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Safety and efficacy of a Bluetongue inactivated vaccine (serotypes 1 and 4) in sheep

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ABSTRACT

A new inactivated vaccine against Bluetongue virus (BTV) serotypes 1 and 4, was developed from field isolates. Safety and efficacy of the vaccine were evaluated in sheep by serological monitoring and virus nucleic acid detection after experimental infection of vaccinated animals. Seroconversion was observed in vaccinated animals at day 14 post vaccination (pv) with neutralizing antibody titer of 1.9 and 1.8 for serotypes 1 and 4, respectively. The titer increase significantly after the booster reaching 2.7 and persist one year >1.5 for both serotypes. After challenge with virulent isolates, vireamia was recorded in control animals, as evident by q-PCR with threshold cycles (Ct) ranging from 24 to 31 and peaked at day 10 post challenge, while no vireamia was detected in vaccinated animals. Vaccinated sheep were fully protected against the disease and infection.

1. Background

Bluetongue (BT) is a vector-borne disease of ruminants, caused by an Orbivirus within the Reoviridae family and transmitted by Culicoides spp (Mertens et al., 2005). The virus cause severe clinical disease and serious economic impact of major importance in the international trade of animals and animal products. The disease is serious in sheep while goats, cattle and camelids usually remain asymptomatic (Dungu et al., 2004; Eschbaumer et al., 2012; Batten et al., 2011). The disease severity varies also between breeds of the same species (DeMaula et al., 2001; DeMaula et al., 2002; MacLachlan, 2004). BTV infection occurs throughout temperate and tropical regions and is characterized by fever, congestion, edema, hemorrhage, hyperthermia and ulceration of the oral mucosa (Roy, 2002). BTV has a high level of antigenic variation, there are 29 serotypes recognized worldwide, distinguishable on the basis of serotype-specific virus neutralization assays (SNTs), with low level of cross-protection (Schwartz-Cornil et al., 2008; Kalyani et al., 2019; Maan et al., 2016; Lakshmi et al., 2018). Vaccination is the preferred method for BT control (Maclachlan and Mayo, 2013). Currently, two types of BT vaccines are used, live-attenuated vaccines (LAVs) and inactivated vaccines (van Rijn, 2019). Live-attenuated vaccines have been extensively used in the past in endemic areas for different serotypes. However, their use is now limited in several countries because of insufficient attenuation leading to clinical disease, risk of diffusion to contact animals and reassortment between the vaccine and the field strains (Van Den Bergh et al., 2018; Savini et al., 2008b; Bréard et al., 2007). The use of inactivated vaccines is an advantageous alternative, they are completely safe and have proved to be highly efficacious in eradication of the disease in many Europeans countries (Stott et al., 1985; Emidio et al., 2004; Savini et al., 2008a). Inactivated vaccines are commercially available for few serotypes 1, 2, 4, 8, 9, 10, 16, 18 and 23. (Ramakrishnan et al., 2006; Pandey et al., 2006; Savini et al., 2007; Savini et al., 2008b; Savini et al., 2009; Wäckerlin et al., 2010; Bréard et al., 2011; Garcia et al., 2011; Moulin et al., 2012; Zientara and Sánchez-Vizcaíno, 2013); A pentavalent inactivated vaccine (BTV-1, 2, 10, 16 and 23 serotypes) have been used in India (Bitew et al., 2017; Bitew et al., 2019).

In this paper, we developed a combined BTV-1 and 4 inactivated vaccine that has been tested for safety and efficacy based on neutralizing antibody response to vaccination and experimental infection in sheep.

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2. Material and methods

2.1. Vaccine preparation

Field isolates of BTV1 and BTV4 have been used to produce viral suspensions after infection of BHK cells on suspension. Virus propagation for antigen preparations were carried out separately for serotype 1 and 4, in stirred bioreactors. A working volume of 6 L containing 2.10^6 cells mL^{-1} of BHK/AC9 suspension cells from European Collection of Authenticated Cell Cultures (ECACC), was inoculated with a 0.01 MOI of BTV serotypes and the virus suspension was harvested three days after, and tested for identity and purity. Virus titration was carried out on Vero cells (African green monkey kidney cells, ATCC No.CCL-81), by serial dilutions of the harvested virus inoculated to cells as described in the OIE Terrestrial Manual (2021). Virus suspensions of BTV1 and BTV4 were inactivated using Binary Ethylenimine (BEI) as described previously (Es-sadeqy et al., 2021). The vaccine was prepared by mixing equal volume of BTV1 and BTV4 inactivated antigens with aluminium hydroxide (8.4 mg) and saponin (0.6 mg) as adjuvants per dose. Formulation was carried out by mixing inactivated antigens with aluminium hydroxide at +4 °C overnight, then saponin was added to complete the vaccine formulation. Three vaccines were prepared, two monovalent ones based on antigens of BTV1 and BTV4 respectively, and a combined one containing both serotypes.

