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Strong protection induced by an experimental DIVA subunit vaccine against bluetongue virus serotype 8 in cattle

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ABSTRACT

Bluetongue virus (BTV) infections in ruminants pose a permanent agricultural threat since new serotypes are constantly emerging in new locations. Clinical disease is mainly observed in sheep, but cattle were unusually affected during an outbreak of BTV seroype 8 (BTV-8) in Europe. We previously developed an experimental vaccine based on recombinant viral protein 2 (VP2) of BTV-8 and non-structural proteins 1 (NS1) and NS2 of BTV-2, mixed with an immunostimulating complex (ISCOM)–matrix adjuvant. We demonstrated that bovine immune responses induced by this vaccine were as good or superior to those induced by a classic commercial inactivated vaccine. In this study, we evaluated the protective efficacy of the experimental vaccine in cattle and, based on the detection of VP7 antibodies, assessed its DIVA compliancy following virus challenge. Two groups of BTV-seronegative calves were subcutaneously immunized twice at a 3-week interval with the subunit vaccine ($n = 6$) or with adjuvant alone ($n = 6$). Following BTV-8 challenge 3 weeks after second immunization, controls developed viremia and fever associated with other mild clinical signs of bluetongue disease, whereas vaccinated animals were clinically and virologically protected. The vaccine-induced protection was likely mediated by high virus-neutralizing antibody titers directed against VP2 and perhaps by cellular responses to NS1 and NS2. T lymphocyte responses were cross-reactive between BTV-2 and BTV-8, suggesting that NS1 and NS2 may provide the basis of an adaptable vaccine that can be varied by using VP2 of different serotypes. The detection of different levels of VP7 antibodies in vaccinated animals and controls after challenge suggested a compliancy between the vaccine and the DIVA companion test. This BTV subunit vaccine is a promising candidate that should be further evaluated and developed to protect against different serotypes.

1. Introduction

Bluetongue virus (BTV) is the causative agent of the primarily vector-borne hemorrhagic bluetongue (BT) disease of ruminants. Since 1998 at least 8 of 26 serotypes have been detected within the European Union [1] and the introduction of new BTV serotypes is a permanent threat to the region. Typically, BT disease most severely clinically affects sheep [2]. However, the 2006 BTV-8 outbreak in central and northern Europe caused clinical signs in cattle including abortion and teratogenic effects [3,4]. The vaccination of cattle, BTV's main amplifying host, along with small ruminants, is important to decrease virus spread [5].

Although modified live virus (MLVs) and inactivated vaccines have been suggested to be effective in controlling BTV in Europe [6–8], MLVs are sometimes associated with viremia, clinical disease, and risk of gene segment reassortment [9–11], while safer inactivated vaccines presently cost more [8] or may be difficult to produce since some serotypes may not replicate well *in vitro* [12]. Neither vaccine type currently allows the differentiation of infected from vaccinated animals (DIVA) nor is easily adaptable to

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target multiple BTV serotypes. The use of DIVA-compliant vaccines could potentially help countries quickly return to BTV-free status [13], and enable surveillance of BTV epidemiology and vaccine efficacy. Vaccine adaptability to novel or multiple BTV serotypes is increasingly necessary given the recent co-circulation of different serotypes within Europe [14]. Many experimental BTV vaccines aim to possess these important qualities, while being as safe and effective as current vaccines (reviewed by [15]).

Vaccine-induced protection against BTV is largely attributed to serotype-specific virus-neutralizing antibodies induced by viral protein 2 (VP2) [16–19]. The virus's non-structural (NS) proteins induce cell-mediated immune responses that may also play a protective role [20–23]. We previously designed and optimized a recombinant subunit vaccine against BTV-8 composed of VP2 from BTV-8 and NS1 and NS2 from BTV-2, with a VP7-based DIVA characteristic [24] that can potentially be used to detect antibodies in samples from animals infected with any serotype [25]. We determined that, in cattle, this vaccine induced strong neutralizing antibody titers, VP2-, NS1-, and NS2-specific antibodies, and cellular immune responses to NS1 [26] that may contribute to a successful multi-serotype vaccine [27]. Here, we aimed to evaluate the clinical and virological protective efficacy of the experimental vaccine against virulent BTV-8 challenge in cattle and to verify its DIVA compliancy using existing diagnostic assays.

2. Materials and methods

2.1. Vaccine production

Recombinant VP2 of BTV-8 and NS1 and NS2 of BTV-2 were produced and purified as described previously [26]. Each 2.5 ml subunit vaccine (SubV) dose contained 150 μg each of purified VP2, NS1, and NS2 and 450 μg AbISCO®-300 (Isconova AB, Sweden), an immunostimulating complex (ISCOM)-based adjuvant.

