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Beta-propiolactone inactivated bivalent bluetongue virus vaccine containing Montanide ISA-71VG adjuvant induces long-term immune response in sheep against serotypes 4 and 16 even after 3 years of controlled vaccine storage

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ABSTRACT

In this study, we developed and evaluated the beta-propiolactone inactivated bivalent bluetongue virus (BTV) serotypes 4 and 16 vaccine delivered with Montanide™ ISA-71VG adjuvant. The safety, stability and immunological profile of the fresh and after three years of long-term storage of the vaccine formulation was analyzed. We observed after long-term storage that the vaccine emulsion was stable as indicated by unchanged pH and viscosity. The stored vaccine formulation induced virus neutralizing antibodies (VNA) in sheep against both the bluetongue virus serotypes at 7–10 day post-vaccination (dpv). VNA titers reached the peak by 60 dpv and detectable during the entire study period. Antibodies against bluetongue virus structural protein VP7 were detected by ELISA in all BTV vaccinated experimental animal groups. Partial clinical protection was observed in vaccinates against challenge virulent BTV-4 and BTV-16 serotypes by 10 dpv, while complete protection was observed at 14 dpv. The levels of viremia was decreased in challenged sheep by 10 dpv while the viremia was undetectable by 14 dpv. In summary, our newly formulated bivalent BTV (BTV-4 and BTV-16) vaccine delivered with Montanide™ ISA-71VG adjuvant was found safe and stable for over three years and induced protective response in sheep.

1. Introduction

Bluetongue (BT) is a hemorrhagic viral disease of ruminants caused by an arbovirus transmitted via *Culicoides* species of biting midges. Infection with bluetongue virus (BTV) is common in a broad geographical area across the world, which until recently stretched from ~35°S to 40°–50°N (Mellor et al., 2008; Wilson and Mellor, 2009). Currently, there are 27 different serotypes of BTV prevalent (Jenckel et al., 2015) and they induce partial cross-protection in infected animals. BTV infects both domestic and wild ruminants and causes severe clinical sickness in sheep (Maclachlan et al., 2009).

Specific prophylaxis measures against BT is the most important and difficult challenge to sheep farmers against the disease outbreaks. Immunization with mono- or polyvalent vaccines containing both live-attenuated and inactivated bluetongue viruses is the most efficient method of controlling BT in endemic regions (Noad and Roy, 2009). Because of certain risks associated with the use of live vaccines such as teratogenicity, reversion to virulence, interfering with immunity and genetic recombination among co-circulating serotypes; inactivated virus vaccines are an important and safe alternative. Such vaccines have been in use in many European countries to control BT outbreaks and reduce BTV viremia and virus circulation among exposed herds (Noad and Roy, 2009).

In this study, we developed and evaluated an inactivated bivalent

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BTV vaccine containing serotypes 4 (BTV-4) and 16 (BTV-16) to control the disease in the Republic of Kazakhstan. Commercially available BT vaccines containing serotype 8 (BTV-8) are not protective in Kazakhstan since this serotype is not epidemiologically relevant in the country, wherein serotypes BTV-4 and BTV-16 are predominent (Abduraimov et al., 2009). The inactivated BTV vaccine developed by us is though not much different from previously developed vaccines, it had commercial adjuvant Montanide™ ISA-71VG to potentiate the vaccine immunogenicity. We analyzed the physical characteristics of the vaccine emulsion including its stability and viscosity. In sheep, we assessed the safety, immunogenicity and protective efficacy of the vaccine formulation after both short and long-term storage. Our results identified immunogenic ability of our long-term storage vaccine indicated by induced protection in sheep against challenge BTV-4 and BTV-16 infections.

2. Materials and methods

2.1. Virus and cells

Serotypes BTV-4 (Khuroson-07/4) and BTV-16 (RT/RIBSP-07/16) from the Microbial Collection of the Research Institute for Biological Safety Problems (RIBSP) were used in this study. These strains were originally isolated from sheep with BT in the Republic of Tajikistan in the course of BT surveillance in 2007. Detailed descriptions of these serotypes have been provided previously (Abduraimov et al., 2009). Virus stock titers were 7.50 ± 0.11 $\log_{10}$ $TCID_{50}$/mL for BTV-4 and 7.62 ± 0.13 $\log_{10}$ $TCID_{50}$/mL for BTV-16. BTV were cultured in Vero cells (Taranov et al., 2010).

2.2. Virus inactivation and analysis of its complete inactivation

Inactivation of BTV with beta-propiolactone (BPL) was performed as described previously (Parker et al., 1975) with slight modifications (Zhugunissov and Zhunushov, 2017). Briefly, the viral suspension (VS) was inactivated with BPL at a final concentration of 0.1% (v/v). To confirm the virus inactivation the inactivated virus suspension was inoculated onto Vero cells grown in 150 ml tissue culture flasks in 50 ml of complete tissue culture media. Infected cells were cultured for 7 days at 37 °C in a 5% CO_2 atmosphere followed by three consecutive passages of supernatants under the same culturing conditions. After passages monolayers were monitored daily for the presence of cytopathic effect (CPE) to reveal the presence of BTV (Zhugunissov and Zhunushov, 2017).

