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Development and Evaluation of an Inactivated Lumpy Skin Disease Vaccine for Cattle

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ABSTRACT

Lumpy skin disease (LSD) of cattle is caused by a virus within Capripoxvirus genus. It leads to huge economic losses in addition to trade and animal movement limitation. Vaccination is the only economically feasible way to control this vector-borne disease. Only live attenuated vaccines have been used so far and no inactivated vaccine has been developed nor tested in cattle.

In this study, we developed an inactivated oily adjuvanted vaccine based on Neethling strain and tested it on cattle. Selected criteria of appreciation were safety, antibody response by Virus Neutralization and protection through challenge. A field trial was also performed in Bulgaria.

The vaccine was safe and did not cause any adverse reaction, high level of specific antibodies was obtained starting from day 7 post-vaccination and protection against virulent challenge strain that caused typical disease in control animals was total. Induced protection was similar to that obtained with live vaccine, without any adverse effect. In addition, the field study confirmed safety and efficacy of the vaccine, which did not show any adverse reaction and induced a high level of antibodies for up to one year.

General prophylaxis based on inactivated vaccine could be of great benefit in endemic countries or at risk regions.

1. Introduction

Lumpy skin disease (LSD) of cattle is a vector-borne disease caused by Lumpy Skin Disease Virus (LSDV), belonging to Capripoxvirus genus (OIE, 2018). LSDV is mainly transmitted by blood-feeding arthropods such us Aedes, Stomoxys, Amblyomma and Rhipicephalus (Milovanović et al., 2019), most outbreaks have been observed following seasonal rains, when arthropod replication increases (Weiss, 1968; Molla et al., 2017; Mulatu and Feyisa, 2018). Fever, skin nodules, emaciation and lesions in the mouth, pharynx and respiratory tract characterize the disease with around 10% mortality rate (Babiuk et al., 2008; Ayelet et al., 2014). It can cause important economic losses within cattle population, such as drop of milk production, weight loss, skin damage and temporary or permanent sterility in both bulls and cows (Ayelet et al., 2014).

LSD was confined in Africa, first reported in Zambia in 1929 (Macdonald, 1930). For decades, the virus spread to the horn of Africa and, in 1989, an outbreak occurred in Israel before spreading to other Middle East countries (Yeruham et al., 1994; Tuppurainen and Oura, 2012). In 2014, the disease appeared in Iran and northern parts of Cyprus. In 2015, first cases of LSD were reported in Europe in Greece, close to Turkish border and in the northern Caucasus region including Azerbaijan, Georgia and Russia (World Animal Health Information Database (WAHID), 2019). In 2016, the disease was notified in many parts of Europe and Asia, including Bulgaria, Serbia, Albania and Kazakhstan (Tuppurainen et al., 2017). In 2019, no LSD outbreaks were notified in southeastern Europe, however the disease was reported for the first time in India, China and Bangladesh (World Animal Health Information Database (WAHID), 2019).

In view of the high risk of LSDV spreading to new territories, the European Food Safety Authority (EFSA) recommended the use of a safe and efficient inactivated or DIVA vaccine for prevention in disease-free countries (EFSA, 2015). Indeed, LSD live vaccines have been reported to cause a local inflammation, drop in milk production and sometimes a

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mild generalized disease with skin lesions called “Neethling disease” (Ben-Gera et al., 2015; Abutarbush et al., 2016; Katsoulos et al., 2017; Bedekovic et al., 2017; Hovari and Beltran-Alcrudo, 2018). Live vaccines also present a potential risk of transmission of extraneous agents, however, they are the only ones which had been used so far to prevent LSD.

The use of inactivated sterile products has the advantage of safety, no replication, no spread in co-habitant unvaccinated animals and no reversion to virulence. In addition, no consequences for the country sanitary status, allowing in principle pursue of trade activities and animal movements.

In this study, we developed an inactivated, oily adjuvanted vaccine based on LSD Neethling strain grown in cell culture. The vaccine was tested for safety, immunogenicity and potency on cattle, in comparison with unvaccinated control animals. We report in this paper results of testing on target species and discuss opportunities of the use at large scale the inactivated LSD vaccine to control global spread of the disease.

2. Material and method

2.1. Good manufacturing practices (GMP)

All procedures, data recording and storage involved in this study were performed in accordance with GMP at MCI Santé Animale facilities in Mohammedia, Morocco, which received the triple certification Quality-Safety Environment (QSE) according to ISO9001, OHSAS 18001 and ISO 14001.

