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Plant-produced Bluetongue chimaeric VLP vaccine candidates elicit serotype-specific immunity in sheep

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ABSTRACT

Bluetongue (BT) is a hemorrhagic non-contagious, biting midge-transmitted disease of wild and domestic ruminants that is caused by bluetongue virus (BTV). Annual vaccination plays a pivotal role in BT disease control in endemic regions. Due to safety concerns of the current BTV multivalent live attenuated vaccine (LAV), a safe efficacious new generation subunit vaccine such as a plant-produced BT virus-like particle (VLP) vaccine is imperative. Previously, homogenous BTV serotype 8 (BTV-8) VLPs were successfully produced in *Nicotiana benthamiana* plants and provided protective immunity in sheep. In this study, combinations of BTV capsid proteins from more than one serotype were expressed and assembled to form chimaeric BTV-3 and BTV-4 VLPs in *N. benthamiana* plants. The assembled homogenous BTV-8, as well as chimaeric BTV-3 and chimaeric BTV-4 VLP serotypes, were confirmed by SDS-PAGE, Transmission Electron microscopy (TEM) and protein confirmation using liquid chromatography-mass spectrometry (LC-MS/MS) based peptide sequencing. As VP2 is the major determinant eliciting protective immunity, the percentage coverage and number of unique VP2 peptides detected in assembled chimaeric BT VLPs were used as a guide to assemble the most appropriate chimaeric combinations. Both plant-produced chimaeric BTV-3 and BTV-4 VLPs were able to induce long-lasting serotype-specific neutralizing antibodies equivalent to the monovalent LAV controls. Antibody levels remained high to the end of the trial. Combinations of homogenous and chimaeric BT VLPs have great potential as a safe, effective multivalent vaccine with the ability to distinguish between vaccinated and infected individuals (DIVA) due to the absence of non-structural proteins.

1. Introduction

Bluetongue (BT) is a non-contagious, infectious, World Organisation for Animal Health (OIE) notifiable disease of ruminants caused by the Bluetongue virus (BTV) (family Reoviridae, genus Orbivirus) and is spread by *Culicoides* spp. biting midges. There are 27 distinct known BTV serotypes determined by the outer viral-capsid protein VP2 encoded for by segment 2 of the dsRNA genome [9]. BT disease is of significant importance globally due to its economic impact and effect on animal welfare. Sheep is the primary host while cattle are carriers of the virus. The eradication of BTV from endemic regions of Africa is virtually impossible due to the role played by widely distributed *Culicoides* spp. midge vectors and the ubiquitous distribution of vertebrate reservoir species [6]. In endemic areas attempts have been made to limit the occurrence of BT disease, and its economic impact, through vaccination [6]. Current effective control against BTV in South Africa is achieved using multivalent, live attenuated vaccine (LAV) supplied by Onderstepoort Biological Products (OBP) SOC (Ltd) and sold in three separate vials A, B and C containing five serotypes each [6,8]. The vaccine elicits strong neutralizing antibodies and confer long-term protection; however there are concerns with regard to the possibility of reversion of live attenuated antigens to virulent forms [20,26]. In addition, prolonged viremia and the possible transmissibility of vaccine antigens to susceptible hosts increase the probability of reassortment to virulent strains. A mono-valent attenuated live BTV-8 vaccine (AL-BTV-8) was previously used and protected against the European BTV-8 outbreak isolate [7] whilst inactivated serotype-specific BT vaccines are used in other countries [19,5]. These first generation vaccines have safety concerns such as teratogenicity, a risk of reassortment, reversion to virulence of the attenuated viral strain (Review, [11]) and are

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prohibited in most developed countries. A reassortant virus containing segments from two BTV serotypes (BTV-9 and BTV-8) was recently detected in experimentally vaccinated cattle [24], rendering the safety concerns valid.

BT VLPs are considered a safe efficacious alternative to LAV. The non-enveloped virus particle contains three layers of viral capsid proteins, namely VP2, VP3, VP5 and VP7 but is void of the viral genome. Homogenous BTV VLPs were successfully assembled in a Baculovirus-insect cell expression system [16] and were able to elicit neutralizing antibodies for complete protection in target animals. In addition, Feenstra and co-workers [10] followed a chimaeric reverse-genetics approach whereby they exchanged VP2 or incorporated chimaeric VP2 in developing a disabled infectious single animal (DISA) vaccine. However, the costs and complexity associated with production and upscaling of insect cell-produced VLPs and reverse-genetics will make only monovalent or bivalent BTV VLP vaccines feasible. In contrast, production of BT VLPs in plants is an attractive affordable alternative with the advantage of facile scalability. Previously, homogenous BTV serotype 8 (BTV-8) VLPs were successfully produced in *N. benthamiana* plants using the cowpea-mosaic virus-based HyperTrans (CPMV-HT) [23,25] and provided protective immunity against challenge with a South African BTV-8 field isolate [23]. Related homogenous viruses in the same Reoviridae family were also successfully produced using the same plant expression platform [4].

Self-assembly of homogenous BT VLPs does not always occur [10], and it is necessary to identify the appropriate core-like particle backbone to facilitate the assembly of chimaeric VLPs in the development of multivalent BT VLP vaccines. In this study, genes encoding the selected capsid proteins from more than one serotype were individually cloned into the plant expression vector, pEAQ-HT, and homogenous BTV-8, chimaeric BTV-4 and chimaeric BTV-3 VLPs were successfully assembled and purified from *N. benthamiana* plants for target animal trials. A single chimaeric was defined as a VLP assembled from four capsid proteins with only one of the four being substituted with that of a second serotype. Most often BTV-8 VP2 was being substituted with VP2 of BTV-3 or BTV-4, resulting in a single chimaeric. A double chimaeric was assembled using the BTV-8 core proteins (VP3 and VP7) whilst both outer capsid proteins (VP2 and VP5) were of another serotype, either serotype 3 or 4. Finally, double chimaeric BTV-4 and BTV-3 VLPs were independently formulated and vaccinated as a prime-boost to evaluate immunogenicity in sheep. Successful assembly of homogenous as well as chimaeric BT VLPs of required serotypes will pave the way towards a next-generation efficacious multivalent vaccine.