2.2. Animals

The experimental protocol was approved by the Internal Laboratory Ethic Committee; the international guidelines were followed for caring and handling of experimental animals as described in Chapter 7.8 of the Terrestrial Animal Health Code and Directive 2010/63/UE of the European commission (Directives EU Commission, 2010; OIE and Terrestrial Animal Health Code, 2016).

Thirty-two sheep, of indigenous Sardi breed, aged about three months, acquired from a recognized breeding farm with no history of BTV infection or vaccination have been selected for this experiment. Animals, maintained in an insect proof building before starting the study, were allowed to acclimatize for two weeks under observations and monitored daily for hyperthermia and appearance of any non-specific clinical signs. Food and water were available *ad libitum*. Prior to vaccination, animals were tested negative for the presence of BTV antibodies by ELISA as described hereafter. Animals were randomly divided into four groups (Gr), Gr. 1 of 8 sheep were vaccinated with the monovalent BTV1 vaccine, Gr. 2 (8 sheep) vaccinated with the monovalent BTV4 vaccine, Gr. 3 (8 sheep) vaccinated with the bivalent BTV1 + BTV4 vaccine and Gr. 4 (8 sheep) kept unvaccinated. Vaccines were injected by subcutaneous route at a dose of 2 mL per animal followed with a booster at day (D) 28. Sheep of each group were housed in separate boxes. Body temperature and general health conditions were monitored for two weeks after each vaccination to evaluate safety. Serum samples were collected by jugular venipuncture at regular intervals at days 7, 14, 21, 28, 35, 42, 2 months and every month up to 12 months pv. Sera were tested for antibody by ELISA and antibody titers were determinated by SNT.

2.3. Laboratory testing

SNT was carried out according to the Chapter 3.1.3 of the OIE Manual of Diagnostic Tests and Vaccines for Terrestrial animals (2019) with slight modifications (Es-sadeqy et al., 2021).

Antibody anti-VP7 of BTV in vaccinated sheep sera were also monitored using a commercial competitive ELISA kit (ID SCREEN® Bluetongue Competition ELISA, IDVet) in accordance to the manufacturer's instructions where sera presenting an inhibition percentage (PI) of $\leq$ 40% were considered positive.

2.4. Challenge study

Virulent strains BTV1 (BTV1 ALG 2006/01) and BTV4 (BTV4 SPA 2004/01) were used in the experimental infection of vaccinated sheep. Strains were kindly provided from Laboratorio Central de Veterinaria at Algete, Madrid. BTV1 was isolated during the 2006 outbreak in Algeria that affected also Morocco and Spain, while BTV4 was isolated during 2004 outbreak in Spain that have also been notified in North Africa the same year.

Virulent serotypes used for challenge were prepared using monolayer BHK-21 cells. Cells maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 5% of irradiated fetal bovine serum (FBS), were infected with a Multiplicity Of Infection (MOI) of 0.01. Inoculums were harvested 5 days post infection (dpi) and titrated on Vero cells. The inoculated doses of the challenge strains BTV1 and BTV4 were adjusted to 10^7 $TCID_{50}$/mL.

At 12 months pv, 8 vaccinated sheep with the bivalent BTV1 + 4 vaccine and 4 unvaccinated control animals were transferred to Biosafety level-3 animal facilities (ABSL-3) for experimental infection. Animals were randomly divided in two groups, Gr. A: 4 vaccinated sheep and 2 controls animals, Gr. B: 4 vaccinated animals and 2 controls animals. Sheep of Gr. A were inoculated with the virulent BTV-1 virus by subcutaneous injection of 2 mL of virus suspension containing 10^7 $TCID_{50}$/mL behind the elbow in each side. Sheep of Gr. B were challenged with virulent BTV-4 virus in the same conditions. Animals were observed for 26 dpi, the body temperature and clinical symptoms were recorded for 14 dpi. Clinical scoring was ranging from 0 to 4 based on the severity of hyperthermia, animal behavior, red eyes and conjunctivitis, mucous membrane congestion, edema, nasal discharge, locomotors problems, redness at the limb and aspect of the tongue, as described in Table 1. A total cumulative score of assessed signs per animal and group per day was calculated.

