Heliyon 8 (2022) e09804

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon

Research article

Dose immunogenicity study of a plant-produced influenza virus-like particle vaccine in layer hens

Celia Abolnik [a,*], Tanja Smith [a,b], Daniel B.R. Wandrag [a], Mark-Anthony Murphy [a], Marizel Rautenbach [a], Olebile Olibile [a], Martha O'Kennedy [a,b]

[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, Brummeria, Pretoria, South Africa

ARTICLE INFO

Keywords:
H6N2 subtype
Avian influenza
Plant-produced VLP
Chicken dose

ABSTRACT

Avian influenza poses one of the largest known threats to global poultry production and human health, but effective poultry vaccines can reduce infections rates, production losses and prevent mortalities, and reduce viral shed to limit further disease spread. The antigenic match between a vaccine and the circulating field influenza A viruses (IAV) is a critical determinant of vaccine efficacy. Here, an *Agrobacterium tumefaciens*-mediated transient tobacco plant (*Nicotiana benthamiana*) system was used to rapidly update an H6 influenza subtype virus-like particle (VLP) vaccine expressing the hemagglutininn (HA) protein of South African H6N2 IAVs circulating in 2020. Specific pathogen free White Leghorn layer hens vaccinated twice with ≥125 hemagglutinating unit (HAU) doses elicited protective antibody responses associated with prevention of viral shedding, i.e. hemaglutination inhibition (HI) mean geometric titres (GMTs) of ≥7 $\log_2$, for at least four months before dropping to approximately 5–6 $\log_2$ for at least another two months. A single vaccination with a 250 HAU dose induced significantly higher HI GMTs compared lower or higher doses, and was thus the optimal dose for chickens. Use of an adjuvant was essential, as the plant-produced H6 HA VLP alone did not induce protective antibody responses. Plant-produced IAV VLPs enable differentiation between vaccinated and infected animals (DIVA principle), and with sucrose density gradient-purified yields of 20,000 doses per kg of plant material, this highly efficacious, safe and economical technology holds enormous potential for improving poultry health in lower and middle-income countries.

1. Introduction

Avian influenza, which is caused by various subtypes of Influenza A virus (IAV), poses one of the largest known threats to global poultry production as well as human health [1]. Most developed countries are well-resourced to detect and control outbreaks of avian influenza early, opting to eradicate the virus in poultry through strict biosecurity with mass culling, but in other regions, subtype H9N2 [2], H6N2 [3,4], H5Nx or H7Nx low pathogenic (LPAI) or high pathogenic (HPAI) strains spread too rapidly and widely to eradicate and eventually became enzootic [5, 6, 7, 8], leaving vaccination as the only option to keep poultry production economically viable and assure food security. However, poor vaccines or poorly-applied vaccines have little effect on controlling disease or reducing virus spread, and can even potentiate the emergence of undesirable variant viruses [3, 9]. Conversely, high-potency influenza vaccines that are applied properly reduce the number of susceptible poultry, increase their resistance to infection, disease, and death, and reduce the amount of virus that infected birds excrete [10, 11], which in turn reduces the viral load in the environment and lateral spread between farms [12, 13, 14].

The IAV hemagglutin (HA) glycoprotein on the outer viral surface contains the cell receptor recognition and binding sites targeted by neutralizing antibodies and is thus the primary antigen incorporated into vaccines, but HA is prone to rapid mutation and antigenic drift, and it has been shown that the level of protection is associated with the relatedness of the vaccine to the challenge strain, therefore, circulating field stains must be monitored constantly, and vaccines need to be updated periodically [3, 9, 15]. The efficacy of an updated IAV vaccine should ideally be evaluated in a live challenge model in the target species, but demonstration of HA-subtype-specific HI antibodies, especially after two vaccinations, has been associated with protection and is considered an adequate metric [16]. The correlates of protection for avian influenza poultry vaccines are well-defined, where experimental studies in Specific

* Corresponding author.
E-mail address: celia.abolnik@up.ac.za (C. Abolnik).

https://doi.org/10.1016/j.heliyon.2022.e09804
Received 16 November 2021; Received in revised form 4 January 2022; Accepted 22 June 2022

Pathogen Free (SPF) chickens established that vaccination-induced HI antibody geometric mean titres (GMTs) of ≥8 or 10 (3 or 3.3 $\log_2$) were associated with survival after HPAI challenge, GMTs of ≥40 (5.3 $\log_2$) prevented oropharyngeal shedding in most vaccinated birds, and GMTs of ≥128 (7 $\log_2$) in antigenically closely-matched vaccines and challenge viruses prevented shedding [17, 18, 19].