2.3. Vaccine formulation and long-term storage conditions

BTV-4 and BTV-16 were pooled at equal virus titers ratio and inactivated as described above. The inactivated BTV suspension pool was mixed with Montanide™ ISA-71VG oil adjuvant at ratio 3:7 (weight/weight) with constant stirring at 800 rpm for 20 min at room temperature. The mixture was then spun at 3500 rpm for 10 min at room temperature until the homogenous emulsion was formed. A total of 100 vials of the vaccine stock were prepared for testing purposes. Fifty of these vaccine stock vials were used to test safety and immunogenicity of the formulation in sheep after production (***Experiment #1***). The additional 50 vaccine stock vials were stored for a period of 3 years at 2–8 °C. After this long-term storage period the vaccine was tested for safety and immunogenicity in sheep (***Experiment #2***).

2.4. Physical properties of the vaccine after short- and long-term storage

pH of the vaccine stock vials was determined by using the pH-meter C830 (Consort, Belgium) at 25 °C in three independent measurements. The viscosity of vaccine stock preparations was measured using a viscosimeter (VPZh-2, Russia) according to the manufacturer's instruction and data were expressed in mm^2/s. Stability of the vaccine emulsion was controlled according to the recommendations provided by SEPPIC company (Castres, France) based on a rapid centrifugation method as previously described (Tabynov et al., 2008). The vaccine emulsion was considered stable if the height of oil fraction in a vial did not exceed 10% of the total emulsion height.

Table 1
Physical parameters of the emulsified bluetongue virus vaccine.

Vaccine composition	Parameters	Short term storage vaccine	Long term storage vaccine	P-value
ISA-71VG + antigen	pH	7.21 ± 0.22	7.01 ± 0.27	ns
	Viscosity (mm^2/s)	35.5 ± 0.18	28.2 ± 0.11	< 0.0001
	Stability of the emulsion	stable	stable	na

2.5. Use of animals in safety and vaccine trials

To test the safety and immunogenicity profiles of the BTV vaccine, Kazakh fine-fleeced sheep of 6–12 months of age were used. Sheep were obtained from herds free of BT and seronegative to BTV. At arrival to the RIBSP animal facility sheep were clinically examined, blood samples collected and ear-tagged for identification. Sheep were acclimated at the facility for 2 weeks prior to start of the experiments. For safety study, a group of five sheep were vaccinated with the inactivated virus (7.5 $\log_{10}$ TCID50/dose) vaccine with adjuvant via intramuscular (IM) route at the inner side of the thigh, while a group of three animals served as a non-vaccinated control group. Sheep were examined individually for local reactions at the site of inoculation and for any signs of systemic adverse effects. For vaccine trials, a group of 30 sheep were vaccinated with the inactivated BTV (7.5 $\log_{10}$ $TCID_{50}$/dose) with adjuvant IM.

2.6. Humoral response analysis in vaccinated sheep

Blood samples were collected at day post-vaccination (DPV) 7, 10, 14, 21 and 28 and once a month during rest of the study period for BTV antibody analysis using a commercial ELISA kit (cELISA, ID-Vet, Montpellier, France). For VNA analysis serum samples collected at 7 dpv (n = 6), 10 dpv (n = 12) and 14 dpv (n = 12) were used. Protective efficacy of the vaccine was assessed by virulent BTV-4 or BTV-16 challenge infection in a ABSL3 facility. Animals were randomly divided into smaller size groups and challenged intravenously with 5.5 $\log_{10}$ $TCID_{50}$ per animal of BTV-4 at 7 dpv (n = 3), 10 dpv (n = 6) and 14 dpv (n = 6); or with BTV-16 at 7 dpv (n = 3), 10 dpv (n = 6) and 14 dpv (n = 6). Non-vaccinated sheep were divided into 2 groups of 9 animals each and exposed to BTV-4 or BTV-16 as described above. Viremia was monitored by RT-PCR in blood samples collected at 2, 4, 6, 8, 10, 12 and 14 days post-challenge (dpc).

2.7. Determining the prolonged efficacy of the BTV vaccine

The duration of protection elicited by the BTV inactivated-adjuvant vaccine in sheep was assessed for a period of 730 days. Sheep were vaccinated as described above, transferred to ABSL3 facility and divided into 8 groups of 5 animals each. The groups were challenged with BTV-4 or BTV-16 via IV with 5.5 $\log_{10}$ $TCID_{50}$/animal at dpv 180, 360, 480 or 730 as described above. Non-vaccinated control animals of the same age were also challenged at similar time points. BTV challenged animals were clinically monitored for 30 days after challenge as previously described (Zhugunissov et al., 2015). Vaccine immunogenicity was evaluated by comparing the reactions of the vaccinated and unvaccinated sheep to infectious virus challenge. The control infected sheep should score at least 10 points after infection. A difference

Table 2
Safety of the vaccine for sheep.

