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Raissa Kuntz Dias

DIVERSIDADE ANTIGÊNICA DE ISOLADOS BRASILEIROS DE PESTIVÍRUS *HOBI-LIKE*

Santa Maria, RS
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Dissertação apresentada ao Programa de Pós-Graduação em Medicina Veterinária, área de concentração em Medicina Veterinária Preventiva da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para obtenção de grau de **Mestre em Medicina Veterinária**.

Orientador: Prof. Rudi Weiblen

Coorientadora: Juliana Felipetto Cargnelutti

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RESUMO

DIVERSIDADE ANTIGÊNICA DE ISOLADOS BRASILEIROS DE PESTIVÍRUS *HOBI-LIKE*

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O vírus da diarreia viral bovina (BVDV) é um dos principais patógenos de bovinos, está distribuído mundialmente e causa grandes perdas econômicas na pecuária bovina. O gênero *Pestivirus*, da família *Flaviviridae*, é composto por quatro espécies virais: os vírus da diarreia viral bovina 1 e 2 (BVDV-1 e BVDV-2), o vírus da doença da fronteira dos ovinos (BDV) e o vírus da peste suína clássica (CSFV). Uma quinta espécie de pestivírus está sendo proposta (pestivírus atípicos bovino, *HoBi-like* ou BVDV-3), devido às suas diferenças genéticas e antigênicas em relação às espécies de BVDV já descritas. Estes vírus foram originalmente identificados em soro fetal bovino de origem brasileira e, posteriormente, isolados de animais infectados em vários países. Este trabalho relata uma caracterização antigênica de oito isolados brasileiros do vírus *HoBi-like*, oriundos de animais infectados persistentemente (PI) e de casos de doença gastroentérica (2007 a 2015). A análise filogenética baseada na região não traduzida 5' (UTR) agrupou esses vírus com outros vírus *HoBi-like* de origem europeia e asiática. Testes de reatividade com anticorpos monoclonais (mAbs) indicaram variabilidade na glicoproteína E2 entre os vírus *Hobi-like* e diferenças significativas das glicoproteínas homólogas do BVDV-1 e BVDV-2. Análise da relação antigênica com base em títulos neutralizantes, utilizando-se antissoros específicos, revelou que os vírus *HoBi-like* são antigenicamente muito diferentes do BVDV-1 e, em menor grau, do BVDV-2. Os ensaios de neutralização cruzada entre pares de vírus *HoBi-like* e seus respectivos antissoros indicaram a existência de variabilidade antigênica entre esses vírus, mesmo para vírus isolados do mesmo rebanho em diferentes ocasiões. Além disso, a identificação de um isolado *HoBi-like* com baixa semelhança antigênica com os outros isolados indica a existência potencial de subgrupos antigênicos entre esses vírus. Finalmente, soro de cordeiros imunizados com vacinas BVDV comerciais mostrou atividade neutralizante baixa ou indetectável contra isolados *HoBi-like*. Estes resultados indicam diferenças antigênicas importantes entre isolados de BVDV e vírus *HoBi-like* brasileiros e a existência de variabilidade antigênica dentro desse grupo de pestivírus. Esses achados ampliam o conhecimento sobre a diversidade antigênica de vírus *HoBi-like* e reforçam a necessidade de sua inclusão em vacinas de BVDV atuais.

Palavras-chave: BVDV-3, doença reprodutiva, soroneutralização, antissoro, anticorpos**.**

ABSTRACT

ANTIGENIC DIVERSITY OF BRAZILIAN ISOLATES OF *HOBI-LIKE* PESTIVIRUSES

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Bovine viral diarrhea virus (BVDV) is a major pathogen of bovine herds and it is distributed globally, causing important economic losses to the cattle industry. The genus Pestivirus of the family Flaviviridae is composed by four viral species: bovine viral diarrhea virus 1 and 2 (BVDV-1 and BVDV-2), border disease virus (BDV) and classical swine fever virus (CSFV). A fifth species has been proposed (atypical pestiviruses, *HoBi-like* pestiviruses or BVDV-3), due to their genetic and antigenic differences in relation to BVDV species. These viruses were originally identified in fetal bovine serum from Brazilian origin and, subsequently, isolated from diseased animals in several countries. Herein we performed an antigenic characterization of eight Brazilian *HoBi-like* viruses isolated from persistently infected (PI) animals and from gastroenteric disease (2007 to 2015). Phylogenetic analysis based on the 5' unstranslated region (UTR) clustered these viruses with other *HoBi-like* viruses from European and Asiatic origin. Monoclonal antibody (mAb) binding indicated variability in the *Hobi-like* virus glycoprotein E2 and significant differences from the homologous BVDV-1 and BVDV-2 glycoprotein. Analysis of antigenic relatedness based on virus-neutralizing titers using virus-specific antisera revealed that *HoBi-like* viruses are antigenically very different from BVDV-1 and, to a lesser extent, from BVDV-2. Cross-neutralizing assays between pairs of *HoBi-like* viruses and their respective antisera indicated the existence of antigenic variability among these viruses, even for viruses isolated from the same herd in different occasions. Moreover, the identification of a *HoBi-like* isolate with low antigenic similarity with the other isolates indicates the potential existence of antigenic subgroups among *HoBi-like* virus isolates. Finally, sera of lambs immunized with commercial BVDV vaccines showed low or undetectable neutralizing activity against *HoBi-like* isolates. These results indicate significant antigenic differences between BVDV genotypes and Brazilian *HoBi-like* viruses and the existence of antigenic variability within this atypical group of pestiviruses. These findings extend the knowledge about the antigenic diversity of *HoBi-like* viruses and reinforce the need for their inclusion in current BVDV vaccines.

Key words: BVDV-3, reproductive disease, seroneutralization, antiserum, antibodies.

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1. INTRODUÇÃO

O Brasil é um dos maiores produtores de carne bovina e leite do mundo e possui um rebanho bovino de aproximadamente 218 milhões de cabeças. Apenas no primeiro trimestre de 2017 o abate chegou a 7,37 milhões de cabeças, cujas exportações injetaram mais de 1,104 milhões de dólares na economia do país. No mesmo período, a indústria processou 5,87 bilhões de litros de leite (INSTITUTO BRASILEIRO DE GEOGRAFIA E ESTATÍSTICA, 2017). Por estas razões, a produção pecuária consiste em uma atividade que desempenha importante papel econômico e social para o país. Diante disso, é primordial estabelecer programas de sanidade eficientes, a fim de prevenir ou controlar enfermidades que possam afetar a produtividade dos animais.

O vírus da diarreia viral bovina (*bovine viral diarrhea virus*, BVDV) está disseminado mundialmente e é considerado o patógeno viral mais importante em bovinos, após o vírus da febre aftosa, causando grandes perdas econômicas na cadeia pecuária. Este vírus causa falhas reprodutivas e uma ampla gama de manifestações clínicas (BAKER, 1995), que implicam na redução da produção, além dos custos na implementação de medidas de controle. Nos Estados Unidos, estima-se que os prejuízos em rebanhos leiteiros devido à infecção por estes vírus girem em torno de 40 a 60 dólares por animal (CHI et al., 2002). Diversos fatores podem alterar o somatório das perdas causadas pela introdução do BVDV em um rebanho. Dentre eles, o estado imunológico dos animais é um dos mais importantes, tendo em vista que um surto em um rebanho completamente susceptível pode causar perdas elevadas em curto prazo, enquanto a circulação contínua do vírus, que resulta em um rebanho com maior nível de imunidade, muitas vezes está associada a perdas moderadas (FOURICHON et al., 2005).