Blood samples collected from challenged sheep, were analysed by quantitative real time reverse transcriptase-polymerase chain reaction (RT-qPCR) to monitor viral load (Hoffman et al., 2008). Antibody titers after challenge, were evaluated using SNT. The sampling dpi were 0, 3, 6, 8, 10, 12, 14, 18, 21 and 26.

2.5. Statistical analysis

Results analysis were performed using Student *t*-test models. Serological response of bivalent vaccine was compared to monovalent vaccines. In addition, a comparison between clinical scoring of vaccinated and unvaccinated animals was carried out. Values of $p \leq 0.05$ were considered significant.

3. Results

3.1. Vaccine safety

Sheep remained healthy and did not show any sign of BT disease after vaccination. A slight increase in temperature was noticed between D2 and D6 pv in sheep of the three groups. Limited local inflammation

Table 1
Clinical scoring of recorded clinical signs.

Clinical signs		Score
Animal behavior	Normal to dead	0 to 4
Red eyes and conjonctivitis	Absence to severe	0 to 3
Mucous membrane congestion	Absence to severe	0 to 3
Edema	Absence to generalized	0 to 2
Locomotors problems	Normal to stiffness	0 to 2
Hyperthermia	Normal to 41	0 to 4
Nasal discharge	Absence to muco-purulent	0 to 3
Redness at the limb	normal to bleeding	0 to 3
Aspect of tongue	Normal to severe cyanocis	0 to 3

(1–2 cm in diameter) at the injection site was observed in vaccinated animals of the three groups starting D3 pv and disappeared in two weeks. The average temperature of each group is reported in Fig. 1.

3.2. Serological response after vaccination

With SNT, 2/8 vaccinated sheep seroconverted at D7 pv and the others 6 by D14 pv in the bivalent vaccine for both BTV1 and BTV4 serotypes. In the monovalent BTV1 vaccine, 1/8 animal seroconverted at D7 and 8/8 at D14 pv. In the monovalent BTV4 vaccine, 2/8 seroconverted at D7 and 8/8 by D14 pv. The titer increase significantly after booster reaching 2.7 and persist one year >1.5 for both serotypes (Fig. 2). By ELISA, at D7 pv 2 or 3 animals were detected positive in each group and all vaccinated animals were detected positive at D14 and remained positive for 12 months (Fig. 3). No significant difference was observed regarding response to BTV1 or BTV4 valences between monovalent and bivalent vaccines (P value > 0.05). Animals of Gr 4 remained negative until the challenge.

3.3. Protection against virulent BTV challenge

Sheep in the control group challenged with BTV1, showed clinical symptoms of the disease, including an elevated body temperature starting at D1 after challenge and which remained high until 14 dpi, with a peak at 9 dpi (41 °C) (Fig. 4). One animal, died at 11 dpi with congestion in eyes and oral mucosa, hyperthermia of 42 °C and a clinical score of 11 (Fig. 5). At necropsy, the following lesions were observed: foam in the nostrils, inflammation in the inoculation site, lung and abdomen congestion, friable spleen, prescapular and retropharyngeal lymph nodes enlargement. The other animal challenged with BTV1, presented hyperthermia, eyes congestion, facial edema and nasal discharge only (Fig. 5). In vaccinated group, no symptoms of BT were observed in any of the 4 challenged animals, except slight congestion of eyes and mild temperature for one day in two sheep.

Regarding BTV 4, on 1 dpi, hyperthermia was recorded in the unvaccinated control group for 14 dpi. Mild nasal discharge, eyes congestion and inflammation of the oral mucosa on 8 dpi were noted in one animal. Eyes congestion and facial edema were also present in the second sheep on 7 dpi (Fig. 5). One vaccinated sheep showed a slight temperature on D9 pi (Fig. 4). The average clinical score in unvaccinated animals was 9.5 for BTV1 and 6 for BTV4 whereas in vaccinated sheep 2.75 and 1.5 respectively (Tables 2 and 3). Clinical scores were significantly higher ($P \leq 0.05$) in unvaccinated sheep for both serotypes 1 and 4 compared with vaccinated animals.