Animals	Animal number	Swelling*	Fever	Stomatitis	Diarrhea	Conjunctivitis	Nasal secretions	Sialorrhea	Depletion
Short-term storage vaccine									
Vaccinated sheep	1	+	+**	–	–	–	–	–	–
	2	+	–	–	–	–	–	–	–
	3	+	–	–	–	–	–	–	–
	4	+	–	–	–	–	–	–	–
	5	+	–	–	–	–	–	–	–
Control sheep	6	+	–	–	–	–	–	–	–
	7	+	–	–	–	–	–	–	–
Long-term storage vaccine									
Vaccinated sheep	1	+	–	–	–	–	–	–	–
	2	+	–	–	–	–	–	–	–
	3	+	–	–	+***	–	–	–	–
	4	+	–	–	–	–	–	–	–
Control sheep	5	+	–	–	+****	–	–	–	–
	6	+	–	–	–	–	–	–	–

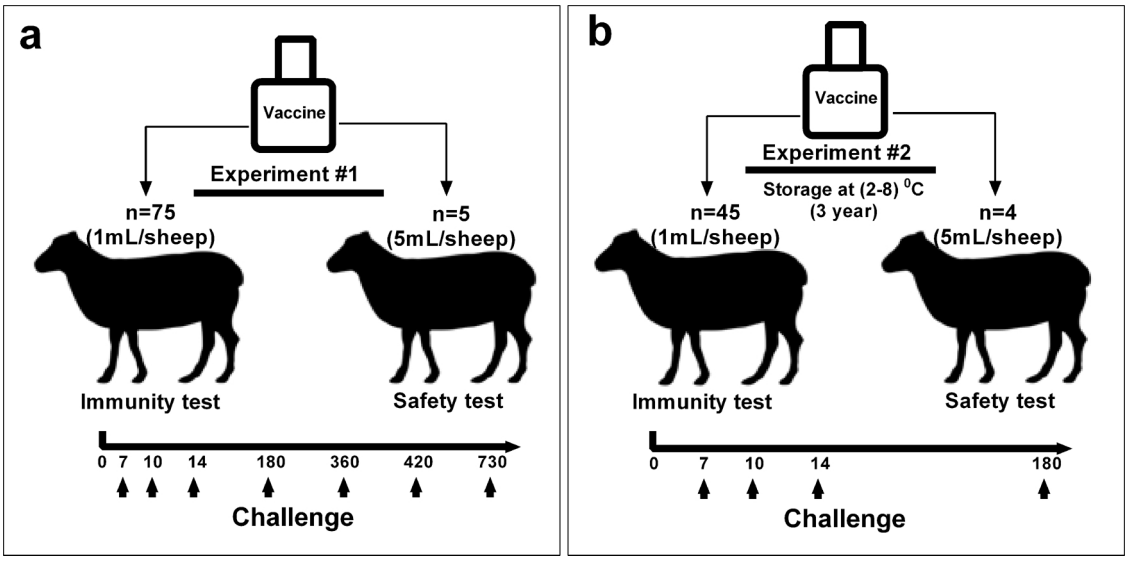

Fig. 1. The experimental design followed in the study. **(a)** The short-term storage vaccine study (experiment #1): During this study in five out of 75 sheep VNA titers were evaluated over a period of 730 days. Early response to vaccine was determined in 30 sheep and the remaining 40 sheep were used for analyzing the duration of vaccine efficacy against virulent challenge strains infection. **(b)** The long-term stored vaccine efficacy was evaluated after storing the formulation for 3-years at 2–8 °C (experiment #2): During this trial five out of 45 sheep were used to evaluate VNA titers over a period of 180 days. Early response to vaccine was determined in 30 sheep and the remaining 10 were used for analyzing the duration of vaccine efficacy against virulent challenge strains infection.

between the vaccinated and unvaccinated sheep was considered insignificant if the average difference in the scores was 0–7 points, weak if the difference was 7–12 points, moderate if the difference was 12–16 points and pronounced if the difference was > 16 points.

2.8. ELISA

BTV-specific antibodies directed against VP7 were screened in sheep sera via a competitive ELISA (cELISA, ID-Screen Bluetongue Early detection ELISA, ID-Vet, Montpellier, France). Tests were performed according to the manufacturer's instructions. For this study, a threshold value of 40% negativity (PN%) was used to discriminate between positive (PN% < 40) and negative (PN% ≥ 40) BTV ELISA results.

2.9. Serum neutralization test (SNT)

SNT was performed to detect VNA titeres according to the method described by Haig and Maraa (1956) using serotypes BTV-4 and BTV-16. Serotype-specific BTV-4 and BTV-16-positive and negative antisera were used as control. Briefly, sera were diluted (1:2 to 1:128) in 96-well plates and VNA titers were estimated against 100 $TCID_{50}$ of BTV-4 or BTV-16. Plates were incubated for 1 h at 37 °C and maintained at 4 °C overnight. After incubation, 50 µL of Vero cells suspension containing 2×10^5 cells/mL was added to each well and plates were incubated for 4–7 days at 37 °C 5% with CO_2 atmosphere. Plates were screened for the presence of BTV induced cpe. Neutralization titers were determined as the inverse of sera dilution giving 50% neutralization end point.

