Research article

Efficacy of an inactivated, recombinant bovine herpesvirus type 5 (BoHV-5) vaccine

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ABSTRACT

Bovine herpesvirus type 5 (BoHV-5) is the causative agent of bovine herpetic encephalitis. In countries where BoHV-5 is prevalent, attempts to vaccinate cattle to prevent clinical signs from BoHV-5-induced disease have relied essentially on vaccination with BoHV-1 vaccines. However, such practice has been shown not to confer full protection to BoHV-5 challenge. In the present study, an inactivated, oil adjuvanted vaccine prepared with a recombinant BoHV-5 from which the genes coding for glycoprotein I (gI), glycoprotein E (gE) and membrane protein US9 were deleted (BoHV-5 gI/gE/US9/C0), was evaluated in cattle in a vaccination/challenge experiment. The vaccine was prepared from a virus suspension containing a pre-inactivation antigenic mass equivalent to 10^7.69 TCID50/dose. Three mL of the inactivated vaccine were administered subcutaneously to eight calves serologically negative for BoHV-5 (vaccinated group). Four other calves were mock-vaccinated with an equivalent preparation without viral antigens (control group). Both groups were boostered 28 days later. Neither clinical signs of disease nor adverse effects were observed during or after vaccination. A specific serological response, revealed by the development of neutralizing antibodies, was detected in all vaccinated animals after the first dose of vaccine, whereas control animals remained seronegative. Calves were subsequently challenged on day 77 post-vaccination (pv) with 10^9.25 TCID50/dose of the wild-type BoHV-5 (parental strain EVI 88/95). After challenge, vaccinated cattle displayed mild signs of respiratory disease, whereas the control group developed respiratory disease and severe encephalitis, which led to culling of 2/4 calves. Searches for viral DNA in the central nervous system (CNS) of vaccinated calves indicated that wild-type BoHV-5 did not replicate, whereas in CNS tissues of calves on the control group, viral DNA was widely distributed. BoHV-5 shedding in nasal secretions was significantly lower in vaccinated calves than in the control group on days 2, 3, 4 and 6 post-challenge (pc). In addition, the duration of virus shedding was significantly shorter in the vaccinated (7 days) than in controls (12 days). Attempts to reactivate latent infection by administration of dexamethasone at 147 days pv led to recrudescence of mild signs of respiratory disease in both vaccinated and control groups. Infectious virus shedding in nasal secretions was
1. Introduction

Bovine encephalitis herpesvirus or bovine herpesvirus type 5 (BoHV-5) is a member of the order Herpesvirales, family Herpesviridae, subfamily Alphaherpesvirinae, genus Varicellovirus (Davison et al., 2009). BoHV-5 is closely related to bovine herpesvirus type 1 (BoHV-1) (Del Médico Zajac et al., 2010).

Cases of BoHV-5-associated encephalitis have been reported worldwide: in Australia (French, 1962; Smith et al., 1995), Europe (Moretti et al., 1964; Bartha et al., 1969; Magyar et al., 1989), North America (Barenfus et al., 1963; Gough and James, 1975; D’Offay et al., 1993; Ely et al., 1996; Ashbaugh et al., 1997), and particularly in South America (Carrillo et al., 1983; Schudel et al., 1986; Riet-Correa et al., 1989; Ridone et al., 1999). In Brazil, outbreaks of BoHV-5 encephalitis seem to be increasing in number (Weiblen et al., 1989; Salvador et al., 1998; Spilki et al., 2003; Silva et al., 2007a; Rissi and Pierzan, 2008). However, determination of BoHV-5 prevalence rates has been hampered initially by the unavailability of methods capable of differentiating BoHV-1 and BoHV-5 infections; secondly, by the lack of a serological method capable of distinguishing between the widely cross-reactive BoHV-1 and BoHV-5 antibodies (Varela et al., 2010; Holz et al., 2009). Moreover, dual BoHV-1 and BoHV-5 infections make such distinction yet more complicated (Campos et al., 2009). Despite these setbacks, in a study carried out in southern Brazil, prevalence of BoHV-5 infections (and co-infections) was in fact higher than the prevalence of BoHV-1 infections (Campos et al., 2009). This suggests that BoHV-5 is widely disseminated in cattle in Brazil, and highlights the need for efficacious BoHV-5 vaccines.

