Vaccine 50 (2025) 126861

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

Protective efficacy of a bivalent equine influenza H3N8 virus-like particle vaccine in horses

Martha M. O'Kennedy [a,b,1,*], Stephanie E. Reedy [c,1], Celia Abolnik [b], Amjad Khan [c,d], Tanja Smith [a], Ilse du Preez [a], Edward Olajide [c], Janet Daly [e], Ann Cullinane [f], Thomas M. Chambers [c]

[a] Council for Scientific and Industrial Research (CSIR), Biomanufacturing Technologies, Future Production: Chemical Cluster, South Africa
[b] Department of Production Animal Studies, University of Pretoria, South Africa
[c] University of Kentucky, Department of Veterinary Science, USA
[d] University of Haripur, Department of Public Health, Pakistan
[e] School of Veterinary Medicine & Science, University of Nottingham, United Kingdom
[f] Irish Equine Centre, Kildare, Ireland

ARTICLE INFO

Keywords:
Equine influenza virus
Virus-like particles
Vaccine
Clinical study
Effective
Safe

ABSTRACT

Equine influenza (EI) is a highly contagious acute respiratory disease of wild and domesticated horses, donkeys, mules, and other Equidae. EI is caused by the Equine Influenza virus (EIV), is endemic in many countries and outbreaks still have a severe impact on the equine industry globally. Conventional EI vaccines are widely used, but a need exists for a platform that facilitates prompt manufacturing of a highly immunogenic, antigenically matched, updated vaccine product. Here we developed a plant-produced bivalent EI virus-like particle (VLP) vaccine candidate which lacks the viral genome and are therefore non-infectious. We conducted a pilot safety/dose response study of a plant produced bivalent VLP vaccine expressing the HA proteins of Florida clade (FC) 1 and FC2 EIV in 1:1 ratio. Groups of three EIV seronegative horses were vaccinated using four antigen levels (0 sham control, 250, 500, 1000 HAU/dose component). Two doses of vaccines were administered one month apart, and horses were observed for adverse reactions, which were minimal. Sera were collected for hemagglutination inhibition (HI) testing using FC1 and FC2 viruses. One month after the second dose, all horses were challenged with the aerosolized FC1 virus. Horses were observed daily for clinical signs, and nasopharyngeal swabs were collected to quantify viral RNA using qPCR and infectious virus by titration in embryonated hens' eggs. Results showed that all vaccinated groups seroconverted prior to challenge. Post-challenge, both clinical scores and virus shedding were much reduced in all vaccinates compared to the sham-vaccinated controls. We conclude that the VLP vaccines were safe and effective in this natural host challenge model. A safe, efficacious, new-generation bivalent EI VLP vaccine produced in plants, which can promptly and regularly be antigenically matched to ensure optimal protection, will pave the way to highly competitive commercially viable vaccine products for all economic environments globally.

1. Introduction

The genus Influenzavirus A are enveloped viruses in the Orthomyxoviridae family and are classified by subtypes based on the antigenicity of the two major surface membrane glycoproteins hemagglutinin (HA) and neuraminidase (NA). Equine influenza (EI), caused by the equine influenza A virus (EIV), is prevalent nearly worldwide: only New Zealand and Iceland have remained continuously free from outbreaks, and some countries only experience sporadic outbreaks related to virus incursions. Two subtypes of EIV are known: H7N7 and H3N8. There has

Abbreviations: EIV, equine influenza virus; VLPs, virus-like particles; HA, hemagglutinin; HI, hemagglutination inhibition; qPCR, quantitative polymerase chain reaction; D, day.

* Corresponding author at: Council for Scientific and Industrial Research (CSIR), Biomanufacturing Technologies, Future Production: Chemical Cluster, South Africa
E-mail address: mokennedy@csir.co.za (M.M. O'Kennedy).

[1] Martha M. O'Kennedy and Stephanie Reedy contributed equally to this work as first authors.

https://doi.org/10.1016/j.vaccine.2025.126861
Received 10 December 2024; Received in revised form 31 January 2025; Accepted 5 February 2025
Available online 11 February 2025

been no confirmed isolation of the H7N7 subtype from horses since 1980 whilst H3N8 is responsible for outbreaks across the globe. Due to antigenic drift caused by point mutations in HA and NA, the evolution of H3N8 EIV has in the 21st century produced two antigenically distinct co-circulating sub-lineages, Florida clade 1 (FC1) and Florida clade 2 (FC2) (reviewed in [1]). FC1 viruses are predominantly found in the US from whence they triggered epizootics in Australia [2], and Asia [3]. More recently, they were associated with widespread outbreaks in South America [4], Europe [5] and Great Britain [6]. In West Africa in 2018 and 2019 thousands of donkeys were infected with an FC1 virus with significant mortality [7]. Until 2019, FC2 viruses predominated in Europe where they were associated with outbreaks in both vaccinated and unvaccinated horses [8,9]. They were also responsible for outbreaks in Mongolia [10], China ([11] and Turkey [12]. In Europe, sporadic introductions of FC1 EIV were noted in 2009–10 but the first major FC1 outbreaks were reported in 2018–19 [5,6], and since then FC1 appears to have replaced FC2 in Europe (https://www.woah.org/en/document/expert-surveillance-panel-on-equine-influenza-vaccine-composition/).

Virus transmission occurs by inhalation through aerosol that can spread fast and effectively through the air, up to 1–2 km of distance according to some studies [13], which contributes silently to the spread of the disease; also horses are frequently transported between continents for performance or breeding purposes. Where geographical isolation does not permit, vaccination of susceptible equids is the most effective prophylactic strategy. Vaccination does not produce long-lasting sterile immunity (protection from infection) and poorly vaccinated horses, whilst clinically protected, can shed virus and serve as sources of disease spread, as happened in Australia in 2007 [2], where EIV silently escaped from a post-importation quarantine facility and spread to 75,000 domestic horses. Despite the development and commercialization of vaccines for almost five decades, H3N8 EIV is still circulating and considered enzootic in numerous countries globally, mainly due to the suboptimal effectiveness of current vaccines. To detect EI vaccine breakdown and associated risk of EI outbreaks, EIV antigenic evolution is closely monitored by the World Organization for Animal Health (WOAH) EI reference laboratories and associated laboratories [14–16]. Vaccine breakdown has been attributed to inadequate vaccine potency and inappropriate vaccination schedules, but historically a major concern has been outdated vaccine viruses not antigenically matched to the most recent viruses [9,17,18]. Antigenic updating of EIV vaccines is often hindered by commercial economic considerations, e.g., the market size does not justify the necessary investment before a major panzootic occurs. Therefore, a need exists for a vaccine that can be easily produced and easily updated.

A plant-produced bivalent virus-like particle (VLP) vaccine based on the HA glycoprotein, representing the most recent H3N8 Florida sub-lineages clade 1 and 2, stimulating both humoral and cellular immune responses for protective immunity in horses, is an attractive alternative. Recurrent vaccination failures against EIV due to antigenic drift [13] can be overcome by a plant-produced (bio-pharmed) recombinant bivalent HA VLP vaccine, which can promptly and regularly be updated to the most recent virus variants as a potentially cost-effective approach to control of EIV. VLPs are robust protein shells that resemble the overall architecture of the native virions but lack the viral genome and are therefore non-infectious. VLPs display highly repetitive target epitopes in their native conformation, resulting in efficacious candidate vaccines to stimulate not only humoral but also cellular immune responses [19]. Numerous pre-clinical studies have proven that influenza VLP vaccines elicit long-lasting (cross-) protective immune responses [20–22].

The aim of this study was to produce a safe, efficacious, plant-produced, antigenically matched EI VLP vaccine candidate, expressing HA representative of recent epidemiologically relevant virus strains recommended by the World Organization for Animal Health (including H3N8 FC1 and FC2 strains, see https://www.woah.org/en/document/expert-surveillance-panel-on-equine-influenza-vaccine-composition/) to stimulate robust immune responses of a magnitude sufficient to effectively protect horses from disease and virus shedding upon exposure to wild-type viruses, with the additional feature of DIVA (differentiation of infected from vaccinated animals) compliance due to the absence of NA and other viral proteins.

