VIROLOGICA SINICA, February 2013, 28 (1):036-042
DOI 10.1007/s12250-013-3283-z
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Research Article

An Inactivated Vaccine from a Field Strain of Bovine Herpesvirus-1 (BoHV-1) has High Antigenic Mass and Induces Strong Efficacy in a Rabbit Model*

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Bovine Herpesvirus-1 (BoHV-1) is a DNA virus belonging to the family *Herpesviridae*, subfamily *Alfaherpesvirinae*; it is a worldwide pathogen, causing serious economic losses in livestock. In Colombia there have been multiple isolates of BoHV-1 that have been subjected to molecular characterization, classifying most of the country isolates as BoHV-1.1. In the present study we developed and evaluated an ethyleneimine binary inactivated isolate from the native BoHV-1 strain (Córdoba-2) in a rabbit model of vaccination and infection. The vaccine was evaluated in two phases, one of immunogenicity with vaccination and a booster after 21 days, and an evaluation phase of protection against challenge with a highly virulent reference strain. The results demonstrate optimum serum-conversion, with protective neutralizing antibody titers 28 days post vaccination and optimal protection against challenge with the reference strain with decreased clinical signs of infection, protection against the onset of fever and decrease of virus excretion post challenge. In conclusion, our results show the enormous potential that an immunogenic inactivated vaccine has produced from the native BoHV-1.1 strain, which produces a high antigen mass to the vaccine to induce optimal immunity and protection, and it is a strong candidate for evaluation and possible future use in different cattle populations.

Antigenic Mass, Adjuvant, Binary Ethyleneimine, Bovine Herpesvirus-1, Vaccine

Bovine herpesvirus-1 (BoHV-1) is a DNA virus belonging to order *Herpesvirales, Herpesviridae* family and the *Alfaherpesvinae* subfamily (Davison A J, et al., 2009) that has a wide diversity serologically indistinguishable strains. However, based on genome analyses with restriction endonucleases it has been classified into 3 subtypes: BoHV-1.1, BoHV-1.2a and BoHV-1.2b (Engels M, et al., 1981). In Colombia, multiple isolates of BoHV-1 have been subjected to molecular characterization, and most have been classified as BoHV-1.1 (Ruiz-Saenz J, et al., 2012).

BoHV-1 control is based on simple hygienic measures for the herd, quarantine periods for new cattle and BoHV-1 seronegative status for new animals. Additionally, recommended vaccines for BoHV-1 can be effective in reducing the clinical manifestations associated with viral infection and reduce virus shedding after infection, thus leading to a reduction in economic losses. However, current vaccines fail to fully protect from field BoHV-1 infection (OIE, 2004) (OIE, World organization for Animal health).

Globally, the market for BoHV-1 vaccines is broad and diverse, allowing the producers, technicians and

Received: 2012-09-07, Accepted: 2013-01-17
* Foundation items: This study was supported by the División de Investigación Universidad Nacional de Colombia, grants No.20201007738 and 202010013254. Julian Ruiz-Sáenz had received a PhD Fellowship from COLCIENCIAS.
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veterinarians to choose from a wide range of vaccines. Commercially, the most widely distributed are the BoHV-1 polyvalent products commonly associated with other viral agents such as bovine viral diarrhea virus, bovine parainfluenza virus type 3, respiratory syncytial virus and bacterial agents such as *Pasteurella spp*, *Leptospira spp*, making difficult to achieve a specific immunization for BoHV-1 in areas where other agents have no epidemiological importance or where vaccination would be used as a mechanism to control a possible outbreak (Ruiz-Saenz J, et al., 2009).

Previous studies from our laboratory have allowed us to isolate and characterize the so-called "Córdoba-2" BoHV-1 strain that belongs to subtype BoHV-1.1. In one step viral growth curves it has a high virulence behavior and good glycoprotein epitope expression by Western blot (Ruiz-Saenz J, et al., 2012), suggesting that the strain is a good candidate to be used as seed for the development of an inactivated vaccine with high immunogenic potential (OIE, 2004).

