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Protective immunity of plant-produced African horse sickness virus serotype 5 chimaeric virus-like particles (VLPs) and viral protein 2 (VP2) vaccines in IFNAR$^{-/-}$ mice

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ABSTRACT

Next generation vaccines have the capability to contribute to and revolutionise the veterinary vaccine industry. African horse sickness (AHS) is caused by an arbovirus infection and is characterised by respiratory distress and/or cardiovascular failure and is lethal to horses. Mandatory annual vaccination in endemic areas curtails disease occurrence and severity. However, development of a next generation AHSV vaccine, which is both safe and efficacious, has been an objective globally for years. In this study, both AHSV serotype 5 chimaeric virus-like particles (VLPs) and soluble viral protein 2 (VP2) were successfully produced in *Nicotiana benthamiana* ΔXT/FT plants, partially purified and validated by gel electrophoresis, transmission electron microscopy and liquid chromatography-mass spectrometry (LC-MS/MS) based peptide sequencing before vaccine formulation. IFNAR$^{-/-}$ mice vaccinated with the adjuvanted VLPs or VP2 antigens in a 10 μg prime-boost regime resulted in high titres of antibodies confirmed by both serum neutralising tests (SNTs) and enzyme-linked immunosorbent assays (ELISA). Although previous studies reported high titres of antibodies in horses when vaccinated with plant-produced AHS homogenous VLPs, this is the first study demonstrating the protective efficacy of both AHSV serotype 5 chimaeric VLPs and soluble AHSV-5 VP2 as vaccine candidates. Complementary to this, coating ELISA plates with the soluble VP2 has the potential to underpin serotype-specific serological assays.

1. Introduction

African horse sickness (AHS) is a severe non-contagious infectious disease of equids which is transmitted by *Culicoides* spp. biting midges and threatens not only sport and companion horses but also working horses in rural communities. The aetiological agent is the African horse sickness virus (AHSV), genus *Orbivirus*, family *Reoviridae* [1]. In Africa, AHSV is maintained in large populations of zebra (*Equus quagga*), which are thought to be the natural vertebrate reservoir host [2,3]. Zebra infected with AHSV, rarely display clinical signs of infection yet in susceptible horses the virus has a mortality rate of up to 95%. African horse sickness is endemic to Sub-Saharan Africa; however, the disease incidentally has spread beyond Africa threatening horse populations in Europe and Asia. Due to its severity, AHS is classified as notifiable by the World Organization for Animal Health (OIE). South Africa and Kenya being the only regions in the world where all nine serotypes of AHSV have been isolated [4]. No effective treatment exists and consequently control of the disease relies on vaccination, control of animal movements and prevention of bites by bloodsucking culicoides midges [5]. Vaccination is mandatory in endemic countries of Southern Africa with the only registered commercially available product being a polyvalent live attenuated viral vaccine (LAV) lacking serotypes 5 and 9, produced by Onderstepoort Biological

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products (OBP). Immunity against all nine AHSV serotypes is essential in an endemic setting [5]. Due to safety [5,6] and efficacy [2] concerns and variable immune response to most serotypes [7] with the LAV vaccine, the need for an alternative safe, efficacious new generation vaccine has become imperative. DIVA compliance, the ability to serologically differentiate between naturally infected and vaccinated horses, is also desirable for sero-surveillance, early detection of localised outbreaks, as well as freedom of movement and trade of horses nationally and internationally.

Vaccine candidates that may hold promise include inactivated viruses [4,8,9], recombinant canarypox and modified Vaccinia Ankara (MVA) viral vectored vaccines [5,10–15], Entry Competent Replication Abortive (ECRA) vaccine strains, previously also referred to as Disabled Infectious Single Cycle (DISC) vaccines [16,17], Disabled Infectious Single Animal (DISA) vaccine [18,19] and VLPs produced in insect cells [20]. The protective efficacy of soluble, adjuvanted AHSV VP2, the most variable viral protein, has been recognised since 1996 [21,22] as an efficacious subunit vaccine candidate in horses. However, the level of protection of the first generation baculovirus-expressed viral protein 2 (VP2) was variable probably due to insolubility. Subsequently, the soluble, biological active form of AHSV-5 VP2 was demonstrated to confer protective efficacy equally well with 10 μg or 50 μg antigen doses when it is delivered with saponin adjuvants [23]. Low production yields in *Spodoptera frugiperda* (Sf9) insect cells of mostly insoluble VP2, resulted in poor immunogenicity [24], whilst elevated production in another insect cell line TnHi5 from *Trichoplusia ni*, yielded soluble AHSV-VP2 protein that conferred protective immunity in IFNAR$^{-/-}$ mice after a single 10 μg dose [25]. Nevertheless, none of these vaccines are commercially available and the initiative to develop safe, efficacious subunit vaccines continued. To this end, plant-produced monovalent virus-like particle (VLP) vaccines, homogenous AHSV-5 and chimaeric AHSV-6 were produced and administered in a prime-boost regime which resulted in high antibody titres in guinea pigs and horses when vaccinated with homogenous AHSV-5 VLPs [26,27] but relatively low titres in horses when vaccinated with the chimaeric AHSV-6 VLPs [28], respectively.

Virus-like particles are similar in size and shape to the wild type virion but lack the viral genome and are therefore non-infectious but highly immunogenic in vaccine formulations. It is well documented that the high-density display of viral epitopes in the native conformation of VLP-based vaccines not only elicits humoral immune responses but is also effective in stimulating CD4+ proliferative and cytotoxic T lymphocyte (CTL) responses [20,29]. Although it is thought that vaccine induced immunity against AHSV is primarily induced by antibodies [12], conserved CD8+ epitopes identified in VP2 that display CTL activity can contribute to protective immunity [33].

Martínez-Torrecuadrada and co-workers (1996) [21] reported that the outer capsid proteins of the AHSV VP2 and VP5 plus inner capsid protein VP7, derived from single or dual recombinant baculovirus expression vectors, are all required for complete protection in horses. Later studies demonstrated that either modified MVA virus or recombinant canarypox virus expressing the AHSV capsid protein VP2 alone or both VP2 and VP5 induced protection against target AHSV serotypes in IFNAR$^{-/-}$ mice and horses [11,15]. In addition, Calvo-Pinilla and co-workers [12] showed that the neutralising antibodies elicited by VP2 alone can confer protection against AHSV upon passive immunization of IFNAR$^{-/-}$ mice. Similarly, protective efficacy was recently demonstrated with a plant-produced soluble Bluetongue virus (BTV) VP2 in IFNAR$^{-/-}$ mice [30]. BTV is the prototype orbivirus and shares many biological and structural characteristics with AHSV [1,31].

The purpose of this investigation was two-fold: firstly, to determine the dose of both plant-produced chimaeric AHSV-1/5 VLPs and soluble AHSV-5 VP2, that will elicit appropriate neutralising antibody titres in IFNAR$^{-/-}$ mice, and secondly to establish protective immunity conferred by both these subunit vaccine candidates when vaccinated animals were challenged with AHSV serotype 5. *Nicotiana benthamiana* ΔXT/FT plants, a glycosylation mutant with a targeted downregulation of xylose and fucose expression that facilitates mammalian-like glycosylation [32] was used in this study as the expression host.

