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SHORT COMMUNICATION

Vaccination of African penguins (*Spheniscus demersus*) against high-pathogenicity avian influenza

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Abstract

Background: High-pathogenicity avian influenza (HPAI) has become a conservation threat to wild birds. Therefore, suitable vaccine technology and practical application methods require investigation.

Methods: Twenty-four African penguins (*Spheniscus demersus*) were vaccinated with either a conventional inactivated clade 2.3.4.4b H5N8 HPAI whole virus or a tobacco leaf-produced H5 haemagglutinin-based virus-like particle (VLP). Six birds received a second dose of the inactivated vaccine. Antibody responses were assessed and compared by employing haemagglutination inhibition tests.

Results: A second dose of inactivated vaccine was required to induce antibody titres above the level required to suppress virus shedding, while a single dose of VLP vaccine produced these levels by day 14, and one bird still had antibodies on day 430.

Limitations: Bacterial contamination of the VLP vaccine limited the monitoring period and sample size in that treatment group, and it was not possible to perform a challenge study with field virus.

Conclusion: VLP vaccines offer a more practical option than inactivated whole viruses, especially in logistically challenging situations involving wild birds.

INTRODUCTION

High-pathogenicity avian influenza (HPAI) has long been recognised as a danger to poultry health, but clade 2.3.4.4b H5 viruses have also become a conservation threat, killing thousands of wild birds on nearly all continents.[1] At least a thousand endangered African penguins (*Spheniscus demersus*) have died in southern Africa,[2–4] but options available to manage the disease are limited.[2] Historically, vaccination of poultry against HPAI has been restricted to a few countries,[5] and vaccination of wild species, including *Spheniscus* spp., has been performed only in zoos.[6–10] The most widely used vaccines comprise inactivated whole viruses, but their application to wild birds seems unrealistic.[11] They are relatively inexpensive to produce but require virus propagation to high antigenic titres in embryonated chicken eggs, parenteral administration and usually two or more doses.[11] Their production is slow,[5] and serological differentiation of infected and vaccinated animals (DIVA) is not possible unless the vaccine contains a neuraminidase

(NA)-type antigen heterologous to the circulating field virus and is used in conjunction with an NA antibody test.

In vitro-produced recombinant antigen vaccines, synthesised in plant leaf tissue, insect or bacterial cells, offer alternatives to conventional inactivated vaccines.[11,12] Rapid synthesis of many doses is possible once a field virus has been genetically sequenced, and no live virus is required.[13] The haemagglutinin (HA) protein expressed on a virus-like particle (VLP) is highly ordered in a dense array, similar to a live virus, which stimulates a strong cellular and humoral response.[14] The absence of internal viral proteins, such as nucleoprotein (NP), allows for DIVA using standard ELISA tests.

The haemagglutination inhibition (HI) test detects antibodies to HA and, as a good correlate of protection against avian influenza viruses (AIV),[5,15] is used to assess the immunogenicity of avian influenza (AI) vaccines. The World Organization for Animal Health (WOAH) Terrestrial Manual[16] recommends an HI serological titre of greater than 32 to protect against death and greater than 128 to reduce challenge virus replication and shedding in chickens.

This trial in African penguins aimed to assess and compare the magnitude and duration of the antibody response induced by two clade 2.3.4.4b H5 HPAI vaccines administered with and without a second dose.

MATERIALS AND METHODS

A pilot trial was performed on two African penguins to confirm the safety of the plant proteins. A volume of 0.25 mL of a 50:50 mixture of tobacco leaf extract (without VLPs) and the oil adjuvant was injected intramuscularly and one bird received a second dose 14 days later.

