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Development of a competitive ELISA for NS3 antibodies as DIVA test accompanying the novel Disabled Infectious Single Animal (DISA) vaccine for Bluetongue

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ABSTRACT

Recently, we have developed a novel vaccine for Bluetongue named BT Disabled Infectious Single Animal (DISA) vaccine. Due to the lack of non-essential NS3/NS3a protein, BT DISA vaccine is a replicating vaccine, but without the inherent risks of live-attenuated vaccines, such as residual virulence or reversion to virulence by mutations, reassortment with field virus, horizontal spread by vectors and vertical transmission. The immune response induced by BT DISA vaccines is rapidly induced, highly protective and serotype specific which is dependent on the immunodominant and serotype determining VP2 protein. The BT DISA vaccine platform provides the replacement of exclusively VP2 from different serotypes in order to safely formulate multivalent cocktail vaccines. The lack of NS3/NS3a directed antibodies by BT DISA vaccination enables differentiation of infected from vaccinated animals (DIVA principle). A highly conserved immunogenic site corresponding to the late domain was mapped in the N-terminal region of NS3. We here established an NS3-specific competitive ELISA (NS3 cELISA) as serological DIVA test accompanying BT DISA vaccines. To this end, NS3 protein missing putative transmembrane regions was produced in large amounts in bacteria and used as antigen in the NS3 cELISA which was investigated with a variety of sera. The NS3 cELISA displayed a high sensitivity and specificity similar to the commercially available VP7-specific cELISA. Results of previously performed vaccination-challenge trials with BT DISA vaccines clearly demonstrate the DIVA system based on the NS3 cELISA and BT vaccine free of NS3 protein.

1. Introduction

Bluetongue (BT) has been listed as a notifiable disease to the World Organisation for Animal Health (OIE) [1]. BT is a non-contagious disease of ruminants caused by bluetongue viruses (BTV) transmitted by *Culicoides* biting midges [2,3]. BTV causes economic losses by morbidity and mortality but mainly by restrictions on movement and trade of animals and animal products [4]. Today, 27 BTV serotypes have been identified [5–9]. BT is endemic in temperate and (sub)tropical climate zones [10], however, has expanded to areas with a milder climate related to competent vectors [11–13].

BTV, the prototype virus species of genus *Orbivirus*, family *Reoviridae* [14,15], is a non-enveloped, multi-layered virus particle that contains a ten-segmented genome of double stranded RNA (Seg-1–10) encoding structural proteins VP1–VP7, and non-structural proteins NS1–NS4 [16–18]. VP7 protein is the major serogroup specific protein used in ELISAs to detect BTV antibodies (Abs). VP2 is the major target for serotype specific neutralising Abs (nAbs) [19,20]. BTV infection also raises non-neutralizing Abs against NS3/NS3a [21].

Vaccination is the most effective measure to control BT. Currently available live-attenuated and inactivated BT vaccines have inherent advantages and disadvantages [22–27]. The replicating BT Disabled Infectious Single Animal (DISA) vaccine exhibits all advantages of live-attenuated vaccine without the inherent risks of live-attenuated vaccines by the deletion of non-essential NS3/NS3a as previously discussed [28–32]. Consequently, BT DISA vaccines potentially enable specific detection of BTV infected animals based

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on NS3 Abs according to the principle of Differentiation of Infected from Vaccinated Animals (DIVA) [33,34].

We here describe the development of a competitive ELISA for BTV NS3 Abs (NS3 cELISA). The diagnostic sensitivity and specificity were compared with a commercial BTV cELISA detecting VP7 Abs, and the DIVA potential was studied in combination with BT DISA vaccine.

2. Material & methods

2.1. Epitope mapping on NS3

BSR cells (a clone of BHK-21 cells; [35]) were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) containing 5% foetal bovine serum, 100 IU/ml penicillin, 100 μg/ml streptomycin and 2.5 μg/ml Amphotericin B.

Plasmids with full length mutated cDNA of Seg-10 for run-off RNA transcription were synthesized by Genscript Corporation (Piscataway NJ, USA), or were constructed according to standard procedures. Seg-10 of strain KUW (BTV26) was based on accession number JN255162. BTV1 based viruses with mutated Seg-10 were rescued using reverse genetics [36]. The following BTV mutants were previously described; MutAUG1 + 2 [28], StyI-rev1, StyI-rev2 and BsiWI-rev1 [37], BTV1(S10)^{25} [38], and ΔD(S2reposition) [39].

Monoclonal antibody (MAb) against BTV-VP7 was produced by hybridoma ATCC-CRL-1875 (American Tissue Culture Collection). MAb 32H2, 32B6, 33H7, 31E9 and 32F1 against BTV-NS3 (generous gifts from Ingenasa, Spain), and rabbit α-mouse serum conjugated to horseradish peroxidase (Dako P0260) were used to map epitopes on NS3 according to standard procedures [40,41].

2.2. NS3 production and purification

Open reading frame (ORF) encoding NS3 from BTV8/NET2006/04 (Genbank accession number AM498060) was codon-optimised for bacterial expression and synthesized (Genscript Corporation (Piscataway NJ, USA), and cloned in pET-51b(+)Ek/LIC vector and transformed to Nova Blue GigaSingles^{TM} competent cells (Novagen) and checked by sequencing. Similarly, expression plasmid with an in-frame deletion from nucleotide position 369 to 568 of Seg-10 corresponding to amino acid (aa) codons 117 to 183 (NS3ΔTM) was constructed (Fig. 1).