The aim of the present study was therefore to develop a monovalent inactivated vaccine from a native strain of BoHV-1, and assess its potential efficiency in a rabbit model, in order to protect the bovine population against BoHV-1 infection, leading to a future possible improvement of the sanitary quality of the livestock.

MATERIALS AND METHODS

Virus and Cells

Mardin-Darby Bovine Kidney (MDBK) cells (ATCC CCL-22) obtained from the Virology Laboratory at the National University of Colombia were used and maintained at 37 ℃ in 5% CO_2 in Minimum Essential Media (MEM; Gibco-Invitrogen, Inc., Grand Island, NY) supplemented with 10% fetal bovine serum (FBS; Gibco-Invitrogen Inc), penicillin-streptomycin (10.000 U/mL and 10 μg/mL respectively) and amphotericin (0.25 μg/mL; Gibco-Invitrogen, Inc.).

Iowa virus (BoHV-1.1) obtained from the Virology Laboratory at the National University of Colombia served as reference virus and the native virus "Cordoba-2", was isolated from a bovine animal under conditions of immunosuppression and was characterized as a BoHV-1.1 strain (Ruiz-Saenz J, et al., 2012). Both viruses were harvested, titrated in MDBK cells and stored at -70 ℃.

Virus Titration

Viruses were titrated in duplicate using a tissue culture infectious dose of 50% (TCID50%) on MDBK cells in 96-well plates (USA Scientific, Inc., Ocala, FL). Briefly, 15.000 MDBK cells per well, inoculated with decimal dilutions of the original stock of virus, and maintenance medium (2% FBS, penicillin-streptomycin (10 000 U/mL and 10 μg/mL respectively) and amphotericin (0.25 μg/mL) were added and incubated for 72 h at 37 ℃ at 5% CO_2, then the plates were inactivated and fixed with 3.7% formaldehyde in PBS and stained with crystal violet. The infectious dose was calculated according to the Spearman Kaerber method (Hierholzer J C, et al., 1996).

Binary Ethyleneimine (BEI) Inactivation

For the inactivation of the Cordoba-2 strain, BEI was used. Briefly, working under biosafety and sterile conditions, a T75 cm^2 MDBK cell culture flask (BD Falcon™ Biosciences) was infected with BoHV-1 strain Córdoba-2 at a MOI of 10 and incubated for 48 h at 37 ℃; the supernatants were then harvested and the infected monolayers were freeze/thawed (-70 / 37 ℃) three times to release intracellular viruses, and then mixed with the supernatant. The suspension was clarified by low speed centrifugation at 5 000 rpm for 10 min. The virus harvest was mixed with 3.2% volume/volume of BEI and incubated at room temperature (16-20℃) under constant agitation for 24 h. Subsequently, BEI was neutralized with a sodium thiosulfate solution to reach 8.7% of the total volume, incubating again at room temperature for 12 h. Finally, a sample (500 μL) of the suspension was inoculated on a monolayer of MDBK cells to verify inactivation of viral culture. Two blind passages were performed to confirm correct inactivation. The inactivated suspension was stored at 4 ℃ until used in the animal model. The solution of BEI was kindly donated by VECOL Inc. (Bogotá-Colombia) Each vaccine dose contained 1 mL of the inactivated viral suspension (total titer per dose: $1x10^{8.25}$ TCID50). No preservatives or adjuvants were added.

Animals

18 New Zealand White (*Oryctolagus cunicullus*) male rabbits, 7-8 weeks of age, with a weight of 2-2.5 kg (Caron L, et al., 2002) and acquired in the "Centre Renewable Natural Resources La Salada" (Medellín, Colombia) were used. Rabbits were kept in separate cages in a conventional animal room with food and water *ad libitum*. Bleeding of the animals was performed by puncture in the marginal ear vein after adequate restraint and disinfection of the area. To be inoculated, the rabbits

were put on their back legs with their nostrils upwards and the inoculum was introduced slowly in each nostril using disposable droppers (Valera A R, et al., 2008). Handling of rabbits and all experimental procedures were performed in compliance with the recommendations of the "Guide for the Care and Use of Laboratory Animals of the National Research Council (Academy Press, 1996, Washington, USA) and previous approval for the Committee for ethics in animal experimentation by the National University.

