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ANÁLISE GENOTÍPICA E FILOGENÉTICA COM BASE NOS GENES DAS GLICOPROTEÍNAS *C* E *D* DE HERPESVÍRUS BOVINO 1 E 5

TESE DE DOUTORADO

Carolina Kist Traesel

Santa Maria, RS, Brasil
2013

ANÁLISE GENOTÍPICA E FILOGENÉTICA COM BASE NOS GENES DAS GLICOPROTEÍNAS *C* E *D* DE HERPESVÍRUS BOVINO 1 E 5

por

Carolina Kist Traesel

Tese apresentada ao Curso de Doutorado do Programa de Pós-Graduação em Medicina Veterinária, Área de Concentração em Medicina Veterinária Preventiva, da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para obtenção do grau de **Doutor em Medicina Veterinária**

Orientador: Prof. Eduardo Furtado Flores

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Elaborada por
Carolina Kist Traesel

Como requisito parcial para obtenção do grau de
Doutor em Medicina Veterinária

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RESUMO

Tese de Doutorado
Programa de Pós-Graduação em Medicina Veterinária
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ANÁLISE GENOTÍPICA E FILOGENÉTICA COM BASE NOS GENES DAS GLICOPROTEÍNAS *C* E *D* DE HERPESVÍRUS BOVINO 1 E 5
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Santa Maria, 8 de março de 2013.

Os herpesvírus bovino 1 (BoHV-1) e 5 (BoHV-5) são agentes genética e antigenicamente relacionados, associados com doença respiratória/genital ou neurológica, respectivamente. Esta tese descreve análises genotípicas e filogenia com base nos genes da glicoproteína C (gC) e D (gD) de isolados de BoHV-1 e BoHV-5. Inicialmente, foi realizada a diferenciação entre BoHV-1 e BoHV-5 pela diferença de tamanho de fragmento obtido por PCR com base na região 5' do gene da gC. Após, é descrita uma análise molecular com base na região 3' do gene da gC de 45 isolados de BoHV, provenientes do Brasil, Uruguai e Argentina (1981-2009). A reconstrução filogenética resultou em uma distinção clara entre BoHV-1.1, BoHV-1.2 e BoHV-5. Os níveis de similaridade de nucleotídeos (nt) variaram de 99,1 a 100% entre as sequências de BoHV-1 (n=12); 96,2-100% entre as sequências de BoHV-5 (n=32) e 77,7-90,3%, entre BoHV-1 e BoHV-5. Um domínio transmembrana de 24 aminoácidos (aa) e uma cauda citoplasmática de oito aa foram também identificados. Além disso, um estudo filogenético com base na região 3' do gene da gD foi realizado para investigar divergência genética entre isolados de BoHV-1 de origem respiratória/genital (n=7), neurológica (n=7) e BoHV-5 de doença neurológica (n=7), e se estas diferenças estariam associadas com a respectiva apresentação clínica. A reconstrução filogenética permitiu a diferenciação de BoHV-1 (n=14) e BoHV-5 (n=7), sendo que os isolados de BoHV-1 de doença neurológica ficaram agrupados dentro do ramo do BoHV-1. Os níveis de similaridade de nt e aa ficaram em 98,3% de média entre os isolados de BoHV-1; 97,8% e 95,8% entre os de BoHV-5; 73,7% e 64,1% entre as espécies virais. Esses resultados indicam que ambos os genes estudados revelaram uma região 3' bastante conservada dentro de cada espécie, porém, uma região menos conservada entre BoHV-1 e BoHV-5. A análise filogenética permitiu diferenciar BoHV-1 e BoHV-5, e até mesmo os subtipos do BoHV-1 agruparam em ramos distintos no estudo com base na região 3' do gene da gC, sugerindo ser uma região mais apropriada para classificação em subgrupos. Assim, é possível concluir que as regiões do genoma estudadas são adequadas para a classificação filogenética de isolados de BoHV-1 e BoHV-5 e, talvez, para a compreensão das relações evolutivas. No entanto, nenhuma conclusão de uma possível associação entre as diferenças nucleotídicas encontradas e diferentes fenótipos pode ser deduzida a partir dessas análises.

Palavras- chave: BoHV-1, BoHV-5, gC, gD, filogenia.

ABSTRACT

Doctoral Thesis
Programa de Pós-Graduação em Medicina Veterinária
Universidade Federal de Santa Maria

GENOTYPIC ANALYSIS AND PHYLOGENY BASED ON GLYCOPROTEINS *C* AND *D* GENES OF BOVINE HERPESVIRUSES 1 AND 5

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ADVISER: EDUARDO FURTADO FLORES
Santa Maria, March 8th, 2013.

Bovine herpesviruses 1 (BoHV-1) and 5 (BoHV-5) are genetic and antigenic closely related pathogens of cattle, historically associated with respiratory/genital and neurological disease, respectively. This thesis reports the genomic and phylogenetic analyzes based on glycoproteins C (gC) and D (gD) genes of BoHV-1 and BoHV-5. Initially, the differentiation between BoHV-1 and BoHV-5 was performed by a differential PCR based on 5' gC gene. Then, a molecular analysis based on the 3' region of the gC gene of 45 BoHV isolates from Brazil, Uruguay and Argentina (1981-2009) was described. The phylogenetic tree reconstruction provided a clear distinction between BoHV-1 and BoHV-5, and BoHV-1 into subtypes BoHV-1.1 and BoHV-1.2. The levels of nucleotide (nt) similarity ranged from 99.1 to 100% among BoHV-1 sequences (n=12); 96.2-100% among BoHV-5 sequences (n=32); and 77.7-90.3% between BoHV-1 and BoHV-5 sequences. A transmembrane domain of 24 amino-acid (aa) and the putative cytoplasmic tail of 8 aa were also identified. In addition, a phylogenetic study was performed to investigate genetic divergences at the 3' region of gD gene of respiratory/genital BoHV-1 (n=7), neurological BoHV-1 (n=7) and neurological BoHV-5 (n=7) isolates, and whether these differences would be associated with the respective neurological presentation. The phylogenetic reconstruction allowed a clear differentiation of BoHV-1 (n=14) and BoHV-5 (n=7), but BoHV-1 isolates from neurological disease grouped within BoHV-1 branch. The nt and aa similarity levels were on average 98.3% among BoHV-1; 97.8% and 95.8% among BoHV-5; 73.7% and 64.1% between viral species. These results indicate that both genes revealed a high conserved 3' region within each species and a less conserved region between BoHV-1 and BoHV-5. The phylogenetic analyzes allowed differentiation of BoHV-1 and BoHV-5 species, and even subtypes grouped in distinct branches in the 3'gC gene-based study, indicating that this region represents a better choice for phylogenetic subgrouping. So, it was concluded that these genome regions represent a suitable target for phylogenetic classification of BoHV-1 and BoHV-5 isolates, and, perhaps, for understanding evolutionary relationships. However, no conclusion of a possible association of genetic differences with phenotypes could be drawn.

Keywords: BoHV-1, BoHV-5, gC, gD, phylogeny.

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SUMÁRIO

1 INTRODUÇÃO

Os herpesvírus bovino 1 (BoHV-1) e 5 (BoHV-5) são classificados na família ***Herpesviridae***, subfamília ***Alphaherpesvirinae***, gênero ***Varicellovirus*** (ROIZMAN et al., 1992). O BoHV-1 está associado a várias manifestações clínicas, incluindo aborto, doença respiratória, genital e eventualmente neurológica (BAGUST, 1972; WEIBLEN et al., 1989; SILVA et al., 2007; FRANCO et al., 2012). Isolados respiratórios de herpesvírus tem sido classificados como BoHV-1.1 e isolados de doença genital como BoHV-1.2 (METZLER et al., 1986; D'ARCE et al., 2002). Já o BoHV-5 é o agente da encefalite herpética, associada à enfermidade neurológica de curso geralmente fatal em bovinos jovens (BAGUST, 1972; CARRILLO et al., 1983; SALVADOR et al., 1998; FRANCO et al., 2012). Este vírus foi anteriormente classificado como BHV-1.3, mas por suas diferenças moleculares e antigênicas, foi posteriormente reclassificado como BoHV-5 (METZLER et al., 1986; ROIZMAN et al., 1992). O BoHV-1 e o BoHV-5 produzem perdas econômicas em rebanhos atingidos por diminuir os índices produtivos e reprodutivos e causar mortalidade de animais (FRANCO et al., 2012). Portanto, esses vírus tem sido alvo de estudos pela importância sanitária e econômica das infecções para rebanhos bovinos.

Infecção e doença causadas por esses agentes ocorrem com frequência em rebanhos brasileiros (WEIBLEN et al., 1989; SALVADOR et al., 1998; COLODEL et al., 2002; RISSI et al., 2008). A infecção pelo BoHV-1 apresenta distribuição mundial e tem sido relatada no Brasil desde a década de 1970 (RIET-CORREA et al., 1989; WEIBLEN et al., 1989). Muitos países europeus implementaram programas de erradicação do BoHV-1, e alguns são reconhecidos como livres do agente pela União Europeia, como a Dinamarca, a Finlândia, a Suíça e a Áustria (OIE, 2010). Surtos de enfermidade neurológica tem sido descritos e associados ao BoHV-5 principalmente na Argentina (CARRILLO et al., 1983), no Uruguai (GUARINO et al., 2008) e no Brasil (WEIBLEN et al., 1989; SALVADOR et al., 1998), com relato de ocorrência de casos no Rio Grande do Sul (WEIBLEN et al., 1989).

Considerando a similaridade genotípica, antigênica e a extensa reatividade sorológica cruzada entre BoHV-1 e BoHV-5, existem dificuldades na realização de diagnóstico, interferência na proteção vacinal e no estudo da epidemiologia das doenças causadas por esses vírus (BRATANICH et al., 1991; TEIXEIRA et al., 1998; CASCIO et al., 1999; DEL MEDICO ZAJAC et al., 2006). As glicoproteínas do envelope viral podem ter uma participação importante nessa problemática, já que estão relacionadas com a indução de

resposta imunológica humoral e celular do hospedeiro (BABIUK et al., 1987; HUTCHINGS et al., 1990; RIJSEWIJK et al., 1999). As principais proteínas inseridas no envelope são a glicoproteína B (gB), a glicoproteína C (gC) e a glicoproteína D (gD) (CHOWDHURY, 1995). Além disso, essas glicoproteínas estão envolvidas no processo de adsorção e penetração dos vírions à superfície das células hospedeiras, servindo como proteínas de ligação a receptores celulares específicos e, dessa forma, contribuindo na determinação do espectro de hospedeiros e do tropismo (CHOWDHURY, 1997; LIMAN et al., 2000). O neurotropismo e a habilidade de causar doença neurológica parece ser a diferença mais marcante entre o BoHV-1 e o BoHV-5, sendo historicamente o BoHV-1 de fenótipo não neurovirulento e o BoHV-5 caracterizado por marcada neurovirulência (BELKNAP et al., 1994; FRANCO et al., 2012). Esse potencial neuropatogênico é uma característica fenotípica que reflete diferenças genéticas e moleculares ainda não esclarecidas (CHOWDHURY, 1995; DELHON et al., 2003). A glicoproteína E (gE) exerce um papel muito importante nessa neuropatogênese e, juntamente com a gC e a proteína do tegumento US9, está associada ao fenótipo neurovirulento do BoHV-5 (CHOWDHURY et al., 2000a; 2000b; 2002; AL-MUBARAK et al., 2004; 2007).

Apesar de serem vírus relacionados genética e antigenicamente, a diferenciação entre o BoHV-1 e o BoHV-5 em nível laboratorial pode ser realizada por técnicas como perfil de reatividade com alguns anticorpos monoclonais (KUNRATH et al., 2004; OLDONI et al., 2004), análise de restrição enzimática do genoma (D'ARCE et al., 2002) e reação em cadeia da polimerase - PCR (ASHBAUGH et al., 1997; ALEGRE et al., 2001; CLAUS et al., 2005). A gC e o seu gene codificante tem sido utilizados em técnicas antigênicas e moleculares para a diferenciação entre BoHV-1 e BoHV-5 (SRIKUMARAN et al., 1990; ASHBAUGH et al., 1997; SILVA et al., 2007) ou até mesmo entre BoHV-1.1 e BoHV-1.2 (RIJSEWIJK et al., 1999). Algumas regiões da gC, que apresentam diferenças entre BoHV-1 e BoHV-5, permitem a sua diferenciação pela variação de extensão dos *amplicons* obtidos por PCR e podem ser utilizadas no diagnóstico e na diferenciação entre as espécies virais (ASHBAUGH et al., 1997; CLAUS et al., 2005).