O BVDV foi dividido em duas espécies virais, devido às diferenças genéticas e antigênicas: BVDV-1 e BVDV-2. Esses vírus pertencem à família *Flaviviridae*, gênero *Pestivirus*, que abriga ainda o vírus da peste suína clássica (*classical swine fewer virus*, CFSV) e o vírus da doença da fronteira dos ovinos (*border disease virus*, BDV) (SIMMONDS et al., 2017). Outros aguardam classificação oficial nesse gênero, oriundos de bovinos e de outras espécies: os pestivírus de girafas (AVALOS-RAMIREZ et al., 2001), de antílopes *Pronghorn* (VILCEK et al., 2005), *Bungowanah* dos suínos (KIRKLAND et al., 2007), pestivírus atípico suíno (HAUSE et al., 2015) e os pestivírus atípicos de origem bovina, também chamados vírus *HoBi-like* ou BVDV-3 (SCHIRRMEIER et al., 2004; LIU et al., 2009a; BAUERMANN et al., 2013b).

Os membros do gênero *Pestivirus* possuem capsídeo icosaédrico composto por múltiplas cópias da proteína C, revestido por um envelope lipoprotéico, no qual se inserem as proteínas E^{rns}, E1 e E2, e medem de 40 a 60nm de diâmetro. O genoma viral é constituído de uma molécula de RNA de fita simples, não segmentado, de polaridade positiva, de aproximadamente 12.3 kilobases (kb), cuja única fase aberta de leitura (*open reading frame*, ORF) é flanqueada por duas regiões não traduzidas (*untranslated regions*, UTR's) próximas às extremidades 5' e 3' (LINDENBACH et al., 2013).

A ligação inicial das proteínas do envelope viral, especialmente da E2, com os receptores celulares CD46, medeia a penetração da partícula vírica pela via endocítica. Com a acidificação do "endossomo", o genoma é liberado no citoplasma possibilitando a ocorrência das próximas etapas do ciclo replicativo (LINDENBACH et al., 2013). O RNA viral é reconhecido pelos ribossomos por meio de um sítio interno de ligação (*internal ribossome entry site*, IRES) na região 5'. Uma longa poliproteína de aproximadamente 4.000 aminoácidos é codificada e clivada durante e após a tradução em 11-12 proteínas maduras, ordenadas da seguinte forma: autoprotease ($N^{pro)}$, proteína do capsídeo (C), proteínas do envelope (E^{rns}, E1 e E2), e proteínas não estruturais (p7, NS2/NS3, NS4A, NS4B, NS5A e NS5B) (MEYERS; THIEL, 1996; RIDPATH, 2005). Durante a replicação do material genético ocorre a síntese de um RNA de sentido antigenômico pela formação do complexo de replicação com a participação de estruturas primárias e secundárias localizadas na extremidade 3' e das proteínas não estruturais sintetizadas (GU et al., 2000; LINDENBACH et al., 2013; NEILL et al., 2013).O RNA negativo serve de molde para a síntese de novas cópias do RNA viral de sentido genômico, que são então encapsidadas formando novas partículas, ou traduzidas em proteínas (GU et al., 2000; NEILL et al., 2013). A associação do genoma de sentido positivo com cópias da proteína C dá início à morfogênese. As partículas recém-formadas brotam para o interior do complexo de Golgi e do retículo endoplasmático; são transportadas, maturadas e fusionam com a membrana plasmática, na qual adquirem o envelope e são liberadas da célula hospedeira por exocitose (LINDENBACH et al., 2013).

Em 1946, o relato de uma doença até então desconhecida ocorreu em um pequeno rebanho bovino de Ithaca, New York, Estados Unidos. Neste episódio os animais apresentaram febre, desidratação, anorexia, diarreia, leucopenia, hemorragia em diversos órgãos, lesões erosivas no trato gastrointestinal e as fêmeas prenhes, especialmente as novilhas abortaram (OLAFSSON et al., 1946). Contudo, o vírus só foi identificado em 1954 (BAKER et al., 1954). Embora os sinais gastroentéricos não sejam os mais frequentes na

infecção pelo BVDV, em virtude da primeira descrição, a doença recebeu o nome de diarreia viral bovina (*bovine viral diahrrea*, BVD) (BAKER, 1995).

Durante a década de 90, surtos de diarreia hemorrágica grave foram relatados na América do Norte. Estudos filogenéticos utilizando isolados obtidos naquela década verificaram que eles possuíam características divergentes daqueles conhecidos até o momento, e foram identificados como BVDV-2 (RIDPATH et al., 1994). Embora hoje os vírus da BVD sejam divididos em duas espécies, inicialmente foram classificados em genótipos, com base em análises filogenéticas da região 5'UTR do genoma, e fenotípicas utilizando-se anticorpos monoclonais (*monoclonal antibodies*, mAb) contra a glicoproteína E2 (PELLERIN et al., 1994). Estes são critérios ainda utilizados para a caracterização genética e antigênica de isolados, o que permitiu a diferenciação de 21 subtipos de BVDV-1 e 3 subtipos de BVDV-2, tendo como base a análise filogenética da 5'UTR e N^{pro} (DENG et al., 2015).

Em 2004, o primeiro pestivírus atípico de origem bovina foi isolado na Alemanha, a partir de um lote de soro fetal bovino (SFB) importado do Brasil e recebeu a denominação D32/00 – HoBi (SCHIRRMEIER et al., 2004). Análises filogenéticas demonstraram que se tratava de um pestivírus diferente daqueles conhecidos, indicando a emergência de uma nova espécie viral. Desde então diversos vírus semelhantes foram identificados em lotes de SFB oriundos de diferentes países (STALDER et al., 2005; STÂHL et al., 2007; LIU et al., 2009b; PELETTO et al., 2012). Estes vírus também estão implicados em diferentes manifestações clínicas pela infecção natural em rebanhos do Brasil (CORTEZ et al., 2006), Itália (DECARO et al., 2011) e Sudeste Asiático (KAMPA et al., 2009).

A região codificadora que compreende a protease N^{pro} geralmente é utilizada para a caracterização inicial de isolados de pestivírus por meios moleculares, uma vez que esta proteína é exclusiva deste gênero. Por meio de análise genética da região 5' UTR, que é altamente conservada no gênero, os isolados são alocados nas espécies virais e respectivos tipos e subtipos (PELLERIN et al., 1994). Um trabalho utilizando oitenta e nove isolados de pestivírus coletados no Brasil entre 1995 e 2014 baseou-se em uma comparação de sequências de genes da 5'UTR, N^{pro} e E2. Destes isolados, 53,9% das sequências pertencem ao BVDV-1, 33,7% ao BVDV-2 e 12,4% aos vírus *HoBi-like*, sendo BVDV-2c e BVDV-1e detectados pela primeira vez no Brasil (SILVEIRA et al, 2017). A homologia da sequência de nucleotídeos entre pestivírus atípicos é superior a 94%, entretanto quando comparadas com sequências de isolados das espécies de BVDV-1/2 a homologia é de aproximadamente 80% (SCHIRRMEIER et al., 2004). Acredita-se que os pestivírus evoluíram a partir de um

ancestral comum e que a diversificação genética destes vírus seja resultante do acúmulo de mutações no genoma ao longo do processo de replicação viral, por erros cometidos pela RNA polimerase e da recombinação com outros RNAs homólogos ou heterólogos no interior de células infectadas (NAGAI et al., 2004).

Em contrapartida, a glicoproteína E2 é altamente variável dentro do gênero *Pestivirus*, uma vez que ela possui os principais epítopos antigênicos (ASFOR et al., 2014), e por isso está em constante pressão de seleção por anticorpos neutralizantes de animais infectados ou vacinados. Estudos demonstram que a similaridade de nucleotídeos no gene desta proteína entre os isolados de pestivírus atípicos HoBi_D32/00, SVA/cont-08, e CH-KaHo é superior a 92%, mas quando comparadas com sequências das cepas NADL (BVDV-1) e NY93 (BVDV-2) a homologia é inferior a 65% (LIU et al., 2009a; LIU et al., 2009b). Embora os pestivírus sejam antigenicamente relacionados entre si, esta variabilidade genética resulta em grandes diferenças antigênicas entre as espécies de BVDV-1, BVDV-2 e vírus *HoBi-like*, repercutindo em reação sorológica cruzada em níveis variáveis entre estas espécies virais (STÅHL et al., 2007; DECARO et al., 2013). Um antissoro produzido contra cepas de BVDV-1 e BVDV-2 apresenta a capacidade em neutralizar o isolado HoBi_D32/00 reduzida em 100 vezes quando comparada à neutralização com antissoro específico (SCHIRRMEIER et al., 2004). Essas variações antigênicas também podem ocorrer dentro de uma mesma espécie viral, uma vez que os isolados de diferentes subtipos de BVDV-1, quando testados frente a um soro hiperimune de ovino, apresentam taxas de similaridade antigênica (R) variando entre 1,1 a 50 (ALPAY; YESILBAG, 2015).

