UNIVERSIDADE FEDERAL DE PELOTAS

Programa de Pós-Graduação em Biotecnologia

Tese

Aplicação da glicoproteína D recombinante de Herpesvírus bovino 5 como antígeno em teste de imunodiagnóstico e vacina de subunidade

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Pelotas, 2013

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Aplicação da glicoproteína D recombinante de Herpesvírus bovino 5 como antígeno em teste de imunodiagnóstico e vacina de subunidade

Tese apresentada ao Programa de Pós-Graduação em Biotecnologia da Universidade Federal de Pelotas, como requisito parcial à obtenção do título de Doutor em Ciências (área de conhecimento: Biologia Molecular e Imunologia).

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Pelotas, 2013

Dados de catalogação na fonte:
Maria Beatriz Vaghetti Vieira – CRB-10/1032
Biblioteca de Ciência & Tecnologia - UFPel

D889a Dummer, Luana Alves
Aplicação da glicoproteína D recombinante de Herpesvírus bovino 5 como antígeno em teste de imunodiagnóstico e vacina de subunidade / Luana Alves Dummer. – 92f. – Tese (Doutorado). Programa de Pós-Graduação em Biotecnologia. Universidade Federal de Pelotas. Centro de Desenvolvimento Tecnológico. Pelotas, 2013. – Orientador Fábio Pereira Leivas Leite ; co-orientador Fabrício Rochedo Conceição.

1.Biotecnologia. 2. Pichia pastoris. 3. Resposta imune. 4. ELISA. 5. Anticorpos neutralizantes. I.Leite, Fábio Pereira Leivas. II.Conceição, Fabrício Rochedo. III.Título.

CDD: 636.2

Banca examinadora:

Prof. Dr. Fabricio Rochedo Conceição, Universidade Federal de Pelotas
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AGRADECIMENTOS

Aos meus pais, Dario e Eloá, pelo amor, exemplo, e incentivo; e aos meus irmãos, seus companheiros e meus sobrinhos pelo exemplo, amizade e carinho;

Ao meu esposo, Otávio Cordeiro, pelo amor, por ter acreditado que era possível e pelos finais de semana e feriados em que passou comigo no laboratório;

Ao meu orientador, Prof. Fábio Leite, por ter acreditado e incentivado. Pela amizade, sinceridade e paciência;

Aos professores Odir A. Dellagostin e Fabrício Rochedo Conceição, pelo incentivo e por terem me acolhido em seus laboratórios;

Aos amigos, que sempre me ajudaram e incentivaram, enfrentando longas noites no laboratório com fermentações e auxiliando com a manipulação de animais e experimentos, que sem ajuda, jamais teriam sido realizados: Patrícia (Bilica), Itauá, Paula Finger, Matheus, Michele Pepe, Paula, Liana, Régis, Alceu, Lívia, Renan, Fernanda Rodrigues, Adalgisa, Marília, Luciana Ávila, Helena e Talita;

Aos demais amigos da Biotecnologia (em especial ao Vílson, Michele Santos, Fábio, Claudinei e Fabiane), do Laboratório de Parasitologia Molecular e Imunologia e do Laboratório de Virologia e Imunologia da Faculdade de Veterinária, pela ajuda e incentivo;

Ao Laboratório de Virologia da UFRGS, em especial ao Fabrício Campos e aos professores Ana Cláudia Franco e Paulo Michel Roehe pelo apoio e amizade;

A equipe responsável pelos animais em experimentação do Biotério, pelo carinho e amizade, em especial a Fabiane, Juliana, Cristina, Patrícia e Cristiano;

A Dra. Sylvia van Drunen Littel-van den Hurk e a todos em seu laboratório, por terem me acolhido com tanto carinho durante o período de Doutorado Sanduíche na Vaccine and Infectious Disease Organization-International Vaccine Centre (VIDO-Intervac);

Ao CNPq pelo apoio financeiro através das Bolsa de Doutorado e Doutorado Sanduíche no Exterior;

Muito Obrigada!

“Experience, that most brutal of teachers.
But you learn, my God, do you learn.”
C. S. Lewis – British novelist. 1898-1963

RESUMO

DUMMER, Luana Alves. **Aplicação da glicoproteína D recombinante de Herpesvírus bovino 5 como antígeno em teste de imunodiagnóstico e vacina de subunidade.** 2013. 92f. Tese (Doutorado) – Programa de Pós-Graduação em Biotecnologia. Universidade Federal de Pelotas, Pelotas.

As novas estratégias no desenvolvimento de vacinas contra o Herpesvírus bovino 1 e 5 focam nas glicoproteínas do envelope viral, como a gD, a qual atua na ligação e fusão do envelope viral com a membrana de células permissivas. A gD está presente em grande quantidade no envelope e estimula resposta imune humoral e celular no hospedeiro. O diagnóstico sorológico através de teste imuno enzimático, como o ELISA Indireto, também utiliza as glicoproteínas do envelope viral, uma vez que estas estão expostas na estrutura viral. Desta forma, o objetivo deste estudo foi aplicar a gD recombinante do BoHV-5 expressa em *Pichia pastoris*, após caracterização antigênica, como antígeno no desenvolvimento de uma vacina de subunidade e de um ELISA Indireto visando o controle da infecção por BoHV-5, o qual é o agente responsável por surtos de meningoencefalite que ocasionam perdas econômicas na pecuária brasileira. A vacina de subunidade desenvolvida foi capaz de estimular resposta imune humoral em camundongos, com a presença de anticorpos neutralizantes, quando administrada sozinha ou com adjuvantes imunológicos. A resposta imune celular foi observada através do aumento da expressão de mRNA de citocinas associadas a este tipo de resposta, como IFN-γ, IL-17 e GM-CSF, quando a vacina foi formulada com o adjuvante oleoso Emulsigen-DDA. O ELISA Indireto desenvolvido demonstrou alta sensibilidade (100%) e especificidade (92.9%) quando comparado a técnica de soroneutralização viral, a qual é considerada a técnica padrão para o diagnóstico sorológico de BoHV-1 e -5. A rgD de BoHV expressa em *P. pastoris* possui conformação tridimensional semelhante a gD nativa de BoHV-5. A rgD é imunogênica, capaz de induzir formação de anticorpos neutralizantes e a vacina de subunidade utilizando a rgD como antígeno induz uma resposta imunológica Th1/Th2 balanceada. A antigenicidade da proteína foi confirmada com o seu uso em um ELISA Indireto, o qual pode ser utilizado para triagem rápida de rebanhos infectados com BoHV-5.

Palavras-chave: *Pichia pastoris*. Resposta imune. ELISA. Anticorpos neutralizantes.

ABSTRACT

DUMMER, Luana Alves**. Aplicação da glicoproteína D recombinante de *Herpesvírus bovino 5* como antígeno em teste de imunodiagnóstico e vacina de subunidade.** 2013. 92f. Tese (Doutorado) – Programa de Pós-Graduação em Biotecnologia. Universidade Federal de Pelotas, Pelotas.

The new strategies in vaccine design against *Bovine herpesvirus 1* and *5* are focused in the viral envelope glycoprotein, such as the gD, which acts in attachment and fusion of viral envelope with the permissive cell membranes. This glycoprotein is present at large quantities in the viral envelope and stimulates strong humoral and cellular immune responses at the host. The serological diagnostic based on immunoenzymatic assays, as the Indirect ELISA, also applies the envelope glycoproteins, once they are expose in the viral structure. Thus, the aim of this study was to apply the gD from BoHV-5 expressed in *Pichia pastoris*, after its antigenic characterization, as antigen in a subunit vaccine and in Indirect ELISA, aiming the control and diagnosis of BoHV-5, which is the causative agent of herpetic meningoencephalitis outbreaks, resulting in economic losses for the Brazilian livestock. The subunit vaccine developed was able to stimulate humoral immune response in mice, with development of neutralizing antibodies when the rgD was administrated alone or in combination with immunological adjuvants. The cellular immune response was observed through the increase in the mRNA expression associated with this kind of responses, as IFN-γ, IL-17 and GM-CSF, when the vaccine was formulated with the oil adjuvant Emulsigen-DDA. The Indirect ELISA developed shown high sensitivity (100%) and specificity (92.9%) when compared with the virus neutralization test, which is considered the gold standard in BoHV-1 and -5 serological diagnosis. The rgD from BoHV-5 expressed in *P. pastoris* possess similar tridimensional conformation from the native BoHV-5 gD. The rgD is immunogenic, capable of inducing neutralizing antibodies development and the subunit vaccine developed with the rgD as antigens induces a balanced Th1/Th2 immune response. The recombinant protein antigenicity was confirmed through the Indirect ELISA, which may be used to a fast screen of BoHV-5 infected herds.

Keywords: *Pichia pastoris*. Immune response. ELISA. Neutralizing antibodies.

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LISTA DE ABREVIATURAS E SIGLAS

ADCC - Citotoxicidade dependente de anticorpos
BAV - Adenovírus bovino
BoHV - *Bovine herpesvirus*
CPE - Efeito citopático
CTL - Linfócitos T Citotóxicos
ELISA - *Enzyme-linked immunosorbent assay*
Em - Emulsigen
FBS - Soro fetal bovino
GM-CSF - *Granulocyte-macrophage colony-stimulatin fator*
HAd - Adenovírus humano
HHV - Herpesvirus humano
HRP - *Horseradish peroxidase*
IBR - Rinotraqueite infecciosa bovina
IFN - *Interferon*
Ig - Imunoglobulina
IL - Interleucina
IPV - Vulvovaginite pustular bovina
MAb - Anticorpo monoclonal
MDBK - *Madin-Darby bovine kidney*
MHC - Complexo principal de histocompatibilidade
NK - *Natural killer*
ODN - Oligodeoxinucleotídeos
PBS - Tampão fosfato-salino
PMN - Polimorfonucleares

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1 INTRODUÇÃO GERAL

O rebanho bovino brasileiro, em 2011, foi estimado em 212,9 milhões de cabeças. Neste mesmo ano, o Brasil destacou-se como segundo maior exportador mundial, atrás dos Estados Unidos (POLL; SANTOS; REETZ, 2012). No entanto, o país ainda possui perdas econômicas relacionadas a doenças que atingem os rebanhos e que podem prejudicar o ganho de peso, índices reprodutivos ou até mesmo ocasionar a perda de animais.

O Herpesvírus bovino 5 (BoHV-5) é o agente responsável por meningoencefalites que afetam principalmente animais jovens, resultando em uma taxa de mortalidade entre 70-100% (RIET-CORREA et al., 2006). Surtos relacionados ao BoHV-5 já foram descritos mundialmente. No entanto, a maioria dos relatos de surtos envolvendo este vírus foram descritos no Brasil (SALVADOR et al., 1998; SPILKI et al., 2003; RISSI et al., 2008; CAMPOS et al., 2009).

Devido as similaridades compartilhadas entre o BoHV-1, agente responsável pela **r**inotraqueíte **i**nfecciosa **b**ovina (IBR), e o BoHV-5, as vacinas convencionais destinadas ao BoHV-1 protegem os animais dos sinais clínicos causados pelo BoHV-5 (DEL MÉDICO ZAJAC et al., 2006). Porém, estas vacinas não impedem estabelecimento de latência pelo BoHV-5 ou a disseminação do mesmo (CASCIO et al., 1999). Vacinas com vírus vivo atenuado podem causar abortos em vacas prenhes (JONES; CHOWDHURY, 2008), ou sofrer reversão da virulência (THIRY et al., 2006), não atendendo requisitos de segurança. Vacinas inativadas podem perder epítopos importantes durante o processo de inativação viral, o que pode afetar a imunogenicidade destas, tornando necessárias imunizações repetidas (COX; ZAMB; BABIUK, 1993).

As novas estratégias vacinais focam na estimulação de imunidade humoral e celular contra as principais glicoproteínas (gB, gC e gD) presentes no envelope viral, uma vez que a infecção ocorre através de interações entre estas glicoproteínas com a membrana das células permissivas no hospedeiro. A glicoproteína D é essencial para a fusão das membranas do envelope viral e das células permissivas (ZHU; WU; LETCHWORTH, 1997) e é o principal alvo do sistema imune do hospedeiro,

estimulando anticorpos neutralizantes e resposta celular, além de ser alvo de células do sistema imune inato (revisado por BABIUK, VAN DRUNEN LITTEL-VAN DEN HURK, TIKOO, 1996).

O diagnóstico sorológico do BoHV-1 ou -5 é realizado através das técnicas de soroneutralização viral e de ELISA, onde antígenos do BoHV-1 são empregados na detecção de anticorpos. A soroneutralização viral é uma técnica laboriosa, a qual necessita de laboratórios com rotina em cultivo celular e manipulação viral. Além disso, os resultados podem demorar até 96 h para serem obtidos. O ELISA, por outro lado, é uma técnica relativamente simples, rápida, segura e que pode empregar antígenos recombinantes, não havendo necessidade de manipulação de agentes infecciosos (PARREÑO et al., 2010).

A tecnologia do DNA recombinante permite o desenvolvimento de vacinas de subunidade seguras, sem a manipulação de agentes virais infecciosos. No entanto, o uso de procariotos, apesar da manipulação simplificada, não permite modificações pós-traducionais, ocasionando perda de epítopos conformacionais na expressão de glicoproteínas, como a gD (VAN DRUNEN LITTEL-VAN DEN HURK et al., 1993). A *Pichia pastoris*, por outro lado, por ser um organismo unicelular, mantém a simplicidade de manipulação dos procariotos, além de realizar modificações pós-traducionais, como a N-glicosilação, e secretar para o meio proteínas solúveis, facilitando procedimentos de purificação (CEREGHINO; CREGG, 1999).