The main trial involved 24 healthy captive African penguins weighing between 2.5 and 4.6 kg. The birds were permanent residents at a seabird rehabilitation centre as they were unsuitable for release. Penguins were randomly assigned to two treatment groups (Supporting Information). Group 1 received 500 haemagglutination units (HAU) of a VLP vaccine displaying an HA protein based on the amino acid sequence of A/Speckled pigeon/South Africa/−9-004B/2017 (clade 2.3.4.4b HPAI H5N8; accession number AVV60712), produced by transient expression in tobacco plant (*Nicotiana benthamiana*) leaves according to the methods described by Abolnik et al.[17] Five percent trehalose, as a stabiliser, and 50% (v/v) commercial mineral oil adjuvant (Montanide ISA 71 VG, Seppic) were included. Group 2 received 512 HAU of an inactivated whole virus vaccine containing A/chicken/South Africa/Villiers/2017 (clade 2.3.4.4b HPAI H5N8; HA protein accession number AVV60593), produced by Deltamune Animal Health Solutions (Pretoria) and adjuvanted with Montanide ISA 71 VG. Half of group 2 (group 2b) received an inactivated vaccine booster on day 56 after the initial vaccination. The vaccines were administered intramuscularly at a volume of 0.25 mL.

A sample size of five per group was calculated using EpiTools[18] (effect size = three $\log_2$ titres, 80% power, equal variances of 2.5[19]) but was increased to six to allow exclusion of birds that became unwell or started their annual catastrophic moult. Half of each group comprised males, except for group 2b, which had four males and two females.

Group 1 was sampled on days 0, 14 and 28 and group 2 was sampled on days 0, 14, 28, 56, 70, 84, 112, 175, 224 and 287. One bird from group 1 was sampled opportunistically on day 430 when blood was drawn for a transfusion. Sampling of a group ceased after all in the group had titres less than 16.

Five millilitres of blood was sampled from the jugular vein and serum was tested at the Western Cape Provincial Veterinary Laboratory. An influenza A NP-based ELISA (part 99-53101, IDEXX Laboratories) was used to assess DIVA capability, as NP should only be present in the whole-virus vaccine. HI tests were performed according to the WOAH-recommended methods for non-chicken species. Additionally, each 0.5 mL of serum was treated with 0.5 μL of NA from *Vibrio cholera* (Sigma–Aldrich, Merck KGaA) and incubated at 37°C overnight, followed by inactivation in a 56°C water bath for 30 minutes and adsorption of penguin red blood cells (RBCs) with chicken RBCs, to avoid non-specific haemagglutination. A/chicken/South Africa/Villiers/2017, with 99.8% HA sequence identity shared with A/Speckled pigeon/South Africa/−9-004B/2017, was employed as the HI antigen. Samples taken on day 0 were also tested with additional H5 antigens to exclude the presence of cross-reacting antibodies from any previous H5 AIV infection.

The penguins underwent a clinical examination, including palpation of the vaccination site, before each vaccination or sampling and were monitored for an hour afterwards to detect any systemic vaccine reaction or haemorrhage. Fish intake and behaviour were recorded during the twice-daily feeds, and any concerning bird was checked by the resident veterinarian if necessary.

For each time point, the number sampled (n), geometric mean HI antibody titre (GMT), $\log_2$ titre standard deviation (SD) and the proportion of each group with titres of 32 or above, based on the H5N8 HI tests, were recorded. Undetectable titres were excluded from the GMT calculation. A pooled t-test, assuming equal variances, was used to compare mean titres between groups (5% significance) and the 95% confidence interval (CI) of the difference was calculated in EpiTools.

RESULTS

All penguins were avian influenza seronegative on day 0 and all NP-ELISAs performed on group 1 were negative (data not shown).