Os anticorpos produzidos contra a E2 são importantes por conferirem proteção (JELSMA et al., 2013), e frequentemente o nível de resposta de anticorpos é utilizado para medir a eficiência das vacinas (HOUE, 1995; RIDPATH et al., 2003). As grandes diferenças antigênicas entre espécies e subtipos de *Pestivirus* geram uma preocupação quanto à eficácia das vacinas comerciais disponíveis, uma vez que a maioria delas possui cepas europeias e americanas em sua composição, que são antigenicamente diferentes das que circulam no Brasil (BOTTON et al., 1998) e nenhuma vacina possui cepas de vírus *HoBi-like*. Animais imunizados com vacina contendo apenas cepas de BVDV-1 apresentam baixos títulos de anticorpos contra o BVDV-2 e *HoBi-like* (RIDPATH et al., 2000; DECARO et al., 2013). Em animais imunizados com vacinas inativadas ou vivas atenuadas contendo cepas de BVDV-1 e BVDV-2, a divergência antigênica do vírus *HoBi-like* com BVDV-1 e BVDV-2 é maior do que a observada entre BVDV-1 e BVDV-2 (BAUERMANN et al., 2013a).

A variabilidade da E2 também pode ser evidenciada pela reatividade dos isolados frente a um painel de anticorpos monoclonais (mAbs) específicos para a proteína. . Pelo uso desta técnica é possível verificar se há epitopos compartilhados entre os diferentes isolados, tanto na E2 quanto em outras proteínas, como a E^{rns} (E0) e a NS2-3, por exemplo. O uso de mAbs que reconheçam determinantes antigênicos variáveis é útil para caracterização e diferenciação dos isolados, enquanto o emprego de mAbs que reajam com um grande número de isolados tem importância para o diagnóstico (CORAPI et al. 1990, EDWARDS; PATON, 1995). Desta forma, o uso de cepas norte-americanas e europeias nas técnicas de diagnóstico sorológico, como a soroneutralização, torna-se questionável pois estas, por serem antigenicamente divergentes dos vírus brasileiros, podem não detectar baixos níveis de anticorpos contra isolados locais (BOTTON et al., 1998). A diferenciação entre espécies de BVDV e do vírus *HoBi-like* por um único mAb ou uma combinação de mAbs seria ideal para a padronização de técnicas diagnóstico (BAUERMANN et al., 2012; 2013). Entretanto, a diferenciação destes vírus pode exigir o uso de vários mAbs combinados (BAUERMANN et al., 2012).

O objetivo do presente trabalho foi caracterizar antigenicamente, por meio de reatividade com mAbs e neutralização cruzada, isolados brasileiros de vírus *HoBi-like.* Os testes realizados demonstraram que esses isolados são antigenicamente diferentes das espécies de BVDV, especialmente de BVDV-1, e que apresentam variabilidade antigênica entre si. Este estudo será apresentado sob a forma de artigo científico, a seguir.

2. CAPÍTULO 1

ANTIGENIC DIVERSITY OF BRAZILIAN ISOLATES OF *HOBI-LIKE* PESTIVIRUSES

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Antigenic diversity of Brazilian isolates of *HoBi-like* pestiviruses

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Abstract

Hobi-like viruses comprise an unclassified group of bovine pestiviruses related to bovine viral diarrhea virus 1 (BVDV-1) and 2 (BVDV-2). These viruses were originally identified in fetal bovine serum from Brazilian origin and, subsequently, isolated from diseased animals in several countries. Herein we performed an antigenic characterization of eight Brazilian *HoBi-like* viruses isolated from persistently infected (PI) animals and from gastroenteric disease (2007 to 2015). Phylogenetic analysis based on the 5' unstranslated region (UTR) clustered these viruses with other *HoBi-like* viruses from European and Asiatic origin. Monoclonal antibody (mAb) binding indicated variability in the *Hobi-like* virus glycoprotein E2 and significant differences from the homologous BVDV-1 and BVDV-2 glycoprotein. Analysis of antigenic relatedness based on virus-neutralizing titers using virus-specific antisera revealed that *HoBi-like* viruses are antigenically very different from BVDV-1 and, to a lesser extent, from BVDV-2. Cross-neutralizing assays between pairs of *HoBi-like* viruses and their respective antisera indicated the existence of antigenic variability among these viruses, even for viruses isolated from the same herd in different occasions. Moreover, the identification of a *HoBi-like* isolate with low antigenic similarity with the other isolates indicates the potential existence of antigenic subgroups among *HoBi-like* virus isolates. Finally, sera of lambs immunized with commercial BVDV vaccines showed low or undetectable neutralizing activity against *HoBi-like* isolates. These results indicate significant antigenic differences between BVDV genotypes and Brazilian *HoBi-like* viruses and the existence of antigenic variability within this atypical group of pestiviruses. These findings extend the knowledge about the antigenic diversity of *HoBi-like* viruses and reinforce the need for their inclusion in current BVDV vaccines.

Key words: BVDV, pestivirus, antigenic diversity, diagnosis, vaccine.

1. Introduction

The genus *Pestivirus*, family *Flaviviridae*, includes four recognized viral species, namely *Bovine viral diarrhea virus* 1 (BVDV-1) and 2 (BVDV-2), *Border disease virus* (BDV) and *Classical swine fever virus* (CSFV) (Simmonds et al., 2012). BVDV-1 and BVDV-2 are distributed worldwide and have been associated with a variety of clinical manifestations including reproductive failure (Baker, 1995). Five other putative pestivirus species with veterinary relevance await for definitive classification, including Giraffe pestivirus (Avalos-Ramirez et al., 2001), Pronghorn pestivirus (Vilcek et al., 2005), Bungowannah virus (Kirkland et al., 2007), Atypical porcine pestivirus and *HoBi-like* virus (Liu et al., 2009a; Bauermann et al., 2013; Hause et al., 2015). The *HoBi-like* viruses, also referred to as atypical bovine pestiviruses or BVDV-3 are recognized as bovine pathogens that cause clinical presentations similar to those observed following BVDV-1 or BVDV-2 infections (Liu et al., 2009b; Bauermann et al., 2013).

The pestivirus genome is a single-stranded, positive sense RNA molecule of 12.3 kb in length. The viral genome contains a long open reading frame (ORF) flanked by two untranslated regions (5' and 3' UTRs, respectively). The ORF is translated into a polyprotein of about 3,988 amino acids, which is processed into 11-12 structural and non-structural proteins (Donis, 1995). Classification of pestiviruses into species and subtypes is based mainly on the nucleotide identity of the highly conserved 5' UTR (Vilcek et al., 2001). Additionally, the region encoding the non-structural proteins N^{pro} (Becher et al., 1997) and NS3 (Ridpath et al., 1994; Pellerin et al., 1994) and the envelope glycoprotein E2 (van Rijn et al., 1997) have also been used for genetic analysis. In addition to genetic criteria, antigenic similarity detected by monoclonal antibody (mAb) binding and cross-neutralization assays

with homologous and heterologous antisera are important criteria for classification of pestiviruses (Paton et al., 1995; Becher et al., 2003).