2. Materials and methods

2.1. Constructs design and gene synthesis

Genes, encoding BTV capsid proteins, were plant codon-optimized, synthesized by BioBasic Inc (Canada) and individually cloned into the pEAQ-HT expression vector [18] using restriction-enzyme sites *Age* I and *Xho* I. These genes encoded the following proteins of serotypes BTV3, 4 and 8 as indicated by respective accession numbers: BTV-3 VP5 AJ586697, BTV-4 VP5 JN255947, BTV-8 VP3 JX272541, BTV-8 VP5 JX272543 and BTV-8 VP7 JX272545 as well as VP2 sequences of the vaccine strains (serotypes 8, 3 and 4), proprietary to OBP.

2.2. Bacterial cultures

Electro-competent *Escherichia coli* DH10B bacterial cells (Thermo Fisher Scientific) were used for propagation of pEAQ-HT gene constructs. Following transformation into DH10B for amplification using a Gene Pulser™ (Bio-Rad) (1.6 kV, 200 Ω and 25 μF), and their growth on Luria-Bertani (LB) agar plates, supplemented with 50 μg ml L^{-1} kanamycin, sequence-validated gene constructs were electroporated into selected *Agrobacterium* cells (1.44 kV, 200 Ω and 25 μF) using a Gene Pulser™ (Bio-Rad). The plant expression vector pEAQ-HT harbouring the genes independently were electroporated into *Agrobacterium* strain LBA4404 and propagated at 28 °C in LB media containing the appropriate antibiotics (50 μg ml L^{-1} kanamycin, 50 μg ml L^{-1} streptomycin and 25 μg ml L^{-1} rifampicin). Relevant constructs were also transformed into *Agrobacterium* strains AGL-1 and GV3101::pMP90 harbouring the binary vector with genes of interest. All reagents were molecular biology grade and obtained from Sigma Life Science unless otherwise indicated.

2.3. Plant agroinfiltration and assembly of VLPs

Prior to plant hand infiltration, *Agrobacterium tumefaciens* (LBA4404) transformed with pEAQ-HT harbouring individual genes were streaked on LB media with the appropriate antibiotics and incubated at 28 °C for 48 hrs. The growing bacteria were scraped off from the plate and inoculated into liquid YMB medium (0.1% yeast extract, 1% Mannitol, 1.7 mM NaCl, 0.8 mM $MgSO_4.7H_20$, 2.2 mM $K_2HPO_4{\cdot}3H_2O$), with the appropriate antibiotics and incubated with rotational shaking (175 rpm) for 24 h at 28 °C. Cells were pelleted by centrifugation at 8000 rpm for 7 min and subsequently resuspended in MMA buffer (10 mM MES hydrate; pH 5.6, 10 mM $MgCl_2$, 100 μM 3,5-dimethoxy-4-hydroxy-acetophe none). The *Agrobacterium* cultures harbouring one of the four capsid proteins were adjusted to OD_{600} of approximately 0.8–2 and mixed in a ratio of 1:1:1:1 for plant infiltration. *N. benthamiana* dXT/FT plants [22] were grown in a growth room facility maintained at 26–28 °C, 16 h day and 8 h dark.

The leaf material was harvested seven to eight days after infiltration using a Matstone Multipurpose juice extractor in VLP extraction buffer (50 mM Bicine, 20 mM NaCl pH 8.4; 0.1% (w/v) and 1 mM dithiothreitol (DTT); protease inhibitor cocktail (Sigma P2714) was added to the VLP extraction buffer immediately before plant leaf extraction).

2.4. Density gradient ultracentrifugation purification of BT VLPs

Particles were purified by either using ultra-high quality sucrose (30–70%) or Iodixanol (20–60%; OptiPrep™, Sigma-Aldrich) density gradient ultracentrifugation independently dissolved in VLP dilution buffer (Bicine, pH 8.4, 20 mM NaCl). The gradients were centrifuged at 32,000*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 analysed on 4–12% Bis-Tris Bolt™ (Life Technologies) sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) protein gels.

2.5. Bench scale production and purification of double chimaeric BTV-4 VLPs

Fifty plants were hand co-infiltrated with *Agrobacterium* harbouring the plasmids of interest to an overall final OD_{600} = 0.8. Hand infiltration of *Agrobacterium* (LBA4404) harbouring BTV serotypes 8 and 4 genes encoding the four capsid proteins (BTV-8 VP3 and VP7; BTV-4 VP2 and VP5; 1:1:1:1 ratio) was conducted. Leaf material was harvested eight days after infiltration in VLP extraction buffer (1:3 ratio). Remaining plant debris was removed by filtering the cell lysate through two layers of cheese cloth before centrifugation (4200*g* for 30 min at 4 °C) in a JA14 rotor using a

Beckman Coulter Avanti J-26 XPI. The plant extract supernatant (23 ml) was layered on top of sucrose density gradients (30%-70%; 3 ml each) and centrifuged at 85,800*g*, at 10 °C for 3 h in a SW-32Ti rotor (Beckman Coulter) in 38.6 ml volume ultra-clear Beckman tubes. The first 6 ml was discarded (60–70% sucrose fractions) and the following 6 ml (45–55%) containing the VLPs, was collected. The VLPs were dialyzed against 2L of VLP dilution buffer at 4 °C and the buffer exchanged twice during 16 h to remove residual sucrose. D-(+)-Trehalose dihydrate (5% m/v) was added before the VLP extract was subsequently filter sterilised through a 0.45 μm + 0.2 μm Sartobran 300 sterile capsule (Sartorius Stedim biotech GmbH) using a peristaltic pump. The BTV-4 VLPs were formulated (1:1) with Alhydrogel® before vaccination on the day of delivery.

2.6. Bench scale production and purification of double chimaeric BTV-3 VLPs

Hand co-infiltration of 25 plants with *Agrobacterium* (LBA4404) harbouring the genes encoding the four capsid proteins (BTV-8 VP3 and VP7, BTV-3 VP2 and VP5) in a 1:1:1:1 ratio with an overall OD_{600} = 2 was conducted. The leaf material was harvested seven days after infiltration in VLP extraction buffer (1:3 ratio) and incubated for 30 min at 4 °C. Remaining plant debris was removed by filtering the cell lysate through two layers of cloth before two successive centrifugations steps (4200*g* for 15 and 40 min at 10 °C). The plant extract was then filtered through a Sartoclean GF sterile midicap (3 μm + 8 μm; Sartorius Stedim Biotech) depth filter using a Masterflex console drive peristaltic pump. Subsequently, the plant extract was filtered through a 300 K Minimate™ Tangential Flow Filtration (TFF) Capsule (Pall Life Sciences) with the pressure not exceeding 2 Bar. The DTT and protease inhibitor were removed from the VLP containing extract through two subsequent wash steps (1 in 10 dilution each) with sterile VLP dilution buffer. D-(+)-Trehalose dihydrate (Sigma Life Science)(5% m/v) was added before filter sterilising through a 0.45 μm + 0.2 μm Sartobran 300 sterile capsule (Sartorius Stedim Biotech GmbH) using a peristaltic pump. Iodixanol density gradient ultracentrifugation was conducted with a 6 ml aliquot of the TFF purified BTV-3 VLP product to determine the concentration of VLPs per ml. The BTV-3 VLPs were formulated as indicated (Table 1).