Experimental Design

Rabbits were divided into three groups, that followed the vaccination schedule reported by Parreno et al., (Parreno V, et al., 2010), receiving two intramuscular doses of 1 mL each at 21 days interval. Group 1 received the inactivated native vaccine (experimental group), group 2 received an inactivated commercial vaccine (positive control group - ViraShield® 6 + VL5HB, Novartis Animal Health U.S., Inc.) and group 3, inoculated with 1 mL of phosphate buffer solution, served as control without vaccination (negative control group). Animals were bled from day zero (pre-vaccination) and every seven days until the end of the experiment at day 50.

On day 30 post vaccination, the animals were challenged with 250 μL of viral suspension (10^7 TCID50%/50 μL) of Iowa reference virus in each nostril (Fig. 1A). After infection, the animals were observed daily for 7 days, rectal temperatures were taken using a digital thermometer (Microlife ® Widnau, Switzerland) and nasal swabs were evaluated for virus isolation. Clinically, rabbits were daily evaluated for the presence of nasal discharge, eye discharge, conjunctivitis and respiratory distress. According to OIE the BoHV-1 vaccine does not pretend to fully avoid field virus infection in animals, thus we did not evaluate virus concentration in blood of the infected rabbits.

Virus Neutralization Test

For this test, the serum was inactivated by incubation for 30 min at 56 ℃, then double serial dilutions were then made from 1 : 2 to 1 : 256 of sera in cell culture plates of 96 wells. These dilutions were then were put in contact with a constant concentration of BoHV-1 strain Iowa (100 TCID50%) and incubated for 2 h at 37 ℃. Subsequently, 10 000 MDBK cells per well were added and incubation was at 37 ℃ in 5% CO_2 for 72 h. Reading was performed and titres were expressed as the reciprocal of the highest serum dilution that completely prevented a cytopathic effect (OIE, 2004).

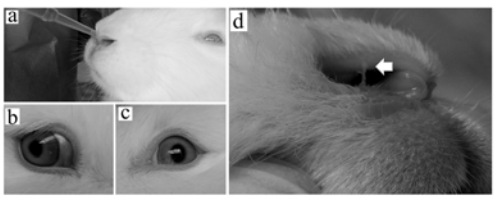

Fig. 1. Intranasal inoculation of BoHV-1 in rabbits. a. Animals were restrained mechanically, and the inoculum was instilled into the nostrils; b. Representative image showing conjunctivitis in the control group without vaccination 3 days post infection; c. Image showing no conjunctivitis in a vaccinated rabbit; d. Mucous secretion in a non-vaccinated rabbit infected with BoHV-1 Iowa.

Indirect ELISA for BoHV-1

SVANOVIR® IBR-Ab (Svanova Biotech AB Sweden), an indirect ELISA that detects IgG antibodies in serum was used, with the following modifications to the recommended procedure. Briefly, serum samples to be tested were diluted and added to test and control wells. This kit is based on an indirect method, and for this reason, the secondary antibodies provided with the kit (horseradish peroxidase conjugated anti-bovine IgG monoclonal antibodies) could not be used, as they would not recognize rabbit antibodies. They were therefore replaced by a horseradish peroxidase conjugated anti-rabbit IgG monoclonal antibody at a 1:200 dilution and incubated for 1 h at 37 ℃. Revealed was achieved using the conventional kit substrate incubated for 10 min in the dark at 20℃. The enzyme reaction was stopped by adding 100 μL of 1 mol/L H_2SO_4 per well and levels were read spectrophotometrically at 450 nm. Serum samples were obtained by subtracting the mean optical density (OD) of the antigen well minus the mean OD of the control well. All samples were run in triplicate.