The first reported isolate of *Hobi-like* virus, D32/00, was detected by a Germany laboratory in a batch of fetal bovine serum (FBS) imported from Brazil (Schirrmeier et al., 2004). Subsequently, genetically similar viruses (and thereafter called *HoBi-like viruses*) were detected in other lots of FBS collected over several decades (Stalder et al., 2005; Liu et al., 2009b; Stahl et al., 2010; Peletto et al., 2012; Giammarioli et al., 2015; Xia et al., 2013). *Hobi-like* viruses have also been associated with a variety of clinical manifestations in cattle in several countries, including Brazil (Cortez et al., 2006; Bianchi et al., 2011; Weber et al., 2016), Italy (Decaro et al., 2011), Thailand (Kampa et al., 2009; Liu et al., 2009a), India (Mishra et al., 2014), and Bangladesh (Haider et al., 2014). A report indicates the involvement of *HoBi-like* viruses in respiratory diseases in small ruminants from China (Shi et al., 2016).

Atypical pestiviruses, subsequently characterized as *HoBi-like*, have been identified in many Brazilian regions. These viruses have been associated with a variety of clinical manifestations, most resembling those classically associated with BVDV infection, including reproductive disorders and respiratory disease (Cortez et al., 2006; Bianchi et al., 2011), and mucosal-like disease (Weber et al., 2016). In addition, a significant part of Brazilian FBS batches are contaminated with pestiviruses, including *HoBi-like* (Xia et al., 2011; Monteiro et al., submitted). These data reinforce that *HoBi-like* viruses are endemic among Brazilian cattle.

The *HoBi-like* viruses identified in Italy are similar to Brazilian isolates, which has led to the suggestion that they were introduced into Europe via contaminated FBS originated in South America (Stahl et al., 2010). The identification of these viruses in several remote regions without apparent epidemiological links and their genetic divergence, however, suggest that *HoBi-like* pestiviruses may not be recently emerged viruses. Rather, they might

have existed long enough to evolve independently (Haider et al, 2014; Bauermann et al 2015; Mishra et al., 2014).

HoBi-like viruses are genetically and antigenically related to BVDV-1 and BVDV-2, yet marked antigenic differences have been demonstrated between these groups of viruses (Schirrmeier et al., 2004; Bauermann et al., 2012; Larska et al., 2012). As a consequence, many BVDV immunodiagnostic and molecular assays may fail to detect *HoBi-like* viruses (Bauermann et al., 2012). Moreover, the low serological cross-reactivity between BVDV species and *HoBi-like* viruses represents a major concern in regards to vaccine protection (Bauermann et al., 2012; 2013; Decaro et al., 2013). To date, no commercial vaccine includes *HoBi-like*.

The objective of the present study was to characterize antigenically eight Brazilian isolates of *HoBi-like* viruses. The isolates were analyzed antigenically based on reactivity with BVDV-1 and BVDV-2 mAbs and by cross-neutralization assays using homologous and heterologous antisera.

2. Material and Methods

2.1. Viruses and cells

The origin of the Brazilian *HoBi-like* viruses characterized in this study is shown in Table 1. Viruses LV01/12, LV02/12, LV03/12, LV04/12, LV/PB22487/12 and LPV-WR_BR11 were isolated at the Veterinary Virology Laboratory (UFRGS). Viruses SV478/07 and SV757/15 were isolated at the Virology Section, Federal University of Santa Maria (SV/UFSM). Singer (BVDV-1a) and 890 (BVDV-2a) are reference strains. Viruses were amplified and quantitated in pestivirus-free Madin Darby bovine kidney cells (MDBK, ATCC – CCL22). Cells were maintained in MEM (minimum essential medium, ThermoFisher

Scientific, Massachusetts, USA), supplemented with 10% equine serum, 100 U/mL of penicillin and 100 µg/mL of streptomycin (Invitrogen, USA). Virus stocks were titrated by limiting dilution in MDBK cells grown in 96-well microtiter plates. Virus replication in indicator cells was revealed by indirect immunofluorescence using a pool of monoclonal antibodies to BVDV (Corapi et al., 1990) as primary antibody and a FITC-conjugated anti-mouse IgG antibody as secondary antibody. Virus titers were calculated according to Reed & Muench (1938) and expressed as $\log_{10}$ median tissue culture infective dose ($TCID_{50}$/mL).

2.2. Generation and phylogenetic analysis from *HoBi-like* viruses

The isolates LV01/12, LV02/12, LV03/12, LV04/12 and LV/PB22487/12 were previously identified as *HoBi-like* viruses based on phylogenetic analysis of the 5'UTR, N^{pro} and E2 coding regions (Silveira et al., 2015). In the present study, the isolates SV478/07 and SV757/15 and LPV-WR_BR11 were genetically characterized. Amplification of a 282 nt sequence of the 5' UTR of the pestivirus genome was performed by a RT-PCR described by Ridpath & Bolin (1998), using total RNA isolated from MDBK cells infected with each isolate. The cDNA synthesis was performed using the SuperScript® III First-Strand Synthesis kit (Thermo Fisher Scientific, Massachusetts, USA). Amplicons were purified using PureLink® Quick Gel Extraction and PCR Purification Combo Kit (Thermo Fisher Scientific, Massachusetts, USA) and sequenced in both directions in an automatic sequencer ABI-PRISM 3100 Genetic Analyzes (Applied Biosystems, Foster City, CA). Consensus sequences were generated using the Staden program (Staden, 1996) and alignment of sequences was performed using BioEdit 7.0.5.3 (http://www.mbio.ncsu.edu/bioedit/bioedit.html). Phylogenetic analysis was conducted in the Molecular Evolutionary Genetics Analysis (MEGA) software 5.0 (Tamura et al., 2011), using the Neighbor-joining method, and the

evolutionary distances were computed using the *p-distance* method. The bootstrap values were calculated using 1,000 replicates.

2.3. Monoclonal antibodies (mAbs) and binding assays

Twenty-seven mAbs raised against BVDV-1 and BVDV-2 strains and reacting with different viral proteins were used to examine the antigenic variability of the *HoBi-like* isolates. These included mAbs against BVDV-1 proteins E2, E^{rns} and NS3 (Corapi et al., 1990); mAbs against BVDV-1 and BVDV-2 E2 (Ridpath et al., 1994) and two mAbs produced in our laboratory against a Brazilian BVDV-1, shown to react with E1 and E2 (Kreutz et al., 2000). For mAb-binding assays, MDBK cells grown in six-well plates were inoculated with each isolate at a multiplicity of infection (m.o.i.) of approximately 0.5-1.0 and 24 h-48 h post-infection were dissociated by trypsin, suspended in culture medium and allowed to attach to multispot glass slides. Cells were fixed in cold acetone for 5 min, air-dried and incubated with each individual mAb for 1 h at 37°C. The mAbs preparation used were either supernatant of hybridoma cultures (pure or diluted 1:10 in MEM) or mouse ascitic fluid (diluted 1:100 to 1:1000, according to each mAb). Incubation with primary antibodies was followed by washing in PBS, distilled water and incubation with an anti-mouse FITC conjugated antibody (1:100, Sigma-Aldrich, USA). Slides were mounted and examined under UV light in an epifluorescence microscope. Cells infected with BVDV-1 Singer, BVDV-2 890 and mock-infected MDBK cells were used as controls.

2.4. Antisera, virus neutralizing (VN) assays and calculation of antigenic relatedness

For virus-neutralizing assays, virus-specific antisera were produced in sheep. The experiments were approved by an Institutional Committee on Ethics and Animal Welfare and Experimentation (UFSM, Comitê de Ética e Experimentação Animal: process 006/2015). Two seronegative lambs were inoculated intramuscularly with $10^{6.0}$ $TCID_{50}$ of each isolate/strain. Immunized animals were maintained in separated barns to avoid cross-contamination. Sera for VN assays were collected at 28-30 day post-inoculation. For the evaluation of the neutralizing activity against *HoBi-like* viruses induced by commercial BVDV vaccines, seronegative lambs were vaccinated twice, with a 30 days-interval, with each of six commercial BVDV vaccines. Vaccines 1, 2 and 3 contain both BVDV-1 and 2; vaccines 4, 5 and 6 contain only BVDV-1 strains in their formulations. The respective viral strains present in the vaccine formulations were not informed by the manufacturers. Two lambs were immunized with each vaccine. Sera for VN assays were collected approximately 30 days after the second vaccine dose. All vaccines were inactivated, adjuvanted vaccines containing either BVDV-1 alone or in combination with BVDV-2 (Anziliero et al., 2015).