BTV-4 and BTV-3 VLP products were subjected to SDS-PAGE and candidate viral proteins were sequenced using LC-MS/MS-based peptide sequencing. Protein bands of interest were in-gel trypsin digested as per the protocol described in [21]. 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. Purified BTV VLP proteins were quantified using a sensitive colorimetric protein assay, the Micro BCA™ Protein Assay Kit (Thermo Fisher Scientific) using Bovine Gamma Globulin (Bio-Rad) protein standards.

2.7. Transmission electron microscopy (TEM)

Both the sucrose and Iodixanol gradient fractions were adsorbed onto holey carbon-coated copper grids and stained as follows: sodium phosphotungstate, pH 7.0 (sucrose samples) or 2% uranyl acetate (Iodixanol samples). The air-dried grid was imaged using either a JEM-2100 Transmission electron microscope (JEOL) or a CM10 Transmission electron microscope (Philips) at the University of Pretoria (UP).

2.8. Live attenuated vaccine production and formulation

The monovalent BTV-3 and BTV-4 LAV supplied by OBP were produced following standard operating procedures for manufacturing commercial BT vaccine. The final freeze-dried pellet was released following quality control methods of the commercial polyvalent vaccine at 4 × 10E4 plaque forming units per ml (PFU) per dose. Prior to injecting sheep, the freeze-dried material was reconstituted with sterile diluent to obtain the required dose for both serotypes.

2.9. Facility and animal care

Merino sheep between 6 and 8 months and BTV-naïve were housed in stable 155 at OBP for the duration of the trial. Merino sheep handling and care were according to standard operating procedures outlined by the Experimental Animal Unit. Briefly animals were fed Lucerne, pellets and Eragrostis and also received water ad lib. The stables were cleaned daily and shavings covering the floor were replaced once a week.

2.10. Animal Pre-screening

Animals were sourced from BT free areas and exposure to BT disease was determined by screening for antibodies before commencement of the trial using a BTV-specific competitive ELISA (c-ELISA) assay as prescribed for testing in international trade [28]. Testing was done at the OIE reference virology laboratory at the Agricultural Research Centre Onderstepoort Veterinary Research (ARC-OVR). In addition to the ELISA, the serum neutralization test (SNT) was also performed as pre-screening method to select BTV free animals.

2.11. Safety and immunogenicity in sheep

Merinos of ages 6–12 months with no previous exposure to BTV were divided into six groups and inoculated subcutaneously (using

Table 1
Clinical experimental design for evaluating immunogenicity of plant-produced double chimaeric BTV-4 and BTV-3 VLPs in sheep. Animals were injected subcutaneously with 1 ml of respective drug sample. The BTV-3 and BTV-4 monovalent LAV were used as controls.

Groups	Animal ID	Drug sample	Dose administered	
			Primary vaccination	Booster
1	521 566 656	Chimaeric BTV-4 VLPs adjuvanted with 50% (v/v) Alhydrogel® (Batch 176)	30 μg VLPs	15 μg VLPs
2	551	LAV monovalent BTV-4 vaccine	5 × 10E4 PFU/dose	N/A
3	634	Bicine buffer with 50% (v/v) Alhydrogel® (Batch 176)	N/A	N/A
4	1646 1636 1655	Chimaeric BTV-3 adjuvanted with 50% (v/v) with Montanide ISA 201 VG (Seppic)	20 μg VLPs	30 μg VLPs
5	1608	LAV monovalent BTV-3 vaccine	5 × 10E4 PFU/dose	N/A
6	1649	Bicine buffer with 50% (v/v) Montanide ISA 201 VG (Seppic)	N/A	N/A

26 gauge needles) on the inner thigh (left) with 1 ml of formulations (Table 1). A booster was administered to animals in group 1 and 4 on day 21. The monovalent BTV-4 and BTV-3 live vaccine controls contained no adjuvant and no boost was administered for animals in these groups. Animals were monitored for reactions at the injection site, and rectal temperatures were recorded twice daily for 14 days following each inoculation and thereafter once daily. Animals were bled on days 0, 7, 14, 21, 28, 35, 42, 49 and 56 for SNT analysis.

2.12. Serological analysis

The SNT assay was used to determine levels of BTV-4 or BTV-3 antibodies induced by respective chimaeric VLPs and live monovalent BTV-4/BTV-3 controls. The SNT method was performed as described in the OIE Terrestrial Manual Standards for Diagnostic Tests and Vaccines (OIE, 2014). Approximately 5–8 ml blood was collected from each of the experimentally inoculated sheep prior to vaccination (day 0) and every 7th day for 56 days. The collected blood was briefly processed as follows; tubes were centrifuged at 4500*g* for 20 min (Beckman Coulter, J6-MI centrifuge with J6-4.2 rotor) and inactivated at 56 °C for 30 min. Neutralizing antibody titers from animal groups 1–3 were determined using BTV-4 specific virus neutralizing antigen. The BT type 3 virus antigen was used for animals in groups 4–6. The cross neutralization activity of BTV-3 chimaeric VLPs against types 6 and 13 and BTV-4 against serotypes 9 and 11 were also assessed. Antibody titers are expressed as the reciprocal of the serum dilution that inhibited 50% of viral cytopathic effect.

2.13. Plaque assays

The amount of live infective BTV-3 and BTV-4 virus particles was determined using a modified plaque method [12] in Vero cells (ECACC). The virus was serially diluted from $1 \times 10E1$ until $1 \times 10E8$. Each of the dilutions was used to infect a confluent monolayer of cells. The cell and virus mix was then incubated at 37 °C for 30 min. A 1% solution of agarose was prepared by melting agarose in Earle's buffer and subsequent addition of cell culture media at a ratio of 1:1. After incubation of the virus and cells, 4 ml of 1% agarose was added to each well and incubated for 5 days at 37 °C with 5% CO_2. Following incubation, the agarose was stained with 2 ml of 1% neutral red solution and incubated for a further 6–8 h. The plaques were visualised under white light and results expressed as (PFU ml^{-1}).