Due to ethical and financial considerations related to the use of horses in high biosecurity installations, the potential of Interferon-α/β receptor knock-out (IFNAR$^{-/-}$) mice as an experimental small animal model for AHSV infection in vaccine and immunology research has been investigated since 2011 [14]. IFNAR$^{-/-}$ mice have extensively been used to test vaccine efficacy against orbiviruses [12,33–35] and they are highly susceptible to clinical disease when infected with AHSV [36].

2. Materials and methods

2.1. Cloning of constructs

pEAQ-HT harbouring genes encoding AHSV-5 VP2 or VP5 (Genbank accession numbers ALM00085.1 and ALL99741.1, respectively) were cloned as described previously [37]. Primers synthesized by Integrated DNA Technologies (IDT, Whitehead Scientific) were designed to produce AHSV-5 VP2^{His} and the gene encoding this protein was cloned into the plant expression vector pEAQ-HT.

2.2. Purification of plant-produced VLPs

N. benthamiana ΔXT/FT plants were grown in a growth room facility maintained at 26–28 °C, 16 h day and 8 h dark. *Agrobacterium* strain AGL-1 (ATCC® BAA-101™) harbouring each of the constructs encoding AHSV-1 VP3/VP7, AHSV-5 VP2 and AHSV-5 VP5 proteins, were adjusted to OD_{600} = 2 and mixed in a ratio of 2:1:1, respectively, for plant infiltration. Leaves were harvested 7–8 days post infiltration in a Bicine buffer (50 mM Bicine, 20 mM NaCl pH 8.4) supplemented with protease inhibitor cocktail (Sigma P2714) and purified using depth filtration, followed by tangential flow filtration (TFF) and filter sterilisation as described [27] but using a 100 K Minimate™ TFF Capsule (Pall Life Sciences). A sample of the TFF purified plant lysate containing the VLPs was subjected to Iodixanol (Optiprep, Sigma) density gradient ultracentrifugation as described previously [37] to quantity the VLPs within 1 ml TFF lysate for subsequent animal studies.

2.3. Purification and validation of AHSV-5 VP2^{His} proteins

AGL-1 harbouring the pEAQ-HT-AHSV-5^{His} construct (OD_{600} = 1.4) was infiltrated in plant leaf tissue. Five days post infiltration (dpi) fresh leaf material (~100 g) was harvested using a Matstone juice extractor followed by a 2 min homogenizing step using an Ultra turrax in a Bicine buffer with the addition of 0.1% (w/v) N-Lauroylsarcosine sodium salt and supplemented with protease inhibitor cocktail in a leaf tissue:buffer ratio of 1:2. Centrifuge clarified supernatant was subjected to immobilized metal ion affinity chromatography (IMAC), with nickel as the metal ion. The column used was a 5 ml bed volume Ni-TED resin packed in a XK16 column (GE Health). Bound protein was eluted isocratically with 5 bed volumes of imidazole-containing buffer. Eluted fractions were pooled and concentrated using Vivaspin 15 columns (Sartorius, VS15T01) in a swing bucket rotor (4 000 g × 60 min). The concentrate was dialyzed twice within 16 h against 2 L PBS buffer (140 mM NaCl, 1.5 mM KH_2PO_4, 10 mM Na_2HPO_4, 2.7 mM

KCl, pH 7.4) at 4 °C before use in the vaccine formulation or in serological tests. To coat ELISA plates, VP2 was further purified using Dynabeads™ (Invitrogen, Catalogue number 10104D) to mitigate antibody detection of contaminating plant proteins.

The iodixanol-purified density gradient VLPs and IMAC purified soluble VP2 were subjected to sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). The assembled VLPs and production of soluble VP2 antigen was confirmed using transmission electron Microscopy (TEM) and LC-MS/MS based peptide sequencing as described before [37]. Protein concentration was determined using the Pierce™ Micro BCA protein assay kit (Catalogue # 23235, Thermo Scientific).

2.4. Production of BEI inactivated vaccine

To prepare the inactivated AHSV-5 vaccine material, an attenuated serotype 5 strain, initially adapted to cell culture was propagated in confluent Vero (African green monkey kidney) cell culture in T175 flasks until 100% cytopathic effect was observed. The cell cultures were originally maintained in Glasgow Minimum Essential Media (GMEM) supplemented with 5% fetal bovine serum (Sigma), 10% tryptose phosphate broth and 1X antibiotic–antimycotic solution (Thermo Scientific, 15240096). Harvested supernatant was clarified by centrifugation, and the virus then serially diluted and titrated on Vero monolayers. A virus harvest of 1.89 $x10^5$ PFU ml^{-1} (plaque forming units per millilitre) was subsequently inactivated using a 1 N solution of binary ethylenimine (BEI) prepared using the method of [38]. The BEI was added to clarified aliquots of virus to a final concentration of 5 mM and incubated for 72 h with agitation at 37 °C. At 72 h the reaction was stopped by adding a 10% v/v solution of 1 M sodium thiosulphate.

Inactivation was assessed by inoculation of a serial ten-fold dilution of the inactivated material into confluent Vero cells in 96-well tissue culture plates. The plates were monitored for 7 days for signs of cytopathic effect or cell toxicity.

2.5. Production of AHSV-5 challenge material

To prepare the challenge material, a freeze-dried low passage isolate of AHSV-5 was retrieved from OBP virus repository (isolate AHS T5 30/62) and resuspended in 1 ml of GMEM. The material was passaged once in confluent Vero cell culture until 100% cytopathogenic effects (CPE) had developed and then titrated in 50% tissue culture infectious dose ($TCID_{50}$ ml^{-1}) and tested for the absence of bacterial and fungal contamination. The final challenge material was sterile and had a titre of 1 $x10^6$ $TCID_{50}$ ml^{-1}.

2.6. Formulation of VLP and VP2 vaccines

D-(+)-Trehalose dihydrate (Sigma-Aldrich)(5 % m/v) was added to the TFF purified VLPs and IMAC purified VP2 antigens before filter sterilisation with a 0.45 μM + 0.2 μM Sartopore 2 sterile capsule (Sartorius, 5441307H4) using a peristaltic pump. The appropriate filter sterilised VLPs and VP2 were mixed with autoclaved adjuvant (5% Montanide GEL 01 PR, Seppic, France) immediately before vaccination. The binary ethyleneimine (BEI) inactivated virus vaccine (positive control) and PBS (negative control) were formulated similarly. A cytotoxicity study in Vero cells was also conducted to confirm the safety of the plant-produced vaccine products (Supplementary data, Fig. S2).