2.2. Preparation of vaccine virus strain

The LSD Neethling attenuated strain of South Africa origin was used to prepare the inactivated vaccine. This virus has been attenuated through 61 passages on chorio-allantoic membrane and used as a vaccine strain for decades in Africa, Middle East and Europe recently (Davies, 1991; Klement, 2018). This strain has advantages to protect properly against the disease despite of reported post-vaccination reaction in vaccinated population (Ben-Gera et al., 2015).

This virus was passed once on 8 seronegative female calves, aged 4-6 months, by subcutaneous injection of $10^{5.0}$ Tissue culture infective dose 50 ($TCID_{50}$)/animal. The virus strain was re-isolated from one cattle that showed generalized mild nodules with an important local inflammation at the injection site. Recovering the virus from nodules was carried out on primary testis cells after inoculation of the filtrated skin lesions homogenate. The first passage was considered as the master seed and was tested for sterility, identity and purity as recommended by international standards (OIE Manual).

2.3. Preparation of the inactivated and live vaccines

For the antigen preparation, the master seed virus was passed three times on primary testis cells maintained in DMEM with 10% irradiated fetal calf serum. Inoculation was carried out using a Multiplicity Of Infection (MOI) of 0.01. The viral suspension was harvested after five days of incubation at 35 °C. Sterility, identity, purity and titration were performed as part of the quality control of the intermediate product. Inactivation was conducted using Bi-ethylimine bromure (BEI) and complete inactivation was confirmed after 3 blind passages of the suspension on susceptible cells.

For the vaccine formulation, the inactivated antigen was mixed with an oily emulsion with Montanide adjuvant from SEPPIC. Formulation was calculated to ensure a dose of the antigen of around 10^6 $TCID_{50}$/animal before inactivation. The final product was tested for stability, emulsion type and other physico-chemical parameters.

A live vaccine was also prepared using the LSD Neethling vaccine strain propagated on primary testis cells. The vaccine was prepared with the virus suspension by the addition of a stabilizer (4% peptone, 8% sucrose and 2% glutamate) and freeze-dried in LSI lyodryer. The live vaccine was tested for sterility, identity, purity and infectious titer. To vaccinate, we used an animal dose of $10^{4.0}$ $TCID_{50}$.

2.4. Animals

Forty cattle, male and female, 4-6 months old, were housed in ABSL3 facility and tested negative for presence of LSD antibodies by Virus Neutralization (VNT). Experiments on cattle were carried out in accordance with International Guidelines for the care and handling of experimental animals as described in a protocol approved by “The MCI Santé Animale Ethic Committee for Animal Experiment”.

2.5. Vaccine safety and immunogenicity evaluation

Safety and serological response to inactivated vaccine were tested on a group of 15 cattle, vaccinated by intramuscular route at day 0 and boosted at day 28. Each animal received 2 ml of the vaccine preparation. Live attenuated vaccine was injected SC on a group of 20 cattle. Animals were monitored two months after vaccination for clinical signs and antibody response. Five cattle were maintained unvaccinated as control animals.

2.6. Efficacy of the vaccine

The experiment was subcontracted with the European Reference Laboratory, Sciensano, in Belgium. Three groups of naïve cattle, 4-6 months old, were vaccinated then inoculated with the virulent LSDV Israeli field isolate ($10^{6.5}$ $TCID_{50}$/100 µl) by intravenous and intradermal route. The first and second group, 7 cattle each, were vaccinated with inactivated and live vaccines, respectively. A third group of 5 cattle was kept unvaccinated as control group. Animals were monitored 21 days for clinical signs, presence of viral DNA in blood and oral swabs. Three weeks following challenge, animals were euthanized and necropsied, organs were analyzed by PCR for presence of LSDV DNA. A clinical scoring was established based on general condition, number and location of nodules, food uptake and lymph node swelling.

2.7. Field trial

Inactivated vaccine was tested in normal conditions of the field on 181 cattle from 4 dairy farms in different regions of Bulgaria. Animals used in the trial were black-pie and Montbeliarde breeds, aged between one and 10 years. Among 181 cattle, 93 were previously vaccinated with a live vaccine and 88 were young naïve calves. The animals were sampled for serology at D0, D28, D60, D120 and D360 post-vaccination and tested by both ELISA and VNT.

2.8. Laboratory analysis

2.8.1. PCR

Blood, oral swabs and organs were analyzed using PCR panel described by Haegeman et al (2016). Screening was carried out with the D5R and confirmation with the E3L and J6R, in case of doubtful results. A number of samples was also tested with the DIVA PCR described by Agianniotaki et al (2017), in order to distinguish between vaccine and wild type LSDV.