3.4. Serological response after challenge

Serology response by SNT post challenge showed seroconversion of unvaccinated sheep for both serotypes BTV1 and BTV4 at day 10 post challenge and an increase in antibody titer in vaccinated animals from 1.38 - 3.0 for BTV1 and 1.74–3.02 for BTV4 (-$\log_{10}$ of 50 % endpoint dilution/mL, Table 4).

3.5. Evaluation of viraemia after BTV challenge

When testing vireamia post challenge by q-PCR, control animals were positive during the observation period with a Ct ranging from 25.0–31.0 for BTV1 and ranging from 24.5–31 for BTV4 and peaked at 10 dpi for both serotypes. All vaccinated animals were negative for vireamia by q-PCR. Tables 5 and 6.

4. Discussion

Bluetongue is a listed vector borne disease of ruminants (World Organization for Animal Health, 2014). Eradication is possible, as was the case in several European countries in which systematic vaccination has been conducted by inactivated vaccines. By means of a mathematical model, it was concluded that when vaccination is applied on 95 % of animals even for 3 years, BT cannot be eradicated and is able to re-emerge. Only after 5 years of vaccination, the infection may be close to the eradication levels (Maclachlan and Mayo, 2013; EFSA, 2017).

Different types of vaccines have been developed to prevent BTV infection in sheep and cattle, live attenuated, inactivated and recently recombinant vaccines (Murray and Eaton, 1996; Boone et al., 2007; Mayo et al., 2017; Calvo-Pinilla et al., 2020). The LAVs have been extensively used to control the clinical disease despite that many studies have been reported the main shortcoming of LAVs, which is reversion to virulence through reassortment phenomena (Batten et al., 2008). Inactivated vaccines have been reported to be safe and effective in preventing clinical disease and infection (Stott et al., 1985; Emidio et al., 2004). However, there are no inactivated vaccines available for all BTV serotypes and very few combined vaccines with more than one serotype have been developed (Savini et al., 2009; Bitew et al., 2017; Bitew et al., 2019). In this study, a combined inactivated BTV-1 and 4 vaccine was developed and tested successfully by experimental infection and antibody response monitoring in sheep, the most sensitive species clinically to the disease showing very often typical symptoms (Maclachlan et al., 2009). In North Africa, sheep population exceed 70 M heads and BT is endemic with repeated outbreaks recorded during the last 2 decades

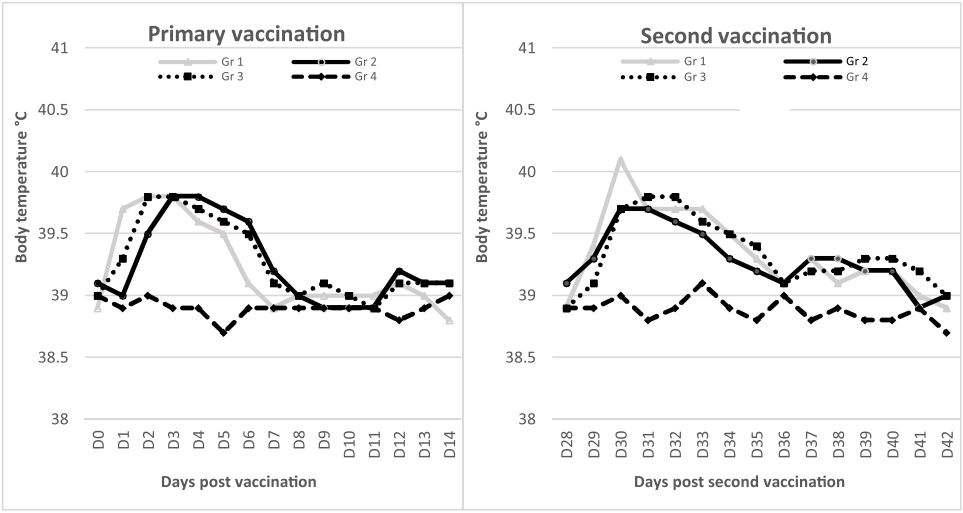

Fig. 1. Average body temperature of the four groups after primary-vaccination and the booster.