2. Materials and methods

2.1. HA expression

EIV HA glycoprotein sequences based on the strains Tipperary/2019 (FC1, EPI1398819|HA|A/equine/Tipperary/1/2019|EPI_ISL_348425||A/H3N8) and Wexford 2014 (FC2, EPI1223791|HA|A/equine/Wexford/0/2014|EPI_ISL_308786|ATY42442|A/H3N8) were codon optimized by the service provider Bio Basic, Canada. These genes were individually cloned by the authors into the plant expression vector pEAQ-HT using restriction digest enzymes *Age* I and *Xho* I [23] using Fast-Link™ DNA ligation (Diagnostech, LK6201H). pEAQ-HT harboring sequence-validated gene inserts (Inqaba, Biotechnical Industries Pty. Ltd) were electroporated into *Agrobacterium tumefaciens* AGL-1 (ATCC1 BAA-101™) cells for transient expression of the HA genes in *N. benthamiana* ΔXT/FT, a glycosylation mutant with a targeted down-regulation of xylose and fucose expression that facilitates mammalian-like glycosylation.

2.2. Design and assembly of EI VLPs

HA sequences were obtained from the WOAH EIV reference laboratory. Based on these, synthetic codon-optimized genes were successfully and individually cloned into the plant expression vector pEAQ-HT. The vector is one of a series of vectors based on Cowpea Mosaic Virus (CPMV) [23,31,32] and utilized under a research & development license agreement. The plasmids are small binary vectors harboring the genes of interest, which are introduced into plant leaves by agroinfiltration. This construct facilitates transient production of the synthesized synthetic H3 HA glycoproteins in the leaves of *Nicotiana benthamiana* ΔXT/FT which facilitates mammalian like glycosylation [33]. *N. benthamiana* is a close relative of tobacco, a non-food/feed plant. The assembly of recombinant EI VLPs were enhanced by co-expression with the M2 influenza ion channel as previously described [34].

2.3. Production, scale up, filtration and ultrafiltration purification of EIV VLPs

The leaf material was harvested 5–6 days after infiltration using a Matstone Multipurpose juice extractor in PBS extraction buffer (140 mM NaCl, 1.5 mM KH_2PO_4, 10 mM Na_2HPO_4, 2.7 mM KCl, pH 7.4). Buffers were supplemented with metabisulfite (0.04 % $Na_2S_2O_5$) and protease inhibitor cocktail (Sigma P2714) immediately before plant leaf extraction. All procedures were conducted at 4 °C or on ice. The plant lysate was filtered through two layers of cheesecloth before centrifugation at 7000 x*g* for 7 min using a Beckman Avanti J-26 XPI centrifuge. VLPs were purified from the supernatant using depth filtration (Sartoclean® GF, Sartorius), followed by tangential flow filtration (TFF, 100 K Minimate™ Capsule, Pall Life Sciences) and filter sterilisation (Sartopore® 2 sterile capsule, Sartorius). The product was subjected to hemagglutination assays to determine the hemagglutination units (HAU) for dosage.

2.4. Transmission electron microscopy (TEM) and LC-MS/MS validation

To characterize the VLPs, filter sterilized TFF purified product was subjected to sucrose (70 %/ 20 %) density gradient ultracentrifugation. The gradients were centrifuged at 32,000 x*g*, at 10 °C for 2 h in a SW-41Ti rotor (Beckman Coulter Optima XE-100 ultracentrifuge). Fractions of 500 µl were collected and aliquots (26 µl) from all fractions were

analyzed on 4–12 % Bis-Tris Bolt™ (Life Technologies) sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) protein gels using SeeBlue™ Plus2 pre-stained protein standard (Thermo Fisher Scientific) as molecular weight marker. Selected bands were subjected to liquid chromatography–mass spectrometry (LC-MS/MS) confirmation of proteins. Proteins of interest were in-gel trypsin or chymotrypsin digested as per the protocol described in [24]. Protein pilot v5 using Paragon search engine (AB Sciex) was used for comparison of the obtained MS/MS spectra with Uniprot Swissprot protein database. Proteins with threshold above ≥99.9 % confidence were reported. LC-MS/MS based peptide sequencing was conducted to confirm that the HA of the two independent clades were indeed detected.

Selected sucrose gradient fractions were adsorbed onto carbon-coated holey copper grids for visualization under TEM. The grids were floated on the undiluted protein sample (15 µl) for 5 min, the excess sample drained off the grid via blotting on filter paper and the grid was then rinsed 5 times in 5 µl distilled water on parafilm. The grid was then stained by floating it on 2 % uranyl acetate (20 µl), pH 4.2 for 15–30 s. The excess stain was drained off by blotting the grid onto filter paper. The grid was air dried and subsequently imaged in a n JEOL JEM- 1400 FLASH Transmission electron microscope.

2.5. Cytotoxicity study to confirm safety features of vaccine in mammalian cell cultures

Vero cells (African green monkey kidney epithelial, ATCC® CCL-81™) were cultured in tissue culture flasks with complete Dulbecco's Modified Eagle Medium (DMEM) (GIBCO), supplemented with 10 % fetal calf serum (FCS) (GIBCO) and 1 % penicillin/streptomycin (Gibco), and maintained at 37 °C in a 5 % CO_2 atmosphere. For the assay, cells (50,000 cells/well) were seeded into 96-well tissue culture plates (flat bottom) in a final volume of 100 µl/well of complete DMEM and incubated for 24 h under the same conditions as previously mentioned. After 24 h, the media was removed, and various compounds or partially purified plant extracts (ranging from 1 µg to 2.3 ng) were added to each well at different concentrations in a final volume of 100 µl/well. The 96-well flat bottom plates were then maintained for an additional 24 h under the same sterile conditions in an incubator.

Following this incubation, 10 µl of the WST-1 cell proliferation reagent (CELLPRO-RO Cell Proliferation Reagent WST-1, Sigma) was added to each well, and the plates were incubated at 37 °C in 5 % CO_2 for 3 h. The absorbance of the formazan product, which forms as a result of metabolic activity in viable cells, was measured at 440 nm using an ELISA plate reader. Negative controls included both wells with (a) media alone and (b) cells, plus WST-1 reagent.

2.6. Vaccine preparation

HA titration of both antigens was repeated upon receipt in USA and again immediately prior to vaccine preparation. Vaccines contained 250 or 500 or 1000 hemagglutinating units (HAU) of each VLP (FC1 and 2) in 1:1 ratio, sterile saline, and 20 % Montanide Gel 01 PR adjuvant (Seppic, France), thoroughly mixed, prepared on the day of vaccination. The sham vaccine (0 HAU) for control horses was prepared in the same manner using saline and adjuvant only. This process was freshly repeated for preparation of the boost vaccine. Each dose was 1 ml in volume.

2.7. Vaccination

Twelve EIV-seronegative weanling horses 8–9 months age, of mixed light horse breed and both sexes (8 male, 4 female) were assigned to four groups (3 horses per group). Horses were identified by microchip. Assignments were made separately for males and females to achieve a distribution of 1 female in each group and randomized by using random-number generator software. All horses were co-pastured throughout the vaccination period, so that the sham-vaccinate group could serve as sentinels for any accidental introduction of EI into the herd. Vaccination was by intramuscular injection (1 in. needle, 20G) into the splenius cervicis/brachiocephalicus muscle of the neck, at a site shaved of hair for ease of visualization of vaccine reactions. Each animal was given a clinical examination (temperature, pulse, respiration rate, parotid and submandibular lymph node palpation, signs of inflammation, depression, anorexia, or signs of swelling at the injection site) on Days (D) 0–3 and again at D7 post vaccination. This was repeated for the second dose (boost, D28). Blood was collected weekly for serology, and peripheral blood mononuclear cell (PBMC) samples were collected bi-weekly for cell-mediated immunity (CMI) testing. A health chart was kept for each horse.