2.2. Challenge virus

To induce both a viremia and clinical signs associated to BTV, the challenge virus consisted of two viral cell suspensions of BTV-8 strain isolated from a BTV-8-viremic cow during a 2007 outbreak in France, on (i) embryonated chicken eggs (ECE) and passaged twice on baby hamster kidney (BHK-21) cells (BHK suspension; 6×10^6 of 50% tissue culture infective dose ($TCID_{50}$)/ml, or (ii) *Culicoides*-derived (KC) cells (kindly provided by the Pirbright Institute, UK) followed by one passage on the same cell line for virus amplification (KC suspension). The KC suspension was analyzed by RT-qPCR (Adiavet™ BTV Realtime ADI352, Adiagene, France) and resulted in a C_t value of 14.1.

2.3. Animals, experimental design, clinical examinations, and sampling

Twelve conventionally reared female Holstein calves aged 6–12 months were housed in the Biosecurity Level 3 animal facilities of the National Institute of Agricultural Research (INRA) Research Center (Nouzilly, France). The calves originated from the same BVDV- and BHV1-free herd, were seronegative for BTV antibodies, and were not previously vaccinated against BTV. Animals were divided randomly into two groups ($n = 6$) and housed in the same room, separated by a fence. All procedures were approved by the ethical review board of Val de Loire (CEEA VdL, committee number n°19, file number 2012-08-01).

Animals were immunized subcutaneously on the left side of the neck at a 3-week interval with SubV or with 450 μg AbISCO®-300 in PBS (Control). Three weeks after second vaccination all animals were subcutaneously inoculated with 2.5 ml each of BTV-8 preparations on the right (BHK suspension) and left (KC suspension) sides of the neck (post-infection day 0 (PID0)). Clinical examinations were performed as shown in Fig. 1, and clinical scoring performed as described previously [28]. Samples for antibody, viremia, and lymphocyte proliferation analyses were collected as indicated in Fig. 1, in dry, ethylene diamintetraacetic acid (EDTA), and heparinized tubes (BD Biosciences, USA), respectively.

2.4. Virus detection by RT-qPCR and ECE inoculation

Viral RNA was extracted using a Magnatrix robot and a pan-BTV qPCR based on segment 1 (VP1) of BTV [29] was performed. The standard curve was obtained by dilution of a viral suspension ($10^{5.9}$ $TCID_{50}$ equivalent units/ml), as performed previously [30]. The quantity of viral RNA is expressed in $\log_{10}$ $TCID_{50}$ equivalent units/ml.

ECE inoculation was performed as described previously [31], in five 12-day-old embryonated specific pathogen-free chicken eggs (Håtunaholm, Sweden) per calf blood sample collected on PID8. Dead embryos were scored as positive if they showed hemorrhages characteristic of BTV infection. Embryos were homogenized after death or on day 7, after placement at +4 °C for at least 4 h. RNA was extracted from swabs of homogenized embryos and RT-qPCR performed as described above.

2.5. BTV-specific antibody detection

Virus neutralizing assays were performed in duplicate on Vero cells, using serially diluted sera from 1:2 to 1:256 (as described previously [32]). BTV-specific CPE were identified under a light microscope after 5 days of incubation. The neutralizing titer was defined as the highest dilution allowing neutralization of 100 $TCID_{50}$ of BTV-8.

Competitive (c) enzyme-linked immunosorbent assays (ELISAs) were used to measure specific serum antibodies to VP2 of BTV-8 and VP7 of any BTV serotype (ID Screen® Bluetongue Serotype 8 Competition and ID Screen® Bluetongue Competition, ID Vet, France, respectively), according to the manufacturer's protocols. Results are expressed as 100% minus competition percentage (100 times [$OD_{sample}/OD_{negative\ control}$]).

Antibodies specific to NS1 and NS2 (BTV-2) were analyzed using indirect ELISAs as described previously [26]. Results are expressed as $\log_{10}$-transformed antibody titers, which were calculated by linear regression to the corrected OD ($COD = OD_{protein} - OD_{background\ control}$) value of negative control sera at a dilution factor of 10. For calculating means and performing statistical analysis, values under the detection threshold were set to that threshold (dilution factor 10).