Avian influenza vaccine technologies include chemically inactivated whole viruses (egg-adapted field strain and reverse-genetics generated) or split vaccines; HA proteins expressed by recombinant heterologous virus-vectored backbones, *in-vitro* expressed soluble HA protein subunits administered alone or within nanoparticles, and DNA vaccines. Each of these systems has advantages and disadvantages in speed of production, safety, scalability, cost, mass application, potency and differentiating infected-from vaccinated animal (DIVA) capability that is reviewed elsewhere [9]. A fifth category of IAV vaccine technology rapidly gaining recognition for its scalability potential and high efficacy in *in vivo* studies is recombinant virus-like particles (VLPs). VLPs are self-assembling macromolecules that mimic the geometry of an actual virus yet lack viral genetic material, rendering them completely non-infectious [20]. VLPs induce broader immune responses than standard split and protein subunit vaccines by directly stimulating dendritic cells, and consequently potent T-cell-mediated immune responses [21]. Recombinant VLPs displaying influenza HA or HA plus other proteins have been produced in *E.coli* [22, 23], insect cells [4, 6, 24, 25], mammalian cells [26, 27] and plants [11, 20, 28]. Of the aforementioned expression systems, plant-based technology leads in its potential for safety, high yields, and low cost vaccine production [28].

In a previous study [11], we used an *Agrobacterium tumefaciens*-mediated transient tobacco plant (*Nicotiana benthamiana*) system to express VLPs displaying the HA protein of a 2016 South African H6N2 strain and tested a high dose (768 hemagglutinating units; HAU) in SPF chickens in a live challenge model. The H6 HA VLP vaccine caused a significant reduction in viral shedding, and reduced the proportion of shedding individuals and the duration of shedding, compared to non-vaccinated chickens. In the present study, we updated the H6 HA in the plant produced VLP vaccine to match a 2020 field strain and compared two purification methods for low-cost production. H6-specific antibody responses measured by HI were then used to establish the minimum effective dose, the intrinsic humoral immune-stimulatory potential of plant-produced VLPs, and the duration of immunity in laying hens over a seven-month period.

2. Materials and methods

2.1. Synthetic gene design and plant expression vector construction

Strain A/chicken/South Africa/411965/2020 (H6N2) was isolated from a commercial flock in the Gauteng Province in June 2020 at the Assurecloud Laboratory in Centurion, South Africa. Extracted RNA was provided for complete IAV genome sequencing as described elsewhere [3] with the data deposited in Genbank under accession numbers MZ410773-MZ410780. The full-length HA gene was codon-optimized and synthesized by Bio Basic Inc., Toronto, Canada, to include an upstream *Mus musculus* monoclonal antibody heavy chain variable region [29], signal peptide, 6 x poly-histidine and linker sequences, 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 [11]. Sequence-verified recombinant pEAQ-HT + H6 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 as before [11].

2.2. VLP extraction, purification and confirmation of expression

Co-expression of HA with M2 increases the yield of H6 VLPs, therefore *A. tumefaciens* cultures containing pEAQ-HT + H6 or pEAQ-HT + M2 [11] were grown overnight at 28 °C in Luria Bertani medium supplemented with 30 μg/ml rifampicin and 50 μg/ml kanamycin. Bacterial cells pelleted by centrifugation at 7,000 x g for 7 min were resuspended in infiltration buffer (10 mM 2-N-morpholino-ethanesulfonic acid (MES), 10 mM $MgCl_2$, pH 5.6) containing 200 μM acetosyringone. The respective infiltration mixes were diluted to an optical density at 600 nm of 1.4, and mixed 1:1. *A. tumefaciens* suspensions were incubated at room temperature for 1 h before syringe-infiltration into the leaves of 5- to 8-week-old *N. benthamiana* plants, modified to allow mammalian-like glycosylation [30]. Six days after infiltration, 76 g of infiltrated *N. benthamiana* leaves was harvested and homogenized in two volumes of 1 x phosphate-buffered saline (PBS) supplemented with 0.04 % (w/v) sodium metabisulfite and proteinase inhibitor cocktail (Sigma-Aldrich). Plant extract clarified through cheese cloth and centrifuged at 7,000 x g for 7 min was loaded onto either an Iodixanol (OptiPrep™; Sigma-Aldrich) density gradient ranging from 20 % to 60 % or a sucrose (Merck, South Africa) cushion consisting of 3 ml 20% sucrose layered on top of 3 ml 70% sucrose. Density gradients were centrifuged for two hours at 32,000 x g at 10 °C before collecting 1 ml and 0.5 ml fractions from base of the iodixanol-purified and sucrose-purified gradients, respectively. For sodium dodecylsulphate–polyacrylamide gel electrophoresis (SDS-PAGE) analysis, partially purified plant extract was separated on an Invitrogen Bolt™ 4%–12% Bis-Tris Plus gel (Thermo Scientific) under reducing conditions and stained with Coomassie G-250 (Merck). For Western blotting, proteins were separated on a 10 % TGX Stain-Free TM FastCast acrylamide gel (Bio-Rad) under reducing conditions and transferred to an Immobilon PVDF membrane. A 1:600 dilution of H6N2 antiserum (Deltamune, Pretoria) and goat anti-chicken IgY horseradish peroxidase conjugated antibody at 1:1500 dilution (Novex Life Technologies, Thermo Scientific) were used as the primary and secondary antibodies, respectively. Proteins were visualized using Clarity™ Western ECL blotting substrate (Bio-Rad) on the ChemiDoc™ MP Imaging System (Bio-Rad). The partially purified plant extracts were examined by transmission electron microscopy (TEM) at the University Of Pretoria Faculty Of Veterinary Science's Electron Microscopy Unit as described previously [11].