2.8. Challenge

On D56 (28 days post-boost), experimental challenges with wild-type EIV were conducted essentially as previously described [25] in a large-animal BSL2 barn. Horses were relocated to this barn 4 days prior to allow for acclimatization, with each horse in its own stall with bedding of wood shavings. Each horse received a daily clinical exam including rectal temperature, auscultation of lungs and intestines as well as evaluation of the clinical parameters noted above. Clinical signs were scored as previously described [25]. Investigators performing the physical exams were blinded as to the horses' group assignments. Horses were individually exposed to 5×10^7 $EID_{50}U$ of influenza A/equine/Kentucky/2014 (KY/14, FC1) using a Flexineb-E2 mask fitting completely over the nose and mouth (Flexineb North America, Union City, Tennessee). Nasopharyngeal swabs were taken daily until completion of the challenge period. Blood was drawn on D56, 63, 70, and 77 (D0, 7, 14, and 21 post-challenge). Treatments for severe disease, in accordance with our Institutional Animal Care and Use Committee (IACUC) protocol, were at discretion of an independent veterinarian blinded to the vaccination status of the animals. All horses were returned in healthy condition to their source farm upon conclusion of the experiment.

2.9. Serology

All sera were tested by hemagglutination-inhibition (HI) assay by using the WOAH HI protocol (https://www.woah.org/app/uploads/2021/03/3-05-07-eq-inf.pdf) for humoral antibody responses, using vaccine cross-reactive antigens, KY/14 (FC1) and A/equine/Richmond/2007 (FC2). Samples were pretreated with potassium periodate as described to reduce non-specific inhibitors of hemagglutination. The lower limit of detection was a titer of 10; therefore, for calculations of geometric means, titers <10 (i.e. undetectable) were arbitrarily set equivalent to 5, and displayed as such in Fig. 1.

2.10. Cellular immune responses

PBMC samples were also collected on Days 0, 14, 28, 42, 56, and 70 (Day 14 post-challenge) for analysis of CMI responses by qRT-PCR as described [35], with samples stimulated using either wild-type EIV (KY/14 strain) or EIV plus stimulated with phorbol 12-myristate 13-acetate (25 ng/ml) and calibrated against group averages as well as individual horses. Samples were analyzed for INF-γ, IL-10, granzyme-B, TNF-α, HPRT1, and perforin. The qPCR assay included an internal positive control to ensure the integrity and efficiency of the amplification process. Additionally, an extraction negative control was included which consisted of a negative sample that underwent all extraction procedures alongside the test samples. The latter served as a safeguard against potential contamination during the extraction process.

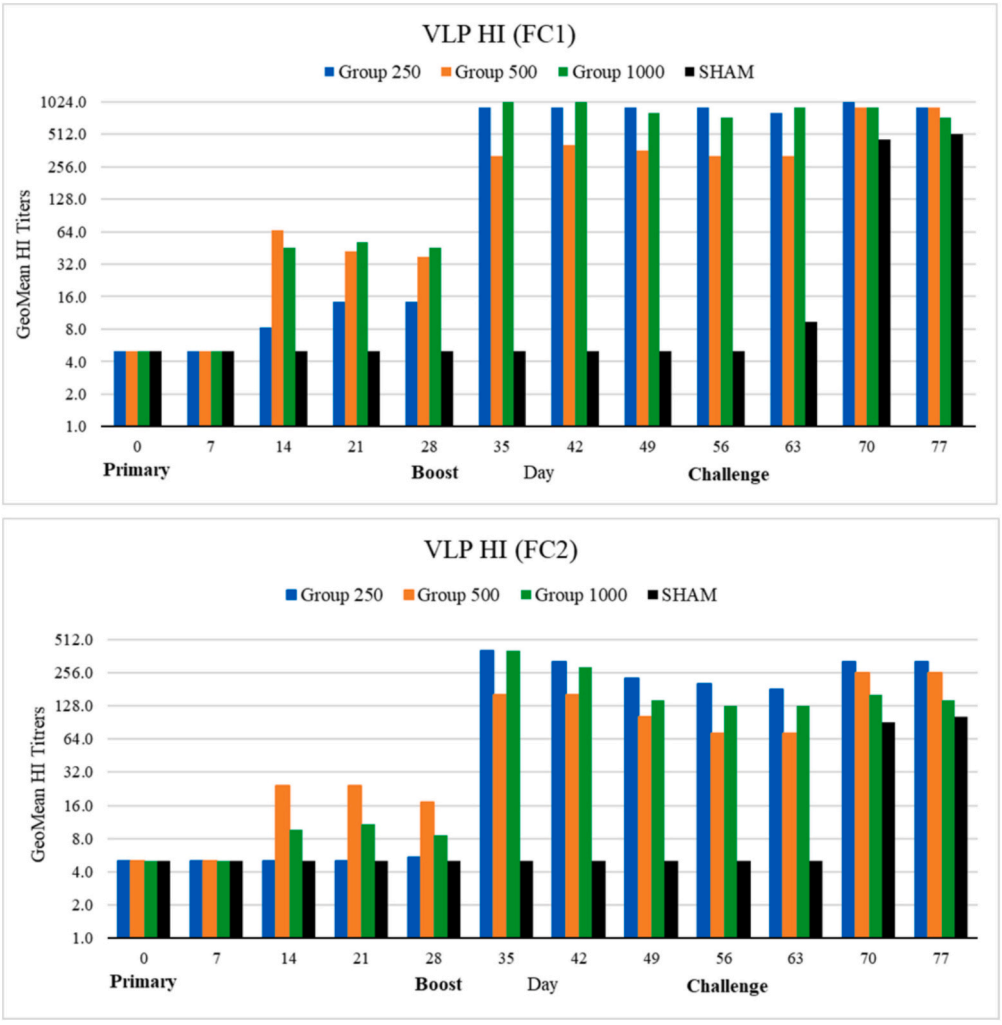

Fig. 1. Serology by HI testing of serum samples collected weekly (D0–77, with boost (second vaccine dose) on D28 and challenge on D56. Groups (250, 500, 1000, sham) correspond to the vaccine doses received. Shown are group mean HI titers; titers <10 (i.e. undetectable) are arbitrarily set to 5. Top panel, titers against Florida clade 1 (FC1) antigen (KY/14 virus strain). Bottom panel, titters against FC2 antigen (eq/Richmond/07 virus strain).

2.11. Virus shedding

All nasopharyngeal swabs in viral transport medium were tested for viral shedding using qPCR as described [26]. Samples were thawed in batches of 11 samples in a random fashion. Individual samples were vortexed for 10 s and centrifuged briefly. RNA from 100 µl of original sample added into 100 µl of PBS was extracted [27] using an extraction kit (QIAamp Viral RNA Mini Kit, Qiagen, USA) as per the manufacturer's recommendations. Extracted RNA was converted into cDNA using SuperScript™ Reverse Transcriptase (ThermoFisher Scientific, Florence, KY, USA) as described previously [28]. Extracted cDNA was tested for the presence of EIV H3N8 genomic copies using quantitative PCR (qPCR) system (Quant Studio 7, Applied Biosystems, Foster City, CA, USA) using a standard thermocycle protocol (26). Master Mix (TaqPath™ qPCR Master Mix, ThermoFisher Scientific) and specific primers and probe for NP gene detection (ThermoFisher Scientific) were mixed with 4.5 µl extracted cDNA using epMotion (epMotion® 5075, Hamburg, Germany). The qPCR EIV positive was reported qualitatively (presence or absence) and semi-quantitatively as cycle threshold (Ct) values. Absolute quantification of the EIV positive samples was done through Applied Biosystems digital (d)PCR (Quant Studio Absolute Q Digital PCR, ThermoFisher Scientific Inc.) using absolute Q DNA Digital PCR Master Mix™ (5×, ThermoFisher Scientific) with the same EIV H3N8 NP-gene primers and probe combination. These tests were done by an investigator (AK) blinded to the group assignments.

To measure live virus titers, EID_{50} assays [29] were performed on swabs collected on D2–4 (expected to be peak shedding) by inoculation of 100 µl of 10-fold serial dilutions of nasal swab supernatant into 10-day-old embryonated hens' eggs, followed by incubation at 34 °C for 72 h. Eggs positive for virus growth were determined by HA assay. Titers were calculated using the method of Reed and Muench [30].

2.12. Statistical analysis

Statistical analysis was done using a mixed-effects model with Geisser-Greenhouse correction and Tukey's multiple comparison test. All tests met normal assumptions based on QQ and residual plots.

3. Results

The aim of this study was to successfully produce EI VLPs in plant leaf tissue, followed by a pilot safety and dose escalating study (prime-boost regime of adjuvanted VLPs representing EIV Florida clades 1 and 2, ratio 1:1) using 3 VLP doses (250, 500, and 1000 HAU; 3 horses per group) and evaluate humoral immune responses as well as clinical responses to challenge with wild-type virus.