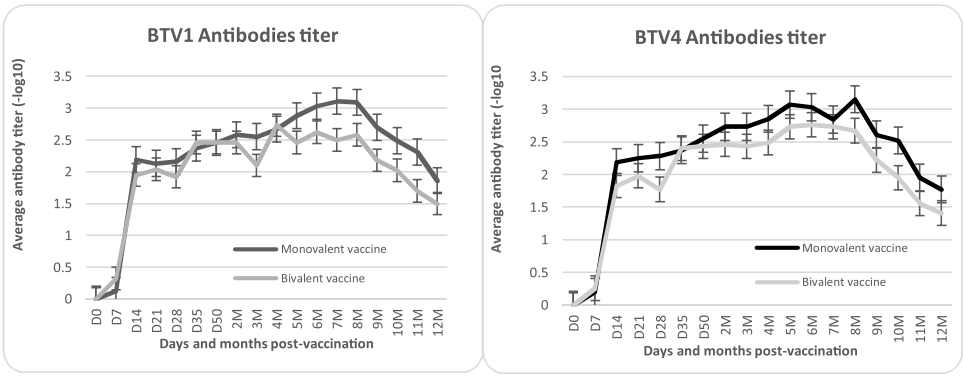

Fig. 2. Average neutralizing antibody after primary-vaccination and booster.

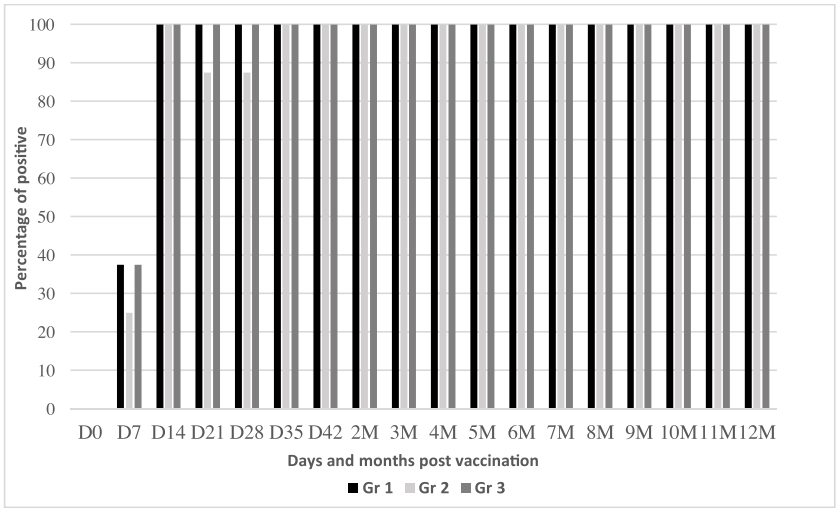

Fig. 3. Percentage of ELISA positives sheep of groups 1, 2 and 3.

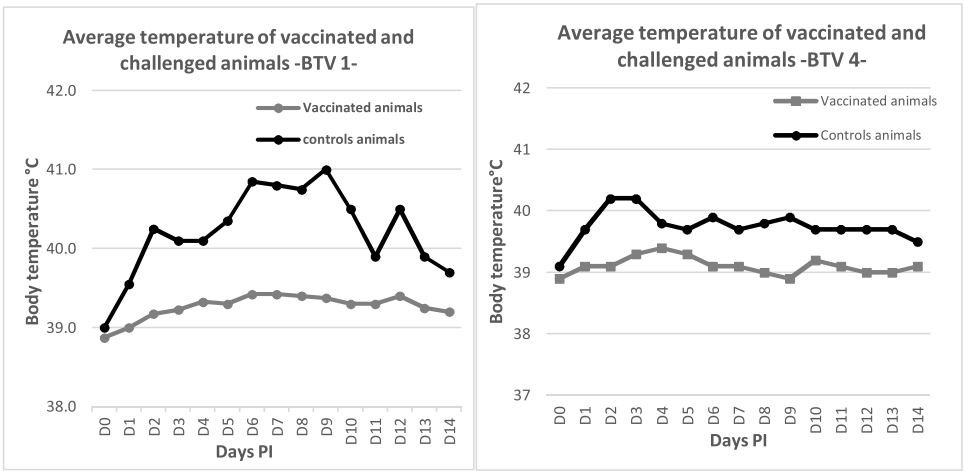

Fig. 4. Average temperature of the four groups after challenge with BTV strains.