In countries where BoHV-5 is prevalent, attempts to vaccinate cattle to prevent clinical signs from BoHV-5-induced disease have relied essentially on vaccination with attenuated vaccines (Kaashoek et al., 1995). BoHV-5 is closely related to bovine herpesvirus type 1 (BoHV-1) (Petzhold et al., 2001). A highly efficacious, experimental gE-negative BoHV-1 vaccine was unable to confer full protection to BoHV-5 infection and had no effect on nasal virus shedding or on the development of encephalitic lesions (Spilki et al., 2003; Silva et al., 2007a). In pathogenicity experiments in calves we demonstrated that such recombinant was still capable of replicating in cells from the basal portions of the central nervous system (Hübner et al., 2005). Therefore, the possibility of using such a recombinant as vaccine virus for an attenuated vaccine was hampered. In addition, safety issues may hinder live attenuated virus vaccines in certain countries. From this point of view, inactivated virus vaccines are safer and may compare favorably to live attenuated vaccines (Kaashoek et al., 1995).

In view of that, in the present study, an inactivated vaccine was prepared with the BoHV-5 gl/gE/US9 recombinant and evaluated in vaccination/challenge experiments, in order to determine whether such vaccine would confer protection to BoHV-5-induced clinical disease. Calves were vaccinated and challenged with a virulent BoHV-5 wild type strain. Subsequently, vaccinated calves were submitted to corticosteroid administration to examine the effect of vaccination on latency.

2. Materials and methods

2.1. Cells and viruses

The cell lineage CRIB (Flores and Donis, 1995), a bovine diarrhea virus-resistant clone derived from Madin-Darby bovine kidney cells (MDBK) was used for virus multiplication, quantification and virus isolation. The cells were maintained in Eagle’s minimal essential medium (E-MEM, Gibco) supplemented with 10% fetal bovine serum (Nutricell), 10 μg/mL enrofloxacin (Baytril®, Bayer), 50 μg/mL gentamicin (Gentamax®, Marcolab), and 2 μg/mL amphotericin B (Cristália). The wild type BoHV-5 strain EVI 88/95 was isolated in 1995 from an outbreak of meningoencephalitis in Mato Grosso do Sul, Brazil, and previously characterized in our laboratory (Roehé et al., 1997; Souza et al., 2002; Esteves et al., 2008). The virus was used for the construction of the recombinant (BoHV-5 gI/gE/US9) as described by Franco et al. (2007). The EVI 88/95 virus was also used as the challenge virus.

2.2. Experimental design

Twelve calves of mixed European breeds, male and female, 4–6 months old, serologically negative for BoHV-5 and BoHV-1 (as determined by serum neutralization tests), were used in the experiment. Eight calves were vaccinated with the recombinant virus BoHV-5 gl/gE/US9 (vaccinated group; VG; see below). Four other calves were mock-vaccinated with sterile vaccine diluents with adjuvant (control group; CG). Both groups were boostered 28 days later. Forty-nine days after the second vaccine dose (on day 77 pv), both vaccinated and control calves were challenged...
with the wild type BoHV-5 strain EVI 88/95 by intranasal inoculation and monitored for 14 days. Seventy days later (on day 147 pv), all calves under study were subjected to dexamethasone administration in attempting to reactivate latent viruses and to observe the effect of such administration on vaccinated and control calves.