Isolation and Virus Titration Post-challenge

After challenge with the reference strain, samples were taken from nasal and conjunctival exudates, using a sterile cotton swab. The swab was transported to the laboratory in plastic vials with 1 mL of transport medium (MEM serum-free medium supplemented with penicillin-streptomycin-amphotericin (10 000 U/mL-10 μg/mL-0.25 μg/mL; Gibco-Invitrogen, Inc.) and frozen at -70 ℃. Subsequently, swabs were washed in medium, centrifuged for 20 min at 1500 rpm and the supernatant was filtered through a 0.45 μm membrane. Tenfold dilutions of filtrate were then put in contact with MDBK cell monolayers previously cultured in 96-well plates so as to try to isolate and titrate the virus excreted (Valera A R, et al., 2008).

Statistical Design

The results were analyzed using descriptive statistics,

determining averages, standard deviation and standard error of the mean. Analysis of variance followed by post-hoc test of least significant difference (LSD-ANOVA) was carried out to compare the neutralizing antibody titers, temperatures and virus excretion among vaccinated groups (commercial and native) and the control group (no vaccine). In all cases, $p < 0.05$ was considered statistically significant.

RESULTS

Safety of Native Vaccine

Prior to inactivation the virus titer of the Cordoba strain was $1\times10^{8.25}$ TCID50. Following inactivation, 500 μL of the solution were removed for culture purposes and incubated with MDBK cells for 48 hours, the time required to confirm the complete inactivation of the agent in the absence of characteristic cytopathic effects of infection. In addition, no cytotoxic effect was observed in the inoculated cell cultures (Fig. 2A). After vaccination, the rabbits presented no change in behavior, in local or systemic reactions, in disease development or in temperature changes, indicating the safety of the product.

Antibody Response in the Rabbits

As shown in Fig. 3A none of the rabbits had antibodies before the beginning of the experiment (day 0). Two weeks after vaccination, experimentally vaccinated groups (with native or commercial vaccine) showed a maximum neutralization titer of 1:4, achieving seroconversion in 4/6 subjects (66%) (Fig. 3B). In commercial vaccinated animals, there was a faster increase in total IgG on day 7 post vaccination (Fig. 3A) ($p<0.05$). Pre-booster antibody titers showed a gradual increase; by day 28 post-vaccination all individuals exhibited seroconverted neutralization titers ranging between 1:4 and 1:16.

After the booster, commercially vaccinated rabbits showed higher IgG levels compared to native vaccinated rabbits ($p<0.05$). One week after the booster, there was a peak of neutralization titers 1:32 for the commercial vaccine and 1:16 for the vaccine prepared with the native strain (Fig. B, day 35). All control subjects without vaccination remained seronegative during this time (Fig. 3A, day 28). However, it was clear that although the commercial vaccine induces a faster generation of neutralizing antibody titers, those generated by the native vaccine may be greater in magnitude at the end of the evaluation period (Fig. 3B).

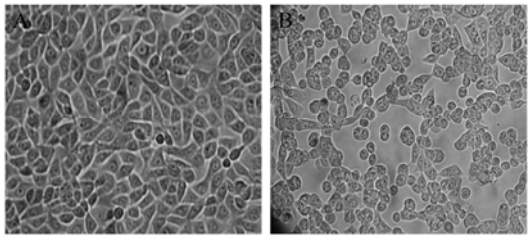

Fig. 2. Complete inactivation of the vaccine Cordoba-2 strain. A. MDBK cultures 48 h post inoculation with BoHV-1 treated with BEI; B. MDBK cells inoculated with untreated virus Cordoba 2. Note in “A” the integrity of the monolayer and in “B” the characteristic BoHV-1 cytopathic effect.