2.3. Preparation of vaccines

The three fractions containing the most abundant H6 HA protein as determined by SDS-PAGE (iodixanol fractions 10 to 12; sucrose fractions 4 to 6) were pooled and dialyzed in 1 x PBS using 3–12 ml Slide-A-Lyzer® Dialysis 3,500 MWCO cassettes (Thermo Scientific, Waltham, USA). Trehalose dihydrate (15 % w/v) (Sigma-Aldrich) was added as a stabiliser [31] to the dialyzed VLPs prior to quantification in a standard hemagglutination assay (HA) using chicken red blood cells (OIE, 2019), and the total protein concentration was determined using a BCA Protein Assay Kit (Pierce). VLP batch sterility was confirmed through aerobic and anaerobic cultivation at the University's Bacteriology Section. Doses of 125, 250 and 500 HAU were diluted in sterile PBS, and mixed with the volumes of Montanide ISA 71 VG adjuvant (Seppic, France) in Table 1 for a total volume of 0.25 ml per dose. The sham control was prepared from non-infiltrated tobacco leaf extract diluted in PBS and mixed 50 % v/v with Montanide ISA 71 VG adjuvant. Vaccines were prepared on the day of the first immunization with storage at 4 °C until the booster [32].

2.4. Animals, housing and experimental design

SPF White Leghorn hens aged 12 weeks (n = 110) purchased from AviFarms, Pretoria, were randomly assigned into six groups marked with coloured, numbered wing tags (Table 1). The hens were kept on sawdust in an open-sided broiler house with nesting boxes and *ad libitum* access to commercial layer feed and water. Egg production was recorded daily. Vaccines were administered by intra-muscular injection of 0.25 ml into the breast and the birds were examined daily for adverse effects. Two mls of blood were collected from the wing veins into 4 ml Vacutainer clot activating tubes (Beckton-Dickinson, UK). Blood was collected prior to the first vaccination (day 0, 12 weeks of age), three weeks after the

Table 1. Vaccine groups and doses.

Group	No. hens (individual bird numbering)	H6 VLP vaccine dose			Adjuvant
		HAU	μl	μg	
White	10 (W1–W10)	0	0	0	50 % v/v
Pink	20 (P1–P20)	500	49	19.6	0
Red	20 (R1-R20)	500	49	19.6	50 % v/v
Orange	20 (O1–O20)	250	25	10	50 % v/v
Blue	20 (B1–B20)	125	13	5.2	50 % v/v
Green	20 (G1-G20)	250	15	10	25 % v/v

HAU- hemagglutinating units; v/v-volume/volume.

booster vaccine (15 weeks of age) and then monthly thereafter when the hens were aged 17 weeks (2 weeks post booster), 22 weeks, 27 weeks, 31 weeks, 35 weeks and 39 weeks. All birds also received a live Newcastle disease vaccine (Nobilis ND C2, Intervet SA) by eye drop at 15 and 23 weeks of age, and live Fowlpox virus (FPV) vaccine (Onderstepoort Biological Products, South Africa) administered in the wing web at 15 weeks of age. The hens were humanely euthanized by cervical dislocation at the end of the study. All animal procedures were approved by the Research and Animal Ethics Committees of the University of Pretoria (approval number REC095-20).

2.5. Antibody detection

Whole blood was left to clot at room temperature for at least one hour and serum was separated by centrifugation at 5,000 x g for 10 min and stored at -20 °C degrees. Influenza nucleoprotein (NP)-specific antibodies were detected by competition ELISA (NP-cELISA) with the IDEXX Influenza A virus Antibody test kit as per the manufacturer's instructions. Sample to negative (S/N) ratios ≥0.50 were considered negative, and S/N ratios <0.5 were positive. H6 HA -specific antibodies were detected in HI tests with the homologous live virus antigen (A/chicken/South Africa/411965/2020 (H6N2) according to the standardized method (OIE, 2019), at the Assurecloud laboratory. HI titres were considered positive if complete inhibition of hemagglutination was observed at a sample dilution of 4 $\log_2$ (2^4 or 1:16) or more. Newcastle disease virus fusion (F) protein specific-antibodies were detected with a Newcastle Disease Virus Antibody Test Kit (BioChek) according to the recommended procedure. Antibody titres were calculated from the sample to positive (S/P) ratio as directed in the kit protocol. Antibody titres ≤992 were considered negative whereas titres ≥993 were positive.

2.6. Statistical analysis

Data expressed as the mean +/- standard deviation (SD) were analysed by one-way ANOVA followed by Tukey's multiple comparison test in GraphPad Prism v 9.1.2 software for Windows (La Jolla, CA, USA). A Pvalue <0.05 was considered significant (see Figure 1).