3.1. Large scale production, validation and dose determination

EI VLPs was successfully produced and purified. Leaf tissue of approximately 200 g of each clade was individually purified using a TFF, 100 K Minimate™ Capsule (Pall Life Sciences). To characterize the VLPs produced, the filter sterilized TFF purified VLPs were overlayed onto a 70 %/20 % sucrose cushion and ultracentrifuged to isolate the VLPs from contaminating plant proteins. Intact VLPs were visualized using transmission electron microscopy (Fig. 2). The VLPs were also subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE, Fig. 2) and candidate viral proteins confirmed by LC-MS/MS based peptide sequencing. Filter sterilized TFF VLP product for EI FC1 yielded an HA titer of 1:65536 (16 Log_2 or 65,536 hemagglutination units (HAU)) per 25 µl of purified VLPs. Filter sterilized TFF VLP product for EI FC2 yielded an HA titer of 1:8192 (13 Log_2 or 8192 HAU) per 25 µl.

3.2. Cytotoxicity testing of VLP product in mammalian cell culture

A cytotoxicity study using Vero (African green monkey kidney) cells as a model, was conducted to confirm the safety of the partially purified plant extracts both with and without VLPs, at concentrations ranging from 2.3 ng to 1 µg per well, alongside the negative control (no plant extract). This was achieved by means of a colorimetric cell proliferation assay (WST-1), as per the manufacturer's instructions. Results from three independent experiments indicated that for both FC1 and FC2 VLPs at all concentrations tested, there was <5 % decrease in cell viability and no statistically significant differences compared to the negative control (Fig. 3). Thus, at the various concentrations of the VLPs and plant extract only that was added, no cytotoxicity was detected, and cells remained equally viable and metabolically active when compared to control.

3.3. Horse vaccination

Physical examinations and intramuscular vaccinations were performed as described above. No adverse reactions were observed with the exception that in one horse only, needle aversion upon primary vaccination produced minor local swelling at the injection site, which had nearly disappeared 2 days later and did not reoccur upon boost. Serum samples were collected at weekly intervals and titrated by HI assay as described above for both FC1 (KY/14) and FC2 (Richmond/07)-reactive antibodies. Results are shown in Fig. 1. All three vaccine groups (250, 500, and 1000 HAU doses) showed small antibody responses following the primary vaccination and strong responses following the booster vaccination 1 month later. Responses were about 2-fold stronger against the Florida clade 1 antigen than Florida clade 2, for reasons unknown. Also, post-boost, the 250- and 1000-HAU doses produced 2- to 3-fold stronger responses than did the 500-HAU dose. Since the source product was the same for each dose level, this is perhaps attributable to that particular 3-horse group. The sham vaccine group showed no antibody response throughout the vaccination period.

While statistically significant increases with time ($P < 0.02$) were observed with IFN-γ, there was no consistent elevation in IFN-γ levels in vaccinates over the sham-vaccinated controls (data not shown). Other cytokine transcript levels, in particular IL-10 and granzyme-B, showed no consistent trend until after challenge and were not correlated with vaccination status.

3.4. Experimental challenge

One month following booster vaccination, all horses were transported into individual stalls in a BSL-2 isolation barn. Following 4 days of acclimatization, horses were challenged (study D56, Day 0 of challenge) with wild-type EIV FC1 (KY/14 strain) as described above. Clinical signs were recorded, and nasopharyngeal swab samples were obtained as described above. Only the sham-vaccinated control horses exhibited pyrexia (rectal temperature > 38.3 °C), on Days 2 and 3 post-challenge (Fig. 4). Mean rectal temperatures in the sham control group were significantly greater ($P < 0.01$) than the overall mean of the 3 vaccine groups on both Days 2 and 3 post-challenge and returned to normal on Day 4. Throughout the infection period no vaccinated horse

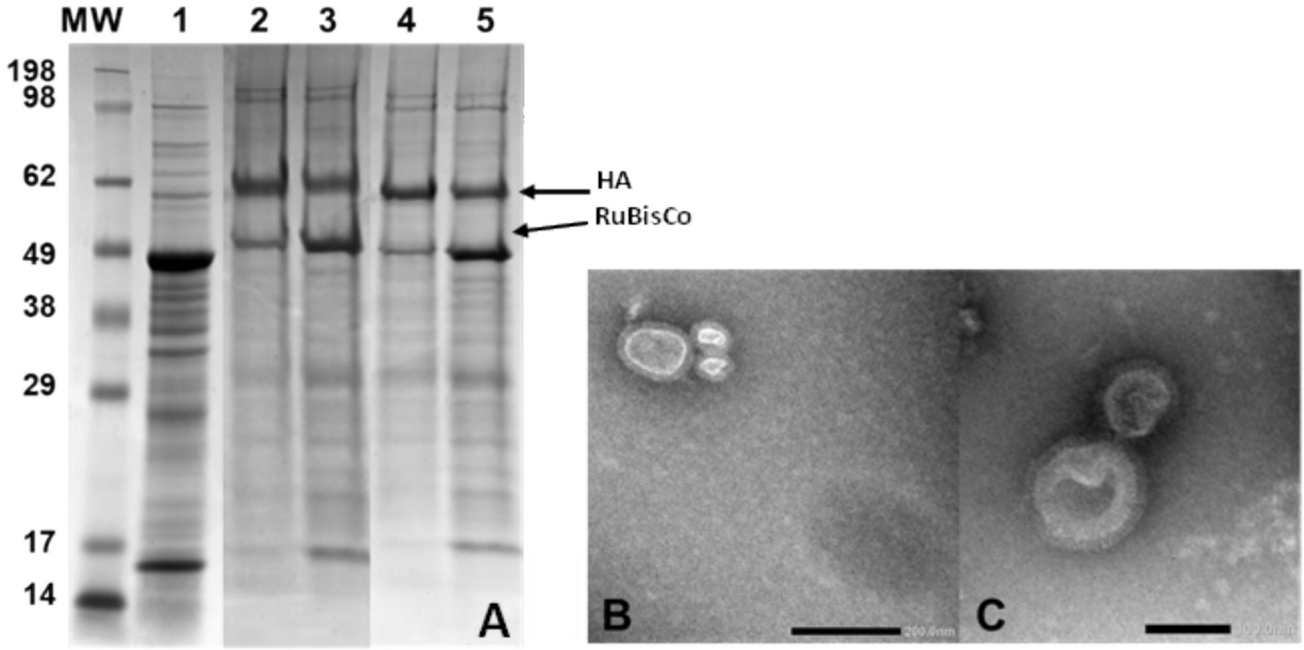

Fig. 2. A. SDS PAGE of plant-produced equine influenza clade 1 and 2 VLP proteins of 65 kDa in size (indicated by the arrow): See Blue® Plus2 pre-stained protein molecular weight marker (MW); lane 1, protein profile of plants agroinfiltrated with pEAQ-HT empty vector; protein profile of clade 1 (lanes 2–3) and clade 2 (lanes 4–5) purified from the TFF purified and filter sterilized sample using sucrose density gradient (70 % and 20 %) ultracentrifugation. **B** and **C,** negatively stained transmission electron microscopy (TEM) images of density gradient purified EIV FC1 and FC2 VLPs, respectively, measuring 80—120 nm diameter. B, 200 nm bar and C, 100 nm bar. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