A comparação genômica entre isolados e a classificação filogenética em espécies (BoHV-1 e BoHV-5) e subtipos (BoHV-1.1 e BoHV-1.2) virais vem sendo estudada para auxiliar na compreensão das bases moleculares dessa diferenciação e sua relação com a apresentação clínica. Nesse sentido, análises filogenéticas com base nos genes da gB (ROS & BELAK, 1999; 2002), da gC (ESTEVES et al., 2008; TRAESEL et al., 2013) e da gD (ROS and BELAK, 1999) tem sido realizadas. No entanto, a associação destes vírus com a sua

respectiva apresentação clínica parece não ser definitiva e mutuamente exclusiva: o BoHV-5 já foi identificado no sêmen bovino (GOMES et al., 2003), em fetos abortados e em bezerros com infecção sistêmica (CARRILLO et al., 1983; SUAREZ HEINLEN et al., 1993). Da mesma forma, BoHV-1 tem sido ocasionalmente isolado de cérebro de bovinos, que sugere que possa também ser associado com doença neurológica (MAGYAR et al., 1993; FURUOKA et al., 1995; ELY et al., 1996; ROELS et al., 2000; PENNY et al., 2002; SILVA et al., 2007; RISSI et al., 2008; FAVIER et al., 2012).

Nesse contexto, a análise genotípica de sequências de nucleotídeos mais conservadas de glicoproteínas do envelope, como a região carboxi-terminal da gC e da gD, podem auxiliar na classificação e identificação das principais diferenças entre as espécies e subtipos virais pela análise filogenética e estudo das relações evolutivas. Já sequências não conservadas na região amino-terminal (extra-vírion) dessas glicoproteínas estariam possivelmente envolvidas na imunogenicidade e determinação do tropismo, podendo ter papel relevante nas diferenças antigênicas, de neurovirulência e reatividade sorológica cruzada entre os isolados de BoHV-1 e BoHV-5. Dessa forma, o estudo de genes dessas proteínas pode auxiliar no entendimento das bases moleculares e antigênicas desses vírus e sua relação com a síndrome clínica apresentada. Pode ainda contribuir na resolução das implicações para o diagnóstico, vacinação e programas de controle e erradicação da doença e disponibilização de técnicas de diagnóstico diferencial entre BoHV-1 e BoHV-5.

2 CAPÍTULO 1

Revisão de Literatura

A subfamília ***Alphaherpesvirinae***, da família ***Herpesviridae***, abriga patógenos importantes de humanos e de animais, incluindo o vírus do herpes simplex 1 e 2 (HSV-1 e -2), o vírus da varicela zoster (VZV), os herpesvírus bovino 1 e 5 (BoHV-1 e -5) e o vírus da pseudoraiva (PRV) de suínos (ROIZMAN et al., 1992). O BoHV-1 e o BoHV-5, juntamente com o VZV, são classificados no gênero ***Varicellovirus***. Os alfaherpesvírus apresentam um amplo espectro de hospedeiros, possuem um ciclo replicativo curto e se disseminam rapidamente em células de cultivo, o que resulta em destruição celular (ROIZMAN et al., 1992; FRANCO et al., 2012). A principal propriedade biológica dos membros dessa subfamília é a capacidade de estabelecer infecções latentes em seus hospedeiros, principalmente em neurônios de gânglios sensoriais e autonômicos, propriedade que é fundamental na epidemiologia e patogenia das infecções, contribuindo para a perpetuação desses vírus na natureza (ROIZMAN et al., 1992; ROCK, 1994).

Os alfaherpesvírus possuem vírions pleomórficos, com 150 a 200 nm de diâmetro, apresentam uma fita dupla e linear de DNA como genoma, com 135-138 kb, um nucleocapsídeo icosaédrico, uma substância amorfa que possui proteínas regulatórias importantes denominada tegumento e são envoltos por um envelope lipoprotéico, no qual são inseridas entre 10 a 12 glicoproteínas virais (ROIZMAN et al., 1992; DELHON et al., 2003). As glicoproteínas do envelope são importantes durante a infecção, pois medeiam a interação do vírus com a célula hospedeira, determinando o tropismo (CHOWDHURY, 1997; LIMAN et al., 2000; SPEAR, 2004), além de serem reconhecidas pelo sistema imunológico do hospedeiro (GLORIOSO et al., 1984; BABIUK et al., 1987; HEROLD et al., 1991). As glicoproteínas mais abundantes presentes no envelope são a gB, gC e gD, previamente denominadas gl, gIll e glV, respectivamente (CHOWDHURY, 1995).

O BoHV-1 e o BoHV-5 compartilham propriedades moleculares, antigênicas e biológicas. Seus genomas apresentam uma similaridade de nucleotídeos (nt) de aproximadamente 85% (ENGELS et al., 1987). O genoma é composto por uma sequência única longa (UL) e uma sequência única curta (US), flanqueada por duas regiões repetidas invertidas, e codifica cerca de 70 proteínas (DELHON et al., 2003; FRANCO et al., 2012). O gene que codifica a gD (US6) está localizado na região US, já a gC é codificada por gene

contido na região UL (UL44) (DELHON et al., 2003). O repertório de proteínas dos dois vírus possui em média 82% de identidade de aminoácidos (aa). A maior divergência entre eles está localizada na região relacionada à latência (LR) e proteínas *immediate early* (BICP0, BICP4, BICP22), com menos de 75% de identidade de aa (DELHON et al., 2003). Essas proteínas são responsáveis pela transcrição dos genes *early*, dando início ao ciclo replicativo produtivo desses vírus, não sendo expressos durante a latência, na qual um único transcrito é detectado nos neurônios infectados, o LR (FRANCO et al., 2012). Já as maiores similaridades entre os dois vírus (mais de 95% de identidade de aa) tem sido descritas nas proteínas envolvidas na replicação e processamento do DNA viral (UL5, UL15, UL29 e UL39), e em certas proteínas estruturais do vírion, como as codificadas pela UL14, UL19, UL48 e US6 (DELHON et al., 2003).

Além da semelhança genotípica, existe uma similaridade antigênica e reatividade sorológica cruzada entre BoHV-1 e BoHV-5 (BRATANICH et al., 1991; TEIXEIRA et al., 1998; CASCIO et al., 1999). Isso dificulta a diferenciação sorológica entre esses vírus e pode interferir nos programas de vacinação e erradicação (D'OFFAY et al., 1995; DEL MÉDICO ZAJAC et al., 2006). Durante muitos anos, essas semelhanças representaram dificuldades para a classificação taxonômica, para o diagnóstico e para o estudo da epidemiologia desses agentes, pois impossibilitavam a sua diferenciação por testes de rotina (BRATANICH et al., 1991; KUNRATH et al., 2004; VOGEL et al., 2004). Além da reatividade sorológica *in vitro*, há possibilidade de proteção cruzada *in vivo* com a vacinação e/ou infecção pelo BoHV-1, seguida de desafio com BoHV-5 (CASCIO et al., 1999; BELTRÃO et al., 2000; DEL MÉDICO ZAJAC et al., 2006). Dessa forma, acredita-se que um animal vacinado contra BoHV-1 estaria protegido da infecção pelo BoHV-5. Isso poderia explicar também a baixa ocorrência da infecção pelo BoHV-5 na Europa e América do Norte, onde a vacinação contra o BoHV-1 é realizada em larga escala (D'OFFAY et al., 1995; ELY et al., 1996; CASCIO et al., 1999).

Apesar das similaridades entre esses dois vírus, eles diferem na habilidade de causar doença neurológica, sendo o BoHV-1 reconhecido historicamente pelo fenótipo não neurovirulento e o BoHV-5 caracterizado por marcada neurovirulência (BELKNAP et al., 1994). O BoHV-1 é associado a diversas manifestações clínicas, incluindo aborto, doença sistêmica em bezerros, doença respiratória (rinotraqueíte infecciosa bovina, IBR) e genital (vulvovaginite e balanopostite infecciosa, IPV e IPB) (BAGUST, 1972; WEIBLEN et al., 1989). O BoHV-5 está geralmente envolvido em surtos de distúrbio neurológico, produzindo

meningoencefalite não-supurativa geralmente fatal em bovinos jovens (BAGUST, 1972; CARRILLO et al., 1983; SALVADOR et al., 1998; RISSI et al., 2008).

2.1. Herpesvírus bovino

O BoHV-1 pode ser classificado em BoHV-1.1 e BoHV-1.2 com base em análise de restrição enzimática, perfil de polipeptídeo viral, reatividade com anticorpos monoclonais específicos ou PCR e sequenciamento (METZLER et al., 1986; RIJSEWIJK et al., 1999; D'ARCE et al., 2002; SPILKI et al., 2005; ESTEVES et al., 2008; TRAESEL et al., 2013). Em geral, o BoHV-1.1 é associado com doença respiratória e reprodutiva e o BoHV-1.2 com doença genital. A rinotraqueíte viral bovina (IBR), produzida pelo BoHV-1.1, é uma enfermidade importante para rebanhos bovinos no Brasil e no mundo inteiro. As infecções respiratórias podem ser subclínicas ou caracterizadas por febre, depressão, anorexia, dispneia, taquicardia, tosse e descarga nasal, apresentando morbidade de até 100% e índices de mortalidade frequentemente baixos (<5%). No entanto, esse agente também é isolado em casos de aborto, além de conjuntivites e infecções sistêmicas em neonatos (CROOK et al., 2012; FRANCO et al., 2012). O BoHV-1.2 é associado a doença genital, produzindo a vulvovaginite pustular infecciosa em fêmeas e a balanopostite pustular infecciosa em machos infectados (IPV e IPB). Essa doença é caracterizada por lesões vesiculares que ulceram e são recobertas por material fibrinoso, localizadas na vulva, pênis e prepúcio dos bovinos afetados. Ambos os agentes apresentam-se amplamente distribuídos no Brasil, com prevalência entre 8 e 82% (FRANCO et al., 2012).

Posteriormente, o BoHV-1.2 foi subdivido em BoHV-1.2a e BoHV-1.2b (METZLER et al., 1986). O subtipo 1.2a já foi descrito associado à doença do trato genital e também a abortos e infecções respiratórias, sendo o subtipo mais isolado no Brasil, e o BoHV-1.2b tem sido associado com doença respiratória leve e IPV/IPB (FRANCO et al., 2012). Ainda, um terceiro subtipo foi descrito anteriormente: isolados de doença neurológica, que produziam semelhante efeito citopático em cultivo de células e possuíam reatividade sorológica cruzada com o BoHV-1, foram inicialmente classificados como uma variante neuropatogênica e designados BoHV-1.3 (BAGUST, 1972; BELKNAP et al., 1994; FRANCO et al., 2012). Esse vírus possui semelhanças genéticas, antigênicas e biológicas com o BoHV-1.1 e BoHV-1.2, no entanto, relevantes diferenças genômicas, no aspecto clínico-epidemiológico e na reatividade com alguns anticorpos monoclonais foram sendo detectadas e justificaram sua

classificação como uma nova espécie viral, distinto, atualmente reconhecido como BoHV-5 (ROIZMAIN et al., 1992; DELHON et al., 2003; FRANCO et al., 2012).

À medida que mais vírus foram sendo isolados, estudados e analisados quanto à associação entre a apresentação clínica e aspectos moleculares, foram sendo relatados casos de BoHV-1 isolado de cérebro de bovinos com doença neurológica (MAGYAR et al., 1993; FURUOKA et al., 1995; ELY et al., 1996; ROELS et al., 2000; PENNY et al., 2002; FAVIER et al., 2012), inclusive no Sul (SILVA et al., 2007; RISSI et al., 2008; BATISTA et al., 2010) e Centro-Oeste do Brasil (ARRUDA et al., 2010). Embora o BoHV-1 também tenha sido implicado como causa de doença neurológica em bovinos, a grande maioria dos casos da doença é associada ao BoHV-5 (SILVA et al., 2007). Os dois vírus são considerados neurotrópicos, mas uma invasão e replicação eficientes no SNC, capaz de produzir encefalite, eram características atribuídas somente ao BoHV-5 (DELHON et al., 2003; SILVA et al., 2007). No entanto, casos de meningoencefalite por BoHV-1 podem não ser tão incomuns como suspeitado anteriormente, pelo menos no Sul do Brasil (RISSI et al., 2008).

O BoHV-5 produz meningoencefalite necrosante não-supurativa, evidenciada em exame histopatológico na maioria dos casos (RISSI et al., 2008). O neurotropismo e a neuroinvasividade são propriedades importantes desse vírus. O BoHV-5 infecta e replica em células epiteliais, invade via retrógrada o sistema nervoso central (SNC) de bovinos, se disseminando e destruindo neurônios e outras células durante a infecção aguda ou estabelecendo infecção latente (CHOWDHURY et al., 1997; LEE et al., 1999; FRANCO et al., 2012). A via olfatória é preferencialmente utilizada pelo BoHV-5 durante a infecção aguda (CHOWDHURY et al., 1997; LEE et al., 1999); a via trigeminal também pode ser utilizada, mas esta parece ser mais importante para o estabelecimento de infecções latentes (LEE et al., 1999; BELTRÃO et al., 2000). A apresentação clínica inclui sinais neurológicos como prostração, falta de coordenação, ranger de dentes, movimentos de pedalagem, andar em círculos, decúbito, além de salivação excessiva, secreção nasal e ocular, de curso geralmente fatal em bovinos jovens (SALVADOR et al., 1998; RISSI et al., 2008).