Em 2009, Dummer et al., descreveram a expressão e caracterização antigênica da gD de BoHV-5 em *P. pastoris* (DUMMER et al., 2009). Anticorpos de animais de diferentes espécies (roedores, ovinos e bovinos) inoculados com BoHV-5 reagiram com a glicoproteína recombinante, demonstrando que esta possui epítopos presentes da glicoproteína nativa. Desta forma, devido as características da gD do BoHV-5, a glicoproteína expressa em *P. pastoris* possui potencial para ser utilizada no desenvolvimento de testes imunológicos para detecção de anticorpos contra o BoHV-5 e no desenvolvimento de vacina de subunidade que auxilie no controle deste agente infeccioso (ANEXO A).

2 OBJETIVOS

2.1 OBJETIVO GERAL

Desenvolver um ELISA Indireto para o diagnóstico sorológico de Herpesvírus bovino 5 e uma vacina de subunidade para o controle da doença causada por este vírus, baseados na utilização da glicoproteína D recombinante expressa em *P. pastoris.*

2.2 OBJETIVOS ESPECÍFICOS

- Padronizar o ELISA Indireto, testando diferentes concentrações do antígeno e diluições sorológicas;
- Determinar os pontos de corte que correspondam a amostras positivas ou negativas com base em resultados de correlação com a soroneutralização viral;
- Vacinar camundongos com o antígeno recombinante associado ou não a diferentes adjuvantes oleosos;
- Avaliar a resposta imune humoral e celular estimulada com as vacinações através de ELISA indireto, soroneutralização viral, isotipagem e análise da expressão de mRNA de citocinas por qPCR.

3 ARTIGO 1

Bovine herpesvirus glycoprotein D: a review of its applications in vaccinology

(Manuscrito a ser submetido ao periódico *Vaccine)*

BOVINE HERPESVIRUS GLYCOPROTEIN D: A REVIEW OF ITS APPLICATIONS IN VACCINOLOGY

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ABSTRACT

The viral envelope glycoprotein D from *Bovine herpesvirus* 1 and 5, two important pathogens of cattle, is a major target in new vaccine development strategies against both viruses due to its capacity to stimulates both humoral and cellular immune responses in the host. The glycoprotein D also acts in virus penetration into permissive cells. Subunit, DNA or vectored vaccines have been developed using this glycoprotein as main antigen, demonstrating that induced antibodies against it has the capacity to neutralize virus entry into permissive cells and also, that the glycoprotein has capacity to stimulate strong cellular mediated immunity. This review highlighted the application of this glycoprotein in new vaccines design, considering its structural characteristics.

Running headline: Review of the application of the gD in vaccinology

1 INTRODUCTION

Herpesviruses are a large and diverse family of enveloped viruses composed of three subfamilies, *Alpha*, *Beta* and *Gammaherpesvirinae*. The viruses classified in the *Alphaherpesvirinae* subfamily share important characteristics: a rapid reproductive cycle and, at least for three genera of this subfamily, the ability for neuronal invasion and latency establishment in sensory nerve ganglia [1]. Important prototypes of this family comprise human viruses, as the *Human herpesvirus* (HHV)-1 and -2 (known as herpes simplex virus (HSV)-1 and -2), and animal herpesviruses. Among alphaherpesviruses infecting ruminants, the prototype is *Bovine herpesvirus 1* (BoHV-1); however, the closely related BoHV-5 is also of great importance for veterinary medicine.

Bovine herpesvirus 1 (BoHV-1) is a pathogen of cattle associated with two major syndromes, called infectious bovine rhinotracheitis (IBR) and infectious pustular vulvovaginitis (IPV) [1]. It is one of the pathogens involved in the Bovine Respiratory Disease complex (BRD), also called “shipping fever”, economically affecting producers by reducing the average daily gain, feed efficiency, and overall performance of calves. The severe damage that the exposure to IBR can cause to the respiratory tract creates opportunities for further fatal secondary bacterial infections [2,3].

The BoHV-5 infection occurs at the same potential entry sites as BoHV-1, i.e. nasal cavity, eyes, oropharynx and genital tract, and the first round of replication usually takes place in the epithelial cells at these entry sites, and then the virus can spread to the neurons. Although BoHV-5 and BoHV-1 are genetically and antigenically related, sharing in average 82% of identity in their amino acids

sequences [4], they differ in their neuroinvasion and neurovirulence capability. BoHV-1 neuroinvasion usually does not progress beyond the first order neuron located in the trigeminal ganglion, where the latent infection is established, whereas BoHV-5 is able to infect different regions of the brain causing lethal encephalitis in young animals (reviewed in Zajac et al., 2010 [5]).

Vaccination is one of the most cost-effective strategies to control infections by these viruses. Although conventional modified live vaccines and killed vaccines have been developed for control of bovine herpesvirus infection and spread, several disadvantages regarding both safety and efficacy makes them unsuitable for vaccination [6]. New vaccine development strategies against both BoHV-1 and BoHV-5 have been based on the immune responses stimulated by the viral envelope glycoproteins.

The glycoprotein D (gD), one of the antigens present in the viral envelope, is involved in virus penetration and has been considered the major target in vaccine development against bovine and human herpesviruses, mainly owing to its ability to stimulate both humoral and cellular immune responses in the host [7]. Subunit vaccines, DNA vaccines or vectored vaccines have been constructed focusing on the immune responses stimulated by this glycoprotein and applied in combination with a variety of immunological adjuvants. This review highlights the application of gD in vaccine strategies developed against bovine herpesviruses, also considering its functional and immunological properties. Although there have been several studies reporting outbreaks of infection by BoHV-5, there are not as many reports on vaccine development strategies against this specific virus as one can find for BoHV-1. The main reason is the molecular homology between these viruses [4]. Considering that,

most of this review will describe studies with BoHV-1 and, when appropriate, with BoHV-5.

2 STRUCTURE OF GLYCOPROTEIN D (gD)

The complex virion structure of the *Herpesviridae* family is common among the viruses of this family. The virion consists of a large double-stranded DNA genome packaged into an icosahedral capsid, which, in turn, is surrounded by a layer of proteins called tegument and an envelope composed of a large number of glycoproteins embedded in a lipid bilayer [8].

The entry of alphaherpesviruses into permissive cells is a complex and not yet fully understood process. These viruses infect cells as free particles and then can spread to adjacent cells through cell contact. Two mechanisms have been proposed for this entry: viral envelope fusion and an endocytic pathway. However, it is known that among the 12 identified envelope glycoproteins, five (gC, gB, gD, gH and gL) participate in viral attachment and entry. The binding of the alphaherpesvirus is attributed to the reversible attachment through gC and/or gB to cell surface heparan sulfate proteoglycans. A detailed description of this interaction has been provided in reviews by [9,10]. Indeed, the infectivity of alphaherpesviruses is increased when gC is attached to its receptor, but gC null mutants are also able to infect the cell [11], suggesting that this attachment alone is not sufficient for virus penetration and that more events are required for entering into the cells .

Fusion of the viral envelope with the cell plasma membrane requires the other four glycoproteins (gD, gB and the gH-gL complex), with the binding of gD to one of its cell surface receptors being an essential event to mediate herpesvirus

entry, targeting a series of interactions between gB and gH-gL that occurs concurrently with fusion [12].

Glycoprotein D of both BoHV-1 and BoHV-5 possesses approximately 417 aa, with 79.9 % amino acids (aa) identity [13], N- and O linked oligosaccharides, and molecular weight of approximately 71 kDa [14]. It is a type I membrane glycoprotein, with signal sequence with cleavage site located between aa 18 and 19 in BoHV-1 gD and between aa 19 and 20 in BoHV-5 gD gD, based on the position, length, relative hydrophobicity and consensus cleavage site characteristic of a signal sequence [15]. This signal sequence is cleaved to yield a mature protein of 399 aa, with an amino-terminal extremity comprising the extracellular domain, while its carboxy terminus comprises a hydrophobic transmembrane anchor sequence and a cytoplasmic tail with approximately 28 aa in BoHV-1 gD and 35 aa in BoHV-5 gD [14-16].

The nucleotide sequence of this glycoprotein gene has a GC nucleotide content of 70% [14], similar to the SuHV-1 (75%) and HHV-1 gD (65%) homologous [17]. The BoHV-1 and -5 gDs aa sequence demonstrates the conservation of 6 cysteine rich residues in its ectodomain, suggesting that these may be disulfide bond linked, which probably plays a role in maintain the proper 3D fold structure and its function [14]. The aligment of both gDs showed that the amino-terminal two-thirds of the protein (1-282 of BoHV-5 gD) is relatively well conserved [15] with the BoHV-1 gD. However, the differences between the gD from both viruses maps to a glycine-rich stretch located in the molecule's C-terminal ectodomain, in close vicinity to the transmembrane region (between amino acids 280 and 330) [13,15]. This region characterizes a major hydrophilic peak in the case of BoHV-1 gD and may be important for interactions of the protein with other molecules of the virus or host cell or both through ionic interactions [16]. This same region in the BoHV-5 gD exhibits

several mismatches with respect to the BoHV-1 gD sequence, with the presence with a series of negatively charged residues from aa 281 to 195 (280 to 292 of BoHV-1 gD), which resulted in a series of hydrophilic peaks compared to one broad peak for BoHV-1 gD [15].

Four antigenic domains have been described for the BoHV-1 gD [18,19] and five epitopes, three interrelated and two independent, were reported as targets of neutralizing antibodies [20]. Monoclonal antibodies that have been developed to epitopes in the amino acids position 52-116 (MAb 3402) and 165-216 (MAb R54) fail in the recognition of BoHV-5 gD, suggesting that these epitopes should be in correspondent altered amino acids in the BoHV-5 gD [15].

The most well characterized is HHV-1 gD, the ectodomain of which is formed by a core with a variable-type immunoglobulin fold (IgV) wrapped by a N-terminal extension and a C-terminal proline-rich extension [21]. In relation of its glycosylation, differences are observed in the N-linked oligosaccharide (N-CHO) distribution for gD. HHV-1, as well as BoHV-1 gD possess three N-CHO (amino acids position 41, 102 and 411 of BoHV-1 gD), while BoHV-5 gD has only two potential sites in its sequence, one been located in its cytoplasmic tail (amino acids position 102 and 411 of BoHV-5 gD) [15]. The importance of the N-CHO sites in the gD is attributed to its structure and antigenic properties rather then to its direct interaction with the cellular receptors [22].

Enzymatic deglycosylation of gD from BoHV-1 suggests that the addition of carbohydrates could mask epitopes involved in T cell recognition. This was observed after inoculation of native or deglycosylated forms of gD in rabbits and measurement of the delayed-type hypersensitivity (DTH) response where a stronger DTH reaction in deglycosylated gD-vaccinated rabbits was observed. In contrast, the total antibody

response to gD after carbohydrate removal was lower than the response observed for native gD-vaccinated animals; however, the neutralizing antibody response and the ability of the antibodies to mediate cell lysis were not significantly reduced, indicating that most functional epitopes on this glycoprotein are carbohydrate-independent [22].

To date, three unrelated molecules have been shown to be gD cell surface receptors: nectin-1, herpesvirus entry mediator (HVEM) and heparan sulfate modified by 3-O-sulfotransferases, at least for HHV-1. Nectin-1 (also known as HveC and Prr1) is the main receptor of gD on epithelial and neuronal cells [23] and can mediate entry of HHV-1 and -2 as well as *Suid herpesvirus* 1 (Pseudorabies virus (PRV) or SuHV-1), BoHV-1 and -5 [24]. However, cells expressing heparin sulfate-modified 3-O-sulfotransferases or the HVEM receptor are not susceptible to BoHV-1 (reviewed by [25].

Nectins are homophilic cell adhesion calcium-independent molecules from the immunoglobulin (Ig)-like superfamily that accumulate at adherent junctions of epithelia, at synapses and puncta adherentia of neurons [23]. All of the nectins have an ectodomain comprising three Ig-like domains (V-C1-C2), a transmembrane and a cytoplasmic tail region. The gD-binding region of nectin-1 is localized at the V-like domain. Studies performed with soluble forms of gD from HHV-1, HHV-2 and SuHV-1 showed efficient binding to truncated forms of nectin-1 retaining only the V-like domain; this binding was blocked by monoclonal antibodies specific for epitopes located in this domain. However, a study performed with chimeras that combine the V-like domain of nectin-1α with C-like domains from nectin-2α demonstrates that the V-like domain is required for full entry activity of BoHV-1, HHV-1 and -2, and SuHV-1, only when this domain is linked to two C-domains. Despite its binding capability with

the V-like domain, attachment does not result in viral entry, suggesting that binding of gD induces conformational changes in both gD and the receptor. This study also suggests that, based on competitively interaction, the regions on nectin-1 to which, gD from different alphaherpesviruses binds, are overlapping but not necessarily identical [26].

The function of nectins as adhesion molecules that are mostly located at cell junctions suggests a key role in cell-to-cell spread of alphaherpesviruses to neurons of the peripheral and central nervous systems as well as with in the epithelium [27] [23]. The gD binding to nectin-1 during later phases of HHV infection correlates with down-regulation of nectin-1 in cells susceptible to HHV endocytosis. Co-culture of nectin-1-null cells expressing gD with target cells that express nectin-1 indicate that the trans-interaction of gD with nectin-1 at contacts between the two cell populations led to down-regulation of nectin-1, consisting of internalization of the receptor from the target cell surface followed by low-pH-dependent degradation suggesting a ligand-induced endocytosis. The cell-bound gD or gD from egressing virions could then mediate internalization of nectin-1 directing HHV to an endocytic pathway during entry [23].