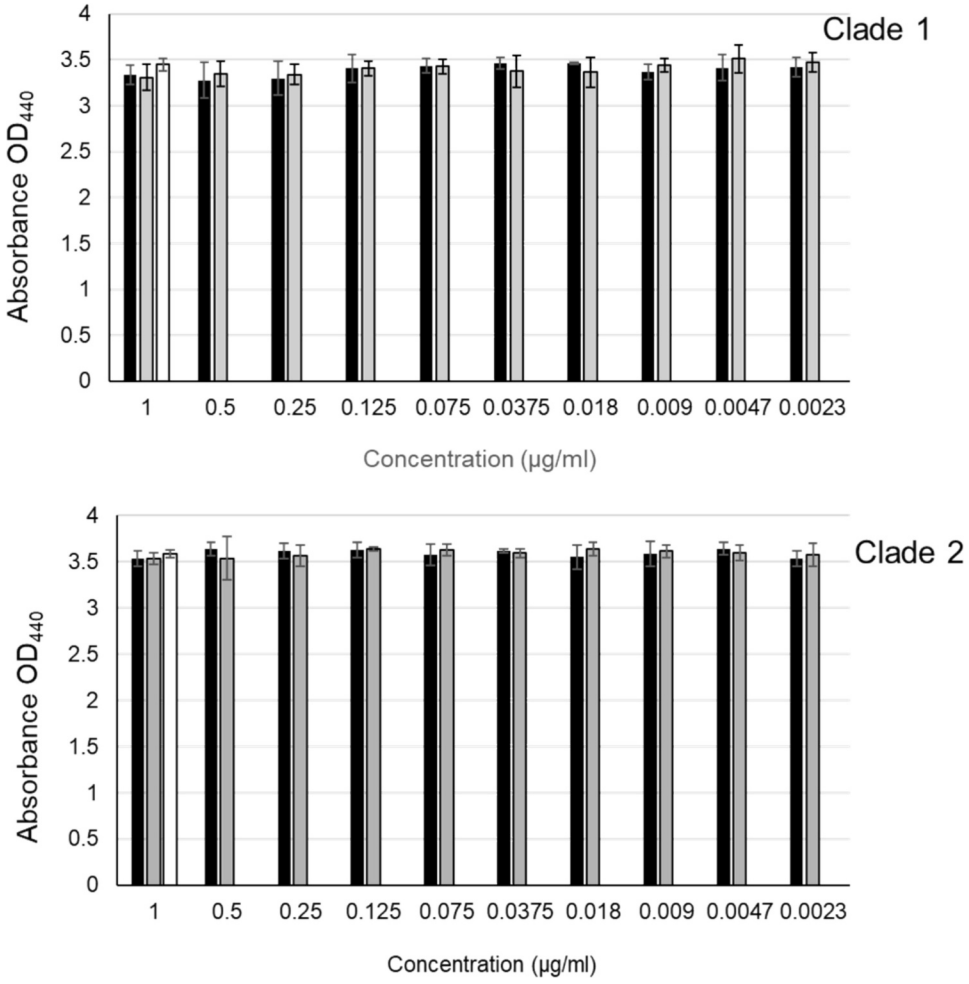

Fig. 3. Cytotoxicity studies in Vero cells validating both plants produced equine influenza clades 1 and 2. Results are shown for different concentrations of VLPs (black bars), and plant extract only (grey bars) compared to cells with WST-1 reagent (as control, white bar). The absorbance value is directly related to the amount of live, metabolically active cells present in each well.

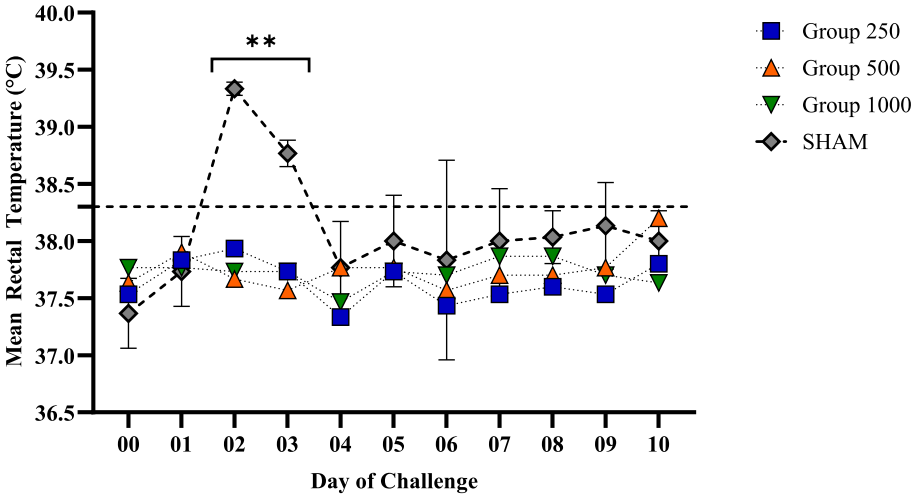

Fig. 4. Rectal temperatures of horses post-challenge. Days 0–10 correspond to D56–65 in text. Groups (250, 500, 1000, sham) correspond to the vaccine doses received. Shown are group means +/− SEM. Rectal temperatures >38.3 °C are considered pyrexic. Sham control group mean temperatures were significantly greater ($P < 0.01$) than vaccine groups both Days 2 and 3, while rectal temperatures in all vaccinated horses remained within normal levels.

in any of the dose groups exhibited a rectal temperature > 38.1 °C.

Clinical scores were compiled and are shown in Fig. 5. Two of the three sham control horses exhibited multiple days of cough and nasal discharge; the third exhibited multiple days of anorexia. Among vaccinates, 1 horse in the dose = 1000 group exhibited 1 day of nasal discharge; 1 horse in the dose = 500 group exhibited 3 non-consecutive

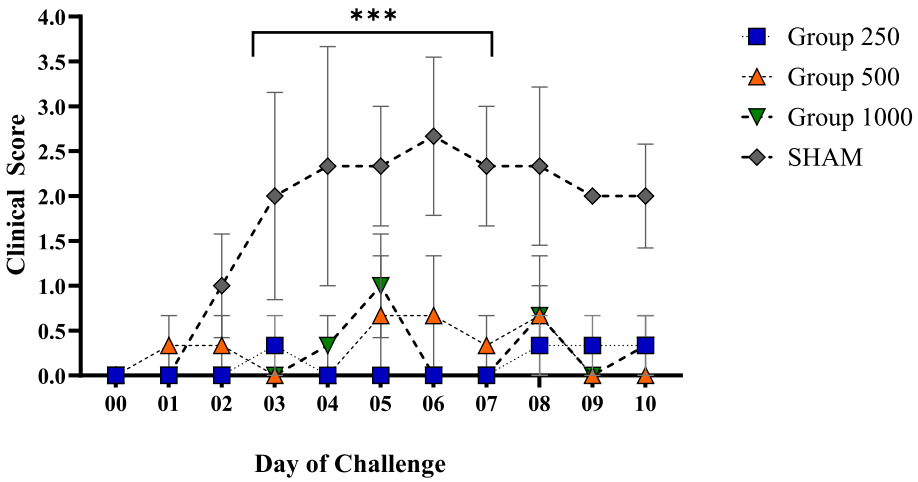

Fig. 5. Clinical scores of horses' post-challenge. Days 0–10 correspond to D56–65 in text. Scores were calculated based on a Clinical Signs Scoring Index described previously (e.g. Blanco-Lobo et al., 2019). Groups (250, 500, 1000, sham) correspond to the vaccine doses received. Shown are group means +/− SEM. Scores for the sham group were significantly elevated over all vaccine groups on Days 3 through 7 ($p < 0.001$). Clinical evaluations were done by an investigator blinded to the group assignments of the horses.

days of cough; and 1 horse in each vaccine group exhibited 2 or more days of anorexia. Mean daily clinical scores for the sham control group were significantly greater ($P < 0.001$) than the overall means of the vaccine groups on Days 3, 4, 5, 6, and 7. Also, when analyzed over the entire infection period (Days 0–10), the sham control group score was significantly greater ($P < 0.05$) than the individual vaccine dose = 250 and 500 group scores (for the sham versus dose = 1000 vaccine group, $P = 0.06$). No horse in the study required treatment intervention for EI, however one horse (the anorexic horse in the vaccine 250 group) suffered a gash to the hock on Day 1 that required repeated sedation/cleaning/wrapping.

Virus shedding was measured by qPCR on daily nasopharyngeal swabs, and titration of infectious virus by EID_{50} assay in the swabs taken on Days 2, 3, and 4 post-challenge, which is typically the period of peak shedding. qPCR results are shown in Fig. 6, where cycle threshold (Ct) value varies inversely with viral RNA quantity. As shown, viral RNA was detected in the sham control horses with peak on Day 2 and remaining strongly positive through Day 6, whereas in all three vaccin250 or e groups viral RNA was not detected or detected at only low levels throughout the post-challenge period (F value = 69.2, $P < 0.001$).