during favourable seasons in this region (Hammami, 2004; Cêtre-Sossah et al., 2011; Kamar et al., 2013; Drif et al., 2014). Serotypes 1 and 4 were the principal causative agents of BTV outbreak in Morocco, the first outbreak was observed in 2004 in sheep caused by BTV-4 and two years later, an outbreak caused by BTV-1 was notified (Drif et al., 2014). BTV-1 was also reported in Algeria (Maan et al., 2008) and in the South of the Iberian Peninsula (García-Lastra et al., 2012; de Diego et al., 2014). The two serotypes also crossed the Mediterranean Sea to cause large outbreaks in Southern Europe. Presently, serotypes 1 and 4 are both endemic in the entire Mediterranean basin (Drif et al., 2014). Inactivated monovalent vaccines against serotypes 1, 2, 4, 8, 9, 11, 18, 16 and 23 have been reported (Berry et al., 1982; Parker et al., 1975; Stevens et al., 1985; Pandey et al., 2006; Savini et al., 2007; Savini et al., 2008b; Savini et al., 2009; Bhanuprakash et al., 2009; Umeshappa et al.,

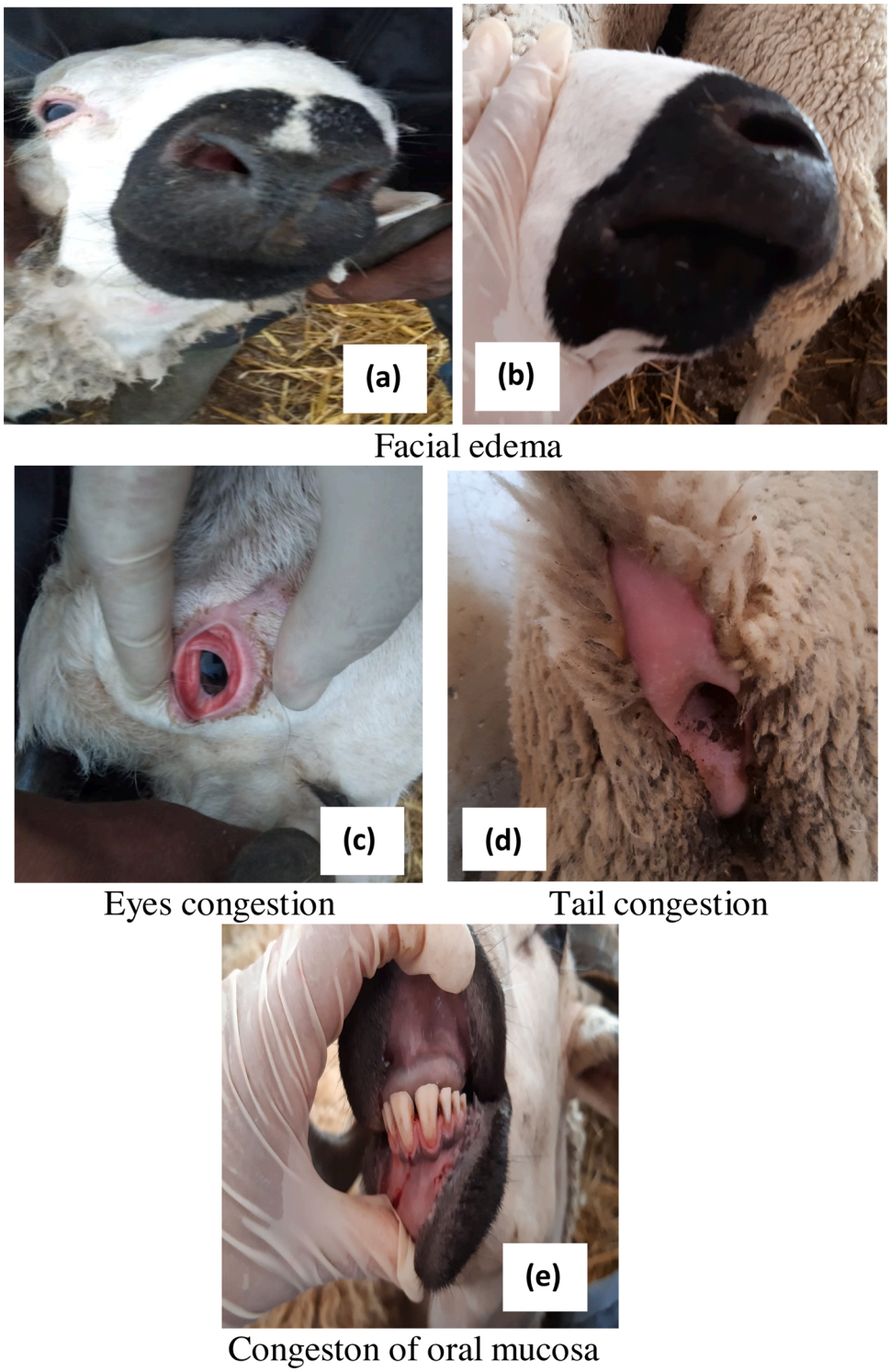

Fig. 5. Signs observed on controls animals.
Facial edema (a; b) eyes congestion (c), tail congestion (d) congestion of oral mucosa (e)