Madin Darby bovine kidney cells (MDBK) expressing gD from BoHV-1 resist infection by heterologous virus, like HHV-1 or SuHV-1, a phenomenon termed interference, which occurs at the level of penetration into cells. However, these cells are still susceptible to BoHV-5, demonstrating an absence of interference, even though it inhibited the enlargement of plaques. This observation suggests that BoHV-5 may use different receptors for entry or that different regions of gD from BoHV-5 may be critical for entry [28]. Indeed, a study showed that BoHV-5 gD can interact with human nectins 1 to 4. Also, a cell type known as J1.1-2 cells, which do not

express any form of nectin and that is resistant to HHV and BoHV-1 infections is susceptible to BoHV-5 infection, reinforcing the idea that BoHV-5 gD can interact with a different range of receptors not available for BoHV-1. The replacement of BoHV-1 gD by the homologous BoHV-5 gD confers an extended host range to BoHV-1 and increased virulence, which, however, does not affect the brain invasiveness of this recombinant viruses [13].

The role of gD in the initial stage of BoHV-1 and -5 infection and its abundance in the viral envelope makes it a target for the host immune system. The host immune responses to these viruses can be divided into humoral (which prevents the infection) and cellular (which, together with humoral responses, aid in recovery) and, furthermore, into specific and nonspecific responses mediated by polymorphonuclear neutrophils (PMN), macrophages, natural-killer cells (NK-cells), etc. The immunology of BoHV-1 infection was reviewed in 1996 [7] and due to the similarities between both viruses, it is expected that most of what was discovered to BoHV-1 also applies to BoHV-5.

The first step in an immune response to a virus infection is to avoid the interaction between virus and permissive cells; however, in primary infection, antibodies are not available to interfere with these interactions. Thus, the first response of the immune system against a BoHV-1 or -5 infection will consist of nonspecific inflammatory and cellular reactions, gD and gB being primary targets for NK-cells. Glycoproteins gC and gD are targets for CD8+ cytotoxic lymphocytes (CTLs) in bovines, and gD possesses specific epitopes which stimulate CD4+ T-lymphocytes [29,30]. Antibody responses, as mentioned, do not prevent cell-to-cell spreading and become detectable when recovery from primary infection is underway. However, during secondary infections they play a pivotal role in preventing infection,

since they are critical in neutralizing extracellular virus and may prevent spread to adjacent animals. Non-neutralizing antibodies may also contribute together with PMN cells, which can cause lysis of BoHV-1 infected cells via antibody-dependent cell cytotoxicity (ADCC) [31,32].

Thus, it becomes clear that not only are the major glycoproteins involved in inducing antibody to prevent infection, but also in stimulating cellular responses. They are also targets for CTLs and ADCC. For these reasons, it is important that new vaccines strategies against BoHV-1 and -5 focus on major glycoproteins, with emphasis on gD [7].

Recent reports regarding a subunit vaccine trial using gD from HHV-2 indicates that while the vaccine prevented HHV-1 disease and infection, it failed in preventing HHV-2 disease or infection [33]. On the other hand, gD from BoHV-1 has been proven to protect cattle from virus challenge in various vaccine strategies [34-37].

3 VACCINES DESIGNED WITH gD

3.1. Subunit vaccines

Subunit vaccines are those containing one or more pure or semi-pure antigens. To develop subunit vaccines, it is important to identify the individual components that are involved in protection from the pathogen. This kind of vaccine may be produced by conventional technologies, such as purification of the protein produced by the pathogen. To develop a safe vaccine against BoHV-1, viral envelope glycoproteins, such as gB, gC and gD, were purified directly from virus-infected cells and retained their antigenic activity, inducing the production of

neutralizing antibodies in cattle and protection against challenge with virulent BoHV-1. Although all three glycoproteins induced neutralizing antibodies, titers were higher in those animals immunized with gD, which also had higher ADCC titers [38].

Purified native glycoproteins from infected cells may keep the characteristics of the antigen; however, this method is generally not cost-effective, so the use of recombinant DNA technology to produce large quantities of proteins for incorporation into vaccines may fulfill the safety and economic requirements. Production of recombinant BoHV-1 gD has been performed in several expression systems, from prokaryotes to eukaryotes. Prokaryotyc systems, although possessing several advantages regarding manipulation, low cost and the possibility to achieve high amounts of protein production, have some disadvantages regarding the expression of viral glycoproteins. As mentioned, the gD conformational structure relies on its correct three dimensional folding, which is dependent on the addition of carbohydrates. Prokaryotic systems do not possess the cell machinery to perform post-translational modifications necessary for proper gD folding. This was confirmed when the BoHV-1 gD was expressed in *Escherichia coli.* Despite the higher antibody levels induced by the *E. coli* recombinant gD (rgD), a small portion of those were capable to neutralize the virus [39].

As a result of the low efficacy of recombinant gD produced in prokaryotes, eukaryotic expression systems were tested, such as yeasts, mammalian, plant and insect cells. Yeast, such as the methylotrophic *Pichia pastoris,* was used as expression system for a secreted form of gD from BoHV-1 alone, or in combination with bovine interleukin-6 as a chimeric protein. Both recombinant proteins induced neutralizing antibodies in mice [40,41]. Also, gD from BoHV-5 was expressed as a secreted form in *P. pastoris* by removing the transmembrane anchor of the native gD

[42]. This recombinant BoHV-5 gD was able to induce neutralizing antibodies in mice after vaccination with oil-based adjuvants (Dummer, Luana Alves; unpublished results).

The expression of recombinant gD without its transmembrane anchor is a strategy based on studies that demonstrated cytotoxicity of full-length BoHV-1 gD in MDBK cells when it was constitutively expressed. Furthermore, the absence of the transmembrane anchor did not change the immunogenic capacity of gD [43].

A Tobacco Mosaic Virus (TMV)-based vector (TMV-30B) were used to express a non-glycosylated form (cytoplasmic) of BoHV-1 gD in plant cells. Mice and cattle immunized with an oil-based gD vaccine formulation induced humoral immune responses and cellular responses in mice and cattle and seemed to be protective after viral challenge; however, no viral neutralization test was performed in this trial [44].

Although yeast, plant and insect cells were able to produce gD with some degree of authenticity, most reports show the expression of a secreted form of BoHV-1 gD (also called tgD) in mammalian cells (MDBK cells) under the control of a inducible a bovine heat shock 70A gene promoter (HSP70 promoter) [45,46].

The BoHV-1 tgD has been combined with several adjuvants and co-adjuvants and administered by different routes to induce mucosal and systemic immune responses. The most important approach seems to be the incorporation of CpG-containing oligodeoxynucleotides in vaccine formulations with classical adjuvants. Several pathogens require cellular immune responses for protection, and, although all currently licensed vaccines are efficient at inducing antibody responses, only modified live vaccines efficiently induce cellular immunity. Formulation of tgD with alum and CpG ODN (or even tgD with CpG DNA alone) induced strong

neutralizing antibodies responses and resulted in higher IgG2/IgG1 ratios as well as strong cellular immunity in calves, resulting in fast recovery from challenge [47]. The incorporation of CpG ODN in oil-based vaccines also resulted in strong Th1-type immune responses, with an increase in production of IFN-γ, or balanced immune responses, in contrast to the immune responses induced by the conventional adjuvants alone, which were Th2 [35,48].

3.2. DNA vaccines

The demonstration that direct transfection *in vivo* with plasmid DNA could be used to express foreign proteins and thereby induce both humoral and cellular immune responses in a variety of murine and primate disease models has engendered considerable excitement in the vaccine community. Immunization by direct transfection *in vivo* with plasmid DNA is an efficient means of eliciting CD8+ CTL, which means that protein antigens produced by DNA vaccination gain access to pathways of antigen presentation via Class I MHC molecules, a system of antigen presentation that is most frequently utilized by live attenuated virus vaccines (reviewed by Donnelly et al., 1997 [49] and Gurunathan et al., 2000 [50]). DNA vaccines attracted attention owing to the immune responses generated, which closely resemble those induced by natural infection, due to endogenous production of viral proteins and glycoproteins [51].

The earliest report of a DNA vaccine developed against BoHV-1 was engineered with pRSV plasmids, which contains transcriptional control sequences from Rous sarcoma virus, being functional in a wide range of animal cell types [52]. Three glycoproteins were tested individually in mice and neutralization titers obtained for gD after intramuscular injection were higher than those obtained for gB or gC [53].

However, intradermal immunization administered by needle injection developed balanced and strong immune responses characterized by the induction of tgD-specific antibody titers and high numbers of IFN-γ-secreting cells, with protection from viral challenge [34].

In 2004 [54] a DNA vaccine encoding a truncated form of BoHV-1 gD was tested in calves through three different routes: intramuscular, intradermal and intranasal. Only intramuscular injection resulted in neutralizing antibodies and in early clearance of viral shedding after challenge. Higher titers of neutralizing antibodies were also obtained by intranasal administration of secreted forms of gD and gB [55].

Incorporation of and multiple copies CpG motif into a vaccine encoding plasmid also resulted in lymphocyte proliferation and cellular immunity and, following challenge high neutralization titers and lower levels of virus shedding, but no difference in clinical symptoms [56]. Plasmid encoding tgD linked to bovine CD154 administered by intradermal immunization was able to bind CD40-expressing dendritic cells present in the skin; however, no enhanced immune response was observed, probably due to the limitation of achieving adequate antigen expression in large animals with current delivery methods [57].

3.3. Vectored vaccines

Vectored vaccines are also expected to induce balanced immune responses once the cell endogenously produces protective antigens expressed by viral vectors. One of the main advantages of this kind of vaccine is the delivery of the antigen directly to the mucosal surfaces and also, the ability to induce humoral and cellular immune responses.

Due to the capability of Bovine adenovirus-3 (BAV-3) replicate in the respiratory tract of cattle, producing mild or no clinical symptoms, and the ability of this virus to grow to high titers in cell culture [58], BAV-3 expressing gD was used to immunize cattle. Intranasal immunization with a replication-competent BAV-3 expressing full-length gD or a secreted form of gD (tgD) demonstrated the induction of gD-specific immune responses, including gD-specific IgA in the nasal secretions. Even though animals were partially protected after the challenge, the magnitude of the IgA response was not sufficient to eliminate BoHV-1 shedding after challenge, but it was sufficient to prime the antigen-specific IgA responses in the nasal passage [59]. The same pattern of response was observed with immunization of cattle through intratracheal/ subcutaneous routes [60].

Human adenovirus 5 (HAd5) has been used as viral vector to delivery BoHV-1 glycoproteins. Replication-defective HAd5 expressing gC or gD under the control of the human cytomegalovirus immediate-early promoter/enhancer (CMV) or human desmin gene 5'regulatory region (DESM) promoters were used to induce immune responses in rabbit model. A single intranasal immunization with the HAd5 expressing gD alone or in combination with HAd5 expressing gC, both under the control of the CMV promoter, elicited high levels of neutralizing antibodies, the titers elicited by gD being much higher than those induced by gC. The same was observed after intramuscular administration of HAd5 expressing gD under the control of DESM promoter; however, the neutralizing antibodies were lower than those induced by intranasal immunization [36]. In cattle, this vector expressing gC and gD, after intranasal administration, induced BoHV-1 neutralizing antibodies and clinically protected cattle after challenge [36]. The same vector construction was further used

in combination with glycol chitosan, demonstrating protection against challenge [37]. No cellular immune responses were evaluated in these trials.

4 CONCLUSION

Glycoprotein D from BoHV-1 and -5 play a major role in viral interactions with permissive cells and are very immunogenic. For these reasons, the BoHV-1 gD has been employed in vaccine strategies over the last decades and may still be applied as long as new immunization strategies are developed. Knowing its characteristics and what has been achieved so far with this glycoprotein is important to develop further trials to minimize economic losses caused by BoHV-1 and -5 in cattle worldwide. The reports presented in literature so far indicates that although the DNA vaccines applying gD are a suitable strategy in the achievement of cellular immune responses, it still needs improvement in achievement of a balanced response whereas both cellular and humoral immunity are present. Thus indicated that subunit vaccines may be a reasonable choice for immunization with recombinant gD, and although new adjuvants able to stimulate a stronger cellular immunity are still needed, the use of CpG-containing oligodeoxynucleotides as adjuvant in subunit vaccine seems to fulfill the requirement of a strong humoral and cellular immunity.

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4 ARTIGO 2

Evaluation of an Indirect ELISA using recombinant gD from *Bovine herpesvirus 5* expressed in *Pichia pastoris*

(Manuscrito a ser submetido ao periódico *Journal of Virological Methods)*

EVALUATION OF AN INDIRECT ELISA USING RECOMBINANT gD FROM *Bovine herpesvirus 5* EXPRESSED IN *Pichia pastoris*

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ABSTRACT

Bovine herpesviruses 1 and *5* (BoHV-1 and -5) are economically important pathogens, causing a variety of diseases manifestations, including respiratory and genital infections and meningoencephalitis. Serological diagnosis is commonly performed using virus neutralization test, which is a time consuming procedure and requires manipulation of non-attenuated virus. In the present study, the development and evaluation of a highly sensitive and specific single dilution Indirect Enzyme-linked immunosorbent assay (ELISA) using the recombinant glycoprotein D (rgD) from BoHV-5 are reported. The comparative evaluation of this rgD Indirect ELISA with virus neutralization test using 239 bovine serum samples revealed 99.4% of accuracy of the test, with sensitivity and specificity of 100 and 92.9%, respectively. The newly developed rgD Indirect ELISA indicates good correlation with the VNT results. Comparison of the results with a commercial blocking ELISA to BoHV-1 shown that the commercial test fail to recognize 10 positive VNT samples, while considered as positive 46 negative VNT samples. The technique described is a simple, convenient, specific and highly sensitive assay for the detection of BoHV-5 antibodies in serum.

1. INTRODUCTION

Bovine herpesviruses 1 and *5* (BoHV-1 and -5) are economically important pathogens causing a variety of diseases manifestations, including respiratory and genital tract infections by BoHV-1 and meningoencephalitis by BoHV-5. Both are closely related viruses and BoHV-5 was regarded as a neuropathogenic variant of BoHV-1 until 1992, when it was recognized as a distinct virus (Roizmann et al., 1992). Both viruses infection occur at the mucosal surfaces and after replication at infection sites, the viruses spread through the central nervous system. Although they are neurotrophic and able to establish latency in the trigeminal ganglion, only the BoHV-5 is neurovirulent and capable of active replication in the central nervous system, causing outbreaks with a mortality rate of 70-100% (Vogel et al., 2003).

