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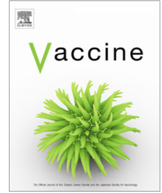

Immunogenicity of adjuvanted plant-produced SARS-CoV-2 Beta spike VLP vaccine in New Zealand white rabbits

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ABSTRACT

The outbreak of the SARS-CoV-2 global pandemic heightened the pace of vaccine development with various vaccines being approved for human use in a span of 24 months. The SARS-CoV-2 trimeric spike (S) surface glycoprotein, which mediates viral entry by binding to ACE2, is a key target for vaccines and therapeutic antibodies. Plant biopharming is recognized for its scalability, speed, versatility, and low production costs and is an increasingly promising molecular pharming vaccine platform for human health. We developed *Nicotiana benthamiana*-produced SARS-CoV-2 virus-like particle (VLP) vaccine candidates displaying the S-protein of the Beta (B.1.351) variant of concern (VOC), which triggered cross-reactive neutralising antibodies against Delta (B.1.617.2) and Omicron (B.1.1.529) VOCs. In this study, immunogenicity of the VLPs (5 μg per dose) adjuvanted with three independent adjuvants i.e. oil-in-water based adjuvants SEPIVAC SWE™ (Seppic, France) and "AS IS" (Afrigen, South Africa) as well as a slow-release synthetic oligodeoxynucleotide (ODN) adjuvant designated NADA (Disease Control Africa, South Africa) were evaluated in New Zealand white rabbits and resulted in robust neutralising antibody responses after booster vaccination, ranging from 1:5341 to as high as 1:18204. Serum neutralising antibodies elicited by the Beta variant VLP vaccine also showed cross-neutralisation against the Delta and Omicron variants with neutralising titres ranging from 1:1702 and 1:971, respectively. Collectively, these data provide support for the development of a plant-produced VLP based candidate vaccine against SARS-CoV-2 based on circulating variants of concern.

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1. Introduction

Severe acute respiratory syndrome coronavirus (SARS-CoV) emerged in 2002 as a highly transmissible pathogenic human Beta-coronavirus [1] with a mortality rate of ~ 3% [2]. Coronaviruses are enveloped, positive-sense, single-stranded RNA viruses. They have the largest genomes (26–32 kb) among known RNA viruses and are phylogenetically divided into four genera (α, β, γ, and δ), with the Beta-coronaviruses further subdivided into four lineages (A, B, C, and D) (3). Of the six known human coronaviruses, four of them (HCoV-OC43, CoV-229E, HCoV-HKU1, and HCoV-NL63) circulate annually in humans and generally cause mild respiratory diseases, although disease severity can be greater in infants, the elderly, and the immunocompromised [3]. Middle East respiratory syndrome coronavirus (MERS-CoV) and severe acute respiratory syndrome coronavirus (SARS-CoV) which belong to Beta-coronavirus lineages C and B, respectively, are highly pathogenic. Both viruses emerged in the human population from animal reservoirs within the last 15 years and caused outbreaks with high case-fatality rates [3]. In contrast to the relatively smaller outbreaks of SARS-CoV in 2002 and MERS-CoV in 2012, SARS-CoV-2 exhibited an unprecedented scale of infection, resulting in

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a global pandemic declaration of Coronavirus Infectious Disease (COVID-19) on 11 March 2020 by the World Health Organization (WHO) [4].

Vaccination remains one of the best approaches to control and eradicate an infectious disease [5]. Despite the SARS-CoV and MERS coronavirus pandemic threat, not a single vaccine for human health was commercially available at the time the COVID-19 pandemic was declared. Since the SARS-CoV-2 outbreak, various vaccine platforms have been adapted for the generation of new vaccines to confer protection against the virus. Consequently, the acceleration of the development, efficacy testing (clinical trials), regulatory approvals, as well as large-scale production of SARS-CoV-2 vaccines were achieved within 12 to 24 months, an unprecedented feat. The global effort has been commendable with 11 vaccines being approved for human use to date, >147 candidate vaccines in clinical phase development and multiple vaccines in various stages of pre-clinical development (WHO vaccine tracker data).

Plant biopharming is a maturing technology in the developed world to produce safe, efficacious new generation vaccines against bacterial and viral infections at companies such as Medicago (Canada) [6], iBio (USA) and Kentucky BioProcessing (KBP, USA). More recently, plant biopharming is also emerging in the developing world with tremendous potential to combat COVID-19 in Low- and Middle-Income Countries (LMICs). Due to the low cost of production, scalability and safety of this platform, there are now several plant-based COVID-19 vaccines in the pipeline [7]. Recently, Medicago and GlaxoSmithKline (GSK) gained approval from Health Canada for Covifenz®, the companies' plant-based COVID-19 virus-like particle (VLP) vaccine. This VLP vaccine is based on the full-length S glycoprotein of SARS-CoV-2, strain hCoV-19/USA/CA2/2020 [8]. Plant-produced VLPs based on the historical Wuhan and more recent Delta variants were also produced [9] but pre-clinical validation of the potential vaccine was still outstanding. VLPs are non-replicating protein shells similar in size and shape to the intact virus but lacking the viral genome and are therefore non-infectious but highly immunogenic in vaccine formulations. Medicago showed that a small quantity of antigen (two doses of 3.75 μg each, three weeks apart) elicited neutralising antibody titres 21 days after the second dose, 10- to 50-fold higher than those seen in human subjects recovering from COVID-19 infection [8].

