Veterinary Microbiology 256 (2021) 109046

Contents lists available at ScienceDirect

Veterinary Microbiology

journal homepage: www.elsevier.com/locate/vetmic

Development of an inactivated combined vaccine for protection of cattle against lumpy skin disease and bluetongue viruses

Youness Es-sadeqy [a,*], Zahra Bamouh [a], Abderrahim Ennahli [a], Najete Safini [a], Soufiane El Mejdoub [a], Khalid Omari Tadlaoui [a], Boris Gavrilov [b], Mehdi El Harrak [a]

[a] Research and Development, MCI Santé Animale, ZI Sud-Ouest B.P: 278, Mohammedia, 28810, Morocco
[b] Biologics Development, Huvepharma, 3A Nikolay Haytov Street, Sofia, 1113, Bulgaria

ARTICLE INFO

Keywords:
Bluetongue virus serotype 4
Combined inactivated vaccine
Lumpy skin disease
Serological response
Vaccination

ABSTRACT

Lumpy Skin Disease (LSD) and Bluetongue (BT) are the main ruminants viral vector-borne diseases. LSD is endemic in Africa and has recently emerged in Europe and central Asia as a major threat to cattle industry. BT caused great economic damage in Europe during the last decade with a continuous spread to other countries. To control these diseases, vaccination is the only economically viable tool. For LSD, only live-attenuated vaccines (LAVs) are commercially available, whilst for BT both LAVs and inactivated vaccines are available with a limited number of serotypes. In this study, we developed an inactivated, oil adjuvanted bivalent vaccine against both diseases based on LSDV Neethling strain and BTV4. The vaccine was tested for safety and immunogenicity on cattle during a one-year period. Post-vaccination monitoring was carried out by VNT and ELISA. The vaccine was completely safe and elicited high neutralizing antibodies starting from the first week following the second injection up to one year. Furthermore, a significant correlation (R = 0.9040) was observed when comparing VNT and competitive ELISA in BTV4 serological response. Following BTV4 challenge, none of vaccinated and unvaccinated cattle were registered clinical signs, however vaccinated cattle showed full protection from viraemia. In summary, this study highlights the effectiveness of this combined vaccine as a promising solution for both LSD and BT control. It also puts an emphasis on the need for the development of other multivalent inactivated vaccines, which could be greatly beneficial for improving vaccination coverage in endemic countries and prophylaxis of vector-borne diseases.

1. Introduction

Bluetongue (BT) and Lumpy skin disease (LSD) are two viral vector-borne diseases of cattle, notifiable by the World Organization for Animal Health (Sprygin et al., 2018; Rojas et al., 2019). LSD is host-specific and caused by the lumpy skin disease virus (LSDV) which belongs to the genus *Capripoxvirus* (CaPVs) within the family of *Poxviridae* (Tuppurainen et al., 2017). The disease is characterized by fever, reduction in milk and meat production, abortion, vulnerability to secondary bacterial infections, skin nodules, mastitis, swelling of peripheral lymph nodes, loss of appetite, increased nasal discharge, high morbidity and low mortality (Madhavan et al., 2016; Sohier et al., 2019). Besides, LSD is endemic throughout Africa except for some northern countries (Morocco, Algeria, Tunisia and Libya) and since 2013, it spread throughout the Middle East and Turkey (Tuppurainen et al., 2017). In 2014, the disease spread to other countries such as Azerbaijan (2014), Armenia (2015) and Kazakhstan (2015), southern Russia and Georgia (2016). In addition, LSD has progressed into the northern part of Cyprus, Greece (2015), Bulgaria, Macedonia, Serbia, Montenegro, Albania and Kosovo (2016). Recently, LSDV was found to affect eastern and south-eastern European countries (Sohier et al., 2019). Vaccination is considered the effective strategy for eradication and control of LSD, which can be ensured by a live attenuated vaccine based on Neethling or KSGP strains and heterologous vaccines based on sheeppox or goatpox viruses (Kononov et al., 2019). Generally, live attenuated vaccines (LAVs) elicit wider protective immunity compared to non-replicating vaccines but could generate adverse reactions such as skin lesions called “Neethling disease” and present the risk of reversion to virulence (Tuppurainen et al., 2018a).

The causative agent of bluetongue disease in domestic and wild ruminants is a virus classified under the genus *Orbivirus* in the *Reoviridae* family (Rojas et al., 2019). Based on the genetic and antigenic features of

* Corresponding author.
E-mail address: y.essadeqy@mci-santeanimale.com (Y. Es-sadeqy).

https://doi.org/10.1016/j.vetmic.2021.109046
Received 13 November 2020; Accepted 19 March 2021
Available online 23 March 2021

the outer capsid proteins, primarily VP2, and neutralizing antibodies generated by the infected host, 27 serotypes were identified along with 2 additional putative ones (Maan et al., 2016). The seriousness of BTV infections varies depending on the pathogenicity of the virus strain and the susceptibility of ruminant host. Sheep are more susceptible and can present severe clinical signs compared to cattle and goats. However, cattle rarely show clinical disease but they are readily infected and are considered as an epidemiologically important BTV reservoir (Van Rijn, 2019). In 2014–2015, the epidemiological situation of BTV4 was remarkable, with high number of outbreaks making it the most circulating serotype in south-eastern and central European countries (Albania with 2463 cases, Bulgaria 2322, Macedonia 1898, Romania 1887, Serbia 649, Croatia 89 and Hungary 86) (Alexandrov and Tchakarova, 2017). BTV-infected animals from the same species present clinical signs ranging from subclinical infection to severe disease. In sheep, usually observed signs include pyrexia, lethargy, tachypnea, buccal ulcers, and edema (of the lips, face, or tongue). Whilst in acute cases, the clinical signs noticed include tongue cyanosis, generalized edema and hemorrhages (especially in the pulmonary artery, lungs, heart, skeletal muscles, and lymph nodes) (Belbis et al., 2017). Vaccination is still the preferred method for BT control. Live-attenuated and inactivated vaccines are available, despite the latter's limited number of serotypes and higher cost (Van Rijn, 2019). The LAVs caused viral diffusion in the environment with reassortment events between field and vaccine strains, while the inactivated BTV vaccines are safe and allowed eradication of the disease in some European countries (Van Rijn, 2019).

In this study, we focus on the development of an inactivated, oil adjuvanted, bivalent vaccine based on LSD Neethling strain and BTV4. The developed combined vaccine was tested for safety, immunogenicity and efficacy on cattle, in comparison with unvaccinated control and vaccinated animals with each valence vaccine.

2. Material and methods

2.1. Good manufacturing practices (GMP) and ethical statement

Vaccine production, storage and data recording were performed according to GMP. Experiments on cattle were executed following the International Guidelines for the care and handling of experimental animals described in chapter 7.8 of the Terrestrial Animal Health Code and Directive 2010/63/UE of the European commission. The protocol was submitted and approved by the Internal Ethic Committee for animal experiment (MCI/CEI/0619).

