Safety and immunogenicity of a glycoprotein E gene-deleted bovine herpesvirus 1 strain as a candidate vaccine strain

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ABSTRACT.- Weiss M., Anziliero D., Martins M., Weiblen R. & Flores E.F. 2016. Safety and immunogenicity of a glycoprotein E gene-deleted bovine herpesvirus 1 strain as a candidate vaccine strain. Pesquisa Veterinária Brasileira 36(11):1067-1074. Setor de Virologia, Departamento de Medicina Veterinária Preventiva, Universidade Federal de Santa Maria, Av. Roraima 1000, Santa Maria, RS 97105-900, Brazil. E-mail: eduardofurtadoflores@gmail.com

A glycoprotein E-deleted Brazilian bovine herpesvirus 1 (BoHV-1gE∆) was tested regarding to safety and immunogenicity. Intramuscular inoculation of young calves with a high virus dose did not result in clinical signs or virus shedding during acute infection or after dexamethasone administration. Calves vaccinated once IM (group I) or subcutaneously (group II) with live BoHV-1gE∆ or twice with inactivated virus plus aluminum hydroxide (group IV) or Montanide™ (group V) developed VN titers of 2 to 8 (GMT: 2); 2 to 4 (GMT: 1.65); 2 to 16 (GMT: 2.45) and 2 to 128 (GMT: 3.9), respectively. All BoHV-1gE∆ vaccinated calves remained negative in an anti-gE ELISA. Lastly, six young calves vaccinated with live BoHV-1gE∆ and subsequently challenged with a virulent BoHV-1 strain shed less virus and developed only mild and transient nasal signs comparing to unvaccinated calves. Thus, the recombinant BoHV-1gE∆ is safe and immunogenic for calves and allows for serological differentiation by a gE-ELISA test.

INDEX TERMS: BoHV-1, cattle, marker vaccine, differential vaccine.

INTRODUCTION

Bovine herpesvirus 1 (BoHV-1) is associated with a variety of clinical manifestations in cattle, including respiratory disease (infectious bovine rhinotracheitis - IBR), genital
Disorders (infectious pustular vulvovaginitis - IPV or infectious pustular balanoposthitis – IPB), transient infertility and abortions (Kahrs 2001). BoHV-1 is an enveloped DNA virus belonging to the family Herpesviridae, subfamily Alphaherpesvirinae, genus Varicellovirus (Muylken et al. 2007). As other alphaherpesviruses, BoHV-1 establishes lifelong latent infection in sensory nerve ganglia after acute infection, from which it can be periodically reactivated and transmitted (Roizmann et al. 1992).

Vaccination has been largely used to prevent and reduce the losses associated with BoHV-1 infection, but traditional vaccines usually contain live attenuated or whole inactivated virus and induce a serological response indistinguishable from that induced by natural infection (Van Drunen Littel-Van den Hurk 2006). In this regard, gene-deleted vaccines that allow serological differentiation - also called DIVA vaccines (differentiating infected from vaccinated animals) - have proven to be attractive alternatives for traditional vaccines (Kaashoek et al. 1994). The envelope glycoprotein E (gE) has been the choice target for deletion towards the production of antigenically marked vaccines for several alphaherpesviruses, including BoHV-1 (Kaashoek et al. 1994, 1995, Chowdhury et al. 1999), BoHV-5 (Brum et al. 2010a,b) and swine herpesvirus 1 or Aujeszky disease virus (Moermann et al. 1990, Van Oirschot et al. 1990).