2.7. $IFNAR^{-/-}$ mice study design to test vaccine safety, immunogenicity, and efficacy

The study designs for the immunogenicity and efficacy testing of plant-produced AHSV-1/5 VLPs and AHSV-5 VP2 as vaccine candidates, are depicted in Table 1 and complemented by a schematic diagram (Fig. 2). In short, 24 and 21 female $IFNAR^{-/-}$ mice were used in these two studies, respectively. Initially, the vaccine was formulated as per the approved volume of 300 μl, but it became evident due to the high concentration of purified VLPs and VP2, that the volume could be reduced to 200 μl and used as such for the booster vaccination.

2.8. $IFNAR^{-/-}$ mice vaccination and challenge

$IFN\alpha/\beta R^{-/-}$ mice (A129, IFNAR type 1) were purchased and imported from B&K Universal, Marshall BioResources and bred at PCDDP with the necessary licence in place. Seven-week-old, female, mice were acclimatised under pathogen-free conditions in the biosafety level 3 (BSL-3) facility for 7 days before vaccination. Feed and water were provided ad libitum. Room temperature and humidity were continuously monitored and kept at 22 °C ± 2 and 55%±15 respectively. Light-dark cycles were set at 12 h each.

Eight groups of $IFNAR^{-/-}$ mice (n = 3) were vaccinated intraperitoneally with 1 μg, 5 μg or 10 μg plant-produced VLPs or VP2 using a fixed needle syringe (25G diameter, 16 mm length). Five groups of mice (n = 6 for vaccinated; and n = 3 for control groups) were used in the challenge study. For ethical reasons only 3 mice were used in the dose escalating study and efficacy study control groups. Mice in the challenge study, were prime boost vaccinated (days 0 and 14) with either 10 μg VLPs (group 1) or 10 μg VP2 (group 2) or PBS buffer (negative control, group 3) or BEI inactivated $5x10^4$ PFU (positive control, group 4). All vaccines were adjuvanted with Montanide GEL 01 PR. Group 0 was not vaccinated and used as naïve control. Groups 1–4 were challenged 28 days after the primary vaccine. No sera were collected from the mice in the challenge study on day 28 to minimise stress. Challenge with a dose containing 1.4 X 10^5 pfu of AHSV-5 per mouse on day 28 (14 days post booster immunization) was administered subcutaneously as it most closely resembles the route of entry during the natural AHSV infection cycle.

2.9. Sera collection and clinical signs

All sera collected were heat inactivated at 56 °C for 30 min before transport to the designated analysis facilities. Clinical scoring was performed as described previously [36]. Mice were humanely euthanised when they showed severe clinical signs (weight loss, dehydration, frequent hunching, severe conjunctivitis, or any other condition that prevented food or water intake).

2.10. qRT-PCR of lung tissue

To assess the presence as well as relative quantity of AHSV-5 segment-2 nucleic acid in harvested lung tissue, 0.1 mg of harvested tissue was homogenised in 1 ml of GMEM. A volume of 0.1 ml of homogenate was subsequently extracted using TRIzol™ reagent (Thermo Fisher Scientific, 15596026) according to the manufacturer's instructions, and the RNA pellet resuspended in a volume of 100 μl nuclease free water and frozen at −80 °C until use. Five microliters of total RNA were subjected to a type-specific RT-qPCR for AHSV-5 as previously described [39], with modification.

The AHSV real-time RT-qPCR assay was performed by adding 5 μl of total RNA from each sample to a qPCR reaction containing 20 μl of VetMax™-Plus One-Step RT-PCR master mix (Applied Biosystems part number 4415328), as well as a final concentration of 0.4 μM of the type-5 specific forward and reverse primer and 0.12 μM of type-5 specific hydrolysis probe (FAM labeled). Non-template control reactions containing nuclease-free water instead

Table 1
Experimental design of a) safety and immunogenicity and b) efficacy trials of plant produced chimaeric AHSV-1/5 VLPs and soluble AHSV-5 VP2 as vaccine candidates in female IFNAR$^{-/-}$ mice (n = 24 or n = 21, respectively). Mice were injected intraperitoneally with 300 µl (primary vaccine) and 200 µl (booster vaccine) of adjuvanted vaccines or PBS buffer as negative control. The AHSV-5 BEI inactivated monovalent vaccine (proprietary to OBP) served as positive control. All vaccines were adjuvanted with 5% Montanide GEL 01 obtained from Seppic (France). IFNAR$^{-/-}$ mice in the efficacy study (groups 1–4) were challenged on day 28 with a low cell culture passaged strain of AHSV-5 at 1.4x10^5 PFU.

a) Safety and immunogenicity study Groups		Dose	Number of mice	Number of doses	Days of vaccination
1–3	Chimaeric AHSV-1/5 VLPs	1,5,10 µg	3/group	2	D0, D14
4–6	Soluble AHSV-5 VP2	1,5,10 µg	3/group	2	D0, D14
7	PBS negative control	–	3	2	D0, D14
8	BEI inactivated positive control vaccine	1.89x10^5 PFU	3	1	D0
b) Efficacy study Groups		Dose	Number of mice	Number of doses	Days of vaccination
0	Control group	–	3	None	None
1	Chimaeric AHSV-1/5 VLPs	10 µg	6	2	D0, D14
2	Soluble AHSV-5 VP2	10 µg	6	2	D0, D14
3	PBS negative control	–	3	2	D0, D14
4	BEI inactivated positive control vaccine	5x10^4 PFU	3	2	D0, D14