2.2. Antigen preparations

Preparation of LSDV antigens was performed using the LSD Neethling attenuated strain of South African origin (Morgenstern and Klement, 2020). The strain was cultured on primary lamb testis (LT) cells in a multilayer cell factory with Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10 % of irradiated fetal bovine serum (FBS). LT cells monolayer were inoculated with the virus at a Multiplicity Of Infection (MOI) of 0.1 and then incubated at 37 °C with 5 % of CO_2. The viral suspension was harvested after six days post-inoculation (pi), when the viral cytopathic effect was generalized. The viral suspension was titrated and tested for identity and purity.

BTV4 antigens were prepared in 10 L stirred bioreactor with a working volume of 6 L. BHK/AC9 suspension cells from European Collection of Authenticated Cell Cultures (ECACC) were used for the propagation of the BTV4. Cells were seeded at a concentration of 0.4×10^6 cells mL^{-1} and the inoculation was carried out at a MOI of 0.01. Parameters of production were adjusted at 37 °C, 38 rpm and dissolved oxygen and pH were controlled at 50 % air saturation and 7.2, respectively. The produced antigen was harvested at third day pi, titrated and tested for identity and purity.

2.3. Inactivation and vaccine formulation

Antigens of LSDV and BTV4 were inactivated using Binary Ethylenimine (BEI) at a concentration of 20 mM. After determination of viral titers, a volume of BEI was added to each live antigen depending of the volume of antigens to be inactivated and inactivation kinetics were performed to determine necessary time for inactivation of each antigen.

An oil emulsion vaccine was prepared with a mixture of the two inactivated antigens and the Montanide™ from SEPPIC as adjuvant. The used antigens were added to ensure a dose of around 10^6 to $10^{6,5}$ $TCID_{50}$/animal before inactivation. Three vaccines were prepared, the first and the second were monovalent LSD and BTV4 vaccines while the third was a bivalent vaccine based on the two antigens. Eventually, the formulated vaccines were monitored for stability, emulsion type and other physico-chemical parameters.

2.4. Animals and vaccination

Forty-four cattle, male and female aged 6–8 months, of a local breed were used for vaccine testing. Animals were housed in ABSL2 facility and tested negative for LSDV antibodies by VNT and for BTV4 antibodies by ELISA as described below. Cattle were divided into three groups and vaccination was performed by intramuscular (IM) route at day 0 and secondary vaccination at day 28. The first group of 20 cattle was vaccinated with 2 mL of the bivalent vaccine (LSDV-BTV4), whilst the second and the third groups of 10 cattle each, received 2 mL of the inactivated monovalent LSDV and BTV4 vaccines, 4 cattle received only the placebo preparation (saline solution). Animals were monitored 14 days for rectal temperature, clinical signs and inflammation at the inoculation site. Serum samples were collected weekly, until day 35 and monthly until the twelfth month for serology monitoring.

2.5. Serological response assessment

Serological response monitoring was carried out by VNT for LSDV, and by ELISA and VNT for BTV4. Blood samples were collected in plain vacuum tubes via jugular venipuncture using an 18 G needle and separated sera from the total blood were stored at −20 °C until analysis.

2.5.1. VNT

VNT was performed as described in the Chapter 3.4.12 and the Chapter 3.1.3 of the OIE Manual of Diagnostic Tests and Vaccines for Terrestrial animals (2019) with slight modifications (World Organization for Animal Health, 2019). Briefly, 50 µL of inoculation medium (DMEM with 1% FBS and 1% antibiotic-antimycotic) were pipetted in all wells. After inactivation for 30 min at 56 °C, 25 µL of serum was subsequently added to 4 wells of the first column to obtain a starting dilution of 1:3. From these wells, three-fold dilutions were made by pipetting 25 µL of each well in the next well, until the 6^{th} column of each sample making 6 dilutions from 1:9 to 1:729. From the final column, 25 µL were discarded. Thereafter, virus dilution (100 $TCID_{50}$ from LSDV or BTV was added to all wells in a volume of 25 µL. Sera and virus were pre-incubated at 37 °C for 1 h to allow neutralization of the virus. Eventually, 18,000 cells per well were added in a volume of 150 µL (cellular suspension of MDBK (NBL-1) ATCC® CCL-22™ for LSDV and Vero ATCC® CCL-81 for BTV4). Plates were then incubated for 5–7 days at 37 °C under 5% of CO_2. The antibodies titers were calculated using the method of Reed & Muench (Saganuwan, 2016), wells displaying no viral foci were designated as positive.

2.5.2. ELISA

Protective antibodies anti-VP7 in cattle sera were assessed with a commercial competitive ELISA kit (ID SCREEN® Bluetongue Competition ELISA, IDVet) in accordance to the manufacturer's instructions. Sera presenting an inhibition percentage (PI) of $\leq$ 35 % were considered positive.

2.6. BTV4 experimental infection

Four cattle were vaccinated at D0 by IM route with the LSDV-BTV4 combined vaccine and re-immunized at D28. Four weeks after the second injection, the cattle were challenged along with 2 unvaccinated control by intravenous route (IV) with a virulent BTV4 strain kindly supplied by the Central Veterinary Laboratory Algete Madrid. The virulent strain of Spain origin was cultivated twice on BHK-21 cells, titrated and tested for purity. Cattle were inoculated with 2 mL, containing 10^6 50 % of Tissue Culture Infectious Dose per mL ($TCID_{50}$/mL), of the virus as previously described by Martinelle et al. (2018). Infected cattle were observed 2 weeks after challenge for clinical signs and rectal temperature and sampled during 4 weeks for detection of viral RNA in blood.

2.7. BTV4 RNA extraction and detection

Blood samples were collected from vaccinated and unvaccinated cattle in EDTA tubes. RNA extraction was carried out on separated erythrocytes. Briefly, 3 mL of total blood were centrifuged at 1500 rpm for 15 min at +4 °C. Plasma was removed and cells were washed twice with PBS (Phosphate Buffered Saline), then lysed with deionized water 5 min, allotted and stored at – 80 °C until use. BTV4 RNA was extracted from 200 µL of red cells suspension using Trizol (Invitrogen) according to manufacturer's instructions. Precipitated RNAs were dissolved in 60 µL nuclease-free water and kept at – 20 °C until use. BTV4 RNA detection was carried out using real time PCR according to the protocol described by Toussaint et al. (2007), targeting the BTV segment 5.

2.8. Statistical analysis

Results analysis were performed using SPSS statistics software version 22 (IBM), a P value ≤ 0.05 was considered significant. LSDV and BTV4 serological response of bivalent vaccine was compared to monovalent vaccines and was evaluated for significance using *t*-test.