2.10. BTV qRT-PCR

Total RNA was extracted from 140 µl of complete blood using the QIAmp Viral RNA Mini Kit (Qiagen, Germany) according to the manufacturer's instructions. Extracted RNA was denatured at 100 °C for 5 min followed by rapid cooling on ice and used immediately for qRT-PCR or stored frozen at −20 °C until tested. A TaqMan based assay was used to amplify BTV genome segment 5. The following primers and a probes were used: BTV-S5-F (5′-ggcaacyaccaaacatgga-3′), BTV-S5-R (5′-aaagtyctcgtggcattwgc-3′) and BTV-S5-probe (5′-FAM-cyccactgatrttg-tattttctcaa-TAMRA-3′) (Toussaint et al., 2007). Amplification of BTV RNA was performed with Superscript® III Platinum One-Step System (Thermo Fisher Scientific, USA) according to the manufacturer's manual in aRotor-Gene 6000 Series thermlcycler (Qiagen, Germany)

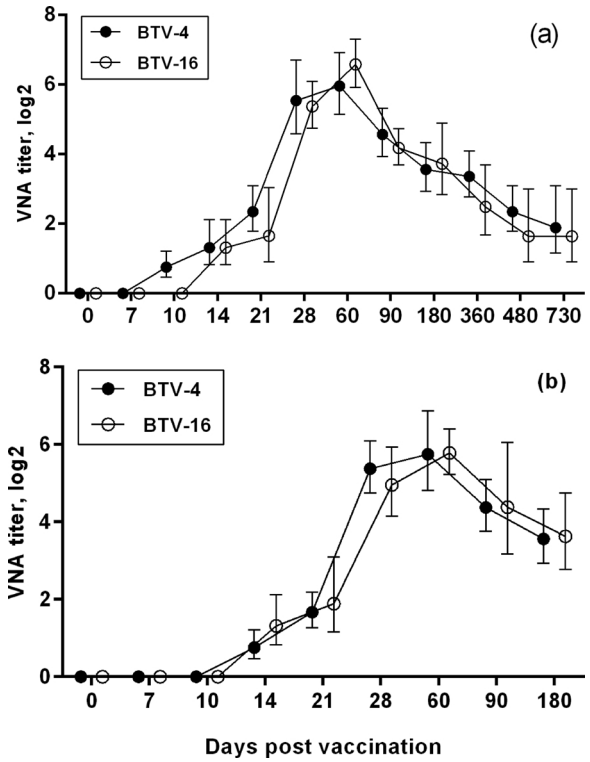

Fig. 2. Serum neutralization titers in the bluetongue virus vaccine received sheep with short-term storage (**a:** experiment #1) and long-term storage (**b:** experiment #2).

under the following cycling conditions: 1 cycle of reverse transcription at 55 °C for 30 min, 1 cycle of 95 °C for 10 min followed by 50 cycles of 95 °C for 15 s, 58 °C for 30 s.

2.11. Statistical analysis

Statistical analyses were performed with Graph Pad Prism® version 6.0. Two-way analysis of variance was used to compare rectal temperatures, clinical scores and serology of BTV exposed groups. A P value ≤ 0.05 was considered statistically significant. Mean values of clinical scores in affected sheep and standard error were also calculated. Difference in the results of scores of sickness signs between the groups was calculated with the Student's criterion where P ≤ 0.05 was considered reliable. Difference in effectiveness between groups was compared by one-sided Fisher's exact test for two proportions at a significance level of alpha < 0.05.

3. Results

3.1. BTV vaccine formulation was found stable after long-term storage

These studies were conducted to compare the changes in the physical characteristics of the vaccine at short-term and long-term storage. pH of the vaccine was decreased after long-term storage from 7.21 ± 0.22 to 7.01 ± 0.27 (Table 1). At the time of production the viscosity of the vaccine was 35.0 ± 0.18 mm^2/s while after long-term storage a lower value of 28.5 ± 0.11 mm^2/s was observed resulting in significant decrease (P < 0.0001) of this parameter. However, the vaccine emulsion remained stable meeting the standards of the adjuvant's manufacturer after storage.

3.2. Inactivated BTV and adjuvant formulation were found safe to use in sheep

Any new vaccine candidates should be tested for safety in susceptible target animals. In vaccinated sheep we recorded results of any general systemic and local reactions at the site of administration. While others also evaluate productivity of the animal after administration of vaccine (Emidio et al., 2004; Gethman et al., 2009). None of the vaccinated animals in all our trials showed anu local or systemic adverse effects to vaccine formulation. Only 1 out of 5 animals vaccinated with a short-term storage vaccine had elevated body temperature of 40.8 °C between 7–8 dpv. At the site of vaccine inoculation in all the vaccinated animals inflammatory effect was minimal which resolved within 5–7 dpv (Table 2). Thus overall, we found safe to use both short-term and long-term stored vaccine formulations in sheep with no untoward reactions.