2.8.2. Serological response monitoring

In order to determine pre and post-vaccination antibody response to LSD, serology was carried out using VNT as described in the OIE Terrestrial Manual (2018). Blood samples were obtained in plain vacuum tubes via jugular venipuncture using an 18-G needle weekly up to 2 months. Paired sera were kept at −20°C until analysis.

Sera were heat inactivated and serial 1:3 dilutions were mixed with

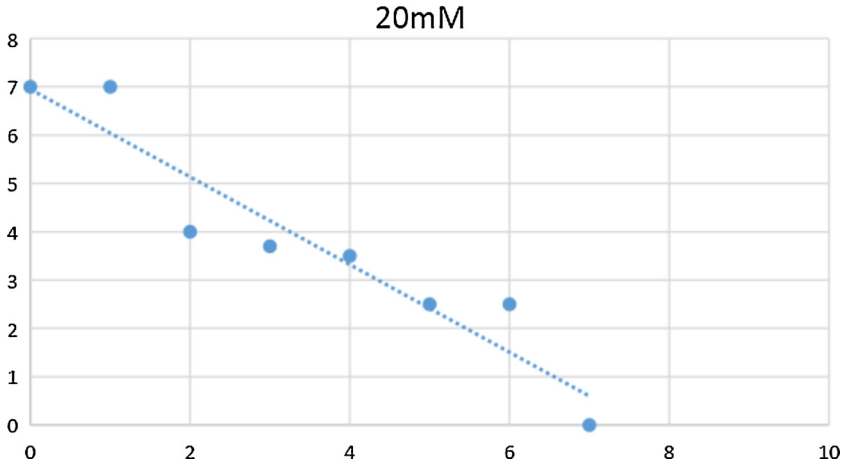

Fig. 1. Inactivation curve of Lumpy Skin Disease virus using BEI.

a constant dose of LSD virus then incubated for one hour. MDBK (ATCC® CCL22™) cell suspension was then added and CPE observed at 7 days post-incubation. To validate the dose of the virus used in VNT, positive control without serum and negative control without virus as well as cells control were introduced in the assay. Neutralizing antibody titers were calculated in accordance with Reed and Muench (1938) method.

The ELISA kit IDScreen® Capripox Double Antigen was also used to detect antibody response during the field trial. Serum samples were added to 96 well plates, coated with Capripoxvirus purified antigen and incubated for 90 min at 21 °C. Wells of the plate were washed with a wash solution, then 100 µl of the conjugate added to each well. After an incubation of 30 min at 21 °C, wells were washed and 100 ml of Tetramethylbenzidine (TMB) substrate solution added to each well. After 15 min of incubation at 21 °C in darkness, reaction was stopped by addition of stop solution and optical density was measured at 450 nm. Sera were considered negative if the ratio S/P $< 30\%$ and positive if the ratio S/P $\geq 30\%$.

2.8.3. Cell-mediated immunity

To evaluate the cell mediated immune response on animals vaccinated with LSDV inactivated vaccine, the Interferon Gamma (IFN-γ) levels upon stimulation of the heparin blood were examined using the Bovigam TB kit. Blood samples were incubated overnight with LSD virus, the derivative of a "pokeweed" protein used as a positive control and a blank (PBS) to stimulate lymphocytes. Then, the IFN-γ present in the plasma supernatant of each blood sample was determined using a sandwich ELISA.

2.9. Statistical analysis

The differences between LSDV antibody titers obtained with vaccinated animals were tested for significant using student t-test. Comparison of vaccinated and unvaccinated cattle at challenge was also performed based on clinical scoring obtained after challenge. Values of $p \leq 0.05$ were considered significant.

3. Results

3.1. A virus seed was prepared from nodules of cattle inoculated with LSDV Neethling strain

To improve immunogenicity of the LSD Neethling strain, the virus was injected into a group of 8 cattle. Two cattle among 8 showed numerous nodules on different parts of the body, starting from day 8 pv. The 6 other animals showed only local inflammation of 2-6 cm ∅ and were discarded from the study. Animal 1545 showed nodules first on day 8 pv, that extended to the whole body 3 days later and remained until euthanasia, 13 days pv. During this period, no hyperthermia was recorded. A small inflammation at the injection site was observed at day 7 (1 x 1 cm) and increased to reach 3 x 10 cm at day 13 pv. Following necropsy, the animal showed a prescapular, pulmonary, mesenteric and inguinal lymph node enlargement. A marked inflammatory subcutaneous tissue kidney shaped at the injection site was also apparent in addition to scattered nodules in the skin and nasal cavity. Moreover, an important swelling of 3 cm was noticed in the lower lobe of the right lung.