SARS-CoV-2 variants of concern (VOC) including Alpha (B.1.1.7), Beta (B.1.351), Gamma (P.1), Delta (B.1.617.2) and Omicron (B.1.1.529) exhibit escape from neutralising antibodies, causing concern about vaccine effectiveness [10] especially vaccines based on the S protein. It was demonstrated that the Beta variant consistently has high resistance to neutralisation of all VOCs [11,12] and the lowest antibody recognition of a Beta virus was determined after vaccination with Wuhan-1 S protein-based mRNA-1273 [13]. SARS-CoV-2 Beta effectively escapes two major classes (class 1 and 2) of neutralising antibodies targeting an immunodominant, highly antigenic site in the receptor binding domain (RBD) of the spike protein (14). In addition, N-terminal domain (NTD) substitutions (D80A, D215G) and a three-amino-acid deletion (Δ242-244) in the Beta variant viruses contributed to the neutralisation escape of three neutralising antibody type classes of therapeutically relevant antibodies [14] with these classes described by Barnes and co-workers [15]. Furthermore, the Beta lineage harbouring mutations E484K, N501Y, and K417N, binds human Angiotensin-converting enzyme 2 (ACE2, also known as the receptor-binding motif, RBM) at nearly five-fold greater affinity than the Wuhan SARS-COV-2 RBD [16]. Neutralisation assays done with plasma from Beta infected individuals showed good neutralisation against first-wave SARS-CoV-2 viruses, whilst the Beta variant was poorly neutralised by plasma from individuals infected by non-VOC viruses [17,18]. Neutralising activity of Moderna and Pfizer-BioNTech mRNA vaccinated patients was significantly lower against Beta (12.4-fold for the Moderna vaccine; 10.3-fold for the Pfizer vaccine) and markedly more resistant to neutralisation by convalescent plasma (~11–33 fold) and vaccinee sera (~6.5–8.6 fold) [19,20]. Similarly, Jalkanen and co-workers [21] demonstrated that sera of prime-boost BNT162b2-vaccinated health care workers (n = 180) effectively neutralise the SARS-CoV-2 variant with the D614G substitution and the Alpha variant, whereas the neutralisation of the Beta variant is five-fold reduced. Complementary to this, Beta and Delta variant infections trigger responses with significantly improved Fc cross-reactivity against global VOCs circulating at the time, suggesting that vaccines based on relevant VOCs such as the Beta variant, might induce broader Fc effector responses [10]. The data suggest that a vaccine based on the S protein of Beta may not only trigger broad-spectrum cross-reactive neutralising antibodies but also Fc effector function which might be important for prevention of severe disease.

The aim or this study was three-fold: firstly, to determine immune responses in New Zealand white rabbits vaccinated with plant-produced SARS-CoV-2 VLPs, comparing VLPs based on the historical Wuhan and Beta variant of concern (VOC); secondly, to determine cross neutralization to other circulating VOCs; and thirdly, to determine immunogenicity of plant-produced Beta S variant VLPs adjuvanted with SEPIVAC SWE™ and two alternative South African produced adjuvants.

2. Material and methods

2.1. Design, cloning and validation of genes encoding rSARS-CoV-2

The Beta variant hCoV-19/South Africa/Tygerberg-461/2020 (SAΔ9) (EPI_ISL_745186), (D80A, D215G, K417N, E484K, N501Y, Δ242-244 and one mutation, A701V near the furin cleavage site) was selected as a suitable representative of Beta variant SARS-CoV-2 as it was reported to escape two major classes of nAbs targeting an immunodominant, highly antigenic site in the RBD of S [14]. Both the Wuhan (MN988668.1, 2019-nCoV WHU01) and Beta based S synthetic genes was designed, codon optimized and synthesized by BioBasic or GeneART, respectively, with the S protein in the antigenically optimal profusion conformation as described before [3]. The modified gene was restriction digested using *Age* I and *Xho* I and cloned into the plant expression vector pEAQ-HT [32] using Fast-Link™ DNA ligation kit (Diagnostech, LK6201H). The original coronavirus S was altered by substituting the native signal peptide with a murine signal peptide and modifying the C-terminal sequences for optimal expression in plant leaf tissue.

2.2. Purification of plant-produced VLP

N. benthamiana ΔXT/FT was hand infiltrated with *Agrobacterium* strain AGL-1 (ATCC® BAA-101™) harbouring each of the constructs encoding rSARS-CoV-2 Beta or Wuhan variant S protein. The modified S protein was co-expressed with the ion channel M2 as previously described for influenza vaccines [22]. Inoculum was adjusted to OD_{600} = 1.5 and mixed in a ratio of 2:1 (rSARS-CoV-2 S protein: M2), respectively, for plant infiltration. Leaves were harvested 6 days post infiltration in a 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 supplemented with sodium metabisulfite (0.04% $Na_2S_2O_5$) and protease inhibitor cocktail (Sigma P2714). Cleared lysate containing the VLPs were purified using depth filtration (Sartoclean GF, Sartorius), followed by tangential flow filtration (TFF) using a 100 K Minimate™ Capsule (Pall Life Sciences) before filter sterilisation with a 0.45 μM + 0.2 μM Sartopore 2 sterile capsule (Sartorius, Germany). A sample

of the filtration/ultrafiltration purified VLPs was subjected to Iodixanol (Optiprep, Sigma) density gradient ultracentrifugation to isolate the VLPs and then subjected to sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE) before densitometry quantification of the S protein. Gel densitometry was used to estimate the concentration of the S protein VLPs using the Chemidoc™ MP imaging system and Image Lab V4.1 software (both Bio-Rad).

The assembled SARS-CoV-2 VLPs were confirmed using Transmission Electron Microscopy (TEM) and the S protein by LC-MS/MS based peptide sequencing as described by Chhiba-Govindjee *et al.* [23]. A competitive SARS-CoV-2 ELISA (Invitrogen, BMS2326), coated with a SARS-CoV-2 Receptor Binding Domain (RBD) antigen, was used to determine antibodies (Abs) in serum samples. Samples with Abs compete with excess amounts of biotinylated ACE2. Gel densitometry was used to estimate the concentration of the S protein VLPs using a Biorad Chemidoc™ MP imaging system.