3. Results

3.1. Expression, purification and quantitation of H6 VLPs

A synthetic gene encoding a 2020 South African H6-subtype HA protein was cloned and agro-infiltrated into *N. benthamiana* leaves for VLP expression, within 14 days. SDS-PAGE analysis of the partially purified plant extract showed a prominent band that corresponded to the expected size of the H6 HA protein of ~64 kDa (Figures 1(a), S1(a)) and was confirmed by Western blot using H6N2-specific chicken antiserum (Figures 1(b), S1(b)). Abundant influenza-like VLP structures ranging from 70 to 140 nm in diameter were visible under the TEM (Figure 2). VLP purification methods using iodixanol or sucrose for differential centrifugation were compared to establish the most cost-effective method for VLP vaccine production. The HA titre of both final products (post dialysis in PBS; with 15 % w/v trehalose added) was 1:256, with a total protein concentration of 403.21 μg/ml (Table 1). Thus, for iodixanol purification, 680 vaccine doses of 250 HAU were obtained from 34 g of infiltrated plant leaves, which corresponds to an estimated 20,000 vaccine doses per kilogram of plant leaves. For the sucrose purification, 740 vaccine doses of 250 HAU (or 10 μg) were obtained from 42 g of leaves, yielding corresponding to 17,620 vaccine doses per kilogram leaf material. The sucrose-purified VLP was used in the *in vivo* dose study.

3.2. Adverse effects and interference with other vaccines

No adverse vaccine effects were observed in any hens at the site of injection, and the birds remained healthy for the duration of the 7-month study, except for three that died from non-vaccine-related causes or were euthanized for humane reasons after sustaining injuries around 32 weeks of age (O4 and B9) or 35 weeks of age (W1). The anticipated scarification of the wing web at the FPV vaccination site was observed on subsequent days. FPV-specific antibodies were not monitored as a routine serological test for determining FPV immunity is not currently available, but no fowlpox lesions were observed in the hens over the 30-week duration of the study, despite observation of the arthropod vectors in the

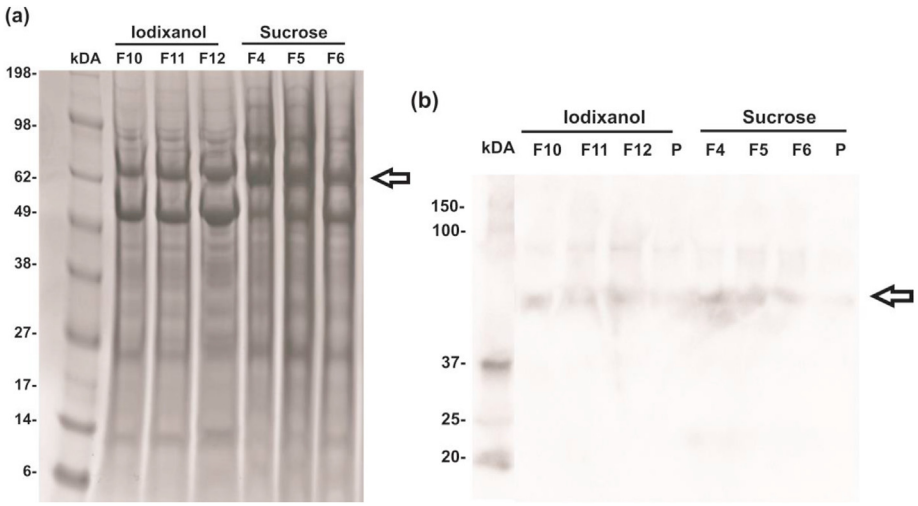

Figure 1. (a) SDS-PAGE and (b) Western blot analysis of plant-produced H6 VLPs partially purified by density gradient centrifugation. F- fraction; P- pooled fractions dialyzed in PBS, with 15 % w/v trehalose added. Hyper-immune chicken H6N2 antiserum was used for HA detection. The arrows indicate the position of the HA protein at approximately 64 kDa. The non-adjusted images are provided as supplementary figures S1(a) and (b), respectively.

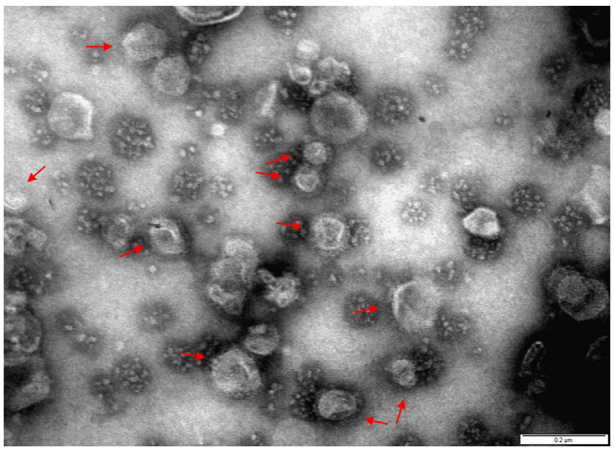

Figure 2. Negative staining transmission electron microscopy of recombinant H6 HA subtype influenza VLPs, indicated by arrows, produced in tobacco (*Nicotiana benthamiana*) leaves.

environment. The hens started laying eggs at 16–18 weeks of age, and there were no adverse effects of vaccination on onset of egg production (Supplemental Figure S2). NDV-specific mean antibody titres >15,000 were detected in hens (n = 10) selected randomly from the 500 HAU dose group (Red) (Supplemental Figure S3), therefore the H6 vaccination did not interfere with live NDV vaccination, or *vice versa*.