Molecular and immunological studies, based on restriction sites mapping of viral DNA, indicates that the viruses differ in their antigenic properties and share 85% DNA identity (Delhon et al., 2003), however traditional serological tests are unable to distinguish between the two of them due to the cross-reactivity induced by BoHV-1 neutralizing antibodies (Vogel et al., 2002; Del Médico Zajac et al., 2006).

The viral envelope glycoproteins of BoHV-1 and -5, specially the glycoproteins C, D and B, are the major targets of the host immune systems against these viruses. Their interaction with permissive cell receptors allow virus attachment and antibodies developed against these glycoproteins prevent virus-cell interaction and entry into host cells (Liang and Babiuk, 1991). Although the gC also interacts with cell receptors, its absence in viral envelope do not prevent infection in cell culture (Kaashoek et al., 1998). The gD is an essential viral glycoprotein, being present at all strains of BoHV-1 and -5, acting on the viral envelope and the plasma

membrane fusion, resulting in conformational changes that allow gH and gL interaction with gB (Geraghty et al., 2000). The recombinant expression of gD from BoHV-5 in the methylotrophic yeast *Pichia pastoris* and its antigenic characterization have been previously reported (Dummer et al., 2009), demonstrating that antibodies against both BoHV-1 and -5 were able to react with the recombinant protein in Western blotting assays. Its antigenic characteristics thus suggest that the recombinant gD (rgD) may be suitable for use as antigen in the Indirect ELISA herein described.

The serological diagnosis of BoHV-1 or -5 is commonly performed using the virus neutralization test (VNT). Changes in the serological response during infection with one of these viruses are readily measured by this assay, however, it lacks sensitivity and demands manipulation of non-attenuated virus, requiring a laboratory with a cell culture routine established and is labor-intensive and time consuming to perform (Collins et al., 1985). The enzyme-linked immunosorbent assay (ELISA) for antibody detection, on the other hand, is a fast, inexpensive and simple method for screening large numbers of test samples. When an end-point titer is unnecessary, for example in qualitative serological test that screen between positive or negatives animals in a herd, the single dilution Indirect ELISA can be applied, testing several animals in a single 96 well plate. The aim of this study was to develop a single dilution Indirect ELISA using recombinant glycoprotein D from BoHV-5 expressed in *P. pastoris*, which could screen with safety positive from negative animals and which would correlate with conventional VNT results.

2. MATERIALS AND METHODS

2.1. Expression and purification of recombinant gD protein

The BoHV-5 glycoprotein D cloned into *Pichia pastoris* expression system strain KM71H Mut^{S} and the recombinant glycoprotein D expression were previously described (Dummer et al. 2009). Briefly, the recombinant clone previously selected was inoculated in culture flasks containing BMGY medium (1% yeast extract, 2% peptone, 1.34% yeast nitrogen base, 4 x 10^{-5}% biotin, 1% glycerol, 100 mM potassium phosphate pH 6.0) and incubated in orbital shaker for 24 h at 28º C with agitation speed of 150 rpm. The cells were harvested and resuspended in BMMY medium (BMGY medium with 0.5% methanol in replacement of 1% glycerol), reducing 10x total medium volume. Induced expression was performed at 28º C with agitation speed of 150 rpm for 48 h. Every 24 h 1% of 100% methanol were added. Cells were then harvested and the collected supernatant concentrated with Centriprep 50YM (Millipore, MA, USA) device at 1500 *x g* for 10 min at 20º C. Recombinant gD was then purified by affinity chromatography using both HisTrap^{TM} HP 1 ml columns pre-packed with pre-charged Ni Sepharose^{TM} and the ÄKTAprime^{TM} Automated Liquid Chromatography system (GE Healthcare, WI, USA). Purified rgD was assayed by Western blot and the protein concentration determined by BCA protein assay (Pierce, IL, USA) method according with the manufacture instructions.

2.2. SDS-PAGE and Western blotting.

Purified proteins were boiled in SDS-PAGE loading buffer and separated on 12% separating gel in Mini-PROTEAN electrophoresis system (Bio-Rad, CA, USA).

The gel was stained with Coomassie Brilliant Blue R250 (Sigma-Aldrich, SP, Brazil). For Western blotting, proteins were transferred onto a nitrocellulose membrane using Bio-Rad Mini Trans-Blot Cell (Sambrook and Russel, 2000). The membrane was blocked with 5% non-fat dry milk and antigenic proteins were detected by incubating membrane with MAb Anti-6xHis HRP conjugated (Life Technologies, SP, Brazil). Recombinant protein was detected with DAB solution (0.6 mg 3,3'-diaminobenzidine, 0.03% nickel sulfate, 50 mM Tris–HCl pH 8.0 and H_2O_2 30 vol.) until a color reaction appeared.

2.3. Cell and viruses

Madin Darby bovine kidney cells (MDBK, ATCC CCL22) were grown in Eagle's Minimal Essential Medium (MEM - Life Technologies, SP, Brazil) supplemented with antibiotics (200 I.U./ml streptomycin and penicillin, 5 μg/ml enrofloxacin and 2.5 μg/ml amphotericin B) and 10% fetal bovine serum (FBS) (CultiLab, SP, Brazil) at 37° C in a 5% CO_2 humidified atmosphere. Virus stocks of *Bovine herpesvirus* 5 was propagated in MDBK cells until a 90% cytophatic effect was visible, stored in liquid nitrogen tank and tittered prior virus neutralization tests.

2.4. Test sera

Bovine blood samples were collected and centrifuged blood serum was stored at -20º C prior to use. Total of 239 samples were screened by virus neutralization test (standard test), rgD Indirect ELISA and a commercial gB ELISA kit. Serum samples were descendant from two different laboratories at UFPel and UFRGS, Brazil.

2.5. Indirect Enzyme Linked Immunosorbent Assay

Indirect ELISA analyses were performed with individual animal serum in duplicates. The overall procedure was performed as follow: ninety-six-well microtiter plates were coated overnight at 4º C with 100 μl/well of purified rgD. The plates were washed three times and incubated for 1 h at 37º C with blocking solution (5% of non fat dry milk + 3% of casein in PBS-T - 137 mM NaCl, 2.7 mM KCl, 100 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4 containing 0.05% Tween-20). After three washes, serum samples diluted with PBS-T were added in duplicate to the wells, and the plates were incubated again for 1 h at 37º C. The HRP-conjugated rabbit anti-bovine IgG whole molecule antibodies (Sigma-Aldrich, SP, Brazil) diluted 1:5000 in PBS-T were added after three washes, followed by incubation at 37º C for 1.5 h and a new five washes step. Reaction were visualized with o-Phenylenediamine dihydrochloride (OPD) (Sigma-Aldrich, SP, Brazil), stopped with 2 N H_2SO_4, and analyzed at O.D 492 nm using ELISA TP-Reader plate spectrophotometer (ThermoPlate, SP, Brazil).

2.5.1. Coating antigen concentration and sera dilution

In order to establish the optimal concentration of antigen to coating plates as well as the optimal serum dilution to be tested, different amounts of purified rgD and different serum concentration were screened to best distinguish positive from negative serum samples in virus neutralization serum assay. Concentrations ranging from 200 to 12,5 ng/well of rgD were used and serum were diluted 1:100, 1:200, 1:300, 1:400 or 1:500 in PBS-T. The Indirect ELISA was performed as described above.

2.6. Virus neutralization test

Serum samples were assayed for neutralizing antibodies against BoHV-5. Briefly, after inactivation of complement proteins at 56º C for 30 min, each serum was serially diluted (2-fold) in 96 well microtiter plate (TPP, Switzerland) in quadruplicate, beginning at 1:2 until 1:256 in MEM. BoHV-5 virus suspension containing 100 CCID50% was added following incubation for 1 or 24 h at 37º C in a 5% CO_2 environment. Approximately $3x10^4$ MDBK cells were then added per well and the microplates were incubated until 100 CCID50% was observed in the retro-titration wells. The absence of cytophatic effect (CPE) was indication of viral neutralization, while its presence resulted from absence of neutralizing antibodies.

2.7. Commercial blocking ELISA test

All serum samples were blindly tested using a commercial blocking BoHV-1 gB-ELISA (IDEXX Laboratories, ME, USA) according with instructions from the manufacturer.

2.8. ROC analysis and graphs

Receiver-operating characteristics (ROC) curves, the area under the curve (AUC) and determination of optimal cut-off point were conducted with the software MedCalc v12.4.0. Graphs were created at GraphPad Prism software version 5.0d.

3. RESULTS

3.1. Expression, purification and quantification of the rgD in Pichia pastoris

After 48 h of methanol induction, the recombinant gD with ~55 kDa was detected in the *P. pastoris* KM71H supernatant by SDS-PAGE (Fig. 1A). The affinity chromatography purification of the rgD was confirmed by Western blotting assay (Fig. 1B). Fractions containing the his-tagged rgD were pooled and average yield of the recombinant protein was ~0.360 mg/ml.

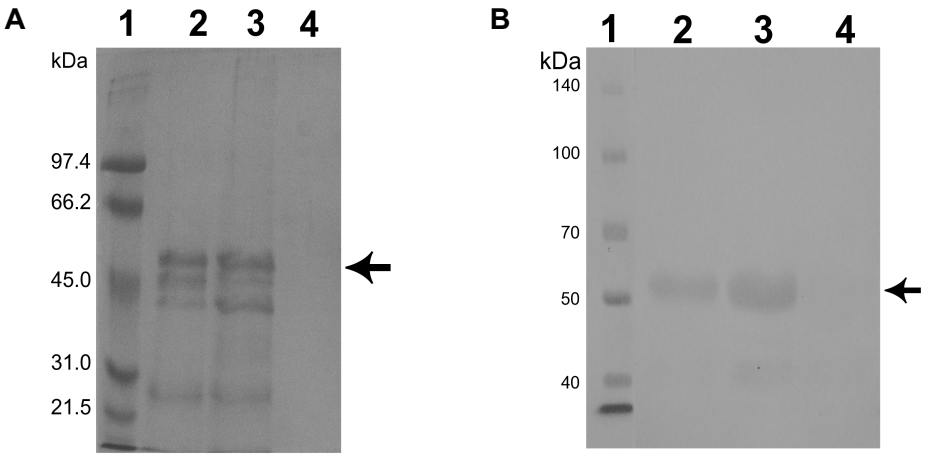

Fig. 1. 12% SDS-PAGE and Western blotting analysis of recombinant gD. A: 12% SDS PAGE from *Pichia pastoris* supernatant; B: Western blotting analysis of purified rgD with MAb Anti-6xHis HRP conjugated. Lane 1: Bio-Rad Low Range Pre-Stained Protein Marker (A) or Pierce Spectra Multicolor Broad Range Protein Ladder (B); Lane 2: Positive control of expression (rgD); Lane 3: rgD expressed in *P. pastoris* supernatant; Lane 4: negative control (KM71H supernatant of non-transformed yeast cells after methanol induction).

3.2. Virus neutralization test

A total of 239 serum samples were tested by virus neutralization test, of which 127 serum samples were found to be seronegative and 112 seropositive.

3.3. Determination of optimal antigen concentration and serum dilution

The optimal coating antigen concentration was established in 50 ng of rgD per well when positive and negative serum samples were tested. At this concentration, the positive and negative serum riches the maximum and minimum absorbance, respectively (Fig. 2A and B). The optimal serum dilution where samples could clearly be differentiated in positive and negative was found at 1/400 (Fig. 2C), when 50 ng of rgD was used as coating antigen.

3.4. ROC analysis of the Indirect ELISA

The AUC indicated that the rgD Indirect ELISA test was on average 99.4% accurate (Fig. 3A). The 95% confidence interval of the AUC for the ELISA ranged from 97.4 to 100% and the significance level P (Area=0.5) was <0.0001. The ROC analysis indicated that the optimal cut-off point was 0.275, which resulted in a sensitivity of 100% and specificity of 92.9% (Fig. 3B). Of 112 virus neutralization test positive sera, all samples were considered to be true positive because the expressed ELISA readings are above the optimized cut-off. Among the 127 virus neutralization test negative sera, 10 samples (7.87%) were considered false positive (Fig. 3C) since they showed ELISA values above the cut-off and 117 samples (92.13 %) showed ELISA readings below the cut-off and were considered to be true negative (Fig. 3C)