Protection against virus challenge with Iowa reference strain

Rabbits were challenged intranasally with 250 μL of viral suspension (>10^7 TCID50%/50 μL) of the reference Iowa virus and were checked daily. There was a slight increase in temperature in the negative control group 24 h

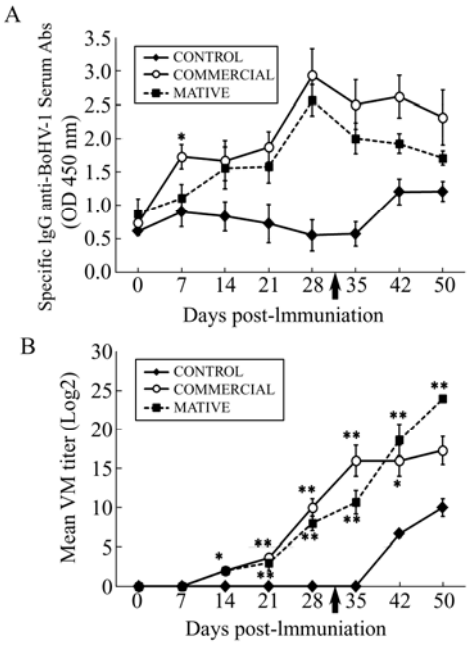

Fig. 3. Humoral immune response induced by intramuscular administration of different inactivated vaccines in rabbits. Rabbits were immunized receiving two intramuscular doses of 1 mL each with 21 days between them (day “0” and day “21”) of a BoHV-1 commercial vaccine, a native experimental vaccine and an additional group was not vaccinated (Control). Ten days after the second (last) immunization the rabbits were intranasally challenged with BoHV-1 (Arrow). Antibody titers were measured before and 4 weeks after the last immunization, as well as after BoHV-1 challenge. A: Serum samples were evaluated by ELISA to detect anti-BoHV-1 antibodies. Curves represent the average values of the OD measured over time. B: Virus neutralizing antibodies were determined in the serum using 100 PFU of BoHV-1. VN: Virus neutralization. Each sample was assayed in triplicate and values represent the mean of each group ± SEM. *$p < 0.05$; **$p < 0.01$.

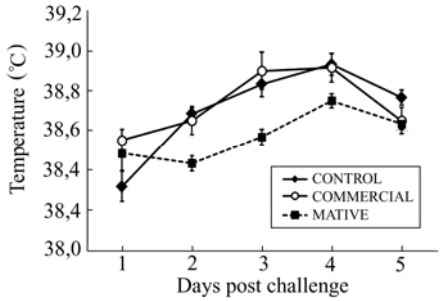

Fig. 4. Mean rectal temperatures of immunized rabbits after challenge with BoHV-1 strain Iowa. Rabbits in groups of 6 were immunized with different vaccines or no vaccination (control) and intranasally challenged on day 30 with 250 μL of viral suspension (10^7 TCID50%/50 μL) of BoHV-1 strain IOWA. Values represent the mean of each group ± SEM. Difference with placebo group *p < 0.05.

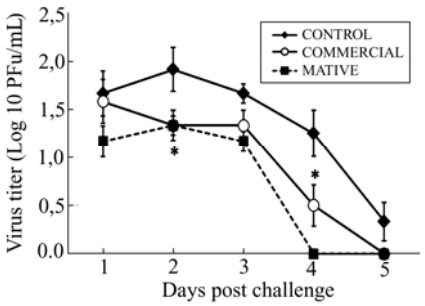

Fig. 5. Shedding of BoHV-1 in nasal swabs following intra-nasal challenge of rabbits that had been immunized twice with native or commercial vaccine, or non-vaccinated as controls. All animals were challenged on day 30 post-immunization with 250 μL of viral suspension (10^7 TCID50%/50 μL) of BoHV-1 strain Iowa. The titers of BoHV-1 were measured by the plaque assay of individual nasal swabs soaked in 500 μL of cold minimum essential medium. Values represent the mean of each group ± SEM. Difference with placebo group *p < 0.05.

post infection (hpi) which reached a peak on day 3 after infection. The commercially vaccinated group presented a similar temperature behavior as the control rabbits reaching a temperature peak on day 3 post-inoculation of 38.5 ℃ (Fig. 4). Native vaccinated animals showed a tendency toward increased body temperature; however they showed a lower temperature increase on days 2 and 3 post-infection ($p<0.05$) compared to the non-vaccinated group and the commercially vaccinated group (Fig. 4).