3. Results

3.1. Assembly of homogenous and chimaeric BT core-like particles and virus-like particles

Transient expression efficiency of the pEAQ-HT plant expression vector harbouring the genes of interest was investigated by agro-infiltration of the RNAi mutant dXT/FT *N. benthamiana* which facilitates mammalian-like glycosylation [22]. *Agrobacterium* strain LBA4404 harbouring the plant expression vector pEAQ-HT with the individual genes of interest was co-infiltrated into plant leaf tissue. BTV-8 core-like particles (CLPs) were assembled by co-expression of the genes encoding BTV8 VP3 and VP7 and used as CLP scaffold for all chimaeric VLPs. Assembly of homogenous BTV-8 VLPs (VP3, VP7, VP2 and VP5) was used as positive control (Fig. 1). It was established that both homogenous BTV-8, chimaeric BTV-3 and BTV-4 virus-like particles (VLPs) assembled with the selected genes cloned and transiently expressed in *N. benthamiana* plants (Fig. 1a and b; supplementary Fig. S1). The presence of all four capsid proteins was detected by SDS-PAGE and protein confirmation using LC-MS/MS-based peptide sequencing (Supplementary Fig. S2). VLPs were visualized with negatively stained transmission electron microscopy (Figs. 1b and S1b) and measured 75–80 nm.

3.2. Assembly of chimaeric BT VLPs

The aim of the following experiment was to compare the stable assembly of double and single chimaeric VLPs of serotypes BTV-4 and BTV-3 using BTV-8 CLPs as a scaffold or inner shell, and to determine the most appropriate combinations to result in stable chimaeric VLPs.

An ELISA diagnostic kit to detect VP2 of only serotype 8 is commercially available (ID Vet, France). The ID Screen® Bluetongue Competition is a competitive ELISA kit for the detection of anti-VP7 antibodies in serum or plasma from multiple species with proven specificity and sensitivity and widespread use in recent outbreaks. Detection of antibodies against all BTV serotypes is due to the highly conserved VP7 protein. Thus, due to the lack of an ELISA kit to detect VP2 of serotypes 3 and 4, a combination of TEM images and protein confirmation using LC-MS/MS-based peptide sequencing was harnessed to give an indication of the fully assembled VLPs and the abundance of VP2 within the assembled VLP. VP2 proteins (150 unique VP2 peptides detected) layered on the inner shell comprised of VP3, VP7 and VP5 were detected when homogenous BTV-8 VLPs assembled (Table 2). The number of unique VP2 peptides in the assembled single or double chimaeric BTV-4 and BTV-3 VLPs (Table 2) were used as a trend to determine the most appropriate combinations for the assembly of chimaeric VLPs for vaccine formulations. In a subsequent experiment (Section 3.4) comparing the assembly of homogenous BTV-8 and chimaeric BTV-3, 2–3 technical replicates were subjected to LC-MS/MS-based peptide sequencing and showed the reproducibility (Supplementary Figs. S3 and S4). The authors do not claim that this method is the ultimate, but rather indicative. Although a weak interaction was suggested between the two outer capsids, VP2 and VP5 [15] preliminary results assembling double chimaeric (VP2, VP5 of the same serotype and VP3, VP7 of a second serotype) rather than a single chimaeric (VP2 of one serotype and the core VP3, VP7 and VP5 of a second serotype) suggested improved assembly of chimaeric BT VLPs in plants. The assembly of double chimaerics, where both VP2 and VP5 of the same serotype are present might enhance the immunity as previously shown [14].

3.3. Safety and immunogenicity sheep trials

Double chimaeric BTV-4 VLPs were purified from the crude plant lysate by traditional sucrose density gradient ultracentrifugation. Sheep were prime-boost vaccinated with 30 and 15 μg adjuvanted purified BTV-4 VLPs, respectively (Table 1). The primary vaccine was administered on day 0 and the booster vaccine on day 21. Animals were bled on days 0, 7, 14, 21, 28, 35, 42, 49 and 56. Sheep 521, 566 and 656 were vaccinated with purified BTV-4 VLPs, sheep 551 with BTV-4 LAV (positive control) and sheep 634 with a formulation of Bicine buffer and Alhydrogel® (1:1) to serve as a negative control and for safety validation.

The SNTs were conducted to determine antibody titers and ≥1:4 is indicative of seroconversion. Primary vaccination of sheep 521 with BTV-4 VLPs (titer of 128) and to a lesser extent sheep 566 (titer of 8) and LAV (sheep 551, titer of 16) resulted in seroconversion within 21 days after vaccination (Table 3). After the booster vaccinations, sheep vaccinated with either VLPs or LAV resulted in titers of 32–128 on day 28 followed by titers of 128–256 from day 35 onwards. Sheep 656 vaccinated with the BTV-4 VLPs had relatively low titers (32–64) yet ≥4 indicating seroconversion.

