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EXPRESSÃO E CARACTERIZAÇÃO DE UM FRAGMENTO DA GLICOPROTEÍNA *E* DO HERPESVÍRUS BOVINO TIPO 1 E USO EM UM TESTE SOROLÓGICO DIFERENCIAL

DISSERTAÇÃO DE MESTRADO

Stephan Alberto Machado de Oliveira

Santa Maria, RS, Brasil
2012

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por

Stephan Alberto Machado de Oliveira

Dissertação apresentada ao Curso de Mestrado do Programa de Pós-graduação em Medicina Veterinária, Área de concentração em Medicina Veterinária Preventiva, da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para a obtenção do grau de
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Orientador: Prof. Rudi Weiblen

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Universidade Federal de Santa Maria
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Como requisito parcial para a obtenção do grau de
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RESUMO

Dissertação de Mestrado
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EXPRESSÃO E CARACTERIZAÇÃO DE UM FRAGMENTO DA GLICOPROTEÍNA *E* DO HERPESVÍRUS BOVINO TIPO 1 E USO EM UM TESTE SOROLÓGICO DIFERENCIAL

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Santa Maria, 5 de março de 2012

O herpesvírus bovino tipo 1 (BoHV-1) é um vírus de distribuição mundial e produz grandes prejuízos econômicos em rebanhos de corte e de leite. A infecção pelo BoHV-1 produz manifestações respiratórias, reprodutivas e também pode cursar com sinais nervosos. Existem diversos testes laboratoriais capazes de diagnosticar a infecção. Contudo, as técnicas sorológicas empregadas atualmente não são capazes de diferenciar anticorpos produzidos pela vacinação daqueles produzidos em resposta à infecção natural. Assim, vacinas diferenciais com deleção da glicoproteína E (gE) têm sido desenvolvidas com essa finalidade. No entanto, necessita-se também de testes capazes de diferenciar a produção de anticorpos vacinais dos induzidos pelo vírus vacinal. Com essa finalidade, essa dissertação relata a expressão e caracterização de um fragmento da glicoproteína E do BoHV-1 e seu uso no desenvolvimento e padronização de um ELISA indireto para detecção de anticorpos anti-gE. Um fragmento de 651 nucleotídeos correspondente ao terço amino-terminal (217 aminoácidos) do gene da gE do BoHV-1, que possui uma alta identidade com o homólogo herpesvírus bovino tipo 5 (BoHV-5), foi clonado com proteína de fusão 6xHis-tag em *Escherichia coli* utilizando vetor de expressão pET16b. Uma proteína solúvel de aproximadamente 25kDa foi purificada a partir de lisados de *E. coli* transformadas. A proteína recombinante foi detectada por *Western blot* (WB) por anticorpos monoclonais anti-histidina e anti-gE do BoHV-1. Anticorpos presentes no soro de animais infectados com BoHV-1 e BoHV-5 reagiram especificamente com a proteína recombinante no WB. Além disso, camundongos imunizados com a proteína purificada desenvolveram anticorpos que reconheceram a gE viral proveniente de lisados de monocamadas celulares infectadas com BoHV-1 e BoHV-5. Um ELISA indireto para detecção de anticorpos anti-gE, baseado na proteína expressa, foi capaz de diferenciar sorologicamente animais vacinados com a cepa gE deletada do BoHV-5 dos animais experimentalmente infectados com BoHV-1 ou BoHV-5. Esses resultados demonstram que o antígeno obtido conservou suas características imunológicas e pode ser utilizado na detecção sorológica das infecções por herpesvírus bovinos. Possui potencial para uso em grande escala como antígeno em testes de ELISA para diferenciar animais naturalmente infectados de animais vacinados com a cepas defectivas na gE

Palavras - chave: teste imunoenzimático, BoHV-1, BoHV-5, antígeno, proteína recombinante.

ABSTRACT

Master's Dissertation
Programa de Pós-graduação em Medicina Veterinária
Universidade Federal de Santa Maria

EXPRESSION AND CHARACTERIZATION OF A TRUNCATED FORM OF BOVINE HERPESVIRUS TYPE 1 ENVELOPE GLYCOPROTEIN E AND ITS USE IN A DIFFERENTIAL SEROLOGICAL TEST

AUTHOR: Stephan Alberto Machado de Oliveira
ADVISER: Rudi Weiblen
Santa Maria, March, 5th , 2012.

Bovine herpesvirus type 1 (BoHV-1) is distributed worldwide and produces high economic losses to the in livestock industry. BoHV-1 infection causes respiratory, reproductive and may also be associated with neurological signs. There are several tests that can diagnose the infection, however, serological techniques currently used are not able to differentiate antibodies produced by vaccination from those produced in response to natural infection. What is sought is a mean to differentiate vaccinated animals of those infected by the field strain. Vaccines with deletion in the glycoprotein E (gE) gene have been developed for this purpose. However, this also requires the development of tests capable to differentiate the serological response between infected and vaccinated animals. To this end, a 651 nucleotide fragment corresponding to the amino-terminal third (217 amino acids) of the BoHV-1 gE gene - that shares a high identity with the homologous BoHV-5 counterpart - was cloned as a 6×His-tag fusion protein in an *Escherichia coli* expression vector pET16b. A soluble protein of approximately 25 kDa was purified from lysates of transformed *E. coli*. The recombinant protein was detected in Western blot (WB) by anti-6-his tag and anti BoHV-1 gE monoclonal antibodies. Antibodies present in the sera of cattle infected with BoHV-1 and BoHV-5 reacted specifically with the 25 kDa recombinant protein in WB. Moreover, mice immunized with the purified protein developed antibodies that recognized the viral gE in lysates of cell monolayers infected with BoHV-1 and BoHV-5. An indirect ELISA for gE antibodies, based on the expressed protein, was able to differentiate serologically calves vaccinated with a gE-deleted BoHV-5 strain from calves experimentally infected with BoHV-1 or BoHV-5. These data demonstrate that the antigen retained its immunological properties and, thus, can be used in serological tests for bovine herpesvirus infections. It has a potential use in a indirect ELISA to differentiate naturally infected animals from those vaccinated whit the recombinant, gE-negative strains.

Key words: Enzyme-linked immunosorbent assay, BoHV-1, BoHV-5, antigen, recombinant protein.