3.3. Dose effects on seroconversion in adjuvanted vaccine groups

All pre-bleed NP-cELISA S/N values were >0.8, verifying that the hens were not exposed to IAV (Table S1). Three weeks after a single vaccination (Figure 3(a); Figure 4; Table S1), all groups that received the H6 VLP vaccine seroconverted with H6-specific HI GMT titres ≥5.05 $\log_2$. The Orange group (250 HAU, 50 % v/v adjuvant) seroconverted with the highest GMT of 6.95 $\log_2$, which was significantly higher than that of the Red group (500 HAU, 50 % v/v adjuvant) at 5.05 $\log_2$ ($P < 0.001$) and the Blue group (125 HAU, 50 % v/v adjuvant) at 5.9 $\log_2$ (both P values <0.001), but there was no significant difference with the 5.15 $\log_2$ GMT of the Green group (250 HAU, 25 % v/v adjuvant) vs. those of the Orange, Blue or Red groups. Two weeks after the booster vaccination (Figure 3(b); Figure 4), GMTs had increased by 1.85–4.2 logs in all H6 VLP-vaccinated groups. The Red group had the highest proportional GMT increase of 4.2 logs after the booster to peak at the maximum of 9.25 $\log_2$, but the titre differences between the Red, Orange, Blue or Green groups' GMTs were not significant. In months 3 and 4 (Figure 3(c) and (d); Figure 4), the GMTs in the Red, Orange, Blue and Green groups remained ≥7.75 $\log_2$ with only a slight month-on-month decrease, but between months 4 and 5 the GMTs dropped suddenly by 2.43 and 3.05 logs. GMTs in the 5–6 $\log_2$ range were however stably