2.6. BTV-specific lymphocyte proliferation assays

Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood of animals as previously described [33], then stored in liquid nitrogen. Cells were restimulated, in duplicate, as described previously [34], with 0.03–1 μg individual proteins (VP2, NS1, NS2) or $10^{3.9}$ $TCID_{50}$/well of UV-inactivated BTV-8 and relevant background controls (Sf9 cell lysate for VP2, NS1; non-transfected BL21-AI™ *E. coli* lysate for NS2; uninfected Vero cell lysate for virus). Absorbances were measured 7–16 h after addition of alamarBlue®-reagent (Invitrogen, UK), at 570 nm and 595 nm. OD ($OD_{570nm} - OD_{595nm}$) and COD values were calculated for all protein- and virus-specific stimulations.

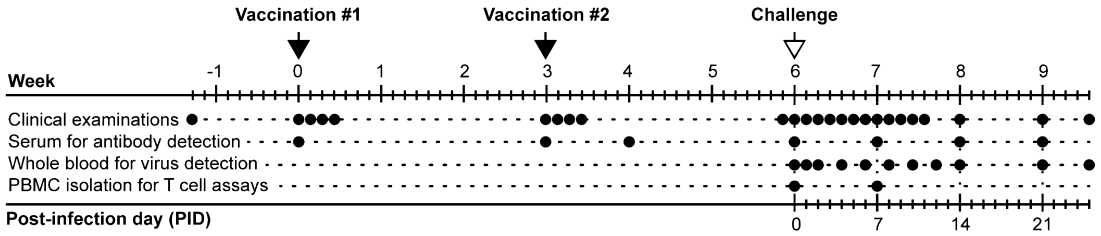


Fig. 1. Timeline of the study. Twelve calves were immunized twice at a 3-week interval with an experimental subunit vaccine against BTV-8 (SubV, $n = 6$) or adjuvant alone (Control, $n = 6$) (black arrows), then challenged with BTV-8 three weeks after the second vaccination (white arrow). Clinical examinations, collection of serum for antibody detection and whole blood for virus detection, and isolation of peripheral blood mononuclear cells (PBMCs) for lymphocyte proliferation assays were performed as indicated by black dots at the indicated week or post-infection day (PID).

2.7. Statistical analyses

According to the sample data distribution, analysis among two groups or time points and three or more time points was performed using non-parametric Mann–Whitney and Kruskal–Wallis tests for independent groups, respectively, in R [35], or using Student's *t*-tests in Excel. Statistical significance was set to $p \leq 0.05$ (*) or $p \leq 0.01$ (**). Where applicable, values are provided as mean ± SD.

3. Results

3.1. Clinical observations

3.1.1. Local and general reactions following vaccination

Mild (<3 cm) localized injection site swellings were observed in 5/6 SubV-immunized calves and in 1/6 controls and lasted 3 days after first vaccination. Following second immunization, mild or mild-to-moderate (<10 cm) injection site swellings were observed in 4/6 controls and in all vaccinated calves, respectively. Slightly elevated rectal temperatures were observed in both groups for 2 days after both immunizations (maximum rectal temperatures mean, SubV: 39.4 ± 0.3 °C; Control: 39.3 ± 0.4 °C) but the groups did not differ significantly ($p = 0.61$).

3.1.2. Clinical signs following BTV-8 challenge

Control calves showed slight general depression with appetite loss (6/6, PID3–4), stiffness (4/6, PID7–8), and lameness (3/6, PID4–6), and had a biphasic rectal temperature pattern that peaked on PID4 and PID7 and reached over 40 °C in 1/6 and 2/6 animals, respectively (PID4 range: 39.1–40.5 °C, mean: 39.6 °C; PID7 range: 38.9–40.3 °C, mean: 39.7 °C). Other clinical signs of BTV infection were observed from PID2–14, including nasal discharge (4/6, PID5–6), congestion with slight edema of the nasal mucosa (2/6, PID5), and moderate edema in the intermandibular space (1/6, PID5–6). Enlargement of right and left prescapular lymph nodes was observed in all controls (PID5–14). The mean clinical scores peaked between PID5–7 and remained elevated through PID14, after which no clinical examinations were performed until PID21 (Fig. 2A).

In contrast to controls, SubV-vaccinated animals showed no significant increase in rectal temperature following challenge (range: 38.4–39.2 °C, $p = 0.29$; Fig. 2B) and 3/6 vaccinated calves demonstrated no clinical signs throughout the study. In the remaining three SubV-vaccinated calves, very slight clinical signs were observed, including slight nasal discharge on PID5 (1/6) and stiff walking in two animals on PID4 (1/6) and PID5 (1/6). Mean clinical scores for vaccinated animals never exceeded 0.5 (PID5) and otherwise remained at 0. Clinical scores of controls were significantly higher ($p \leq 0.05$ or $p \leq 0.01$) than those of vaccinated calves on each day from PID4–14 (Fig. 2A).