Animal 5064 showed smaller and milder nodules starting from day 10 pv and distributed mainly in the flank and the neck. No hyperthermia was recorded until its euthanasia and swelling at inoculation site was of 2 x 10 cm at day 14 pv. Following necropsy, the animal showed a pulmonary emphysema and pulmonary and mesenteric lymph node enlargement.

Isolate from the animal 1545 was selected for seed preparation because of early and typical cytopathic effect presence and high titer registered ($10^{6.4}$TCID$_{50}$/ml). Inflammatory tissue from the skin and nodules were sampled, homogenized and passed on cells for virus isolation.

3.2. An inactivated oily adjuvanted LSDV vaccine was produced

For antigen preparation, a characteristic CPE on primary cells appeared at day 3 pi with LSD strain isolated from the animal 1545 nodules. CPE generalized in cells at day 5 pi, the obtained titer of viral suspension was between $10^{7.0}$ and $10^{7.2}$ TCID$_{50}$/ml. The harvested viral suspension was inactivated using BEI after 7 hours (Fig. 1).

Vaccine formulation resulted in white colored emulsion, one phase water in oil type. Injected volume was 2 ml in deep intramuscular at day 0 and day 28.

For the live attenuated vaccine, the virus showed cytopathic effect starting from day 3 and was harvested at day 5 pi. Titration revealed a titer of $10^{6.8}$ to $10^{7.0}$ TCID$_{50}$/ml.

3.3. The inactivated LSDV vaccine was safe and immunogenic

A group of 15 cattle was used for safety and immunogenicity of the inactivated LSD vaccine and another group of 20 cattle was vaccinated with the live vaccine and tested comparatively. After vaccination with the inactivated vaccine, animals remained healthy and a moderate temperature rise (39.6 °C) was observed at day 4 pv on 2 cattle among 15 after the first injection. Another cattle showed a local reaction which was observed starting from day 4 after the booster and the swelling

Table 1
Animals vaccinated with inactivated and live vaccines serologically positive using Virus Neutralization Test.

Vaccine	Number of animals	D0	D7	D14	D21	D28
Inactivated	15	0	3	6	10	13
		0%	**20%**	**40%**	**67%**	**87%**
Live	20	0	0	5	9	10
		0%	**0%**	**25%**	**45%**	**50%**

persisted during 10 days. After vaccination with the live attenuated vaccine, average body temperature reached 39.5 °C at 3 to 4 days post-vaccination then returned to normal. No local inflammation was observed at the injection site and no nodules were observed after vaccination in the 20 cattle vaccinated with the live vaccine.

Serology by VNT performed on the two groups of inactivated and live vaccine is reported in Table 1. Antibody response was significantly higher ($P \leq 0.05$) for cattle vaccinated with the inactivated vaccine compared to live vaccine. Percentage of reactors with inactivated vaccine was of 20% starting from day 7 pv and reached 87% at day 28 pv. Live attenuated vaccine induced antibody response starting from day 14 pv (25%) and reached 50% at day 28 pv.

The booster is well marked in the Fig. 2 that reports neutralization titer of positive animals in inactivated vaccine group.

Thirteen animals among 15 vaccinated with inactivated LSD vaccine responded to vaccination when tested by IFN-γ, 9 strongly and 4 moderately. No responsiveness was seen in 2 animals.

3.4. Inactivated LSDV vaccine provided complete protection against challenge with virulent LSDV

Efficacy of the vaccine was subcontracted with Sciensano European reference laboratory. Groups of cattle were vaccinated and challenged with a highly virulent LSD strain. Cattle were monitored for clinical signs and presence of viral DNA in blood, swabs and organs.

In both groups of vaccinated animals, body temperature remained normal and no animal showed nodules on the body after challenge with the virulent LSDV strain. Blood and oral swabs showed absence of LSDV DNA and at necropsy, only traces of LSDV genome were found in the skin of 2 animals (CT 38.5).

In the unvaccinated control group, 3 out of 5 animals showed a severe disease with generalized nodules over the whole body between 7 and 8 days pi, viremia and positive oral swabs. At necropsy, typical LSD lesions were observed and confirmed positive by PCR. Clinical scores were significantly higher in group of unvaccinated animals comparing to vaccinated cattle ($P \leq 0.05$).