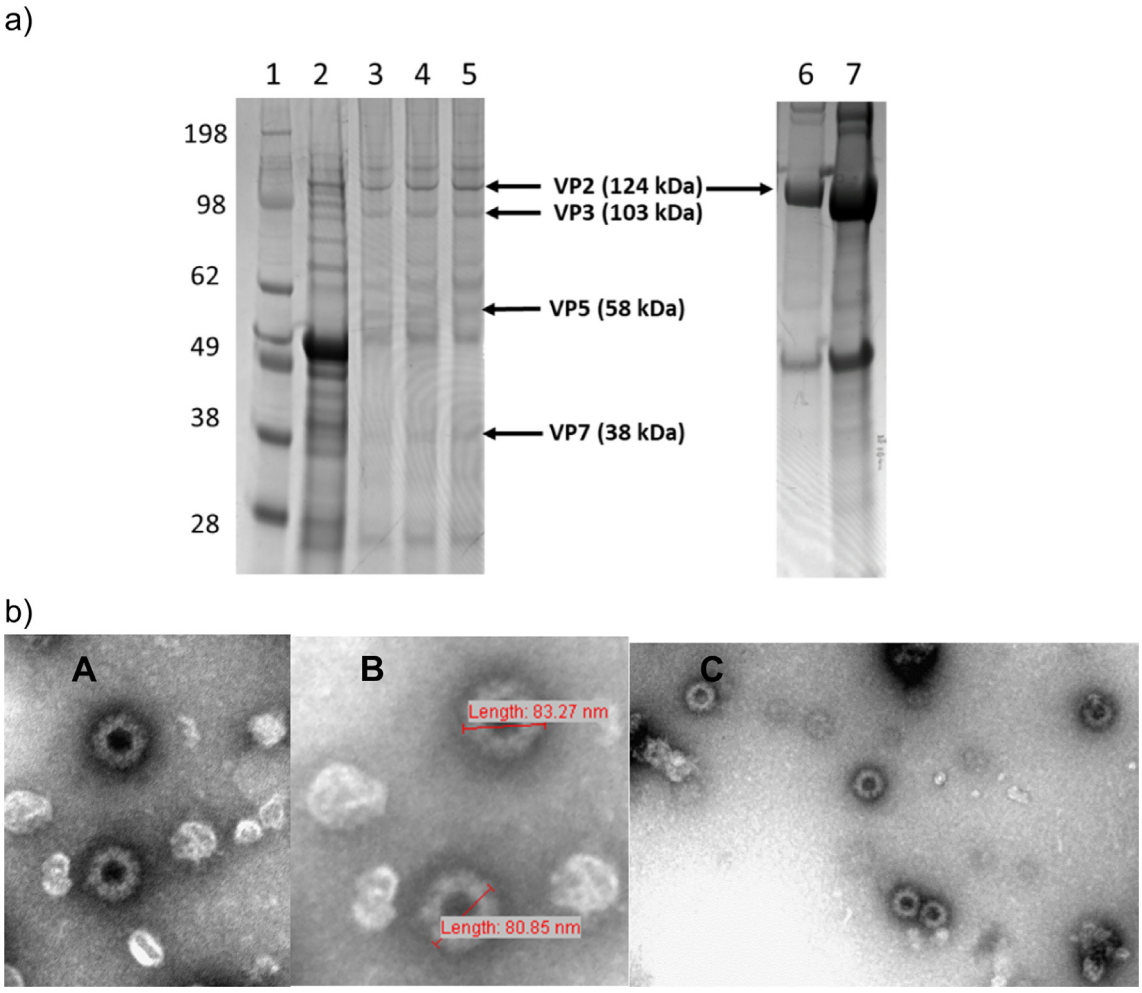

Fig. 1. SDS-PAGE and TEM images of plant produced chimaeric AHSV-1/5 VLPs and AHSV-5 VP2. Six-week-old *N. benthmiana* (ΔXT/FT) were co-infiltrated with *Agrobacterium* AGL-1 harbouring relevant constructs to assemble chimaeric AHSV-1/5 VLPs. (a) Iodixanol density gradient fractions 40–35% were collected as well as IMAC purified soluble VP2 and separated by 4–12% Bolt Tris-Bis Plus precast gels (Life Technologies), followed by Coomassie blue staining: lane 1, MW, SeeBlue® Plus2 Pre-stained Protein Standard; lane 2, pEAQ-HT-empty vector expressed in plant leaf tissue; lanes 3–5, chimaeric AHSV-1/5 VLPs fractions 10–12; 40–30% Iodixanol; lanes 6–7, IMAC partially purified VP2. Viral capsid proteins VP2 (124 kDa), VP3 (103 kDa), VP5 (58 kDa) and VP7 (38 kDa) are indicated with arrows. (b) VLPs in Iodixanol fractions were stained using uranyl acetate and visualised using TEM. Fully assembled VLPs measure ~ 80 nm (A-C).

of RNA template as well as positive control reactions containing RNA that was extracted from the OBP AHSV-5 live attenuated vaccine strain, were included in addition to the test samples. The reactions were cycled on a Corbett Rotor Gene 6000 system using the following cycling parameters [48 °C 10 min; 95 °C 10 min; 40 × 95 °C for 15 sec, 55 °C for 45 sec], with the detection threshold set at 0.1. Lung and control samples were tested in duplicate and average cycle threshold (Ct) values calculated.

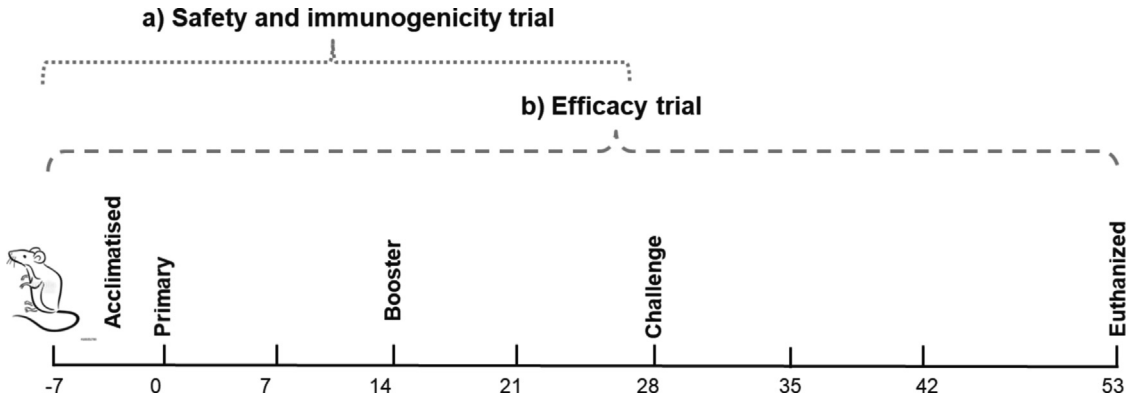

Fig. 2. Schematic diagram of a) safety and immunogenicity and b) efficacy study designs in IFNAR$^{-/-}$ mice.

2.11. Isolation of splenocytes, antigen stimulation and flow cytometry

Mice were euthanised and their spleens aseptically collected when the study was terminated. Spleen cells were released into incomplete RPMI media by mashing the organs and filtering through 25 mm sterile cell strainers (Fisherbrand^{TM}) directly into 50 ml falcon tubes. After red blood cell lysis with RBC lysis buffer (BioLegend), splenocytes were re-suspended in complete RPMI media supplemented with 10% fetal bovine serum (FBS), 1% L-glutamine. Isolated cells were stimulated with either: 1) PBS buffer or 2) the relevant antigen or 3) lipopolysaccharides (*Eschericia coli* produced, Sigma) and technical duplicate 24 well cell culture plates (TPP) and incubated at 37 °C in 5% CO_2 for 48 h. Unstained cells served as negative control. After 48 h of incubation, the cells were harvested by centrifugation and resuspended in 0.1% BSA/PBS with the addition of mouse Fc blocker (Biorad) for the cells to be stained.

Following antigen stimulation, cells were recovered to perform surface staining with FITC-labelled anti-CD3, PE anti-mouse CD8a and PerCP/Cyanine5.5 anti-mouse CD4 antibodies (BioLegend 100204, 100708 and 116012, respectively). After incubation, the cells were fixed in 3% paraformaldehyde, and then once more centrifuged before resuspending in 0.1% BSA/PBS and transferred to FACS tubes. Samples were analysed with the BD FACSLyric flow cytometer (BD Biosciences) equipped with a 488 nm laser for excitation of CD3-FITC, CD8-PE and, CD4-PerCP-Cy5.5. A lymphocyte gate was used during analysis to capture 15 000 cells. Data were analysed with FCSExpress version 7 (De Novo Software, Pasadena, CA, USA) and GraphPad Prism version 9 (San Diego, CA, USA). Lymphocytes were identified on a forward scatter (FSC) and side scatter (SSC) density plot. To ensure stringent single-cell gating, doublets were excluded using SSC and FSC Height and Width. Single events were gated on the FSC-H vs. FSC-W density plots.