3.2. Quantification of viral RNA in blood and virus isolation in ECE

Using RT-qPCR analysis, no BTV RNA was detected in blood collected from vaccinated calves between PID0 and PID25 (Fig. 3A). In contrast, BTV RNA was detected in blood of 1/6 controls on PID2, 2/6 controls on PID4, and in all controls on PID6–25 (experiment

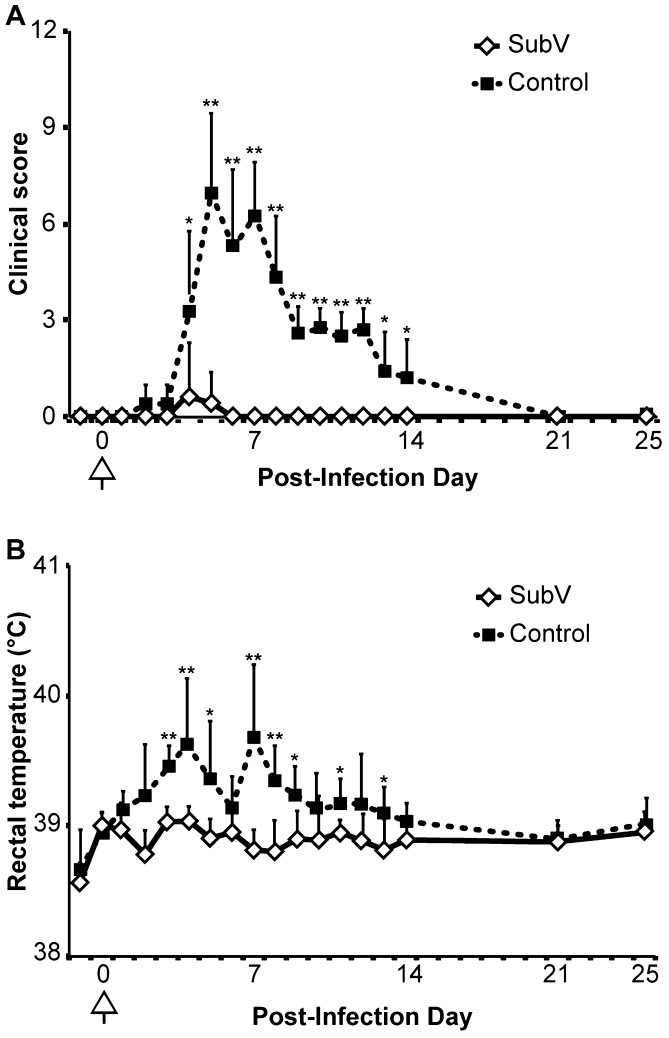
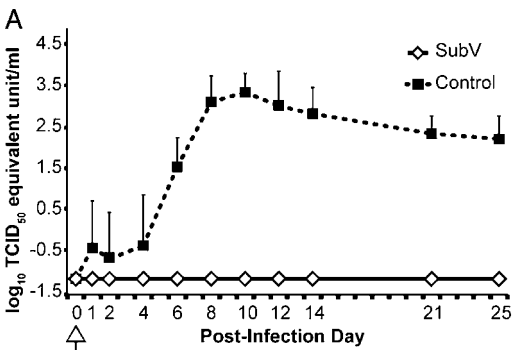


Fig. 2. Kinetics of clinical scores and rectal temperatures of calves following challenge with BTV-8. Animals were vaccinated with SubV (white diamonds) or Control (black squares), challenged with BTV-8 (white arrow), and clinical examinations were performed as indicated in Fig. 1. Clinical scores (A) and rectal temperatures (B) are presented as group means. Standard deviations are indicated by upward deflection lines and statistical significance between groups is indicated by asterisks ($p \leq 0.05$ [*] or $p \leq 0.01$ [**]).



B

Calf	Embryo lesion	RT-PCR
SubV 1	0/5	-
SubV 2	0/5	-
SubV 3	0/5	-
SubV 4	0/5	-
SubV 5	0/5	-
SubV 6	0/5	-
Control 1	5/5	+
Control 2	3/5	+
Control 3	4/5	+
Control 4	2/5	+
Control 5	4/5	+
Control 6	4/5	+

Fig. 3. Detection of BTV RNA in whole blood of immunized calves after challenge with BTV-8. Calves were vaccinated with SubV (white diamonds) or Control (black squares), then challenged with BTV-8 (white arrow), as indicated in Fig. 1. The quantity of viral RNA detected by RT-qPCR analysis is indicated as group means expressed as $\log_{10}$ $TCID_{50}$ equivalent units/ml, with standard deviations indicated as upward deflection lines (A). Embryonated chicken eggs were inoculated, in quintuplicates, with whole blood from individual calves on post-infection day (PID) 8, evaluated for embryo lesions after 1 week of incubation, and the number of eggs with embryo lesions indicated (B). BTV RT-qPCR was performed on swabs from homogenized embryos and positive ($27 < C_t < 38$) and negative samples (no C_t value) are indicated by (+) or (−), respectively (B).

termination). Peak viremic levels were observed on PID10 (mean: 3.26 ± 0.44 $\log_{10}$ $TCID_{50}$ equivalent units/ml).

