



VP2-serotyped live-attenuated bluetongue virus without NS3/NS3a expression provides serotype-specific protection and enables DIVA

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ABSTRACT

Bluetongue virus (BTV) causes Bluetongue in ruminants and is transmitted by *Culicoides* biting midges. Vaccination is the most effective measure to control vector borne diseases; however, there are 26 known BTV serotypes showing little cross protection. The BTV serotype is mainly determined by genome segment 2 encoding the VP2 protein. Currently, inactivated and live-attenuated Bluetongue vaccines are available for a limited number of serotypes, but each of these have their specific disadvantages, including the inability to differentiate infected from vaccinated animals (DIVA).

BTV non-structural proteins NS3 and NS3a are not essential for virus replication *in vitro*, but are important for cytopathogenic effect in mammalian cells and for virus release from insect cells *in vitro*. Recently, we have shown that virulent BTV8 without NS3/NS3a is non-virulent and viremia in sheep is strongly reduced, whereas local *in vivo* replication leads to seroconversion. Live-attenuated BTV6 without NS3/NS3a expression protected sheep against BTV challenge. Altogether, NS3/NS3a knockout BTV6 is a promising vaccine candidate and has been named Disabled Infectious Single Animal (DISA) vaccine.

Here, we show serotype-specific protection in sheep by DISA vaccine in which only genome segment 2 of serotype 8 was exchanged. Similarly, DISA vaccines against other serotypes could be developed, by exchange of only segment 2, and could therefore safely be combined in multi-serotype cocktail vaccines with respect to reassortment between vaccine viruses.

Additionally, NS3 antibody responses are raised after natural BTV infection and NS3-based ELISAs are therefore appropriate tools for DIVA testing accompanying the DISA vaccine. To enable DIVA, we developed an experimental NS3 ELISA. Indeed, vaccinated sheep remained negative for NS3 antibodies, whereas seroconversion for NS3 antibodies was associated with viremia after heterologous BTV challenge.

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1. Introduction

Bluetongue virus (BTV), a *Culicoides* borne orbivirus in the family *Reoviridae*, causes Bluetongue (BT) in ruminants, characterized by fever, oral and nasal erosions and discharge, oedema, coronitis, anorexia and death, due to damage of the vascular endothelium [1,2]. At least 26 serotypes, hardly showing cross protection, are known [3,4]. Historically, BT is endemic in regions with

temperate and tropical climate, and more recently with moderate climate, related to the presence of competent vectors [5]. In Europe, outbreaks of BTV serotypes 1, 2, 4, 8, 9 and 16 have been reported and serotypes 2, 10, 11, 13 and 17 are endemic in the USA [6], causing large economic losses [7,8].

BTV has a 10 segmented double-stranded (ds) RNA genome encoding seven viral proteins (VP1–VP7) and four non-structural proteins (NS1–4) [9–11]. The outer capsid protein VP2, encoded by Seg-2, is the major serotype determining protein. Membrane associated NS3/NS3a, encoded by Seg-10, is important for virus release and is an inhibitor of the cellular interferon response [12–16].

Vaccination is effective in controlling BT outbreaks [17]. Currently marketed vaccines are either conventionally live-attenuated or chemically inactivated and each has specific disadvantages,

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such as incomplete protection and required re-vaccinations for inactivated vaccines, and residual virulence, reassortment, and horizontal and vertical vaccine spread for live-attenuated vaccines [18–21]. Promising vaccine candidates have been described, but none of these are marketed yet [22]. In addition to efficacy and safety, the ability to differentiate infected from vaccinated animals (DIVA) is important to detect infections in vaccinated livestock [17].

Recently, we described the rational design of the next-generation Disabled Infectious Single Animal (DISA) vaccine [23]. BT DISA vaccine is based on live-attenuated vaccine virus BTV6/net08 [24,25], without NS3/NS3a expression and is serotyped by exchange of immunodominant VP2. NS3/NS3a knockout results in avirulence and strongly reduced viremia, whereas protection is dependent on local replication. Here, we demonstrate that exchange of only VP2 induces serotype-specific protection at nine weeks post vaccination in sheep, and we show the ability of DIVA with an experimentally developed NS3 ELISA.

2. Material and methods

2.1. Cell culture and viruses

BSR cells (a clone of BHK-21 cells [26]) were cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen), containing 5% fetal bovine serum (FBS), 100 IU ml⁻¹ Penicillin, 100 µg ml⁻¹ Streptomycin and 2.5 µg ml⁻¹ Amphotericin B.

DISA vaccine for serotype 8 has been described previously [23]. Briefly, it is based on live-attenuated BTV6/net08 with Seg-2 from BTV8/net07 generated using reverse genetics [27], and with a deletion in Seg-10 [28] leading to an NS3/NS3a negative phenotype.

DISA vaccine was produced by infection of BSR cells at low multiplicity of infection (MOI). When >50% of cells were immunostained positive for BTV VP7 in a duplicate well using anti-VP7 monoclonal antibody (MAb) ATCC-CRL-1875, DISA vaccine was harvested by freeze thawing and centrifugation. Vaccine in clarified supernatant was used for vaccination.