2.3. Vaccine preparation

CRIB cells were multiplied and 16–24 h after seeding, when monolayers were nearly confluent, the medium was removed and cells inoculated with the recombinant virus (BoHV-5 gl/gl/US9) at a multiplicity of infection equal to 1. After 1 h at 37°C, the inoculum was removed, the bottles replenished with E-MEM without fetal calf serum and incubated for 16–24 h at 37°C. When cytopathic effect was evident in 90% of the monolayers, bottles were shaken to fully detach cells, frozen and stored at −70°C until use. After thawing, infectious titers of the supernatants of infected cultures were determined following standard procedures (Reed and Muench, 1938). The viral suspension was inactivated with binary ethylenimine (BEI) as described previously (Bahnemann, 1975). The vaccine was prepared in a 50% low viscosity water-in-oil-in-water emulsion with oil (MontanideTM ISA 206) and subjected to usual controls, as recommended (Petzhold et al., 2001). Each 3 mL dose of the vaccine contained an antigenic mass equivalent to \(10^{7.69}\) 50% tissue culture infective doses (TCID_{50}). The inactivation process was evaluated by titration of the inactivated suspension in 96-well cell culture plates and by the inoculation of serial 10-fold dilutions of the suspension in 25 cm² cell culture flasks. As positive controls, aliquots of the viral suspension previous to inactivation were included in the tests. The vaccine was stored at 4°C until use.

2.4. Animal inoculation

Eight calves were vaccinated (day 0) subcutaneously in the neck region with a 3 mL dose of the vaccine. Four other calves were mock-vaccinated with 3 mL of the oil-in-water emulsion without antigen. Both groups were boosted 28 days later using the same administration protocol. Clinical observations and collection of specimens for virus isolation are described below.

2.5. Challenge

On day 77 post the first vaccination, all calves were challenged with \(10^{1.25}\) TCID_{50} of the wild-type BoHV-5 strain EVI 88/95, administered intranasally in 10 mL (5 mL into each nostril). Seventy days after challenge (on day 147 pv), all calves were subjected to corticosteroid administration in attempting to reactivate latent infections. Dexamethasone (0.2 mg per kg of body weight) was administered intravenously for 5 consecutive days as described (Silva et al., 2006). Calves were kept under observation and samples collected as below. All procedures involving animal care, and handling were performed under veterinary supervision and according to the recommendations of the Brazilian Law (No 11.794, 2008) (Marques et al., 2009). The project was approved by the Ethics Commission of the Institute for Veterinary Research “Desidério Finamor” under protocol number 02/ 2009.

2.6. Clinical examination

Clinical examinations were performed daily from day 12 prior to vaccination up to day 14 pv. The same examination protocol was followed from day 12 prior to dexamethasone administration (reactivation) up to day 14 pr. Signs recorded were rectal temperature (fever was defined as a rectal temperature of more than 39.5°C), respiratory rate, coughing, congestion of the nasal mucosa, conjunctivitis, ocular and nasal discharges, lesions on the nasal and oral mucosa and changes in behavior and appetite. The respiratory clinical scoring method was adapted from Collie (1992), with modifications based on our previous experience with BoHV-1 experimental inoculations (Franco et al., 2002). Thus, scores were determined by attributing different weights to different signs of disease. Mean clinical scores were calculated daily for each group. Neurological symptoms were detected by the observation of depression, circling, tremors, bruxism and incoordination. Seventy days later, during reactivation attempts, clinical and virological examinations were performed as described by Franco et al. (2002).

2.7. Virological examination

Samples for virological examination were collected daily from days 1 to 14 pv, as well as on days 1–14 pr. Nasal swabs were eluted in 2 mL of E-MEM supplemented with 10 times the usual concentration of antimicrobials and incubated for one hour at 4°C. The samples were vigorously shaken, the swabs removed, drained, the medium clarified by low speed centrifugation and stored at −70°C. Virus titrations were performed on 96-well microtitre plates. Infectious titers were calculated and expressed as log_{10} TCID_{50} per 50 μL using the method of Reed and Muench (1938).

2.8. Virus neutralization tests (VNTs)

Serum samples were collected by jugular venipuncture at weekly intervals from the first vaccination (day 0) onwards till the end of the experiment (on day 161). VNTs were performed according to the protocol recommended by OIE (Kramps, 2008). Sera were tested in serial twofold dilutions in a standard BoHV-5 neutralizing antibody test (VNTs). Tests were carried out in duplicate in 96-well microplates, with 50 μL per well of each serum dilution against 100 TCID_{50} of the BoHV-5 strain EVI 88/95. After a 24 h incubation at 37°C in a 5% CO₂ incubator (Forma Scientific), 100 μL of a cell suspension containing 3 × 10^4 cells were added to wells and the plates further incubated at 37°C in a 5% CO₂ incubator. Up to 4 days after the start of the VNTs the cells were examined for the presence of the characteristic BoHV-5 cytopathic effect. Neutralizing antibody titers were calculated by the method of Reed and Muench (1938).
2.9. Necropsied and histopathology

Tissues from necropsied animals collected for histological examination were fixed in 10% buffered formalin, embedded in paraffin, sectioned at 6 μm and stained with haematoxylin-eosin following routine protocols (Prophet et al., 1992). The brain sections were collected following the protocol described by Vogel et al. (2003).