EID_{50} results are shown in Table 1. Infectious virus was detected only in the sham control horses, with peak on Day 2 post challenge (Day 58). Based on these data, all horses in all three vaccine groups possessed sufficient immunity at one month post second dose of vaccine to completely prevent shedding of live virus (sterilizing immunity).

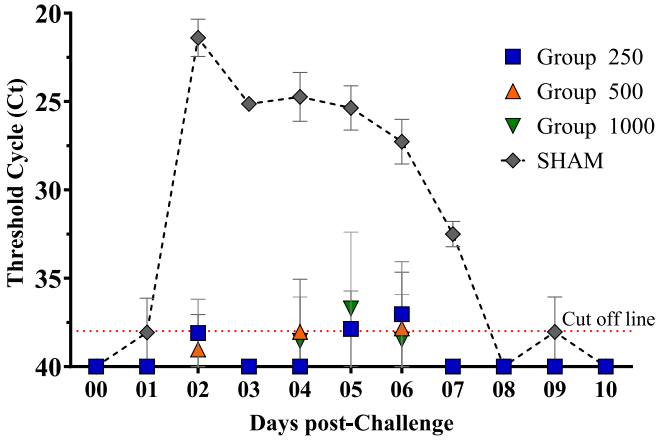

Fig. 6. Viral RNA in nasopharyngeal swabs as measured by RT-qPCR. Day 00 post-challenge is D56 in text. Cycle threshold is considered negative at Ct = 36. Groups (250, 500, 1000, sham) correspond to the vaccine doses received. Shown are group means +/− SEM. RT-qPCR testing was done by an investigator blinded to the group assignments of the horses.

Table 1
Quantification of virus shedding in nasopharyngeal swabs by EID50 assay. Groups (left column) correspond to the vaccine doses received. ID, the specific identifier of each individual horse. Only swabs from Days 2, 3, and 4 (D58, 59, 60 in text) were tested as this was expected to cover the period of peak virus shedding (see Fig. 5). The lower limit of detection was 1 × 103/ml. Virus was detected only in swabs from the sham (unvaccinated) controls; no vaccinated horse shed detectable virus.

Group	Animal ID's	D2	D3	D4
	W3	<	<	<
	W4	<	<	<
250	**W7**	<	<	<
	W2	<	<	<
	W10	<	<	<
500	**W123**	<	<	<
	W1	<	<	<
	W6	<	<	<
1000	**W8**	<	<	<
	W9	4.64×10^5	1×10^4	1×10^5
Sham	**W121**	1×10^5	3.16×10^5	1×10^3
	W122	1×10^6	3.16×10^5	4.64×10^3

Post-challenge, by Day 14 post-challenge (D70 in Fig. 2) all sham control horses seroconverted against both FC1 (KY/14) and FC2 (Richmond/07) antigens. Among the 9 vaccinates, which possessed generally high serum antibody titers post-vaccination, only 1 horse showed a further post-challenge increase in titer >2-fold against the FC1 antigen, and only 3 horses against the FC2 antigen.

4. Discussion

We demonstrate the generation of a safe, DIVA-compliant, antigenically matched, plant-produced EI VLP vaccine that produces serum anti-HA antibody titers of sufficient magnitude to effectively protect horses against H3N8 EIV, with sterile immunity (no detectable virus shedding) at one month post second (booster) dose.

Horse vaccinations with this bivalent vaccine produced serum antibody responses against the FC1 HA that exceeded those against the FC2 antigen by typically 2-fold. Possibly this was due to antigen interference. Antigen interference has not been carefully studied in horses, but multivalent influenza vaccines have been used in horses for >30 years [17] so other explanations, such as incorrect matching of HA unit doses, should also be evaluated. This might also account for the non-linear dose response post-boost (dose = 500 group had lower titers than either the 250 or 1000 groups). Also, our expectation that vaccination would induce detectable pre-challenge CMI responses was not supported by the results obtained from PBMC samples.

Further work will be needed for refinement of the effective dose (250 HAU or less); verification of efficacy against FC2 virus challenge; duration of immunity in vaccinated horses; and duration of VLP stability post-production when using a large scale GMP production methodology. As the plant-produced VLP is non-replicative, there is no reason to expect factors such as pregnancy to affect its safety or efficacy. CSIR is currently constructing a current Good Manufacturing Practise (cGMP) facility for plant produced biopharmaceuticals to be operational by 2025, so a future study will be quite feasible.

Plant biopharming as an emerging manufacturing platform is an increasingly promising molecular pharming platform for both human and veterinary vaccines, recognized for its scalability, prompt update to the latest circulating strains, versatility and low production costs (recently reviewed in [36]). With a vaccine dose of 250 HAU and FC1 VLPs production yields of almost 118 million HAU per 223 g leaf tissue, it was conservatively estimated that more than 800,000 horses can be prime boost vaccinated per kilogram of leaves. Similarly, with a 250 HAU vaccine dose of FC2 VLPs at a production yield of almost 15 million HAU per 206 g leaf tissue, 145,000 horses can be prime boost vaccinated per kilogram of leaves.

Plant-produced VLP vaccines for human influenza have proven safe and efficacious in mice and in human clinical trials (e.g., [37,38]). A safe, efficacious, new-generation bivalent EI VLP vaccine produced in plants, which can promptly and regularly be antigenically matched to ensure optimal protection, will pave the way to highly competitive commercially viable vaccine products for all economic environments. VLPs display highly repetitive target epitopes in their native conformation, yielding prospective vaccine candidates designed to stimulate not only humoral but also cellular immune responses [19]. Plant surface glycans differ from those in mammalian cells, but this has not affected plant-derived VLP efficacy and glyco-engineering may facilitate production efficiency [36]. Plant glycans with core β1–2 xylose and α1,3 fucose may be allergens although human subjects reportedly do not develop signs of allergy or hypersensitivity (e.g., [39]). Whether this is also true in equids has not been determined. We used a glycosylation mutant of *N. benthamiana* (ΔXT/FT) for targeted knock-down of endogenous xylosyl and fucosyltransferases, reported to yield plant surface glycans functionally and electrophoretically similar to those of mammalian Chinese hamster ovary cells [33], with the aim of ameliorating allergy-like immune responses. Our team has previously generated plant-produced VLP vaccine candidates for agents including bluetongue virus and African horse sickness virus. These VLP vaccines elicit serotype-specific immunity in sheep and horses, respectively [40–43], and protective neutralizing antibodies in a mouse vaccination/challenge model [44]. More relevantly, the team developed highly efficacious influenza A VLP candidate vaccines of the H6 low pathogenicity avian influenza subtype and the clade 2.3.4.4B H5Nx high pathogenicity avian influenza (HPAI) subtype for poultry protection. The H6 VLP vaccine resulted in a significant reduction of viral shedding in chickens by >100-fold in the oropharynx and > 6-fold in the cloaca and shortened the period of oropharyngeal viral shedding by at least a week when compared to the commercially available vaccine [45]. The clade 2.3.4.4B H5 VLP vaccine provided complete protection against clinical signs and mortality, with similar significant reductions in HPAI viral shedding [45–47]. In addition, numerous pre-clinical studies have proven that influenza VLP vaccines elicit long-lasting (cross-) protective immune responses [20–22]. These efficacious plant-produced influenza H5 and H6 VLP vaccines for poultry facilitated the design and production of a bivalent EI VLP vaccine candidate for horses described here. If the need arose, the H5 VLP which is already produced and tested in chickens, might serve as an equine candidate anti-H5 vaccine if the avian/bovine H5N1 influenza virus detected in humans and other mammals in the USA in 2024 (e.g., [48]) were to spread to horses.