Challenge virus BTV8/net07 [29] was isolated on eggs and passaged three times on BHK-21 cells (BTV8/net07/e1/bhkp3) [27]. BTV2 challenge virus (BTV-2/SAD2001/01) was isolated from sheep (Pirbright Institute, UK) and was grown for one passage in embryonated chicken eggs, two passages on BHK-21 cells and three passages on KC cells (BTV2/SAD01/01/e1/bhkp2/kcp3).

Virus titers were determined by endpoint dilution on BSR cells and expressed as 50% tissue culture infectious dose per ml (TCID₅₀ ml⁻¹).

2.2. Animal experiment

All animal experiments were performed under the guidelines of the European Community and were approved by the Committee on the Ethics of Animal Experiments of the Central Veterinary Institute (permit number 2013.016). Sixteen female Blessumer sheep of 6–24 months old were obtained from a Dutch farm and were free of BTV and BTV antibodies. Sheep were randomly allocated in four groups of four animals. After one week of acclimatization, sheep were vaccinated with 2 × 1 ml of 10⁵ TCID₅₀ ml⁻¹ DISA vaccine. Sheep were injected subcutaneously (s.c.) in the back between the shoulder blades at both sides of the spinal cord. At 21 days post vaccination (dpv), animals received a booster vaccination. At 84 dpv, one vaccinated and one non-vaccinated group were infected s.c. with 4 × 1 ml 10⁵ TCID₅₀ ml⁻¹ BTV8/net07/e1/bhkp3 or BTV2/SAD01/01/e1/bhkp2/kcp2. At 21 days post challenge (dpc), all sheep were sacrificed.

Body temperature and clinical signs were examined, and EDTA blood and serum were collected at indicated days (Figs. 1 and 2).

Clinical signs were scored according to the clinical score table for Bluetongue in sheep (Table S1 [25]). Statistical differences in body temperature were calculated using a split plot ANOVA and maximal temperatures were compared using a pairwise *T*-test with *p* < 0.05 indicating significance.

2.3. Detection of BTV RNA

Samples of EDTA blood were examined for BTV RNA by panBTV real-time reverse transcription (RT) PCR testing targeting Seg-1. After isolation of BTV-RNA using the MagNA Pure isolation robot (Roche) [30], Seg-1 RT PCR was performed using primers F-pan-S1, R-pan-S1, and probe P-pan-S1 [31] according to the all-in-one method for the panBTV Seg-10 RT PCR [30] (Table 1). Crossing point (Cp) values were calculated, and samples without Cp value showing increase of the OD_{640/530} were interpreted as 40 and negative samples were set at 45. Statistical differences in Cp value were calculated using a split plot ANOVA and minimal Cp values of each animal were compared using a pairwise *T*-test with *p* < 0.05 indicating significant differences.

2.4. VP7 ELISA

The Bluetongue competition VP7 enzyme-linked immunosorbent assay (VP7 ELISA) was used to detect VP7 antibodies in serum samples according to the supplier's instructions (ID.Vet). The percentage of blocking was displayed as 100 minus value.

2.5. Experimental NS3 ELISA

Optimal dilutions of coated NS3 antigen, serum, mouse MAb 33H7, and conjugated rabbit anti-mouse MAb were determined in advance by a brief validation with positive and negative control sera (not shown). *E. coli* produced BTV NS3 antigen was dissolved in coating buffer (100 mM bicarbonate/carbonate, pH9.6) and bound overnight at 4°C to Nunc maxi sorp plates. After incubation for 1 h at 37°C with dilution buffer (PBS 0.1% Tween 20 and 5% FBS), 100 µl of serum samples (1:2 in dilution buffer) were incubated in coated wells for 1 h at 37°C. After washing, wells were incubated with 100 µl 1:1000 MAb 33H7 (Ingenasa, Madrid, Spain) for 1 h at 37°C. After washing, 100 µl 1:5000 rabbit anti-mouse MAb (DAKO, P0260) was added and incubation was continued for 1 h at 37°C. After washing, wells were incubated at room temperature with TMB substrate (ID.Vet) for 10 min. Coloring was stopped by stop solution (ID.Vet). OD₄₅₀ was determined and percentage blocking was displayed as 100 minus value. Seroconversion cut-off is 30%, based on the mean blocking percentage of negative samples plus three times the standard deviation.

2.6. Serum neutralization test

Serotype-specific neutralizing antibody (nAb) titers were determined by serum neutralization tests (SNTs) with serum from 0, 21, 42, 84 and 105 dpv according to Haig [23,32] using BTV8/net07/e1/bhkp3 and BTV2/SAD01/01/e1/bhkp2/kcp2.

3. Results

3.1. Temperature and clinical signs

After the first and booster vaccination, none of the animals experienced clinical signs or fever, indicating that the DISA vaccine is non-pathogenic. At one day and four days after booster vaccination (22 dpv and 26 dpv), two different sheep developed a slightly elevated body temperature (not shown).

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