The nature of BTV inactivated-adjuvant vaccine formulation was tested in sheep after a short-term storage (Fig. 1a) and long-term storage of 3 years at 2–8 °C (Fig. 1b). For safety studies, a group of 4 sheep were vaccinated by IM route and 3 were kept as non-vaccinated negative control. Sheep were observed individually at the site of inoculation for adverse reactions and recorded signs of systemic adverse reactions. Immunogenicity studies of the vaccine in sheep including duration of antibody response, early response and protective efficacy against wild-type virulent challenge BTV infection were evaluated under the same experimental conditions described above (Fig. 1).

3.3. Candidate BTV vaccine induced humoral response even after long-term storage

Study of the levels of VNA titers is important in assessing the effectiveness of vaccine in animals. Vaccine-induced VNAs were detected early by 7 dpv with titers raging between 0.1-0.2 log_2. These titers increased between 10 dpv to 60 dpv from 0.83-0.9 log_2 to 6.1–6.4 log_2,

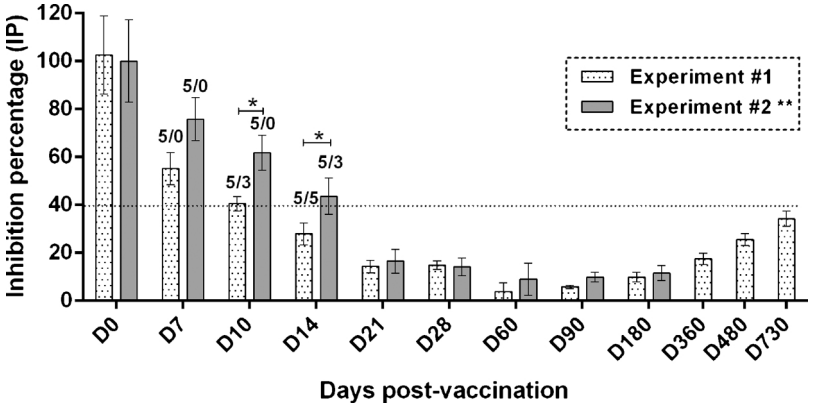

Fig. 3. Humoral immune response analysis in serum samples of sheep. Inhibition percentage of **< 40%** was considered positive and **≥ 40%** was considered as negative. Asterisk determines statistical significance P < 0.05.

Table 3
Onset of immunity and duration of the protective immune response in sheep immunized with the short-term and after 3-years of controlled storage of inactivated bivalent bluetongue virus vaccine.

Terms of the challenge post vaccination	Mean clinical scores in challenged sheep						% Vaccine effectiveness (number of sheep in the experiment/affected animals)			
	BTV-4			BTV-16			BTV-4		BTV-16	
	Vaccinated	Control	Points (score) difference	Vaccinated	Control	Points (score) difference	Vaccinated	Control	Vaccinated	Control
Short-term storage vaccine										
D7	19.6 ± 2.08	23.3 ± 1.53	3.7	20.3 ± 0.57	23.6 ± 2.31	3.3	0 (3/3)	0 (3/3)	0 (3/3)	0 (3/3)
D10	7.3 ± 10.31*°	24.0 ± 0.00	16.7	7.8 ± 8.7*°	24.3 ± 3.33	16.5	50 (6/3)	0 (3/3)	50 (6/3)	0 (3/3)
D14	0 ± 0.00*	26.3 ± 1.15	26.3	0 ± 0.00*	22.0 ± 2.66	22.0	100 (6/0)	0 (3/3)	100 (6/3)	0 (3/3)
D180	0.0 ± 00*	25.8 ± 0.84	25.8	0.0 ± 0.00*	25.2 ± 1.30	25.2	100 (5/0)	0 (5/5)	100 (5/0)	0 (5/5)
D360	0.0 ± 00*	25.8 ± 1.09	25.8	0.0 ± 0.00*	26.2 ± 1.09	26.2	100 (5/0)	0 (5/5)	100 (5/0)	0 (5/5)
D480	0.2 ± 0.45*	25.8 ± 1.09	25.6	0.2 ± 0.45*	26.2 ± 0.83	26.0	80 (5/1)	0 (5/5)	80 (5/1)	0 (5/5)
D730	0.40 ± 0.89*	26.0 ± 0.71	25.6	0.6 ± 1.34*	26.2 ± 1.30	25.6	80 (5/1)	0 (5/5)	80 (5/1)	0 (5/5)
Long-term storage vaccine										
D7	18.6 ± 2.82	25.0 ± 1.00	6.4	19.0 ± 1.00	25.0 ± 1.00	6.0	0 (3/3)	0 (3/3)	0 (3/3)	0 (3/3)
D10	13.7 ± 9.45*	25.0 ± 2.65	11.3	15.8 ± 7.88*	25.5 ± 1.04	9.7	33.4 (6/4)	0 (3/3)	16.7 (6/5)	0 (3/3)
D14	0 ± 0.00*	23.0 ± 2.65	23.0	0 ± 0.00*	24.5 ± 2.17	24.5	100 (6/0)	0 (3/3)	100 (6/0)	0 (3/3)
D180	0.0 ± 00*	24.2 ± 1.64	24.2	0.0 ± 00*	25.2 ± 1.30	25.2	100 (5/0)	0 (5/5)	100 (5/0)	0 (5/5)