Virus-neutralizing (VN) assays were performed in quadruplicates in 96-well plates, by incubating two-fold dilutions (starting at 1:5) of each serum with approximately 100-200 $TCID_{50}$ of the respective virus for 1 h at 37°C in a 5% CO_2 incubator, followed by addition of a suspension of MDBK cells and incubation for 96 h. Readings were performed by fluorescent antibody assay (FA). The titer were expressed as the reciprocal of the highest serum dilution that prevented virus replication in 50% of the wells.

The titers obtained for each pair of virus x antiserum in VN assays were used to calculate the coefficient of antigenic similarity *R* for each pair of viruses, according to the formula proposed by Archetti & Horsfall (1950): $R = 100 \times \sqrt{(\text{titer strain A with antiserum B} \times$

titer strain B with antiserum A)/ (titer strain A with antiserum A) x (titer strain B with antiserum B). According to this calculation, *R* values of 25 indicate 4-fold differences in VN titers of homologous and heterologous antisera.

3. Results

3.1. Genetic relationships among Brazilian *HoBi-like* isolates

The isolates LV01/12, LV02/12, LV03/12, LV04/12 and LV/PB22487/12 were previously identified as *HoBi-like* viruses based on phylogenetic analysis of the 5'UTR, N^{pro} and E2 coding regions (Silveira et al., 2015). In this study, 282 nt from the 5'UTR of the isolates SV478/07 and SV757/15 and LPV-WR_BR11 were amplified by PCR and sequenced. Subsequent phylogenetic analysis showed that these isolates cluster together with the other Brazilian *HoBi-like* isolates previously sequenced (Figure 1). The nucleotide sequence identity among the Brazilian *HoBi-like* isolates ranged from 97.6 to 100%. Overall, the 5'UTR identity between Brazilian *HoBi-like* isolates and BVDV-1 (Singer, NADL, Oregon and Osloss) ranged from 76.4 to 79%; and with BVDV-2 (890, Giessen 6, New York 93, Soldan and VM96) from 83 to 85.6%.

3.2. Variability of *HoBi-like* virus glycoprotein E2 detected by mAb binding

The results of the binding assays of BVDV-1 and BVDV-2 mAbs against the *HoBi-like* virus isolates are presented in Figure 2. MAbs to BVDV proteins E^{rns} (15c5), E1 (6f11) and NS3 (20.10.6) showed a broad range of recognition, binding to BVDV-1, BVDV-2 and to all *HoBi-like* isolates. Most BVDV-1 and BVDV-2 E2 mAbs failed to recognize the majority of *Hobi-like* virus isolates. The exceptions were mAb 3.1c4 (BVDV-1) which recognized all *HoBi-like* isolates/strains, mAb CA-36 (BVDV-1) which failed to react with one *HoBi-like*

isolate (SV478/07) out of seven, and mAb BZ72 (BVDV-2) which failed to recognize two *HoBi-like* isolates (LV02/12 and SV478/07). These results suggest a high conservation in E^{rns} and NS3 epitopes and a high variability among E2 mAb epitopes. While most E2 epitopes were more variable, the binding of mAb 3.1c4 to all *HoBi-like* isolates indicate the existence of conserved epitopes in E2 of pestiviruses.

3.3. Antigenic variability of Brazilian *HoBi-like* isolates detected by virus-neutralizing assays

The neutralizing activity of each of the 10 antisera was determined against each isolate/strain (Table 2). BVDV-1 Singer was poorly neutralized by *HoBi-like* antisera (GMT = 13.75 [5 to 40]) and *HoBi-like* isolates were neutralized by BVDV-1 antiserum in low titers (GMT=33.7 [10 to 80]). *HoBi-like* antisera neutralized BVDV-2 890 in lower titers than the homologous viruses (GMT= 135 [40 to 160]) and the neutralizing activity of BVDV-2 antiserum against *HoBi-like* isolates was also lower than the homologous neutralization (GMT = 115 [40 to 160]).

The antigenic relatedness for each pair of viruses was calculated based on homologous and heterologous VN titers and expressed as the coefficient of antigenic similarity (*R*) (Archetti & Horsfall, 1950) (Table 3). According to this calculation, *R* values of ≥25 indicate ≤4-fold differences in VN titers of homologous and heterologous antisera. Hence, *R* values of ≤25 are indicative of significant antigenic differences between the viruses of each pair, since they reflect ≥4-fold differences in titers of homologous and heterologous antisera.

The coefficient of antigenic similarity *R* of BVDV-1 Singer with all *HoBi-like* isolates was very low (average = 2.15 [1.1 – 4.4]). Seven out of eight *R* values between BVDV-1 and *HoBi-like* isolates correspond to ≥32 differences in homologous versus heterologous VN titers. Moreover, the lowest *R* value calculated for a pair of BVDV-1 and *HoBi-like* (1.1)

represents a > 64-fold difference in VN titers. The *R* values for BVDV-2 890 and *HoBi-like* viruses were variable, yet they were generally higher than those for BVDV-1 and *HoBi-like* viruses (average 16.6 [6.3 to 25]). Nonetheless, all *R* values for pairs of BVDV-2 x HoBi viruses were ≤25 and two pairs presented *R* values below 10 (6.3 and 8.8). The *R* values for most pairs of *HoBi-like* viruses were generally moderate to high (35.4 to 100), reflecting minor antigenic differences. An exception was isolate SV478/07, which yielded very low *R* values with all other *HoBi-like* isolates. Taken together, these results indicate that Brazilian *HoBi-like* isolates are antigenically more distant to BVDV-1 than to BVDV-2 and suggest the existence of antigenic variability among *HoBi-like* virus isolates.

3.4. Sera of sheep immunized with commercial BVDV vaccines present low or undetectable neutralizing activity against Brazilian *HoBi-like* viruses

The VN titers against *HoBi-like* viruses in the sera of lambs vaccinated with commercial BVDV vaccines are shown in Figure 3. Each group/vaccine was composed by two lambs whose sera collected at day 30 after the second vaccine dose was mixed 1:1 and submitted to VN assays against BVDV and *HoBi-like* isolates. In general, the VN titers against *HoBi-like* isolates were much lower than to BVDV-1 and BVDV-2 – and even undetectable – in most groups. The low – and many times undetectable – VN activity was more pronounced in vaccines containing only BVDV-1 strains.

4. Discussion

The results presented herein extend previous observations demonstrating that *HoBi-like* pestiviruses present important antigenic differences from both BVDV-1 and BVDV-2. Previous studies have already demonstrated marked antigenic differences between BVDV

genotypes and the prototype *HoBi-like* virus, D32/00_HoBi strain (Schirrmeier et al., 2004; Bauermann et al., 2012). Our results extend these observations for a number of Brazilian *HoBi-like* isolates. The main differences were evidenced by mAbs directed to E2, the major and most variable pestivirus glycoprotein, and a major target for neutralizing antibodies (Ridpath et al., 1994; Donis, 1995). The lack of recognition of *HoBi-like* viruses by most BVDV-1 and BVDV-2 E2 mAbs reflects the low sequence similarity between the E2 of BVDV species and *HoBi-like* viruses (Silveira et al., 2015). In addition, differential binding by some E2 mAbs on *HoBi-like* isolates indicates the existence of variability of E2 among Brazilian *HoBi-like* isolates. This variability has been already suggested by partial nucleotide sequencing of E2 of some *HoBi-like* isolates (Silveira et al., 2015). Our sequencing data showed that the amino acid identity among the studied *HoBi-like* viruses ranged from 90.9 to 98.7% in the analyzed segment (nucleotide position 3,001 to 3,225; data not shown). Interestingly, even viruses isolated from PI animals in the same herd a few months apart (LV01-04/12) presented some variation in the recognition by E2 mAbs.