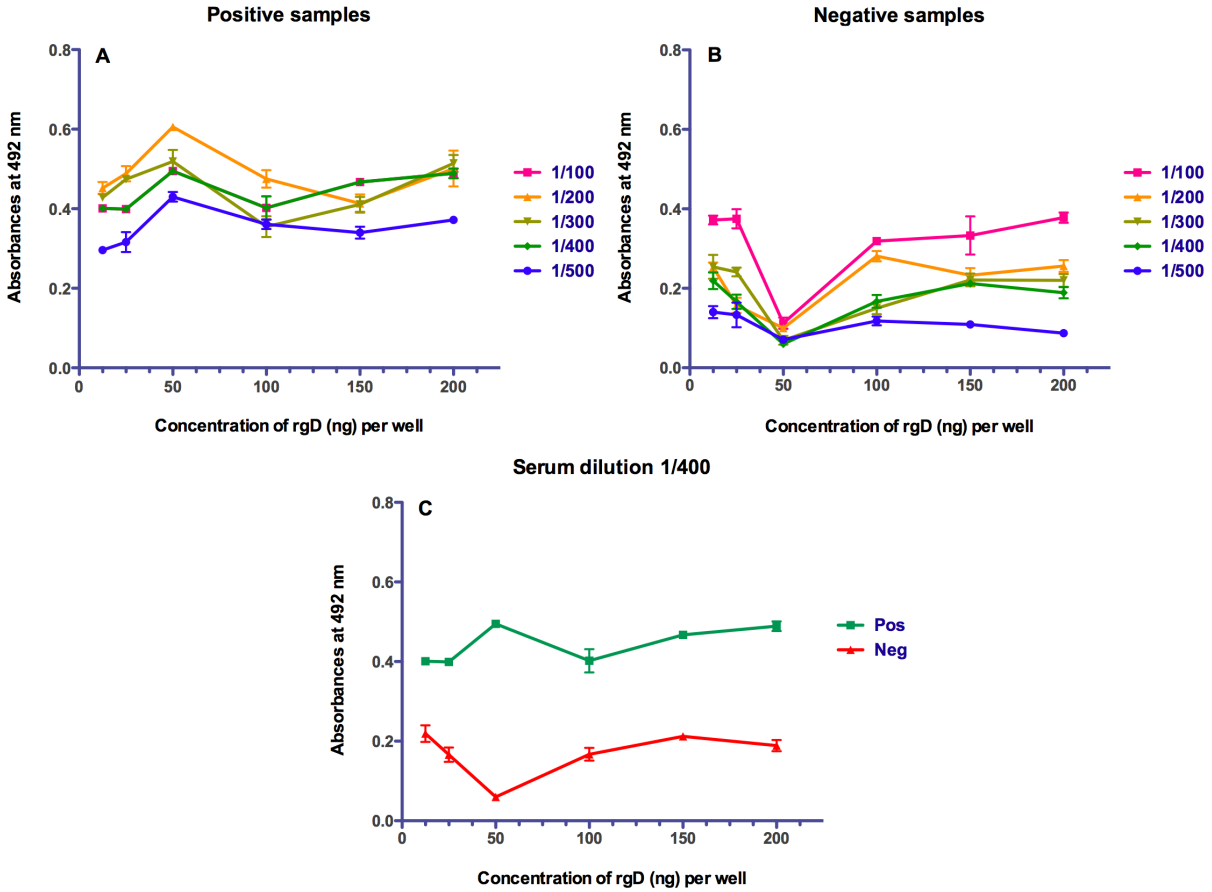

Fig. 2. Indirect ELISA to determination of coating antigen concentration and serum dilution. The rgD was used as coating antigen in the Indirect for selection of optimal combination of antigen and serum dilution to further experiments. Absorbance results at 492 nm for positive control serum (A) and negative control serum (B) dilution with coating antigen concentration ranging from 12.5 – 200 ng of rgD; and the optimal condition established (C).

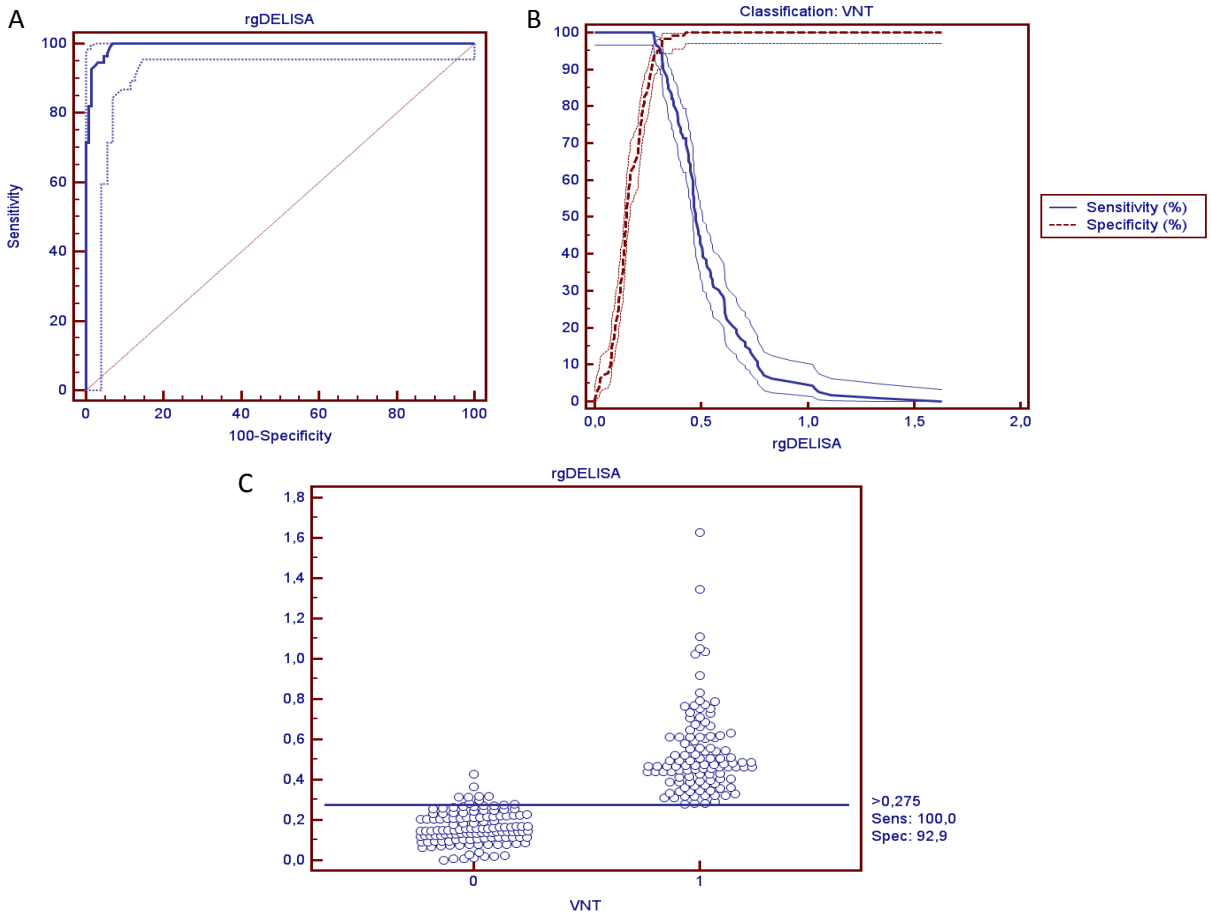

Figure 3: ROC analysis of rgD Indirect ELISA test. A- ROC curves based on test results obtained for the rgD Indirect ELISA test; B- Relationship between ROC-based estimates for test sensitivity and specificity and ELISA cut-offs; C- Interactive dot diagram based on ELISA outcomes in relation to virus neutralization test (VNT negative = 0 and VNT positive = 1).

3.5. Comparison with commercial ELISA

Virus neutralization screened samples were also screened with the developed rgD Indirect ELISA and with a commercial blocking gB-ELISA for diagnosis of IBR antibodies. The diagram on Figure 4 show the results obtained for all individual samples. The commercial test failed to recognize 10/112 positive 1h VNT samples, while 1/112 were considered as suspect. The rgD Indirect ELISA

recognized as positive all 112 positive VNT samples. The major discrepancy between testes occurred when VNT negative samples were tested with commercial ELISA. The rgD Indirect ELISA recognized as positive 10/127 samples while the commercial test recognized as positive 46/127 samples (39 from a 1h VNT and 7 from a 24h VNT) and 7/127 were considered as doubtful readings (4 from 24h VNT and 3 from a 1h VNT negative results).

Figure 4: Diagram of the results from individual samples tested with VNT and two ELISAs. rgD-ELISA represents the in Indirect ELISA using rgD as antigen, whereas gB-ELISA represents the available commercial kit. Green boxes – positive; Red boxes – negative; Slash boxes – suspect samples.

4. DISCUSSION

The recombinant glycoprotein D from *Bovine herpesvirus 5* expressed in the methylotrophic yeast *Pichia pastoris* expression system was successfully used as antigen for the Indirect ELISA. The antigenic characteristics of the rgD had already been described by our research group, whereas serum from both BoHV-1 and BoHV-5 positive animals were able to recognize the rgD in Western blotting techniques, suggesting that the rgD possess the antigenic characteristics present in native gD (Dummer et al., 2009).

The glycoprotein D, among others envelope glycoproteins from BoHV-5, is a glycoprotein required for the recognition of the viral envelope through its cellular receptors in permissive host cells, being essential for envelope fusion with membrane cells and infectivity. The gD is also a major target from the host immune systems, stimulating the production of neutralizing antibodies. As it is essential in BoHV-5 infectivity, it is unlikely that this protein will be absent in wild-type strains. Because gDs of both BoHV-1 and BoHV-5 show a strong sequence homology (Abdelmagid et al., 1995; Gabev et al., 2010), the possibility that sera containing antibodies directed to one of these viruses give positive responses in the Indirect ELISA due to immunologic cross-reactivity cannot be excluded (Kramps et al., 1994).

Several studies reported the development of ELISAs using proteins expressed in *Pichia pastoris* system as antigens in past decade. In 2003, Ao et al. described the expression of pseudorabies virus gE and its utilization in an indirect ELISA (Ao et al., 2003). In 2005, Huang and colleagues applied the yeast expressed gD from HSV-1 as a coating antigen in an ELISA, which as specific and sensitive for the diagnosis of the HSV-1 infection (Wang et al., 2005). A sandwich blocking ELISA

for detection of the Classical Swine Fever Virus glycoprotein E^{rns} was described in 2006 (Huang et al., 2006). In 2012, Li and colleagues described an indirect ELISA for the detection of reticuloendotheliosis virus using gp90 protein, showing a highly correlation with the virus neutralization test results (Li et al., 2012). Also in 2012, (Shehata et al., 2012) the successfully expression and application of two H5N2 proteins in a single dilution Indirect ELISA had been described. The advantage of the *P. pastoris* expression system is that the recombinant protein can be produced in large quantities at low cost. Also, *P. pastoris* are as easy to manipulate as prokaryotic systems, growing at large cell density, and as most aspects of the post-translational modification of proteins in *P. pastoris* cells are similar to those in higher eukaryotic cells (Daly and Hearn, 2005), antigenic sites from native structures may be conserved in recombinant proteins.

Also, there are studies reporting the development of ELISAs aiming the detection of antibodies against BoHV-1 (Kramps et al., 1994; Bashir et al., 2011; Oliveira et al., 2013). However none of those tests have been applied for detection of anti-BoHV-5 antibodies and, to our knowledge, no ELISA test employing the gD from BoHV-5 had been described so far.

The single dilution rgD Indirect ELISA described herein was optimized and both positive and negative sera were tested. The specificity and sensitivity of the test were 92.9% and 100%, respectively. Single dilution Indirect ELISA system offers considerable savings over more labor-intensive tests, such as virus neutralization and ELISA end-point titration, that are conventionally used for testing serum pairs and that require testing of serial dilutions of each sample.

Among a panel of 239 serum samples, none was considered as false negative and ten were found to be false positive when considered the established

cut-off and the virus neutralization test results as “gold standard” diagnose procedure. The serum samples tested classified as false positives, once readings above the established cut-off was obtained, may be explained due to the ability from the ELISA to detect lower antibody levels than the VNT and not only neutralizing antibodies. So, these samples should be considered as positives in a screening procedure.

Major discrepancies were found within results obtained from the commercial gB blocking ELISA. Among negative samples in serum neutralization, 46 out of 127 were considered positive in the commercial ELISA. As the commercial blocking ELISA applied bovine serum without dilution, it is able to detect weak positive samples that may not be detected in conventional VNT. The modified VNT, which allow virus contact with serum for 24 h, may be as sensitive as the commercial ELISA (Kramps et al., 1994; Kramps et al., 1996), however, 11/46 samples gB ELISA positive/suspect were considered negative in 24 h VNT as well. The fact that the rgD Indirect ELISA applied a 1:400 dilution in serum samples may contribute to inability to detect weakly positive samples, however, undiluted samples may not be applied in Indirect ELISA due to the unacceptable high background readings.

Validation of ELISA test to detect antibodies to BoHV-1 should be performed with three European Union standard bovine reference sera of IBR-EU1 (strong positive), IBR-EU2 (weakly positive) and IBR-EU3 (negative) used as external reference standards (Parreño et al., 2010). However, as we use a recombinant gD from BoHV-5, we could expect slightly differences in readings of BoHV-1 weakly positive serum due to the amino acid sequences and epitope differences between the two glycoproteins (Abdelmagid et al., 1995; Gabev et al., 2010).

The newly developed rgD Indirect ELISA indicate that bovine serum can be tested as single dilution with a defined cut-off point, with a correlation with the VNT results. The technique described is a simple, convenient, specific and highly sensitive assay for the detection of BoHV-1 or -5 antibodies in serum, allowing a fast screen of herds for the presence or absence of antibodies against those viruses.

5. ACKNOWLEDGES

We thank Brazilian National Counsel of Technological and Scientific Development (CNPq) for the financial support, Mr. Fabricio Torres and Mr. Eduardo Masuda from Axys Análises (Porto Alegre, RS, Brasil) and Mr. Frank Schoonjans from MedCalc Software for technical support.

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5 ARTIGO 3

Mice immune response against recombinant *Bovine herpesvirus 5* glycoprotein D

(Manuscrito a ser submetido ao periódico *Vaccine*)

MICE IMMUNE RESPONSE AGAINST RECOMBINANT *Bovine herpesvirus 5* GLYCOPROTEIN D

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ABSTRACT

Glycoprotein D (gD) is essential for attachment and penetration of *Bovine herpesvirus 5* (BoHV-5) into permissive cells, and is a major target of host immune systems, inducing strong humoral and cellular immune responses. The immunogenicity of recombinant BoHV-5 gD expressed in *Pichia pastoris* was evaluated in mice. Vaccines formulated with rgD and oil adjuvanted vaccines (Montanide 50 ISA V2; Emulsigen; Emulsigen-DDA) or rgD without adjuvants were administered intramuscularly or subcutaneously. All vaccine formulations stimulated humoral immune responses after three doses, and higher titer of neutralizing antibodies were obtained in all three oil-based adjuvants formulations when compared to non-adjuvanted vaccine formulations. rgD + Emulsigen-DDA stimulated the higher mRNA expression levels of Th1 (INF-γ) and pro-inflammatory cytokines (IL-17, GM-CSF). The results demonstrated that the recombinant gD conserved important epitopes for viral neutralization from native BoHV-5 gD and was able to stimulate cellular mediate immunity, stimulating a balanced Th1/Th2 immune response in mice.