2.10. Virus isolation from tissues

Tissues from necropsied animals collected were submitted to virus isolation. The tissues were homogenized with sterile sand at 10% (w/v) with E-MEM supplemented with 10-fold usual concentration of antibiotics and clarified at 2500 × g for 20 min at 4 °C. Volumes of 200 μL of each supernatant were inoculated onto CRIB monolayers prepared on 96-well plates in quadruplicate (50 μL/well). Infected cultures were checked for the presence of virus for 7 days. All negative cultures were frozen at -70 °C, thawed and passaged once more on fresh CRIB monolayers. When viral cytopathic effect was detected, 10-fold dilutions of the original suspensions were titrated on CRIB cells. Recovered viruses were titrated and its identity confirmed by a polymerase chain reaction (PCR) as described below. Infectious titers were determined, calculated and expressed as log10 TCID50 per 50 μL after 72 h of incubation at 37 °C as previously described.

2.11. PCR amplification

Detection of viral DNA from tissues was performed by PCR. At first, samples were submitted to the extraction of total DNA following a standard phenol (Invitrogen) extraction and ethanol (Synth) precipitation (Sambrook and Russell, 2001), later the purified DNA was dissolved in 200 μL TE (10 mM Tris pH 7.4; 1 mM EDTA pH 8.0). The primers used in the PCR assay anneal to a region of glycoprotein C (gC) shared by both BoHV-1 and BoHV-5 (Esteves et al., 2008; Campos et al., 2009). To identify false positive results, negative control reactions were added to each set of four PCRs and an internal control was added to each reaction. This protocol was also applied to confirm the identity of isolated viruses from tissues.

2.12. Statistical analysis

Analysis of variance for repeated measures (ANOVA) was performed using mixed procedure in SAS v. 9.2 and the multiple comparisons were performed using the LSMeans Proc with Tukey-Kramer Adjustment. The graphs were performed with Data Analysis Supplement for ExcelTM (Office System 2007 for WindowsTM, Microsoft Corp., Seattle, USA). Differences of p < 0.05 were considered significant.

3. Results

3.1. Vaccination

No significant clinical signs or adverse effects were seen during or after vaccination. A post-vaccination granulo-

matous reaction was detected in 50% of the inoculated calves (4/8 on VG and 2/4 on CG). Apart from that, all animals were clinically healthy and no signs of respiratory disease were observed up to challenge on day 77 pv.

3.2. Clinical and virological findings pc

Between days 3 and 11 pc, vaccinated calves displayed mild clinical signs of respiratory disease (mild nasal lesions, nasal discharge, sneezing, coughing). Pirexia was observed among vaccinated calves only on days 3 and 4 pc (Fig. 1a). All animals on VG shed BoHV-5 in nasal secretions from days 1 to 7 pc. The virus titers ranged from 100.53 to 105.55 TCID50/mL (Fig. 2a). BoHV-5 shedding after challenge in nasal secretions was significantly lower (p < 0.05) in vaccinated calves than in control calves on days 2, 3, 4 and 6 pc (Fig. 2a). The CG calves developed clinical signs of respiratory disease during the pc period, albeit much more intense than those on VG. Additionally, 2/4 calves on CG developed severe clinical neurological signs of illness which started on day 10 pc (calf no. 9) and 12 (calf no. 12). Due to the severity of the clinical signs, these calves were euthanized on humanitarian grounds. The intensity and the range of neurological signs were different between calves. In calf no. 9 the signs were characterized by muscle trembling, ataxia followed by lateral recumbence, paddling and opisthotonus. In calf no. 12 a progressive weight loss, difficulties of standing and walking, circling,
disorientation and blindness were observed. In all of the CG calves, on day 2 pc, the rectal temperatures started to rise beyond 39.5°C. Fever was recorded from days 2 to 4 and on day 12 (Fig. 1a). Virus shedding was detected in nasal secretions from day 1 to 12 pc on all CG calves. However, the period of viral excretion was longer on CG than on calves from VG. Infectious virus titers of the CG calves ranged from 10\(^{0.58}\) to 10\(^{4.80}\) TCID\(_{50}\)/mL in nasal secretions (Fig. 2a).