These data were confirmed by ECE inoculation of blood. Virus was detected by induction of embryonic mortality between 2 and 5 days post-inoculation in 2–5 of 5 inoculated eggs per calf blood sample collected on PID8 from controls (Fig. 3B). The embryo mortality and observed hemorrhagic characteristics were attributed to BTV since BTV RNA was detected only in swabs from homogenized embryos that had been inoculated with blood from controls. In contrast, no dead or hemorhaggic embryos were observed following inoculation with blood from vaccinated calves and no BTV RNA was detected in these embryos (Fig. 3B).

3.3. Detection of neutralizing and protein-specific antibodies

BTV-8-specific neutralizing antibodies were detected in the sera of 5/6 vaccinated calves 1 week after second vaccination and in all vaccinated calves 2 weeks later (mean: 4.5 ± 1.4 $\log_2$ titers) (Fig. 4A). These titers remained high 3 weeks after challenge. In contrast, BTV-8 neutralizing antibodies were only detected in the sera of controls after challenge.

BTV-8 VP2-specific serum antibodies were detected by ELISA in all vaccinated calves 1 week after second immunization, continued to increase through 1 week after challenge, and remained stable 2 weeks later (Fig. 4B). VP2-specific antibodies were detected

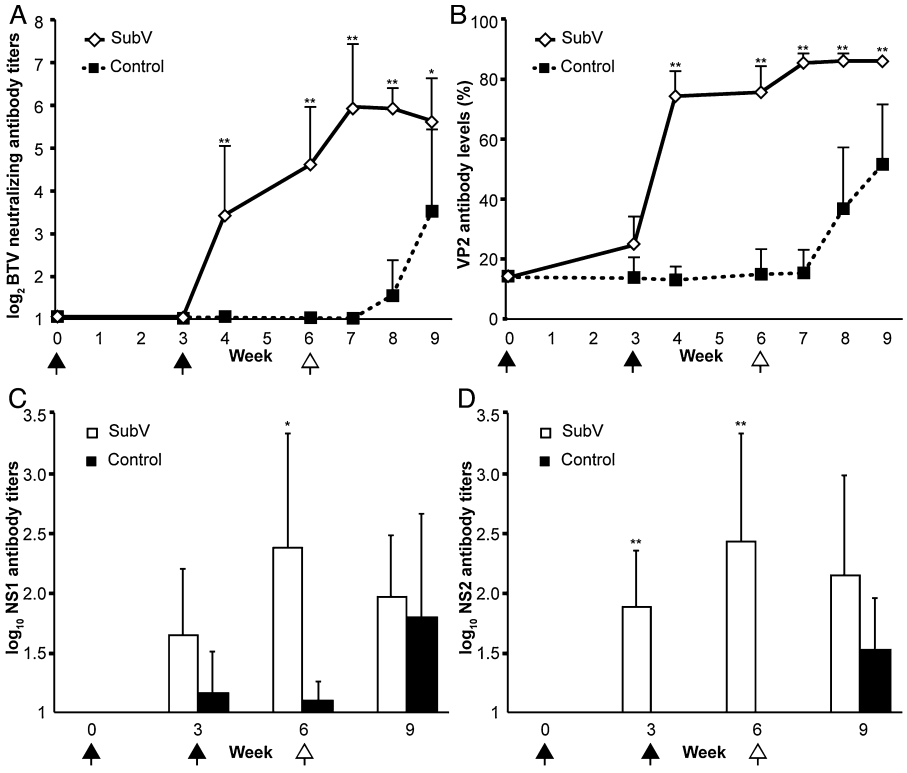


Fig. 4. Kinetics of BTV-8-neutralizing antibodies and protein-specific serum antibodies against VP2 of BTV-8 and NS1 and NS2 of BTV-2 in calves. Calves were immunized (black arrows) with SubV (white diamonds) or Control (black squares) and challenged with BTV-8 (white arrow), as indicated in Fig. 1. Titers of neutralizing antibodies directed against BTV-8 (A) were calculated using the Reed–Muench method and are expressed as $\log_2$ values. VP2-specific antibodies were detected by competitive ELISA and expressed as 100% minus the competition percentage (100 times $OD_{sample}/OD_{negative}$), (B) while antibodies directed against NS1 (C) and NS2 (D) were detected using indirect ELISAs and are expressed as $\log_{10}$ antibody titers. Upward deflection lines indicate standard deviations and statistical significance is indicated by asterisks ($p \leq 0.05$ [*] or $p \leq 0.01$ [**]).