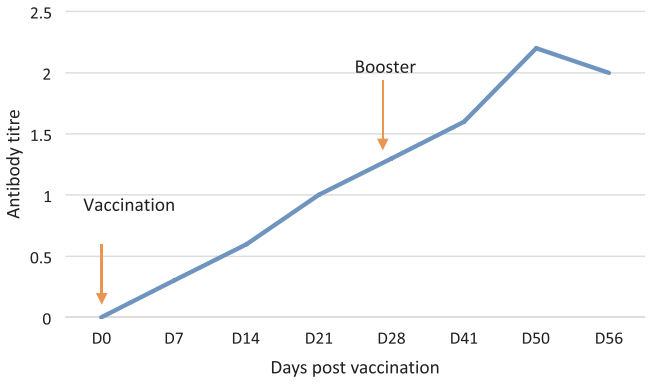

Fig. 2. Mean neutralizing antibody titers of 15 cattle vaccinated with inactivated LSD vaccine. The arrow correspond to the booster.

Table 2
Animals vaccinated with the inactivated LSD vaccine in the field and tested positive by ELISA.

Animals	Number	Seroconverted	Percentage
Naïve	88	69	78%
Previously vaccinated	91	72	79%
Total	179	141	78,7%

3.5. Field trial showed a high seroconversion of cattle vaccinated with LSDV inactivated vaccine

The inactivated LSD vaccine was tested at large scale, under natural field conditions in Bulgaria. Response showed that 141 cattle out of 179 animals (80%) seroconverted at 28 dpv and 124 out of 179 (68%) seroconverted at 120 dpv using ELISA test (Table 2). Using neutralization test, the percentage recorded was of 70%.

4. Discussion

Because of its recent expansion in the Middle East, Europe and Central Asia, the LSD became an important threat to the bovine production chain, which requires appropriate and effective solutions the earliest.

Only live attenuated vaccines are commercially available in the market. Live vaccines create a strong and long-lasting immune response and have certainly contributed to control disease in many regions (Klement, 2018; Tuppurainen et al., 2018), however, because of side effects, they have not been well tolerated by farmers in infected areas (Tuppurainen et al., 2018). Indeed, live vaccines have been reported to cause local vaccine inflammation and a mild generalized disease with skin lesions (Ben-Gera et al., 2015; Bedekovic et al., 2017; Lojkić et al., 2018; Hovari and Beltran-Alcrudo, 2018). In addition, isolation of the vaccine strain from nodules in vaccinated animals is considered a risk of potential spreading by insects (Bedekovic et al., 2017). The origin of this reaction is still unknown, probably due to incomplete attenuation or presence in the vaccine of non-homogenous particles of different virulence level. Some authors have supported the hypothesis that Neethling disease is more related to the animal health status and probable existence of recurrent infection, while others have reported genetic exchanges between closely related strains of capripoxviruses and vaccine strain in the field (Sprygin et al., 2018).

In addition, live vaccines, especially those produced in primary cells, present the risk of contamination by adventitious agents, such as detection of BTV in LSDV and SPPV commercial batch vaccines reported respectively by Bumbarov et al (2016) and Rajko-Nenow et al. (2020).

Inactivated vaccines are known to be safe, more stable in the tropics, they allow combination with other antigens to make polyvalent vaccines and could be applied in disease-free countries without loss of free status. Moreover, with inactivated vaccines, there is no reversion to virulence or transmission of the virus between vaccinated and co-habitant. However, inactivated vaccines are generally costly and require multiple administrations.

Advantages of such vaccines remain enormous in countries at risk of LSD introduction with huge cattle population. Inactivated vaccines could also be used in the final phase of disease eradication as part of overall strategy that uses live vaccines first to reduce prevalence. Besides, the development of new model technology can substantially reduce the price and number of injections needed.

To date, inactivated LSD vaccine has never been developed nor tested to our knowledge. An inactivated vaccine has been successfully tested recently against Sheeppox virus, which is a disease caused by a virus of the same genus of LSD. The authors reported that oily adjuvanted vaccine showed complete protection against virulent strain

that caused disease in control animals. Antibody response was also significantly high and long lasting (Boumart et al., 2016). In the family of Poxviridae, Camelpox inactivated vaccine has also been developed and used in Morocco and other countries in mass vaccination for years and contributed to limit expansion of the disease in camel population (El Harrak and Loutfi, 2000).