Escherichia coli strain BL21 (DE1) with pET-51b(+)Ek/LIC-NS3 or with pET-51b(+)Ek/LIC-NS3ΔTM were grown in 400 ml of LB medium (supplemented with 100 μg/ml Ampicillin) at 37 °C to an optical density at 600 nm of approximately 0.6. Protein expression was induced by adding Isopropylthio-β-D-galactoside (IPTG) to 1 mM final concentration. After 4 h at 37 °C, bacteria were harvested by centrifugation at 4000 × *g* for 15 min and stored at −20 °C.

His-tagged NS3 proteins were purified with Ni–NTA resin-packed columns according to the instructions of the supplier (Qiagen). The eluted fractions with purified NS3 proteins were pooled and repeatedly washed with PBS with 150 mM NaCl using 3 K Amicon ultra plus filter devices (Merck Millipore Ltd.). The protein concentration was estimated according to the Bradford procedure using bovine serum albumin as standard, and analysed by SDS–PAGE according to standard procedures. His-tagged NS3 proteins were identified by westernblot analysis using commercial α-His MAb conjugated to horseradish peroxidase (1:1000)(Roche) or NS3 directed MAbs.

2.3. NS3 competitive ELISA

First, dilutions of purified NS3ΔTM antigen, MAb 33H7, and rabbit α-mouse serum conjugated to horseradish peroxidase (Dako P0260) were optimized in a chessboard format with positive and negative sera (not shown). Approximately 150 ng/well purified NS3ΔTM in coating buffer (100 mM bicarbonate/carbonate, pH 9.6) was bound into maxi sorp plates (Nunc) overnight at 4 °C. Coated wells were incubated for 1 h at 37 °C with PBS containing 0.1% Tween 20 and 5% FBS to reduce non-specific binding. Sera were diluted (1:2) in PBS 0.05%, Tween 20, 0.5% FBS, and 100 μl was added to wells and incubated for 1 h at 37 °C. Unbound serum Abs were removed by washing with 0.05% Tween 80. Then, wells were incubated for 1 h at 37 °C with 100 μl MAb 33H7 (1:1000). After washing, wells were incubated with 100 μl conjugated rabbit α-mouse serum (1:4000) for 1 h at 37 °C. After washing wells were incubated for 10 min at room temperature with TMB substrate (ID.VET), and colouring was stopped by stop solution (ID.VET). Optical density at 450 nm was determined for wells without adding

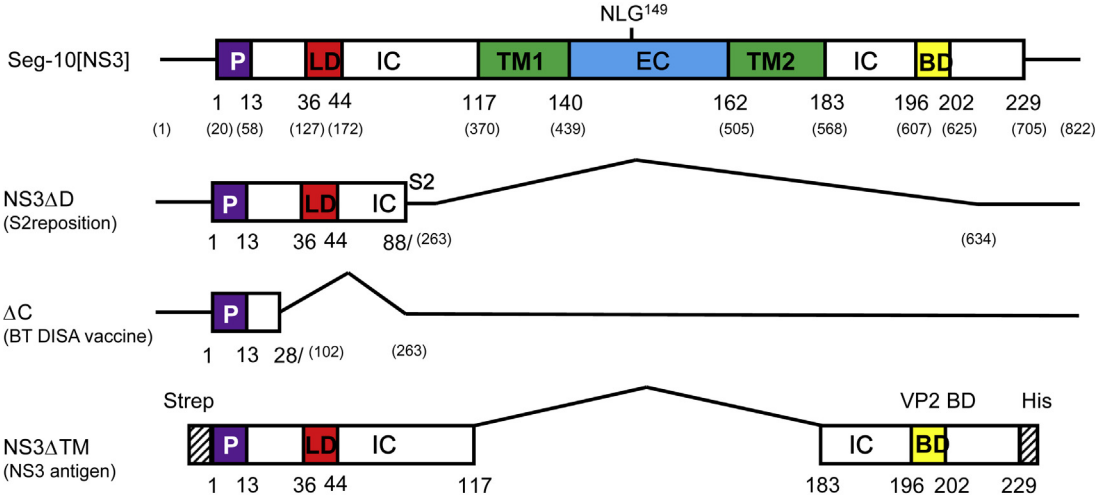

Fig. 1. *Schematic representation of Seg-10 and the encoded NS3/NS3a proteins.* Calpactin p11 binding domain (P), late domain (LD), two transmembrane regions (TM1 and TM2) with a conserved glycosylation sites in-between (NLG^{149}), the VP2 binding domain (BD) are indicated. The topological orientation is indicated by intracellular (IC) and extracellular (EC). Putatively expressed NS3 related proteins are indicated by boxes, whereas untranslated sequences are shown by lines. Numbers indicate the amino acid position, and numbers between brackets indicate the nucleotide position. Seg-10 NS3ΔD(S2reposition) was used to map epitopes on NS3 (Supplemented data A), and Seg-10 mutant ΔC was incorporated in BT DISA vaccines. NS3ΔTM protein was used as antigen, and contains an in-frame deletion encompassing TM1, EC, and TM2. Streptavidin tag (Strep) and His$_{10}$ tag (His) were linked in-frame to the N- and C-terminus, respectively, and are indicated by striped boxes.