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INTRODUÇÃO

O herpesvírus bovino tipo 1 (BoHV-1) é um vírus pertencente a família *Herpesviridae*, subfamilia *Alphaherpesvirinae*, gênero *Varicellovirus,* com 150 a 200 nm de diâmetro, pleomórfico, envelopado e possui como genoma uma molécula de DNA de fita dupla de aproximadamente 137 mil pares de base (kpb) (ROIZMANN et al., 1992). O vírus apresenta um ciclo de replicação rápido e lítico *in vitro,* capacidade de infectar uma ampla gama de hospedeiros e estabelecer infecções latentes em gânglios do sistema nervoso periférico e em outros tecidos neurais (ROIZMAN et al., 1992; MUYLKENS et al., 2007). O BoHV-1 foi agrupado em subtipos 1.1 e 1.2 (ENGELS et al., 1981). O BoHV-1.1 é o agente responsável pela forma respiratória da enfermidade, conhecida como rinotraqueíte infecciosa bovina (IBR), enquanto o BoHV-1.2 causa a forma genital, vulvovaginite em fêmeas (IPV) e balanopostite em machos (IPB) (KAHRS, 2001). Além disso, pode estar envolvido na etiologia do complexo respiratório bovino, infectando inicialmente as células do trato respiratório superior, permitindo a infecção secundária grave com o envolvimento de outros agentes (MUYLKENS et al., 2007). Existem também relatos da associação do BoHV-1 com doença neurológica, porém a maioria dos casos da doença estão relacionados ao herpesvírus bovino tipo 5 (BoHV-5) (SILVA et al., 2007).

O (BoHV-5) é um vírus com genoma DNA fita dupla de aproximadamente 138 quilobases (kb), envelopado e pertencente à família *Herpesviridae*, subfamília *Alphaherpesvirinae*, gênero *Varicellovirus* (ROIZMAN et al., 1992). O BoHV-5 foi descrito em vários continentes, no entanto, parece ocorrer com maior frequência na América do Sul, sobretudo no Brasil e na Argentina (CARRILLO et al., 1983; SALVADOR et al., 1998; SILVA et al., 2007). É o agente etiológico da meningoencefalite herpética bovina, que contitui-se por ser a enfermidade de ocorrência mais frequente em animais jovens submetidos a situações de estresse (SALVADOR et al., 1998; RISSI, et al., 2007). A manifestação clínica dessa doença caracteriza-se por tremores musculares, andar cambaleante, bruxismo, flexionamento do pescoço, opistótono, salivação excessiva, pressionamento da cabeça contra anteparos, ataxia, decúbito, convulsões e morte (RISSI et al., 2007). O BoHV-5 possui aproximadamente 82% de identidade de aminoácidos com o BoHV-1 (DELHON et al., 2003). Devido a essa similaridade e à extensa reatividade sorológica cruzada, parte dos surtos envolvendo doença neurológica por herpesvírus foram atribuídos ao BoHV-1, o que contribui

para a indefinição sobre a real prevalência e distribuição das infecções (DELHON et al., 2003).

A transmissão do BoHV-1 e 5 ocorre por contato direto e indireto, e também de forma indireta, por veículos ou fômites (ENGELS & ACKERMANN, 1996). A penetração do agente ocorre no trato respiratório superior onde ocorre a replicação inicial e penetração nos neurônios regionais, onde o vírus pode replicar ativamente ou estabelecer infecção latente sem produção de progênie viral. A invasão e replicação do sistema nervoso central (SNC) pelo vírus causa meningoencefalite de curso frequentemente fatal (CHOWDHURY et al., 1997; SILVA et al., 1999; DIEL et al., 2005).

A grande diversidade de manifestações clínicas causadas pelo BoHV-1 gera prejuízos econômicos para a pecuária mundial, exceto para alguns países europeus que já erradicaram a infecção, como Suíça e Dinamarca (ACKERMANN & ENGELS, 2006). A doença respiratória causada pelo BoHV-1 tem grande importância econômica (ENGELS & ACKERMANN, 1996) e tem sido descrita no Brasil em diversos estados, seja pelo isolamento do agente ou por detecção de anticorpos. A real prevalência da infecção no Brasil é de difícil estimativa, pelo elevado número de bovinos no rebanho brasileiro, e já que boa parte dos estudos utiliza um pequeno número de amostras. Entretanto, alguns estudos regionais citam uma soroprevalência elevada, como 51,9 % em Goiás (BARBOSA et al., 2005), e 18,8 % no rebanho leiteiro do Rio Grande do Sul (LOVATO et al., 1995).

A vacinacão representa o método mais eficaz de controle das infecções por herpesvírus bovino, e é implementada nos programas de controle e erradicação dessas infecções (VAN OIRSCHOT, 1999). As vacinas para os herpesvírus podem ser inativadas ou atenuadas, convencionais ou diferenciais (VAN OIRSCHOT et al., 1996; VAN DRUNEN LITTEL-VAN DEN HURK, 2007). As vacinas convencionais, inativadas ou atenuadas, tem sido amplamente utilizadas no controle e erradicação do BoHV-1 em vários países (VAN OIRSCHOT, 1999; VAN DRUNEN LITTEL-VAN DEN HURK, 2007). As vacinas são caracterizadas por estimularem preferencialmente a resposta humoral e induzirem uma resposta imune em níveis moderados e passageiros, necessitando assim, reforços periódicos (VAN OIRSCHOT, 1999; VAN DRUNEN LITTEL-VAN DEN HURK, 2007). Por outro lado, as vacinas atenuadas estimulam tanto a resposta humoral quanto celular, e induzem uma imunidade de maior magnitude e duração (VAN DRUNEN LITTEL-VAN DEN HURK, 2007). Uma importante restrição tanto das vacinas inativadas quanto das atenuadas é a

incapacidade na diferenciação sorológica entre animais vacinados e infectados naturalmente (VAN DRUNEN LITTEL-VAN DEN HURK, 2006).

Por outro lado, existem as vacinas diferenciais, inativadas ou atenuadas, que são baseadas em cepas virais contendo deleções de um ou mais genes não-essenciais que codificam proteínas imunogênicas, o que torna possível a diferenciação sorológica dos animais vacinados (VAN OIRSCHOT et al., 1996). Em alguns países, as vacinas diferenciais para o BoHV-1 e PRV vêm sendo utilizadas no controle e erradicação destas enfermidades (VAN OIRSCHOT, 1999; ACKERMANN & ENGELS, 2006).

O genoma do BoHV-1 codifica várias glicoproteínas que são expressas no envelope viral (SCHWYZER & ACKERMANN, 1996). Dentre elas, a gE tem sido alvo de deleção para a produção de vacinas diferenciais tanto para o BoHV-1 quanto para o BoHV-5 (KAASHOEK et al.,1996; FRANCO, 2002; HÜBNER et al., 2005). A deleção da gE não prejudica a replicação viral *in vitro*, o que possibilita a obtenção de grandes quantidades de inóculo para produção vacinal em escala industrial (BRUM et al., 2010). No BoHV-5 a gE desempenha um papel importante na neuroinvasividade e neurovirulência (METTENLEITER, 2003). Além disso, a sua deleção constitui-se em um adequado marcador antigênico (VAN OIRSCHOT, 1996). Cepas de diferentes herpesvírus, como BoHV-1, BoHV-5 e vírus da doença de Aujeszky, contendo deleção na gE apresentaram significativa redução da virulência, podendo ser utilizadas em vacinas atenuadas diferenciais (KAASHOEK et al.,1996; BABIC et al., 1996).