Several efforts have been made to produce and to make commercially available BoHV-1 marker vaccines in Brazil (Franco et al. 2002a, 2002b, Spilki et al. 2005). A gE-negative BoHV-1 strain has been constructed and evaluated regarding to safety, immunogenicity and potential serological differentiation (Spilki et al. 2005, Weiss et al. 2010). A gE and thymidine kinase (TK) double deletion BoHV-5 recombinant strain was constructed and proposed as a candidate vaccine strain (Brum et al. 2010b). Nonetheless, no BoHV-1 or BoHV-5 marker vaccine is currently available in Brazilian market. To fill this gap, we constructed a gE-deleted strain (BoHV-1gEΔ) out of a well characterized genital Brazilian BoHV-1 strain, intended to be used as a vaccine strain (Weiss et al. 2015).

This article reports an investigation on the safety and immunogenicity of the recombinant BoHV-1gEΔ in calves and on its ability to confer protective immunity against challenge with a heterologous BoHV-1 strain.

**MATERIALS AND METHODS**

**Viruses and cells**

The recombinant BoHV-1gEΔ was constructed out of a Brazilian BoHV-1 SV56/90 strain (Weiblen et al. 1992). All procedures of construction and in vitro characterization of the recombinant were published recently (Weiss et al. 2015). The experiments described herein used the clone #3, passage # 3 of the recombinant BoHV-1gEΔ. The BoHV-1 strain EV1123 was used in the challenge experiment (virus kindly provided by Dr. Paulo Michel Roehle, Universidade Federal do Rio Grande do Sul, Brazil). Madin Darby bovine kidney cells (MDBK, ATCC - CCL-22) maintained in MEM (Eagle's Minimum Essential Medium, HIMedia Laboratories, India), supplemented with 10% inactivated and γ-irradiated fetal bovine serum (Nutricell, Brazil), 100U/mL of penicillin and 100μg/mL of streptomycin (Invitrogen, USA) were used in all procedures.

**Animals, virological and serological monitoring**

All experiments used calves free of BoHV-1 antibodies, as ascertained by two negative VN assays 30 days apart (Weiss et al. 2015). The breed and age of the animals varied according to the experiment (safety, immunogenicity and vaccination-challenge) and are specified in the respective sections. Animals were maintained in native grass and/or supplemented with alfalfa and given water ad libitum.

Nasal swabs were collected and processed for virus isolation in MDBK cells as described previously (Anziliero et al. 2011) and positive samples were quantitated by limiting dilution and virus titers were expressed as TCID\(_{50}\)/mL. Serum samples obtained at different times after virus inoculation or immunization were tested for virus neutralizing (VN) antibodies against BoHV-1 by virus neutralization assay, using the parental virus as the challenge virus (Weiss et al. 2010). Titers were expressed as the reciprocal of the highest dilution that prevented virus replication, were transformed in geometric mean titers (GMT - log\(_{2}\)) for the calculation of the mean antibody titers of each group (Thrusfield 1986). Anti-gE antibodies in sera of inoculated-vaccinated calves were tested using a commercial anti-gE antibody ELISA test according to instructions (Bovine Rhinotracheitis Virus gE Antibody Test - IDEXX - the Netherlands).

All procedures of animal handling and experimentation were conducted under veterinary supervision and according to recommendations by the Brazilian Committee of Animal Experimentation (COBEA, law #6.638 of May, 8th, 1979). The experiment was approved by an Institutional Animal Ethics Committee (UFSM, approval #34/2014).

**Safety test**

Five 3 to 4-months-old Holstein calves, seronegative to BoHV-1, were inoculated by the intramuscular route (IM) with the recombinant BoHV-1gEΔ in a dose of 10\(^{6.5}\) TCID\(_{50}\) per animal. Two calves were kept in contact with the inoculated animals. Inoculated and sentinel animals were monitored in a daily basis (clinical signs, body temperature) and submitted to collection of nasal swabs up to day 14 post-inoculation (pi). At day 42pi, inoculated and sentinel animals were submitted to dexamethasone administration (Dx, Decadronal, Achê, Brazil, 0.1mg.kg.day during 5 days) and monitored for virus shedding and clinical signs up to day 14 post-Dx.