Key Words: oil adjuvant; *Pichia pastoris; Bovine herpesvirus;* glycoprotein D; subunit vaccine

Running headline: Immune response of recombinant BoHV-5 gD in mice.

1. INTRODUCTION

Outbreaks of meningoencephalitis can reach a mortality rate of 70-100% [1] when associated with *Bovine herpesvirus 5* (BoHV-5). The disease affects young cattle and is responsible for economic losses in South America [2]. First reported as a neuropathogenic variant of BoHV-1 [3], the etiological agent of infectious bovine rhinotracheitis and vulvovaginitis/balanopostitis, due to its biologic, morphologic, and antigenic properties, the BoHV-5 was recognized in 1992 as distinct virus [4]. Molecular and immunological studies based on restriction sites mapping of viral DNA and monoclonal antibody reactivity indicated that the viruses differ in their antigenic properties, despite having 85% DNA identity [5].

Although previous vaccination of cattle with BoHV-1 resulted in protection against neurological disease caused by the BoHV-5, due to cross-reactivity induced by BoHV-1 neutralizing antibodies [6], it does not prevent infection and establishment of latency by BoHV-5 [7]. Immunization of cattle with current modified live and killed BoHV vaccines may protect against the disease, however, live attenuated vaccines have been associated with the induction of abortions and reactivation of the vaccine strain [8,9]. Killed vaccines not always offer the same protection levels of the live attenuated, inducing only humoral immunity, depending on the inactivation method, antigens may be denatured [10,11].

Novel subunit vaccines strategies against herpesviruses have been based on the viral major envelope glycoproteins owing its important biological and immunological functions, since they effect attachment and penetration of the virus into the cell and, also, mediate the immune responses of the host to the infection [12]. Three BoHV-1 glycoproteins, the gC, gB and gD have been tested as potential

antigens individually, and although they conferred protection from disease [13], gD is essential for viral attachment and penetration into host permissive cells [14] and has the ability to induce strong cellular immune response when compared to that induced by the other mentioned glycoproteins [15]. The BoHV-1 gD has been used as antigen for bovine immunization since past decades, applied in subunit or DNA vaccines strategies, being efficient in reduction of viral replication, shedding and clinical signs [16].

The gD from BoHV-1 and -5 has 79.9% amino acid identity and the gD from BoHV-5 provide the ability of this virus to infect cells that are not accessible to BoHV-1, once is suggested that gD from BoHV-5 can attach to all four nectins (1-4) and appeared to increase the virulence of a recombinant BoHV-1 carrying the gD from BoHV-5 [17]. The glycoproteins also differ in an N-linked glycosylation site, which is absent in the BoHV-5 [18]. To our knowledge, the glycoprotein D from BoHV-5 has never been used in subunit vaccine research trials to date. Our group has previously reported its recombinant expression in *Pichia pastoris* and its antigenic characterization [19]. So, the aim of this study was to evaluate the immunological responses of mice against recombinant glycoprotein D from BoHV-5 expressed in the yeast *P. pastoris* alone or in formulations with mineral-oil adjuvants.

2. MATERIAL AND METHODS

2.1. Expression of recombinant glycoprotein D

The BoHV-5 glycoprotein D was cloned into *Pichia pastoris* expression system strain KM71H Mut^S and the recombinant glycoprotein D were expressed as described previously [19]. Briefly, the recombinant clone previously selected was

inoculated in culture flasks containing BMGY medium (1% yeast extract, 2% peptone, 1.34% yeast nitrogen base, 4 x 10^{-5}% biotin, 1% glycerol, 100 mM potassium phosphate pH 6.0) and incubated in orbital shaker for 24 h at 28º C with agitation speed of 150 rpm. The cells were harvested and resuspended in BMMY medium (BMGY medium with 0.5% methanol in replacement of 1% glycerol), reducing 10x total medium volume. Induced expression was performed at 28º C with agitation speed of 150 rpm for 72 h. Every 24 h 1% of 100% methanol was added. Cells were then harvested at 10.000 x g for 10 min at 4ºC and the collected supernatant concentrated with Centriprep 50YM (Millipore, MA, USA) device at 1500 x g for 10 min at 20º C. The concentrated supernatant was used for vaccines formulations. For Indirect ELISA, recombinant gD was purified by affinity chromatography using both HisTrap™ HP 1 ml columns pre-packed with pre-charged Ni Sepharose™ and the ÄKTAprime™ Automated Liquid Chromatography system (GE Healthcare, WI, USA). Purified and concentrated rgD was assayed by Western blot and the protein concentration determined by BCA protein assay (Pierce, IL, USA) method according with the manufacturer's instructions.

2.2. Cells, animals and viruses

Madin Darby bovine kidney cells (MDBK, ATCC CCL22) were grown in Eagle's Minimal Essential Medium (Life Technologies, SP, Brazil) supplemented with antibiotics (200 I.U./ml streptomycin and penicillin, 5 μg/ml enrofloxacin and 2.5 μg/ml amphotericin B) and 10% fetal bovine serum (CultiLab, SP, Brazil) at 37° C in a 5% CO_2 humidified atmosphere. Virus stock of *Bovine herpesvirus* 5 were propagated in MDBK cells until a 90% cytophatic effect was visible, and the stored in

liquid nitrogen tank. Prior virus neutralization test, the virus stock was tittered and stored at -80º C.

Female Swiss mice six-to-nine weeks of age were maintained and handled at the animal care facility from Universidade Federal de Pelotas (UFPel), Brazil. The mice were housed in autoclaved cages (Alesco, Brazil) with no food or water restrictions. All procedures were performed in accordance with the Brazilian Committee for animal care and use (COBEA) guidelines and approves by the UFPel Ethics Committee for animal research (project number 002129).

2.3. Western blot

Proteins were boiled in SDS-PAGE loading buffer and separated on 12% separating gel in Mini-PROTEAN electrophoresis system (Bio-Rad, CA, USA). Then, proteins were transferred onto a nitrocellulose membrane using Bio-Rad Mini Trans-Blot Cell [20]. The membrane was blocked with 5% non-fat dry milk and then, MAb Anti-6xHis HRP conjugated (Life Technologies, SP, Brazil) replaced the blocking solution. Recombinant gD was detected by color reaction when the membrane was incubated in DAB solution (0.6 mg 3,3'-diaminobenzidine, 0.03% nickel sulfate, 50 mM Tris-HCl pH 8.0, and hydrogen peroxide 30 vol).

2.4. Immunizations

Six groups of six outbreed six-to-nine-week-old female Swiss mice were immunized three times, within 14 days intervals, subcutaneously (s.c.) or intramuscularly (i.m.) with 40 µg recombinant BoHV-5 gD in PBS (pH 7.4). Intramuscular vaccines were formulated either with 50% v/v Montanide ISA 50 V2 (Seppic Adjuvants, France) or with PBS. Control groups were immunized with PBS

only. Subcutaneous vaccines were formulated either with PBS, 30% Emulsigen (Em) or 30% Emulsigen-DDA (Em-DDA) (MVP Laboratories, NE, USA). Retro-orbital bleeding was performed every 14 days after primary vaccination until day 42.

2.5. Enzyme-Linked Immunosorbent Assays (ELISA)

Indirect ELISA analyses were performed with individual animal serum from each collection. Sera were assayed for rgD specific total IgG, IgG1 and IgG2a. Ninety-six-well microtiter plates were coated overnight at 4º C with of 0.2 μg of purified rgD per well. The plates were washed three times in PBS (pH 7.4) containing 0.05% Tween-20 (PBS-T) and incubated 1 h at 37º C with serum samples serially diluted with PBS-T, beginning at 1:10 (days 0 and 14) or 1:100 (days 28 and 42), and continuing in a 2-fold dilutions. After three washes, HRP-conjugated goat anti-mouse IgG whole molecule antibodies (Sigma-Aldrich, SP, Brazil) diluted 1:4000 were added, followed by incubation at 37º C for 1.5 h and five washes. Reaction were visualized with o-Phenylenediamine dyhydrochloride (OPD) (Sigma-Aldrich, SP, Brazil), stopped with 2 N H_2SO_4, and analyzed at O.D 492 nm using ELISA TP-Reader plate spectrophotometer (ThermoPlate, SP, Brazil). Results were expressed as the reciprocal of the highest dilution resulting in a reading of three standard deviations above the value of the negative control serum. To assay the IgG isotype, pooled serum from days 0, 14, 28 and 42 were diluted 1:1000 and ELISA was performed according with the instructions of isotyping kit from Sigma-Aldrich for IgG1 and IgG2a detection.

2.6. Virus neutralization assay (VNT)

Serum samples were tested for neutralizing antibodies against BoHV-5 [21] at day 42, fourteen days after third inoculation. Briefly, each serum was serially diluted (2-fold) in 96 well microtiter plate (TPP, Switzerland) in quadruplicate, beginning at 1:2 until 1:256 in MEM (no FBS). BoHV-5 virus suspension containing 100 CCID50% was added following incubation for 1h at 37º C in a 5% CO_2 environment. Approximately $3x10^4$ MDBK cells were then added per well and the microplates were incubated until 100 CCID50% was observed in the control cells. The absence of cytophatic effect (CPE) was indication of viral neutralization, while its presence resulted from absence of neutralizing antibodies. Antibodies titers were calculated by the Behrends & Kärber method [22] and expressed as the reciprocal of the highest dilution

2.7. Spleen cells

Spleens were collected from immunized mice at day 42 post-vaccination as described elsewhere [23]. Briefly, spleen cells suspensions were prepared by mechanical dissociation in wash medium (RPMI 1640 supplemented with 10 $mg.L^{-1}$ gentamycin and 25 mM HEPES). Splenocytes cells obtained from animals of the same groups were separately pooled. Cells were washed and spleen erythrocytes were removed by osmotic lysis with Tris-buffered ammonium-chloride pH 7.2 (0.16 M ammonium chloride, 0.17 M Tris buffer pH 7.65). Splenocytes were cultured in 24-well plates at a density of $2x10^6$ cells/well in RPMI 1640 medium (supplemented with 10% FBS, 0.1 mM non-essential amino acids, 25 mM HEPES, 1 mM sodium pyruvate, 50 µM 2-mercaptoehanol, 10 $mg.L^{-1}$ gentamycin). After ~12 h incubation at 37º C in a 5% CO_2 humidified atmosphere, lymphocytes were restimulated with either

10 µg of rgD, whole live BoHV-5 ($10^{6.25}$ CCID 50.ml^{-1}), 5 µg of ConA (Sigma-Aldrich, SP, Brazil) or RPMI 1640 culture medium alone for ~18 h at same incubation conditions. ConA and RPMI 1640 stimuli were used as controls for the cells stimulation. Cells were then harvested with TRIzol Reagent (Life Technologies, SP, Brazil) according to the manufacturer's instructions for total RNA isolation and pooled lysates were frozen at -80º C until further processing.

2.8. RNA isolation, cDNA synthesis, and qPCR

Total RNA was extracted using the protocol supplied by the manufacturer of TRIzol Reagent (Life Technologies, SP, Brazil). After removal of DNA with Amplification Grade DNAse I (Sigma-Aldrich, SP, Brazil), approximately 1 µg of RNA was reverse transcribed with High Capacity cDNA Reverse Transcription Kit (Life Technologies, SP, Brazil), according with the manufacturer's instruction. Approximately 500 ng of the resulting cDNA was subjected to qPCR to analysis of relative expression of the following cytokines: IL-4, IL-10, IL-17, GM-CSF and INF-γ. GAPDH was used as endogenous reference genes and animals in pre-immune conditions were used as calibrator. qPCR reactions were performed on a MX3005P QPCR System (Agilent Technologies, CA, USA) using SYBR Green I (Life Technologies, SP, Brazil) as a double-strand DNA-specific binding dye.

Table 1. Primer sequences for qPCR.

Gene	Forward (5'- 3')	Reverse (5'- 3')	TM
GAPDH	AAC GAC CCC TTC ATT GAC 3	TCC ACG ACA TAC TCA GCA C	60 ºC
IL-4	CCA AGG TGC TTC GCA TAT TT	ATC GAA AAG CCC GAA AGA GT	60 ºC
IL-10	TTT GAA TTC CCT GGG TGA GAA	ACA GGG GAG AAA TCG ATG ACA	60 ºC
IL-17	GCT CCA GAA GGC CCT CAG A	AGC TTT CCC TCC GCA TTG A	60 ºC
GM-CSF	GCC ATC AAA GAA GCC CTG AA	GCG GGT CTG CAC ACA TGT TA	63 ºC
IFN-γ	GCG TCA TTG AAT CAC ACC TG	TGA GCT CAT TGA ATG CTT GG	60 ºC

2.9. Statistical analysis

Data were analyzed using the statistical software program GraphPad Prism v5.0d for Mac OSX 10.6.8 (GraphPad Software, CA, USA). Analysis of differences in serum ELISA antibody titers and in serum neutralizing antibodies between treatment groups was performed by one-way ANOVA on $\log_{10}$ or $\log_2$-transformed titer data, and pairwise comparisons were made using Dunnett's or Tukey's Multiple Comparison posttest. Differences in relative cytokine expression were also investigated using one-way analysis of variance (ANOVA) with Dunnett's Multiple Comparison posttest. Data were compared to non-treated control group.

3. RESULTS

3.1. *Recombinant gD expression*

The expression of the ~55 kDa recombinant protein, which is in agreement with our previous results [19], was successfully detected by Western blotting using MAb Anti-6x-HIS HRP conjugated after concentration (Fig 1). Purified rgD was also detected with the same procedure (Fig 1) and used as ELISA antigen.