2.12. Detection of antibodies against plant-produced VP2 by ELISA

MaxiSorp plates (Thermo Scientific) were coated with 1.25 µg IMAC purified VP2 and incubated overnight at 4 °C. Plates were saturated with blocking buffer (PBS-0.05% Tween 20 and 4% casein hydrolysate). The animal sera diluted in blocking buffer (1:200 or 1:500) were added and incubated for 1 h at 37 °C. After three washes in PBS-0.05% Tween 20, plates were incubated for 1 h at room temperature with a goat anti-mouse-IgG/IgM-HRP secondary antibody (Sigma) at a 1:5000 dilution in blocking buffer. Finally, after three washes in PBS-0.05% Tween 20, the reaction was developed with substrate 3,3′,5,5′-Tetramethylbenzidine (TMB)(Sigma) and stopped by adding 50 ml of 3 N H_2SO_4. Results were expressed as optical densities (ODs) measured at 450 nm. Background OD values were obtained from wells containing blocking buffer without serum samples. This background OD was subtracted from the OD measurements of the sample wells.

2.13. Serum neutralising tests (SNTs)

A 1:5 start dilution of each serum was made in PBS + containing 1% Genta 50. Each serum sample was inactivated at 56 °C for 30 min before testing. Twofold serum dilutions (initial dilution 1:10) were made in duplicate rows in minimum essential medium with 2 g l^{-1} $NAHCO_3$, gentamycin sulfate (Gentamycin 50) 0.05 mg ml^{-1} and 5% fetal calf serum. Equal volume of virus with a predetermined titre was added and incubated at 37 °C in a 5% CO_2 gassed incubator for 1 h. A Vero cell suspension containing 480 000 cells ml^{-1} were added in a volume of 80 µl. Plates were incubated at 37 °C in 5 % CO_2 and the progress of the CPE of the virus was recorded daily for up to 4–5 days of incubation. Titres were determined as the reciprocal of the highest serum dilution that provided > 50% protection of the cell monolayers.

2.14. Statistical analysis

For statistical considerations, statistical difference at $P < 0.05$ were considered significant in a two tailed Student's *t* test.

3. Results

3.1. Production and purification of plant-produced AHSV-1/5 VLPs and AHSV-5 VP2

AHSV-1/5 VLPs and AHSV-5 VP2 were produced in *N. benthamiana* ΔXT/FT. The AHSV virion is a triple layered particle formed by the outer capsids (VP2 and VP5), the middle layer (VP7), and the inner shell (VP3, subcore) [23]. In this study, chimaeric AHSV-1/5 VLPs displaying VP2 and VP5 of serotype 5 as the outer shell layered on the AHSV serotype 1 core (VP3/VP7), measured 80 nm (Fig. 1) and were similar in size to the homogenous AHSV-5 VLPs previously used to vaccinate horses [27]. Iodixanol-purified VLPs and histidine tagged AHSV-5 VP2 proteins were subjected to SDS PAGE (Fig. 1a) and the appropriate band size of 124 kDa, representing VP2, subjected to LC-MS/MS based peptide sequencing. VP2 that forms part of assembled VLPs and VP2 antigens were confirmed by 73–77.4% coverage (133–144 unique peptides) and 77–86.8% coverage (206–235 unique peptides), respectively (Supplementary data, Fig. S1). Only VP2 assembled in VLPs, or histidine tagged VP2 were subjected to LC-MS/MS based peptide sequencing as VP2 is the major determinant eliciting serotype specific neutralising antibodies.

3.2. Cytotoxicity study

A cytotoxicity study in Vero cells was conducted to confirm the safety of the plant-produced and partially purified VLPs and VP2 at concentrations ranging from 1 μg to 2.3 ng serial dilutions per well prior to the pre-clinical trials (Supplementary data, Fig. S2).

3.3. Formulation of monovalent chimaeric AHSV-1/5 VLPs and soluble AHSV-5 VP2

In previous studies, horses were vaccinated by homogenous AHSV-5 VLPs [27] or triple chimaeric VLPs [28] adjuvanted with 5% Montanide Pet Gel A (Seppic, France). In this study, both chimaeric AHSV-1/5 VLPs and AHSV-5 VP2 were adjuvanted with 5% Montanide GEL 01 PR (Batch 36067D, 200623010550, Seppic, France). The latter was recommended by the manufacturer as substitute for Pet Gel A which was discontinued.

3.4. Immunogenicity study

Mice were vaccinated in a dose escalating manner with 1, 5 or 10 μg of either monovalent chimaeric AHSV-1/5 VLPs or monovalent AHSV-5 VP2 vaccine candidates on days 0 and 14, in a prime-boost regime for the initial safety and immunogenicity study. Mice vaccinated with a single dose of plant-produced chimaeric AHSV-1/5 VLPs seroconverted at a 5 μg and 10 ug vaccine dose, within the first 14 days (Table 2). The primary vaccination of AHSV-1/5 VLPs led to seroconversion of 1:40 (1.6 $\log_{10}$) and 1:28 (1.45 $\log_{10}$) when vaccinated with 5 and 10 μg AHSV-1/5 VLPs already on day 14, respectively. A booster vaccine was however necessary to elevate the neutralising antibodies (nAbs) to 1:320 (2.5 $\log_{10}$) on day 28 for all VLP vaccine doses (1, 5 and 10 μg). Ten micrograms of soluble VP2 per mouse were required to equal this immune response after prime-boost vaccination (Table 2). Mice vaccinated with the positive control vaccine, BEI inactivated AHSV-5, seroconverted within the first 14 days at 1:44 (1.64 $\log_{10}$) whereafter the titre dropped to 1:14 (1.15 $\log_{10}$) on day 21 and 0 on day 28. A booster vaccine was not administered due to an adverse effect leading to the death of one of the three mice in the group. Although 1, 5 and 10 μg adjuvanted chimaeric AHSV-1/5 VLPs resulted in SNTs of 1:320 (2.5 $\log_{10}$), only 10 μg of soluble VP2 resulted in a similar 1:320 (2.5 $\log_{10}$) titre. An ELISA using VP2 as antigen (coated with 0.6 μg Dynabead purified VP2) corroborated the SNT data as serum from seroconverted mice resulted in an OD_{450} detection of $\geq$ 0.19, whilst antibody negative control mice were below 0.15 (Table 2). As mentioned before, the BEI inactivated virus vaccine was not administered as a second dose and therefore resulted in an ELISA detection of 0.17 on day 28. As small quantities of serum were collected from the 20–25 g IFNAR$^{-/-}$ mice, the samples within the same groups were pooled to facilitate analysis, particularly for the SNTs.