**Immunogenicity tests**

The immunogenicity tests were performed in 8 to 10-months-old cross-breed calves maintained in extensive conditions on natural grass. The immunogenicity of BoHV-1gEΔ was evaluated both as a live virus and in an inactivated vaccine preparation. In the first, 17 calves were inoculated IM (group I, n=8) or SC (group II, n=9) with 10\(^{7.0}\) TCID\(_{50}\) of viable BoHV-1gEΔ. As controls, three calves were inoculated IM with the parental virus (SV56/90 strain - group III). Sera collected at the day of vaccination (day zero) and at day 42 post-vaccination (pv) were tested for VN and gE antibodies.

In the second test, a BoHV-1gEΔ virus stock was inactivated with binary ethylenimine according to standard protocols (Brum et al. 2010a). Then, two vaccine formulations were prepared, either using 15% of aluminum hydroxide (Omega, Brazil - group IV) or 50% of Montanide™ gel 1 (high molecular weight polyacrylic polymer - Seppic, USA - group V), both in an aqueous formulation. Each viral dose contained inactivated virus correspondent to 10\(^{7.0}\) TCID\(_{50}\) before inactivation in a final volume of 5mL. Calves were immunized IM twice (30 days apart) and serum for VN tests and ELISA was collected at day 30pv and 30 days later (day 60pv).
Vaccination-challenge experiments

This experiment used 3 to 4-months-old Holstein calves, seronegative to BoHV-1. Six calves were vaccinated IM with BoHV-1gEΔ in a dose of $10^{7.5}$TCID$_{50}$ and four were kept as non-vaccinated controls. Forty-seven days pv, vaccinated and controls were challenged by IN instillation of a highly virulent BoHV-1 strain – EV1123 – [20] in a dose of $10^{7.5}$TCID$_{50}$ per animal, following by swabbing the inoculum against the nasal mucosa. Clinical signs, body temperature and nasal swabs for virus isolation and quantitation were collected in a daily basis for 14 days. Blood for serology (VN and gE-ELISA) was obtained at the day of vaccination, at the day of challenge (47 days pv) and 14 days after challenge. The clinical monitoring consisted of daily clinical examination by two veterinarians who were not aware of the experimental groups, who attributed scores to the following parameters for each animal/day: nasal secretion (0 = absence to 4 = abundant, mucopurulent); ocular secretion (0 = absence or 1 = presence); dyspnea (0 = absence or 1 = presence); conjunctivitis (0 = absence or 1 = presence); nasal signs (0 = normal nasal mucosa to 6 = generalized pustules). The clinical scores were adapted from a previous paper (Anziliero 2011).

Statistical analysis

The results shown in the text, tables and figures are expressed as the mean standard error (SEM). Differences among the treatments were tested by Student’s t-test in one way analysis of variance (ANOVA), using the Assista program, version 7.7. The least significance difference between groups for $p<0.05$ was calculated to determine whether treatments were statistically different.

RESULTS

Safety of the recombinant BoHV-1gEΔ in calves

Even using such a high virus dose, correspondent to 10- to 100-times the highest commercial vaccine dose, none of the inoculated animals showed any clinical sign suggestive of BoHV-1 infection. Inoculated animals remained healthy and with normal temperature throughout the monitoring period (Table 1). Likewise, no infectious virus was recovered from nasal swabs collected daily following virus inoculation. In addition, the sentinel in-contact calves remained healthy, nasal swabs were negative for virus and the animals remained seronegative, demonstrating lack of transmission of the vaccine virus. To confirm efficient replication of the vaccine virus, all vaccinated animals developed VN antibodies on day 42pi (4-16, GMT: 2.6). As expected, these calves remained negative for gE antibodies (Table 1).