3. Results

3.1. LSDV and BTV4 live antigens were prepared

LSDV and BTV4 live antigens were prepared using the suitable cultivation method for each virus. Live LSDV Neethling strain was produced in stationary cell-factory system. The virus showing a CPE from day 3 was harvested at day 6 pi with a titer of $10^{7,7}$ $TCID_{50}$/mL. Separately, BTV4 antigens were produced using a suspension batch system, the viral suspension was harvested after 3 days pi with a titer of $10^{7,6}$ $TCID_{50}$/mL.

3.2. An inactivated oil adjuvanted LSDV-BTV4 vaccine was formulated

An inactivated combined LSDV-BTV4 vaccine was prepared in parallel with the monovalent vaccines of each valence. The harvested viral LSDV and BTV4 suspensions were inactivated after a period of around 7 h and 12 h, respectively (Fig. 1). LSDV inactivated antigen was used for the formulation of LSDV monovalent and LSDV-BTV4 bivalent vaccines, while the BTV4 antigen was used to formulate the BTV4 monovalent and the LSDV-BTV4 bivalent vaccines. Vaccines were characterized by a white colored, water in oil emulsion with a pH around 7,2. Vaccines successfully passed analytical QC testing for sterility and absence of extraneous agents.

3.3. The inactivated LSDV-BTV4 vaccine was innocuous

Cattle were vaccinated at day 0 and re-immunized at day 28 in a volume of 2 mL applied through deep intramuscular. The first group of 20 cattle was vaccinated with LSDV-BTV4 bivalent vaccine, the second with LSDV vaccine and the third group received BTV4 vaccine. Cattle remained healthy without any adverse reactions with an average body temperature ranging from 38,7 to 39,7 °C between the second- and fifth-days pi in groups 1 and 2. The temperature remained normal in-group 3 and unvaccinated control (Fig. 2A).

3.4. The inactivated LSDV-BTV4 vaccine elicited satisfactory serological response

The pv monitoring was carried out using VNT for anti-LSDV and by VNT and ELISA for anti-BTV4. VNT results of LSDV and BTV4 vaccines are reported in Table 1, while BTV4 ELISA results were summarized in Table 2 as number of positive cattle. Serological response for LSDV started at day 21 pv in the bivalent and the monovalent vaccines (Fig. 2B). High antibodies (Abs) titers were reported for both vaccines from the second injection to the end of the observation of one year, with no significant difference between monovalent and bivalent vaccines (P value < 0.05) (Fig. 2B). Remarkably, serological response in the bivalent vaccine was slightly higher than the monovalent vaccine after the second injection but not statistically significant (P-value < 0.05). Specifically, from the D35 to 12 M, Abs titers were ranging from $10^{1,42}$ to $10^{1,82}$ and from $10^{0,8}$ to $10^{1,6}$ 50 % logarithm dilution per mL (LD_{50}/mL) for the bivalent and monovalent vaccines, respectively (Fig. 2B).

Regarding the serological ELISA response to BTV4, Abs were detected earlier in the monovalent vaccine compared to the bivalent and the percentage of positives ranged from 75 % to 95 % for the LSDV-BTV4 following the second injection (Fig. 3A and Table 2). VNT antibody peaked at 4 months and 3 months pv in the bivalent and the monovalent vaccines, respectively. Interestingly, BTV4 VNT of the bivalent vaccine

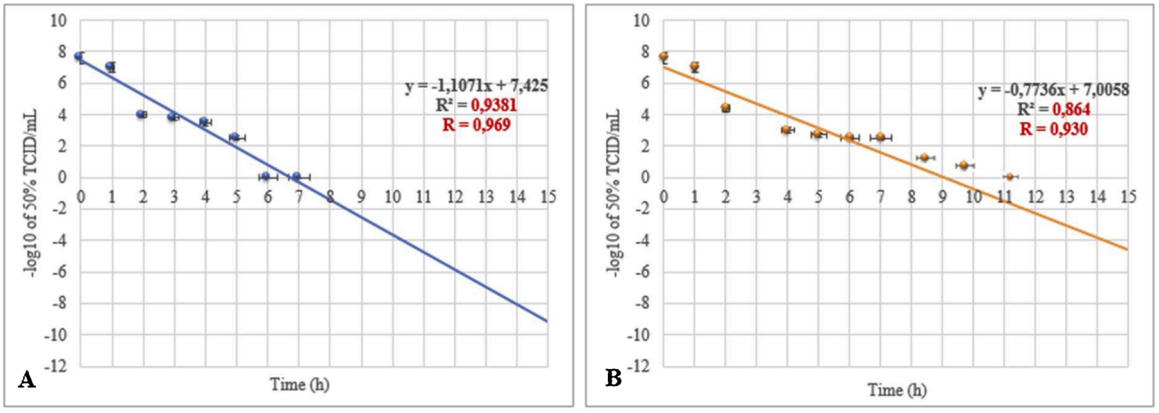

Fig. 1. Inactivation kinetics of LSDV and BTV4 using 20 mM of BEI as determined by viral titration. A: Inactivation Kinetics of LSDV; the estimated time for inactivation is 7 h. B: Inactivation Kinetics of BTV4; estimated time for inactivation is 12 h.

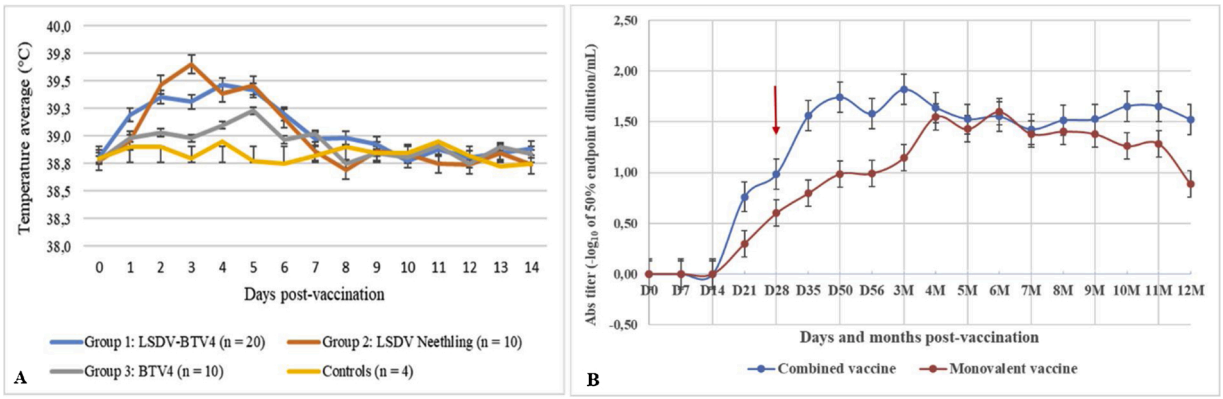

Fig. 2. Temperature monitoring of vaccinated versus unvaccinated cattle during 14 days post vaccination and mean neutralizing antibody titers in respond to vaccination with LSDV valence. A: Body temperature of the 40 vaccinated cattle and controls. B: Mean protective LSDV-antibody titers in cattle vaccinated with the combined vaccine versus the monovalent vaccine titrated by VNT. Red arrow indicates the time period in which cattle from G1 and G2 were received the second injection.