Apesar de ter sido descrito pela primeira vez na Austrália (FRENCH, 1962; JOHNSTON et al., 1962) e já terem sido relatados casos de infecção por BoHV-5 nos EUA (D´OFFAY et al., 1995), Itália (MORETTI et al., 1964) e Hungria (BARTHA et al., 1969), surtos de enfermidade neurológica associados ao BoHV-5 tem sido descritos principalmente na Argentina (CARRILLO et al., 1983), Uruguai (GUARINO et al., 2008) e Brasil (WEIBLEN et al., 1989; SALVADOR et al., 1998). No Brasil, existem relatos da ocorrência de surtos da infecção pelo BoHV-5 em vários Estados, incluindo o Rio Grande do Sul (RIET-

CORRÊA et al., 1989; WEIBLEN et al., 1989), Mato Grosso (COLODEL et al., 2002), Mato Grosso do Sul, São Paulo (SALVADOR et al., 1998), Paraná (CLAUS et al., 2000), Rio de Janeiro (SOUZA et al., 2002) e Minas Gerais (GOMES et al., 2002). Acredita-se que esse vírus esteja amplamente distribuído nos rebanhos bovinos do país. Contudo, informações sobre prevalência e distribuição geográfica são escassas pela reatividade sorológica cruzada com o BoHV-1 (SOUZA et al., 2002).

Da mesma forma que o BoHV-1, análises de restrição enzimática do genoma permitiram a classificação do BoHV-5 em subtipos "a", "b" e "c" (também denominado "não a, não b"). A maioria das amostras isoladas e caracterizadas foram classificadas como BoHV-5 "a", mas ainda não há informação disponível sobre possíveis relações entre os subtipos e diferentes quadros clínicos ou níveis de virulência (FRANCO et al., 2012). O BoHV-5 é geralmente recuperado de cérebro de bovinos que apresentavam doença neurológica - muitas vezes como diagnóstico diferencial de raiva - e a partir de secreções e tecidos de animais infectados experimentalmente, principalmente coelhos, que são tidos como modelos experimentais da doença pela apresentação clínico-patológica semelhante (BELTRÃO et al., 2000; CHOWDHURY et al., 2000a; 2000b; 2002; RISSI et al., 2008). No entanto, o BoHV-5 também vem sendo associado com doença do trato reprodutivo: já foi identificado em amostras de sêmen bovino no Brasil e na Austrália (GOMES et al., 2003; KIRKLAND et al., 2009), com evidências de transmissão natural a partir de sêmen contaminado (FAVIER et al., 2012); e o DNA encontrado em ovários, embriões e em tecidos de fetos abortados e bezerros com infecção sistêmica (CARRILLO et al., 1983; SUAREZ HEINLEN et al., 1993). Esses dados, da mesma forma que os casos de BoHV-1 isolado de cérebro de bovinos com doença neurológica, sugerem que a associação entre esses vírus e a sua apresentação clínica parece não ser definitiva (SILVA et al., 2007).

2.2. Glicoproteína C (gC)

A gC é uma glicoproteína dimérica presente no envelope viral, não-essencial para a replicação em cultivo celular. A gC está envolvida na penetração dos vírions nas células e na determinação do tropismo, mediando a ligação das partículas virais com os receptores do tipo heparan sulfato presentes na membrana celular das células alvo (CHOWDHURY, 1997). Aparentemente, a gC possui um importante papel na modulação da neurovirulência em coelhos (CHOWDHURY et al., 2000a). É uma proteína transmembrana do tipo I, pertencente à superfamília das imunoglobulinas, constituindo-se em um antígeno importante, que

influencia interações imunológicas vírus-hospedeiro, sendo expressa na membrana das células hospedeiras após a infecção (FITZPATRICK et al., 1989).

A gC do BoHV-1 (gC1) possui 521 resíduos de aa, e 471 aa no BoHV-5 (gC5), e é constituída por um peptídeo sinalizador entre os resíduos 7 e 21, uma região hidrofílica projetada extra-vírion amino(N)-terminal, um domínio transmembrana entre os resíduos 467 e 500, seguida por uma região hidrofílica carboxi(C)-terminal (FITZPATRICK et al., 1989; CHOWDHURY, 1997). Outro estudo relata que a gC1 codifica 508 aa e a gC5, 486 (DELHON et al., 2003). Sítios potenciais de glicosilação já foram descritos na região N-terminal e epítopos da gC do BoHV-1 já foram mapeados entre os resíduos 22 e 287 (FITZPATRICK et al., 1990; RIJSEWIJK et al., 1999).

Devido ao alto grau de variabilidade na gC N-terminal, esta região pode ser utilizada para o desenvolvimento de testes diferenciais (CHOWDHURY, 1995; RIJSEWIJK et al., 1999; SPILKI et al., 2005). A gC do BoHV-1 e a do BoHV-5 diferem nas qualidades de ligação ao receptor heparan, o que pode modular a habilidade desses vírus de se disseminar no sistema nervoso central (LIMAN et al., 2000). Ao contrário da região N-terminal, a porção C-terminal da gC é mais conservada, e permite a diferenciação entre espécies e subtipos (RIJSEWIJK et al., 1999; ESTEVES et al., 2008; TRAESEL et al., 2013).

A sequência de aminoácidos da gC apresenta pequena identidade entre o BoHV-1 e o BoHV-5 (DELHON et al., 2003). A janela aberta de leitura (ORF) da gC do BoHV-5 é mais curta que a do BoHV-1, com ausência de dois sítios de glicosilação no BoHV-5. Apesar dos dois terços C-terminal serem bem conservados entre os dois vírus (CHOWDHURY, 1995; DELHON et al., 2003), incluindo a região central da gC que é o domínio de ligação ao receptor no BoHV-1 (OKAZAKI et al., 1994), a região C-terminal hidrofílica é consideravelmente menor no BoHV-5. Além disso, diferenças nas sequências da região N-terminal da gC do BoHV-1 e do BoHV-5 já foram relatadas e podem contribuir parcialmente para a determinação das propriedades biológicas desses vírus e para a patogenia das doenças associadas (CHOWDHURY, 1995).

Pequenas diferenças na gC parecem determinar o tropismo diferencial entre os dois vírus. Assim, a substituição da gC do BoHV-1 pela gC do BoHV-5 não afetou a capacidade de ligação dos vírions aos receptores. Entretanto, a qualidade da ligação foi alterada, o que pode modular a capacidade do vírus se disseminar no SNC (LIMAN et al., 2000). Da mesma forma, o BoHV-5 recombinante contendo a gC do BoHV-1 replica com menor eficiência no SNC e não recupera o fenótipo neurovirulento do BoHV-5 parental. Isso indica que a gC

pode regular o neurotropismo do BoHV-5 e é importante para a neurovirulência. Entretanto, não é essencial e determinante desse fenótipo (CHOWDHURY et al., 2000a).

2.3. Glicoproteína D (gD)

A gD é uma glicoproteína essencial e crítica para a ligação com os receptores em muitos alfaherpesvírus (SPEAR, 2004; CAMPADELLI-FIUME et al., 2007), principalmente receptores das famílias do fator de necrose tumoral, como o mediador de entrada de herpesvírus HveA, e do poliovírus (HveB ou nectin 2 e HveC ou nectin 1) (MONTGOMERY et al., 1996; GERAGHTY et al., 1998; WARNER et al., 1998), além de uma forma modificada do heparan sulfato (SHUKLA et al., 1999). A região N-terminal da gD é importante na neutralização viral, na geração de resposta imune. A maioria dos anticorpos reage com a região 5' da ORF da gD (entre os resíduos 1 a 216), correspondendo à região N-terminal. Alguns anticorpos monoclonais (Mabs) estudados podem impedir a penetração do BoHV-1 na célula, ou seja, neutralizam o BoHV-1, mas não neutralizam o BoHV-5 (ABDELMAGID et al., 1995). Além disso, a gD induz resposta imune celular de maior magnitude que a gB ou a gC (HUTCHINGS et al., 1990).

A gD é uma glicoproteína típica tipo 1 e seu gene codifica 417 aa, distribuídos em um peptídeo sinal de 18 aa, um domínio extracelular, uma região transmembrana (361-389 aa) e uma cauda citoplasmática de 28 aa (TIKOO et al., 1990). A sequência de aa da gD entre o BoHV-1 e -5 foi relatada como tendo 98% de identidade (DELHON et al., 2003). No entanto, GABEV et al. (2010) apresentou um nível de similaridade de aa de 79,9% entre as gD dos dois vírus, com diferenças maiores mapeadas em uma região rica em glicina no ectodomínio da molécula, entre os aa 280 e 330 da gD do BoHV-5, próxima a região transmembrana. Já segundo ABDELMAGID et al. (1995), as maiores diferenças na gD entre o BoHV-1 e -5 estão na região C-terminal, entre os aa 283 e 354 do BoHV-5. Essa região pode ser importante nas interações entre proteína e a região entre 245 a 320 aa é importante no processamento e transporte da gD até a superfície celular (TIKOO et al., 1993). Essas diferenças podem contribuir para a determinação das propriedades biológicas do BoHV-5, inclusive a neuroinvasividade. Sabe-se que a gD do HSV está envolvida na patogênese da doença neurológica (IZUMI & STEVENS, 1990). No entanto, segundo GABEV et al. (2010) em estudo utilizando mutantes do BoHV-1 e -5 contendo a gD da espécie heteróloga, a gD do BoHV-5 confere um amplo espectro celular de hospedeiros para o BoHV-1 e pode ser considerada como um fator de virulência, mas não contribui para a invasão do cérebro.

Essa tese relata o estudo filogenético e análise de sequências de nt e aa dos genes da gC e gD de isolados de BoHV-1 e BoHV-5. No capítulo 2, são apresentadas análises filogenética e genotípica da região 3' do gene da gC, além do perfil de hidrofobicidade, envolvendo 47 BoHV. No capítulo 3, estão descritas a filogenia e análise genética da região 3' do gene e da ORF da gD de sete isolados de BoHV-1 típicos, provenientes de doença respiratória ou genital, sete BoHV-1 isolados de encéfalo de bovinos com doença neurológica e sete BoHV-5 típicos de doença neurológica.

3 CAPÍTULO 2

Nucleotide sequencing and phylogenetic analysis of the 3' region of glycoprotein C gene of South American bovine herpesviruses 1 and 5

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Nucleotide sequencing and phylogenetic analysis of the 3' region of glycoprotein C gene of South American bovine herpesviruses 1 and 5

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Abstract

We herein describe a molecular analysis based on the 3'region of the glycoprotein C gene of 45 bovine herpesvirus (BoHV) isolates from Brazil (n=41), Uruguay (n=2) and Argentina (n=2). Nucleotide (nt) sequencing and alignment of 333 nt revealed levels of similarity ranging from 99.1 to 100% among BoHV-1 sequences (n=12); 96.2-100% among BoHV-5 sequences (n=32); and 77.7-90.3% between BoHV-1 and BoHV-5 sequences. The phylogenetic tree reconstruction provided a clear distinction between BoHV-1 and BoHV-5, and BoHV-1 into subtypes BoHV-1.1 and BoHV-1.2. The isolate SV 453/93 (BoHV-1 associated with genital disease) could not be included within BoHV-1 subtypes since it presented a markedly distinct nt and amino acid (aa) deduced sequences. A transmembrane domain of 24 aa and the putative cytoplasmic tail of 8 aa were identified and mapped. These results indicate that this genome region represents a suitable target for phylogenetic subgrouping of BoHV-1 and BoHV-5 isolates and, perhaps, for understanding evolutionary relationships.

*Keywords***:** BoHV-1, BoHV-5, gC, transmembrane helix, cytoplasmic tail.

1. Introduction

Bovine herpesviruses types 1 (BoHV-1) and 5 (BoHV-5) are major pathogens of cattle, belonging to the family *Herpesviridae*, subfamily *Alphaherpesvirinae*, genus *Varicellovirus* (Roizman et al., 1992). BoHV-1 is distributed worldwide and has been associated with a variety of clinical manifestations, including respiratory disease (infectious bovine rhinotracheitis – IBR), systemic disease in newborn calves, genital disorders (vulvovaginitis and balanoposthitis – IPV and IBP) and abortions (Bagust, 1972; Weiblen et al., 1989). In general, respiratory BoHV-1 isolates have been classified as BoHV-1.1 and

genital isolates as BoHV-1.2, based on differences in restriction enzyme analysis, viral polypeptide profile, reactivity with monoclonal antibodies (MAbs) and virus neutralization assays (Metzler et al., 1986; Rijsewijk et al., 1999; D'Arce et al., 2002; Spilki et al., 2005). In addition, BoHV-1.2 can be further divided into subtypes "a" and "b" (D'Arce et al., 2002). Bovine herpesvirus 5 (BoHV-5), previously classified as BoHV-1.3, has been historically associated with outbreaks of neurological disease, mainly in calves (Bagust, 1972; Carrillo et al., 1983; Salvador et al., 1998). In contrast with the widespread distribution of BoHV-1, BoHV-5-associated disease has been more frequently reported in South American countries as Brazil (Weiblen et al., 1989; Salvador et al., 1998), Argentina (Carrillo et al., 1983) and Uruguay (Guarino et al., 2008). Based on antigenic and/or molecular differences, BoHV-5 can be sub-classified into "a", "b" and "non-a non-b" subtypes, the latter displaying an unusual MAb binding pattern and restriction endonuclease profile (D'Arce et al., 2002).