To investigate whether the vaccine virus would reactivate latent infection, vaccinated animals were submitted to Dx treatment at day 42pi and monitored for virus shedding and VN antibodies. Again, none of the vaccinated animals showed clinical signs of BoHV-1 infection, shed virus in nasal secretions or had an increase in VN titers, indicating lack of reactivation. Taken together, these results showed that the recombinant BoHV-1gEΔ is safe for calves after IM administration – even using an excessively high virus titer - and is not excreted in nasal secretions during acute replication or after Dx administration.

Immunogenicity of the recombinant BoHV-1gEΔ in calves

The immunogenicity of the recombinant BoHV-1gEΔ was evaluated in beef calves (8 to 10-months-old) under field conditions, testing either a live virus vaccine (one dose) or an inactivated, adjuvanted vaccine preparation (two doses). Serological tests (VN and anti-gE ELISA) were performed in sera collected day 42pv (live virus) or after the second vaccine administration, at day 60pv (inactivated preparation). Calves immunized with live virus IM (group I) developed VN titers of 2-8 (GMT: 2), whereas calves immunized with live virus SC (group II) or the group immunized with the parental virus (group III) developed VN titers of 2-4 (GMT: 1.65). Both groups (group I and II) remained negative for gE antibodies at day 42pv and the three control calves inoculated with the parental virus developed gE antibodies (Table 2). These results showed that calves immunized with live recombinant virus developed VN titers comparable to those developed by calves immunized with wild type virus, yet remained negative for gE antibodies.

All calves immunized with inactivated virus, regardless the adjuvant, developed VN titers and remained negative for gE antibodies at day 30 post-revaccination (Table 3). Calves of group V (adjuvant Montanide™ gel 1) developed

### Table 1. Results of the safety test. Clinical, virological and serological findings in calves inoculated intramuscularly with the recombinant BoHV-1gEΔ and monitored during the acute phase and after dexamethasones (Dx) administration

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Animal #</th>
<th>Virus shedding/clinical</th>
<th>Acute infection</th>
<th>Virus shedding/clinical</th>
<th>Dexamethasone treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>VN antibodiesa</td>
<td>Anti-gE ELISAb</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>D0 pi D47pi</td>
<td>D0 pi D47pi</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BoHV-1gEΔ</td>
<td>117</td>
<td>-a</td>
<td>-b 2</td>
<td>-a</td>
<td>-</td>
</tr>
<tr>
<td>108.5TCID50/animal</td>
<td>127</td>
<td>-</td>
<td>-c 1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Intramuscular</td>
<td>129</td>
<td>-</td>
<td>-d 1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>132</td>
<td>-</td>
<td>-e 2</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>136</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Control animals</td>
<td>118</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>135</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

a Virus neutralizing antibodies were measured by virus neutralization assay as described in material and methods; b Sera submitted to a commercial anti-gE antibody ELISA test (Bovine Rhinotracheitis Virus gE Antibody Test (IDEXX) the Netherlands); c pi - post-inoculation; d Dx = post-dexamethasone; e No clinical signs or virus shedding; f Sample negative in VN assay at its lower dilution (1:2); g Samples negative in the anti-gE ELISA test; h The average of serology results is expressed as geometric mean titers (GMT - log).
Protection conferred by the recombinant BoHV-1gEΔ

The protection conferred by vaccination with the recombinant BoHV-1gEΔ was evaluated in 3 to 4-months-old Holstein calves, challenged IN with a highly virulent BoHV-1 at day 47pv. The body temperature of vaccinated calves remained within normal limits up to day 6 post-challenge (pc), in contrast with the controls, which presented an increase in temperature (Fig.1). From day 7pc to the end of monitoring, both groups presented a drop in body temperature, accompanying a drastic drop in ambient temperature (not shown). Regardless, the temperatures of vaccinated animals remained generally below the temperature of controls.

The duration of virus shedding was significantly reduced (p<0.05) in vaccinated animals (6.8 days ±2.4 days), comparing with controls (11 days ±2.6 days). Virus shedding was no longer detected after day 9pc in vaccinated animals whereas it continued up to day 13 pc in the controls. The differences in virus titers in nasal secretions (controls versus vaccinated) were 3 log10 (3 days); 2 log10 (3 days); 1 log10 (3 days) whereas in three days there was no difference (Fig.2).