Table 1
VNT results of cattle vaccinated with LSDV-BTV4, LSDV and BTV4 monovalent vaccines in addition to unvaccinated cattle (number of positives).

		Days and months pv																	
Vaccine	Tested valence	D0	D7	D14	D21	D28	D35	D50	D56	3M	4M	5M	6M	7M	8M	9M	10M	11M	12 M
LSDV-BTV4	**LSD**	0	0	0	12	20	20	20	20	20	20	20	20	20	20	20	20	20	19
(20 cattle)	**BT4**	0	0	4	8	11	14	20	20	20	20	20	19	18	18	18	15	9	7
LSD (10 cattle)		0	0	0	4	9	9	9	10	10	10	10	10	10	10	10	10	10	9
BT4 (10 cattle)		0	0	4	10	10	10	10	10	10	10	10	9	9	9	9	8	5	4
Control (4 cattle)		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Table 2
BTV4 ELISA results of cattle vaccinated with LSDV-BTV4, BTV4 monovalent and control (number of positives).

	Days and months pv																	
vaccine	D0	D7	D14	D21	D28	D35	D50	D56	3M	4M	5M	6M	7M	8M	9M	10M	11M	12 M
LSDV-BTV4 (BTV4 valence) n = 20	0	0	4	8	9	18	18	18	17	19	18	15	17	18	18	16	18	16
BTV4 Mono n = 10	0	0	0	10	10	10	9	10	10	10	10	10	10	10	10	9	9	9
Control n = 4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

presented a percentage of positives ranged from 35 % to 100 % during the year and following the second injection.

For presentation of ELISA results, a % Inhibition $\leq$ 35 % is considered a positive sample, but in Fig. 3(A and B), ELISA results are expressed as 100-% I to make results more comparable to VNT results. Values of positives were ranging from 65,41 to 87,77 % and from 82,43 to 99,17 % for the bivalent and the monovalent, respectively (Fig. 3A and B).

Assessment of BTV4 neutralizing antibodies was carried out by both recognized standard methods (VNT and ELISA). While ELISA method is qualitative, it allows the detection of one type of antibody (Anti-VP7 IgG). VNT, on the other hand, is considered a quantitative method and detects all types of IgG. In this regard, a correlation was observed between VNT and ELISA with a consistent correlation coefficients (R) = 0,9040 and 0,8263 for the bivalent and the monovalent vaccines, respectively (Fig. 4). No significant difference was observed regarding response to BTV4 valence present in the monovalent or the bivalent vaccines (P value $<$ 0.05).

3.5. The combined LSDV-BTV4 vaccine induced full protection after challenge with BTV4 virulent strain

Regarding BTV4 challenge, vaccinated cattle did not show any clinical symptoms or viraemia after challenge. The 2 unvaccinated cattle showed increase of body temperature from D3 to D6 pi but no clinical signs were observed (Fig. 3C). Real time PCR performed from D0 to D28 pi showed no Ct values in vaccinated cattle, while unvaccinated controls revealed a Ct ranged from 23,7 to 27,7 with a peak at D10 pi (Fig. 3C).

4. Discussion

Control of vector-borne diseases have a tremendous interest worldwide. Among these diseases, LSDV and BTV4 have recently known extensive and concerning expansion around the globe, especially in Africa, Middle East, Eastern Europe and Central Asia where both diseases coexist in endemic form (Bouchemla et al., 2018; Teffera and Babiuk, 2019). Vaccination is the most efficient tool to control vector-borne pathogens since stamping out and movement restriction failed to limit the spread of the virus transmitter. Current vaccination schemes do not allow simultaneous vaccination with monovalent vaccines which creates significant issues such as of the need for multiple manipulations, animals stress, and reduced performance (milk production etc.). Another issue is the unknown effect of both vaccines, when applied separately, on the immune response against the 2 diseases. From an economic and logistic point of view, the development of a combined LSDV-BTV4 could be advantageous to reduce vaccination cost by