BoHV-1 and BoHV-5 are very closely related and display a remarkable similarity in genetic, antigenic and biological aspects. Their double stranded DNA genomes (135 kb in BoHV-1; 138 kb in BoHV-5) present around 85% nucleotide (nt) similarity and 82% amino acid (aa) identity (Belknap et al., 1994; Delhon et al., 2003). The viral genome encodes approximately 70 proteins, from which eleven are glycosilated. Most BoHV-1 and BoHV-5 gene products share high levels of aa identity (≥ 95%), such as proteins involved in viral DNA replication and processing and some virion proteins; yet some genes also present discrete differences (Delhon et al., 2003). The gene encoding glycoprotein C (gC), also called UL44, is one of the least conserved genes (Roizman et al., 1992; Delhon et al., 2003). Biologically, the major difference between these viruses is the neuropathogenic potential of BoHV-5 (Belknap et al., 1994). Despite the high similarity, differentiation among BoHV-1.1, BoHV-1.2 and BoHV-5 can be achieved by antigenic or molecular analysis (Ashbaugh et al., 1997; Claus et al., 2005; Silva et al., 2007). Most PCRs and sequencing analysis to differentiate among BoHV-1 subtypes (and these from BoHV-5) are based on the less-conserved regions, such as the gC gene (Spilki et al., 2005; Esteves et al., 2008).

Glycoprotein C of bovine herpesviruses is a dimeric, non-essential, type I integral envelope glycoprotein containing a signal peptide, a highly hydrophilic glycosilated amino-terminal (NH_2-t) ectodomain, a single hydrophobic transmembrane anchor domain and a carboxy-terminal (COOH-t) hydrophilic region (Fitzpatrick et al., 1989; Skoff and Holland, 1993). BoHV-1 gC (gC1) gene encodes for 508 aa residues whereas gC5 gene codes for 486 aa (Delhon et al., 2003). Glycoprotein C plays an important role in the attachment of virions to target cells by binding to heparin surface receptors and may, therefore, contribute to cell

tropism and neurovirulence (Chowdhury, 1997). The gC gene product displays a low aa identity (75%) between BoHV-1 and BoHV-5 (Delhon et al., 2003) and differs substantially at the 5' third (Chowdhury, 1995; Delhon et al., 2003). The variability of gC between BoHV-1 and BoHV-5 has been exploited for the development of differential diagnostic tests (Ashbaugh et al., 1997; Rijsewijk et al., 1999; Claus et al., 2005; Silva et al., 2007). In contrast, the 3' region of gC gene (corresponding to the COOH-t of the protein) is moderately conserved and, as such, would be suitable for phylogenetic analysis to allow classification of BoHV into types and subtypes.

We herein describe a phylogenetic analysis of South American BoHV-1 and BoHV-5 isolates based on the 3' region of the gC gene. This study extends previous study by Esteves et al. (2008), by analyzing a higher number of isolates and including BoHV-1 isolates associated with neurological disease, a trait historically attributed only to BoHV-5. In addition, amino acid deduction and analysis were performed to investigate the position of transmembrane and cytoplasmic domains in the target gC region.

2. Material and methods

Forty five BoHV isolates (13 BoHV-1 and 32 BoHV-5) obtained from clinical specimens from Brazil, Uruguay and Argentina (1981-2009) were analyzed. Two American and one European BoHV-1 strains (Los Angeles, Cooper and LAM) were included in the analysis. Viruses were initially submitted to differentiation (BoHV-1 x BoHV-5) by a PCR described by Ashbaugh et al. (1997) and modified (Silva et al., 2007). Then, viral DNA was submitted to a second PCR using primers for the 3' region of gC gene and the amplicons were submitted to nt sequencing. Phylogenetic analysis of the nt sequences was then performed for subdivision into types and/or subtypes. In addition, aa sequences were predicted for the hydropathicity and transmembrane region analysis.

2.1. Viruses and cells

The information concerning the isolates is shown in table 1. The isolates were identified based on the typical cytopathic effect (cpe) produced in cell cultures followed by indirect fluorescent assay using a pool of BoHV-1- and BoHV-5 MAbs. The viruses were amplified and submitted to three rounds of biological cloning; cloned viruses were further

amplified for characterization. Virus isolation, amplification and cloning were performed in a MDBK-derived cell line named CRIB (Flores and Donis, 1995). Cells were maintained in minimal essential medium containing 1.6 mg/L penicillin, 0.4 mg/L streptomycin, 2.25 mL/L amphotericin B, and 5% fetal calf serum. During characterization, the reference strains BoHV-1 (Cooper) and BoHV-5 (SV 507/99) were used as positive controls, and mock-infected CRIB cells were used as the negative control.

2.2. DNA extraction for PCR

Viral DNA for PCR was extracted from CRIB cells infected with each isolate at a multiplicity of infection of 1. For this purpose, inoculated cells were harvested when the cpe reached about 80% of the monolayers and then centrifuged (5 min, 5000 x g). Total DNA was extracted from the cell pellets using DNAzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol, resuspended in 80 µL of TRIS-EDTA buffer and stored at -18 ºC until use.

2.3. PCR amplification

A gC gene-based PCR able to differentiate BoHV-1 from BoHV-5 (Ashbaugh et al., 1997) was performed, with modifications described by Silva et al. (2007). Briefly, the use of primers (forward 5'-GCGGGGGCTCGCCGAGGA-3' and reverse 5'-GGAGCGCACGGTCAGGGC-3') complementary to the coding region of the 5' region of gC gene results in a 653 base-pair (bp) product for BoHV-1 and a 589 bp amplicon for BoHV-5.

Isolates classified as BoHV-1 or BoHV-5 were then submitted to another PCR, based on the 3' region of gC gene. Two pairs of primers were used: one set for BoHV-1 (forward 5'-GTACATGCGTGGTCTTTG-3' and reverse 5'-TCTTTACGGTCGACGACTC-3') and other set for BoHV-5 (forward 5'-AGGTGGAGGCTCACCCTTTC-3' and reverse 5'-CGACGTCTTTACGGTCGATG-3'). PCR resulted in amplicons of 625 bp for BoHV-1 (genome position 17251-16627) and 610 bp for BoHV-5 (genome position 18161-17552). Approximately 1 µg of total DNA extracted from infected cells was used as a template in 48 µL of a mix containing ultrapure water, 25 µM of each primer, 0.4 mM of deoxyribonucleotides, 10% of DMSO, 6 mM of $MgCl_2$, 10% of Taq 10x buffer and 1U of Taq DNA polymerase (Invitrogen, Carlsbad, CA, USA). PCR conditions were as follows: a

denaturation step of 5 min at 94 ºC, followed by 40 cycles of denaturation at 94 ºC for 50 sec, annealing at 52 ºC (BoHV-1) or 58.5 ºC (BoHV-5) for 55 sec and extension at 72 ºC for 1 min. Cycling was followed by one last extension step of 10 min at 72 ºC. PCR products were analyzed under UV light after electrophoresis in a 1.5% agarose gel stained with GelRed (Biotium, Hayward, CA, USA), using a 1 kb DNA ladder (Invitrogen, Carlsbad, CA, USA). In all procedures, total DNA extracted from mock-infected CRIB cells and from cells infected with the reference strains of BoHV-1 and BoHV-5 was used as control.

2.4. Nucleotide sequencing and analysis

For sequencing, PCR products were purified using the PureLink PCR kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions and eluted in ultrapure water. The quality of DNA preparations was monitored by agarose gel electrophoresis stained by GelRed. Sequencing reaction was performed in duplicates in a MegaBACE 500 automatic sequencer (GE Healthcare) and used 50-100 ng of amplicon DNA, 5 µM of forward and reverse primers (described above, according to virus type). Thus, four sequences were obtained; each product was sequenced twice in both directions to obtain a consensus sequence.

The quality of the obtained DNA sequences was analyzed by the GAP software implemented in the Staden Package (Staden, 1996) and overlapping fragments of each sequence were assembled. At least three sequences of high quality were used to form the consensus sequence of each isolate. The consensus sequence was submitted to comparison with BoHV-1.1 gC (strain Cooper; GenBank ID AJ004801) and BoHV-5 – SV 507/99 – (GenBank ID AY261359) complete genomes, using NCBI database and BLAST software (http://www.ncbi.nlm.nih.gov/BLAST/) (Altschul et al., 1997). Nucleotide sequences were deposited in GenBank (accession numbers: JN173204 up to JN173251).

2.5. Phylogenetic analysis

Assembled consensus sequences of the isolates and reference BoHV-1 and 5 sequences (GenBank ID AJ004801 and AY261359) were aligned and edited by BioEdit Sequence Alignment Editor software suite, version 7.0.5.3 (Hall, 1999). The sequence alignments were performed using Clustal W software (Thompson et al., 1997) included in the BioEdit suite. The MODELTEST software was used to determine the best evolutionary

model of nucleotide substitution in the group of analyzed sequences (Posada and Crandall, 1998). Evolutionary analysis and phylogenetic reconstruction were conducted in MEGA 5 (Tamura et al., 2007). The evolutionary history was inferred by using the maximum likelihood method based on the data-specific model/general time reversible model (Nei and Kumar, 2000), and the bootstrap values were calculated using 1000 replicates. In this reconstruction, the reference sequences of BoHV-1 and BoHV-5 (GenBank ID AJ004801 and AY261359, respectively) recorded in the GenBank were used for comparison along with the gC 3' region sequences of each BoHV subtype (GenBank ID DQ173717, DQ173719, DQ173721, DQ173723, DQ173725, DQ173726, DQ173728 to DQ173732, DQ173735, DQ173737, DQ173739, DQ173741, DQ173742 and DQ184912) as well as a PRV genomic sequence (GenBank ID AF403051). To confirm the SV 453/93 identity, a phylogenetic comparison with some ruminant alphaherpesvirus (AJ00481, BoHV-1 genome; AY26135, BoHV-5 genome; AY821804, CapHV-1; DQ333389, ElkHV; DQ333390, CervidHV-1 and DQ333391, CervidHV-2) and a similarity matrix were performed. Preliminary studies of 5' region of gC gene and 3' region of gD gene were performed to further analyze SV 453/93. Phylogenetic trees were reconstructed for both genes by the same methodology described for gC 3' region, using nt sequences of five isolates of each type/subtype (BoHV-1.1, BoHV-1.2 and BoHV-5) and SV 453/93 (data not shown).

2.6. Hydropathic profile and predicted transmembrane domain

Based on nt sequences, the aa sequence of the corresponding gC region of each isolate/strain was predicted by The Sequence Manipulation Suite software (http://www.bioinformatics.org/sms/). The nt sequence translations were submitted to homology comparison using the protein BLAST software (http://www.ncbi.nlm.nih.gov/BLAST/). One isolate representing each type or subtype and SV 453/93, were selected to verify the hydropathicity and the prediction of the transmembrane domain. The hydropathic profile of the deduced aa of BoHV-1.1 and BoHV-5 gC reference sequences (AJ004801 and AY261359), BoHV-1.2 isolate SV 228/06, BoHV-1 isolate SV 453/93 and BoHV-5 isolate SV 102/07 were produced by the ProtScale program and by the Protein Hydrophobicity Plots, using the Kyte and Doolittle (1982) scale to delineate the protein hydrophobic character (http://expasy.org/tools/protscale.html and http://www.vivo.colostate.edu/molkit/hydropathy/index.html). The location of transmembrane helices in proteins of the same isolates/strains was predicted by TMHMM

Server version 2.0 (http://www.cbs.dtu.dk/services/TMHMM-2.0/) and the HMMTOP Server Version 2.0 (Tusnady and Simon, 1998; 2001).

3. Results

3.1. Nucleotide sequences

Glycoprotein C genes of BoHV-1 and BoHV-5 isolates were partially sequenced. The sequenced fragments encompassed the coding region of the COOH-terminus of gC; the length of the edited sequences varied from 393 to 559 nt. A consistent alignment provided by 333 nt in the 3' region of gC gene (BoHV-1.1 gC reference position: nt 1105 to 1438) revealed a high degree of similarity among all BoHV isolates, mainly within the same type or subtype, indicating a moderately conserved region.

Nucleotide insertions, deletions and substitutions (mostly transversions, a few transitions) were observed within the analyzed region. These changes are partially demonstrated in Fig. 1A and B. The presence of three guanines in all BoHV-1 isolates that are absent in all BoHV-5 isolates was observed at positions 1196 to 1198 of BoHV-1.1 gC (Fig. 1B). Most changes were observed in isolates SV 226/06, SV 229/06, A 613, Uruguay T2, SV 41/06, SV 190/00 A, SV 136/88, EVI 340 and EVI 88 when compared with reference sequences. A BoHV-1 isolate (SV 453/93) stood out because it presented a different nt pattern, with many nt substitutions.