The vaccinated animals presented milder and transient clinical signs comparing to the control group. The main differences concerned to the amount and aspect of nasal discharge, presence of ocular discharge and conjunctivitis, and lesions (vesicles, pustules, fibrinous membrane) in the nasal mucosa. The animals from the control group presented higher amount of nasal discharge and the secretion evolved to mucopurulent in 3 out of 4 animals. These animals also presented serious ocular discharge and conjunctivitis in five of the 14 days of monitoring; in addition to higher number...
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Table 4. Serological response of calves immunized with the recombinant BoHV-1gEΔ and challenged intranasally with a heterologous, virulent BoHV-1 strain at day 47 post-vaccination

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Animal #</th>
<th>Post-vaccination (pv)</th>
<th>Post-challenge (pc)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>D0</td>
<td>D47pv</td>
</tr>
<tr>
<td>Immunized</td>
<td>109</td>
<td>&lt;1(^a)</td>
<td>2(^d)</td>
</tr>
<tr>
<td>BoHV-1gEΔ</td>
<td>130</td>
<td>&lt;1</td>
<td>2</td>
</tr>
<tr>
<td>10(^7.5)TCID(_{50})/animal Intramuscular</td>
<td>131</td>
<td>&lt;1</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>134</td>
<td>&lt;1</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>139</td>
<td>&lt;1</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>142</td>
<td>&lt;1</td>
<td>1</td>
</tr>
<tr>
<td>Control animals</td>
<td>01</td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
<tr>
<td></td>
<td>03</td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
<tr>
<td></td>
<td>108</td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
<tr>
<td></td>
<td>105</td>
<td>&lt;1</td>
<td>&lt;2</td>
</tr>
<tr>
<td>GMT: 2(^f)</td>
<td></td>
<td></td>
<td>GMT: 5.5</td>
</tr>
<tr>
<td>GMT: 3(^f)</td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

\(^a\) Virus neutralizing antibodies measured by virus neutralization assay as described in material and methods;
\(^b\) Sera submitted to a commercial anti-gE antibody ELISA test (Bovine Rhinotracheitis Virus gE Antibody Test - IDEXX - the Netherlands);
\(^c\) Sample negative in VN assay at its lower dilution (1:2);
\(^d\) Samples negative in the anti-gE ELISA test;
\(^e\) Samples positives in the anti-gE ELISA test;
\(^f\) The average of serology results is expressed as geometric mean titers (GMT - log2).

and size of pustules in the nasal mucosa. Two of the control animals presented coalescent pustules covering most of the nasal mucosa and the other two controls presented localized coalescent pustules. In contrast, the vaccinated group presented only serous nasal discharge, did not show ocular discharge or conjunctivitis and developed few, small and localized pustules in the nasal mucosa. The overall daily clinical score developed by control and vaccinated groups are shown in Figure 3.

All vaccinated animals remained negative to gE antibodies up to the day of challenge (day 47pv), seroconverting to gE thereafter. At day 30pc all vaccinated and control animals were positive in the gE ELISA, remaining positive up to day 90pc (Table 4).