2.3. Cytotoxicity

Vero cells (African green monkey kidney, ATCC® CCL-81™) were cultured in tissue culture flasks in complete DMEM (10% Fetal Calf Serum, 100 I.U./ml penicillin and 100 μg ml^{-1} streptomycin) at 37 °C and 5% CO_2(g). Cells (50 000 cells/well) were seeded in microplates (tissue-culture grade, 96 wells, flat bottom) in a final volume of 100 μl/well culture medium (complete DMEM) and incubated in a humidified atmosphere, 37 °C and 5% CO_2 for 24 h. After 24 h the media was removed and the various compounds and partially purified plant extracts (1 – 0.0023 μg ml^{-1}) were added to a final volume of 100 μl per well. The plates were again incubated as described before for 24 h. After this incubation period, 10 μl of the cell proliferation reagent WST-1 (CELLPRO-RO Cell Proliferation Reagent WST-1, Sigma) was added to each well. The method was done as per the manufacturer's instructions. The plates were incubated at 37 °C and 5% CO_2(g) for 3 h after which the absorbance of the formazan product was measured at 440 nm in an ELISA plate reader. Both media alone and cells with WST-1 served as negative controls. The assay is based on the cleavage of the slightly red tetrazolium salt WST-1 to form a dark red formazan dye by metabolically active cells and thus only occurs in viable cells. As the formazan dye formed is soluble in aqueous solutions, it is directly quantified using a scanning multiwell spectrophotometer (ELISA reader).

2.4. Formulation of SARS-CoV-2 VLP vaccines

D-(+)-Trehalose dihydrate (Sigma-Aldrich)(15% m/v) was added to the TFF-purified VLPs 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 were mixed with sterile adjuvant (50% SEPIVAC SWE™, Seppic, France), or 50% Afrigen "AS IS" or Disease Control Africa's slow-release adjuvant, immediately before vaccination.

2.5. New Zealand white rabbit study design to test vaccine safety and immunogenicity

The study designs for the immunogenicity testing of plant-produced SARS-CoV-2 VLPs (Wuhan and Beta variants) as vaccine candidates, are depicted in Table 1 and complemented by a schematic diagram (Fig. 3). In short, 7 and 12 male rabbits were used in these two pilot studies, respectively. Seven rabbits were vaccinated in the first pilot study on days 0 and 21, administering 5 μg per dose (1 ml volume; 50% SEPIVAC SWE™) of the Wuhan (n = 3) or Beta variants (n = 3) and one rabbit received the adjuvant only. In a second pilot study, rabbits were vaccinated only with SARS-CoV-2 Beta VLPs and adjuvanted with either SEPIVAC SWE™, Afrigen "AS IS" or Disease Control Africa's slow-release adjuvants (n = 3 per group), plus one rabbit for each of the adjuvants. Serum was collected on days 0, 7, 14, 21, 28, 35 and 42 in both studies.

2.6. New Zealand white rabbit vaccination

The Animal Ethics Committee (AEC) of La-Bio Research verified that the animal facility operated within the standards and rules of the National Laboratory Animal Ethical Code of Conduct and the OECD guidelines for animal testing. All efforts were made to ensure that the animals were kept according to recognized international standards in animal husbandry practice.

The protocol was reviewed and approved for ethical clearance by the AEC of La-Bio Research.

New Zealand white rabbits were sourced from an approved animal breeder and their health monitored by the facility veterinarian according to the approved criteria. After 14 days of acclimatisation, the animals were subjected to a full veterinary inspection, and certified as suitable for use in the study. A temperature of 20 °C ± 3 °C and a humidity of 30–70% was maintained in the housing room for the duration of the study. A 12-hour day/night light cycle was constant in the housing room. The light intensity was kept between 40 and 100 lx. Rabbit feed and water was available *ad lib*. Each animal received a daily food enrichment, which alternated between carrots and cabbage. At day 0 and 21, rabbits were injected with a volume of 1 ml intramuscularly in the lumbar muscle with the different vaccine candidates using a 21-gauge needle (1 in. in length). Blood samples were collected under anaesthesia from the saphenous vein on day 0, 7, 14, 21, 28, 35 and 42. A total of 4 ml of blood was collected for serum isolation. Animals were anaesthetized by short acting isoflurane inhalation anaesthesia calibrated to deliver a 4% induction and 2% maintenance concentration. Terminal blood was collected via cardiac puncture on anaesthetized animals using a 21-gauge needle (1 in. in length). For cardiac puncture, the animals were anaesthetised using 0.4 mg/kg Butorphanol/ 15 mg/kg Ketamine/ 0.25 mg/kg Medetomidine combination intramuscularly. Following terminal blood collections animals were killed by intrahepatic overdose administration of pentobarbital 150 mg/kg. All animals showed a normal weight gain profile for the duration of observation. All animals were monitored for acute adverse vaccine reactions for 7 days following primary and booster vaccinations. No gross adverse reactions as a result of the vaccinations were observed in any of the study animals.

2.7. Lentiviral pseudovirus production and neutralisation assay.

The 293 T/ACE2.MF cells modified to overexpress human ACE2 were kindly provided by M. Farzan (Scripps Research). Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco BRL Life Technologies) containing 10% heat-inactivated serum (Fetal Bovine Serum) and 3 μg ml^{-1} puromycin at 37 °C, 5% CO_2. Confluent cell monolayers were disrupted by treatment with 0.25% trypsin in 1 mM EDTA (Gibco BRL Life Technologies). The SARS-CoV-2, Wuhan-1 spike, cloned into pCDNA3.1 was mutated using the QuikChange Lightning Site-Directed Mutagenesis kit (Agilent Technologies) to include D614G (original) or K417N, E484K, N501Y, D614G (RBD only) or L18F, D80A, D215G, Δ242-244, K417N, E484K, N501Y, D614G, A701V (Beta). Pseudoviruses were produced by co-transfection with a lentiviral backbone (HIV-1 pNL4.luc encoding the firefly luciferase gene) and either of the SARS-CoV-2 spike plasmids with PEIMAX (Polysciences). Culture supernatants were separated from cells with a 0.45 μM filter and stored at − 70 °C. Plasma/serum samples were heat-

Table 1
SARS-CoV-2 VLP vaccine formulations for immunogenicity studies in New Zealand white rabbits.