The sequence alignment revealed a moderately conserved 3'region, confirming previous observations in a limited number of isolates (Esteves et al., 2008). The nt similarity ranged from 77.7 to 90.3% among BoHV-1 and BoHV-5 isolates. BoHV-1 isolate SV 453/93 presented an unusual nt pattern and could not be typed as BoHV-1.1 or BoHV-1.2. This virus was separately compared to all other isolates/strains, resulting in identity ranging from 77.7 to 85.2%. This low identity indicates that SV 453/93 is rather atypical concerning nt sequences of the 3' region of gC gene and comparing to other isolates. When this isolate was excluded from the comparison, the similarity levels increased, ranging from 87.3 to 90.3% between BoHV-1 and BoHV-5. Moreover, a conserved pattern with 96.2 to 100% nt identity was observed among BoHV-5 sequences, noticeably at the central region of the amplified fragment. The degree of similarity among all BoHV-1 sequences ranged from 84.4 to 100%. However, with the exclusion of SV 453/93, a highly conserved region was evidenced among

239 BoHV-1 isolates (99.1 to 100% of similarity). The consistent alignment of 333 nt,
240 corresponding to the central region of the amplified fragment, showed punctual mutations
241 throughout the entire sequences of the nearby isolates, revealing a moderately conserved 3'
242 region. When isolate SV453/93 was excluded, the gC 3' region was shown to be moderately
243 conserved between BoHV-1 and 5, more conserved among BoHV-5 and highly conserved
244 among BoHV-1 sequences.
245
246 3.2. Phylogenetic analysis
247
248 The phylogenetic tree was constructed by Maximum Likelihood method and yielded
249 two major clusters, BoHV-1 (n=16) and BoHV-5 (n=32) (Fig. 2). The first branch was
250 divided into subtypes BoHV-1.1 (n=9) and BoHV-1.2 (n=6), and SV 453/93 felt into a
251 distinct branch. The BoHV-1 reference sequence and Cooper, LAM and Los Angeles strains
252 were grouped in the BoHV-1.1 cluster together with five isolates. The BoHV-5 cluster was
253 not clearly separated into distinct subtypes, but the ISO 97/45 isolate was grouped separately
254 from the other isolates. A similar tree topology was previously described (Esteves et al.,
255 2008) after analysis of a smaller number of isolates. The phylogenetic comparison with
256 ruminant alphaherpesvirus and the identity matrix (data not shown) confirmed that SV 453/93
257 is more similar to BoHV-1 than to other alphaherpesvirus. The phylogenetic analysis of 5'
258 region of gC gene showed that SV 453/93 grouped in a separate branch within the BoHV-1
259 cluster, close to SV 47/05. The phylogenetic tree reconstruction based on 3' region of gD
260 gene demonstrated that SV 453/93 clustered with another isolate in a distinct branch
261 separately from BoHV-1 and BoHV-5 groups.
262 In summary, BoHV-1 isolates were grouped in BoHV-1.1 (along with reference
263 sequence) and BoHV-1.2 clusters, revealing a clearly classification in subtypes as reported
264 earlier (Rijsewijk et al., 1999; Esteves et al., 2008). The SV 453/93 was located in a distinct
265 branch inside the BoHV-1 cluster. The BoHV-5 grouping did not allow a subtype
266 differentiation; yet ISO 97/45 seems to belong to a different subgroup together with the "non-
267 a non-b" BoHV-5 sequences obtained from GenBank (D'Arce et al., 2002; Esteves et al.,
268 2008).
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270
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3.3. Hydropathicity and transmembrane region analysis

The predicted aa sequences of all isolates showed homology with BoHV gC, i.e., all studied fragments potentially codify for the gC COOH-t region. A few residue modifications in the predicted aa were observed in comparison to the predicted aa of the reference sequences. The major changes were observed in isolate SV 453/93 (24 aa changes in a sequence of 168 residues in length when compared with the predicted aa of AJ004801 gC). These changes include five changes for proline and three for glycine.

The hydropathicity analysis and prediction of transmembrane helices of BoHV-1 or BoHV-5 aa sequences demonstrated three distinct regions: an extense (136 to 154 aa) and predominantly hydrophilic extra-virion region; a hydrophobic transmembrane helix containing 24 aa and; eight hydrophilic aa at the 3' end, corresponding to the cytoplasmic tail. The alignment of the deduced aa of BoHV-1 isolates showed a highly conserved cytoplasmic domain. Moreover, we identified a conserved cysteine at residue 499 of BoHV-1 gC but not in the BoHV-5 gC, where a cysteine is present in almost all isolates at residue 477 but is absent in other strains such as EVI 345/96, SV 102/07 and A 613.

4. Discussion

We analyzed the 3' region of the gC gene of 45 BoHV-1 and BoHV-5 isolates obtained from clinical specimens in Brazil, Uruguay and Argentina (1981-2009). The levels of nt similarity were compared to determine whether this region is suitable for phylogenetic classification. Among the 45 isolates, 32 were classified as BoHV-5 and 13 as BoHV-1. The higher number of BoHV-5 isolates compared with BoHV-1 does not necessarily reflect a higher prevalence or circulation of this virus in the region. Rather, it may reflect the higher impact of neurological disease over other clinical manifestations, which calls more attention of farmers and veterinarians leading to more frequent diagnosis submissions. Although other reports of the genetic characterization of BoHV-1 and BoHV-5 gC gene are available (Esteves et al., 2008), the present study provides the most comprehensive analysis of a larger number of isolates. Furthermore, it includes the phylogenetic study of four BoHV-1 isolated from cases of neurological disease, an association otherwise attributed to BoHV-5 (Silva et al., 2007).

Bovine herpesvirus 1 gC gene is homologous to genes found in other alphaherpesviruses (e.g. HSV-1 gC and PRV gIII) and its product displays features of a typical type I integral membrane glycoprotein. It contains a signal peptide, an intermediary region with a highly hydrophilic and glycosylated NH_2-t ectodomain, a single hydrophobic transmembrane anchor and a COOH-t hydrophilic tail (Fitzpatrick et al., 1989; Solomon et al., 1990; Skoff and Holland, 1993; Okazaki et al., 1994). Based on the hydropathicity profile and the prediction of transmembrane helices, the regions identified in the sequenced gC 3' fragment were: 1) An extra-virion region, predominantly hydrophilic, composed of 136 to 154 aa residues (depending on the length of the obtained edited sequence); 2) A highly hydrophobic (likely transmembrane) domain of 24 aa, located between residues 477 and 500 of BoHV-1 gC (based on the deduced aa of reference sequence) and between residues 455 and 478 of BoHV-5 gC; 3) A short intravirion (cytoplasmic) domain composed of eight hydrophilic residues at the COOH-end, corresponding to residues 501 – 508 of BoHV-1 gC and 479 – 486 of BoHV-5 gC. It has been previously shown that the gC COOH-t hydrophilic region of BoHV-5 is smaller than that of BoHV-1 (Chowdhury, 1995).

It should be noted that our findings on the size and positions of gC domains differ substantially from previous data (Fitzpatrick et al., 1989). These authors located the transmembrane domain of BoHV-1 gC (Cooper) between residues 467 and 500 (34 aa in length) and the putative cytoplasmic tail between residues 501 and 521 (21 aa in length). Thereafter, these data have been used by others (Okazaki et al, 1994; Chowdhury, 1995; 1997; Rijsewijk et al., 1999). On the other hand, our findings are compatible with previous studies on the length of gC1 and gC5 (Delhon et al, 2003) and are corroborated by studies on other alphaherpesvirus, namely HSV and PRV (Holland et al., 1988; Solomon et al., 1990). In this sense, a potential mistake in the sequence obtained of Fitzpatrick et al. (1989) was already pointed out by Hecht et al. (1995) and, thus, might explain the differences found at the C terminus. Moreover, the cysteine in residue 499 of gC1 and 477 of almost all gC5 is conserved in nearly all herpesvirus gC homologues (exceptions are HSV-1 and -2) and it is located near the beginning of the cytoplasmic domain. This residue was considered the key feature defining the transition between the hydrophobic transmembrane region and the cytoplasmic tail of PRV gIII. However, the structural or functional role of this residue in herpesvirus gC remains to be determined (Solomon et al., 1990).

Glycoprotein gC presents a 75% aa identity between prototypes BoHV-1 and BoHV-5, with major variation at the NH_2-t third (Delhon et al., 2003). The COOH two-thirds portion of gC is more conserved among alphaherpesviruses such as HSV-1, HSV-2, varicela zoster

virus, PRV, equine herpesvirus type 1 and BoHV-1 (Fitzpatrick et al., 1989; Solomon et al., 1990). In addition, it is highly conserved among BoHV-1 and BoHV-5 types and subtypes (Okazaki et al., 1994; Esteves et al., 2008). This suggests an important biological function, not yet completely known. The number of positively charged residues in the small COOH-t cytoplasmic domain of HSV-1 gC suggests a role for this domain in protein processing (Holland et al., 1988; Skoff and Holland, 1993). The cytoplasmic domain of HSV-1 gC might interact with capsid or tegument proteins during budding of the nucleocapsid through the inner nuclear membrane to promote specific incorporation of gC into nascent virions, but it is not essential for the process (Holland et al., 1988). Similarly, this cytoplasmic domain is not required for membrane anchoring; the stable anchoring of the HSV-1 gC in the plasma membrane is provided by the transmembrane domain (Skoff and Holland, 1993). The putative cytoplasmic domain of PRV gIII is also not required for transport to the cell surface, for stable membrane anchoring or for incorporation of gIII into virions (Solomon et al., 1990). Regardless of the function, the high level of conservation of gC COOH-t has enabled its use for grouping of alphaherpesviruses into types and subtypes (Ros and Belák, 1999; Esteves et al., 2008).

In the present study, the phylogenetic analysis based on nt sequences yielded clusters corresponding to viral types and subtypes. Supported by high bootstrap values (>50%), three groups were evident: BoHV-1.1, BoHV-1.2 and BoHV-5 (Fig. 2). This grouping agrees with that in another phylogenetic study using the gC COOH-t region (Esteves et al., 2008) and with phylogenetic studies using gB and gD coding regions (Ros and Belák, 1999). Thus, phylogenetic analysis based on conserved regions of the three major envelope glycoproteins apparently results in similar grouping/classification of BoHV-1 and BoHV-5 isolates.

The phylogenetic analysis based on 3' region of gC gene clearly grouped the BoHV-1 isolates into two clusters corresponding to BoHV-1.1 and BoHV-1.2 (Fig. 2). The bovine herpesvirus reference strains Cooper, LAM, and Los Angeles as well as the BoHV-1 reference sequence, BoHV-1.1 (NCBI; Esteves et al., 2008), were grouped together with five BoHV-1.1 isolates. The BoHV-1.1 subtype has been classically associated with respiratory isolates, and BoHV-1.2 comprises mostly viruses associated with genital disease (Metzler et al., 1986; Rijsewijk et al., 1999; D'Arce et al., 2002; Spilki et al., 2005). Thus, the classification of BoHV-1 into subtypes BoHV-1.1 and 1.2 based on the phylogenetic analysis of gC COOH-t (and gB, gD as well) appears to correlate with specific biological properties, determinants of tissue tropism and pathogenesis. However, this classification may not reflect a definitive association between genotypes and phenotypic traits. For example, isolate SV