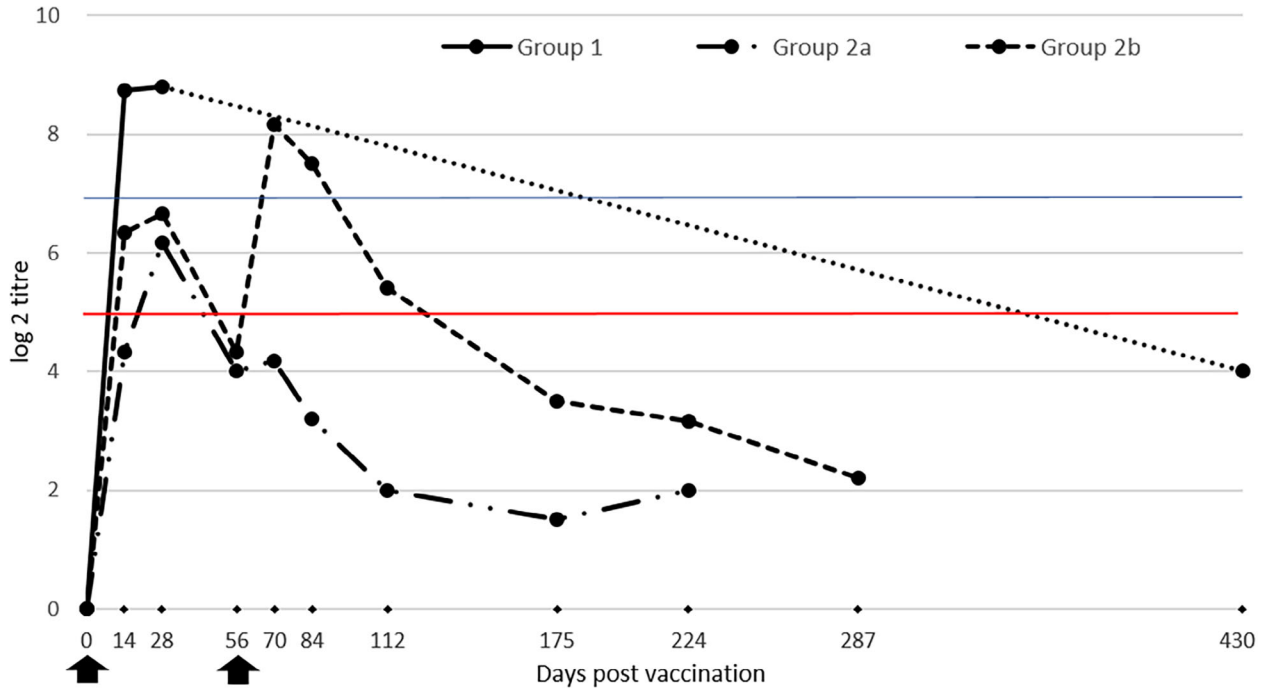

FIGURE 1 H5 influenza subtype-specific antibody response in African penguins vaccinated with one of two clade 2.3.4.4b high-pathogenicity avian influenza vaccines: an H5 avian influenza virus-like particle vaccine (group 1) or an inactivated H5N8 vaccine administered as one (group 2a) or two (group 2b) doses. Days on which vaccine was administered are indicated by arrows. The red and blue lines indicate the minimum antibody titres expected to provide protection against death and reduction in virus shedding, respectively

The two penguins in the pilot trial showed no adverse effects, and group 2 demonstrated few side effects from the inactivated vaccine, besides some weight loss after vaccination. However, the VLP vaccine caused swelling at the vaccination site and systemic illness in 11 of the 12 birds in group 1. Bacteria common in the environment, including *Pantoea (Enterococcus) agglomerans*, *Enterococcus* spp. and *Escherichia vulneris* were cultured from the VLP vaccine intended for the booster, as well as from the stock solution. *Corynebacterium amycolatum*, *Streptomyces* spp. and *Streptococcus* spp. were cultured from an abscess. All birds recovered with antibiotics and, in two cases, surgical treatment, but their illness prevented the administration of a booster and limited sampling to days 14 and 28.

The single dose of the inactivated vaccine induced protective anti-H5N8 antibody levels (GMT = 40, n = 12) by day 14 after vaccination. However, levels dropped by day 56 (GMT = 18, n = 10) and were never high enough to be considered able to reduce virus shedding (Figure 1, Table 1 and Supporting Information). In group 2b, the GMT rose to 287 by day 70, 14 days after the booster dose, which was significantly higher than in group 2a (GMT = 18, p = 0.0002, difference 95% CI = 2.4–5.6), and remained above the levels required to both provide protection and suppress virus shedding until at least day 84. Penguins that received the VLP vaccine had a GMT of 424 on day 14, which was significantly higher than group 2 (p = 0.0008, difference 95% CI = 1.6–5.2), and one bird sampled on day 430 had a titre of 16.