2011; Zientara and Sánchez-Vizcaíno, 2013). Protection by challenge was confirmed for BTV 8 inactivated vaccine (Moulin et al., 2012) and pentavalent BT vaccine (BT-1, 2, 10, 16 and 23) (Bitew et al., 2019).

In this study, serological monitoring pv showed that all vaccinated sheep with monovalent or bivalent vaccines developed an immune response against both BTV serotypes by SNT as animals were all seropositive at D14 pv. Presence of neutralizing antibodies have been shown to correlate with protection against BTV (Huismans et al., 1987; Roy et al., 1990), and in our experiment protective antibodies are present and detectable with significant titer 12 months after vaccination. An inactivated vaccine against BTV-2 also led to 100 % seroconversion in sheep following the first injection, and conferred full protection against challenge infection as reported by (Hamers et al., 2009). Limited inactivated vaccines are available for serotypes 1 and 4 control, to our knowledge only SYVAZUL which is a commercial vaccine produced by SYVA. The challenge carried out on vaccinated and control animals

Table 2
Clinical scoring of animals challenged with BTV serotype 1.

Group	Animal	Behavior	Ocular	Cong	Edema	Loco	T°	Resp	Tongue	Total	Average
Vaccinated	1	0	2	0	0	0	1	0	0	3	2.75
	2	0	2	0	0	0	0	0	0	2	
	3	0	1	2	0	0	0	0	0	3	
	4	0	2	0	0	0	1	0	0	3	
Controls	5	0	2	0	1	0	4	1	0	8	9.5
	6	4	2	1	0	0	4	0	0	11	

Table 3
Clinical scoring of animals challenged with BTV serotype 4.

Group	Animal	Behavior	Ocular	Cong	Edema	Loco	T°	Resp	Tongue	Total	Average
Vaccinated	1	0	0	0	0	0	0	0	0	0	1,5
	2	0	2	0	0	0	1	0	0	3	
	3	0	1	0	0	0	0	0	0	1	
	4	0	0	1	0	0	1	0	0	2	
Controls	5	0	2	0	1	0	3	0	0	6	6
	6	0	2	1	0	0	2	1	0	6	

Table 4
Seroconversion after BTV challenge in vaccinated and control animals, assessed by SNT.

Group	Animal	BTV-1		BTV-4	
		D0	D26	D0	D26
Vaccinated	1	1.26	2.46	1.5	2.7
	2	1.74	3.18	1.98	2.94
	3	1.26	3.18	1.98	3.18
	4	1.26	3.18	1.5	3.42
Average antibody titer of vaccinated sheep		**1.38**	**3.00**	**1.74**	**3.02**
Control	5	0	1.98	0	1.98
	6	0	2.22	0	2.22
Average antibody titer of unvaccinated sheep		**0**	**2.1**	**0**	**2.1**

showed that the viral titer of 10^7 $TCID_{50}$/mL for BTV-4 and BTV-1 was sufficient to reproduce typical clinical signs in animals, which was also confirmed in previous studies (Savini et al., 2008a). Other authors (Ramakrishnan et al., 2006) demonstrated that 1.2×10^5 was sufficient to induce a clinical disease in sheep infected with BTV-1 or 8, and dose of 10^6 also induced a clinical signs in animals infected with BTV-8 (Eschbaumer et al., 2009) and BTV-4 or BTV-16 (Kalyani et al., 2019). However, the North African sheep breed are known to be less susceptible to BTV than European breeds (Verwoerd and Erasmus, 2004), which justify the high dose used for challenge in this study. Capability to reproduce the disease is however not depending only on the dose but also the serotype and the viral strain inside the same serotype. Indeed, some BTV serotypes are known to be more virulent than others and many serotypes are circulating in ruminants population without causing clinical disease (Gard, 1984; Parsonson, 1990; Coetzer and Tustin, 2004; Maclachlan et al., 2009). In this experiment, clinical signs obtained after challenge with serotype 1 were more pronounced compared to those observed with BTV-4, confirming that the virulence is serotype dependent. In the field, during the BTV4 outbreak of 2004 in Morocco, clinical signs observed in sheep were less severe than those observed during BTV1 outbreaks in 2006 (Drif et al., 2014).

