Whereas a double vaccination (prime-boost) with a heterologous whole inactivated Gs/Gd H5 vaccine failed to prevent H5 HPAI virus transmission to the eggs after challenge (Abdelwhab et al., 2011), a homologous whole inactivated Gs/GD vaccine (Bertran et al., 2015) and our plant-produced H5 VLP vaccine completely prevented HPAI H5 virus transmission to eggs.

The second concern about vaccination is its effect on serologic surveillance, specifically the inability to distinguish vaccine-induced antibodies from those elicited by a virus circulating in the flock (DIVA) (Tatár-Kis et al., 2019, Suarez, 2005). Unlike conventional whole inactivated viruses, most recombinant VLPs including the plant-produced H5 vaccine in the present study do not contain the NP protein, or any viral nucleic acid. cELISAs that detect NP antibodies can therefore be used for screening at least 14 days post challenge infection on a flock basis (Cavalcanti et al., 2017), indeed, 75% of the vaccinated hens in the present study had already developed NP-specific antibodies ten days after challenge. In countries where HPAI or LPAI is endemic, NP cELISA can be used in conjunction with serotype specific HIs or ELISAs and viral RNA detection methods to demonstrate DIVA and ensure to trading partners that flocks that have been vaccinated are free of HPAI H5 infection. Unfortunately, in the past a country's vaccination status has been used to justify instituting a non-tariff trade barrier for the introduction of poultry or poultry products, on the presumption that HPAI infections are prevalent there (Suarez, 2005).

Thirdly, the long-term utilization of vaccines for the control of H5 HPAI have been associated with accelerated genetic and antigenic drift and the emergence of vaccine-resistant field viruses (Bertran et al., 2015; Suarez, 2005). As discussed above and as we demonstrated in this study, technological advancements that allow the rapid updating of the HA in a vaccine can effectively reduce the viral load in the enviroment, and consequently break the transmission cycle before further variants emerge. Recent alternative strategies have focused on HPAI H5 vaccines that can protect against antigenically distinct and diverse co-circulating viruses as well as future drift variants. Chickens vaccinated with multivalent VLPs expressing several clades of H5 HA in a single VLP shed less virus and were better protected against challenge than chickens vaccinated with monovalent vaccine (Kang et al., 2021 Jul 2, Kapczynski et al., 2016 Mar 18). Another approach used the consensus HA protein sequence of diverse H5 clades and sub-clades ("COBRA") expressed in an HVT vector (Bertran et al., 2021) or as VLPs (Ross et al., 2019; Wu et al., 2017). COBRA vaccines were even able to elicit potent HAI responses against antigenically distinct recent drift viruses that were not included in the design (Ross et al., 2019). However, there is a prevailing risk that such broad-based vaccines could more rapidly drive the evolution of immunity-escaping antigenic variants than a monovalent vaccine. Veterinary authorities would likely still require *in vivo* demonstration of efficacy of any such commercial broad-based vaccine against the current outbreak strain before authorizing its use in a particular country.

In conclusion, antigenically-matched homologous or "autogenous" HA VLP vaccines produced rapidly in plants could maximise the chances for controlling future HPAI epidemics in poultry, which is pivotal to global food security, and reduce the risks for an emergent human pandemic virus, aligning with the global one health initiative. Plant expression systems for vaccines have significant advantages, and here we showed that a highly efficacious, safe and non-toxic plant-produced clade 2.3.4.4b H5 VLP vaccine for chickens could be produced with a yield of at least 85,000 doses per Kg of plant leaf material. Furthermore, within two weeks of receiving the synthetic clone, we had already produced an updated clade 2.3.4.4b H5 VLP based on the South African 2021 H5N1 virus (data not shown). It is important that developing countries like South Africa become self-sufficient in their vaccine production capabilities, but national investment in the infrastructure for plant based large-scale production is still required to make this a reality.

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.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.vetvac.2022.100001.

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