Complementary to the nAbs response in mice, harvested splenocytes were stimulated for 48 h with the respective antigens to measure the cellular immune responses by means of flow cytometry (Figs. 3 and 4). Compared to unstimulated PBS controls of the same mice, all the test antigens indicated a measure of CD4+/CD8+ stimulation which is required to induce cell memory. Mice vaccinated with plant-produced AHSV-1/5 VLPs (1, 5 and 10 μg dose, Fig. 3) showed a larger increase in stimulation as compared to the OBP BEI inactivated vaccine as positive control and to a lesser extent for the soluble VP2 vaccinated mice (1, 5 and 10 μg dose, Fig. 4). This suggests a role in cell-mediated immunity contributing to the protection.

3.5. Efficacy study in IFNAR$^{-/-}$ mice

The prime-boost vaccine regime of 10 μg was chosen for the subsequent efficacy study as both 10 μg VLPs or 10 μg VP2 resulted in strong seroconversion by day 28 (1:320, 2.5 $\log_{10}$, Table 2). Vaccinated mice (groups 1–4) were challenged with AHSV-5 at $1.4x10^5$ PFU on day 28 and all groups of mice were monitored daily for clinical signs of AHS for the 25-day period after challenge until the planned study termination on day 53. No clinical signs were observed in mice, potentially due to low challenge dose of $1.4x10^5$ PFU per mouse, slow onset of viraemia (not determined), or low strain virulence. Nevertheless, protection against AHSV-5 conferred by both plant-produced adjuvanted VLPs and VP2 vaccines correlated strongly with SNTs determined during the immunogenicity study (Table 2) and mice (n = 6) of each group survived until day 25 post challenge when the study was terminated. The negative control group (PBS with adjuvant) succumbed within 8–11 days after challenge. One of the BEI-vaccinated positive control group mouse was euthanized on day 11. Viral infection in lungs showed a trend of reduced viral load (VLPs < VP2 < BEI inactivated) but were not statistically significant (statistical difference at $P < 0.05$ for averages obtained with n = 3 in a two tailed Student's *t* test, Table 3 and supplementary Table S1).

4. Discussion

Biopharming, developing biopharmaceutical products in plants, is a feasible option to produce veterinary vaccines. Plant-produced vaccines are considered cost-effective, safe, highly immunogenic, DIVA compliant and facilitate the prompt production of synthetic VLPs and VP2 antigens resembling the latest field strains. The authors anticipate that initial mono- and bivalent vaccines will pave the regulatory pathway for the ultimate polyvalent AHS vaccine to induce protective immunity across all nine serotypes.

Table 2
Safety and immunogenicity study: serum neutralizing test (SNT) titres of IFNAR$^{-/-}$ mice vaccinated with plant produced chimaeric AHSV-1/5 VLPs or AHSV-5 VP2, PBS buffer as negative control and BEI inactivated AHSV-5 monovalent vaccine as positive control. Vaccines and PBS buffer were formulated with 5% Montanide GEL 01®. ELISA plates were coated with plant-produced soluble VP2 purified by IMAC* (1.25 μg per well) followed by a second purification step using Dynabeads** (0.6 μg per well).

		Titres after primary vaccination			Titres after booster vaccination		ELISA* OD_{450} VP2 antigen	ELISA** OD_{450} VP2 antigen
Groups	**Vaccination status**	**D0**	**D7**	**D14**	**D21**	**D28**		
1	1 μg chimaeric AHSV-1/5 VLPs	0	0	0	0	320	1.25 ± 0.08	0.313 ± 0.01
2	5 μg chimaeric AHSV-1/5 VLPs	0	0	40	0	320	1.45 ± 0.07	0.384 ± 0.03
3	10 μg chimaeric AHSV-1/5 VLPs	0	0	28	0	320	1.21 ± 0.13	0.387 ± 0.022
4	1 μg soluble AHSV-5 VP2	0	0	0	0	0	1.33 ± 0.05	0.154 ± 0.009
5	5 μg soluble AHSV-5 VP2	0	0	0	0	0	1.42 ± 0.25	0.096 ± 0.005
6	10 μg soluble AHSV-5 VP2	0	0	0	0	320	1.27 ± 0.4	0.194 ± 0.004
7	BEI inactivated AHSV-5 positive control	0	0	44	14	0	0.17 ± 0.01	0.080 ± 0.005
8	PBS negative control	0	0	0	0	0	0.36 ± 0.02	0.091 ± 0.002

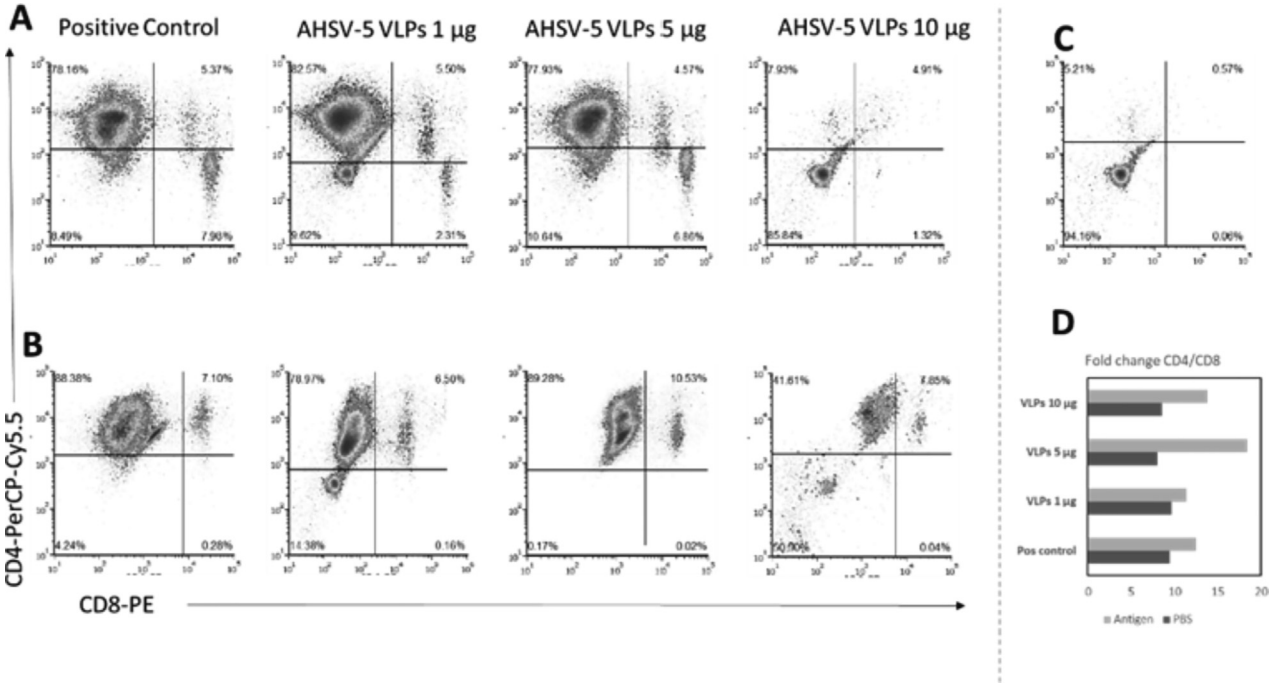

Fig. 3. T-cell responses of mice splenocytes harvested 28 days after vaccination with different doses of AHSV-1/5 VLPs (1, 5, 10 µg). Splenocytes stained with CD4- and CD8-specific antibodies and analysed by flow cytometry. Representative flow cytometry plots are illustrated. The % splenocytes positive for both CD4+ and CD8+ were quantified and presented in the upper right quadrant. A) Splenocytes stimulated with PBS buffer or B) AHSV-5 VLPs. C) Unstained cells served as a negative control. D) the fold change relative to the negative control of the respective groups.