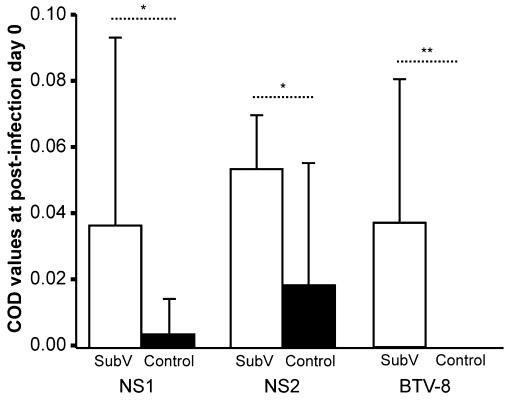


Fig. 5. Specific lymphocyte proliferation following NS1 (BTV-2), NS2 (BTV-2) and UV-inactivated BTV-8 restimulation of isolated PBMCs from calves. Animals were vaccinated with SubV (white bars) or Control (black bars) and challenged with BTV-8 as indicated in Fig. 1. PBMCs were isolated from whole blood of calves 3 weeks after second vaccination and restimulated against NS1, NS2, or inactivated BTV-8 and corresponding control antigens. Proliferation is expressed as group means of corrected OD ($COD = OD_{protein\ or\ virus} - OD_{background}$) values after 4 days of stimulation and addition of alamarBlue® reagent. Standard deviations and statistical significance are indicated by upward deflection lines and asterisks ($p \leq 0.05$ [*] or $p \leq 0.01$ [**]), respectively.

in controls 2 weeks after challenge and had increased 1 week later.

Increases in NS1-specific and NS2-specific serum antibody titers were detected in vaccinated calves 3 weeks after first and second vaccinations. Antibody titers to NS2 were significantly higher than those detected in controls 3 weeks after first vaccination ($p \leq 0.01$) and to NS1 and NS2 3 weeks after second vaccination ($p \leq 0.05$ and $p \leq 0.01$, respectively) (Fig. 4C and D). Antibodies to NS1 and NS2 (BTV-2) were observed 3 weeks after BTV-8 challenge in the sera of controls and vaccinated calves, but did not differ significantly ($p = 0.94$ and $p = 0.23$, respectively).

3.4. Detection of protein- and BTV-8-specific lymphocyte proliferative responses

In vitro NS1-specific and NS2-specific lymphoproliferative responses were detected in PBMC of vaccinated calves (means: 0.04 ± 0.06 and 0.05 ± 0.02 COD, respectively) 3 weeks after second vaccination, at statistically higher levels than controls (means: 0.00 ± 0.01 and 0.02 ± 0.04 COD, respectively; $p \leq 0.05$ for both) (Fig. 5). Furthermore, BTV-8 specific lymphoproliferation was detected in vaccinated calves (mean: 0.04 ± 0.04 COD) at this time point but not in any controls (mean: 0.00 ± 0.00 COD, $p \leq 0.01$). No VP2-specific lymphoproliferatives responses were observed.

3.5. Assessment of DIVA compliancy by VP7 antibody detection

VP7-specific serum antibodies were not detected in any calf before challenge, but were detected at high levels (≥75%) in 5/6 controls 2 weeks after challenge and in all controls 1 week later (mean: $92 \pm 3\%$) (Fig. 6). Vaccinated calves also developed VP7-specific serum antibodies following challenge, but antibody levels remained significantly lower than those in controls (peak mean: $44 \pm 22\%$ at 2 weeks after challenge, $p \leq 0.01$).

4. Discussion

In this study, we demonstrated that the experimental vaccine based on VP2 of BTV-8 combined with NS1 and NS2 of BTV-2 and an ISCOM–matrix adjuvant provided strong clinical and virological protection against virulent BTV-8 challenge in calves. This protection was mediated by specific immune responses directed against all or certain proteins included in this vaccine, in agreement with our previous findings [26]. Furthermore, the potential of the DIVA characteristic based on VP7 was confirmed.