The immune reaction was assessed according to 30-points scale of clinical (sickness) signs (Zhugunissov et al., 2015). The levels of immunity was assessed by clinical reaction (in points) in control and vaccinated animals: 0–7 points – no immunity; 7–12 points – weak immunity; 12–16 points – moderate immunity; over 16 points – pronounced immunity.
(*) – from $P \leq 0.05$ to $P < 0.0001$ vs. appropriate control groups.
(°) – $P < 0.001$ vs. appropriate day post-vaccination in experiment #2.

respectively. A decline in VNA titers was observed by 90 dpv to 5.2-4.5 $\log_2$ dropping steadily towards 730 dpv to 3.0-2.8 $\log_2$ (Fig. 2a). A significant difference in VNA titers between BTV-4 and BTV-16 was not observed ($P \geq 0.05$). Animals vaccinated with the long-term stored vaccine showed a slightly different pattern of VNA response (Fig. 2b). For instance, the onset of VNAs was observed three days later by 10 dpv with titers ranging between 0.5-0.6 $\log_2$. VNA titers peaked by 60 dpv (5.6–5.8 $\log_2$) and decreased thereafter. Collectively, VNA titers were not different ($P \geq 0.05$) among animals vaccinated with both the BTV serotypes.

Animals vaccinated with the stored vaccine showed BTV antibodies by 10 dpv (3/5 sheep) or 14 dpv (3/5 sheep) (Fig. 3). The number of reactors differ significantly from 10 dpv to 14 dpv with animals vaccinated, with the stored vaccine being overall less reactive between those time points. However, by 21 dpv all vaccinated animals tested positive in VP7-based ELISA. Furthermore, sheep tested positive until 730 dpv.

3.4. Inactivated BTV-adjuvant formulation induced protective efficacy in virus challenge trials

We evaluated both early response and duration of protective efficacy in naturally susceptible animals of BTV against virulent challenge virus infections. Onset of specific immune response induced by the vaccine formulation against BTV-4 and BTV-16 challenge infection was noticed by 10 dpv and reached the protective levels against clinical BT by 14 dpv which lasted until 360 dpv (Table 3). A decline in the protective capability induced by vaccination was observed by 480 dpv wherein approximately 80% of the animals were protected. However, protection against BTV challenge was still noticed at 730 dpv. Animals vaccinated with long-term stored vaccine showed similar levels of protection, and they were protected against BTV-4 or BTV-16 challenge at 10 dpv. Overall the number of protected animals was relatively less when the stored vaccine was used. In all cases non-vaccinated control sheep developed BT showing increased body temperature (41.1–41.5 °C), stomatitis, hyperemia of the visible mucosa, nasal and ocular discharge, dyspnea, edema in the head and neck, lameness, diarrhea, anorexia and cachexia.

3.5. Challenge BTV load was reduced in vaccinated sheep

We analyzed the levels of viremia in animals during early days post-vaccination. Generally, in sheep infected with BTV viremia persists for 7 days (Ramakrishan et al., 2005), and others reported replication of the challenge virus in vaccinated animals (Savini et al., 2007; MAPA Report, 2006; Eschbaumer et al., 2009). Blood samples collected from vaccinated and non-vaccinated sheep were negative for BTV RNA by qRT-PCR. While blood samples collected from virus challenged sheep at 7 dpv were tested positive for BTV RNA between dpc 4 to 14 (Fig. 4a, c). Whereas viral RNA was not detected in blood from animals challenged either at 10 dpv or at 14 dpv (Fig. 4f, h, k, m). Viral RNA was readily detected in blood of non-vaccinated control animals between dpc 4 to 14. A similar trend was observed with sheep vaccinated with the stored vaccine and challenged with BTV. Viral RNA was detected in animals challenged at 7 dpv (Fig. 4b, d), as well as in blood of 4 sheep challenged at 10 dpv in 6 dpc and 8 dpc (Fig. 4g, h). No BTV RNA was detected in sheep that were challenged at 14 dpv (Fig. 4l, n). As expected, BTV RNA was detected in blood of non-vaccinated/challenged control animals as early as 4 dpc.