3.3. Clinical and virological findings following reactivation attempts

Calves were treated with dexamethasone to determine if inactivated BoHV-5 gI/gE/US9^C0 vaccine influenced reactivation of latent virus. All animals were clinically normal before dexamethasone treatment. After administration of the corticosteroid, calves from both VG and CG developed signs of respiratory disease similar to those observed on the pc period. However, such signs started on day 3 pr and remained detectable until the end of reactivation. Calves on VG were pyrexic on days 7 and 8 pr, whereas on the CG, on days 6–8 pr (Fig. 1b). The increase in rectal temperatures correlated with virus shedding (Figs. 1b and 2b). BoHV-5 shedding was detected in nasal secretions of all CG calves from days 4 to 13 pr (10\(^{2.13}\) to 10\(^{4.55}\) TCID\(_{50}\)/mL, Fig. 2b), and on VG calves from days 3 to 11 pr (10\(^{0.50}\) to 10\(^{5.30}\) TCID\(_{50}\)/mL, Fig. 2b). Infectious virus shedding in nasal secretions was significantly lower (p < 0.05) between groups after reactivation on day 11–13 (Fig. 2b). In addition, during reactivation, no signs of neurological disease were noticed in both groups.

3.4. Neutralizing antibody responses

None of the calves had detectable BoHV-5 neutralizing antibodies previous to the experiments. Calves on CG remained seronegative to BoHV-5 until challenge. After the first vaccination, the calves nos. 2 and 6 (VG) had detectable neutralizing antibodies to BoHV-5 (on day 7 pv). On day 21, all calves seroconverted to BoHV-5. The mean neutralizing antibody titer detected after the second dose of vaccine in all vaccinated animals increased in comparison to the mean titer obtained after the first dose of vaccine. After challenge, again a boost in BoHV-5 neutralizing antibody titers was detected in all VG calves, which produced higher antibody titers after challenge than the CG calves (Fig. 3). On days 84–119 pv and on day 161 pv the mean neutralizing antibody titer was significantly higher on VG than on CG (p < 0.05; Fig. 3).

3.5. Necropsy and histopathology

Two CG calves were severely affected and displayed evident signs of neurological disease after challenge. These were euthanized in extremis, one on day 14 pc (calf no. 9) and another on day 16 pc (calf no. 12). On day 9 pr, one vaccinated calf (no. 6) was necropsied. Post-mortem examinations revealed that only the necropsied calves on CG had typical BoHV-5 lesions in brain tissues, with foci of malacia and sinking areas on the frontal, parietal and temporal lobes. Mononuclear meningoencephalitis with perivascular infiltrates was consistent microscopical findings. However, typical BoHV-5 lesions in the brain the calf no. 6 were not detected. Instead, the lung parenchyma revealed congestion, intra-alveolar and interstitial edema, fibrin deposition and extensive hemorrhages.

3.6. Virus isolation

Infectious virus was recovered from all tissues of CG calf no. 9, euthanized in extremis on day 14 pc (Table 1). From the CG calf no. 12, euthanized in extremis on day 16 pc, infectious virus was recovered from tonsils, adrenal glands, kidneys and all brain sections (Table 1). On the other hand, infectious virus was not recovered from the vaccinated calf (no. 6), euthanized in extremis on day 9 pr (Table 1).

3.7. PCR amplification

The PCR assay was applied on the tissues of CG calves with neurological signs (calves nos. 9 and 12) and the vaccinated calf (no. 6) that died during the reactivation.