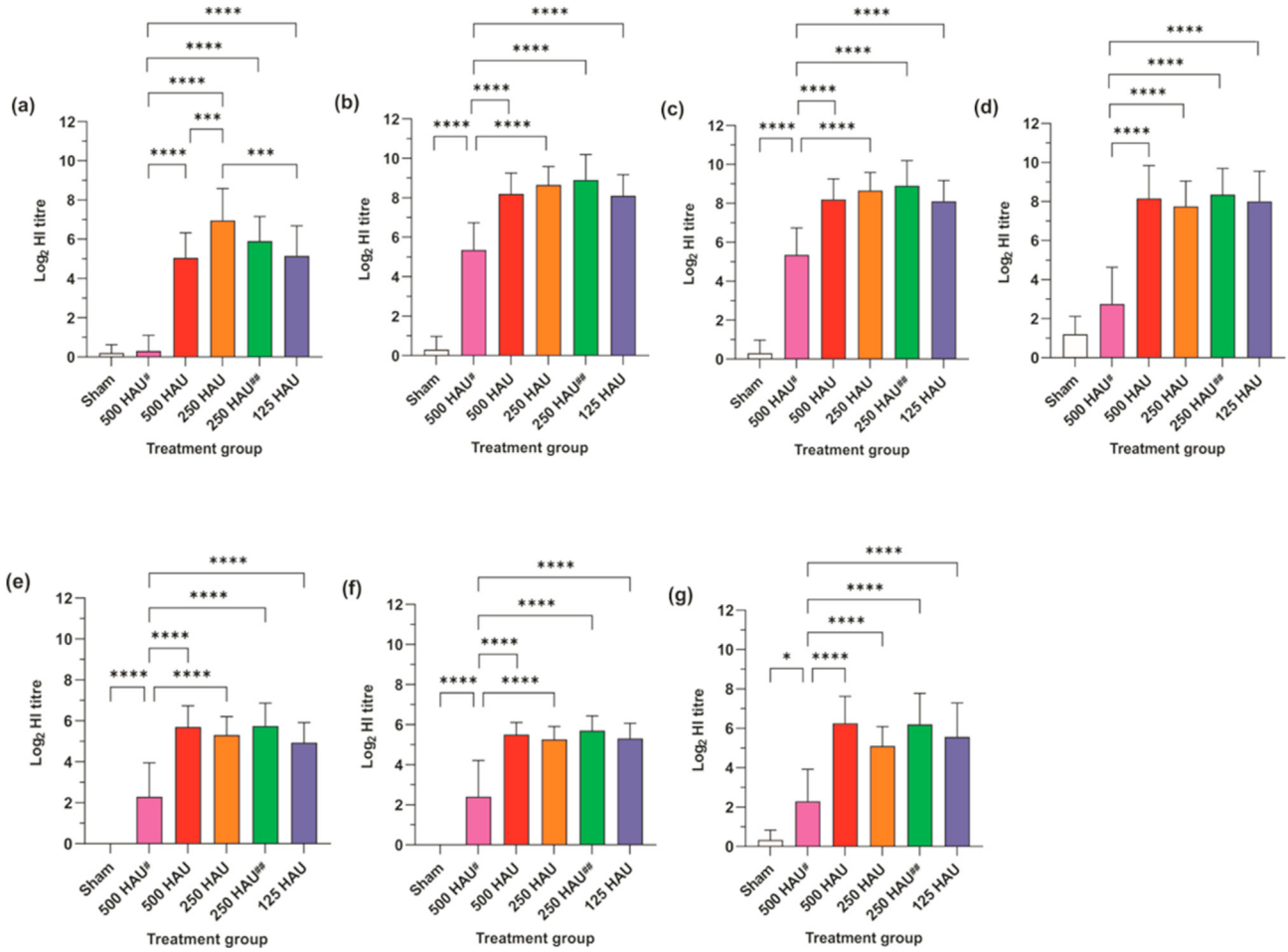

Figure 3. H6 influenza subtype-specific antibody responses in hens aged (a) 15 weeks (month 1), three weeks after a single vaccination with a recombinant plant-produced VLP vaccine; (b) 17 weeks (month 2), two weeks after a booster vaccination; (c) 22 weeks (month 3); (d) 27 weeks (month 4); (e) 31 weeks (month 5); (f) 35 weeks (month 6) and (g) 39 weeks (month 7). Vaccines were formulated with 50 % v/v adjuvant apart from # and ## with zero and 25 % v/v adjuvant, respectively. Statistical significance between groups is indicated as: *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001. Only the statistical comparison between Sham and 500 HAU# is shown; the comparison between Sham and all other groups were very significant at P < 0.0001.

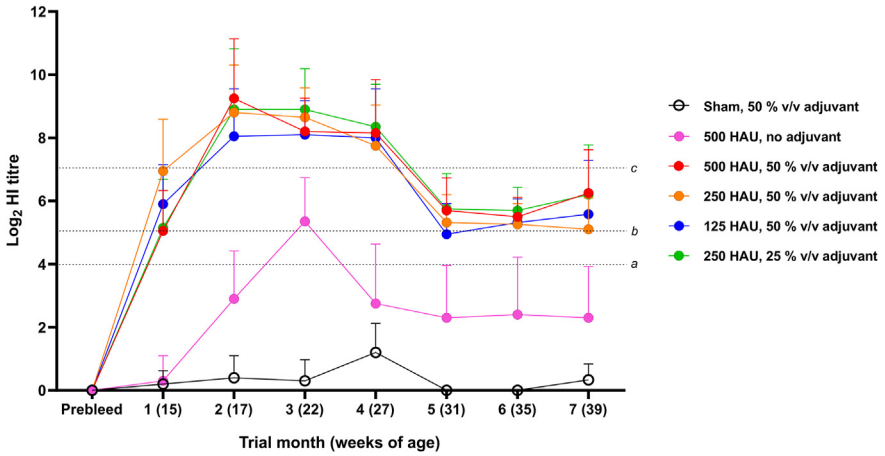

Figure 4. Seroconversion in layer hens vaccinated with a plant-produced H6-subtype VLP vaccine at 12 weeks of age, with a booster given at 15 weeks of age. Grey dotted lines represent the threshold hemaglutination inhibition (HI) assay titres associated with (a) positive HI assay, (b) protection against mortality and (c) prevention of viral shedding.

maintained in these four groups until the termination of the study in month 7 (Figures 3(e)–(g); Figure 4).

3.4. Intrinsic immune-stimulatory potential of plant-produced VLPs in chickens

After a single vaccination with 500 HAU of a non-adjuvanted VLP (Pink), the GMT increased from 0.3 to 2.9 $\log_2$, but this was not significant compared to the sham-vaccinated control group (White) (Figure 3(a); Table S1). Two weeks after the booster vaccination, the Pink group GMT increased significantly ($P < 0.01$) compared to the sham-vaccinated birds but still remained below the 4 $\log_2$ threshold for positivity. H6-specific antibodies in this group continued to increase in month 2 and peaked in month 3 with a GMT of 5.35 $\log_2$ (Figure 3(c)), but the antibodies rapidly declined again to GMT <3 $\log_2$ in month 5 and stayed in the 2.3–2.4 $\log_2$ range for the remainder of the study (Figures 3(d)–(g); Figure 4).