Nesse contexto, objetivando o controle e erradicação das infecções por herpesvírus, tem-se buscado o desenvolvimento de vacinas que permitam a diferenciação sorológica entre animais naturalmente infectados e vacinados viabilizando o monitoramento sorológico do rebanho após a vacinação (VAN OIRSCHOT, 1996).

Cepas brasileiras mutantes do herpesvírus bovino tipo 5 (BoHV-5) com deleção nos genes da enzima timidina quinase (TK) e glicoproteína E (gE), ou dupla, em ambos os genes, foram desenvolvidas no Setor de Virologia da UFSM, como alternativa às cepas vacinais existentes (BRUM et al., 2010). Estudos prévios utilizando coelhos como modelo experimental demonstraram que os recombinantes com deleção única na TK e dupla na gE e TK foram atenuados, enquanto o recombinante com deleção única na gE apresentou virulência residual (SILVA et al., 2010). Posteriormente, a vacinação de bovinos com o recombinante gE e TK deletado inativado induziu níveis satisfatórios de anticorpos neutralizantes. Estudos em bezerros têm demonstrado que o recombinante apresenta-se como um candidato para uso em formulações vacinais (ANZILIERO et al., 2011).

Para que vacinas diferenciais possam ser utilizadas comercialmente, é necessário um teste de diagnóstico capaz de diferenciar um animal vacinado de outro com infecção natural (MORENKOV et al., 1997). Esta diferenciação pode se realizada por um teste de ELISA que detecta anticorpos contra a proteína ausente no vírus vacinal (gE), mas presente no vírus de campo. Os testes de ELISA podem ser facilmente empregados, pois são de fácil automação, possibilitam o teste de várias amostras simultaneamente e a obtenção de resultados rápidos e objetivos (CANAL & VAZ, 2008).

A técnica de ELISA é de fácil execução, possibilita análise de um grande número de amostras e permite a obtenção de resultados em poucas horas. Com base nessas qualidades, o ELISA é o método mais usado para a detecção de anticorpos para o BoHV-1 em soro bovino (MÉDICI et al., 2000; CORTEZ et al., 2001). Muitos dos testes disponíveis para detecção de anticorpos contra gE do BoHV-1 são ELISA de bloqueio, baseados em anticorpos monoclonais contra um ou dois epítopos (VAN OIRSCHOT et al., 1988; MORENKOVS et al., 1997; GUT-WINIARSKA et al., 2000). No Brasil, para a realização de sorologia para o BoHV-1 por ELISA indireto são disponíveis apenas *kits* comerciais importados, o que aumenta consideravelmente o custo do diagnóstico (MÉDICI et al., 2000; CORTEZ et al., 2001).

Com esse fim, o presente trabalho descreve a clonagem e expressão de um fragmento da glicoproteína E do BoHV-1 para uso potencial em testes de diferenciação sorológica de animais vacinados de animais infectados com o vírus de campo.

1. CAPÍTULO 1

A truncated bovine herpesvirus 1 envelope glycoprotein E retains its immunological properties and is suitable for use in an ELISA for gE antibodies.

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Abstract

We herein describe the expression of a truncated form of bovine herpesvirus 1 (BoHV-1) glycoprotein E (gE) for use as an immunodiagnostic reagent. A 651 nucleotide fragment corresponding to the amino-terminal third (217 amino acids) of the BoHV-1 gE gene - that shares a high identity with the homologous BoHV-5 counterpart - was cloned as a 6×His-tag fusion protein in an *Escherichia coli* expression system. A soluble protein of approximately 25 kDa was purified from lysates of transformed *E. coli*. The recombinant protein was detected in Western blot (WB) by anti-6-his tag and anti BoHV-1 gE monoclonal antibodie. Antibodies present in the sera of cattle infected with BoHV-1 and BoHV-5 reacted specifically with the 25 kDa recombinant protein in WB. Moreover, mice immunized with the purified protein developed antibodies that recognized the viral gE in lysates of cell monolayers infected with BoHV-1 and BoHV-5. An indirect ELISA for gE antibodies, using the expressed protein as antigen, performed comparably to a commercial anti-gE ELISA was able to differentiate serologically calves vaccinated with a gE-deleted BoHV-5 strain from calves infected with BoHV-1. Thus, the truncated gE retains most of its immunological properties and, thus, may be useful for use in serological tests designed to differentiate between animals vaccinated with gE-negative marker vaccines from naturally infected animals.

Keywords: BoHV-5, bovine herpesvirus, vaccine, DIVA, recombinant.

1. Introduction

Bovine herpesvirus type 1 (BoHV-1) is an enveloped double stranded DNA virus belonging to the family *Herpesviridae,* subfamily *Alphaherpesvirinae*, genus *Varicellovirus* (Roizmann et al., 1992). Bovine herpesvirus type 1 is an important pathogen of cattle; it is distributed worldwide – with the exception of some European countries that eradicated the infection - and is associated with a variety of clinical conditions, including respiratory disease (infectious bovine rhinotracheitis, IBR), reproductive disease (vulvovaginitis or balanopostitis IPV/IBP) and abortions (Kahrs, 2001; Ackermann and Engels, 2006). Bovine herpesvirus type 5 (BoHV-5) is the agent of herpetic meningoencephalitis, a severe and important disease of cattle in South American countries, where numerous outbreaks are reported every year (Carrillo et al., 1983; Salvador et al., 1998; Rissi et al., 2007). BoHV-1 and BoHV-5 are closely related viruses and, as such, share a high genetic and antigenic similarity. These agents display an extensive serological cross-reactivity and, consequently, cannot be distinguished by routine serological or immunodiagnostic tests (Bratanich et al., 1991; Cascio et al., 1999; Vogel et al., 2002; Del Médico Zajac et al., 2006).

A number of BoHV-1 vaccines are available in South America, the majority based on inactivated virus and only one containing a modified live virus. In contrast, few vaccines contain BoHV-5 antigens in their formulation, none of them based on live virus (Flores, E.F. unpublished). The antigenic similarity and serological cross-reactivity between BoHV-1 and -5 has led to the concept that vaccines containing either virus could be of value to prevent disease upon homologous and heterologous virus challenge (Del Médico Zajac et al., 2006; Brum et al., 2010a; Anziliero et al., 2011). In any case, an important restriction for the use of conventional vaccines is the impossibility of serological differentiation between vaccinated and naturally infected (latently infected) animals (Van Oirschot, 1999; Van Drunen Littel-van

den Hurk, 2006), a restriction that assumes special importance concerning programs of control/eradication (Van Oirschot, 1999; Van Drunen Littel-van den Hurk, 2006).