![Fig. 2. Nasal virus shedding: (a) after challenge with wild type virus (EVI 88/95); (b) during reactivation with dexamethasone administration. Infectious virus titers expressed in log\(_{50}\) of 50% tissue culture infectious doses per mL (TCID\(_{50}\)/mL). Black circles: vaccinated calves (vaccinated group, VG); empty squares: mock-vaccinated calves (control group, CG). Vertical bars = standard deviation. *Mean nasal virus shedding was significantly differed (p < 0.05) between of groups on day 2, 3, 4 and 6 during challenge and on day 11, 12 and 13 during the reactivation.](image-url)
period. PCR assay was performed with a series of brain sections. Most of the samples from infected calves which died from meningoencephalitis during primary infection had detectable BoHV-5 genomes in different parts of the brain (Table 2). In contrast, vaccinated calf no. 6 had BoHV-5 DNA only in the trigeminal ganglia and three regions of the brain (thalamus, midbrain and olfactory cortex; Table 2).

4. Discussion

In view of the high degree of antigenic relatedness between BoHV-1 and BoHV-5, the need for a vaccine with BoHV-5-specific antigens still remains. It has been speculated that BoHV-5 infections would not occur in countries where BoHV-1 vaccination is massively employed (Ackermann and Engels, 2006). However,
Despite the apparent epidemiological evidence, no experiments have been made to support such hypothesis. Vaccination to BoHV-1 has been employed in attempting to control outbreaks of BoHV-5 encephalitis (Silva et al., 2006, 2007b). In a previous study, we have demonstrated that an experimental gE-negative BoHV-1 vaccine would not confer satisfactory protection to BoHV-5 challenge (Silva et al., 2006). This highlights the need for efficacious BoHV-5 vaccines. Currently, in Brazil, some bovine herpesvirus vaccines include BoHV-5 antigens in its preparations (SINDAM, 2010). Yet, to our knowledge, data on the efficacy of such vaccines have not been made publically available. In this study, a vaccination/challenge experiment was conducted with an inactivated vaccine prepared with a recombinant BoHV-5 (gI/gE/US9-). Vaccinated and control groups were monitored for 14 days prior to vaccine administration and then from vaccination (day 0 and 28) to challenge (on day 77) up to reactivation attempts (on day 147–161). The vaccine was safe for use in calves since it did not cause any relevant adverse effects on inoculated animals. Moreover, it was shown to confer protection to encephalitis in cattle upon challenge with a high dosage of virulent BoHV-5. None of the vaccinated calves showed any clinical signs of encephalitis. In contrast, two of four non-vaccinated calves infected with BoHV-5 displayed evident signs of neurological disease. Moreover, vaccinated calves shed wild type virus in nasal secretions for a shorter period of time than did mock-vaccinated calves. Thus, the vaccine was also efficacious in reducing the duration of nasal virus shedding in vaccinated calves.

Hübner et al. (2005) examined the effect of the intranasal inoculation of the infectious, triple BoHV-5 deletion mutant (BoHV-5 gI/gE/US9-) in calves. Those authors observed some degree of neural damage in the CNS of inoculated calves; such undesirable effect would compromise its potential use as an attenuated vaccine. On the other hand, as shown here, the inactivated oil adjuvanted vaccine prepared with the same recombinant caused no damage to the CNS of vaccinated animals. This was of course expected since no infectious virus was present in the inactivated vaccine preparation.

Despite protecting from encephalitis, the vaccine was not capable of preventing virus infection upon challenge, since the challenge virus was still able to replicate in nasal tissues at the site of inoculation, as detected by virus isolation. Therefore, here, as reported on a number of occasions for other herpesvirus vaccines, vaccination did not prevent infection, though conferring protection to the major clinical signs of disease (Cascio et al., 1999; Meyer et al., 2001; Hübner et al., 2005; Del Médico Zajac et al., 2006; Silva et al., 2006).