3.5. DIVA capability of the plant-produced H6 VLP vaccine

Thirty blood samples from the final bleed (month 7) were randomly selected and tested with the NP-cELISA. As expected, all S/N values were negative (Table S1), because the plant-produced VLP vaccine does not contain the NP protein. An increase in the mean S/N value at the final bleed compared to the pre-bleed was likely caused by the long-term storage of the ELISA kit, but the kit was still within its expiration date and the kit positive and negative controls were still within the acceptable recommended OD range. Exposure to a live virus from a field infection as well as immunization with whole inactivated virus vaccines induces anti-NP antibodies in the host, therefore using a combination of NP-cELISA and HI, that are validated tests used widely within the accreditation systems of the national testing laboratories, enables the easy distinction between vaccinated and infected birds if HA VLP vaccines are used.

4. Discussion

H6N2 strains have been endemic and causing economic losses in the South African poultry sector since 2002. An inactivated whole virus vaccine derived from a South African 2004 H6N2 strain is the only IAV vaccine registered for use in the country, but despite marked antigenic drift in H6N2 field viruses [33] the vaccine has not been updated, and its use complicates the national surveillance program because whole-virus based technology generally does not enable DIVA [3]. The importance of antigenic matching between an avian influenza vaccine and the circulating field viruses cannot be overstated; this is the primary determinant of vaccine efficacy [9]. Previously, we produced an H6 VLP vaccine in tobacco plants that was 95.8 % identical in the HA protein to a 2016 H6N2 challenge virus. After two immunizations, this VLP vaccine reduced the proportion of infected chickens as well as the magnitude of mean viral shedding by $>$ 100-fold in the oropharynx and $>$ 6-fold in the cloaca, and reduced shedding time by at least a week compared to non-vaccinated chickens. However, although the commercial inactivated whole virus vaccine elicited high HI GMTs of 8.8 $\log_2$ after two vaccinations, it only shared 91.5 % amino acid identity in the HA with the challenge virus, and consequently failed to reduce viral shedding at all compared with non-vaccinated birds -alarmingly, it even seemed to increase it [11].

In the present study, we updated a DIVA-compliant H6 HA VLP vaccine produced in tobacco plants to match field strains that circulated in South Africa in 2020. In an initial phase we compared the yields of sucrose and iodixanol-purified VLPs. Approximately 20,000 doses (250 HAU) of this H6 HA VLP vaccine can be produced per kilogram of plant material, and although the yield of the sucrose purified VLP was slightly lower than iodixanol, the purification costs for 20,000 doses using iodixanol was estimated at $52.50, whereas the cost for preparing the equivalent doses with sucrose was $1.12 (current pricing).

In the subsequent immunogenicity study in layer hens, birds that received a single vaccination with the 250 HAU dose developed significantly higher GMTs compared to the 125 HAU dose group and surprisingly, the 500 HAU dose group, a phenomenon previously recorded with similar doses of an insect cell-produced H9 HA VLP vaccine [24]. Hens vaccinated twice with 125, 250 or 500 HAUs of the plant-produced vaccine seroconverted with GMTs $>$8 $\log_2$, but the differences were not significant. Compared to equivalent doses of VLPs produced in insect cells, the immunogenicity in chickens of the plant produced VLP after single vaccination was similar to an H7 HA VLP vaccine [6] but approximately one log lower than H5, H6 or H9 HA VLP vaccines [4, 7, 24, 34]. However, after two vaccinations, the GMTs elicited by the plant-produced H6 HA VLP were comparable with other VLP vaccines. As a stronger early protective response in the field is ideal, 250 HAU of the plant-produced VLP vaccine was considered to be the optimal dose in chickens.

Hens vaccinated twice with $\geq$125 HAU doses of the plant-produced vaccine elicited protective antibody responses associated with prevention of viral shedding (i.e. HI GMTs of $\geq$7 $\log_2$). In live challenge studies, even though pre-challenge titres of VLP-vaccinated chickens were $>$8 $\log_2$, viral shedding was not completely abolished, but usually by 5 days post challenge the virus titres had dropped below the threshold for infectivity in eggs. In contrast, up to100 % of the non-vaccinated controls excreted high titres of virus, and in the case of H6N2, viral shedding in the non-vaccinated controls lasted up to 21 days [4, 6, 11, 24, 25].

All HPAI challenge studies with VLP vaccines reported 100 % protection against morbidity and mortality, but even GMT HI thresholds as low as 3 to 3.3 $\log_2$ are associated with survival [16, 25]. In LPAI challenge models in SPF chickens, the correlation of a specific antibody titre with prevention of morbidity has been harder to define. In the field LPAI viruses like H9N2 and H6N2 can cause respiratory disease and weight loss with increases in weekly mortalities, egg quality drop, and production drops of between 5 and 60 %, that are exacerbated by concomitant infections with other pathogens [3, 35], which is difficult to duplicate under experimental conditions. Studies on efficacy of vaccines in preventing egg production losses due to LPAI infection are few, and all thus far have used inactivated whole virus vaccines [35, 36, 37]. Dharmayanti and co-workers [35] performed an efficacy study in laying hens with inactivated whole virus vaccines, comparing a vaccine that was homologous with the H9N2 challenge strain they used to a commercial bivalent vaccine that contained H9N2, but was not matched to the challenge virus. Pre-challenge titres were 10.5 $\log_2$ for the bivalent vaccine, and 8.3 $\log_2$ for the antigen-matched vaccine. Two weeks post challenge, egg production dropped by 6.67 % in the bivalent vaccine group, 12.14 % in the antigen matched vaccine group, and 60.12 % in the non-vaccinated controls. The ability of the bivalent vaccine to better mitigate against the initial drop in egg production was correlated with its higher pre-challenge GMT, however, the antigen-matched vaccine hens recovered egg production to pre-challenge levels within two weeks, whereas in the non-matched vaccine egg production only recovered to pre-challenge levels at 7 weeks post challenge.