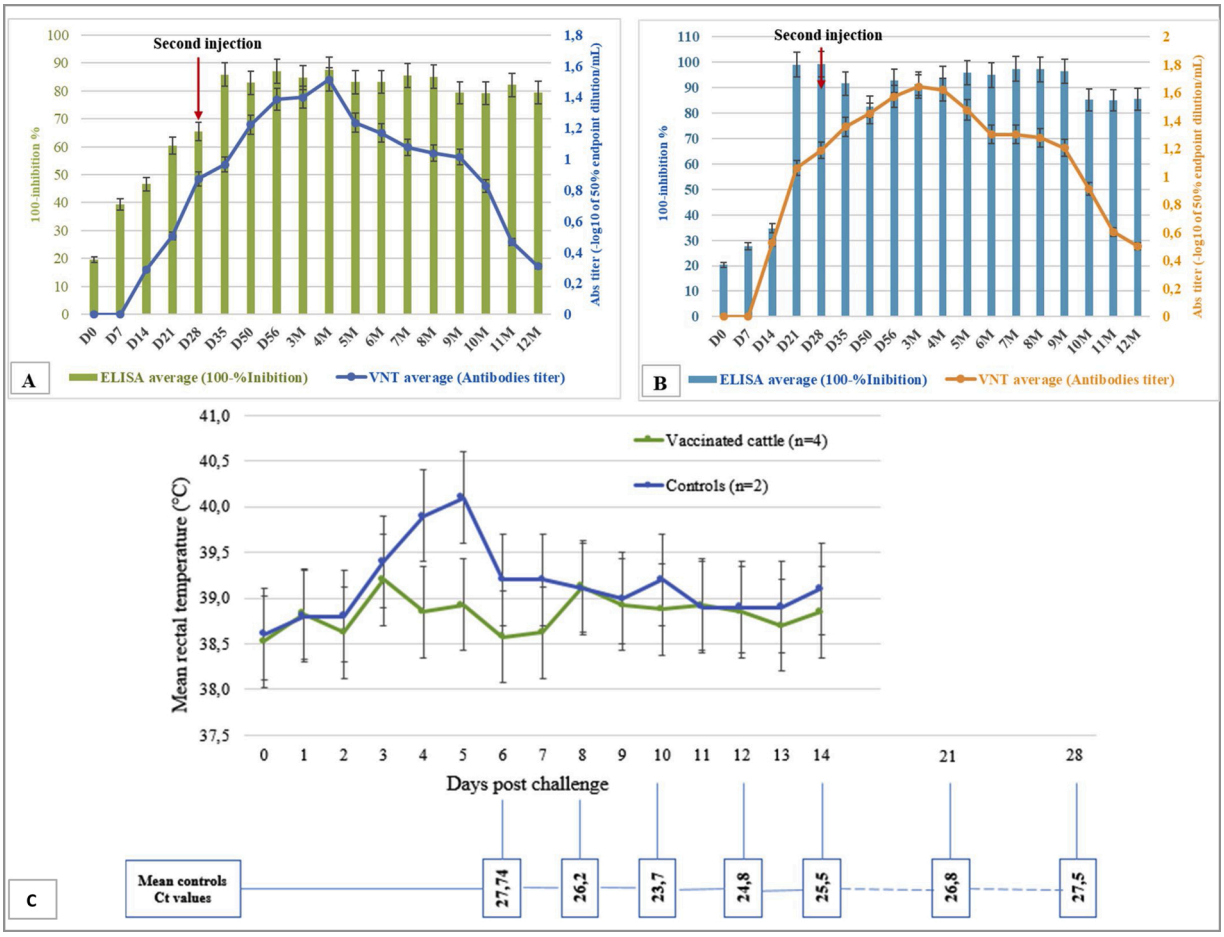

Fig. 3. Average anti-BTV4 titration of the combined and the monovalent vaccines by VNT and ELISA. A: protective antibody response to BTV4 valence of the combined vaccine, B: protective antibody response to BTV4 monovalent vaccine (VNT results are expressed as $-\log_{10}$ of 50 % endpoint dilution/mL and ELISA results are presented as 100-inhibition percentage). C: rectal temperature monitoring and controls Ct values following the challenge.

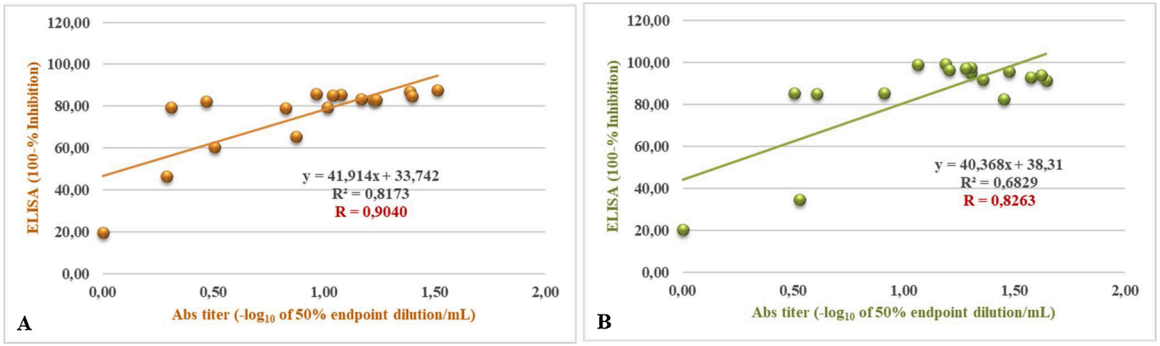

Fig. 4. Correlation of BTV4 VNT and ELISA results. A: BTV4 neutralizing antibodies of the bivalent vaccine, B: BTV4 neutralizing antibodies of the monovalent vaccine.

protecting cattle against two diseases in one shot. We also emphasize the fact that, to the best of our knowledge, this combination has never been tested before neither in laboratory nor in animals.

LAVs must replicate in animals to be efficient. Combination of live viruses raises constraints related to interference between each component of the vaccine, which leads to vaccination failure. Interference between viruses in co-infection has been reported by many authors as amply detailed in a recent review by Kumar et al. (2018). As an example, *poliovirus* vaccine strain is known to inhibit the growth of standard wild-type viruses; following vaccination this interference is manifested by stimulating antibody production that restricts the growth of the second virus. Especially, enteroviruses interfered with the *poliovirus* vaccine and led to vaccine failure (Kumar et al., 2018). This may explain the fact that very few live combined vaccines are available in the market and no multivalent LAVs exist.

Inactivation was successfully achieved by 20 mM of BEI for both antigens, which is comparable to data reported previously by other authors (Bitew et al., 2016, 2019; Hamdi et al., 2020). The inactivated vaccines are known to be safe, preferred for disease control, and could be preemptively applied in disease-free countries. Until today, poxvirus disease control was ensured only by LAVs because of their capacity to elicit a strong and long-lasting immunity (Klement et al., 2018;

Tuppurainen et al., 2018b). However, these vaccines create adverse reactions characterized by local inflammation and mild generalized diseases with skin lesions, not completely tolerated by farmers (Bedeković et al., 2018; Ben-Gera et al., 2015; Lojkić et al., 2018; Tuppurainen et al., 2018a). There are limited studies in the development of *poxvirus* inactivated vaccines. An inactivated vaccine against Camelpox virus was developed and then used in Morocco and other countries for decades in order to control the disease expansion (El Harrak and Loutfi, 2000). Furthermore, Boumart et al. (2016) successfully developed and tested an inactivated vaccine for Sheeppox virus, which belongs to the same genus as LSDV. The authors concluded that the oil adjuvanted vaccine showed complete protection against a virulent challenge with significant antibody response. Finally, Hamdi et al. (2020) reported recently that an inactivated vaccine based on LSDV Neethling strain was safe and provided complete protection against challenge with interesting neutralizing antibodies titers in comparison to LAV. In addition, LSDV inactivated vaccine did not show any adverse side effects and no risk of reversion to virulence or diffusion to unvaccinated cohabitant animals. These results of Hamdi et al. (2020) were confirmed by Wolff et al. (2021) who reported the innocuity and the efficacy of an inactivated LSDV vaccine against virulent strain after the challenge infection.

On the other hand, BT inactivated vaccines have been massively and successfully used in Europe to control and eradicate the disease. Different inactivated vaccines based on BTV1, BTV2, BTV4 or BTV8 are available in the market on oil, aluminum gel hydroxide and saponin forms (Savini et al., 2008; Calistri et al., 2010; Pérez de Diego et al., 2012; Van Rijn, 2019). Although inactivated vaccines are more expensive than LAVs due to higher antigen concentration per dose and the second injection requirement, they provide the only viable tool for control and prevention of LSD in disease-free countries (Wolff et al., 2021). The high cost could be solved through combination of multiple antigens in one vaccine, and the use of cell cultivation novel-technologies (Lesch et al., 2020).

For this purpose, the present study focused on the development of an inactivated bivalent vaccine based on LSDV and BTV4 antigens. This vaccine was evaluated in term of safety and immunogenicity in comparison with the inactivated monovalent vaccines. We used in our experiment BTV4 serotype as an example, which could be replaced, in others studies to be performed, by another BTV serotype or other bacterial/viral antigens.

Vaccinated cattle with the bivalent vaccine represent a sufficient number of animals to evaluate the safety and immunogenicity of the product. Complete safety was obtained in cattle that remained normal and healthy without any excessive local swelling at the injection site, barring slight inflammation, usually observed with oil adjuvanted vaccines.