After dexamethasone administration, the vaccinated calf no. 6 (VG) was euthanized on day 10 pr on humanitarian grounds; the calf displayed clinical signs which were consistent with a secondary pulmonary infection, probably related to the immunosuppressive effects of the corticosteroid administration. No signs of neurological disease were observed on this calf. To rule out encephalitis by BoHV-5 as the cause of illness in this animal, we examined in detail tissues samples obtained during necropsy. Virus isolation attempts from post-mortem tissue samples were negative. PCR tests in search for BoHV-5 DNA were also negative, except in the trigeminal ganglion and three brain regions (thalamus, midbrain and olfactory cortex) in the CNS. The presence of viral DNA in these regions, the absence of infectious virus in tissues, plus the absence of detectable clinical signs and lesions in brain tissues of this calf confirmed that it was latently infected with the challenge virus. Reactivation in fact took place, as detected by the presence of infections virus in nasal secretions; however, infectious virus apparently did not spread to other brain tissues. This is interesting since it shows that BoHV-5 may also be reactivated at peripheral sites and produce infectious virus and not necessarily lead to encephalitis – despite the virus’ recognized neurovirulence.

The vaccine induced high titers of neutralizing antibodies. After challenge, vaccinated animals responded with a significant secondary response, indicating that it was highly effective in inducing neutralizing antibodies and in stimulating memory cells. These results were similar to those obtained in other vaccination experiments using BoHV-5 vaccines (Hübner et al., 2005; Cascio et al., 1999). In addition, the inactivated vaccine induced higher antibody titers than, most likely due to the addition of the adjuvant.

The data presented here show that the inactivated, oil adjuvanted vaccine prepared with the BoHV-5 gI/gE/US9- recombinant was safe and protected vaccinated calves against encephalitis when challenged with a high infectious dose (10^6.25 TCID_{50}) of virulent wild type BoHV-5. The vaccine did not prevent infection, but vaccinated calves displayed only mild respiratory clinical signs after such a massive infectious virus dosage. Such vaccine preparation seems promising and must be evaluated further in field studies in order to determine its applicability to control BoHV-5 disease in the field. Moreover, the recombinant BoHV-5 tested here in an inactivated formulation has the potential to be used as a marker vaccine, which would allow the serological differentiation of vaccinated and naturally infected animals, crucial to an efficient control of infections in animal populations. It may be argued that the absence of gE in the vaccine might make it less prone to induce protection to encephalitis. There is strong evidence highlighting the importance of gE in neurovirulence (Al-Mubarak et al., 2004; Meyer et al., 1996). Actually, the three proteins not coded by the BoHV-5 gI/gE/US9- evaluated here seem to play some role in neurovirulence, as pointed out previously (Hübner et al., 2005). Nonetheless, despite the absence of such genes, the inactivated recombinant was still able to induce protection to neurovirulence. Thus, it is very likely that other proteins beyond gE, gI and US9 might also be involved in neuropathogenicity. In a rabbit model, this triple recombinant was also shown to retain some neuropathogenicity (Silva et al., 2009). In fact, the removal of any residual neuropathogenicity – clearly unacceptable in a vaccine – was one of the main reasons for testing the inactivated preparation described here. Nevertheless, the reason why such a severely deleted recombinant was still able to protect cattle against neurological disease remains...
unknown. These findings provide more evidence to support the involvement of other mechanisms than those already explored to justify neurovirulence. In view of recent previous reports (Gabev et al., 2010), it might also be suggested that gD might be the main immunological target of the recombinant vaccine here reported. This, however, can merely be hypothesized since such demonstration was beyond the scope of the present study.

5. Conclusions

The efficacy of BoHV-5 gI/gE/US9 recombinant was evaluated as an inactivated, oil adjuvanted vaccine in vaccination/challenge experiments in cattle. After challenge with the parental virus strain (BoHV-5 EVI 88/95), vaccinated calves were protected from encephalitis. Virus shedding was significantly shortened in vaccinated calves (7 days) when compared to controls (12 days). Dexa-methasone administration at 147 days post-vaccination led to recrudescence of mild respiratory clinical signs in both vaccinated and control groups, though nasal virus shedding differ significantly between groups at reactivation. In view of its immunogenicity and protective effect upon challenge with a virulent BoHV-5, it is concluded that the recombinant vaccine evaluated here is suitable to be further evaluated in larger field experiments.

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