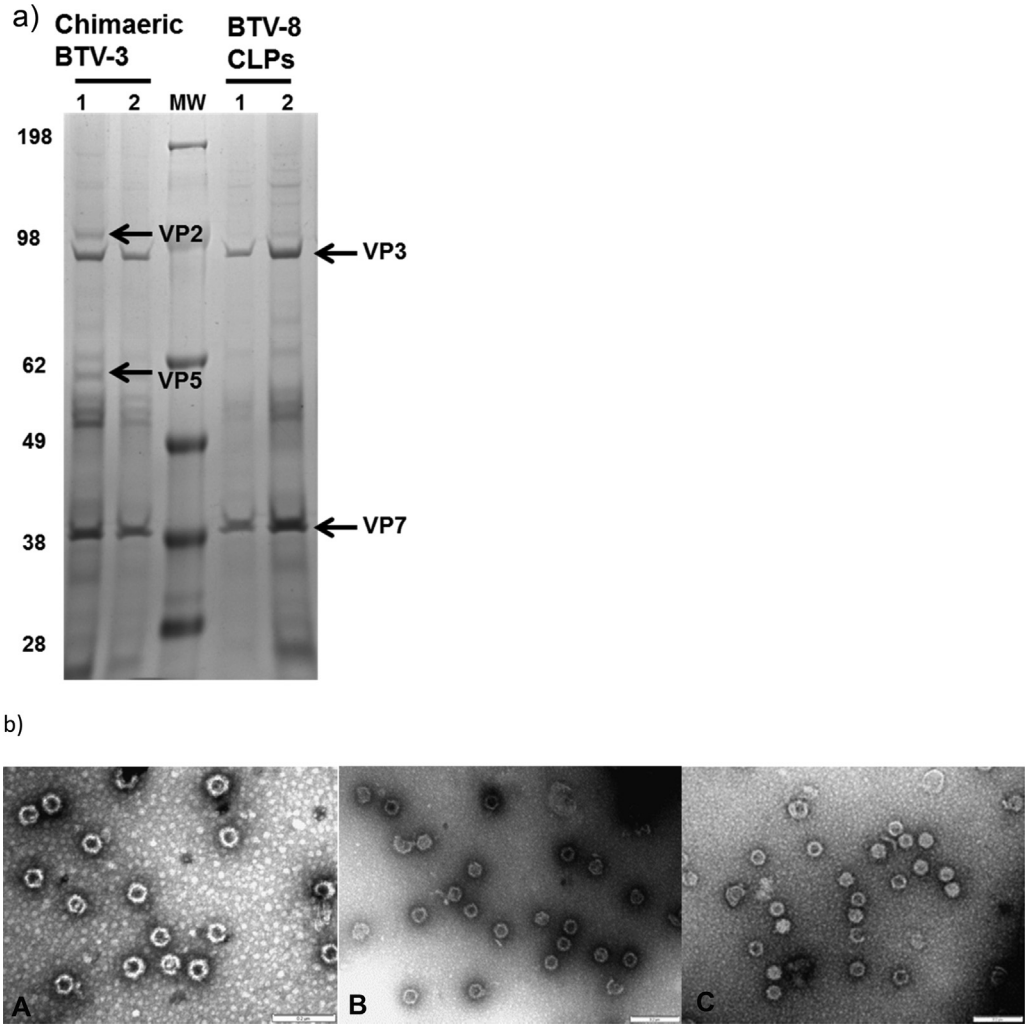

Fig. 1. SDS-PAGE and TEM images of plant-produced BTV-8 and chimaeric BTV-3 VLPs. Six weeks old *N. benthmiana* (dXT/FT) were co-infiltrated with all four BTV-8 or chimaeric BTV-3 *Agrobacterium* recombinants, leaves were harvested 7 days post infiltration and the crude plant extracts were subjected to iodixanol density gradient ultracentrifugation. (a) Gradient fractions 30–40% were collected and separated by 4–12% Bolt™ Bis-Tris Plus precast gels followed by Coomassie blue staining. MW, SeeBlue® Plus2 Pre-stained Protein Standard. Chimaeric BTV-3 VLP capsid proteins, lanes 1 and 2 and BTV-8 core-like proteins, lanes 1–2. Viral capsid proteins VP2 (111 kDa), VP3 (102 kDa), VP5 (59 kDa) and VP7 (38 kDa) are indicated with arrows. (b) Gradient fraction 35–40% was imaged by TEM revealing the presence of fully assembled VLPs with minor assembly intermediates: plant produced BTV-8 VLPs (A), BTV-3 double chimaeric VLPs (B) and BTV-3 single chimaeric (C) VLPs. Scale bar, 200 nm.

Table 2
LC-MS/MS-based peptide sequence analysis of VP2 in homogenous BTV-8, as well as chimaeric BTV-4 and BTV-3 VLPs showing the number of unique peptides identified with >95% confidence. BTV-3 single chimaeric VLPs (sc) were assembled by substituting VP2 of BTV-8 VLPs with VP2 of serotype 3. BTV-3 double chimaeric VLPs (dc) were assembled by substituting both outer capsid proteins BTV-8 VP2 and VP5 with BTV-3 VP2 and VP5 whilst retaining the BTV-8 core (VP3 and VP7). Similarly, BTV-4 dc VLPs was assembled by substituting both outer capsid proteins BTV-8 VP2 and VP5 with both VP2 and VP5 of serotype 4 whilst retaining the BTV-8 core (VP3 and VP7). BTV-4 sc VLPs were assembled by substituting either (a) BTV-8 VP2 with VP2 of serotype 4 or (b) using only BTV-8 VP3 assembled with BTV-4 VP7, VP5 and VP2. All capsid proteins were assembled in a 1:1:1:1 ratio.

BTV VLPs	# of unique VP2 peptides
BTV-8 homogenous	150
BTV-3 sc	22
BTV-3 dc	81
BTV-4 sc[a]	18
BTV-4 dc	48
BTV-4 sc[b]	42

Sheep vaccinated with the buffer-adjuvant (sheep 634) were zero throughout.

A large scale VLP purification system, consisting of depth filtration followed by tangential flow ultrafiltration (TFF) processes, was established for biomass BTV-3 VLP (double chimaeric) production for the purpose of subsequent target animal (sheep) immunogenicity studies (Table 4). Hand infiltration of *Agrobacterium* harbouring the genes encoding the four capsid proteins (BTV-8 VP3 and VP7; BTV-3 VP2 and VP5) was conducted as described. In order to quantify the VLPs within a given volume of TFF partially purified VLPs, the TFF lysate was subjected to Iodixanol density ultracentrifugation and the purified VLP capsid proteins subsequently quantified. Conservatively estimated, 26 mg BTV-3 VLPs were produced per Kg of plant leaf tissue. The sheep were prime-boost vaccinated with 20 and 30 μg adjuvanted VLPs, respectively (Table 1). Once more sheep were pre-bled to confirm the absence of pre-exposure to BTV. The primary vaccine was administered on day 0 and the booster vaccine on day 21.

Table 3
Serum neutralizing test (SNT) titers of sheep vaccinated with plant-produced chimaeric BTV-4 VLPs, Bicine buffer as negative control and OBP BTV-4 monovalent live attenuated vaccine strain (LAV) as positive control. Vaccines and buffer were formulated with Alhydrogel (1:1).

		Day								
		Vaccination				Booster				
Group	Animal ID	0	7	14	21	28	35	42	49	56
Sucrose*	521	0	0	4	128	128	256	256	256	256
	566	0	0	0	8	32	256	256	256	256
	656	0	0	0	0	2	32	32	32	64
Bicine	634	0	0	0	0	0	0	0	0	0
OBP LAV	551	0	0	0	16	128	128	256	256	256

* Sucrose = sucrose density gradient ultracentrifugation purified chimaeric BTV-4 VLPs.