Differential vaccines (DIVA for *differentiating infected from vaccinated animals*) have been widely used to control/eradicate BoHV-1 infections in several countries (Van Oirschot, 1999; Ackermann and Engels, 2006; Van Drunen Littel-van den Hurk, 2006). DIVA vaccines are usually based on viral strains deleted in genes encoding envelope glycoproteins and, thus, induce a serological response differentiable from that induced by natural infection. The serological differentiation of DIVA-vaccinated from naturally infected animals relies upon the use of an immunoenzymatic test specific for antibodies to the deleted viral protein (Van Oirschot, 1999; Van Drunen Littel-van den Hurk, 2006). The non-essential viral envelope glycoprotein E (gE) has been shown to be the most suitable antigenic marker towards production of BoHV-1 differential vaccines (Kaashoek et al., 1995; Van Oirschot, 1999; Van Drunen Littel-van den Hurk, 2006). In addition to provide an antigenic marker, gE deletion from BoHV-1 and BoHV-5 genomes contributes for virus attenuation (Kaashoek et al., 1995; Kaashoek et al., 1998; Chowdhury et al., 2000; Brum et al., 2010b).

Following the trend of North America and European countries, South American countries, especially Brazil and Argentina are now embarking on the development and use of BoHV-1 DIVA vaccines (Brum et al., 2010a). A few years ago, a gE-deleted BoHV-1 strain was produced and characterized (Franco et al., 2002). Recently, our group described the construction and characterization of a vaccine candidate BoHV-5 strain lacking gE and thymidine kinase (tk) genes (BoHV-5gEΔTKΔ) (Brum et al., 2010b). The double mutant was shown to be fully attenuated for calves (Santos et al., 2011; Anziliero et al., 2011) and conferred protection upon homologous and BoHV-1 challenge (Anziliero et al., 2011).

The present article describes the expression and characterization of a truncated form of BoHV-1 gE and its use as a coating antigen for an indirect anti-gE-ELISA to be used as a companion test for gE-deleted BoHV-1/BoHV-5 vaccine strains.

2. Material and methods

2.1. Cells, virus and bacterial strains

A MDBK-derived cell line named CRIB (Flores and Donis, 1995) was used for virus amplification. Cells were grown on minimal essential medium (MEM), containing ampicillin (1.6 mg/L), streptomycin (0.4 mg/L), amphotericin (2 mg/L), supplemented with 10% fetal bovine serum (Cultilab, Brazil). The virus used for gE cloning was the BoHV-1.2 isolate SV56/90 (Weiblen et al., 1992; Silva et al., 2007). The viruses used in the characterization of the recombinant proteins were Brazilian BoHV-1 (SV-265 and 265gE-) and BoHV-5 (SV507/99) strains described elsewhere (Silva et al., 2007; Franco et al., 2002). *Escherichia coli* strain BL21 (DE3) was used for initial cloning, sequencing and maintenance of DNA fragment. For expression of the recombinant protein, *E. coli* BL21 (DE3) RP codon plus were used. *E. coli* was grown in Luria- Bertani (LB) media (Sambrook, 2001).

2.2. Gene amplification and cloning

Total DNA was extracted from CRIB cells infected with BoHV-1 SV56/90 using DNAzol Reagent (*Invitrogen*). A fragment of 651 bp of the gE coding region was amplified by PCR using the following primers: forward 5' AAACCCCGCATATGGCTTCGGTCGACACGGTCTTCA and *reverse* 3' GTCGAAGGATCCAGACTGCAGACGCACCGATAG. These primers contain restriction sites for *Nde*I and *Bam*HI, respectively. The PCR conditions were as follows: initial denaturation for 15 min at 95°C, and 30 cycles of 30 sec at 95°C, 30 sec at 60°C and 45 sec at 72°C, followed by an extension of 10 min at 72°C. The 651 bp amplicon was digested with

*Nde*I and *Bam*HI (*Invitrogen)* and ligated to the pET16b vector (*Novagen*) after digestion with the same enzymes. Ligation products were used to transform *E. coli* BL21 DE(3) RP codon plus by heat shock (Sambrook, 2001). The recombinant clones were selected and plasmid DNA was extracted and characterized by digestion with restriction enzymes and PCR amplification. The location of the cloned fragment within the gE gene and the cloning strategy are depicted in Figure 1.

For sequencing, PCR product was purified using the PureLink kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The sequencing reaction used 50-100 ng of amplicon DNA with 5 μM of forward and reverse primers (described above, according to viral type) and was performed in duplicate in a MegaBACE 500 automatic sequencer (GE Healthcare). Thus, four sequences were obtained; each product was sequenced two times in both directions, aiming at obtaining a consensus sequence. The consensus sequence was submitted to homology analysis performed with NCBI database and BLAST software (http://www.ncbi.nlm.nih.gov/BLAST/) (Altschul et al., 1997) by comparison with BoHV-1 (accession number AJ004801.1) and BoHV-5 (accession number AY261359.1) sequences deposited in GenBank.

2.3. Expression and purification of recombinant gE fragment

E. coli strain BL21 (DE3) transformed with pET16b-*gE* was grown in LB broth supplemented with ampicillin (100 mg/ml) at 37°C with agitation. When the log phase was reached (OD_{600}=0.6), expression was induced with 1.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 3 h. To monitor protein expression, *E. coli* lysates were resolved in a 8.5% SDS-PAGE and the gel was Coomassie blue stained. For protein purification, selected clones were grown, IPTG-induced and bacterial pellet was collected. The purification was done by affinity chromatography using HisTrap™ HP 1 ml columns prepacked with precharged Ni Sepharose™ using the ÄKTAprime™ automated liquid

chromatography system (GE Healthcare). Fractions containing recombinant proteins were identified by SDS-PAGE and quantified by comparison with different concentrations of bovine serum albumin (BSA). Stocks of the purified protein typically contained approximately 0.25 mg/ml. The antigen was stored at −80 ◦C for further use.