Upon clinical examination, the non-vaccinated group showed strong signs of conjunctivitis accompanied by mild ocular discharge (Fig.1B), strong nasal discharge with a serous to mucoid aspect (Fig. 1D), signs of respiratory distress, lethargy and anorexia. None of the vaccinated individuals showed signs of disease, respiratory or ocular secretions (Fig. 1C).

Post-challenge, seroconversion was detected in individuals belonging to the unvaccinated control group, achieving mean peak titers of 1 : 10 (Fig. 3A and 3B, day 50) by the time of the end of the study. Antibody titers of vaccinated individuals also increased, with the highest neutralizing titers appearing in rabbits vaccinated with native vaccine, showing statistical differences ($p<0.05$) compared to the commercially vaccinated group.

Decrease of viral excretion

As shown in Fig. 5, there was a reduction in virus excretion both in vaccinated groups compared to non-vaccinated rabbits. In the control group, it was possible to recover infectious virus up to day 5 post-infection, while in the group vaccinated with the native vaccine this was only possible until up to day 3 post-infection, with significant differences when compared with the control group ($p <0.05$). Similarly, in the group vaccinated with the commercial vaccine, it was only possible to recover infectious virus until day 4 post-infection.

DISCUSSION

It is a fact that according to OIE, BoHV-1 vaccination is the most efficient way to control and eradicate the disease; however, in areas with high serological prevalence, the use of attenuated vaccines may promote viral recombination between vaccine strains and field isolates generating highly virulent strains (Muylkens B, et al., 2006), together with other well-known disadvantages such as the risk of reversion to virulence, induction of latency of the vaccine strain and the inability to apply them to pregnant cows, due to the risk of inducing abortion (Miller J M, et al., 1991).

Inactivated vaccines, as evaluated here, have minor biological risks; they are safe and efficient for application in pregnant cows and do not cause abortion or viral shedding after vaccination. Also, the establishment of latency by the vaccine strain is impossible and there is no interference with the production of antibodies against other inactivated antigens (Pastoret P P, et al., 1985; Rossi C R, et al., 1982). However, it has been reported that a full mucosal immunity or cell-mediated immunity is not reached and therefore adjuvants and/or multiple doses of vaccine are required to induce optimal protection. Despite this, many researchers postulate that inactivated vaccines are highly effective in controlling BoHV-1 infection (Schudel A A, et al., 1986). Consistent with OIE and literature (OIE, 2004; Patel J R, 2005), our native vaccine showed complete inactivation *in vitro* (Fig. 2A) and it was

impossible to detect the presence of infectious virus in the suspension, a fact that was corroborated *in vivo* by inoculating rabbits and showing no signs of local or systemic infection post vaccination (Fig. 3A, B).

It has been reported that commercially-produced BoHV-1 vaccines differ between each other by the quality of the protection they afford, such as in reduction of the spread of the virus and in the onset of clinical signs after challenge (DesCoteaux L, et al., 2003; Patel J R, 2005). The inactivated native vaccine tested here induced good levels of humoral immunity. Considering that this vaccine had not been mixed with an adjuvant, and taking in account that the induction of antibodies is similar in both magnitude (Fig. 3A) and neutralizing capacity of immunity as generated by the commercial vaccine (Fig. 3B), our native vaccine has a great immunogenic potential. Probably, as has been described, BEI reacts directly on nucleic acids and preserves the conformation and accessibility of the epitopes to a much greater extent than other inactivating agents used commercially, such as formalin and β-propiolactone (Bahnemann H G, 1990; Duque H, et al., 1989).