Test item	Dose	Adjuvant	Animal numbers	Number of doses	Days of vaccination
Study 1					
Wuhan VLPs	5 µg	SEPIVAC SWE	3	2	D0, D21
Beta VLPs	5 µg	SEPIVAC SWE	3	2	D0, D21
PBS	–	SEPIVAC SWE	1	2	D0, D21
Study 2					
Beta VLPs	5 µg	SEPIVAC SWE	3	2	D0, D21
Beta VLPs	5 µg	Afrigen "AS IS"	3	2	D0, D21
Beta VLPs	5 µg	DCA slow release	3	2	D0, D21
PBS	–	SEPIVAC SWE or Afrigen "AS IS" or DCA slow release	3	2	D0, D21

*DCA = Disease control Africa.

inactivated and clarified by centrifugation. Pseudovirus and serially diluted plasma/sera were incubated for 1 h at 37 °C, 5% CO_2. Cells were added at 1×10^4 cells per well after 72 h of incubation at 37 °C, 5% CO_2, luminescence was measured using PerkinElmer Life Sciences Model Victor X luminometer. Neutralisation was measured as described, by a reduction in luciferase gene expression after single-round infection of 293 T/ACE2. MF cells with spike-pseudotyped viruses. Titres were calculated as the reciprocal plasma dilution (ID_{50}) causing 50% reduction of relative light units. Equivalency was established through participation in the SARS-CoV-2 Neutralising Assay Concordance Survey Concordance Survey 1 run by EQAPOL and VQU, Duke Human Vaccine Institute. Cell-based neutralisation assays using live virus or pseudovirus have demonstrated high concordance, with highly correlated 50% neutralisation titres (Pearson r = 0.81–0.89) [33].

2.8. Statistical analysis

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

3. Results

Virus-like particles of the SARS-CoV-2 Beta and Wuhan variants were successfully assembled in *Nicotiana benthamiana* ΔXT/FT, a glycosylation mutant with a targeted downregulation of xylose and fucose expression that facilitates mammalian-like glycosylation [34]. Assembled VLPs were visualised using transmission electron microscopy indicating reproducibility of different batches (Fig. 1 B&C) and the presence of the S protein was confirmed using LC-MS/MS based peptide sequencing. A cytotoxicity study in Vero cells was conducted to confirm the safety of the plant-produced and partially purified SARS-CoV-2 VLPs of concentrations ranging from 1 µg to 2.3 ng per well, prior to the pre-clinical trials (Fig. 2). This was achieved by means of a colorimetric cell proliferation assay based on tetrazolium salt (WST-1), which is reduced to water-soluble orange formazan in viable cells by cellular mitochondrial dehydrogenase. Cell viability varied<20% when either plant extract or plant extract containing the VLPs were compared to the control standard.

Plant-produced SARS-CoV-2 VLPs (Wuhan or Beta) at 5 µg per 1 ml dose were formulated with SEPIVAC SWE™ (Seppic, France) and adjuvanted in a 1:1 (v/v) ratio. In an exploratory study with only seven rabbits, the animals were prime boost vaccinated intramuscularly with either Wuhan or Beta variant S VLPs (n = 3 per group; single rabbit control) on days 0 and 21 and serum samples collected once a week (Table 1, Fig. 3). The levels of SARS-CoV-2 antibodies in serum samples were detected using a commercially available competitive ELISA kit coated with a SARS-CoV-2 RBD antigen and calculated as percentage inhibition (≥20% being positive) as described by the manufacturer (Invitrogen, Catalog Number BMS2326) (Fig. 4). Signals were inversely proportional to the level of inhibitory antibodies. Adjuvanted SARS-CoV-2 Beta variant VLPs elicited nAbs after primary vaccination on day 21 (48%, 47% and 46%, animals 10–12, respectively) and doubled after booster vaccination (94%, 93% and 92%, animals 10–12, respectively). Thereafter we observed a gradual decline during the following 14 days to 90%, 88% and 67%. The Wuhan variant VLP vaccine resulted in a poorer immune response with percentage neutralisation of 65%, 58% and 61% on day 28 (animals 1–3) dropping to 38%, 25% and 43% on day 42 for individual animals, respectively.

A similar trend was demonstrated with pseudovirus neutralising assay titres represented as ID_{50}, the inhibitory dilutions at which 50% neutralisation is attained, for a panel of pseudoviruses representing Wuhan, Beta, Delta and Omicron variants (Table 2, Fig. 5). The sera of three rabbits in each treatment group were pooled in the first study for preliminary ID_{50} determination. Rabbits vaccinated with Beta variant VLPs neutralised pseudoviruses of Beta on days 14 and 21 (titres of 211 and 775, respectively), which increased to 18,204 (86-fold increase) after booster vaccination followed by a gradual drop to 6262 and 1337 within 14 days after booster vaccination. Rabbits vaccinated with Beta variant

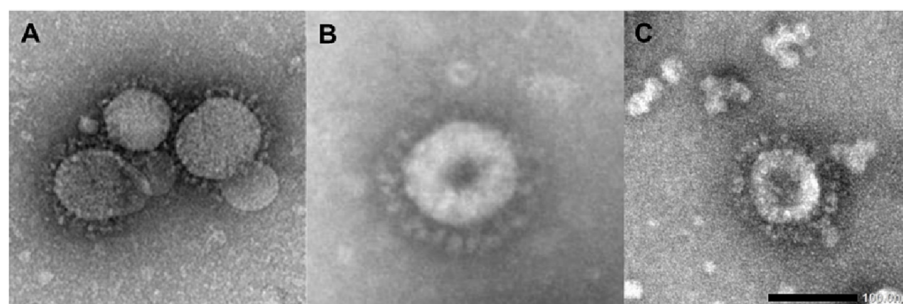

Fig. 1. Negatively stained transmission electron microscopy (TEM) images of live coronaviruses (Wikipedia) (A) versus plant-produced and density gradient purified Coronavirus rSARS-CoV-2 Beta S protein VLPs (B & C). Bar 100 nm.