4. Discussion

Significant increase in the immunogenicity induced by inactivated vaccines are due to the inclusion of adjuvants or immunostimulants in the vaccine formulation (Aucouturier et al., 2001). Adjuvants such as aluminium hydroxide, saponin, Montanide™ ISA-206 and Montanide™ ISA-50 have been in use in the production of inactivated bluetongue virus vaccines (Savini et al., 2007, 2009; Emidio et al., 2004; Ramakrishan et al., 2005; Pandey et al., 2006; Bhanuprakash et al., 2009). These vaccine preparations have been proven safe and immunogenic in sheep and cattle. To develop a potent BT vaccine that suits the needs of Kazakhstan, we focused on two epidemiologically relevant BTV serotypes; BTV-4 and BTV-16. These viral serotypes were chemically inactivated by using beta-propiolactone and combined with a mineral oil adjuvant Montanide™ ISA-71VG as per the recommendation of Seppic Co. (France). The adjuvant role of Montanide™ ISA-71VG has been shown before for vaccines used in sheep and rabbits

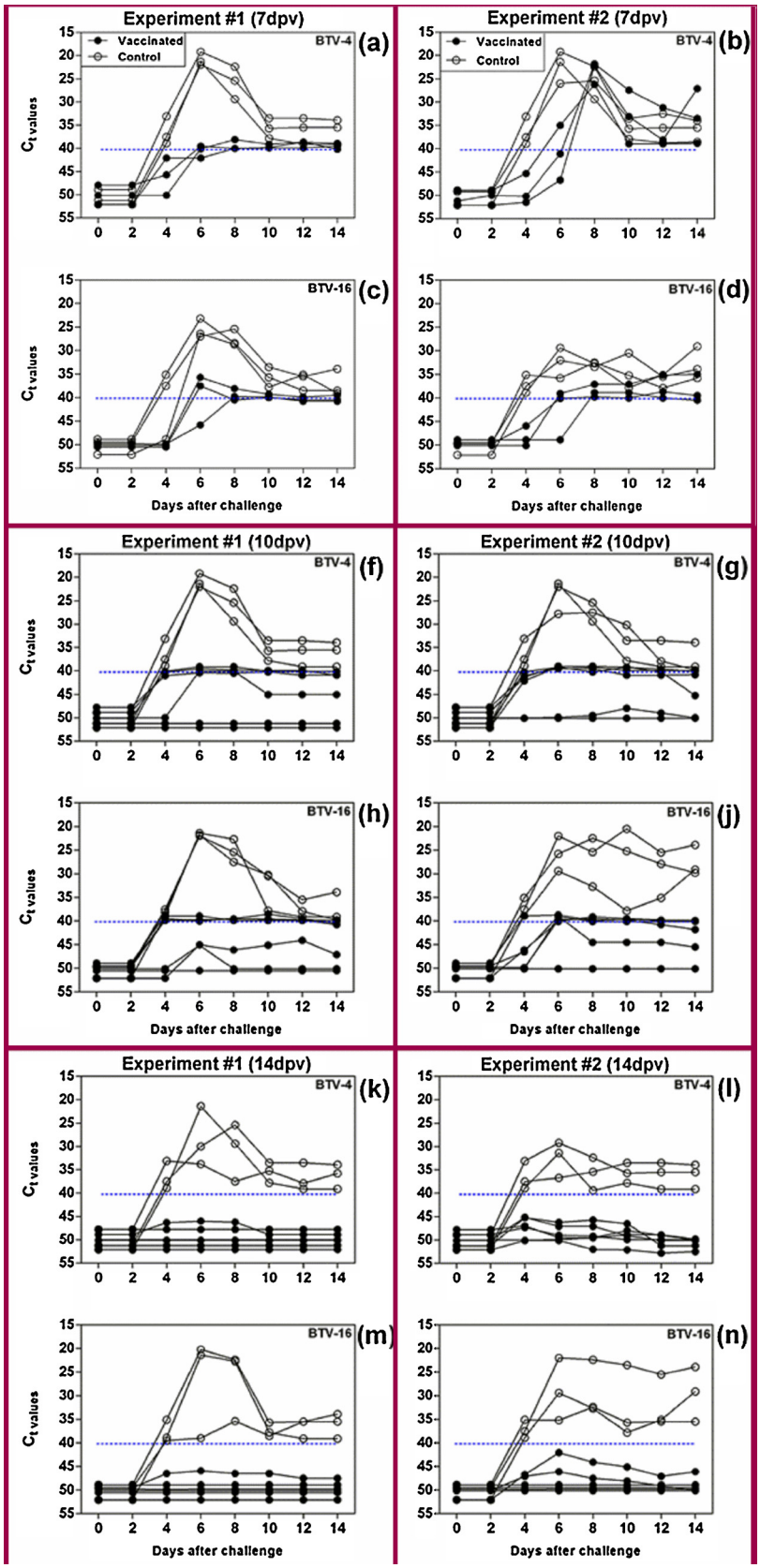

Fig. 4. Determination of virulent BTV-4 and BTV-16 load in experimental vaccinated sheep at different days post-vaccination (dpv) by RT-PCR. **(a)** Experiment #1 - vaccinated and control sheep challenged with BTV-4 at dpv 7. **(b)** Experiment #2 - vaccinated and control sheep challenged with BTV-4 at dpv 7. **(c)** Experiment #1 - vaccinated and control sheep challenged with BTV-16 at dpv 7. **(d):** Experiment #2 - vaccinated and control sheep challenged with BTV-16 at dpv 7. Each vaccinated and control sheep group had three animals each. **(f):** Experiment #1 - vaccinated and control sheep challenged with BTV-4 at dpv 10. **(g)** Experiment #2 - vaccinated and control sheep challenged with BTV-4 at dpv 10. **(h)** Experiment #1 - vaccinated and control sheep challenged with BTV-16 at dpv 10. **(j)** Experiment #2 - vaccinated and control sheep challenged with BTV-16 at dpv 10. Number of animals in vaccinated group (n = 6) and control group (n = 3). **(k)** Experiment #1 - vaccinated and control sheep challenged with BTV-4 at dpv 14. **(l):** Experiment #2 - vaccinated and control sheep challenged with BTV-4 at dpv 14. **(m):** Experiment #1 - vaccinated and control sheep challenged with BTV-16 at dpv 14. **(n):** Experiment #2 - vaccinated and control sheep challenged with BTV-16 at dpv 14. Number of animals in vaccinated group (n = 6) and control group (n = 3). **C_t value up to 40 –** positive; **C_t value 41** and **above** – negative result.

(Zhugunissov et al., 2017) and birds (Jang et al., 2013; Lone et al., 2017). A potent vaccine against Bovine Respiratory Syncytial Virus has included this adjuvant (Riffault et al., 2010).

Besides safety, an effective BT vaccine should induce a rapid onset of immune response to control viral transmission and spread of the disease in an outbreak situation. At the same time, the vaccine should induce a response that can eliminate or minimize the length of viremia and virus titers in blood. It has been reported that a single