The clinical signs and viremia observed in controls were comparable to those observed in natural or experimental infections in ruminants [30,36,37] and consequently show the efficacy of SubV in preventing both clinical and virological disease. In contrast to previously reported challenge studies where no clinical signs were observed [32,38], here, clinical signs including fever and some congestion or mucosal edema were demonstrated in controls, but not vaccinated calves, from 2 to 14 days post-infection. This could be explained by passage of the challenge virus in KC cells, which may better mimic natural infection *via Culicoides* compared to virus passaged in other cell cultures [39,40] as observed previously [41]. Furthermore, BTV was only detected in the blood of controls. The very limited clinical signs observed in three vaccinated animals were probably unrelated to BTV since we did not detect any viremia in these animals by RT-qPCR analyses nor by isolation in ECE.

The strong protection observed in the vaccinated calves corresponds with diverse humoral and cellular immune responses induced by SubV. Importantly, BTV-8-neutralizing antibodies were detected in sera of vaccinated calves as soon as 1 week after second vaccination. These antibodies were likely directed against VP2 since it is the only protein included in the experimental vaccine known to induce them [16,19] and because the presence of VP2 antibodies was also confirmed by cELISA. Our results support recent suggestions that VP2 alone induces sufficient neutralizing antibody titers, without the aid of VP5 [42,43]. Additionally, SubV induced specific antibody production to NS1 and NS2 following vaccination. Although the protective contribution of cellular immune responses against the non-structural proteins has previously been indicated for both BTV and the related African horse sickness virus [44,45], the role that these antibodies may play against BTV infection remains to be evaluated.

Low but specific T cell responses against NS1 and NS2 were observed 3 weeks after second vaccination, which confirms previous findings for NS1 and adds new information about NS2. Compared to previously [26], the NS2-specific lymphoproliferative responses were detected by increasing the concentration of this protein for PBMC restimulation. NS1 and NS2 have been reported to induce cross-serotype helper T cell [44] and cytotoxic T cell responses [21,44,46,47]. Here, helper T cell proliferation was likely induced by the killed antigens used for *in vitro* restimulations, while *in vivo* cross-presentation may have facilitated possible induction of cytotoxic T cell responses. The ISCOM–matrix adjuvant included in the vaccine has also been demonstrated to induce T cell responses in cattle [34] and cross-priming leading to cytotoxic T cell responses [48]. Since T cell responses were only detected against NS1 and NS2 (BTV-2), but not VP2 (BTV-8), the observed lymphocyte proliferation to UV-inactivated BTV-8 *in vitro* suggests cross-serotype reactions induced by the NS proteins, although responses induced by VP2, but not detected in peripheral circulation by the VP2-specific assay employed herein, cannot be excluded. Furthermore, species differences in T cell responses to the same protein, such as VP2-specific lymphoproliferation observed following vaccination in mice but not cattle [24], highlights the importance of performing vaccine studies in the target species. Specific T cell responses from samples collected on PID7 could not be determined because of poor viability, likely due to storage of this batch of cells in liquid nitrogen (data not shown).

Taken together, the vaccine-induced protection was probably due to serotype-specific neutralizing antibodies against VP2 and cross-serotype immune responses to NS1 and NS2. Even though the roles of NS1 and NS2 in protection need further investigation, we believe that the diverse immune responses induced by the