EI is enzootic in the USA and is considered to be either enzootic or periodically epidemic in much of the world. Conventional vaccines also provide immunity to the viral NA antigen, but updating of these vaccines has proven to be a cumbersome process and, from a commercial standpoint, sometimes considered to be of dubious cost-effectiveness. A highly efficacious antigenically matched H3N8 EIV VLP vaccine for annual or six-monthly booster vaccination to confer protective immunity and curtail regional EIV outbreaks in horses globally will be advantageous. The VLP vaccine technology allows rapid antigenic updating of the EI vaccine as required, and production within 10 days is relatively inexpensive, with 1 kg of plant leaf material yielding thousands of doses of vaccine. The bio-pharmed EIV VLP vaccine product with distinct attributes of DIVA compliance, the absence of live virus and anti-vector immunity, make the product a promising market contender globally. The feature of DIVA compliance of the vaccine candidate will, in addition, assist in disease surveillance and outbreak management. DIVA compliance is an important goal of future vaccine development, as it facilitates disease control efforts and epidemiological investigation, but among the current generation of commercially available EIV vaccines only the canarypox-vectored vaccine (no longer available in the USA) has DIVA capability; this is why the canarypox-vectored vaccine was the only EIV vaccine approved for use in Australia during their 2007 outbreak. The effectiveness of the canarypox vaccine as well as other genetically engineered EI vaccines (e.g., [49,50]) demonstrates that immunity to HA alone is protective if titers are high enough. One EI Modified live virus (MLV) vaccine is currently available in the USA but not licensed in Europe [51]. EIV MLV vaccines pose a hypothetical risk because of the possibility of reassortment between the MLV and a co-infecting wild-type virus; although it is doubtful whether this would expand either host range or pathogenicity. Using non-replicative plant derived VLP to stimulate an efficient protective immune response is a safe alternative and, if effective in the field, may open up a new prospect in terms of immunity, protection and management, for global eradication of EI.

Ethics approvals

A South African Department of Agriculture, Land Reform and Rural Development (DALRRD) Section 20 approval reference number 12/11/1/1/12 (1470) and CSIR Research Ethics Committee (REC) approval Ref number 311/2020 were in place to clone, express and assembly the VLPs in plants. CSIR REC Reference number 409/2022 to produce the bivalent VLP vaccine for a foal trial.

Funding

This project was generously supported by a grant from the Grayson-Jockey Club Research Foundation. CSIR Parliamentary grant funding supported the original development and optimisation of the EIV vaccine. SER and TMC were supported by Project No. KY-14067 of the Kentucky Agricultural Experiment Station, and AK was supported by the Lucille P. Markey Charitable Trust.

Ethical statement for animal care and use

Horse experiments were conducted under TMC's IACUC Protocol no. 2022–4028 and IBC protocol no. B21–3721-M2, approved respectively by the University of Kentucky's Institutional Animal Care & Use

Committee and Institutional Biosafety Committee. Dr. Allen Page, a member of IACUC, provided independent veterinary oversight of the horse experiments.

CRediT authorship contribution statement

Martha M. O'Kennedy: Writing – original draft, Supervision, Resources, Investigation, Funding acquisition, Data curation, Conceptualization. **Stephanie E. Reedy:** Writing – review & editing, Writing – original draft, Visualization, Investigation, Formal analysis, Data curation, Conceptualization. **Celia Abolnik:** Writing – review & editing, Data curation, Conceptualization. **Amjad Khan:** Writing – review & editing, Investigation, Formal analysis, Data curation, Conceptualization. **Tanja Smith:** Methodology, Investigation. **Ilse du Preez:** Investigation, Data curation. **Edward Olajide:** Investigation. **Janet Daly:** Writing – review & editing, Funding acquisition, Conceptualization. **Ann Cullinane:** Writing – review & editing, Funding acquisition, Conceptualization. **Thomas M. Chambers:** Writing – review & editing, Supervision, Resources, Project administration, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

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

Acknowledgements

The pEAQ-HT and pEAQ-express vectors were used under a license agreement from Plant Bioscience Limited (PBL), UK. We thank Albert Mabetha for technical assistance producing the plants for vaccine production, Sharon Kgasago for technical assistance, Antoinette Lensink (University of Pretoria Onderstepoort, Laboratory for Microscopy and Microanalysis) for TEM imaging, and Gugu Mkhize for administrative support. The authors are grateful to Dr. Allen Page from the Department of Veterinary Science, University of Kentucky for veterinary oversight of the horse challenge. The Dept. of Veterinary Science Farm Crew, consisting of Felicia Stamper, Millard Stamper, Chad Tucker, Chris Floyd, Pedro Deluna, and Courtney Floyd, were integral to the animal work. We thank Amanda Adams (University of Kentucky) for CMI analyses.

Data availability

Data will be made available on request.

References

[1] Chambers, T.M.: Equine Influenza. In: **Influenza: The Cutting Edge**; Cold Spring Harbor Perspectives in Medicine, G. Neumann and Y. Kawaoka, eds., Cold Spring Harbor Laboratory Press, 2021, pp. 465-481. Advanced Online Article. doi: 10.1101/cshperspect.a038331.

[2] Cowled B, Ward MP, Hamilton S, Garner G. The equine influenza epidemic in Australia: spatial and temporal descriptive analyses of a large propagating epidemic. Prev Vet Med 2009;92(1–2):60–70.

[3] Yamanaka T, Niwa H, Tsujimura K, Kondo T, Matsumura T. Epidemic of equine influenza among vaccinated racehorses in Japan in 2007. J Vet Med Sci 2008;70 (6):623–5.

[4] Olguin-Perglione C, Vissani MA, Alamos F, Tordoya MS, Barrandeguy M. Multifocal outbreak of equine influenza in vaccinated horses in Argentina in 2018: epidemiological aspects and molecular characterisation of the involved virus strains. Equine Vet J 2020;52(3):420–7.

[5] Fougerolle S, Fortier C, Legrand L, Jourdan M, Marcillaud-Pitel C, Pronost S, et al. Success and limitation of equine influenza vaccination: the first incursion in a decade of a Florida clade 1 equine influenza virus that shakes protection despite high vaccine coverage. Vaccines 2019;7(4).

[6] Whitlock F, Grewar J, Newton R. An epidemiological overview of the equine influenza epidemic in Great Britain during 2019. Equine Vet J 2023;55(1):153–64.

[7] Shittu I, Meseko CA, Sulaiman LP, Inuwa B, Mustapha M, Zakariya PS, et al. Fatal multiple outbreaks of equine influenza H3N8 in Nigeria, 2019: the first introduction of Florida clade 1 to West Africa. Vet Microbiol 2020;248:108820.

[8] Woodward AL, Rash AS, Blinman D, Bowman S, Chambers TM, Daly JM, et al. Development of a surveillance scheme for equine influenza in the UK and characterisation of viruses isolated in Europe, Dubai and the USA from 2010-2012. Vet Microbiol 2014;169(3–4):113–27.

[9] Gildea S, Garvey M, Lyons P, Lyons R, Gahan J, Walsh C, et al. Multifocal equine influenza outbreak with vaccination breakdown in thoroughbred racehorses. Pathogens 2018;7(2).

[10] Yondon M, Heil GL, Burks JP, Zayat B, Waltzek TB, Jamiyan B-O, et al. Isolation and characterization of H3N8 equine influenza a virus associated with the 2011 epizootic in Mongolia. Influenza Other Respir Viruses 2013;7(5):659–65.

[11] Yang H, Xiao Y, Meng F, Sun F, Chen M, Cheng Z, et al. Emergence of H3N8 equine influenza virus in donkeys in China in 2017. Vet Microbiol 2018;214:1–6.

[12] Gahan J, Garvey M, Gildea S, Gur E, Kagankaya A, Cullinane A. Whole-genome sequencing and antigenic analysis of the first equine influenza virus identified in Turkey. Influenza Other Respir Viruses 2018;12(3):374–82.

[13] Singh RK, Dhama K, Karthik K, Khandia R, Munjal A, Khurana SK, et al. A comprehensive review on equine influenza virus: etiology, epidemiology, pathobiology, advances in developing diagnostics, vaccines, and control strategies. Front Microbiol 2018;9:1941.

[14] Paillot R, Rash NL, Garrett D, Prowse-Davis L, Montesso F, Cullinane A, et al. How to meet the last OIE expert surveillance panel recommendations on equine influenza (EI) vaccine composition: a review of the process required for the recombinant canarypox-based EI vaccine. Pathogens 2016;5(4).

[15] Cullinane A, Elton D, Mumford J. Equine influenza - surveillance and control. Influenza Other Respir Viruses 2010;4(6):339–44.

[16] Legrand LJ, Pitel P, Marcillaud-Pitel CJ, Cullinane AA, Couroce AM, Fortier GD, et al. Surveillance of equine influenza viruses through the RESPE network in France from November 2005 to October 2010. Equine Vet J 2013;45:776–83.

[17] Paillot R. A systematic review of recent advances in equine influenza vaccination. Vaccines 2014;2(4):797–831.