Also, as has been seen by others (Patel J R, 2004; Patel J R, 2005), there was not a perfect correlation between total serum IgG and viral neutralizing titers. However it is clear that neutralizing antibody activity fully correlates with protection against challenge (Patel J R, 2004) as could be seen in our experiments.

In addition to the inactivation method used, it has been reported that the antigenic mass of the vaccine is one of the most important characteristics when inducing good antiviral immunity (Patel J R, 2004; Patel J R, 2005). Specifically, it has been reported that an anti-BoHV-1 vaccine must have at least $1\times10^{7,7}$ TCID50% prior to inactivation to induce an acceptable neutralizing immune response (Kamaraj G, et al., 2009; Patel J R, 2004). This dose was exceeded by nearly a log by the Córdoba-2 strain used here as seed vaccine (TCID50 $1\times10^{8.25}$ prior to inactivation). This could explain why although our vaccine does not use an adjuvant, it results in efficient induction of humoral immunity, mainly in higher neutralizing antibody titers (Fig. 3B). Our results, as also described by others, support the conclusion that the antigenic mass is vital for efficient production of vaccines against BoHV-1 (Kamaraj G, et al., 2009; Lesko J, et al., 1993).

Post-challenge, animals vaccinated with native vaccine showed a decrease in the presentation of fever compared to unvaccinated rabbits and to commercially vaccine controls (Fig. 4); this correlates with high neutralizing titers after vaccination and after challenge (Fig. 3) and with decreased titers in viral shedding (Fig. 5). We postulate that this is the expected response in a vaccine that would be considered useful to induce protection and allow controlled infection in the host animal population.

There has been much discussion about the usefulness of the BoHV-1 rabbit model of infection (Duque H, et al., 1989). However, given the difficulty of establishing groups of seronegative animals for vaccine evaluation, this has led to the use of animal models for immunization such as rabbits (Valera A R, et al., 2008) and guinea pigs (Parreno V, et al., 2010), which allow evaluation of the potential response to a vaccine and have high correlation with the expected immune response in cattle (Parreno V, et al., 2010). In this study, a rabbit model was used to show that vaccines are able to induce good humoral immunity, reduce viral shedding and prevent onset of BoHV-1 disease.

The results also reinforce the importance of using boosters, as has been widely reported elsewhere (Ioannou X P, et al., 2002; Toussaint J F, et al., 2005), these proved useful in this study to achieve a strong increase in antibody titers, to 35 dpi (Fig. 3) and showed that challenge with a reference virus (Iowa) could act as a new immunogenic booster, inducing an increase in antibody titers and protecting against disease.

There remains the need to explore the potential of the native vaccine in assessments of long-term immunity in which, no doubt, the presence of an adjuvant is essential to achieve slow release of antigen and long-term protection (Patel J R, 2005; Peters A R, et al., 2004). The commercial multivalent vaccine used as control has been previously evaluated and has proven useful in preventing infection in cattle (Zimmerman A D, et al., 2007). It uses a commercial adjuvant called Xtend III® (Novartis Animal Health U.S., Inc.), which is an oil emulsion that enhances the immune response and maintains the antigens of the vaccine in the injection site, slowly releasing them for longer-lasting immunity (Chung H, et al., 2001; Zimmerman A D, et al., 2007). However, as has been reported, short-term immunity may be similar to that produced by other commercial vaccines. Thus our native vaccine which, even without adjuvant, shows enormous potential for future studies.

In conclusion, our results show the enormous immunogenic potential of inactivated vaccine produced from the Córdoba-2 strain, a native strain of BoHV-1.1; in a rabbit model, it provides a high antigenic mass to the

vaccine and induces optimal humoral immunity and protection against challenge with a highly virulent reference strain, decreasing the rate of infection and viral shedding which could be very useful in controlling outbreaks.

Acknowledgements

The authors thank Anne-Lise Haenni for revision of the English and critical reading of the manuscript.

Author Contributions

Conceived and designed the experiments: JRS, JJ, VV. Performed the experiments: JRS. Analyzed the data: JRS, JJ, VV. Wrote the paper: JRS.

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