The mAb binding assays also allowed for the identification of shared epitopes, mainly in the conserved pestivirus proteins NS3 (mAb 20.10.6) and E^{rns} (15c5) but in the glycoprotein E2, as well (mAb CA-36). These findings are important towards choosing mAbs for use in immunodiagnostic assays, as they recognize a wide range of pestiviruses, including D32/00_HoBi (Bauermann et al., 2012). In particular, two mAbs (6f11 and 3.1c4) produced in our laboratory to a Brazilian BVDV-1 isolate (Kreutz et al., 2000) showed a broad range of reactivity over BVDV-1 and BVDV-2 isolates, including all *HoBi-like* isolates. The differentiation of BVDV species from *HoBi-like* viruses by a single mAb or a combination of mAbs would be helpful for diagnosis and control strategies (Bauermann et al., 2012; 2013). From our panel, no single mAb was able to differentiate between BVDV species and *HoBi-like* viruses. These data confirm previous findings that differentiation of this group of viruses

from BVDV-1 and BVDV-2 may require the use of several mAbs combined (Bauermann et al., 2012).

The antigenic differences indicated by mAb binding (Figure 2) and E2 sequencing (Silveira et al., 2015) reflected in low cross-neutralization between most pairs of BVDV-1/BVDV-2 and *HoBi-like* viruses. As a consequence, low coefficients of antigenic similarity *R* were determined for pairs of *HoBi-like* viruses and BVDV-1, and, to a lesser extent, for *HoBi-like* and BVDV-2 pairs (Table 3). Very low *R* values were determined for all pairs of BVDV-1/*HoBi-like* viruses (Table 3). Seven out of eight of these pairs yielded *R* values < 3.12 (reflecting > 32-fold differences in VN titers) and one *R* value (1.1) corresponded to a > 64-fold difference. Hence, the *R* values for most pairs of BVDV-1/*HoBi-like* viruses indicate antigenic differences more pronounced than for some pairs of BVDV-1 and BVDV-2 isolates/strains (Botton et al., 1998; Becher et al. 2003). Thus, our data indicate that antigenic divergences between *HoBi-like* and BVDV-1 isolates may be higher than those observed between BVDV-1 and BVDV-2 (Ridpath et al., 1994; Pellerin et al., 1994; Becher et al. 2003; Bauermann et al., 2013). However, it should be pointed out that significant antigenic differences – yielding *R* values as low as 1.9 – may even be observed for pairs of BVDV-1 strains belonging to different sub-groups (Becher et al., 2003; Bachofen et al. 2008). Thus, the antigenic differences observed between BVDV-1 and *HoBi-like* isolates might be puzzled by the high variability observed among BVDV-1 isolates as compared to BVDV-2.

The coefficient of antigenic similarity *R* for pairs of BVDV-2 and *HoBi-like* viruses were more variable, ranging from low (as low as 6.3, reflecting approximately 16-fold differences in VN titers) to moderate (*R* = 25 or 4-fold differences). Regardless, all *R* values for pairs of BVDV-2/HoBi like viruses were ≤25, indicating significant antigenic differences between viruses from these groups. In general, the *R* values for pairs of BVDV-2/*HoBi-like* were higher than those for BVDV-1/*HoBi-like* (Table 3). Thus, antigenic analysis based on

VN titers and coefficients of antigenic similarity (*R* values) confirmed previous antigenic studies with D32/00_Hobi strain showing that *HoBi-like* viruses are more distantly related to BVDV-1 than to BVDV-2 (Bauermann et al., 2012; 2015). Analysis of Indian *HoBi-like* isolates by VN assays and mAb binding also suggested a closer proximity to BVDV-2 than to BVDV-1 (Misrha et al., 2014). Moreover, some of the differences between HoBi and BVDV-1/BVDV-2 may be even higher than those observed between BVDV-1 and BVDV-2 isolates (Bauermann et al., 2012, 2015; Mishra et al., 2014).

The VN titers and *R* values calculated for pairs of *HoBi-like* viruses indicate the existence of antigenic variation among field isolates. Most *R* values indicate minor to moderate antigenic differences, reflecting 2-to-4-fold differences in VN titers (Tables 3 and 4). Antigenic differences – even minor – were observed between viruses isolated in the same herd in different occasions, distanced four months (LV01-04/12). Interestingly, the isolate SV478/07 presented very low *R* values when matched to the other *HoBi-like* isolates. *R* values of 4.4 and 8.8 correspond to approximately 22-fold and > 10-fold differences in VN titers, respectively. These *R* values are comparable to some values observed for pairs of *HoBi-like* viruses and BVDV-2 and suggest the potential existence of antigenic subgroups among *HoBi-like* viruses. Although preliminary and based on a few isolates, these are new and interesting findings, since previous studies addressing the antigenic relationship between HoBi-viruses and BVDV were based on a single or fewer HoBi-isolates (Bauermann et al., 2012, 2013; Decaro et al., 2013). The extent of the antigenic diversity among *HoBi-like* isolates – and the possible existence of antigenic subgroups - and how it may influence immunological diagnosis and vaccine cross-protection would depend upon the examination of a higher number of isolates from different geographical locations. Unfortunately, the limited number of *HoBi-like* isolates available to date precludes a more comprehensive analysis of their antigenic variability.

A major concern after the identification of *HoBi-like* viruses and their antigenic differences from BVDV was the uncertainty about the cross-protection conferred by current BVDV vaccines. Some studies have shown that either killed or modified live vaccines containing strains of BVDV-1 and BVDV-2 did not induce high levels of neutralizing antibodies to *HoBi-like* virus in cattle, and that a significant number of animals did not seroconvert (Bauermann et al., 2012; 2013). Our data reinforce this concern since sera of sheep vaccinated with commercial BVDV vaccines showed very low (or even undetectable) neutralizing activity against *HoBi-like* viruses. Although the experiment was conducted in sheep, these data demonstrate that the current Brazilian BVDV vaccines induce antibody titers with low neutralizing activity *in vitro* against *HoBi-like* viruses. As the protection conferred by inactivated vaccines is predominantly based on neutralizing antibodies, it is unlikely that these vaccines would afford adequate protection upon exposure to these viruses *in vivo.* Upon the confirmation of the wide circulation of *HoBi-like* viruses in Brazilian cattle – as of other countries as well -, the inclusion of representatives of these virus in vaccines seems mandatory.

The results presented herein extend previous observations on the antigenic relationship/differences between *HoBi-like* pestiviruses and BVDV-1/BVDV-2. In addition, mAb binding and VN assays indicate the existence of antigenic variation among *HoBi-like* isolates. Further studies using a higher number of isolates from different countries/continents are needed to investigate the potential existence of antigenic subgroups. Finally, the results showed that inactivated BVDV vaccines induce weak (or even absent) neutralizing activity against *HoBi-like* viruses. These findings are relevant towards the evaluation and eventual reformulation of immunodiagnostic tests and vaccines used in endemic regions.

Conflict of interest

The authors declare no conflict of interest. EFF, RW, CWC and JFC are recipients of fellowships by the Brazilian Council for Research (CNPq).