374 56/90, herein and elsewhere classified as BoHV-1.1 (Roehe et al., 1997), was isolated from
375 an outbreak of balanoposthitis in bulls (Weiblen et al., 1992). This isolate was shown to be
376 very virulent for bulls and heifers upon genital inoculation (Vogel et al., 2004; Henzel et al.,
377 2008). Similarly, isolates SV 265/96 and SV 261/07 were recovered from animals with
378 respiratory disease and were here classified as BoHV-1.2, which is a subtype historically
379 associated with genital disease (Metzler et al., 1986; D'Arce et al., 2002). The same
380 classification of SV 265/96, i.e., as a respiratory BoHV-1.2a isolate, was suggested by others
381 (D'Arce et al., 2002; Esteves et al., 2008). SV 265/96 was grouped together with SV 169/06,
382 a typical genital isolate, in a distinct branch within BoHV-1.2; SV 261/07 was grouped
383 together with two BoHV-1.2 isolates recovered from animals with neurological disease (SV
384 63/03 and SV 1613/93) (Silva et al., 2007) (Fig. 2). The other two neurological BoHV-1
385 isolates characterized in the present study, SV 609/03 and SV 47/05, were grouped in the
386 BoHV-1.1 subtype cluster (Fig. 2). Hence, the neurological BoHV-1 isolates grouped
387 together with typical BoHV-1 as ascertained by nt analysis of gC 3' region. Thus,
388 phylogenetic grouping based on 3' region of gC gene may provide a suitable means of
389 classification of BoHV-1 and BoHV-5 into subtypes but it does not necessarily reflects a
390 definitive and absolute association with phenotypic traits.
391 In the same phylogenetic analysis, the BoHV-5 Australian strain N569 obtained from
392 GenBank was located separately from BoHV-5 isolates originating from Brazil, Argentina
393 and Uruguay, which were grouped together (Fig. 2). The segregation of South American
394 isolates and the Australian BoHV-5 reference strain was already described (Esteves et al.,
395 2008). In that study, the nt analysis showed that South American BoHV-5 isolates were more
396 closely related to each other than to N569. The location of the Australian strain in another
397 branch indicates that the BoHV-5 grouping is apparently related to the geographical origin of
398 this virus (Esteves et al., 2008). Another possible explanation is provided by the temporal
399 occurrence: the N569 strain was isolated many decades apart from the other BoHV-5 isolates
400 (French, 1962). Regardless, the Australian prototype strain N569 was characterized as
401 BoHV-5 "a", similarly to the Brazilian isolates SV 136/88, EVI 88/95 and EVI 340/96.
402 Argentinean strain A 663, retrieved from GenBank, was identified as BoHV-5 "b" by
403 reactivity with MAbs and REA (D'Arce et al., 2002). Thus, according to the topology of the
404 BoHV-5 cluster found here and depicted in Fig. 2, a specific classification into "5a" or "5b"
405 subtypes could not be determined. However, the ISO 97/45 isolate was grouped separately
406 from other BoHV-5 isolates of this study (Fig. 2) but together with ISO 45 and ISO 87
407 sequences obtained from GenBank, which seems to result in a grouping distinct from the "a"

and “b” subtypes (D'Arce et al., 2002). Thus, these viruses were considered previously to be “non-a non-b” BoHV-5 (D'Arce et al., 2002). A similar pattern of tree topology was already demonstrated (Esteves et al., 2008). Thus, the BoHV-5 cluster was not clearly separated into distinct subtypes, and the grouping of BoHV-5 isolates that were supported by bootstraps values higher than 50% (A613 and SV 437/04; Uruguay T4 and SV 102/07; Uruguay T2 and SV 136/88; 002/00 and 97/642) (Fig. 2) apparently does not reflect either biological or temporal trends. The presence of three guanines only in the BoHV-1 isolates as observed in this study (Fig. 1B) was reported previously (Esteves et al., 2008).

A particular BoHV-1 isolate, SV 453/93, which was recovered from a cow with vulvovaginitis in Brazil, presented a different nt and aa pattern on the 3’ region of gC gene (Fig. 1A) and was located in a branch distinct from BoHV-1.1 and 1.2 clusters (Fig. 2). To confirm its identity, a phylogenetic comparison with some ruminant alphaherpesvirus and a similarity matrix were performed. The results confirmed a higher level of similarity with BoHV-1. Indeed, this isolate has been already classified as BoHV-1.2 by MAb binding (Roehe et al., 1997). The predicted aa of SV 453/93 showed the highest number of aa changes (24 in total) in comparison with all other sequences. Ongoing phylogenetic analysis on 5’ region of gC and 3’ region of gD gene showed that SV 453/93 tends to cluster separately (out or within) from BoHV-1 group, but not necessarily distant. Therefore, based on gC 3’ region, SV 453/93 was the most dissimilar and tentatively classified as a nontypeable BoHV-1 isolate. Nevertheless, preliminary studies on 5’ region of gC and gD gene do not support this finding yet further studies are needed for a definitive classification of this isolate.

5. Conclusion

The amplification, sequencing and phylogenetic analysis of the 3’ region of the gC gene of bovine herpesvirus isolates allowed molecular differentiation and phylogenetic classification of BoHV-1.1, BoHV-1.2 and BoHV-5. These results reinforce gC COOH-t as a suitable target for classification of BoHV into types and subtypes, even with a large number of isolates and including BoHV-1 isolates associated with neurological disease. Genetic grouping based on this region would probably be of genetic (and perhaps evolutionary) value but might not have a clear and definitive biological significance. The mapping of potential structural domains allowed the prediction of unusual length and location of BoHV gC

transmembrane helix and putative cytoplasmic tail. For a better understanding and a more accurate phylogenetic classification (mainly for the isolate SV 453/93 and the BoHV-5 cluster), the 5' gC region and other envelope glycoproteins genes are currently being studied.

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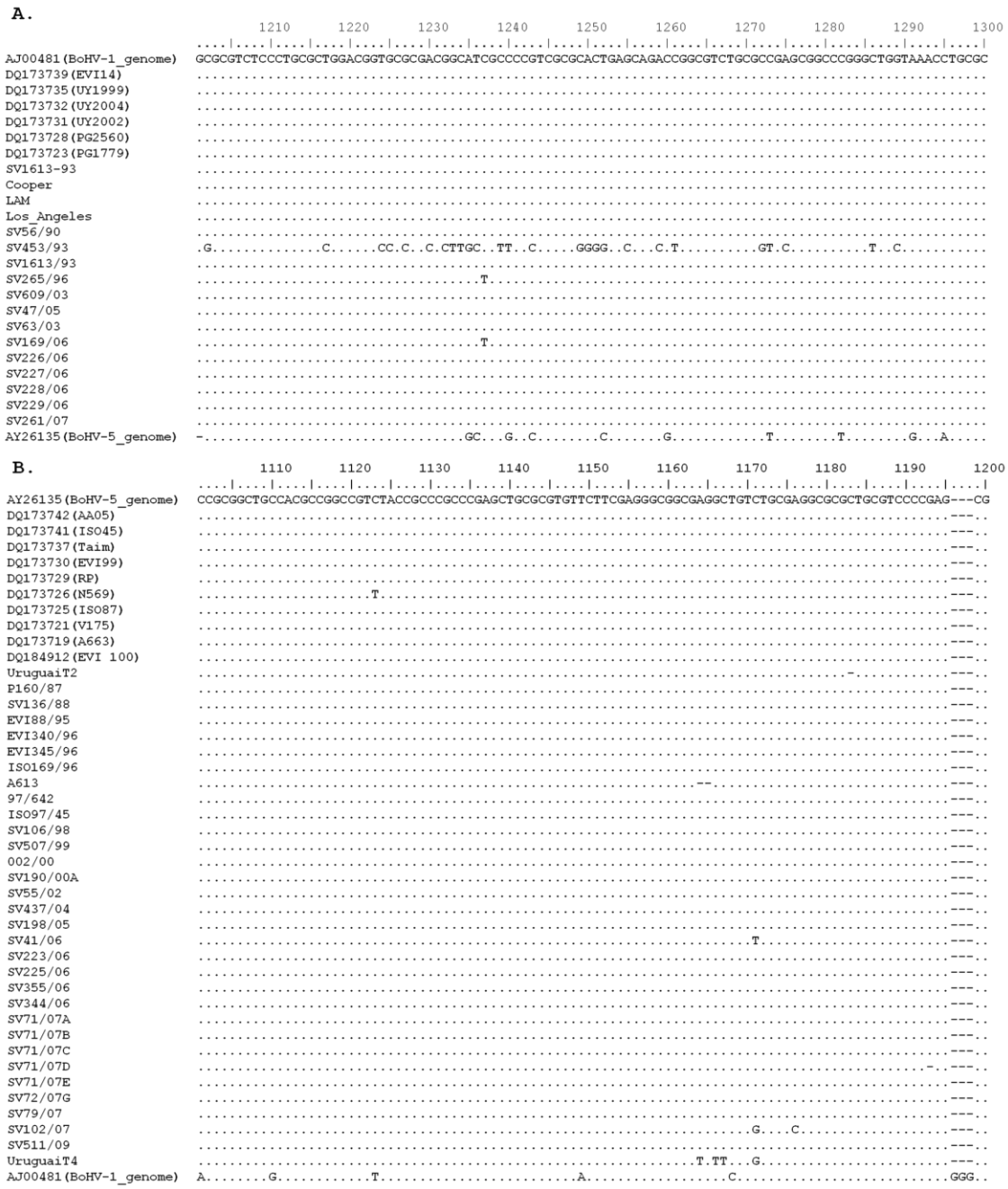
618 **Table 1**

619 Bovine herpesvirus type 1 (BoHV-1) and BoHV-5 isolates from Brazil (BR), Uruguay (UY)
620 and Argentina (AR) and reference strains included in the present study

Virus	Classification	Clinical sings/material	Country/state	Year	GenBank ID
Cooper	BoHV-1.1	Respiratory	US	(-)	JN173204
LAM	BoHV-1.1	Respiratory	NL	(-)	JN173205
Los Angeles	BoHV-1.1	Respiratory	US	(-)	JN173206
SV 56/90	BoHV-1.1	Genital/preputial lavage	BR/RS	1990	JN173207
SV 453/93	BoHV-1	Genital/vaginal swab	BR/RS	1993	JN173208
SV 1613/93	BoHV-1.2	Neurological/brain	BR/RS	1993	JN173209
SV 265/96	BoHV-1.2	Respiratory/nasal swab	BR/RS	1996	JN173210
SV 609/03	BoHV-1.1	Neurological/brain	BR/RS	2003	JN173211
SV 47/05	BoHV-1.1	Neurological/brain	BR/RS	2005	JN173212
SV 63/06	BoHV-1.2	Neurological/brain	BR/RS	2006	JN173213
SV 169/06	BoHV-1.2	Genital/vaginal swab	BR/RS	2006	JN173214
SV 226/06	BoHV-1.1	Routine monitoring/semen	BR/SP	2006	JN173215
SV 227/06	BoHV-1.1	Routine monitoring/semen	BR/SP	2006	JN173216
SV 228/06	BoHV-1.2	Routine monitoring/semen	BR/SP	2006	JN173217
SV 229/06	BoHV-1.1	Routine monitoring/semen	BR/SP	2006	JN173218
SV 261/07	BoHV-1.2	Respiratory/nasal swab	BR/RS	2007	JN173219
Uruguay T2	BoHV-5	Neurological/brain	UY	1981	JN173220
P 160/87	BoHV-5	Neurological/brain	BR/RJ	1987	JN173221
SV 136/88	BoHV-5	Neurological/brain	BR/RS	1988	JN173222
EVI 88/95	BoHV-5	Neurological/brain	BR/MS	1995	JN173223
EVI 340/96	BoHV-5	Neurological/brain	BR/MS	1996	JN173224
EVI 345/96	BoHV-5	Neurological/brain	BR/MS	1996	JN173225
ISO 169/96	BoHV-5	Neurological/brain	BR/SP	1996	JN173226
A 613 (97/613)	BoHV-5	Neurological/brain	AR	1997	JN173227
97/642	BoHV-5	Neurological/brain	AR	1997	JN173228
ISO 97/45	BoHV-5	Neurological/brain	BR/MG	1997	JN173229
SV 106/98	BoHV-5	Neurological/brain	BR/RS	1998	JN173230
SV 507/99	BoHV-5	Neurological/brain	BR/RS	1999	JN173231
002/00	BoHV-5	Systemic/spleen	BR/RS	2000	JN173232
SV 190/00A	BoHV-5	Neurological/brain	BR/MS	2000	JN173233
SV 55/02	BoHV-5	Neurological/brain	BR/RS	2002	JN173234
SV 437/04	BoHV-5	Neurological/brain	BR/RS	2004	JN173235
SV 198/05	BoHV-5	Neurological/brain	BR/RS	2005	JN173236
SV 41/06	BoHV-5	Neurological/brain	BR/MS	2006	JN173237
SV 223/06	BoHV-5	Routine monitoring/semen	BR/SP	2006	JN173238
SV 225/06	BoHV-5	Routine monitoring/semen	BR/SP	2006	JN173239
SV 355/06	BoHV-5	Neurological/brain	BR/RS	2006	JN173240
SV 344/06	BoHV-5	Neurological/brain	BR/MS	2006	JN173241
SV 71/07 A	BoHV-5	Neurological/brain	BR/MS	2007	JN173242
SV 71/07 B	BoHV-5	Neurological/brain	BR/MS	2007	JN173243
SV 71/07 C	BoHV-5	Neurological/brain	BR/MS	2007	JN173244
SV 71/07 D	BoHV-5	Neurological/brain	BR/MS	2007	JN173245
SV 71/07 E	BoHV-5	Neurological/brain	BR/MS	2007	JN173246
SV 72/07 G	BoHV-5	Neurological/brain	BR/MS	2007	JN173247
SV 79/07	BoHV-5	Neurological/brain	BR/RS	2007	JN173248
SV 102/07	BoHV-5	Neurological/brain	BR/RS	2007	JN173249
SV 511/09	BoHV-5	Neurological/brain	BR/SP	2009	JN173250
Uruguay T4	BoHV-5	Neurological/brain	UY	(-)	JN173251

621 US – United States of America; NL – Netherlands. BR states: RS – Rio Grande do Sul; SP – Sao Paulo; RJ –
622 Rio de Janeiro; MS – Mato Grosso do Sul; MG – Minas Gerais; (-) – no information.
623



624

625 **Fig. 1.** Nucleotide sequence alignment of the carboxy-terminal (COOH-t) region of
626 glycoprotein C (gC) gene of bovine herpesvirus 1 (BoHV-1, n=15; A) and BoHV-5 (n=32;
627 B). Reference sequences of BoHV-1 and BoHV-5 (GenBank ID AJ004801 and AY261359)
628 and gC COOH-t nucleotide sequences of each subtype (seven of BoHV-1 and 10 of BoHV-
629 5), recorded in the GenBank, were included for comparison. The gC gene position is shown
630 above.