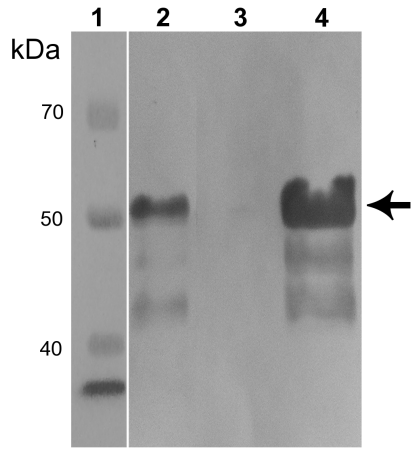

Fig. 1. Western blotting analysis of recombinant gD. Lane 1: Pierce Spectra Multicolor Broad Range Protein Ladder; Lane 2: rgD Purified with affinity chromatography; Lane 3: negative control (KM71H supernatant of non transformed yeast cells)*;* Lane 4: rgD from concentrated supernatant.

3.2. *Humoral immune responses against rgD*

To determine the immunogenicity of BoHV-5 rgD, mice were vaccinated with formulation of rgD with PBS only or oil adjuvants and stimulated humoral immune responses evaluated by ELISA and virus neutralization test. Fourteen days following primary vaccination, all the formulations with oil adjuvant significantly increased the antibody response to rgD ($P<0.001$) when compared to the non-treated group. The group immunized intramuscularly with the combination of rgD + PBS also shown significant difference ($P<0.001$) when compared to non-vaccinated group. Animals vaccinated with rgD + Em+DDA produced higher levels of total IgG when compared to levels induced by other treatments after the first dose. After the second vaccination, an increase in magnitude of the response was observed, including the formulations with rgD with PBS administrated intramuscularly. Fourteen days after

the third and last vaccine dose administration, all combinations produced detectable levels of IgG (Fig. 2).

Neutralizing antibodies were investigated fourteen days after the last vaccination. Although subcutaneously administration of rgD plus PBS did not produced any neutralizing antibody, the intramuscularly administration of the same formulation were able to produce a significant titer of 1 $\log_2$ ($P<0.05$) (Fig. 3). Combinations of rgD with Em-D, Em or ISA 50 V2 stimulated significantly different neutralizing IgG titers ($P<0.001$) when compared with non-vaccinated control, although no statistical difference was observed between these groups.

The Th bias of the immune response was analyzed by determination of the IgG isotype, reflecting Th1 or Th2 polarization (Fig. 4). While the absorbance readings of IgG1 increases right after the first dose at high levels (Fig. 4a), the IgG2a production was less pronounced but increases after second and third vaccination (Fig 4b). The ratio IgG2a/IgG1 was calculated after fourteen days post each vaccination (Fig. 4c). The IgG2a/IgG1 was enhanced in all but the animals vaccinated with rgD + PBS administered subcutaneously. This was expected, once this group did not demonstrate any detectable levels of IgG before. Fourteen days after the last vaccination, the ratio of IgG2a/IgG1 of animals vaccinated with rgD with PBS administered intramuscularly shown a decrease when compared to the same period after second vaccination.

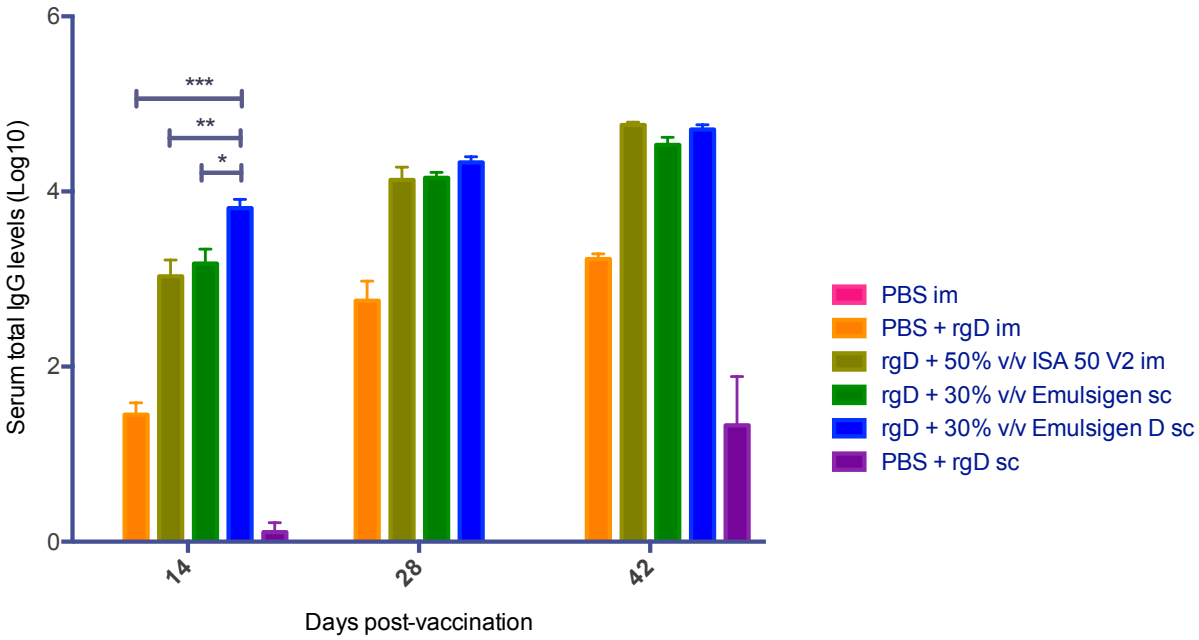

Fig. 2. Total serum IgG levels of mice vaccinated with 40 µg rgD associated or not with oil adjuvants. Indirect ELISA of sera collected fourteen days after each vaccination performed at days 0, 14 and 28. Formulations inoculated subcutaneously are indicated by sc. The data represents the mean ± S.E.M of $\log_{10}$ transformed data and are expressed as the reciprocal of the highest dilution whereas the absorbance at 492 nm was higher then the reading of three standard deviations above the value of the negative control serum. * P<0.05, ** P<0.01 and *** P<0.001.

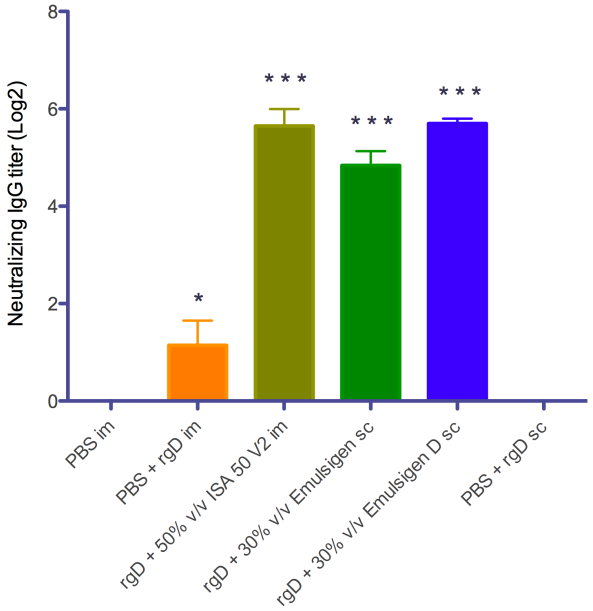

Figure 3 – Neutralizing antibody titer of mice vaccinated with 40 µg rgD associated or not with oil adjuvants. The titer was determined by viral neutralization assay fourteen days after the third inoculation. Formulations inoculated subcutaneously are indicated by sc. The data represents the mean ± S.E.M of log_2 transformed data and are expressed as the reciprocal of the highest dilution that completely inhibited virus-induced CPE. * P<0.05; *** P<0.001.

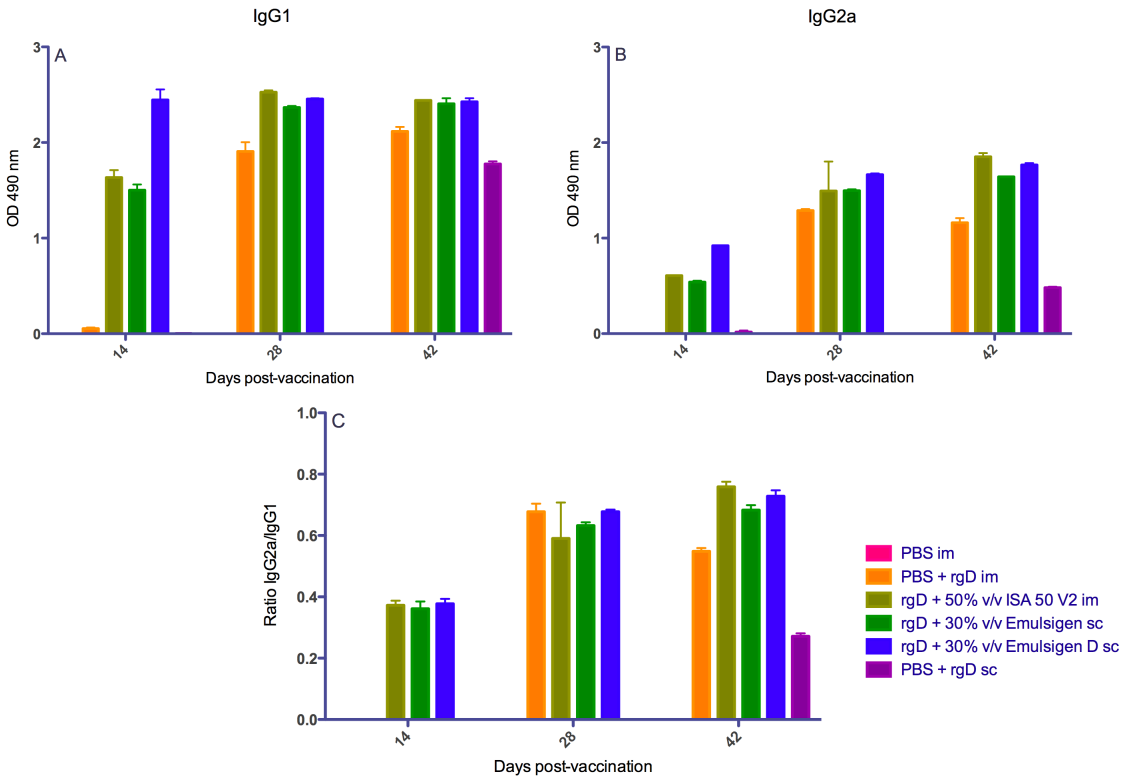

Figure 4 – Immunoglobulin isotype profiles and ratio IgG2a/IgG1. A- IgG1 isotype response; B- IgG2a isotype response; C- Any ratio >1 is associated with Th1 response while any ratio <1 is associated with a Th2 response. The data represents the mean ± S.E.M.

3.3. *Cytokine mRNA expression*

The relative expression of cytokines mRNAs in splenocytes, such as IL-4, IL-10, IL-17, IFN-γ and GM-CSF, was determined to assess the cellular immune responses of mice vaccinated with rgD (Fig. 5). All vaccines formulations were compared to animals in pre-immune status, which did not receive any antigen. Data is expressed as relative quantification in fold increase. Animals vaccinated with rgD + ISA 50 V2 increased 14-fold ($P<0.01$) the mRNA expression of IFN-γ when spleen cells were stimulated *in vitro* with whole live BoHV-5 (Fig. 5b). The group that received rgD + Em subcutaneously up regulated in all cytokines studied when spleen

cells were stimulated with rgD however, significant difference was observed in the mRNA expression of IL-4 ($P<0.05$), IL-10 ($P<0.01$) and GM-CSF ($P<0.001$) with 3.6, 1.7, 13-fold increase, respectively (Fig. 5a; 5c; 5e). On the other hand, spleen cells from animals immunized with rgD + Em-D stimulated with rgD up regulated the expression of IFN-γ, IL-17 and GM-CSF. The fold increase observed was 122 ($P<0.01$) for IFN-γ and 24 ($P<0.001$) for both IL-17 and GM-CSF (Fig. 5b; 5d; 5e). Cells from the same group also demonstrate up-regulation when stimulated with BoHV-5 of 91-fold increase for IFN-γ ($P<0.001$), 1.3 for IL-10 ($P<0.05$), 20 for IL-17 ($P<0.001$) and 13 for GM-CSF ($P<0.001$) (Fig. 5b; 5c; 5d; 5e). Splenocytes of groups immunized with formulations of rgD + PBS administered intramuscularly or subcutaneously up regulated the mRNA expression of IFN-γ when cells were stimulated with BoHV-5, showing 57 ($P<0.01$) and 91-fold increase ($P<0.001$), respectively (Fig. 5b).