In the present research, the vaccination dosage per cattle was fixed on 10^6 and $10^{6,5}$ $TCID_{50}$ before inactivation for LSDV and BTV4 respectively. These dosages were chosen based on previous studies. Concerning LSD, we used the same dose as described in a recent study that proved the efficiency of an inactivated LSDV against virulent strain on cattle (Hamdi et al., 2020), whereas for BTV, we have chosen a dose commonly used in commercially available vaccines (specifically BTV1 (Zulvac 1) containing $\geq 10^{6.4}$ $TCID_{50}$, and BTV8 (Zulvac 8) containing $\geq 10^{6.7}$ $TCID_{50}$, both produced by Fort Dodge Animal Health Division) (Pérez de Diego et al., 2012; Wäckerlin et al., 2010). Consequently, a dose between 10^6 and $10^{6,5}$ $TCID_{50}$ for both valences seems efficient for eliciting satisfactory humoral responses.

To assess the conferred immunity, we selected the only validated serological test for CaPVs that is described in the OIE Manual (Milovanović et al., 2019). VNT is the gold standard technique, despite being time-consuming and requiring high technicity, as it allows the detection of protective IgG antibodies that reflect the potency of the inactivated vaccines (Hamdi et al., 2020). Titers obtained in response to vaccination were high and stable during one year of pv monitoring, proving that the inactivated vaccine efficiently induced production of a significant number of neutralizing antibodies with satisfactory immunity duration.

In our study, protective antibody titer was detected from D21 to D28 after vaccination (60%–100% of positives) for the bivalent vaccine, before the second injection, which is in accordance with what was reported previously (Hamdi et al., 2020). LAV elicits both specific immunity pathways (humoral and cell-mediated) (Norian et al., 2017), while inactivated vaccines are believed to have a lesser effect on the stimulation of cellular immunity (Cohen and Bordin, 2015). Thus, we could consider that VNT testing is sufficient to assess conferred immunity and there is no need to analyze cell-mediated immunity using complex techniques. In our experiments, we avoided performing the challenge for LSD due to ethical considerations related to animal welfare, associated high cost, and biosafety issues. Indeed, it has been demonstrated that a neutralizing antibody titer $\geq 10^1$ LD_{50}/mL, following the first week after the second immunization, could be correlated with full protection at the challenge (Boumart et al., 2016; Hamdi et al., 2020). These results are well corroborated by our serological results of the bivalent vaccine, where protective antibodies titers were ranging from 10^1 to 10^2 LD_{50}/mL from one week after the second injection until the end of the year.

Concerning BT vaccines, two types are commercially available and used in many countries around the world. Both LAVs and inactivated vaccines are based on complete BT virus and induce immune response against BTV proteins (Van Rijn, 2019). Similarly, to LSDV, LAVs of BTV are cheap and effective but show adverse effects with a high risk of reversion to virulence through reassortment phenomena (Savini et al., 2008; Van den Bergh et al., 2018). Generally, BTV vaccine provides serotype-specific protection although cross-protection between some serotypes remains possible albeit hard to predict (Martinelle et al., 2018). Only inactivated vaccines are currently used in Europe despite their availability for a limited number of serotypes.

We evaluated in this experiment safety and immunogenicity of an oil adjuvanted BTV4 inactivated vaccine on cattle, either alone or in combination with LSDV antigens. Serological response to BTV4 was acceptable and no significant difference was observed between the monovalent and the bivalent vaccines (P-value < 0.05). Concerning Abs production, our results are in accordance with other studies performed in BTV1 and BTV8 inactivated vaccines (Pérez de Diego et al., 2012; Wäckerlin et al., 2010).

It should be mentioned that BT protection is predominantly evaluated through neutralizing antibody response and often described to be a significant determinant of protection (Feenstra and Van Rijn, 2017). Interestingly, even if antibody level is below the cut-off in the competitive ELISA, challenged animals 12 months after vaccination, were protected against virus replication (Wäckerlin et al., 2010). Comparatively, our results were above the ELISA cut-off following the second injection until the end of the year, suggesting a full immunity status of cattle.

Many authors reported full protection against virulent BTV strains, concluding that seropositivity correlate perfectly with the protection (Ries et al., 2019). Savini et al. (2009) reported that the administration of a bivalent BTV2-BTV4 inactivated vaccine resulted in complete protection in calves challenged with a high dose of virulent BTV2 or BTV4. Oura et al. (2012) noticed that neutralizing Abs produced in response to BTV8 vaccine persisted in cattle for at least 3 years pv, suggesting that the presence of these Abs could lead to a protection for at least 3 years. Furthermore, a significant correlation was obtained between the presence of neutralizing antibodies and full protection in cattle herds vaccinated with BTV8 and challenged by a virulent strain (Hamers et al., 2009). Indeed, Celma et al. (2013, 2017) reported that cattle vaccinated with Disabled infectious single-cycle (DISC) viruses (BTV2, BTV4, and BTV8) induced neutralizing Abs production and ensured full protection at challenge in both sheep and cattle. Consequently, based on antibody response to vaccination, LSDV-BTV4 could induce full protection against BTV4 infection. In addition, it could reduce the risk of BTV4 infection in

sheep and goats, since cattle the major BTV reservoir.

In this study, experimental infection showed full protection of vaccinated animals following the challenge carried out one month after vaccination. The combined vaccine was proven to be efficient for prevention of BTV4 replication in cattle. Unvaccinated and challenged cattle have not shown any clinical signs except a transient hyperthermia confirming the poor sensitivity of cattle to BT, indeed these animals are more involved as a virus carrier (Van Rijn, 2019). Vireamia was detected in control calves four weeks post-challenge with low Ct values which represent a significant source of the virus in natural conditions. Vaccinated cattle were protected from BTV4 viraemia, which is in accordance with previous reported studies on commercial BTV vaccines (BTV8, (Hamers et al., 2009) and BTV2-BTV4, (Savini et al., 2009)).

To summarize, the inactivated combined LSDV-BTV4 vaccine provides a beneficial solution to protect livestock against two vector-borne diseases in one solution. The vaccine is completely innocuous and efficient for use in disease-free and endemic countries as a promising inactivated combined vaccine.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Declaration of Competing Interest

The authors report no declarations of interest.

Acknowledgements

Authors are grateful to all the staff of the Central Veterinary Laboratory Algete Madrid and especially to Dr Rubén Villalba Martínez, the Head of Virology department, and to Monserrat Agüero García, the technical director for the BTV4 challenge strain. In addition, authors would also like to acknowledge the veterinary and technical staff of MCI animal facilities for help in animal surveillance and sample collection.

References

Alexandrov, T., Tchakarova, S., 2017. Bluetongue in the Region. Presented at the Lumpy Skin Disease and Bluetongue Training of Trainers in FYR of Macedonia. Retrieved from. http://www.fao.org/fileadmin/user_upload/reu/europe/documents/events2017/LSD_TTT/2.pdf.