631

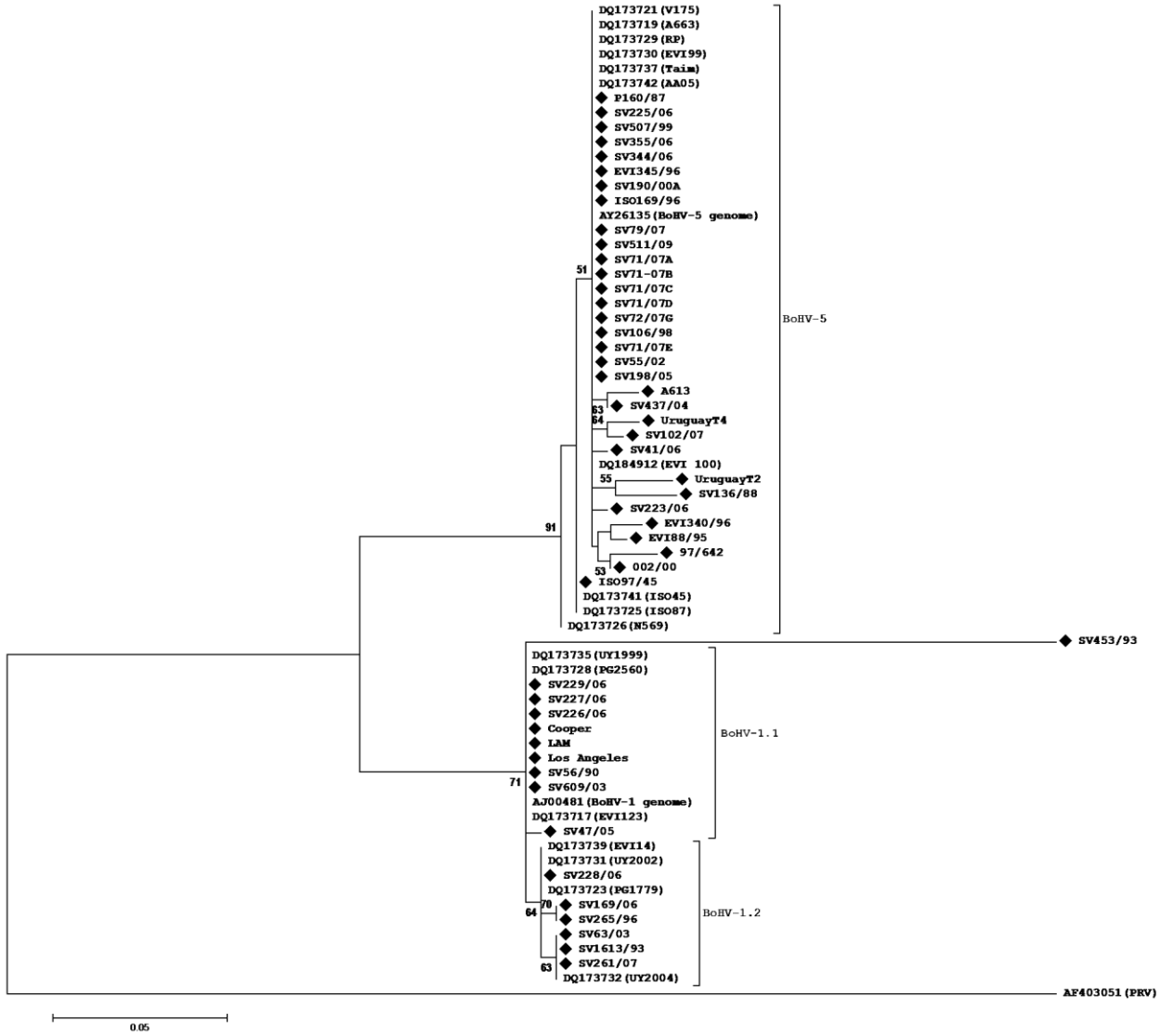


Fig. 2. Phylogenetic tree based on Maximum Likelihood method for the nucleotide sequence of the 3' region of the glycoprotein C gene of 32 bovine herpesvirus type 5 (BoHV-5), 13 BoHV-1 isolates and three BoHV-1 strains (♦; 226 positions). Only bootstraps values higher than 50% of 2000 replicates are shown.

4 CAPÍTULO 3

Glycoprotein D-based phylogeny of typical and neurological bovine herpesviruses 1 and 5[1]

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Bovine herpesviruses 1 (BoHV-1) and 5 (BoHV-5) are closely related pathogens of cattle, associated with respiratory/genital and neurological disease, respectively. Nonetheless, cases of encephalitis caused by BoHV-1 have been occasionally reported. Envelope glycoprotein D of BoHV-1 (gD1) and BoHV-5 (gD5) is involved in virus attachment, penetration and cell fusion, and may also participate in viral neuropathogenesis. In addition, gD gene is well conserved and, as such, may be used for phylogenetic analysis. In this study, a genetic and phylogenetic study was performed to investigate genetic divergences at the 3' region of gD gene of respiratory/genital BoHV-1 (n=7), neurological BoHV-5 (n=7) and neurological BoHV-1 (n=7) isolates. Differences in this region could be associated with the respective clinical phenotype. In addition, this genomic region might serve for phylogenetic classification. The isolates/strains were initially differentiated as BoHV-1 or BoHV-5 by a differential PCR. Then, 3' region of gD gene was sequenced and analyzed; and the amino acid (aa) sequence was deduced. The phylogenetic reconstruction based on nucleotide (nt) and aa allowed for a clear differentiation of BoHV-1 (n=14) and BoHV-5 (n=7) in different clusters. The seven BoHV-1 isolates from neurological disease grouped within BoHV-1 branch. A consistent alignment of 310 nt revealed a highly conserved region within each specie and a least conserved between gD1 and gD5. The nt and aa similarity levels were on average 98.3% among gD1; 97.8% and 95.8% among gD5 and 73.7% and 64.1% between both viruses. Thus, the phylogenetic and identity similarity levels allowed for differentiation/classification of BoHV-1 and BoHV-5 species. However, no conclusion of a possible involvement of gD 3' nucleotide sequence in determination of the neurovirulent phenotype could be drawn.

INDEX TERMS: BoHV-1, BoHV-5, gD, genetic analysis, meningoencephalitis.

INTRODUCTION

Bovine herpesviruses 1 (BoHV-1) and 5 (BoHV-5) are major pathogens of cattle, members of the family *Herpesviridae*, the subfamily *Alphaherpesvirinae* and the genus *Varicellovirus* (Roizmann et al. 1992). These viruses are very similar in genetic, antigenic and biological aspects, what pose problems for diagnosis and disease control. Their DNA genome and protein repertoire display around 85% nucleotide (nt) and 82% amino acid (aa) identity, respectively (Engels et al. 1987, Delhon et al., 2003). The major divergence between these viruses seems to be in specific biological aspects (Belknap et al. 1994). BoHV-1 has been historically associated with respiratory (infectious bovine rhinotracheitis – IBR) and genital/reproductive disease (vulvovaginitis/balanoposthitis – IPV/IPB and abortions) (Bagust 1972, Weiblen et al. 1989, Crook et al. 2012). The neuropathogenic BoHV-5 is frequently involved in outbreaks of encephalitis, reported mainly in South American countries (Guarino et al. 2008, Salvador et al. 1998, Weiblen et al. 1989).

Respiratory and reproductive BoHV-1 isolates have been classified as BoHV-1.1 and genital isolates as BoHV-1.2 by restriction enzyme analysis, viral polypeptide profile or reactivity with monoclonal antibodies (D'Arce et al. 2002, Metzler et al. 1986, Rijsewijk et al. 1999, Spilki et al. 2005). BoHV-1.2 was further divided in “a” and “b” (Metzler et al. 1986). Due to the high virological and antigenic similarity, the neurological isolates were previously classified as BoHV-1.3 (Belknap et al. 1994, Metzler et al. 1986), however, the clinical, epidemiological and genetic differences subsequently detected justified the classification as a new virus specie, named BoHV-5 (Roizmann et al. 1992). Likewise, BoHV-5 can be sub-classified into “a”, “b” and “non-a non-b” subtypes based on antigenic and/or molecular differences (D'Arce et al. 2002).

As more BoHV isolates were being obtained and characterized at the molecular level, it has been shown that some isolates may escape the classical classification. For instance, a phylogenetic analysis based on the 3’ region of glycoprotein C gene, identified BoHV-1.1 and 1.2 DNA in specimens from respiratory, genital or neurological disease, and BoHV-5 DNA was demonstrated in bull semen (Traesel et al. 2013). BoHV-1 was already isolated from the brain of cattle with neurological disease (Ely et al. 1996, Furuoka et al. 1995, Penny et al. 2002, Roels et al. 2000, Rissi et al. 2008, Silva et al. 2007). It is known that both viruses are neurotropic, but efficient replication in the central nervous system (CNS) leading to with clinical encephalitis is primarily attributed to BoHV-5 (Belknap et al. 1994, Chowdhury et al. 1997, Metzler et al. 1986, Silva et al. 2007). Therefore, more phylogenetic and molecular

studies are needed to better refine the BoHV classification and, as a consequence, to define the possible association between genetic traits and clinical aspects.

Despite of the fact that some BoHV-1 and BoHV-5 genes present genetic divergence, most of approximately 70 proteins encoded by the viral genome share high levels of aa identity, such as the genes encoding proteins involved in viral DNA replication and virion protein processing (Delhon et al. 2003). The gene encoding glycoprotein D (gD) is located in the unique short region (Us6) of the genome and encodes a polypeptide of 417 aa (Delhon et al. 2003). Glycoprotein D is a typical type 1 glycoprotein and the open reading frame (ORF) consists of an 18 amino acids signal peptide, the extracellular domain, a hydrophobic transmembrane domain between residues 361 and 389 and, finally, a 28 amino acid cytoplasmic tail (Tikoo et al. 1990). BoHV-1 and BoHV-5 gD (gD1 and gD5) gene products are 79.9 (Gabev et al. 2010) to 98% identical (Delhon et al. 2003). The major differences between gD1 and gD5 were mapped to the carboxy-terminal (COOH-t) region, including a glicin-rich ectodomain, between residues 280 and 354 of gD5 (Abdelmagid et al. 1995, Gabev et al. 2010). The COOH-t region can contribute to the protein-protein interactions (Tikoo et al. 1993) and these differences could be involved in the determination of biological properties of the two viruses. Herpes simplex virus (HSV-1) gD is believed to be involved in the neuroinvasiveness (Izumi & Stevens 1990). However, this may not occur with BoHV as a previous study showed that gD5 may not contribute to invasion to the brain, but it would confer an extended host range to BoHV-1 (Gabev et al. 2010).

Glycoprotein D is critical for virus-host interactions, acting as an important determinant of host range and is a major antigen recognized by the immune system (Abdelmagid et al. 1995, Mettenleiter 2003). This glycoprotein mediates infection of target cells being an essential receptor binding protein for many alfaherpesviruses (Campadelli-Fiume et al. 2007, Spear 2004) and, thus, influencing cell and tissue tropism. Members of the tumor necrosis factor (TNF) receptor family (HveA) and of the poliovirus receptor family (HveB or nectin 2 and HveC or nectin 1), and specific sites in 3-O-sulfated heparan sulfate, can mediate herpesvirus entry (Spear et al. 2000). Each viral serotype could have somewhat different receptor preferences (Spear et al. 2000). Studies indicate that nectin 1 can serve as a receptor for gD1 (Connolly et al. 2001), but it seems not to be essential for BoHV-5 productively replication *in vitro* (Gabev et al. 2010). Moreover, gD is involved in cell to cell transmission (Liang et al. 1995, Tikoo et al. 1995). The binding to cell receptors appears to occur at the amino-terminal region of gD (Spear & Longnecker 2003). This region is also important for the generation of the immune response, inducing virus-neutralizing antibody

activity (Abdelmagid et al. 1995) and a stronger and consistent cellular immune response to BoHV-1 (Hutchings et al. 1990).

Although previous studies disagree about the level of aa similarity between gD1 and gD5 (98% - Delhon et al. 2003; 79.9% - Gabev et al. 2010), gD-based phylogenetic analysis of ruminant alphaherpesvirus was already performed and allowed for the classification in viral species and subtypes (Ros & Belak 1999). To clarify this disagreement on similarity levels (Delhon et al. 2003, Gabev et al. 2010), to identify major differences in the COOH-t region (Abdelmagid et al. 1995, Gabev et al. 2010) and to investigate possible associations with the clinical presentations, we conducted a phylogenetic study based on the 3' region of gD gene, including BoHV-1 respiratory and genital isolates, BoHV-5 neurological isolates and BoHV-1 isolates recovered from the brain of cattle with neurological disease.