DISCUSSION

Although the VLP vaccine described here still requires individual injection of birds, a single dose may provide sufficient protection during the peak of an epidemic and involves far less expense, effort and stress to the birds than the two doses required with inactivated vaccines. The development of droplet or spray vaccines that can be applied via the mucosa could address the logistical challenges posed by mass parenteral vaccination.[20]

The VLP vaccine is believed to have been contaminated via the phosphate-buffered saline diluent or the sucrose density gradient purification, which meant that only one dose was administered and limited sample sizes and sampling points were achieved for group 1. However, the day 14 GMT was already above the level required to suppress virus shedding and the persistence of antibodies to day 430 in one bird, albeit below protective levels, is encouraging. The same low antibody titres were reached much earlier, by day 56 and 175 respectively, in groups 2a and 2b. In addition, the negative NP-ELISAs in group 1 illustrate the H5 VLP vaccine's DIVA capability. Serology could become valuable in the study of subclinical AIV circulation in wild birds and DIVA vaccines would then be required.

The bacterial infections from the VLP vaccine may have affected antibody production, so a sterile vaccine may have performed differently. Evidence that concurrent bacterial infections affect vaccine immunogenicity is limited, although invasion of immune cells,[21,22] as with some *Enterococcus* spp.,[23] could have a suppressive effect. However, two 250 HAU

TABLE 1 Antibody titres of African penguins vaccinated with one of two clade 2.3.4.4b high-pathogenicity avian influenza vaccines: an H5 avian influenza virus-like particle vaccine (group 1) or an inactivated H5N8 vaccine administered as one (group 2a) or two (group 2b) doses

	Days after primary vaccination										
	0	**14**	**28**	**56**	**70**	**84**	**112**	**175**	**224**	**287**	**430**
n (*n* titres >0)											
Group 1	12	11	5	0	0	0	0	0	0	0	1
Group 2a	5[a]	6	6	4	6	5	5	5 (4)	6 (2)	0	0
Group 2b	6	6	6	6	6	4	5	6	6	6 (5)	0
Proportion protected											
Group 1	0	0.91	0.80	–	–	–	–	–	–	–	0
Group 2a	0	0.33	1.00	0.25	0.33	0	0	0	0	–	–
Group 2b	0	0.83	1.00	0.67	1.00	1.00	0.80	0.17	0.17	0	–
GMT											
Group 1	–	424	446	–	–	–	–	–	–	–	16
Group 2a	–	20	72	16	18	9.2	4.0	2.8	4.0	–	–
Group 2b	–	81	102	20	287	181	42	11	9.0	4.6	–

Note: Sample size (*n*), with the number of titres greater than 0, used to calculate geometric mean titre (GMT), in parentheses; proportion protected: the proportion of the group that seroconverted with antibody titres greater than 32.
[a]Blood could not be obtained from one penguin, but it was vaccinated.

doses, three weeks apart, of the same VLP vaccine completely protected specific pathogen-free chickens against challenge with the homologous virus and significantly reduced virus shedding.[17] This demonstrates the immunogenic properties of the vaccine, even though they could not be confirmed here with a challenge trial or another in vitro assay such as virus neutralisation. Furthermore, just a kilogram of leaves would be sufficient for more than 42,000 500 HAU doses of penguin vaccine.

Plant-produced AI VLP vaccines offer a more effective and feasible alternative to inactivated whole viruses, especially when considering the additional logistical challenges posed by vaccinating wild birds. However, the protection conveyed by the vaccine to vulnerable species should be confirmed and more practical application methods should be investigated.

AUTHOR CONTRIBUTIONS

Data curation, formal analysis, investigation, methodology, project administration, visualization and writing—original draft (lead): Laura Christl Roberts. *Conceptualisation (equal), supervision (supporting) and writing—review and editing (equal)*: Darrell Abernethy. *Investigation, project administration (supporting) and writing—review and editing (equal)*: David Gordon Roberts. *Project administration (supporting), resources and writing—review and editing (equal)*: Katrin Ludynia. *Resources and writing—review and editing (equal)*: Martha Magaretha O'Kennedy. *Conceptualisation (equal), funding acquisition (lead), methodology (supporting), resources (equal), supervision (lead) and writing—review and editing (equal)*: Celia Abolnik.

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CONFLICT OF INTEREST STATEMENT

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

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ETHICS STATEMENT

Research approval was obtained from the University of Pretoria, Faculty of Veterinary Science Research Ethics and Animal Ethics committees (REC256-19).

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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