DISCUSSION

Our results showed that the recombinant BoHV-1gEΔ - a candidate vaccine strain constructed out of a Brazilian BoHV-1 isolate - is safe and immunogenic for calves and, as expected, induces a serological response that can be differentiated from that induced by wild type virus. Highly susceptible calves inoculated iM with a viral dose higher than 10 to
fety test, performed in highly susceptible young calves and
nes, a safety would be required to assess its inocuity. The sa-
ther in modified live vaccine (MLV) or in inactivated vacci-
1 recombinants (Franco et al. 2002a, Romera et al. 2014).
ly introduced in other continents. In South America, a
- vaccine preparations. Additional safety tests in pregnant cows, large-
identical BoHV-1 strain. Four calves were kept as
s demonstrated that the recombinant was able to
duce satisfactory protection upon stringent challenge with
sh shadow and clinical protection. These results are
om towards the use of this recombinant in vaccine
ations. Additional safety tests in pregnant cows, large-
duration of immunity are currently underway to confirm the
of this recombinant for vaccine use.
DIVA vaccines – most of them based on gE deletion -
have long been used for the control/prevention of BoHV-1
fection and disease in several European countries (Van
Littel-Van den Hurk 2006) and have been gradu-
other continents. In South America, a
number of gE-deleted BoHV-1 and BoHV-5 recombinants
have been constructed and proposed as candidate vacci-
strains (Franco et al. 2002a, Brum et al. 2010b, Romera
et al. 2014). Nonetheless, no such vaccine is yet com-
ially available in South American countries (Anziliero
et al. 2015). The reasons for the delay in making the BoHV-
recombinants (Franco et al. 2002a, Romera et al. 2014)
commercially available are unknown. For this reason, we
decided to construct and to evaluate another BoHV-1 gE-
deleted recombinant.

As the recombinant BoHV-1gEΔ is intended to be used ei-
ther in modified live vaccine (MLV) or in inactivated vacci-
ines, a safety would be required to assess its inocuity. The sa-
afety test, performed in highly susceptible young calves and
using such a high virus dose (as recommended by the Euro-
pean Pharmacopea for live viral vaccines) confirmed the sa-
fety of the recombinant. Upon IM inoculation, no infectious
virus was recovered from nasal secretions nor seroconver-
sion of sentinel calves was observed. In this sense, a number
of studies have demonstrated that gE deletion contributes
for BoHV-1 attenuation and, thus, reinforces the choice for
gE deletion as an antigenic marker for BHV-1 vaccines (Kaa-
et al. 2000, Franco et al. 2002b, Romera et al. 2014). On the
other hand, the serological response developed by inocula-
ted calves demonstrated that proper vaccine virus replica-
did ensue upon IM inoculation in these calves.

In addition to the safety during acute infection, the re-
combinant was not excreted upon Dx treatment, confirming
previous findings that gE-deleted BoHV-1 recombinants are
deficient in reactivation and/or in shedding upon corti-
esteroid treatment (Kaashoek et al. 1998, Mars et al. 2000,
Brum et al. 2009, Chowdhury et al. 2010). Regardless the
reasons for the lack in virus shedding upon Dx treatment
(deficient establishment of latency, deficient reactivation
or deficient anterograde transport), this is a highly desire-
ble phenotype in a herpesvirus candidate vaccine strain.

Glicoprotein E-deleted BoHV-1 mutants usually produ-
se smaller plaques in cell culture than wt virus and show
a reduced immunogenicity in vivo (Kaashoek et al. 1994,
Chowdhury et al. 1999). In our experiments, however, im-
munization of calves with the recombinant induced a VN
response equivalent to that induced by the parental virus
(Table 2). Nonetheless, as the protection induced by live
herpesvirus vaccines also rely upon the cellular response,
it would be interesting to assess whether the cellular res-
pone induced by gE-deleted virus would differ from that
induced by wt virus (Romera et al. 2014).

The immunogenicity of the recombinant was also de-
monstrated in inactivated vaccine preparations. In this
assay, the VN response in calves immunized with inactiva-
ted virus plus Montanide™ gel 1 was significantly higher
than the VN response to virus combined with aluminum
hydroxide (Table 3). As the licensing of recombinant live
vaccines for livestock is a time-consuming and laborious
process in Brazil, this recombinant would probably be used
first in inactivated formulations. Regardless, in both cases,
the serological response induced by vaccination could be
differentiated from that induced by parental virus by the
use of a commercial ELISA (Table 2 and 3). In addition, An-
ziliero et al. (2015) showed that the VN response induced
by inactivated, aluminum adjuvanted Brazilian commercial
vaccines was comparable, yet generally lower, than that
observed in this study.