[18] Woodward A, Rash AS, Medcalf E, Bryant NA, Elton DM. Using epidemics to map H3 equine influenza virus determinants of antigenicity. Virology 2015;481: 187–98.

[19] Crisci E, Barcena J, Montoya M. Virus-like particles: the new frontier of vaccines for animal viral infections. Vet Immunol Immunopathol 2012;148(3–4):211–25.

[20] Quan FS, Huang C, Compans RW, Kang SM. Virus-like particle vaccine induces protective immunity against homologous and heterologous strains of influenza virus. J Virol 2007;81(7):3514–24.

[21] Song JM, Wang BZ, Park KM, Van Rooijen N, Quan FS, Kim MC, et al. Influenza virus-like particles containing M2 induce broadly cross protective immunity. PLoS One 2011;6(1):e14538.

[22] Schwartzman LM, Cathcart AL, Pujanauski LM, Qi L, Kash JC, Taubenberger JK. An intranasal virus-like particle vaccine broadly protects mice from multiple subtypes of influenza a virus. mBio 2015;6(4):e01044.

[23] Sainsbury F, Saxena P, Geisler K, Osbourn A, Lomonossoff GP. Using a virus-derived system to manipulate plant natural product biosynthetic pathways. Methods Enzymol 2012;517:185–202.

[24] Chhiba-Govindjee VP, Mathiba K, van der Westhuyzen CW, Steenkamp P, Rashamuse JK, Stoychev S, et al. Dimethylformamide is a novel nitrilase inducer in Rhodococcus rhodochrous. Appl Microbiol Biotechnol 2018;102(23):10055–65.

[25] Blanco-Lobo P, Rodriguez L, Reedy S, Oladunni FS, Nogales A, Murcia PR, et al. A bivalent live-attenuated vaccine for the prevention of equine influenza virus. Viruses 2019;11(10).

[26] Khan A, Olajide E, Friedrich M, Holt A, Goehring LS. Evaluation of non-invasive sampling techniques for the molecular surveillance of equid herpesviruses in yearling horses. Viruses 2024;16(7).

[27] Couturier BA, Bender JM, Schwarz MA, Pavia AT, Hanson KE, She RC. Oseltamivir-resistant influenza a 2009 H1N1 virus in immunocompromised patients. Influenza Other Respir Viruses 2010;4(4):199–204.

[28] Park GS, Ku K, Baek SH, Kim SJ, Kim SI, Kim BT, et al. Development of reverse transcription loop-mediated isothermal amplification assays targeting severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). J Mol Diagn 2020;22(6): 729–35.

[29] Chambers TM, Reedy SE. Equine Influenza Culture Methods. Methods Mol Biol 2020;2123:393–400.

[30] Reed LJ, Muench H. A simple method of estimating fifty per cent endpoints. Am J Hyg 1938;27(3):493–7.

[31] Sainsbury F, Canizares MC, Lomonossoff GP. Cowpea mosaic virus: the plant virus-based biotechnology workhorse. Annu Rev Phytopathol 2010;48:437–55.

[32] Sainsbury F, Thuenemann EC, Lomonossoff GP. pEAQ: versatile expression vectors for easy and quick transient expression of heterologous proteins in plants. Plant Biotechnol J 2009;7(7):682–93.

[33] Strasser R, Stadlmann J, Schahs M, Stiegler G, Quendler H, Mach L, et al. Generation of glyco-engineered *Nicotiana benthamiana* for the production of monoclonal antibodies with a homogeneous human-like N-glycan structure. Plant Biotechnol J 2008;6(4):392–402.

[34] Jutras PV, D'Aoust MA, Couture MM, Vezina LP, Goulet MC, Michaud D, et al. Modulating secretory pathway pH by proton channel co-expression can increase recombinant protein stability in plants. Biotechnol J 2015;10(9):1478–86.

[35] Adams AA, Horohov DW. The effect of an immunomodulator (parapoxvirus ovis) on cell-mediated immunity (CMI) in abruptly weaned foals. Vet Immunol Immunopathol 2013;153(1–2):118–22.

[36] Strasser R. Plant glycoengineering for designing next-generation vaccines and therapeutic proteins. Biotechnol Adv 2023;67:108197.

[37] D'Aoust MA, Lavoie PO, Couture MM, Trepanier S, Guay JM, Dargis M, et al. Influenza virus-like particles produced by transient expression in *Nicotiana*

benthamiana induce a protective immune response against a lethal viral challenge in mice. Plant Biotechnol J 2008;6(9):930–40.
[38] Ward BJ, Makarkov A, Seguin A, Pillet S, Trepanier S, Dhaliwall J, et al. Efficacy, immunogenicity, and safety of a plant-derived, quadrivalent, virus-like particle influenza vaccine in adults (18-64 years) and older adults (>/=65 years): two multicentre, randomised phase 3 trials. Lancet 2020;396(10261):1491–503.
[39] Ward BJ, Landry N, Trepanier S, Mercier G, Dargis M, Couture M, et al. Human antibody response to N-glycans present on plant-made influenza virus-like particle (VLP) vaccines. Vaccine 2014;32(46):6098–106.
[40] Dennis SJ, Meyers AE, Guthrie AJ, Hitzeroth II, Rybicki EP. Immunogenicity of plant-produced African horse sickness virus-like particles: implications for a novel vaccine. Plant Biotechnol J 2018;16(2):442–50.
[41] Dennis SJ, O'Kennedy MM, Rutkowska D, Tsekoa T, Lourens CW, Hitzeroth II, et al. Safety and immunogenicity of plant-produced African horse sickness virus-like particles in horses. Vet Res 2018;49(1):105.
[42] Mokoena NB, Moetlhoa B, Rutkowska DA, Mamputha S, Dibakwane VS, Tsekoa TL, et al. Plant-produced bluetongue chimaeric VLP vaccine candidates elicit serotype-specific immunity in sheep. Vaccine 2019;37(41):6068–75.
[43] Rutkowska DA, Mokoena NB, Tsekoa TL, Dibakwane VS, O'Kennedy MM. Plant-produced chimeric virus-like particles - a new generation vaccine against African horse sickness. BMC Vet Res 2019;15(1):432.
[44] O'Kennedy MM, Coetzee P, Koekemoer O, du Plessis L, Lourens CW, Kwezi L, et al. 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. Vaccine 2022;40(35):5160–9.
[45] Smith T, O'Kennedy MM, Wandrag DBR, Adeyemi M, Abolnik C. Efficacy of a plant-produced virus-like particle vaccine in chickens challenged with influenza a H6N2 virus. Plant Biotechnol J 2020;18(2):502–12.
[46] Abolnik C, O'Kennedy MM, Murphy M-A, Wandrag DBR. Efficacy of a plant produced clade 2.3.4.4 H5 influenza virus-like-particle vaccine in layer hens. Veterinary. Vaccine 2022;1:1–10.
[47] Abolnik C, Smith T, Wandrag DBR, Murphy MA, Rautenbach M, Olibile O, et al. Dose immunogenicity study of a plant-produced influenza virus-like particle vaccine in layer hens. Heliyon 2022;8(6):e09804.
[48] Burrough ER, Magstadt DR, Petersen B, Timmermans SJ, Gauger PC, Zhang J, et al. Highly pathogenic avian influenza a(H5N1) clade 2.3.4.4b virus infection in domestic dairy cattle and cats, United States, 2024. Emerg Infect Dis 2024;30(7):1335–43.
[49] Ault A, Zajac AM, Kong WP, Gorres JP, Royals M, Wei CJ, et al. Immunogenicity and clinical protection against equine influenza by DNA vaccination of ponies. Vaccine 2012;30(26):3965–74.
[50] Paillot R, Kydd JH, Sindle T, Hannant D, Edlund Toulemonde C, Audonnet JC, et al. Antibody and IFN-gamma responses induced by a recombinant canarypox vaccine and challenge infection with equine influenza virus. Vet Immunol Immunopathol 2006;112(3–4):225–33.
[51] Chambers TM, Holland RE, Tudor LR, Townsend HG, Cook A, Bogdan J, et al. A new modified live equine influenza virus vaccine: phenotypic stability, restricted spread and efficacy against heterologous virus challenge. Equine Vet J 2001;33(7):630–6.