2.4. Antibodies and immunoblot analysis

For Western blot (WB) analysis, 2.5 µg of purified recombinant protein was used per well, along with lysates of transformed and induced *E. coli* BL21. To assess the reactivity of mouse immune serum with native gE, lysates of CRIB cells infected with either BoHV-1 (SV56/90) or BoHV-5 (SV507/99) were also submitted to SDS-PAGE and blotting. The gels were blotted onto Nitrocellulose Supported Transfer Membrane (Gibco®) using a semi-dry system (Bio-Rad), using transfer buffer containing 25 mM Tris (pH = 8.3), 192 mM glycine and 20% methanol at 55 V for 1 h at 4°C. The blotted membrane was blocked with 5% (w/v) non-fat dry milk in TRIS buffer saline tween 20 (TBST buffer) (0.5 M NaCl, 0.02 M Tris pH = 8.5, 0.05% Tween 20) for 1 h at room temperature (RT). The primary antibodies used to probe the membranes were as follows: an anti-BoHV-1 gE monoclonal antibody (kindly provided by Dr. Geoffrey Lecthworth) diluted 1:200 in TBST (incubated 1 h at 37°C); sera of cattle experimentally infected with BoHV-1 or BoHV-5 (Anziliero et al, 2011) diluted 1:1000 in TBST (incubated overnight at 4°C); mouse gE-specific antiserum (1:5000) incubated overnight at 4°C. Mouse gE-specific antiserum was produced by immunizing two-months-old female *Mus musculus* subcutaneous with the purified protein mixed with Freunds' Complete and incomplete adjuvant, three times with a 15 days interval. Each inoculation consisted of 70 µg of the protein + 280 µL of adjuvant. Sera were collected 15 days after the third immunization. Mouse pre-immune serum and bovine negative serum were used as negative control in WB analysis. After incubation with the primary antibodies, the membranes were washed three times with TBST (10 min each) and incubated with peroxidase conjugated anti-

mouse or anti-bovine IgG antibodies (Sigma, Inc) at an 1:2500 dilution in TBST. The blots were then washed three times with TBST and reactions were developed with luminescent substrate (Thermo Scientific) and exposed to an X-ray film.

2.5 Indirect anti-gE ELISA

An indirect ELISA for gE antibodies (thereafter named ELISA gE^r) was set up using the recombinant protein to coat the plates. For this, 96-Well Microtiter Plates (Costar®) were coated with 100 µl of purified gE diluted at 1µg/mL in carbonate-bicarbonate buffer (pH 9.5) and incubated at 4°C overnight. Plates were then washed with phosphate-buffered saline containing 0.05% Tween-20 (PBS-T) and blocked with blocking solution (0.5% skim milk in PBS-T)) at 25ºC for 2 h. Subsequently, the plates were washed and incubated with serum samples diluted 1:8 in blocking solution at 37◦C for 1 h. The plates were washed five times and incubated for 1h at 37°C with 100 µl of an anti-bovine IgG-Alkaline Phosphatase conjugate (Sigma, Inc., diluted 1:15.000). After washing, a chromogenic substrate (10 mg/mL of 4-nitrophenylphosphatase) was added and the plates were incubated at room temperature in the darkness for 20 min. Then, 0.5% EDTA was added to stop the colorimetric reaction. Optical density (OD_{405nm}) was read using a Molecular Devices Microplate Reader Model Spectramax M5. In each plate, standard gE positive and negative samples were added in duplicates, as positive and negative controls, respectively. For determination of the cut-off value, 118 bovine sera obtained from a BoHV-1-free herd (and tested negative for BoHV-1/5 antibodies by VN) were tested in duplicate by the ELISA gE^r. The cut-off value was defined as the mean of all negative samples OD_{405}nm values plus three standard deviations (OD mean+3SD/99.8% confidence). For validation of the test, 152 bovine sera were tested in duplicate and in parallel by the ELISA gE^r and by a commercial BoHV-1 gE-Antibody Test Kit (IDEXX). The sensitivity and specificity of the ELISA gE^r were calculated according to

Jacobson (1999). Samples with OD within the "suspect" limits in both test kits were not included in the calculation of sensitivity and specificity.

2.6 Ability of the ELISA gE^r to detect anti-gE antibodies

Then, we accessed whether the ELISA gE^r would specifically detect anti-gE antibodies and, thus, would be able to differentiate animals vaccinated with a gE-negative strain from BoHV-1 infected animals. Serum samples from 34 calves lacking BoHV-1/BoHV-5 antibodies were obtained at day zero; at day 42 days after intramuscular immunization with a gE-negative recombinant BoHV-5 (BoHV-5gEΔTKΔ) and 30 days after challenge with a gE-positive BoHV-1 strain (SV56/90; Anziliero et al., 2011). All sera were tested in duplicate by the ELISA gE^r , by the ELISA gE IDEXX and by VN. Virus-neutralizing (VN) tests were performed according to standard protocols, using 96-well plates and testing two-fold dilutions of sera against a fixed virus titer (100-200$TCID_{50}$). MDBK cells were used as indicators and readings were performed after 7h.

3. Results

3.1. Cloning and expression of a truncated form of gE

The cloning strategy and the location of the cloned fragment within the gE gene are depicted in Figure 1A. The cloned fragment is 651 bp in length and codes for a polypeptide of 217 amino acids, corresponding to the amino-terminal third of gE. The recombinant fragment has an estimated MW of 24 kDa. The 6×His-tag fused to the amino-terminus confers an additional 0.66 kDa. Thus, the predicted mass of the expressed protein plus the histidine tag would have approximately 25 kDa. Nucleotide sequencing of the cloned fragment revealed a DNA sequence with 93% identity with a BoHV-1 sequence deposited in GenBank and 82% identity with the homologous BoHV-5 sequence (Figure 1B).

SDS-PAGE examination of lysates of *E. coli* revealed a discrete protein band of approximately 25 kD among the proteins of transformed bacteria (Figure 2, lanes 3 and 4).

The product of transformed, IPTG induced *E. coli* submitted to purification through a Ni column resulted in an abundant protein of approximately 25 kDa (Figure 2, lane 5), with the same migration pattern of the protein visualized in *E. coli* lysates (lanes 3 and 4). The purified protein (and the other bands as well) correspond to the expected mass of the cloned fragment plus the histidine tag. Therefore, transformation of *E. coli* with the plasmid pET16b containing a fragment of the gE gene fused to a 6×His tag resulted in expression of a protein of approximately 25 kDa, corresponding to the expected size.

3.2. Identification of the expressed protein

In order to unequivocally confirm the identity of the 6×His tagged protein, lysates of *E. coli* (non-transformed, transformed and IPTG-induced) were submitted to SDS-PAGE, blotted onto a nitrocellulose membrane and probed with an anti-BoHV-1 gE Mab. The result of this WB is shown in Figure 3. The gE-MAb specifically recognized a protein of approximately 25 kDa in lysates of transformed *E. coli*, induced or not (lanes 3 and 4), failing to react with lysates of non-transformed bacteria (lane 2), as expected. The gE Mab also reacted strongly with the purified recombinant protein (Figure 3, lane 5). These results demonstrate that the expressed protein is indeed the expected gE fragment, and this fragment is recognized by an anti-BoHV-1 gE Mab.