The vaccination-challenge experiments demonstrated a
satisfactory degree of protection conferred by vaccination
with live recombinant virus, as demonstrated by reduction
in virus shedding and clinical protection after challenge.
It should be emphasized that we performed a highly strin-
gent challenge, using a high dose of a highly virulent virus
(Spilki et al. 2004). Unfortunately, no BoHV-1 vaccine has
been shown to completely abolish virus shedding upon
challenge, raising a concern about possible selection of
escape mutants. Regardless, vaccinated animals shed the

Fig.3. Clinical score of the challenge test. Six calves were vaccinat-
ed with BoHV-1gEΔ and 47 days later were challenged with a
heterologous virulent BoHV-1 strain. Four calves were kept as
controls. Bars represent the mean standard error.

100-times an usual vaccine dose remained healthy, did not
shed virus following virus inoculation nor after Dx treatment.
Immunization of calves with live virus or with inactivated,
adjuvanted virus preparations resulted in a VN response of
aqueate magnitude. In addition, vaccination-challenge ex-
periments demonstrated that the recombinant was able to
produce satisfactory protection upon stringent challenge with
a heterologous BoHV-1 strain, as measured by reduction
in viral shedding and clinical protection. These results are
promising towards the use of this recombinant in vaccine
preparations. Additional safety tests in pregnant cows, large-
scale immunization in field conditions and evaluation of the
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using such a high virus dose (as recommended by the Euro-

challenged virus in much lower titers and for a shorter period of time (in most of the time). These animals also developed only mild signs of nasal/respiratory disease and recovered faster than the controls (Fig.2 and 3). In contrast, the animals from the control group showed more severe clinical signs, most of them correlated with the presence of mucopurulent nasal discharge and the number and size of the pustules in the nasal mucosa. It is reasonable to speculate that, under natural conditions, in which challenge would be likely significantly lower/weaker, the vaccine would confer a higher degree of protection.

Bovine herpesvirus 5 (BoHV-5) is a neuropathogenic virus, closely related to BoHV-1 and highly prevalent in South American countries (Del Medico Zajac et al. 2010). The high antigenic similarity and extensive cross-neutralization between BoHV-1 and 5 has led to the concept that proper immunization with either virus would confer cross protection (Del Medico Zajac et al. 2010, Anziliero et al. 2011). Thus, an adequate BoHV-1 vaccine accompanied by a reliable vaccination program would probably confer protection to both viruses in areas where they co-circulate. Regardless these hypothetical considerations, it should be advisable to test this vaccine against BoHV-5 challenge to generate confirmatory data that would allow confirmation of this hypothesis.

Regarding to safety, vaccine tests involving cows in different stages of pregnancy need to be performed to demonstrate that the vaccine strain is safe to be used in these animals. Likewise, vaccination-challenge experiments after immunization with an inactivated virus formulation still have to be performed in order to ensure the safety and efficacy of this vaccine candidate.

In summary, our experiments demonstrated that the recombinant BoHV-1gΔ virus is safe (was not shed by inoculated animals or transmitted to sentinel animals; did not reactivation infection upon Dx treatment and is immunogenic for calves both in live and inactivated preparations. Vaccination-challenge tests demonstrated that immunization of calves with the recombinant conferred partial virological and clinical protection upon challenge. Further, both live and inactivated virus preparations induced a serological response that could be differentiated from that induced by wt virus. Thus, the recombinant BoHV-1gΔ presents properties that candidates it as a vaccine strain, upon additional experiments to confirm its safety and immunogenicity. The availability of a commercial gE-ELISA test for serological differentiation would favor its use as a vaccine strain in control/eradication programs of BoHV-1/5 infection in Brazil.

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