In the present study, we developed an inactivated vaccine based on Neethling attenuated strain that was passed once on animal to generate complete immunogenic particles. The produced seed showed a titer of $10^{6.4}$ $TCID_{50}$/ml, an early CPE and complied with quality control testing. The harvested suspension titer was between 10^{7} and $10^{7.2}$ $TCID_{50}$/ml, which is higher than what is usually obtained with live vaccine ($10^{6.8}$ $TCID_{50}$/ml).

Inactivation was successfully accomplished by BEI with a concentration of 20 mM, comparable to data reported by Awad et al. (2003) for the Sheeppox virus. The use of other inactivate agents is not recommended because of safety concerns as reported for β-propiolactone or formaldehyde (International Agency for Research on Cancer (IARC, 1974; IARC, 2006).

A sufficient number of animals was vaccinated with the inactivated vaccine under controlled conditions. Complete safety was demonstrated in vaccinated cattle, general health status remained normal and no excessive local swelling was noted at the injection sites, only a limited inflammation, normally observed with oily adjuvanted vaccines. With the inactivated vaccine, the local inflammation seemed more marked after the booster than in primary injection. This may be avoided if the two injections are performed in longer delay (3 months instead of 21 days).

The selected inactivated vaccine dose (10^{6}) seems to procure a good protection level. In this study, we did not perform a dose escalation test, but decided on the dose based on previous experimentations and manufacturing feasibility. Indeed, this titer is easily obtained without any concentration of the harvested antigen which may increase the total cost of the vaccine.

Regarding immunogenicity, positive reactors were recorded starting from day 7 and 13 among 15 cattle vaccinated with the inactivated vaccine. Those results are in accordance with previous reports which state that the onset of immunity is around 10 days and complete by 3 weeks (Hovari and Beltran-Alcrudo, 2018).

The inactivated vaccine induced higher humoral antibody response comparated to what is normally observed with the live vaccine, suggesting that the inactivated antigens are essentially conferring humoral immunity. However, we have also observed a response to IFNγ which is almost the same than response to live vaccine even if the latter is known to induce cell-mediated immunity essentially. Indeed, serological response to live vaccine we obtained is around 50% positive animals which is in concordance with other authors who reported a seroconversion rate between 34% and 65% with live attenuated LSD vaccine (Milovanović et al., 2019; Samojlović et al., 2019). This response depends probably on the vaccine dose, animal status and serological technique used.

Results of the potency test showed full protection obtained with the developed inactivated vaccine. The test was validated since at least 3 out of 5 control animals showed characteristic symptoms of LSD. The normally obtained results after challenge specify that 50% of unvaccinated control animals should display clinical symptoms of the disease. LSDV was found in all organs of necropsied unvaccinated animals while in contrast, only traces of DNA were detected in few organs by qPCR in cattle vaccinated with the inactivated vaccine.

Field trial conducted with the inactivated LSD vaccine showed a percentage of 88% of positive animals, sampled at day 28, 60 and 120 post-vaccination. Sera were evaluated by ELISA test, which is less-time consuming and allows analysis of large number of samples in mass screening activities, compared to standard serological test (Milovanović et al., 2019). Sera were also evaluated by Virus neutralization test which detected up to 70% of positive animals. Obtained percentage of positive animals was higher with ELISA, which can be explained by the high sensitivity of the test, detecting all types of antibodies, not only IgG as with VNT. Results allowed us to conclude on a satisfactory immunological response of animals that received inactivated vaccine.

5. Conclusion

To summarize, the inactivated vaccine could provide a good alternative and a valuable tool to protect livestock against LSD, particularly in disease-free areas at risk of introduction or as part of an eradication program. The inactivated LSD vaccine developed in this study and tested in laboratory and field conditions, showed positive results of protection and antibody response in target species. The product is completely safe and as protective as live attenuated vaccine. Further studies are in progress to explore immunity duration. Reduction of the vaccine dose or immunization with a single injection as well as DIVA potential of this vaccine should also be explored.

Author's contribution

JH performed experiments, analyzed data and wrote the manuscript. SD, AE, ZBo, ZBa and MJ carried out the experiments, KT and OF participated in the design and the follow up of the study, BG participated in the design and analysis of data of field trial. ME participated in the design of the study, manuscript drafting, data analysis and interpretation. All authors read and approved the final manuscript.

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Declaration of Competing Interest

All authors declared no conflict of interest.

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Appendix A. Supplementary data

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