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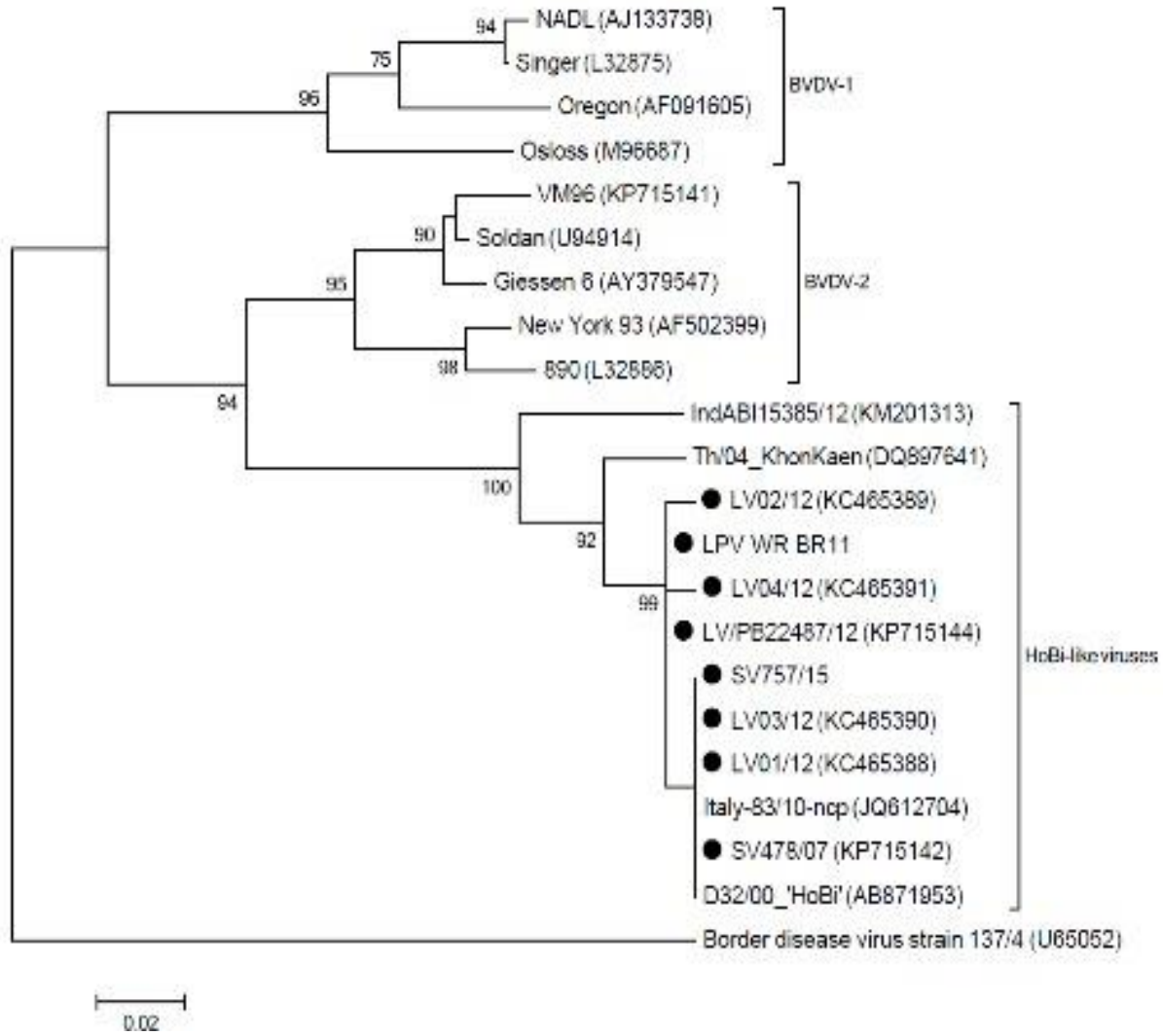

Figure 1. Phylogenetic tree based on 5'UTR region of pestiviruses. The analysis was conducted in the MEGA software 5.0, using the Neighbor-joining method, and the evolutionary distances were computed using the p-distance method. The bootstrap values were calculate using 1,000 replicates. The *HoBi-like* viruses of this study are marked with a black circle.

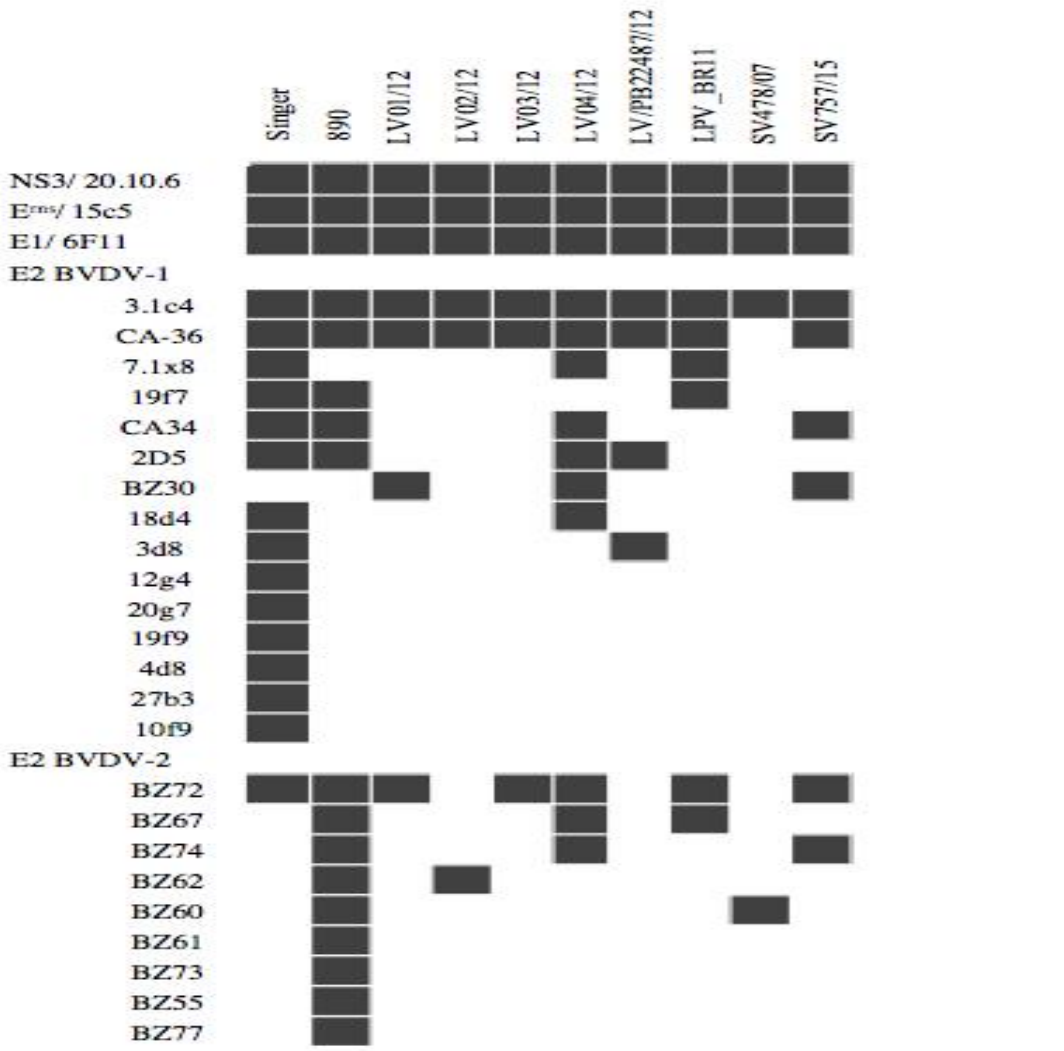

Figure 2. Reactivity of monoclonal antibodies raised against BVDV-1 and BVDV-2 with antigens of Brazilian *HoBi-like* virus isolates. Black boxes indicate monoclonal antibody reaction.