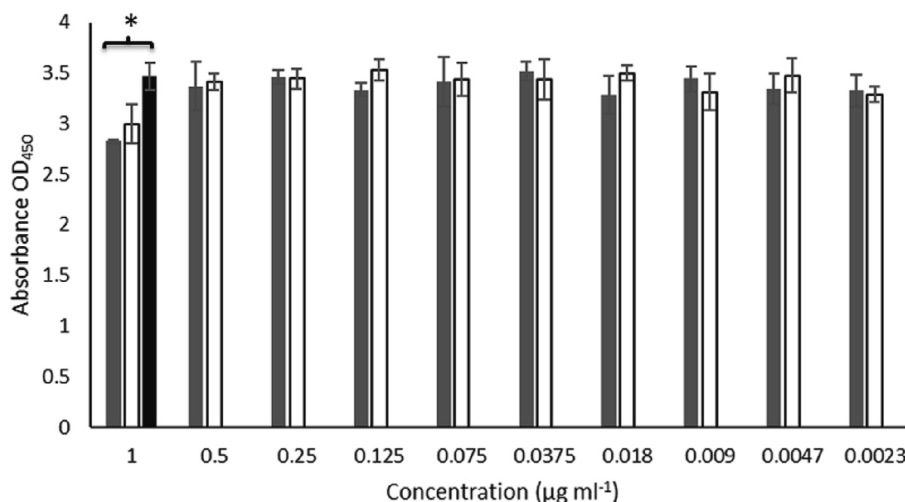

Fig. 2. Cytotoxicity studies in Vero cells validating SARS-CoV-2 Beta VLPs. Cells incubated with SARS CoV-2 VLPs (grey bars) or plant extract (white bars) and cells with WST-1 alone served as negative control (black bar). Statistical significance between mean OD values at P < 0.05 (denoted by *).

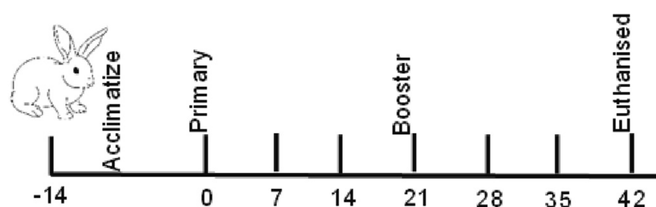

Fig. 3. Schematic diagram of immunogenicity studies of the VLP vaccine candidates in New Zealand white rabbits.

VLPs also cross-neutralised pseudoviruses of Delta variant (titres of 1702, 412 and 138) and Omicron (971, 736 and 186) on days 28, 35 and 42, respectively. In contrast, antibodies elicited by Wuhan variant VLP vaccination resulted in less favourable titres. Rabbits vaccinated with Wuhan variant VLPs cross-neutralised Beta variant resulting in titres of 286, 1348 and 211 on days 21, 28 and 35, respectively. Rabbits vaccinated with Wuhan variant VLPs cross-neutralised the Delta variant only after booster vaccination on day 28, with a titre of 65. The ID_{50} values were presented in graph format (Log_{10}) for ease of comparison (Fig. 5). Thus, adjuvanted plant-produced SARS-CoV-2 Beta variant VLPs elicited antibodies