Table 4
Serum neutralizing test (SNT) titers of sheep vaccinated with plant-produced chimaeric BTV-3 VLPs, Bicine buffer and naïve untouched animal as negative control and OBP BTV-3 monovalent live attenuated vaccine strain (LAV) as positive control. Vaccines and buffer were formulated with Montanide ISA 201 VG (1:1).

		Day								
		Vaccination				Booster				
Group	Animal ID	0	7	14	21	28	35	42	49	56
TFF*	1646	0	0	0	2	256	256	256	256	256
	1639	0	0	0	2	256	256	256	256	256
	1655	0	0	0	4	256	256	256	256	256
Bicine	1649	0	0	0	0	0	0	0	0	0
Naïve untouched	1629	0	0	0	0	0	0	0	0	0
OBP LAV	1608	0	128	256	256	256	256	256	256	256
	1614	0	0	128	Δ	256	256	256	256	256

* TFF = Tangential flow filtration purified chimaeric BTV-3 VLPs.

This time, seroconversion was shown as early as 7 days after the primary vaccination of BTV-3 LAV for one of the sheep (1608) followed by titers of 256 (14 and 21 days after vaccination) (Table 4). Only one of the three sheep vaccinated with BTV-3 VLPs seroconverted 21 days after vaccination. After booster vaccination, all three sheep vaccinated with BTV-3 VLPs and BTV-3 LAV vaccinated sheep seroconverted with consistent titers of 256 up to day 56. Sheep vaccinated with the buffer-adjuvant (1649) or non-vaccinated naïve sheep (1629) were zero throughout.

Serum collected prior to inoculation was free of BTV antibodies when tested using ELISA and SNT methods, animals naïve to BTV were used for immunogenicity testing. The SNT assay was used to determine levels of BTV-4 or BTV-3 antibody levels induced by respective chimaeric VLPs and live monovalent BTV-4 or BTV-3 controls. Primary vaccination with BTV-4 or BTV-3 chimaeric VLPs induced low levels of specific neutralizing antibodies from day 14 and 21, respectively (Tables 3 and 4). However, antibody titers increased on day 28 following booster on day 21. The titers remained high in all vaccinated animals untill day 56. Vaccination with live monovalent vaccines induced high titers from as early as day 7 with BTV-3 (Table 4). The antibodies were consistently high till the end of the trial on day 56 (Tables 3 and 4). Neutralizing antibody levels after booster were significantly comparable to the LAV. Animals injected with adjuvanted Bicine buffer induced no antibodies as anticipated.

Animal temperatures were maintained following vaccination with VLP formulations (Fig. 2). All animals in the vaccination groups had a maximum temperature variation of less than 1 °C. The chimaeric VLP vaccine formulations did not induce any temperatures reactions in the first 14 days proving the vaccine to be safe for use in sheep. A temperature increase was observed with Bicine control animal following injection. However since the increased temperature did not exceed 40 °C and animal had low temperature prior, this adjustment was deemed normal. Furthermore, the specific animal was healthy during the 14 day observation period after injection. No mortalities or reduced mobility were observed following injection with chimaeric BTV-3 and BTV-4 VLP vaccine formulations. The vaccine doses administered

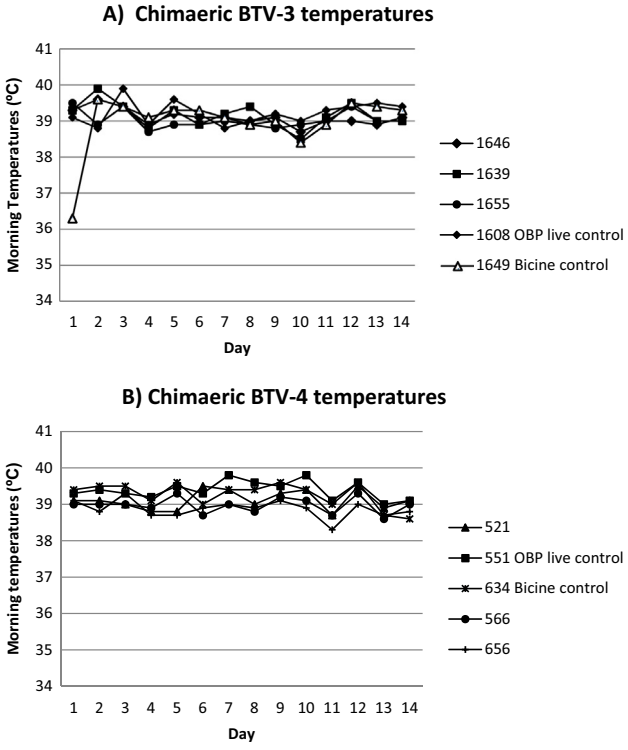

Fig. 2. Vaccine temperature reaction in sheep. Morning rectal temperatures of the first 14 days following vaccination with chimeric BTV-3 and BTV-4 formulated vaccines. (A) Represents temperature data obtained following vaccination chimaeric BTV-3 and (B) indicate chimaeric BTV-4 VLP formulation.

were safe for use in target species and no lesions were observed at the injection site with merino sheep.

Finally, experiments to determine the ability of BTV-4 VLPs to elicit a cross neutralizing immune response against serotypes 9 and 11 and, similarly, the ability of BTV-3 VLPs to elicit a cross neutralizing immune response against serotypes 6 and 13 were inconclusive. These assays were conducted more than three years after completion of the sheep trials and the serum storage conditions may not have been optimal. The data was therefore not included.

3.4. Various Agrobacterium strains as delivery agents

Assembly of homogenous BTV-8, as well as double chimaeric BTV-3, in *Agrobacterium* strains LBA4404, AGL-1 or GV3101::pMP90 were compared as a vehicle to deliver the expression vector pEAQ-HT, harbouring the selected genes, to the plant cells. The hand infiltration inoculum of all strains had an OD_{600} = 2 and eight microgram of protein containing the VLPs were separated per SDS-PAGE and the VP2 protein bands subjected to LC-MS/MS-based peptide sequencing. VP2 of the homogenous BTV-8 VLPs remained almost constant harnessing all three independent *Agrobacterium* stains to assemble the VLPs, whereas VP2 within the double chimaeric BTV-3 VLPs diminished significantly using AGL-1 and to an almost non-detectable level using GV3101::pMP90 as reflected in SDS PAGE and LC-MS/MS results (Supplementary data, Figs. S3 and S4). Thus, strains AGL-1 or GV3101 did not result in an elevated assembly of chimaeric VLPs when compared to LBA4404 (Supplementary data, Figs. S3 and S4).