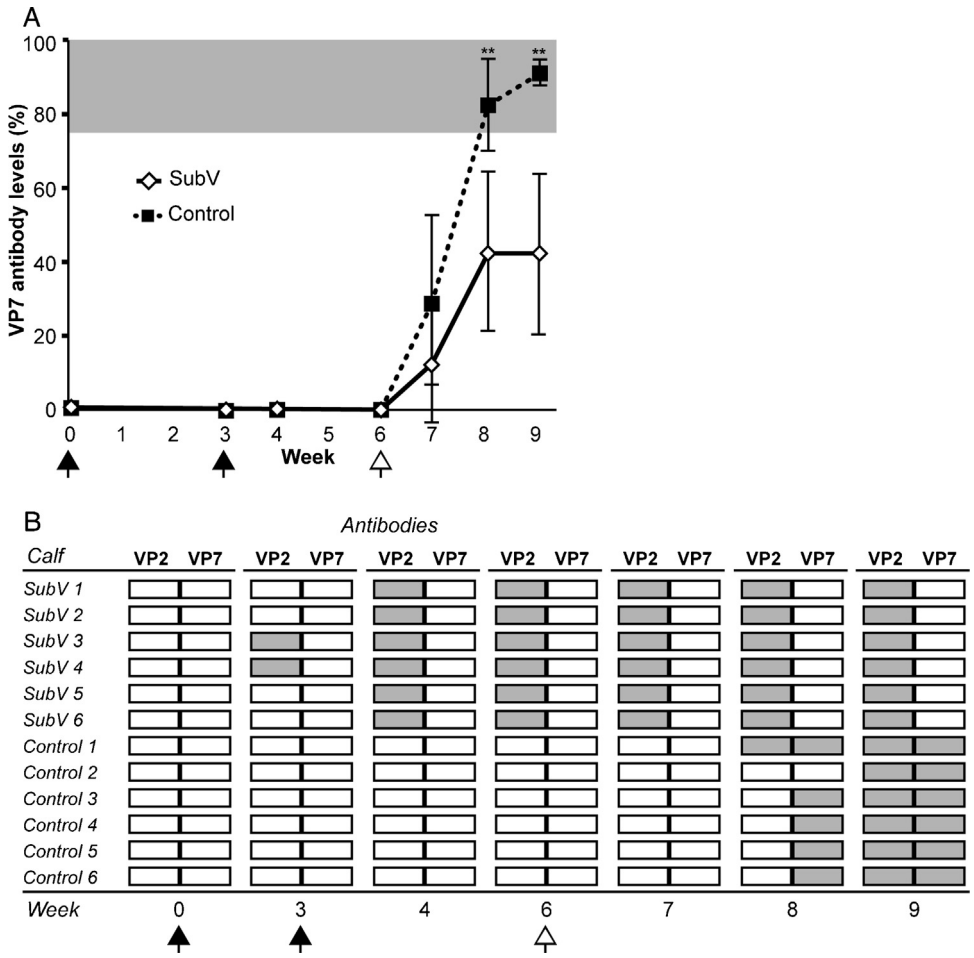


Fig. 6. Kinetics of BTV VP7- and VP2-specific serum antibodies for DIVA compliancy of SubV following BTV-8 infection. Calves were vaccinated (black arrows) with SubV (white diamonds) or Control (black squares) and challenged with BTV-8 (white arrow), as indicated in Fig. 1. VP7-specific antibodies were detected by cELISA and mean percentages (100%-competition percentage) for VP7 antibodies are presented in (A) with standard deviations indicated by deflection lines and statistical significance between groups indicated by asterisks ($p \leq 0.01$ [**]). Individual results for VP2- and VP7-specific serum antibodies are presented in (B). Gray boxes indicate samples that are seropositive for BTV-8 VP2-specific antibodies (≥30%) or have high levels of VP7-specific antibodies of any BTV serotype (≥75%), indicating BTV replication.

mixture of BTV proteins included in SubV may contribute to its efficacy against different BTV-8 strains and perhaps to a long duration of immunity, by potentially stimulating a broader pool of memory B and T cells and long-lived plasma cells. This would have to be investigated since it has direct consequences on vaccine use in livestock such as cattle, which have a long economical life compared to shorter-lived agricultural animals such as swine and poultry. It is notable that compared to the preceding study [26], we decreased the adjuvant quantity in SubV by 25% and observed less systemic and local reactions following vaccination, yet still observed similar immunological responses.

The DIVA characteristic of SubV is based on the detection of VP2 antibodies, to prove serotype-specific infection or vaccination, and differences in VP7 antibody levels, to distinguish between infection and vaccination with any serotype. VP7 has been shown to induce good immune responses that do not seem to be essential for protection [16,43,49] and therefore is a good DIVA candidate. All calves were BTV-8 seropositive within 3 weeks following BTV-8 vaccination or infection. Furthermore, following BTV-8 challenge, high VP7-specific antibody levels were rapidly detected in the sera of all controls. VP7 antibodies were also detected in vaccinated calves, but at lower levels than controls and therefore the vaccinated and unvaccinated animals could be distinguished. Since no virus replication was detected in vaccinated calves, we believe that the observed antibody induction was due to the quantity of VP7 antigen present in the challenge virus, as has already been observed with the use of a commercial inactivated vaccine [26,50], or to limited local replication at the injection site. Based on this data, a cut-off of ≥75% can be defined to suggest BTV replication and to identify animals in which the virus can replicate sufficiently to transmit, as soon as 2–3 weeks after infection. This cut-off would probably be lower under field conditions. Our results indicate that SubV is potentially DIVA compliant under these conditions but would need to be validated with samples from naturally infected animals.

In conclusion, an experimental BTV vaccine consisting of VP2, NS1, and NS2 induced diverse immune response and is a promising candidate vaccine that provides strong clinical and virological protection against experimental BTV-8 infection in cattle. Further investigations of SubV should be performed, including exchanging or combining VP2 of other serotypes to test the vaccine's adaptable nature and evaluating the duration of immunity. The DIVA compliancy of this vaccine should also be evaluated under field conditions.

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