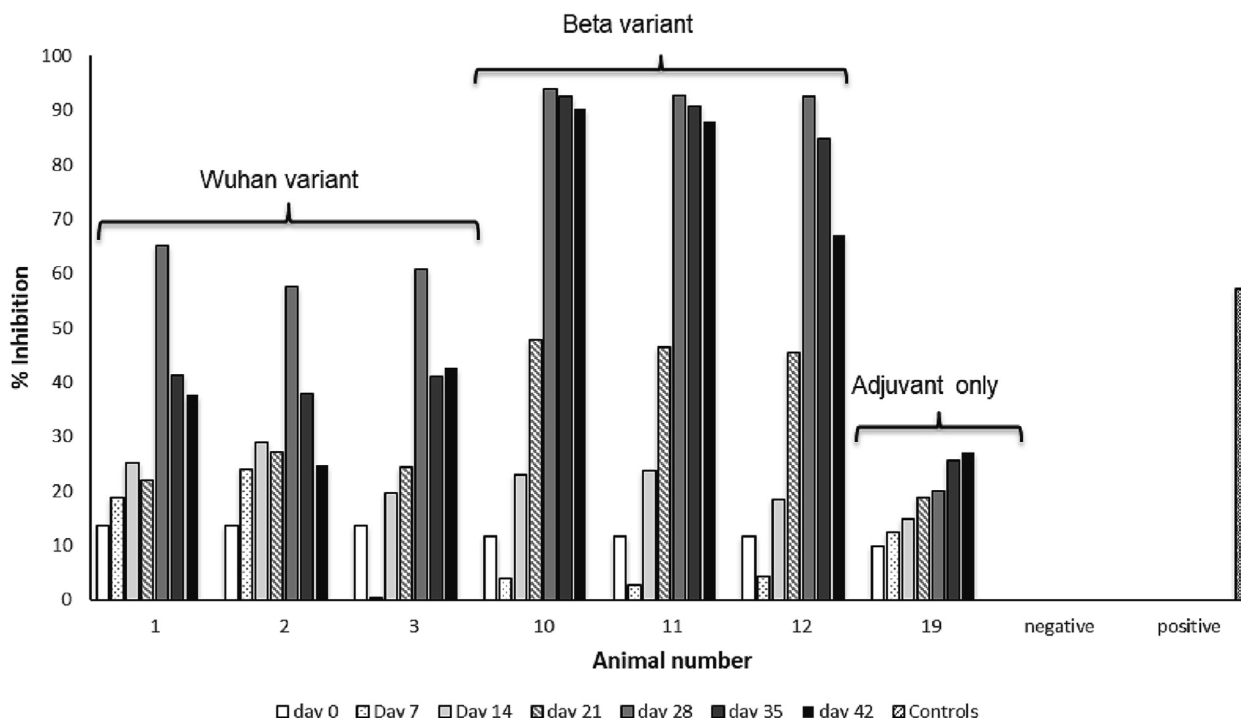

Fig. 4. Percentage inhibition of serum antibodies (collected weekly) to the RBD antigen of a commercial competitive SARS-CoV-2 ELISA kit. New Zealand white rabbits vaccinated with SEPIVAC SWE™ adjuvanted VLPs based on either the Wuhan (animals 1–3) or Beta (animals 10–12) S proteins. A single rabbit was vaccinated with the adjuvant only and served as negative control. The positive control was provided in the ELISA kit. Signals were inversely proportional to the level of antibodies and calculated as percentage, with a ≥ 20% regarded as positive inhibition.

Table 2
Cross-neutralising titres of serum elicited in rabbits prime boost vaccinated (days 0 and 21) with plant-produced SARS-CoV-2 Beta or Wuhan variant S protein based VLPs and adjuvanted with (SEPIVAC SWE). ID_{50} values are the inhibitory dilutions at which 50% neutralisation is attained. A value < 50 is indicated as 0.

A	**Beta VLPs vaccinated rabbits**						**Wuhan variant vaccinated rabbits**					
	Days											
	7	**14**	**21**	**28**	**35**	**42**	**7**	**14**	**21**	**28**	**35**	**42**
Beta B.1.351	0	211	775	18204	6262	1337	0	0	286	1348	211	0
Delta B.1.617.2	0	0	0	1702	412	138	0	0	0	65	0	0
Omicron B.1.1.529	0	0	0	971	736	186	0	0	0	0	0	0

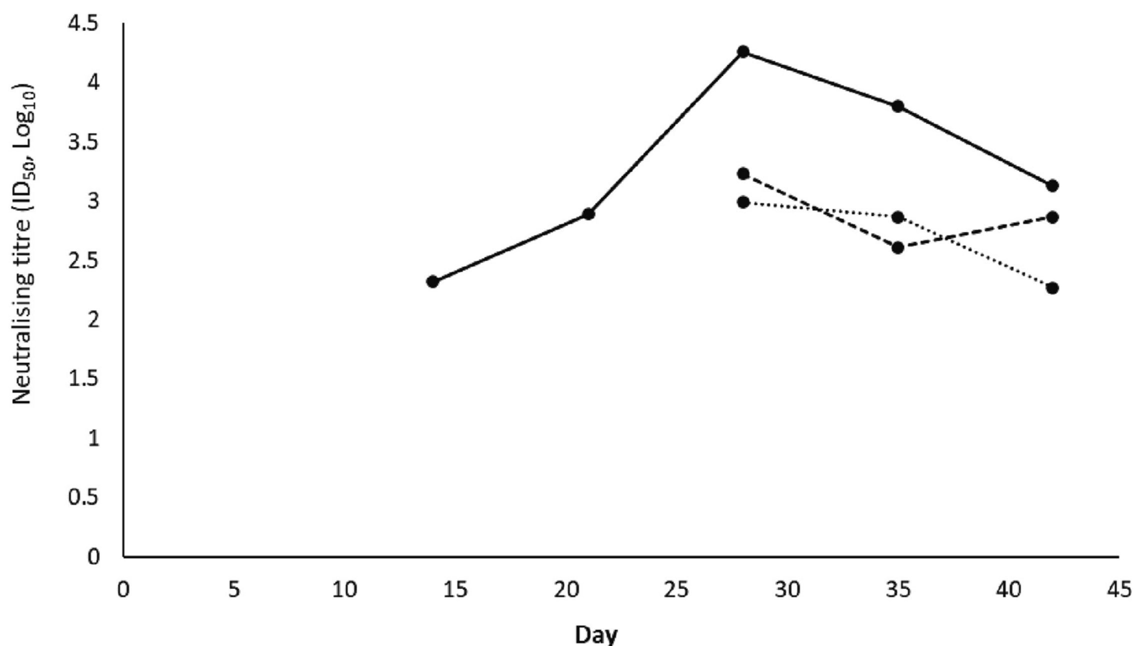

Fig. 5. Serum obtained from rabbits, prime boost vaccinated (days 0 and 21) with plant-produced SARS-CoV-2 Beta VLPs and adjuvanted with (SEPIVAC SWE). Rabbits vaccinated with Beta VLPs neutralised pseudoviruses of Beta (solid line) as from days 14 to 42 but also cross-neutralise Delta (days 28–42, striped line) and Omicron (days 28–42, dotted line) pseudoviruses. ID_{50} values are the inhibitory dilutions at which 50% neutralisation is attained.

in rabbits that neutralised pseudoviruses of Beta, but also cross neutralised Delta and Omicron variants (Fig. 5).

In a second pilot study, plant-produced Beta variant VLPs were formulated with SEPIVAC SWE^{TM} (formulation 1:1) as positive control and compared with "AS IS" (Afrigen, South Africa) (formulation 1:1) and a slow-release adjuvant from Disease Control Africa (DCA, South Africa) (n = 3 per adjuvant group; one rabbit for each of the adjuvants served as negative control). Rabbits were prime boost vaccinated with Beta variant VLPs on days 0 and 21 (5 µg per 1 ml dose) and serum samples collected once a week. The geometric mean titres (GMT) represented as ID_{50} values are the inhibitory dilutions at which 50% neutralisation was attained. Beta variant

Table 3
Neutralising titres of serum elicited in rabbits prime boost vaccinated (days 0 and 21) with plant-produced SARS-CoV-2 Beta VLPs and formulated with three different adjuvants namely SEPIVAC SWE, Afrigen "AS IS" or DCA slow release. ID_{50} values are the inhibitory dilutions at which 50% neutralisation is attained. A value < 50 is indicated as 0.