4. Discussion

Virus-like particle (VLP) vaccines are non-replicating multiprotein shells similar in size and shape to the intact virus. 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 its native conformation, resulting in efficacious candidate vaccines to stimulate not only humoral but also cellular immune responses [3]. In addition, plant expression systems offer distinct posttranslational modifications, cost-effectiveness, production speed and scalability [2]. The authors envisage that the work done in this study will underpin a future multivalent vaccine comprised of plant-produced homogenous BT VLPs complemented by chimaeric BT VLPs of serotypes recalcitrant to the formation of homogenous VLPs.

This study reports on the successful assembly of all four capsid proteins from two different serotypes to assemble chimaeric BT VLPs. Although TEM images and size measured for chimaeric VLPs suggest that the VLPs might not contain the full complement of VP2 triskelions (180 trimers) in all VLPs assembled, the strong serotype-specific neutralization antibodies reflected in the consistent high titers of 256 immediately after booster vaccination, is indicative that sufficient numbers of VP2 trimers are present in the outer shell. It was previously shown that BTV-8 CLPs, consisting of assembled VP3 and VP7 alone does not elicit neutralizing antibodies and fail to produce protective immunity [23]. Thuenemann and co-workers adjuvanted with 50% Montanide ISA 70 VG whereas in this study the chimaeric BTV-3 VLPs were adjuvanted with 50% Montanide ISA 201 VG (water-in-oil-in-water (W/O/W) inducing short and long-term immunity). Here we report on next-generation chimaeric BTV-4 and BTV-3 VLP subunit candidate vaccines that elicit titers equal to the well-established first generation live-attenuated vaccines and plant-produced homogenous BTV-8 VLPs [23]. Although efficacy of the adjuvanted chimaeric BTV-4 and BTV-3 VLPs still need to be established, the authors anticipate that the vaccine administered as a prime-boost vaccine, will protect against these Bluetongue virus serotypes. In addition, the prospect that the plant-produced BTV-4 VLP vaccine can potentially cross-protect against BTV-9 and 11, as previously shown in challenge studies in sheep [27], will be advantageous. Furthermore, cross-protection of BTV-8 and BTV-4, and between BTV-1 and BTV-8 were reported in challenged calves [13].

An estimated 1.5% of TFF purified proteins were conservatively determined to be BTV-3 assembled VLPs. In this study, sheep were vaccinated with an estimated 20 and 30 μg BTV-3 VLPs prime-boost vaccination, respectively. Conservatively estimated, 570 sheep can be vaccinated with partially purified TFF chimaeric BT VLPs produced per kilogram of plant leaf tissue. Thus plant-produced chimaeric BT VLPs can be purified by scalable commercially viable downstream processes, resulting in seroconversion in sheep.

In South Africa, multiple serotypes are endemic and a vaccine must protect against multiple serotypes. Future developments of the multivalent vaccine would include a challenge study to establish the efficacy of plant-produced VLPs and also minimum-dose of monovalent and multivalent VLP vaccines formulations. In addition, subsequent studies with multiple BT VLP serotypes combined with non-structural protein 1 (NS1) to elicit a humoral and enhanced cell-mediated immune response, is in progress. NS1 and NS2 have been reported to induce cross-serotype helper T-cell and cytotoxic T cell responses [17] and T-lymphocyte responses cross-reactive between BTV-2 and BTV-8 [1]. Thus cross-protective immunity against other serotypes will also pave the way for fewer serotypes per vial without compromising the immunity against the disease.

5. Conclusion

In this study, we report on the assembly of plant-produced chimaeric BTV-4 and BTV-3 VLP vaccines eliciting seroconversion in sheep. Both adjuvanted sucrose density gradient ultracentrifugation as well as scalable depth filtration/TFF ultrafiltration purification of BT VLPs resulted in seroconversion in sheep. The antibody titer levels were comparable to that of monovalent LAV controls and lasted for the duration of the 56 days of the trial. As both homognous BTV-8 VLPs and chimaeric BTV-3 and BTV-4 VLPs were successfully produced in plants, the development and validation of a multivalent vaccine would be a logical next.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

The authors Drs Nobalanda Mokoena, Daria Rutkowska and Martha O'Kennedy filed a patent application protecting the production of chimaeric orbivirus virus-like particles in plants (PCT/IB2017/052236). Full PCT filed on 19 April 2017 and entitled: "Plant-produced chimaeric Orbivirus VLPs" (PCT/IB2017/052236), WO 2017/182958 A1).

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(P0079617) issued by the South African Department of Agriculture, Forestry and Fisheries (DAFF) and from the American type culture collection (ATCC® BAA-101TM, *Rhizobium radiobacter*). The authors would like to thank Antoinette Buys for the Transmission electron microscopy work conducted at the University of Pretoria, South Africa. We are greatly indebted to Albert Mabetha, Gugu Mkhize and Sharon Kgasago for technical assistance. The authors would like to acknowledge members of Quality Control and Experimental Animal Department at OBP for conducting quality control tests and handling animals during clinical trials, respectively. The ELISA tests were subcontracted to Agricultural Research Council Onderstepoort Veterinary Research (ARC-OVR), South Africa.

Author's contribution

MOK and NM designed the study and prepared the manuscript. NM selected and both NM and BM cloned the genes into the plant expression vector. MOK produced the Bluetongue VLPs under optimised parameters and determined the chimaeric BT VLP combinations. TT guided the filtration and ultrafiltration processes. MOK, DR and TT prepared the primary and booster vaccines. Both MOK and NM participated in the vaccine formulation and designed the sheep trial. VD conducted serum neutralization assays and was responsible for the acquisition of animal clinical data. SM conducted the LC-MS/MS-based peptide sequencing and analysis.

Ethics approval and consent to participate

The protocol for the sheep trial was submitted and approved by both Onderstepoort Biological Products Animal Ethics Committee (South African Veterinary Council Facility Registration Number: FR1514054) and CSIR Research Ethics Committee (REC) registration number: 130/2015. The trial was further approved under Section 20 of Animal Diseases Act (Act 35 of 1984) of the Department of Agriculture, Forestry and Fisheries. The CSIR Biosciences has registered laboratory, growth rooms and pilot plant facilities (Registration number 39.2/CSIR – 17/099 from 13 November 2017 until 13 November 2020) from DAFF to clone, *Agrobacterium* infiltrate and produce the vaccine antigens in plants.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vaccine.2019.08.042.