3.3. Recognition of the recombinant protein by BoHV-1 and BoHV-5 antisera

As the recombinant protein fragment is intended to be used in immunodiagnostic tests, we next investigated whether it would be recognized by anti-gE antibodies present in the sera of seropositive cattle. To this end, we probed the membrane containing the purified protein, after SDS-PAGE and blotting, with sera obtained from calves experimentally infected with BoHV-1 or BHV-5. These sera were shown to harbor virus-neutralizing (VN) antibody titers of 1024 and 256, respectively (Anziliero et al., 2011). The result of this WB is shown in Figure 4. Antibodies present in both sera reacted specifically with a protein of approximately

25 kDa, corresponding to the expressed protein (Figure 4, lanes 2 and 3). The weaker signal observed upon incubation with the BoHV-5 antiserum (lane 3 versus lane 2) may reflect its lower antibody titer (VN 256 versus 1024) or a suboptimal binding to the BoHV-1 gE, or both. No reaction was observed by probing the membrane with bovine pre-immune serum (lane 1), attesting the specificity of the reaction. Thus, the recombinant gE fragment seems to retain most of the gE epitopes responsible for recognition by bovine immune serum. Moreover, the recombinant protein is recognized by bovine antibodies developed against both BoHV-1 and BoHV-5.

3.4. Immunogenic properties of the recombinant gE fragment

In order to assess the immunogenic properties of the recombinant protein, mice were immunized three times with the purified protein mixed with complete Freunds' (1x) followed by incomplete (2x) adjuvant and sera collected after the third immunization were used as primary antibody in WB analysis. Serum of mice immunized with the recombinant protein – and not mouse preimmune serum - recognized the purified protein in WB (not shown). Moreover, antibodies present in the mouse immune serum specifically recognized the viral, native gE in lysates of BoHV-1 and BoHV-5 infected CRIB cells. Mouse antibodies bound to a protein of approximately 90 kDa, the expected size of viral gE (Figure 5, lanes 3 and 5). Mouse immune serum failed to bind to viral proteins present in cells infected with gE-defective BoHV-1 and BoHV-5 (Figure 5, lanes 4 and 6), attesting the specificity of the reaction. Thus, the recombinant gE fragment appears to retain most of its immunogenic properties and epitopes as mouse antibodies raised against it recognize the native gE of both BoHV-1 and BoHV-5 in lysates of infected cells.

3.5 Indirect ELISA gE^r

The optimal antigen concentration, serum and secondary antibody dilution were defined as 1 µg/mL; 1:8 and 1:15.000, respectively. On the basis of endpoint values of negative samples, the cut-off value (OD_{405}nm) was 0,465 (mean +3SD). Based on this data we defined an interval of results, classified as: negative, inconclusive and positive samples OD_{405} values of: ≤465, 466-565, ≥566, respectively. Testing 152 bovine sera in parallel by ELISA gE^r and IDEXX ELISA revealed a sensitivity of 69,2% and specificity of 85,4% (not shown). Although both parameters should be improved, these results are encouraging towards the use of the truncated gE fragment in serological tests for gE antibodies. In particular, the relatively high OD values yielded by negative samples, probably reflecting inadequate purification of the recombinant protein (see inespecific bands in Figure 2, lane 5), could be lowered by improving the purification and/or blocking steps.

We then sought to investigate the ability of the ELISA gE^r to specifically detect anti-gE antibodies and, thus, to differentiate serologically infected animals from animals immunized with a gE-negative strain (Table 1). At the day of vaccination (day zero), all calves were negative in both ELISAs and harbored VN titers <2. At day 42pv all vaccinated calves seroconverted to the vaccine BoHV-5 strain, presenting VN titers from 2 to 8 (GMT 1.8). As expected, these animals remained negative for gE antibodies, as ascertained by both ELISAs. Thirty days after challenge with a gE-positive BoHV-1 (SV56/90), 32 out of 34 calves had developed gE antibodies. From these, 28 were positive in the ELISA gE^r and 27 in the commercial ELISA (Table 1); two samples remained negative in both tests. VN titers in these animals ranged from 16 to 1024 (GMT 5.8). As the experiment was discontinued, we were unable to retest the negative animals at a larger interval to search for late gE seroconversion, as described by Brum et al. (2010b). Thus, the developed ELISA gE^r was able to specifically detect gE antibodies and to differentiate serologically animals vaccinated with

a gE-negative strain from those infected with gE-positive BoHV-1. Moreover, the results obtained with the ELISA gE^r were comparable to those obtained with a commercial gE antibody kit.

4. Discussion

We herein describe the cloning and expression of a truncated form of BoHV-1 glycoprotein E (gE). A fragment of 651 bp (217 aa), corresponding to the amino-terminal third of the protein, was cloned as a 6×His tagged protein in *E. coli* and its expression resulted in an abundant, easily purified polypeptide of approximately 25 kDa. The recombinant protein was recognized in Western blot by a gE-specific Mab, and by antibodies present in the sera of BoHV-1 and BoHV-5 positive cattle. In addition, mice immunized with the recombinant protein developed antibodies that reacted with native gE in BoHV-1 and BoHV-5 infected cells. An ELISA based on the expressed fragment performed comparably to a commercial gE antibody kit in detecting anti-gE antibodies in sera of naturally infected cattle and in differentiating gE- vaccinated from infected calves. Thus, the recombinant, truncated form of BoHV-1 gE expressed in *E.* may be useful as antigen in immunodiagnostic assays for BoHV-1 and BoHV-5.

Antigenically marked vaccines for bovine herpesvirus 1 and 5 have been developed in the last decade in Brazil. Our collaborators reported the construction of a gE-deleted BoHV-1 recombinant virus intended to be used as a vaccine strain (Franco et al., 2002). As BoHV-1 and BoHV-5 co-circulate in Brazilian cattle (Silva et al., 2007), a commercial vaccine for use in the country would require the inclusion of both viruses. To this end, our group recently described the construction of a gE- and tk-deleted BoHV-5 vaccine candidate strain (Brum et al., 2010a). Hence, a gE-specific ELISA – for serological differentiation of vaccinated from naturally infected animals - would be required as a companion test. Commercial gE ELISAs using a crude viral preparation as immobilized antigen are available in the United States and

Europe. However, restrictions to these ELISAs include lack of specificity and yield of false positive results when sera collected from multivaccinated cattle are tested (Lehmann et al., 2004). In addition, commercial gE-antibody kits are expensive and subjected to laborious and time-consuming import procedures. Therefore, aiming at producing an immunoenzymatic companion test for our differential, gE-deleted BoHV-1 and BoHV-5 strains, we cloned and expressed a fragment of BoHV-1 gE. Based on the similarity between BoHV-1 and BoHV-5 gE homologues (Chowdhury et al., 1999; Delhon et al., 2003), we expected that the recombinant protein would cross-react with BoHV-5 antibodies and, thus, would serve for immunodiagnostic for both viruses.