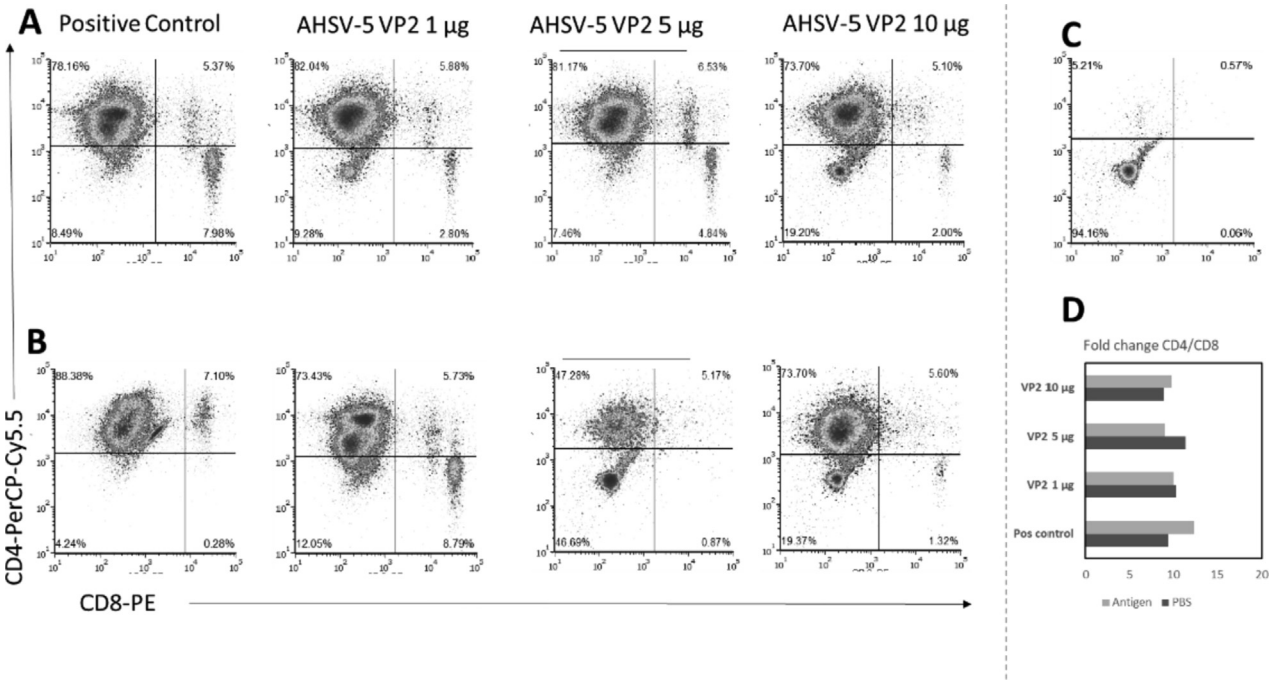

Fig. 4. T-cell responses of mice splenocytes harvested 28 days after vaccination with different doses of AHSV-5 VP2 (1, 5, 10 µg). Splenocytes stained with CD4- and CD8-specific antibodies and analysed by flow cytometry. Representative flow cytometry plots are illustrated. The % splenocytes positive for both CD4+ and CD8+ were quantified and presented in the upper right quadrant. A) Splenocytes stimulated with PBS buffer or B) AHSV-5 VP2. C) Unstained cells served as a negative control. D) the fold change relative to the negative control of the respective groups.

In this study, plant-produced chimaeric AHSV-1/5 VLPs elicited a humoral immune response of 1:320 (2.5 Log_{10}) in $IFNAR^{-/-}$ mice when prime-boosted with 1 µg, 5 µg and 10 µg, whereas only 10 µg doses of VP2 resulted in seroconversion (1:320 (2.5 log_{10})). VLPs consist of all four capsid proteins assembled (VP2, VP3, VP5 and VP7) whilst soluble VP2 is a single protein, which might be the reason why lower doses of VLPs resulted in equally high titres. The authors speculate that a single inoculation of 5 or 10 µg VLPs, which resulted in 1:40 and 1:28 (1.6 and 1.45 log_{10}), might even confer protection as it is beyond the OBP LAV manufacturer

Table 3
Summary table of efficacy study. Clinical protection and survival of IFNAR$^{-/-}$ mice vaccinated with plant-produced chimaeric AHSV-1/5 VLPs (Group 1) or AHSV-5 VP2 (Group 2), PBS buffer as negative control (Group 3) and OBP BEI inactivated AHSV-5 monovalent vaccine as positive control (Group 4). Vaccines and PBS buffer were formulated with 5% Montanide GEL 01. Group 0, non-vaccinated, non-challenged but serum samples collected at specific time points. Total RNA (5 μg) extracted from harvested lung tissue were subjected to a type-specific RT-qPCR for AHSV-5. Higher cycle threshold (Ct) values are indicative of virus suppression.

Groups	Vaccination status	Day 53, RT-qPCR viral detection in lung cells, Ct values	Clinical protection and survival
0	Naïve mice, not challenged	–	3/3
1	10 μg chimaeric AHSV-1/5 VLPs	38.34 ± 0.554	6/6
2	10 μg soluble VP2	34.61 ± 3.005	6/6
3	1X PBS negative control	–	0/3
4	OBP BEI inactivated positive control	33.45 ± 7.167	2/3

minimum protective titre of 1:16, although the real protective SN titre is still unknown and likely variable [2]. Nevertheless, vaccination with both plant-produced AHSV serotype 5 chimaeric VLPs and soluble VP2 resulted in protective immunity in IFNAR$^{-/-}$ mice. The protective efficacy of VP2 alone has been shown by numerous authors [25], but this is the first study showing that a plant-produced soluble VP2 can confer protective immunity in IFNAR$^{-/-}$ mice and has potential as a vaccine candidate for horses. Earlier studies in horses underpinned that nAbs is dependent on the presence of a soluble form of VP2 [23].