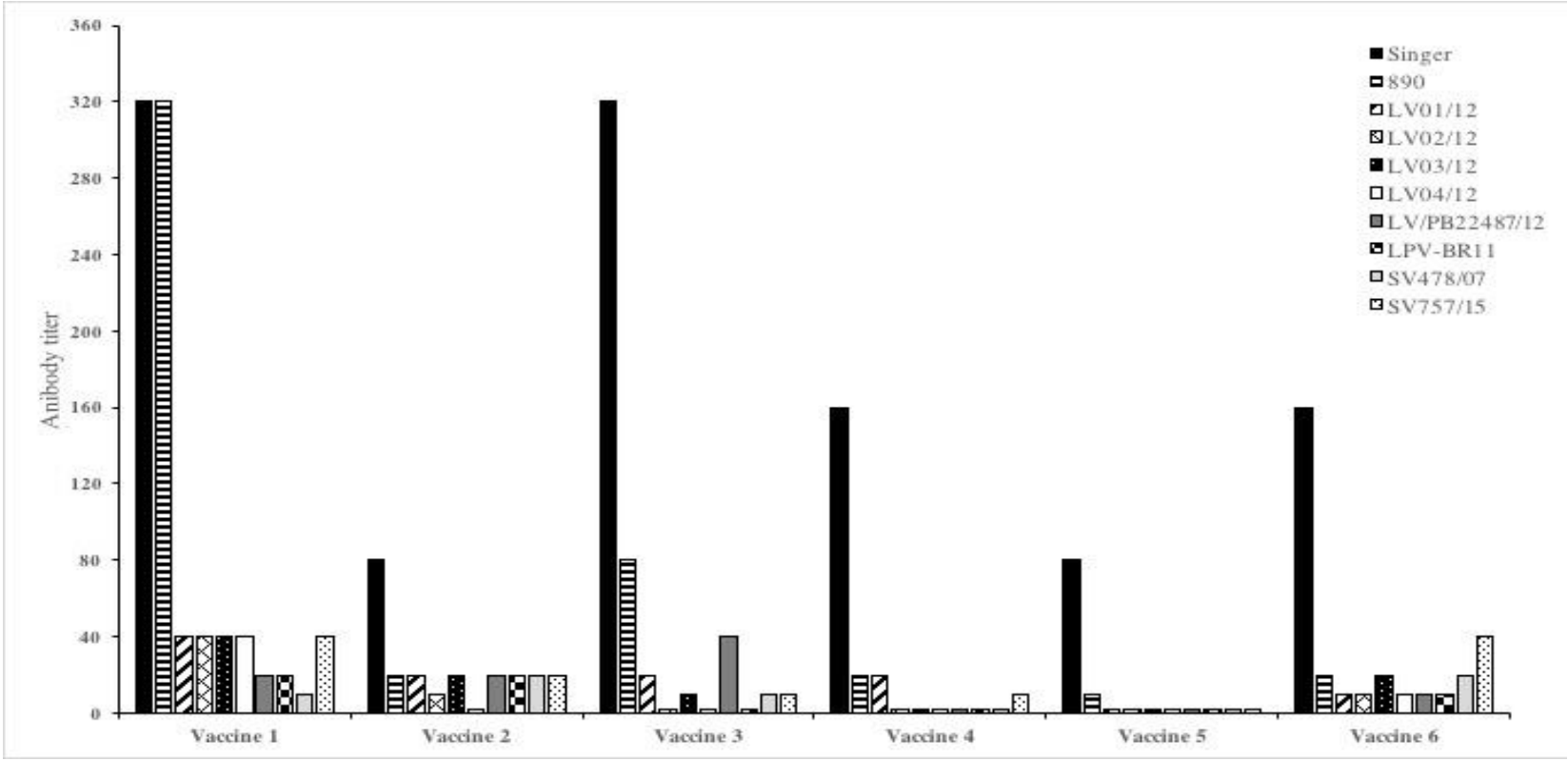

Figure 3. Virus-neutralizing antibody titers in the sera of lambs immunized with commercial BVDV vaccine.

Table 1. Origin and clinical history of the eight Brazilian *HoBi-like* virus isolates included in this study.

Identification	Biotype	State[1] (region)	Year	Clinical history
LV01/12	Ncp	PB (northeast)	2011	PI[2], farm with reproductive disorders
LV02/12	Ncp	PB (northeast)	2011	PI, farm with reproductive disorders
LV03/12	Ncp	PB (northeast)	2011	PI, farm with reproductive disorders
LV04/12	Ncp /cp	PB (northeast)	2012	PI calf; died with signs resembling mucosal disease
LV/PB22487/12	Ncp	PB (northeast)	2012	PI, farm with reproductive disorders
SV478/07	Ncp	MS (midwest)	2007	PI, farm with reproductive disorders
LPV-WR_BR11	Ncp/cp	MS (midwest)	2011	Died with signs resembling mucosal disease
SV757/15	Ncp	SP (southeast)	2015	Heifer/gastroenteric disease

[1] PB – Paraiba; MS – Mato Grosso do Sul; SP – São Paulo; [2]PI - Persistently infected animal.

Table 2. Virus neutralizing antibodies titers against Brazilian *HoBi-like* viruses in the sera of lambs inoculated with BVDV-1, BVDV-2 and *HoBi-like* viruses.

Isolate	Antiserum to:									
	Singer	890	LV01/12	LV02/12	LV03/12	LV04/12	LV/PB22487/12	LPV-BR11	SV478/07	SV757/15
Singer	640	40	10	10	10	10	5	5	20	40
890	20	640	160	160	160	80	160	40	160	160
LV01/12	20	80	1280	640	1280	640	640	640	80	640
LV02/12	40	160	320	1280	640	640	320	320	80	640
LV03/12	40	160	640	320	640	320	640	160	40	640
LV04/12	20	80	320	640	320	1280	160	320	40	320
LV/PB22478/12	80	160	320	320	640	320	640	160	20	640
LPV-BR11	10	40	160	160	320	320	320	640	20	640
SV478/07	20	80	40	40	80	20	20	20	320	40
SV757/12	40	160	640	1280	1280	640	320	320	40	2560

Table 3. Coefficients of antigenic similarity (R) between pairs of viruses based on the neutralizing titers of their antiserum against the respective viruses.

Isolate	Antiserum to:									
	Singer	890	LV01/12	LV02/12	LV03/12	LV04/12	LV/PB22487/12	LPV-BR11	SV478/07	SV757/15
Singer	100	4.4	1.6	2.2	3.1	1.6	3.1	1.1	4.4	3.1
890		100	12.5	17.7	25	8.8	25.0	6.3	25	12.5
LV01/12			100	35.4	100	35.4	50.0	35.4	8.8	35.4
LV02/12				100	50	50	35.4	25	8.8	50
LV03/12					100	35.4	100	35.4	12.5	70.7
LV04/12						100	25.0	35.4	4.4	25
LV/PB22478/12							100	35.4	4.4	35.4
LPV-BR11								100	4.4	35.4
SV478/07									100	4.4
SV757/12										100

* R =100 x √(titer strain A with antiserum B x titer strain B with antiserum A)/ (titer strain A with antiserum A) x (titer strain B with antiserum

3. CONCLUSÃO

Os resultados apresentados ampliam as observações anteriores sobre as relações e diferenças antigênicas entre os pestivírus *HoBi-like* e BVDV-1 / BVDV-2. Embora exista resposta sorológica cruzada entre esses vírus, esta resposta é baixa. Isso demonstra que os isolados de vírus *HoBi-like* utilizados neste trabalho são antigenicamente diferentes das espécies de BVDV, especialmente de BVDV-1. Além disso, os testes de reatividade com mAbs e soroneutralização indicam a existência de variação antigênica entre os isolados *HoBi-like*. São necessários mais estudos com um maior número de isolados *HoBi-like* de diferentes países e/ou continentes para investigar a potencial existência de subgrupos antigênicos. Finalmente, os resultados mostraram que as vacinas de BVDV inativadas induzem atividade neutralizante fraca (ou mesmo ausente) contra vírus *HoBi-like*. Essas informações são relevantes para a avaliação e eventual reformulação de testes de imunodiagnóstico e vacinas utilizadas em regiões endêmicas para esses vírus.

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