Down-regulation was observed in animals vaccinated with rgD + PBS im and stimulated with BoHV-5, for IL-4 ($P<0.001$) and IL-10 ($P<0.01$) with 0.87 and 48-fold decrease, respectively (Fig. 5a; 5c). When stimulated with rgD, the spleen cells from animals of this same group down-regulated the levels of GM-CSF ($P<0.05$), with a 0.55-fold decrease (Fig. 5e). The mRNA levels of IL-10 ($P<0.05$) was also down regulated when spleen cells of animals that were vaccinated with rgD + ISA 50 V2 were stimulated with BoHV-5, showing a 0.39-fold decrease (Fig. 5c). Animals vaccinated with rgD + Em down regulated IL-4 ($P<0.05$) and IL-10 ($P<0.05$) when cells were stimulated with BoHV-5, 0.47 and 0.5-fold decrease, respectively (Fig. 5a; 5c). When stimulated also with BoHV-5, spleen cells from animals vaccinated with rgD + Em-D and rgD + PBS sc down regulated the levels of IL-4 ($P<0.001$), with a 0.93 and 0.82-fold decrease, respectively (Fig. 5a)

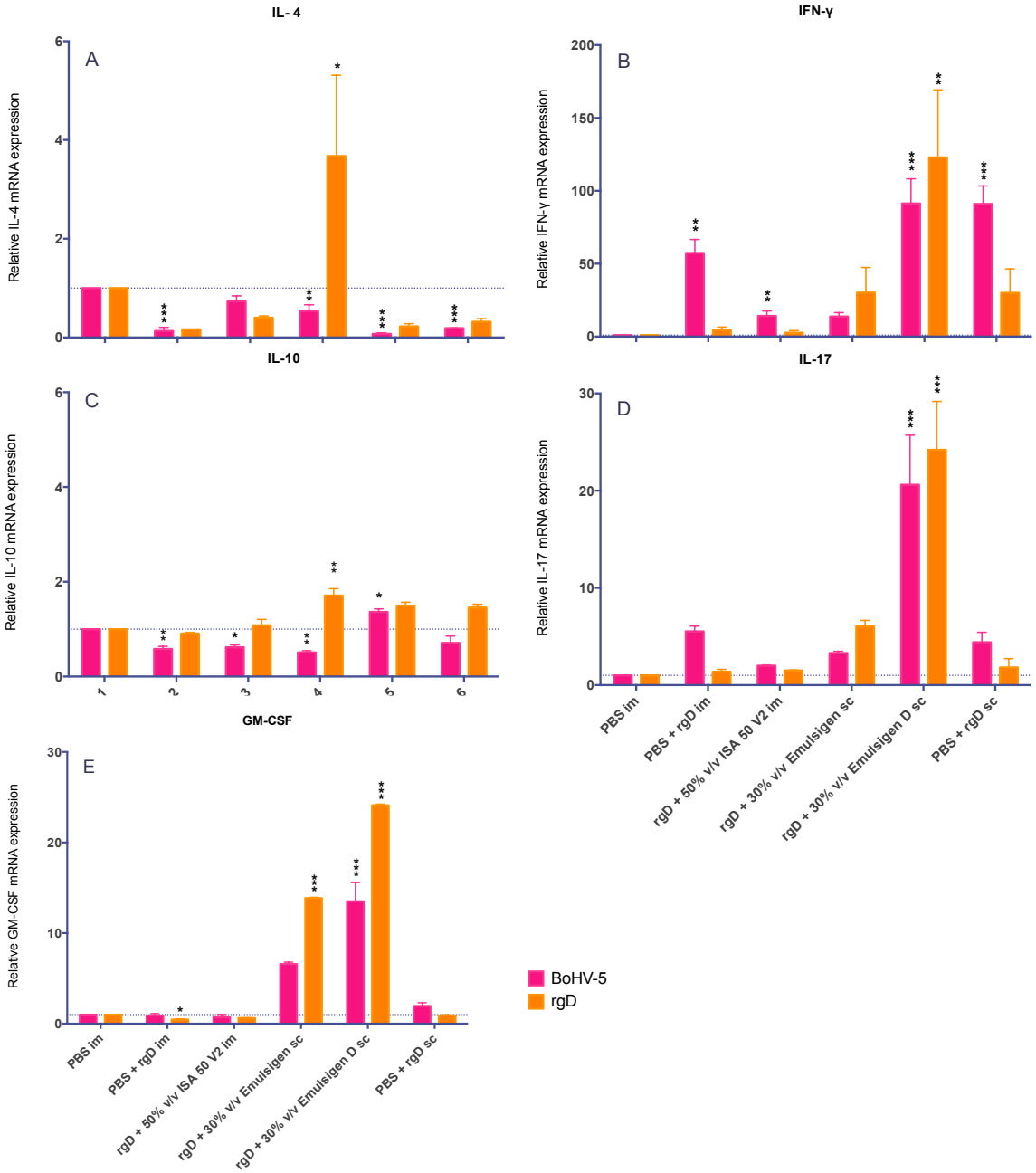

Figure 5 – Cytokines mRNA expression in spleen cells from mice. Two weeks after the third vaccination, cells were stimulated *in vitro* with whole live BoHV-5 and rgD (10 µg) for 18 h. Total RNA was extracted and the correspondent cDNA subjected to qPCR. The fold changes for IL-4 (A); IFN-γ (B); IL-10 (C); IL-17 (D) and GM-CSF (E) were calculated from the threshold cycle (Ct) values normalized to Ct values obtained form non-vaccinated mice spleen cells. Th results were expressed as mean ± S.E.M. *P<0.05; ** P<0.01; *** P<0.001.

4. DISCUSSION

In this study, the ability of the recombinant glycoprotein D from BoHV-5 expressed in *Pichia pastoris* to elicit immune responses was evaluated first time, as a vaccine in mice. The expression of the gD from BoHV-5 has been previously described by our research group, and we demonstrated that the recombinant truncated form of gD is antigenic [19]. However, no vaccine trial was performed at that study, so the capacity of the recombinant gD from BoHV-5 to elicit neutralizing antibodies and the immunomodulation stimulated by this recombinant protein is first described herein.

Pichia pastoris as expression system of heterologous proteins has advantages for production of subunit vaccine antigens, as the growing at large cell density in simple culture medium and at low costs, and the post-translational modification necessary for recombinant glycoprotein expression [24]. The expression of glycoprotein D from other alphaherpesviruses in this expression system has been described. In 1997 and 1999, the BoHV-1 gD expression alone or as a chimeric protein with bovine IL-6 was applied as a subunit vaccine, inducing neutralizing antibodies capable of avoiding BoHV-1 infection of cell cultures [25,26]. Mice immunized with recombinant gD from *Equine herpesvirus 1* (EHV-1) were protected against EHV-1 challenge, although this recombinant protein was hyperglycosylated by *P. pastoris* [27]. High yields of recombinant gD from *Human herpesvirus 1* and *2* (HHV-1 and -2) were expressed with this systems. These recombinant proteins were able to interfere with the HHV-1 and -2 infection process due to its capability to recognize HHV entry mediator [28].

To evaluate the immune responses elicited by rgD, the recombinant protein were delivered to mice in combination of PBS only, intramuscularly or subcutaneously, and in combination with oil adjuvants. The water-in-oil Montanide ISA 50 V2 was administered intramuscularly whereas the oil-in-water Emulsigen or Emulsigen DDA was administered subcutaneously. Indirect ELISA and viral neutralization test evaluated humoral immune responses. Indirect ELISA shown that mice vaccinated with rgD from BoHV-5 expressed in *P. pastoris* stimulated high levels of total IgG and all groups seroconverted after the third vaccination. The rgD also were able to stimulate neutralizing antibodies to BoHV-5, even when administered intramuscularly in a formulation with PBS only, although, as expected, at low levels when compared to the titers stimulated by formulations with adjuvants. These results were consistent with those reported after mice vaccination with recombinant gD from BoHV-1 expressed in *Pichia pastoris* alone or as chimeric protein with bovine IL-6, which were in the same range as neutralization titers engendered in calves by native gD [25,26]. Among adjuvants, no difference was observed in the neutralizing titers.

Cellular immune responses were evaluated by relative quantification of mRNA expression of a panel of five cytokines. Surprisingly, as our results show (Fig. 5), after vaccination, all the animals developed detectable levels of all cytokines, with predominance of IFN-γ over IL-4, suggesting that rgD induced Th1 immune response. The low levels of IL-10 also corroborate these findings, as IL-10 acts as a regulatory cytokine that typically down-regulates the expression of Th1 cytokines [29,30]. Although higher levels of IgG1 were found, IL-4 was not up regulated in all but one group (Fig 5a). As suggests in Figure 4, the immunomodulation after the first vaccine administration seems to be Th2, however, it switches to Th1 after

subsequent vaccinations, suggesting a balanced immune response. The higher levels of IFN-γ in spleen cells stimulated *in vitro* with whole live BoHV-5 after vaccination with rgD suggests that the recombinant gD was able to prime those cells against the virus, which was also observed in animals vaccinated with rgD + Em-D, who had higher stimulation levels of GM-CSF and IL-17 when spleen cells were stimulated with BoHV-5.

No differences between adjuvants were observed in the humoral immune response, when cytokines mRNA levels of *in vitro* stimulated spleen cells were evaluated, the animals immunized with the ISA 50 V2 only up regulated the expression of IFN-γ, however at lower levels when compared to the other adjuvants used. On the other hand, spleen cells of mice immunized with the combination of rgD + Em-D demonstrated the highest mRNA expression levels of IFN-γ, IL-17 and GM-CSF when re-stimulated with both rgD and BoHV-5. IL-17 is a proinflammatory molecule produced by Th17 T-cells and known to stimulate granulocyte macrophage (GM)-CSF, promoting expansion of myeloid lineages [31]. IL-17 also contributes to the efficacy of immune responses induced by vaccine administration. Vaccination with whole-cell pertussis induces IL-17 production, which is involved in cell-mediated immunity by enhancing the bactericidal activity of macrophages [32]. This cytokine has also been important in mice vaccinated with antigens from *Mycobacterium bovis* [33], which demonstrate increased levels of IFN-γ. GM-CSF, on the other hand, has been shown to induce Th1 responses by increasing cellular immunity without having a detrimental effect on the humoral immune responses [34].

Despite the many advantages of subunit vaccines regarding its safety, it still requires powerful adjuvants to induce strong humoral and cellular immune response. Oil emulsion adjuvants cause the innate immune system to elicit the signal required

for the initiation of an adaptive immune response. They act forming a depot effect, by trapping the antigen at the site of administration, increasing the surface area available to the antigen and by attracting various kind of cells, including antigen-presenting cells (APC) and macrophages [35]. This kind of adjuvant is commonly associated with strong Th2 responses, however, the enhancement of Th1 type of response observed in animals immunized with Em-D may be explained by the incorporation of the cationic dimethyldioctadecylammonium bromide (DDA), a non-specific immune activator, since it is an inducer cell-mediated immunity [36,37].

Although T-cell responses are considered to be critical for recovering from BHV-1 infection, humoral immune responses appear to be very important for protection against BHV-1 infection in cattle, with neutralizing antibodies been correlated with protection. Cellular responses in the absence of high antibody titers may not fully protect against infection [38]. Based on this information, our data suggest that rgD is able to elicit neutralizing antibodies and a Th1 type immune response. Our findings demonstrated that the recombinant gD conserved important epitopes for viral neutralization from the native gD from BoHV-5 and animals immunized with rgD were able to respond against the whole live BoHV-5 in *in vitro* restimulation of splenocytes cells. New trials are been conducted in both mice and cattle for further determination of dose and adjuvant to increase the response herein observed.

5. ACKNOWLEDGES

We are grateful to Éverton Fagonde da Silva, Fabiane Chaves de Carvalho, Juliana da Silva Latorre and all Animal Care technicians from Universidade Federal

de Pelotas (RS, Brazil) for provide and care of the animals; and José Carlos Sandrini for technical assistance. We thank Brazilian National Counsel of Technological and Scientific Development (CNPq) for the financial support.

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6 CONCLUSÃO

A partir do resultados obtidos pode-se concluir que:

- foi padronizado um ELISA Indireto utilizando a gD recombinante de BoHV-5;
- a diluição dos soros de bovinos para o ELISA Indireto utilizando a gD recombinante de BoHV-5 ficou estabelecida em 1:400;
- o ELISA Indireto demonstrou correlação com a soroneutralização, apresentando sensibilidade de 100% e especificidade de 92.9%;
- as vacinas de subunidade utilizando a rgD do BoHV-5 induziram a produção de anticorpos neutralizantes;
- a vacinação estimulou uma resposta celular Th1/Th2 balanceada;
- a vacinação com rgD associada a um adjuvante oleoso acrescido de um imunoestimulador estimulou resposta imune celular.

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ANEXO

ANEXO A – DEPÓSITO DE PEDIDO DE PATENTE

INSTITUTO NACIONAL DA PROPRIEDADE INDUSTRIAL
PROTOCOLO GERAL
DIRETORIA DE PATENTES
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< Uso exclusivo do INPI >

Espaço reservado ao protocolo

Espaço para etiqueta

DEPÓSITO DE PEDIDO DE PATENTE OU DE CERTIFICADO DE ADIÇÃO

Ao Instituto Nacional da Propriedade Industrial:
O requerente solicita a concessão de um privilégio na natureza e nas condições abaixo indicadas

1. Depositante (71):

1.1 Nome: Universidade Federal de Pelotas

1.2 Qualificação

1.3 CNPJ/CPF: 92242080/0001-00

1.4 Endereço Completo: Rua Gomes Carneiro N 01

1.5 CEP: 96010-610 1.6 Telefone: (53)32293090 1.7 Fax:

1.8 E-mail: agtpi@hotmail.com

☐ continua em folha anexa

2. Natureza: ◉ Invenção ○ Modelo de Utilidade ○ Certificado de Adição

Escreva, obrigatoriamente, e por extenso, a Natureza desejada: Invenção

3. Título da Invenção ou Modelo de Utilidade ou Certificado de Adição(54):

PROCESSO DE PREPARAÇÃO DE VACINA CONTRA HERPESVÍRUS BOVINO TIPO 5 E VACINA OBTIDA E DE KIT DE DIAGNÓSTICO PARA HERPESVÍRUS

☐ continua em folha anexa

4. Pedido de Divisão: do pedido Nº Data de Depósito:

5. Prioridade: ☐ interna ☐ unionista

O depositante reivindica a(s) seguinte(s):

País ou organização de origem	Número de depósito	Data do depósito

6. Inventor (72):

☐ Assinale aqui se o(s) mesmo(s) requer(em) a não divulgação de seu(s) nome(s)

6.1 Nome: Luana Alves Dummer

6.2 Qualificação Doutorando 6.3 CPF: 010940580-33

6.4 Endereço completo General Osório, 971

6.5 CEP: 96020-000 6.6 Telefone: (53)32259826 6.7 Fax:

6.8 E-Mail: dummer@gmail.com

☒ continua em folha anexa