Adjuvanting plant-produced monovalent VLPs or VP2 with 5% Montanide GEL 01 and administering the vaccine candidates at 10 μg prime-boost doses elicited efficient nAbs (1:320, 2.5 $\log_{10}$) to confer protection in IFNAR$^{-/-}$ mice. A detailed study conducted by Scanlen and co-workers (2002) [23] demonstrated that the type of adjuvant used had a greater contribution to the protective effect as opposed to dose variances of the insect cell-produced VP2 in horses. In addition, Aksular and co-workers (2018) [25] determined that both MVA-VP2 or baculovirus-expressed VP2 induced protective immunity against AHSV in IFNAR$^{-/-}$ mice, even after using a single vaccine dose of 10 μg VP2 adjuvanted with 50% alum. Based on these studies, we hypothesize that increasing the Montanide GEL 01 adjuvant to 10–20% as per the manufacturer's recommendation (Seppic, France) will potentially improve efficacy of single dose and or prime-boost vaccinations for both VLP and VP2 vaccine candidates.

In this pilot study, the authors demonstrated protective immunity of IFNAR$^{-/-}$ mice when prime-boost doses of 10 μg each of VLPs or VP2 were administered. The challenge with AHSV serotype 5 of $1.4x10^5$ PFU rather than 10^6 PFU used in most studies [12,14,34,36], might have resulted in the slow onset of detectable clinical signs and viraemia (not determined). Nevertheless, the control group vaccinated only with adjuvanted PBS buffer succumbed within 8–11 days post challenge whilst the vaccinated mice survived until the study was terminated, 25 days after challenge. Although two of the mice (66% survival rate) in the positive control group, vaccinated with BEI inactivated AHSV-5, survived until the study was terminated, their blood was hypercoagulable, and therefore the serum could not be collected for analysis. Nevertheless, using a semi-quantitative RT-qPCR, the viral load, in lung samples progressively decreased in the VLP vaccinated, VP2 vaccinated and positive control groups. The limited duration of protective immunity afforded by the inactivated vaccine might be due to a decreased potency of the VP2 antigen that is available after inactivation. A repeat inoculation could have resulted in a stronger immune response. However, the two surviving BEI vaccinated mice presented with hypercoagulability and some hepatosplenomegaly with the liver somewhat anaemic.

The plant-produced AHSV-1/5 VLPs (1 μg, 5 μg and 10 μg) and plant-produced soluble VP2 (10 μg) resulted in nAb titres of 1:320 in IFNAR$^{-/-}$ mice within 14 days after booster vaccination in this study. Similar titres were obtained in horses after vaccination with plant-produced homogenous AHSV-5 VLPs in 100 μg and 200 μg soluble protein vaccine doses (prime-boost) [27]. Although the commercially available polyvalent LAV vaccine lacks serotype 5, it was shown that one of the 15 foals vaccinated with bottle 1 and 2 of the LAV vaccine of six months seroconverted to AHSV serotype 5 (titre 80 or 1.9 Log_{10}) [7]. Von Teichman and co-workers (2010) [40] reported cross protection between serotype 5 and 8. This phenomenon was confirmed when horses vaccinated with plant-produced AHSV serotype 5 VLPs, seroconverted to AHSV serotype 8 [27]. The authors anticipate that nAbs elicited by plant-produced chimaeric AHSV-1/5 VLPs and VP2 will similarly cross-neutralise AHSV-8 but it was not experimentally confirmed due to the limited serum obtained from the IFNAR$^{-/-}$ mice. Future immunogenicity studies planned in horses will necessitate SNTs of individual horses as it is well documented [7] that seroconversion to various AHSV serotypes is highly variable in horses.

Following the successful production of homogenous AHSV-5 VLPs [27], triple chimaeric AHSV-1/3/6 [28] and now the double chimaeric AHSV-1/5 as well as soluble AHS VP2 vaccine candidate, a direct comparison between VLPs and VP2 was feasible. Based on the results of this study, it is anticipated that both VLPs and VP2 subunit vaccine candidates might have greater advantage over existing mammalian or baculovirus-expressed recombinant products due to high production levels, cost-effectiveness, speed to produce antigenically matched antigens and facile scalability. Here we report that both plant-produced VLP and VP2 of serotype 5 vaccine candidates show great promise as future commercial vaccines. The potential to utilise VP2 in serotype-specific ELISA test kits is also envisaged.

5. Concluding remarks

This is the first report demonstrating protective efficacy of plant-produced, monovalent vaccine candidates of serotype 5, based on chimaeric VLPs and VP2, in IFNAR$^{-/-}$ mice. In this study, IMAC partially purified soluble AHSV-5 VP2 was used as vaccine candidate and further purified for serological tests. Commercially available AHS LAV vaccine has a limited market mostly in Africa, whereas the plant-produced VLP and VP2 vaccines have the potential to become the vaccine of choice globally. Once efficacy has been established, producing monovalent vaccines for localised AHS outbreaks locally and globally is feasible and a polyvalent vaccine protecting horses against all nine AHSV serotypes is envisaged.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: 'Plant-produced chimaeric Orbivirus VLPs underpinning the development of the VLP vaccine was patented (PCT/IB2017/052236, PCT publication number WO2017/182958, US 11,053,509 B2, 2021 granted) with co-inventors from CSIR (Dr Rutkowska, Dr Stark and Dr O'Kennedy), OBP (Dr Mokoena) and inventors from the University of Cape Town whilst the design of the plant-produced soluble VP2 is proprietary to CSIR. A provisional patent protecting the design of the plant-produced AHS VP2 fusion

protein and uses thereof, filed (Inventors: MM O'Kennedy and Y Lemmer).

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Author contribution

MO and YL conceptualised, designed, conducted and interpreted the IFNAR$^{-/-}$ mice studies and ELISA results. JV assisted with interpretation of immune responses. MO designed, cloned all relevant genes and produced both plant-produced chimaeric AHSV-1/5 VLPs and soluble VP2 proteins and formulated the vaccines for the mice studies. PC and OK produced the inactivated AHSV-5 vaccine and AHSV-5 challenge material whilst PC also determined viral load in lung samples of IFNAR mice challenged with the live virus. CL conducted the SNTs. LdP conducted the flow cytometry and interpreted the FACS data. LK guided the bench scale VP2 antigen purification using the AKTA instrument and IdP conducted the cytoxicity studies in Vero cells. NM, Orbivirus project leader at OBP. DR provided the cloned pEAQ-express AHSV-1 VP3/VP7 construct [28]. SM conducted the LC-MS/MS and processed the data. All authors proofread the manuscript before submission.

Ethics approvals

Department of Agriculture, Land Reform and Rural Development (DALRRD) approvals (12/11/1/1/12-1377) and (12/11/1/1/12-1317KL1). The trials were conducted according to the procedures and schedule for the BSL3 facility of the Department of Science and Innovation (DSI)/ Northwest University (NWU) Preclinical Drug Development Platform (PCDDP), South Africa (NWU-AnimCareREC reference number NWU-00599-19-A5). All animal manipulations were performed by skilled and South African Veterinary Council (SAVC) authorised staff. Supplementary CSIR Research Ethics Committee (REC) 285-2019 approval.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vaccine.2022.06.079.

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