Glycoprotein E of BoHV-1 is a 575 amino-acid, type I transmembrane glycoprotein with a calculated molecular weight (MW) of 61 kDa and an apparent MW of 92 kDa (Jacobs et al., 1994; Fitzpatrick et al., 1989). The native alphaherpevirus gE contains an extravirion amino-terminal region, a transmembrane domain and a short, intravirion carboxy-terminal tail (Jacobs et al., 1994; Fitzpatrick et al., 1989). Deletion of gE gene does not affect the ability of BoHV-1 replicate *in vitro or in vivo*, and does not affect the immunogenicity of the virus (Kaashoek et al., 1996). Moreover, gE induces a fast and long-lasting immune response for at least 3 years (Kaashoek et al., 1996). These properties have candidated gE as a suitable target for deletion towards the production of antigenically marked herpesvirus strains (Kaashoek et al., 1996; Van Oirschot, 1999; Van Drunen Littel-van den Hurk, 2006).

Instead of expressing the whole protein, we decided to express a gE fragment corresponding to the amino-terminal third of the protein (Figure 1). The amino-terminal domain is exposed on the surface of virions and is also expressed on the plasma membrane of infected cells (Jacobs et al., 1994; Fitzpatrick et al., 1989). Therefore, it is expected to be exposed to the immune system and to elicit an antibody response in infected animals. In this

sense, the host antibody response to viral gE is the basis for serological differentiation of DIVA vaccinated animals (Van Oirschot, 1999; Van Drunen Littel-van den Hurk, 2006).

Heterologous proteins expressed in *E. coli* would not undergo some post-translational modifications (e.g. glycosylation, phosphorylation) that influence protein folding and, as a consequence, recognition by antibodies (Nature Methods, 2008). In addition, the cloned fragment could lack sequences important for 3-D structure determination and protein folding, and might also lack important epitopes. Hence, the suitability of our protein fragment for the desired purposes was uncertain. Fortunately, the truncated form of gE seemed to retain most 3-D structural features and critical epitopes as it was recognized by a gE specific Mab (Figure 3) and by gE antibodies present in the sera of BoHV-1 seropositive animals (Figure 4) – a critical property towards its use for diagnostic purposes. In addition, the recombinant protein fragment was recognized by gE antibodies present on sera of BoHV-5 seropositive cattle (Figure 4).

The recognition of the expressed fragment by both BoHV-1 and BoHV-5 antisera was somewhat expected since the homologue proteins – and the amino terminal domain as well - share high nucleotide and amino acid similarity (Delhon et al., 2003). This similarity has been demonstrated by nucleotide sequencing and also by cross-reactivity with polyclonal antiserum and several monoclonal antibodies – including the Mab used herein (Chowdhury, S.I., unpublished; Flores, E.F. unpublished). Furthermore, gE antibodies present in sera of cattle experimentally challenged with BoHV-5 (Anziliero et al., 2011) or immunized with an inactivated BoHV-5 vaccine (Brum et al., 2010b) cross-reacted with BoHV-1 antigens in a commercial ELISA kit for BoHV-1 gE antibodies with sensitivity and specificity undistinguishable from that of BoHV-1 antibodies (Brum et al., 2010b; Anziliero et al., 2011). The lower intensity of the band observed upon incubation with BoHV-5 antisera may reflect its lower antibody titer and/or partial recognition of the recombinant BoHV-1 protein. Thus,

the recognition of the recombinant gE fragment by BoHV-1 and BoHV-5 antisera would enable its use in ELISA tests – as a companion test for BoHV-1 and BoHV-5 differential gE-deleted vaccines - in cattle populations where both viruses co-circulate.

In order to assess the suitability of the expressed protein fragment as antigen in an immunodiagnostic test for gE antibodies, an indirect ELISA (ELISA gE^r) was standardized and the results were compared with a commercial gE-antibody kit. Our preliminary data showed that the purified protein is suitable for use in such ELISA, as it was recognized by sera from naturally infected animals. As the validation of the assay used a limited number of samples (n= 152, being 52 VN positive and 100 VN negative), testing a higher number of sera and adjusting some assay conditions would be necessary to increase its sensitivity (69.4%) and specificity (85.4%).

The ELISA gE^r also performed comparably to the commercial ELISA kit in the detection of gE antibodies in sera of calves challenged with BoHV-1, allowing their differentiation from calves vaccinated with the BoHV-5gEΔTKΔ strain (Table 1). Thus, the truncated gE fragment seems suitable for use in immunoenzimatic tests, allowing the specific detection of gE antibodies and, therefore, the serological differentiation of animals vaccinated with gE-negative marked vaccines. A few samples remained negative for gE antibodies in either or both ELISAs at day 30 after challenge, probably reflecting delayed seroconversion to gE as observed earlier (Brum et al., 2010; Anziliero et al., 2011). As the differential ELISAs are mainly intended to be used for herd screening – rather than for individual diagnosis – a few false-negatives scattered among seropositive animals would have little impact for herd diagnosis and control. Retesting these samples 30 to 45 days later would probably yield positive results in most samples.

In summary, we reported the expression of a truncated form of BoHV-1 gE that retains most of its immunological properties, is easily purified and is recognized by BoHV-1 and

BoHV-5 antisera. The recombinant protein may be useful as immunodiagnostic reagent, especially for the development of a companion ELISA for gE-deleted BoHV-1 and BoHV-5 vaccines.

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Figure 1. Cloning and expression of the amino-terminal region of BoHV-1 glycoprotein E. **A**. Representation of BoHV-1 genome, gE gene and the cloned fragment fused with 6×His

tag in the expression vector. **B.** Sequence comparison between the cloned sequence and the corresponding regions of BoHV-1 and BoHV-5 gE. Top: sequence of BoHV-1 gE deposited in GenBank (AJ004801.1); middle: consensus sequence of the cloned fragment; bottom: BoHV-5 SV507/99 gE (AY261359.1).

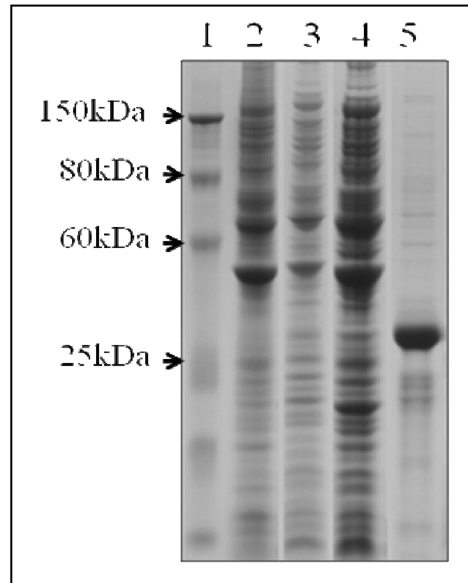

Figure 2. Expression of a truncated form of BoHV-1 gE in *E. coli*. Coomassie blue stained SDS-PAGE gel. Lane 1: Molecular weight marker; lane 2: lysate of non-transformed *E. coli*; lane 3: transformed, non-induced *E. coli*; lane 4: transformed and IPTG-induced *E. coli*; lane 5: purified protein.