Bedeković, T., Šimić, I., Krešić, N., Lojkić, I., 2018. Detection of lumpy skin disease virus in skin lesions, blood, nasal swabs and milk following preventive vaccination. Transbound. Emerg. Dis. 65 (2), 491–496. https://doi.org/10.1111/tbed.12730.

Belbis, G., Zientara, S., Bréard, E., Sailleau, C., Caignard, G., Vitour, D., Attoui, H., 2017. Bluetongue virus: from BTV-1 to BTV-27. Adv. Virus Res. 99, 161–197. https://doi.org/10.1016/bs.aivir.2017.08.003.

Ben-Gera, J., Klement, E., Khinich, E., Stram, Y., Shpigel, N.Y., 2015. Comparison of the efficacy of Neethling lumpy skin disease virus and x10RM65 sheep-pox live attenuated vaccines for the prevention of lumpy skin disease - the results of a randomized controlled field study. Vaccine 33 (38), 4837–4842. https://doi.org/10.1016/j.vaccine.2015.07.071.

Bitew, M., Nandi, S., Ravishanka, C., Sharma, A., 2016. Humoral immune response and protective efficacy of binary ethylenimine (BEI) inactivated pentavalent bluetongue vaccine after challenge with homologous virus in sheep. Int. J. Virol. 13 (1), 43–52. https://doi.org/10.3923/ijv.2017.43.52.

Bitew, M., Ravishankar, C., Chakravarti, S., Kumar Sharma, G., Nandi, S., 2019. Comparative evaluation of T-cell immune response to BTV infection in sheep vaccinated with pentavalent BTV vaccine when compared to unvaccinated animals. Vet. Med. Int., 8762780 https://doi.org/10.1155/2019/8762780.

Bouchemla, F., Agoltsov, V.A., Larionov, S.V., Popova, O.M., Shvenk, E.V., 2018. Epizootiological study on spatiotemporal clusters of Schmallenberg virus and Lumpy skin diseases: the case of Russia. Vet. World 11 (9), 1229–1236. https://doi.org/10.14202/vetworld.2018.1229-1236.

Boumart, Z., Daouam, S., Belkourati, I., Rafi, L., Tuppurainen, E., Tadlaoui, K.O., El Harrak, M., 2016. Comparative innocuity and efficacy of live and inactivated sheeppox vaccines. BMC Vet. Res. 4–9. https://doi.org/10.1186/s12917-016-0754-0.

Calistri, P., Giovannini, A., Savini, G., Bonfanti, L., Cordioli, P., Lelli, R., Caporale, V., 2010. Antibody response in cattle vaccinated against bluetongue serotype 8 in Italy. Transbound. Emerg. Dis. 57 (3), 180–184. https://doi.org/10.1111/j.1865-1682.2010.01136.x.

Celma, C.C.P., Boyce, M., van Rijn, P.A., Eschbaumer, M., Wernike, K., Hoffmann, B., et al., 2013. Rapid generation of replication-deficient monovalent and multivalent vaccines for bluetongue virus: protection against virulent virus challenge in cattle and sheep. J. Virol. 87 (17), 9856–9864. https://doi.org/10.1128/JVI.01514-13.

Celma, C.C., Stewart, M., Wernike, K., Eschbaumer, M., Gonzalez-Molleda, L., Breard, E., et al., 2017. Replication-deficient particles: new insights into the next generation of bluetongue virus vaccines. J. Virol. 91 (1) https://doi.org/10.1128/JVI.01892-16.

Cohen, N.D., Bordin, A.I., 2015. Principles of vaccination. Equine Clinical Immunology. Wiley and Sons, Ltd., pp. 263–278. https://doi.org/10.1002/9781119086512.ch28

El Harrak, M., Loutfi, C., 2000. Camel pox in the calf in Morocco: identification of the isolated virus, development and application to prophylaxis. Revue d'Elevage et de Médecine Vétérinaire Des Pays Tropicaux 53 (2), 165–167. https://doi.org/10.19182/remvt.9745.

Feenstra, F., van Rijn, P.A., 2017. Current and next-generation bluetongue vaccines: requirements, strategies, and prospects for different field situations. Crit. Rev. Microbiol. 43 (2), 142–155. https://doi.org/10.1080/1040841X.2016.1186005.

Hamdi, J., Boumart, Z., Daouam, S., El Arkam, A., Bamouh, Z., Jazouli, M., et al., 2020. Development and evaluation of an inactivated lumpy skin disease vaccine for cattle. Vet. Microbiol. 245 (Nov 2019), 108689. https://doi.org/10.1016/j.vetmic.2020.108689.

Hamers, C., Galleau, S., Chery, R., Blanchet, M., Besancon, L., Cariou, C., et al., 2009. Use of inactivated bluetongue virus serotype 8 vaccine against virulent challenge in sheep and cattle. Vet. Rec. 165 (13), 369–373. https://doi.org/10.1136/vr.165.13.369.

Klement, E., Broglia, A., Antoniou, S.-E., Tsiamadis, V., Plevraki, E., Petrović, T., et al., 2018. Neethling vaccine proved highly effective in controlling lumpy skin disease epidemics in the Balkans. Prev. Vet. Med. https://doi.org/10.1016/j.prevetmed.2018.12.001.

Kononov, A., Byadovskaya, O., Kononova, S., Yashin, R., Zinyakov, N., Mischenko, V., et al., 2019. Detection of vaccine-like strains of lumpy skin disease virus in outbreaks in Russia in 2017. Arch. Virol. 164 (6), 1575–1585. https://doi.org/10.1007/s00705-019-04229-6.

Kumar, N., Sharma, S., Barua, S., Tripathi, B.N., Rouse, B.T., 2018. Virological and immunological outcomes of coinfections. Clin. Microbiol. Rev. 31 (4) https://doi.org/10.1128/CMR.00111-17.

Lesch, H.P., Valonen, P., Karhinen, M., 2020. Evaluation of the single-use fixed-bed bioreactors in scalable virus production. Biotechnol. J. 16 (1) https://doi.org/10.1002/biot.202000020.

Lojkić, I., Šimić, I., Krešić, N., Bedeković, T., 2018. Complete genome sequence of a lumpy skin disease virus strain isolated from the skin of a vaccinated animal. Genome Announc. 6 (22) https://doi.org/10.1128/genomeA.00482-18.

Maan, S., Maan, N.S., Belaganahalli, M.N., Potgieter, A.C., Kumar, V., Batra, K., et al., 2016. Development and evaluation of real time RT-PCR assays for detection and typing of bluetongue virus. PLoS One 11 (9). https://doi.org/10.1371/journal.pone.0163014 e0163014.

Madhavan, A., Venkatesan, G., Kumar, A., 2016. Review article capripoxviruses of small ruminants: current updates and future perspectives. Asian J. Anim. Vet. Adv. 11 (12), 757–770. https://doi.org/10.3923/ajava.2016.757.770.