		Beta VLP vaccinated rabbits						
			Days					
Adjuvants		Rabbit no.	7	14	21	28	35	42
SEPIVAC SWE		M1	294	76	110	1870	2635	436
		M2	0	0	0	3022	5804	402
		M3	0	0	0	2049	7585	160
	GMT		98 ± 170	25 ± 44	36 ± 64	2313 ± 620	5341 ± 2507	332 ± 151
Afrigen "AS IS"		M5	0	99	51	1333	0	579
		M6	0	0	0	453	285	99
		M7	0	0	0	218	147	0
	GMT			33 ± 57	17 ± 29	668 ± 588	144 ± 143	226 ± 310
DCA slow release		M9*	643	97				
		M10	0	0	0	126	95	0
		M11	0	0	0	991	478	226
	GMT		214 ± 371	32 ± 56		558 ± 612	287 ± 271	113 ± 160

* Cage injured animal euthanised after day 14.

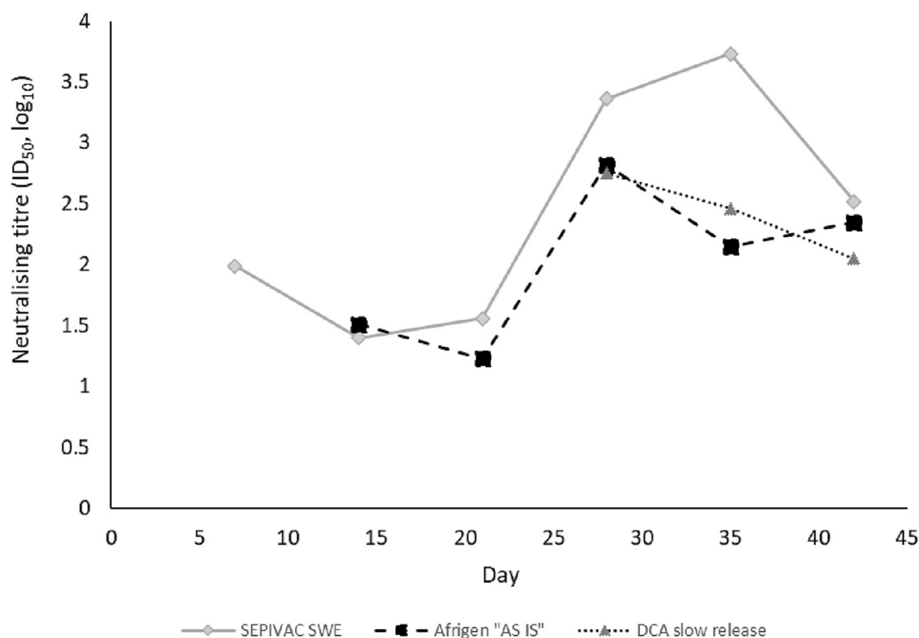

Fig. 6. Serum obtained from rabbits, prime boost vaccinated (days 0 and 21) with plant-produced SARS-CoV-2 Beta VLPs and adjuvanted with SEPIVAC SWE, Afrigen "AS IS" or Disease Control Africa (DCA) slow release. Rabbits vaccinated with Beta VLPs neutralised pseudoviruses of Beta when adjuvanted with SEPIVAC SWE (days 7 – 42, solid grey line), Afrigen (days 14 – 42, striped line) and DCA (days 28 – 42, dotted line). ID_{50} values (presented as Log_{10}) are the inhibitory dilutions at which 50% neutralisation is attained.

VLPs adjuvanted with SEPIVAC resulted in GMTs of 98 – 2313 on days 7 – 28, elevated to 5341 after booster vaccination whereafter it dropped to 332 on day 42 (Table 3, Fig. 6). Beta variant VLPs adjuvanted with Afrigen "AS IS" resulted in GMTs of 33 – 668 on days 14 – 28 whereafter it dropped to 144 and 226 on days 35 and 42, respectively. Beta VLPs adjuvanted with DCA slow-release adjuvant resulted in GMTs of 558 on day 28 whereafter it dropped to 287 and 113 on days 35 and 42, respectively. Once more, the ID_{50} values were also presented as Log_{10} in graph format for ease of comparison (Fig. 6). The SEPIVAC SWE™ adjuvant was considered superior to the other adjuvants tested.

4. Discussion

Virus-like particles (VLPs) are known to be safe, efficacious vaccine candidates as they are non-replicating protein shells lacking the viral genome but mimicking the native virion with repetitive virion epitopes to induce both innate and adaptive immunity. VLP vaccine products already commercially available for human health are produced in traditional expression systems such as yeast, *Escherichia coli* and insect cells include hepatitis B virus (HBV, Recombivax HB®, Engerix®), human papillomavirus (HPV, Cervarix® and Gardasil®) and hepatitis E virus (HEV, Hecolin Xiamen Innovax Biotech) and paved the way for seasonal and pandemic influenza, as well as malaria VLP vaccines which are already in advanced clinical trials [24,25]. More recently, Medicago produced the first biopharmed COVID-19 VLP vaccine Covifenz® based on a variant that circulated in the USA approved by Health Canada.