References

[1] Anderson J, Hägglund S, Bréard E, Riou M, Zokari S, Comtet L, et al. Strong protective induced by an experimental DIVA subunit vaccine against bluetongue virus serotype 8 in cattle. Vaccine 2014;32:6614–21. https://doi.org/10.1016/j.vaine.2014.09.066.
[2] Chen Q, Lai H. Plant-derived virus-like particles as vaccines. Human Vaccines Immunotherap 2013;9(1):26–49.
[3] Crisci E, Bárcena J, Montoya M. Virus-like particles: The new frontier of vaccinesor animal viral infections. Vet Immunol Immunopathol 2012;148:211–25.
[4] 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;49:105. https://doi.org/10.1186/s13567-018-0600-4.
[5] Calvo-Pinilla E, Castillo-Olivares J, Jabbar T, Ortego J, de la Poza F, Marín-López A. Recombinant vaccines against bluetongue virus. Virus Res 2014;182:78–86.
[6] Dungu B, Gerdes T, Smit T. The use of vaccination in the control of bluetongue in southern Africa. Veterinaria Italiana 2004;40(4):616–22.
[7] Dungu BK, Louw I, Potgieter C, von Teichman BF. Attenuated live Bluetongue virus vaccine protects sheep from challenge with the European BTV-8. Open Vet Sci J 2008;2:130–3.
[8] Dungu B, von Teichmann B, Louw I. Inactivated live-attenuated Bluetongue virus vaccine WO 2009/128043 A1; 2009.
[9] Fay P, Attouri H, Mohd-Jafaar F, Batten C, Nomikou K, Daly J, et al. Evidence of a protective immune response using transiently expressed Bluetongue protein, VP2, from a Plant-based system and cross-reactive studies. In: 12th International dsRNA Virus Symposium, Goa, India, 6-10 October 2015.
[10] Feenstra F, Pap JS, van Rijn PA. Application of Bluetongue disabled infectious single animal (DISA) vaccine for different serotypes by VP2 exchange or incorporation of chimaeric VP2. Vaccine 2015;33:812–8.
[11] Feenstra F, van Rijn PA. Current and next-generation bluetongue vaccines: Requirements, strategies, and prospects for different field situations. Crit Rev Microbiol 2017;43:142–55.
[12] Howell PG, Verwoerd DW, Oellermann RA. Plaque formation by bluetongue virus. Onderstepoort Vet Res 1967;34:317–32.
[13] Martinelle L, Dal Pozzo F, Thys C, De Leeuw I, CampeW Van, De Clercq K, et al. Assessment of cross-protection induced by a bluetongue virus (BTV) serotype 8 vaccine towards other BTV serotypes in experimental conditions. BMC Vet Res 2018;49:63. https://doi.org/10.1186/s13567-018-0556-4.
[14] Mohd-Jaafar F, Belhouchet M, Vitour D, et al. Immunization with bacterial expressed VP2 and VP5 of bluetongue virus (BTV) protect alpha/beta interferonreceptor knock-out (IFNAR(-/-) mice from homologous lethal challenge. Vaccine 2014;32:4059–67.
[15] Nason EL, Rothagel R, Mukherjee SK, Kar AK, Forzan M, Venkataram Prasad BV, et al. Interactions between the Inner and Outer capsids of Bluetongue virus. J Virol 2004;78(15):8059–67. https://doi.org/10.1128/JVI.78.15.8059-8067.2004.
[16] Pearson LD, Roy P. Genetically engineered multi-component virus-like particles as veterinary vaccines. Immunol Cell Biol 1993;71:381–9.
[17] Rojas JM, Peña L, Martín V, Sevilla N. Ovine and murine T cell epitopes from the non-structural protein 1 (NS1) of bluetongue virus serotype 8 (BTV-8) are shared among viral serotypes. BioMed Central, Vet Res 2014;45:30.
[18] Sainsbury F, Saxena P, Geisler K, Osbourn A, Lomonossoff GP. Using a virus-derived system to manipulate plant natural product biosynthesis pathways. Methods Enzymol 2012;517(2012):185–202.
[19] Savini G, MacLachlan NJ, Sanchez-Vizcaino J-M, Zientara S. Vaccines against bluetongue in Europe. Comp Immunol Microbiol Infect Dis 2008;31:101–20.
[20] Shaw AE, Ratinier M, Nunes SF, Nomikou K, Caporale M, Golder M, et al. Reassortment between two serologically unrelated Bluetongue virus strains is flexible and can involve any genome segment. J Virol 2013;87(1):543–57.
[21] Shevchenko A, Tomas H, Havliš J, Olsen VJ, Mann M. In-gel digestion for mass spectrometric characterization of proteins and proteomes. Nat Protoc 2007;1:2856–60.
[22] Strasser R, Stadlmann J, Schähs M, Stiegler G, Quendler H, Mach L, et al. Generation of glyco-engineered *Nicotiana benthamiana* for the production of monoclonal antibodies with a homogenous human-like N-glycan structure. Plant Biotechnol J 2008;6:392–402.
[23] Thuenemann EC, Meyers AE, Verwey J, Rybicki EP, Lomonossoff GP. A method for rapid production of heteromultimeric protein complexes in plants: assembly of protective bluetongue virus-like particles. Plant Biotechnol J 2013:1–8.
[24] Van den Bergh C, Coetzee P, Guthrie AJ, le Grange M, Venter EH. Complete genome sequence of five Bluetongue virus (BTV) vaccine strains from a commercially live attenuated vaccine, a BTV-4 field strain from South Africa, and a reassortant strain isolated from experimentally vaccinated cattle. Am Soc Microbiol 2016;4(3):1–2.
[25] Van Zyl AR, Meyers AE, Rybicki EP. Transient Bluetongue virus serotype 8 capsid protein expression in *Nicotiana benthamiana*. Biotechnol Reports 2016;9:15–24.
[26] Zhugunissov K, Yershebulov Z, Barakbayev K, Bulatov Y, Taranov D, Amanova Z, et al. Duration of protective immunity after a single vaccination with a live attenuated bivalent bluetongue vaccine. Vet Res Commun 2015;39:203–10.
[27] Zulu GB, Venter EH. Evaluation of cross-protection of bluetongue virus serotype 4 with other serotypes in sheep. J S Afr Vet Assoc 2014;85(1):1041. https://doi.org/10.4102/jsava.v85i1.1041.
[28] World Organisation for Animal Health (OIE), 2014 https://www.oie.int/standard-setting/terrestrial-manual/access-online/.