Only one prior study [7] investigated the longer-term duration of immunity conferred by VLP vaccines in chickens. Here, we demonstrated that HI GMTs peaked two weeks after the booster vaccination, and remained high for approximately four months before dropping suddenly to around 5–6 $\log_2$, but then remained stable at least another two months. Similar results were reported in a study with a single immunization of an H5 HA VLP vaccine [7]. GMTs of at least 5.3 $\log_2$ prevent oropharyngeal shedding in most vaccinated birds [16], but only partial protection from shedding can be expected at lower titres. Indeed, an H9 HA VLP vaccine's pre-challenge GMT of 4.3 $\log_2$ was insufficient to prevent shedding from the tracheas and cloacae in 70 % and 30 % of chickens, respectively [24]. Thus, a third immunization with a VLP vaccine should be administered after 4 months to elevate the antibody titres to levels that would prevent virus shedding in the event of a field challenge, and possibly every four months in long-lived birds, however the practicality of this remains a challenge.

Most studies testing insect cell or plant-produced VLPs have used proprietary oil-based adjuvants to elicit maximal and longer-lasting protective immune responses in chickens, and there is scope to investigate, optimise and improve the adjuvants used with VLPs to extend high levels of protection beyond four or five months. For example, a single vaccination with a 640 HAU dose of an inactivated whole virus H5N1 vaccine, formulated with an oil adjuvant containing 3.9 % anhydromannitol-octadecenoate-ether (AMOE), elicited a peak GMT of 11 $\log_2$ after four weeks, and after seven months the GMT was still above 10 $\log_2$ [38].

VLPs are superior to inactivated whole virus vaccines in eliciting cell-mediated immune responses in animal models [21, 39], and their effects on stimulating the humoral response of chickens without adjuvant were explored for the insect-cell expressed VLPs. Single doses of H6 HA (432 HAU) or H9 HA (20 μg) VLPs induced peak HI GMTs of ~4 and 4.3 $\log_2$, respectively, after 3 weeks [4, 24], and chickens that received two doses of H5 HA VLP (512 HAU) seroconverted with peak GMTs of 6.5 $\log_2$ after three weeks, which was sufficient to prevent 100 % of mortalities after challenge with H5N1 HPAI virus [5]. Similarly, a booster of the insect-cell produced H6 HA VLP increased the GMT ~7 $\log_2$, and the titre remained >6 Log_2 for at least another two months thereafter [4]. In contrast, we found that without an adjuvant, the plant-produced H6 HA VLP did not induce protective antibody responses. At least two immunizations with a 500 HAU dose were required to produce the peak GMT of 5.35 $\log_2$ that declined rapidly within a month to 2.75 $\log_2$. The importance of including an adjuvant in plant-produced VLPs for chickens was also evident in the group that received a 250 HAU dose with only 25 % v/v adjuvant, where the peak GMT was reached a month later than dose groups with 50 % v/v adjuvant.

In conclusion, two doses of as little as 125 HAU or 5 μg of an adjuvanted plant-produced VLP vaccine induced protective antibodies against H6-subtype avian influenza in layer hens for up to 7 months, which should be verified in future field challenge studies. Mass-application however remains a major drawback for VLP vaccines. Influenza VLP vaccines administered intra-nasally in mice and ferrets demonstrated 100 % efficacy against challenge [40, 41], therefore further research into adjuvants that allow intra-nasal application of VLPs vaccines in chickens for spray administration is warranted. *In-ovo* application of HA VLP vaccines as a primer to live recombinant HA vaccines also remains to be investigated. DIVA-compliant VLP vaccines in general but plant produced VLPs vaccines in particular hold enormous potential for improving poultry health in lower and middle-income countries.

Declarations

Author contribution statement

Celia Abolnik: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Tanja Smith, Martha O'Kennedy: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Daniel B.R. Wandrag: Performed the experiments; Wrote the paper.

Mark-Anthony Murphy, Marizel Rautenbach, Olebile Olibile: Performed the experiments.

Funding statement

This study was funded by the Department of Science and Innovation/National Research Foundation SARChI grants no. 114612 and 129242.

Data availability statement

Data included in article/supp. material/referenced in article.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

Supplementary content related to this article has been published online at https://doi.org/10.1016/j.heliyon.2022.e09804.

Acknowledgements

Thlasila Aphane and Peter Smith are thanked for assistance with the procedures on hens.

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