Martinelle, L., Dal Pozzo, F., Thys, C., De Leeuw, I., Van Campe, W., De Clercq, K., et al., 2018. Assessment of cross-protection induced by a bluetongue virus (BTV) serotype 8 vaccine towards other BTV serotypes in experimental conditions. Vet. Res. 49 (1), 63. https://doi.org/10.1186/s13567-018-0556-4.

Milovanović, M., Dietze, K., Milićević, V., Radojičić, S., Valčić, M., Moritz, T., Hoffmann, B., 2019. Humoral immune response to repeated lumpy skin disease virus vaccination and performance of serological tests. BMC Vet. Res. 15 (1), 80. https://doi.org/10.1186/s12917-019-1831-y.

Morgenstern, M., Klement, E., 2020. The effect of vaccination with live attenuated neethling lumpy skin disease vaccine on milk production and mortality - an analysis of 77 dairy farms in Israel. Vaccines 8 (2), 324. https://doi.org/10.3390/vaccines8020324.

Norian, R., Afzal ahangaran, N., Varshovi, Hreza, Azadmehr, A., 2017. Evaluation of cell-mediated immune response in PBMCs of calves vaccinated by capri pox vaccines using ELISA and real-time RT-PCR. J. Mol. Med. 5 (2), 3–8. https://doi.org/10.29252/rmm.5.2.3.

Oura, Ca.L., Edwards, L., Batten, C.A., 2012. Evaluation of the humoral immune response in adult dairy cattle three years after vaccination with a bluetongue serotype 8 inactivated vaccine. Vaccine 30 (2), 112–115. https://doi.org/10.1016/j.vaccine.2011.10.100.

Pérez de Diego, A.C., Sánchez-Cordón, P.J., de las Heras, A.I., Sánchez-Vizcaíno, J.M., 2012. Characterization of the immune response induced by a commercially available inactivated bluetongue virus serotype 1 vaccine in sheep. Transfus. Apher. Sci. 2012, 147158 https://doi.org/10.1100/2012/147158.

Ries, C., Beer, M., Hoffmann, B., 2019. BTV antibody longevity in cattle five to eight years post BTV-8 vaccination. Vaccine 37 (20), 2656–2660. https://doi.org/10.1016/j.vaccine.2019.03.082.

Rojas, J.M., Rodríguez-Martín, D., Martín, V., Sevilla, N., 2019. Diagnosing bluetongue virus in domestic ruminants: current perspectives. Vet. Med. Res. Rep. 10, 17–27. https://doi.org/10.2147/VMRR.S163804.

Saganuwan, S.A., 2016. The new algorithm for calculation of median lethal dose (LD50) and effective dose fifty (ED50) of Micrarus fulvius venom and anti-venom in mice. Int. J. Vet. Sci. Med. 4 (1), 1–4. https://doi.org/10.1016/j.ijvsm.2016.09.001.

Savini, G., Maclachlan, N.J., Sanchez-vizcaino, J., 2008. Vaccines against bluetongue in Europe. Comp. Immunol. Microbiol. Infect. Dis. 31, 101–120. https://doi.org/10.1016/j.cimid.2007.07.006.

Savini, G., Hamers, C., Conte, A., Migliaccio, P., Bonfini, B., Teodori, L., et al., 2009. Assessment of efficacy of a bivalent BTV-2 and BTV-4 inactivated vaccine by

vaccination and challenge in cattle. Vet. Microbiol. 133 (1–2), 1–8. https://doi.org/10.1016/j.vetmic.2008.05.032.

Sohier, C., Haegeman, A., Mostin, L., De Leeuw, I., Campe, W.V., De Vleeschauwer, A., et al., 2019. Experimental evidence of mechanical lumpy skin disease virus transmission by Stomoxys calcitrans biting flies and Haematopota spp. Horseflies. Sci. Rep. 9 (1), 20076. https://doi.org/10.1038/s41598-019-56605-6.

Sprygin, A., Artyuchova, E., Babin, Y., Prutnikov, P., Kostrova, E., Byadovskaya, O., Kononov, A., 2018. Epidemiological characterization of lumpy skin disease outbreaks in Russia in 2016. Transbound. Emerg. Dis. 65 (6), 1514–1521. https://doi.org/10.1111/tbed.12889.

Teffera, M., Babiuk, S., 2019. Potential of using capripoxvirus vectored vaccines against arboviruses in sheep, goats, and cattle. Front. Vet. Sci. 6, 450. https://doi.org/10.3389/fvets.2019.00450.

Toussaint, J.F., Sailleau, C., Breard, E., Zientara, S., De Clercq, K., 2007. Bluetongue virus detection by two real-time RT-qPCRs targeting two different genomic segments. J. Virol. Methods 140 (1–2), 115–123. https://doi.org/10.1016/j.jviromet.2006.11.007.

Tuppurainen, E., Alexandrov, T., Beltrán-Alcrudo, D., 2017. Lumpy Skin Disease - Field Manual for Veterinarians. Retrieved 19 June 2020 from. http://www.fao.org/3/a-i7330e.pdf.

Tuppurainen, E.S.M., Antoniou, S.-E., Tsiamadis, E., Topkaridou, M., Labus, T., Debeljak, Z., et al., 2018a. Field observations and experiences gained from the implementation of control measures against lumpy skin disease in South-East Europe between 2015 and 2017. Prev. Vet. Med. https://doi.org/10.1016/j.prevetmed.2018.12.006.

Tuppurainen, Eeva, S.M., Babiuk, S., Klement, E., 2018b. Lumpy Skin Disease. Springer International Publishing. https://doi.org/10.1007/978-3-319-92411-3.

Van den Bergh, C., Coetzee, P., Venter, E.H., 2018. Reassortment of bluetongue virus vaccine serotypes in cattle. J. S. Afr. Vet. Assoc. 89 (0) https://doi.org/10.4102/jsava.v89i0.1649.

Van Rijn, P.A., 2019. Prospects of next-generation vaccines for bluetongue. Front. Vet. Sci. 6 https://doi.org/10.3389/fvets.2019.00407.

Wäckerlin, R., Eschbaumer, M., König, P., Hoffmann, B., Beer, M., 2010. Evaluation of humoral response and protective efficacy of three inactivated vaccines against bluetongue virus serotype 8 one year after vaccination of sheep and cattle. Vaccine 28 (27), 4348–4355. https://doi.org/10.1016/j.vaccine.2010.04.055.

Wolff, J., Moritz, T., Schlottau, K., Hoffmann, D., Beer, M., Hoffmann, B., 2021. Development of a safe and highly efficient inactivated vaccine candidate against lumpy skin disease virus. Vaccines 9 (1), 4. https://doi.org/10.3390/vaccines9010004.

World Organization for Animal Health, 2019. Manual of Diagnostic Tests and Vaccines for Terrestrial Animals. In: chapter 3.4.12 (LSD) and chapter 3.1.3 (BT). Retrieved June 28, 2020, from. https://www.oie.int/en/international-standard-setting/terrestrial-manual/access-online/.