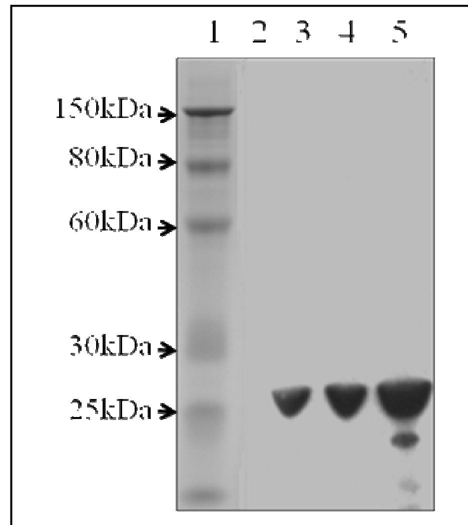

Figure 3. Western blot of the recombinant protein recognized by an anti- BoHV-1 gE Mab. Proteins from *E. coli* lysates were separated by SDS-PAGE, blotted onto a nitrocellulose membrane and probed with a gE-specific Mab followed by incubation with an anti-mouse IgG HRP-conjugated antibody. Reaction was revealed with chemiluminescent substrate and captured in an X-ray film. Lane 1: MWM; lane 2: *E. coli* lysate; lane 3: *E. coli* transformed with the plasmid pET16b-gE; lane 4: *E. coli* transformed and induced by IPTG induced; lane 5. Purified protein.

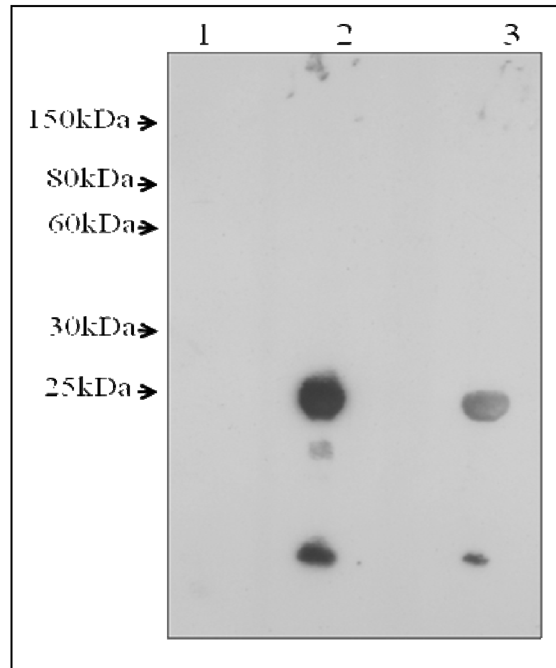

Figure 4. Recognition of the recombinant protein by antibodies present on sera of seropositive cattle by Western immunoblot. The purified protein was submitted to SDS-PAGE, blotted onto a nitrocellulose membrane and probed with bovine preimmune serum (lane 1); or sera of cattle seropositive to BoHV-1 (lane 2) or BoHV-5 (lane 3) as primary antibodies, followed by incubation with an anti-bovine IgG HRPO-conjugated antibody. Reaction was revealed with chemiluminescent substrate and captured in an X-ray film.

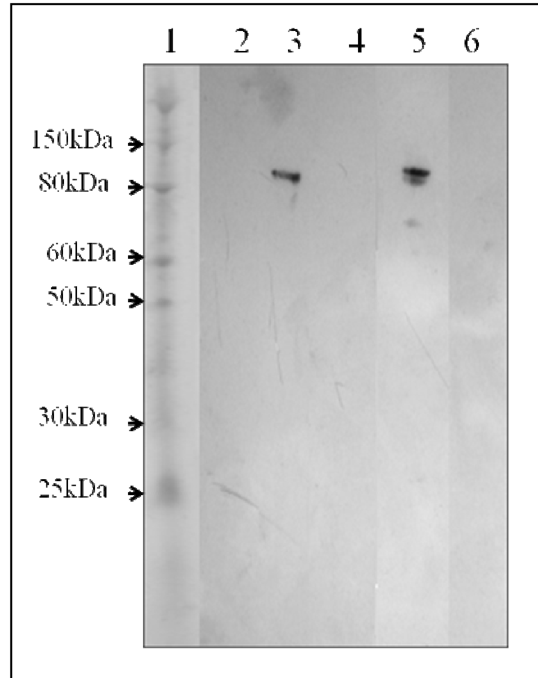

Figure 5. Recognition of native BoHV-1 and BoHV-5 gE by mouse antisera raised against the recombinant fragment by Western immunoblot. Lysates of CRIB cells infected with BoHV-1 and BoHV-5 were separated by SDS-PAGE, transferred to a nitrocellulose membrane and probed with serum of a mouse immunized with the purified recombinant protein, followed by incubation with an anti-mouse IgG HRPO-conjugated antibody. Lane 1: MWM; lane 2: lysate of CRIB cells; lane 3: BoHV-1 (SV265) infected CRIB cells; lane 4: BoHV-1 gE- (SV265gE-) infected cells; lane 5: BoHV-5 (SV507/99) infected CRIB cells; lane 6: BoHV-5 gE- (SV 507/99gE-) infected CRIB cells. Reaction was revealed with chemiluminescent substrate and captured in an X-ray film.

Table 1. Ability of the indirect ELISA gE^r to differentiate animals vaccinated with a gE-negative BoHV-5 mutant from BoHV-1 infected animals.

	Day of vaccination			Day 30 post-vaccination (BoHV-5gEΔ)			Day 30 post-challenge (WT BoHV-1)		
	ELISA gE^r	ELISA IDEXX	VN	ELISA gE^r	ELISA IDEXX	VN	ELISA gE^r	ELISA IDEXX	VN
Negative	34	34	34	34	34	0	6	7	-
Positive	0	0	0	0	0	34 (GMT=1,8)	28	27	34 (GMT=5,8)

CONCLUSÕES

Com os resultados obtidos no presente trabalho pode-se concluir que:

- Foi possível obter, por meio de clonagem e expressão em *E.coli.*, um fragmento da glicoproteína E do BoHV-1, com aproximadamente 25kDa.

- A proteína recombinante conservou as suas propriedades imunológicas, foi imunogênica para camundongos, e foi reconhecida por anticorpo monoclonal específico para a gE e por anticorpos presentes no soro de animais infectados com BoHV-1 e BoHV-5.

- A proteína recombinante é adequada para uso em teste imunoenzimático designado a diferenciar animais infectados com BoHV-1 ou 5 de animais vacinados com cepa viral defectiva na gE.

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