immunization of sheep with BTV inactivated vaccine induced a strong immune response that protected animals against virulent BTV for a period of one year (Hamers et al., 2009). In terms of rapid onset of immunity, a single immunization with an inactivated vaccine prevented viremia in animals upon challenge with a virulent BTV at 14 dpv (MAPA Report, 2006). The length of immunity induced by this type of vaccines can be significant, and cattle immunized with a BTV-8 inactivated vaccine induced VNAs for up to 3 years (Oura et al., 2012), whereas sheep and cattle showed a cellular immune response for up to 2 years post-vaccination (Alexandra et al., 2012). Together these data suggest that effective protection against BT in livestock can be achieved by the use of inactivated vaccines.

Our vaccine formulation proved safe in sheep since no systemic or significant local adverse reactions were observed, and further even after using the vaccine formulation stored for 3 years. Moreover, this vaccine formulation retained its ideal features even after 3-years of storage under controlled conditions. In this study, BTV inactivated-adjuvant vaccine provided only partial protective immunity in sheep challenged at 7 dpv. VNAs were detected by 7 dpv albeit at low titers that may not suffice to control the IV challenge. A BTV-18 based vaccine induced detectable antibodies by 7 dpv (Ramakrishan et al., 2005), while VNAs against BTV-2 and BTV-4 based vaccines were detectable in cattle and sheep between 14–28 dpv (Savini et al., 2008; Hamers et al., 2009). However, the protective efficacy of induced VNAs was not confirmed via challenge with a virulent BTV in those studies. VNAs indeed play a key role on protection against BT clinical signs and BTV viremia, however the correlation between neutralizing antibody titers and protection is not well established (Savini et al., 2008). It has been observed that inactivated vaccines may provide protection even in the absence of detectable VNAs (Stott et al., 1979).

In our study, viremia was detected in the group of animals challenged at 7 dpv. The length of viremia and virus titers in blood were similar in both vaccinated and non-vaccinated groups. However, animals challenged at 10 dpv had no detectable viral RNA in blood. Interestingly, vaccine storage seems to affect viremia since BTV RNA was detected in blood of animals challenged at 10 dpv albeit for a brief period of time. Consistent to our study, BTV RNA in blood was detected by others after early time point challenge (Ramakrishan et al., 2005) or not detected (Savini et al., 2007; MAPA Report, 2006; Eschbaumer et al., 2009).

Our BTV inactivated-adjuvant vaccine induced complete protection in challenged sheep by 14 dpv. This protective response by using one dose of the inactivated vaccine extended up to 480 dpv, wherein 80% of sheep were protected against BT challenge infection. Importantly, the protection achieved by one dose of the vaccine was noticeable up to 730 dpv (~2 years). The extent of protection is a key feature of the vaccine in the context of Central Asia where breeding sheep, goats and cattle under free-range management is rather a common practice. The inclusion of Montanide™ ISA-71VG as adjuvant in the formulation of vaccine proved critical as it helped in rapid and long-lasting protection against BTV, and observed even when the vaccinated animals were subjected to an highly stringent IV challenge infection.

Conflict of interest

The author(s) declared no potential conflicts of interest with respect to the research, authorship and/or publication of this article.

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