In this pilot study, effective cross-neutralisation of VOCs by serum obtained from rabbits vaccinated with adjuvanted plant-produced Beta variant VLPs was demonstrated which aligns with results for other VOC-based vaccines in more advanced preclinical studies. For example, the prototype-Beta chimeric RBD-dimer tandem repeat protein subunit vaccines developed induced broader nAbs against SARS-CoV-2 variants than its antigenically homotypic counterparts, decreasing viral loads of 3.4-log_{10} (1:2520) in lung and prevented virus-induced lung lesions in rhesus macaques [26]. The latter vaccine also had higher cross-neutralisation towards Omicron but also improved protection against SARS-CoV-2 in animal models when compared to both homotypic prototype and Beta vaccines [26]. It was not surprising that subsequent Delta-Omicron RBD-dimer vaccine [26] and Omicron-specific mRNA vaccine [27,28] prevented Omicron infection in mice but induced antibodies with limited capacity to neutralise historical variants. In addition, Corbett and co-workers [12] could not discern a significant difference in neutralising antibody titres or reduction in viral replication between the homologous Moderna's mRNA-1273 or heterologous mRNA-1273 Beta boost of nonhuman primates (NHPs). Yet, a primary vaccination series with mRNA-1273Beta yielded a unique repertoire (both qualitative and quantitative) with marked increases in reactivity to epitopes associated with broad and potent neutralisation of VOCs [12]. In addition, priming with mRNA-1273.Beta in naïve NHPs not only induced potent neutralising antibody responses against Beta but also provided high-level protection in the upper and lower airways after challenge [11]. The authors reasoned that future mRNA vaccines can be designed to imprint B cell repertoires in naïve individuals for increased potency and breadth of neutralising activity.

In this study, Beta variant VLP vaccinated rabbits at a vaccine dose of 5 µg resulted in GMT of 211, 14 days after primary vaccination and this increased 86-fold after booster vaccination to 18 204. In addition, the serum conferred cross-immunity against Delta and Omicron VOCs resulting in titres of 1702 and 971, 28 days after primary vaccination, respectively, whereafter it declined to 138 and 186. The authors anticipate that similar titres will result in protective immunity in a follow up study in golden Syrian hamsters. In support of this, Khoury and co-workers [29] demonstrated that neutralising antibody levels are highly predictive of immune protection from symptomatic SARS-CoV-2 infection. Individuals vaccinated with seven registered vaccine candidates varying in mean titres of 28 to 654; or 35 to 983 in convalescent individuals, resulted in 50% protective neutralisation titres between 7 and 199. Thus, antibody titre thresholds indeed align with protective immu-

nity in humans. Earle and co-workers [30] also provided evidence for post-immunisation antibodies as a protective correlate for mRNA, adenoviral vector, protein subunit, and inactivated virus Covid-19 vaccines in clinical studies. A French team [31] demonstrated that neutralising antibody titres in vaccinated healthcare workers below 8 provided no protection against a BA.1 infection (one of the most prolific sublineage, detected worldwide at the time), whereas titres of 16 or 32 gave 73.2% protection, and a titer of 64 or 128 provided 78.4% protection. Although the plant-produced Beta VLP vaccine needs to be tested in clinical studies, we provide compelling support for future clinical studies.

In this study, alternative adjuvants were also tested to stimulate robust and durable neutralising-antibody responses to confer protective immunity against SARS-CoV-2 as different adjuvants presumably induce different cellular and humoral responses. To this end, plant-produced Beta VLPs were adjuvanted with three different adjuvants. Adjuvanting with SEPIVAC SWE^{TM} (squalene-based, oil-in-water) resulted in higher neutralising GMT (5341) whereas the highest GMTs induced by the DCA slow release or Afrigen "AS IS" were 558 and 668, respectively. Medicago adjuvanted their plant-produced VLP subunit vaccine with either CpG 1018 adjuvant, composed of cytosine 227 phosphoguanine (CpG) motifs (Dynavax) or AS03 adjuvant, an oil-in-water emulsion containing tocopherol (vitamin E) and squalene (GlaxoSmithKline), showing the latter to be superior in eliciting IL-4 responses, IFNγ responses and antibody titres with evidence of both Th1- and Th2-type activation. The authors thus chose SEPIVAC as the preferred adjuvant for future efficacy studies in golden Syrian hamsters.

5. Concluding remarks

In this pilot study, low doses (5 μg) of plant-produced SARS-CoV-2 Beta variant VLPs triggered robust neutralising antibodies in rabbits which cross-reacted to Delta and Omicron variants of concern, with SEPIVAC SWE^{TM} being superior as adjuvant. WHO advised the development of multivalent vaccines as the next generation of COVID-19 vaccine candidates for induction of broader immune responses against both circulating and emerging variants (WHO, 2022). Thus, formulating a multivalent vaccine consisting of a cocktail of selected VLPs might result in broad spectrum protection. Collectively, these data provide compelling support in the development of a plant-produced VLP based candidate vaccine protecting against SARS-CoV-2 VOCs. The authors opted to produce rSARS-CoV-2 VLPs in *N. benthamiana* ΔXT/FT to mitigate potential allergy/hypersensitivity and development of antibodies against plant glyco-epitopes as considered before for plant-made influenza VLPs [35]. Detailing the glycosylation profile of clinical grade rSARS-CoV-2 VLPs is envisaged for future studies but not conducted for this pre-clinical study.

Author contribution.

MO, PM and YL conceptualized, designed, conducted, and interpreted the rabbit study, ELISA and SNT results. JV assisted with interpretation of immune responses. MO, CA, YL, TS, KS and RR designed and cloned all relevant genes, and MO and AT produced the VLP proteins using Agrobacterium AGL-1 and formulated the vaccine for the rabbit studies. MM and OM supported the work with substantial funding, IP assessment and contributed to identifying appropriate adjuvants. TM conducted the SNTs and interpreted the data. IdP conducted and interpreted the cytotoxicity studies in Vero cells. All authors proofread the manuscript before submission.

Ethics approvals

CSIR Research Ethics Committee (REC) approval (Reference number 251/2018) to clone the coronavirus synthetic genes into a plant expression vector obtained in a BSL-1 facility. Immunization of New Zealand white rabbits was also approved by CSIR REC 355/2021, LaBio LBR-12-F01 and UP Animal ethics committee approval (REC084-21).

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Data availability

The data that has been used is confidential.

Declaration of Competing Interest

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

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