Vaccinated animals remained healthy after challenge during the observation period and no vireamia was detected by PCR in the group of vaccinated and challenged animals which is in accordance with previous reported studies on BTV2 vaccines and BTV2-BTV4 (Hamers et al., 2009; Savini et al., 2008a). Viraemia was detected in unvaccinated and challenged sheep, at low Ct values, which represent a significant source of

Table 5
Individual Ct values per animal after BTV-1 challenge.

Group	Animal	D0	D3	D6	D8	D10	D12	D14	D18	D21	D26
Vaccinated	1	0	0	0	0	0	0	0	0	0	0
	2	0	0	0	0	0	0	0	0	0	0
	3	0	0	0	0	0	0	0	0	0	0
	4	0	0	0	0	0	0	0	0	0	0
Average Ct values of vaccinated sheep		**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
Control	5	0	29.5	27.8	26.9	26.6	27.6	29.5	28.6	29.6	30,1
	6	0	28.4	27.1	25.1	23.4	26.5	27.1	28.5	29.5	31,8
Average Ct values of control sheep		**0**	**29,0**	**27.5**	**26.0**	**25.0**	**27.1**	**28.3**	**28.6**	**29.6**	**31.0**

Table 6
Individual Ct values per animal after BTV-4 challenge.

Group	Animal	D0	D3	D6	D8	D10	D12	D14	D18	D21	D26
Vaccinated	1	0	0	0	0	0	0	0	0	0	0
	2	0	0	0	0	0	0	0	0	0	0
	3	0	0	0	0	0	0	0	0	0	0
	4	0	0	0	0	0	0	0	0	0	0
Average Ct values of vaccinated sheep		**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
Control	5	0	28.5	26.8	25.9	25.6	26.2	27.5	28.6	29.9	30,1
	6	0	27.4	25.5	23.8	23.4	25.8	27.6	27.9	28.3	31,8
Average Ct values of control sheep		**0**	**27,95**	**26.15**	**24.85**	**24.5**	**26.0**	**27.55**	**28.25**	**29.1**	**30.95**

the virus in natural conditions, as stated with an inactivated BTV8 vaccine study (Eschbaumer et al., 2009). Monitoring the level of viraemia in vaccinated animals after challenge is considered the most effective way to evaluate vaccine efficacy, because experimental infection with BTV is not able to induce regularly typical symptoms of the disease in animals (Savini et al., 2008a; EMEA, 2008). In recent studies, the clinical signs reported after BTV experimental infection were mild compared to those reported from the field, and the most obvious hypothesis to explain it is the use of culture grown viruses (Martinelle et al., 2018; Martinelle et al., 2019).

These results give evidence that the combined BTV 1 + 4 inactivated adjuvant vaccine is efficient to protect at least for one year the animals against infection by the corresponding serotypes BTV, and also indicate that the SNT titer of, at least 1.5 is correlated to protection. The obtained protection is related to both the amount of antigen used per dose and also the adjuvant effect to stimulate the immunity system. Aluminium hydroxide and saponin are extensively used in inactivated vaccines formulation because of safety and capability to enhance immunity post vaccination.

5. Conclusion

Vaccination with the inactivated combined vaccine BTV1+4, provided full protection of sheep against experimental infection with virulent strains BTV1 and 4 carried out 12 months after vaccination. The vaccine also induced strong response in neutralizing antibodies and prevented vireamia at challenge.

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Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analyzed during the current study available from the corresponding author on reasonable request. All recorded raw data are archived in MCI Santé Animale.

Authors' contributions

ZBa performed the experiment and drafted the manuscript. YE performed SNT testing. NS performed ELISA, inoculum preparation and PCR tests and.. LD serology data analysis and interpretations. KT design of the study. RA reviewing the manuscript. VA challenge results analysis and interpretation. OF validation. ME participated in the design of the study, supervising and manuscript revision. All authors read and approved the final manuscript.

Declarations of Competing Interest

The authors declare that they have no competing interests.

Acknowledgments

All the authors have seen and approved the content and have contributed significantly to the work. The authors gratefully acknowledge the support for this study by MCI (Multi-Chemical Industry) Santé animale.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vetmic.2021.109212.

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