MATERIALS AND METHODS

Twenty-one BoHV isolates (7 typical BoHV-1, 7 BoHV-1 recovered from animals with neurological disease and 7 BoHV-5 from neurological disease) obtained from clinical specimens from Brazil, one Argentinean and one North American strain, isolated from 1990-2012, were analyzed. BoHV-1 and BoHV-5 viral DNA samples were initially submitted to a differential PCR already described by Ashbaugh et al. (1997) and modified by Silva et al. (2007) and, then, submitted to a second PCR specific for the 3' region of gD gene. The amplicons were sequenced and a phylogenetic study was performed with the nt and predicted aa sequences.

Viruses, cells and DNA extraction

Seven BoHV-1 isolates recovered from respiratory (Cooper, SV265/96, EVI123/98, SV261/07) or genital disease (SV56/90, SV453/93, SV169/06), seven BoHV-5 isolates from neurological disease (EVI88/95, EVI345/96, A613, ISO97/45, SV507/99, SV198/05, SV387/12) and seven BoHV-1 isolates recovered from the brain of cattle with neurological signs (SV1613/93, SV609/03, SV47/05, SV63/06, SV167/10, SV178/10 and SV194/10) were used for this study. The information about the viral specie, clinical presentation, local and date of isolation of BoHV isolates/strains included in this study are shown in table 1. Virus isolation, amplification and cloning were performed in a MDBK-derived cell line named

CRIB (Flores & Donis 1995). Total DNA was extracted from CRIB cells infected with each isolate using DNAzol reagent (Invitrogen, Carlsbad, CA, USA). The procedures with viruses and DNA extraction were already described in details (Traesel et al. 2013).

PCR amplification

A first 5' gC gene-based PCR able to differentiate BoHV-1 from BoHV-5 by different amplicon lengths already described (Ashbaugh et al. 1997) and modified (Silva et al. 2007) was performed. Isolates classified as BoHV-1 or BoHV-5 were then submitted to another PCR, based on the 3' region of gD gene. A pair of specific primers was used: forward 5'-TCTCACGCAGTACTACCC -3' and reverse 5'- CGCGCTGTAGTTGACGTTG -3'. PCR resulted in amplicons of 556 base pairs (bp) for BoHV-1 (genome AJ004801 position 119582-120137) and 553 bp for BoHV-5 (AY261359 genome position 121819-122370). Approximately 1 µg of total DNA extracted from infected cells was used as a template in 47 µL of a mix containing ultrapure water, 25 µM of each primer, 0.4 mM of deoxyribonucleotides, 10% of DMSO, 4 mM of $MgCl_2$, 10% of Taq 10x buffer and 1U of Taq DNA polymerase (Invitrogen, Carlsbad, CA, USA). PCR conditions were as follows: a denaturation step of 5 min at 94 ºC, followed by 40 cycles of denaturation at 94 ºC for 50 sec, annealing at 59 ºC for 55 sec and extension at 72 ºC for 1 min. Cycling was followed by one last extension step of 10 min at 72 ºC. PCR products were analyzed under UV light after electrophoresis in a 1% agarose gel stained with GelRed (Biotium, Hayward, CA, USA), using a 100 pb DNA ladder (Ludwig Biotec, Alvorada, RS, BR). In all procedures, total DNA extracted from mock-infected CRIB cells and from cells infected with the reference strains of BoHV-1 (Cooper) and BoHV-5 (SV507/99) was used as controls.

Nucleotide sequencing

For nucleotide sequencing, PCR products were purified using the Illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare, Buckinghamshire, UK), according to the manufacturer's instructions, and 30-60 ng of DNA amplicon, 4.5 pmol of forward or reverse specific 3' gD primers (described in 2.2) and ultrapure water to a final volume of 6 µl were used. Sequencing reactions were performed in duplicates (each product was sequenced twice in both directions) by ACTGene Analises Moleculares Ltd. (Centro de Biotecnologia, UFRGS, Porto Alegre, RS, Brazil) using the automatic sequencer ABI-PRISM 3100 Genetic

Analyzer armed with 50 cm capillaries and POP6 polymer (Applied Biosystems, Carlsbad, CA, USA). DNA templates (30 to 45 ng) were labeled with 3.2 pmol of the specif primer and 3 μL of BigDye Terminator v3.1 Cycle Sequencing RR-100 (Applied Biosystems, Carlsbad, CA, USA) in a final volume of 10 μL. Labeling reactions were performed in a GeneAmp PCR System 9700 (Applied Biosystems, Carlsbad, CA, USA) thermocycler with a initial denaturing step of 96 ºC for 3 min followed by 25 cycles of 96 ºC for 10 sec, 55 ºC for 5 sec and 60 ºC for 4 min. Labeled samples were purified by isopropanol precipitation followed by 70% ethanol rinsing. Precipitated products were suspended in 10 μL formamide, denatured at 95 ºC for 5 min, ice-cooled for 5 min and electroinjected in the automatic sequencer. Sequencing data were collected using the software Data Collection v 1.0.1 (Applied Biosystems, Carlsbad, CA, USA) programmed with the following parameters: Dye Set "Z"; Mobility File "DT3100POP6{BDv3}v1.mob"; BioLIMS Project "3100_Project1"; Run Module 1 "StdSeq50_POP6_50cm_cfv_100"; and Analysis Module 1 "BC-3100SR_Seq_FASTA.saz".

Nucleotide sequences analysis and deduced amino acid sequences

The quality of the obtained DNA sequences was analyzed by the GAP software implemented in the Staden Package (Staden 1996) and overlapping fragments of each sequence were assembled. The consensus sequence was submitted for comparison with BoHV-1.1 (strain Cooper; GenBank ID AJ004801) and BoHV-5 (SV 507/99; GenBank ID AY261359) complete genomes, using NCBI database and BLAST software (http://www.ncbi.nlm.nih.gov/BLAST/) (Altschul et al. 1997).

Assembled consensus sequences of the isolates and reference BoHV-1 and 5 sequences (GenBank ID AJ004801 and AY261359) were aligned and edited by BioEdit Sequence Alignment Editor software suite, version 7.0.5.3 (Hall 1999). Based on nt sequences, the aa sequences of the corresponding gD region (COOH-t) of each isolate/strain were translated in reading frame 1 on the direct strand by The Sequence Manipulation Suite software version 2 (http://www.bioinformatics.org/sms2/). The nt sequence translations were submitted to homology comparison using the protein BLAST software (http://www.ncbi.nlm.nih.gov/BLAST/). The sequences alignments (nt and deduced aa) were performed using Clustal W software (Thompson et al. 1997) included in the BioEdit suite (Fig. 1A and 1B). The identity matrix was also obtained by BioEdit software. The

MODELTEST software was used to determine the best evolutionary model of nucleotide substitution in the group of analyzed sequences (Posada & Crandall 1998).

Phylogenetic analysis

Evolutionary analysis and phylogenetic reconstructions were conducted in MEGA 5 (Tamura et al. 2011), based on Maximum Likelihood and Neighbor-joining methods for both nt and aa sequences (data not shown), and the most appropriate method for each analysis was chosen. The evolutionary history based on nt sequences was inferred by using the Maximum Likelihood method based on the data-specific model (Nei & Kumar 2000). The Neighbor-Joining method (Saitou & Nei 1987) was used for predicted aa sequences study and the evolutionary distances were computed using the p-distance method (Nei & Kumar 2000). The bootstrap values were calculated using 2000 replicates (Felsenstein 1985). In these reconstructions, the gD gene from the complete genomes of BoHV-1 and BoHV-5 (GenBank ID AJ004801 and AY261359, respectively) or its translations were used as reference sequences for comparison along with the gD 3' region or corresponding COOH-t gD. Likewise, gD genomic or deduced aa sequences of each BoHV subtype recorded in the GenBank (ID Z23068.1 and U14656.1) and a PRV outgroup (GenBank ID AF403051) were used in this study (Fig. 2 and 3).

RESULTS

In this study, the 3' regions of gD gene from BoHV-1 and BoHV-5 were sequenced. Nucleotide sequences will be deposited in the GenBank. The edited sequences length varied from 402 to 532 nt. A consistent alignment of approximately 346 nt positions with gaps (nt 799 to 1145 of AJ00481) revealed a high degree of similarity among all nt sequences of isolates/strains within the same specie (BoHV-1 or BoHV-5) and a less conserved region when comparing BoHV-1 and BoHV-5. The nt similarity was on average 98.3% ± 1.8 (values ranged from 93.8 to 100%) among BoHV-1 and 97.8% ± 2.4 (92.5-100%) among BoHV-5 isolates/strains. The level of genomic similarity between BoHV-1 and BoHV-5 averaged 73.7% ± 0.8 (minimum 70.9% and maximum 74.5%) (Table 2).

A few nt substitutions, mostly tranversions, were observed in the alignment of BoHV-1 and BoHV-5 isolates, analyzing each specie separately and in relation to respective

reference sequence (AJ00481, AY26135). Among BoHV-1 isolates, most changes were found in SV265/96, SV261/07, SV56/90, SV169/06 and SV63/06, with major differences observed at the 3' third of SV169/06. EVI88/95, EVI345/96, ISO97/45 were the most dissimilar among BoHV-5 isolates. This alignment also revealed differences between viral species: a deletion of six nt in all BoHV-1 isolates (position 846-851) and three insertions in BoHV-1 that were absent in BoHV-5 isolates (Fig. 1A).

Phylogenetic reconstructions based on Maximum Likelihood and Neighbor-joining methods for both nt and aa sequences showed similar result; thus, one method for each analysis are shown (Fig. 2 and 3). The phylogenetic tree reconstruction by Maximum Likelihood method, based on nt sequences of 3' gD gene from BoHV isolates, yielded two major clusters, BoHV-1 (n=14) and BoHV-5 (n=7) (Fig. 2). A clear subtype differentiation was not possible; respiratory, genital and neurological isolates clustered together, without distinct classification, in BoHV-1 cluster. Isolate ISO97/45 grouped separately from others, within the BoHV-5 cluster.

The prediction of aa sequences, alignment and phylogenetic study of the 3' region of gD were also performed. The level of aa identity observed was on average: 98.3% ± 2.4 (92.2-100%) among gD1; 95.8% ± 4.8 (84.4-100%) among gD5 isolates/strains and 64.1% ± 1.6 (58.2-66%) between gD1 and gD5, lower that first showed with nt (Table 2). The nt changes, described above, reflected the aa differences between sequences of both viral species, with change in reading frame (Fig. 1B). The phylogenetic analysis of deduced aa sequences based on Neighbor-joining method (Fig. 3) provided a similar tree configuration to those observed for nt (Fig. 2), with two major clusters, BoHV-1 (n=14) and BoHV-5 (n=7). The separation of ISO97/45 within the BoHV-5 cluster was also noticed, being the most dissimilar gD5 (≤ 89.3% of aa identity among other gD5). The main difference between nt and aa phylogenetic analysis was observed with SV169/06, isolate with the lowest aa identity compared to other gD1 (≤ 93.2%), that grouped separately within BoHV-1 branch in the aa tree, but not separatedly in the nt tree. Curiously, gD1 of SV169/06 and gD5 of ISO97/45 presented the lowest identity level (58.2%).

In summary, this study revealed a high degree of nt and aa similarity within the same viral specie and a less conserved 3' gD gene and ORF, between BoHV-1 and BoHV-5. The genomic analysis and the phylogenetic reconstructions allowed for the differentiation between virus species, however a classification according to subspecies or clinical presentation was not possible.

DISCUSSION

We studied the 3' region of gD gene, the coding region for COOH-t region of gD, of 21 BoHV-1 and BoHV-5 isolates/strains. The DNA of samples was sequenced and aa were deduced to determine the levels of similarity and a phylogenetic classification. Other reports of molecular characterization of BoHV-1 and BoHV-5 or a broader group of ruminant alphaherpevirus, based on gB, gC and/or gD gene, are available (Esteves et al. 2008, Ros & Belak 1999, Traesel et al. 2013). However, this study aimed to compare genetic and clinical aspects, especially related to neurological presentation. Thus, seven isolates from each of the three groups were selected: typical BoHV-1, typical BoHV-5 and BoHV-1 from neurological disease. The analysis of gD gene is part of a broader study on BoHV-1 and 5 glycoproteins, that is being conducted to establish genetic divergences involved in phylogenetic classification, clinical presentation and antigenic behavior (Traesel et al. 2013). The 3' region of gD has been reported to contain many genetic differences between BoHV-1 and BoHV-5 (Abdelmagid et al. 1995, Gabev et al. 2010), and these divergences might be associated to biological properties of these viruses.

Both nt and aa based phylogenetic trees built similarly, allowing for differentiation between BoHV-1 (n=14) and BoHV-5 (n=7), in agreement with the previous differential PCR results. Phylogenetic reconstructions based on Maximum Likelihood and Neighbor-joining methods were performed for nt and aa sequences intending to investigate both evolutionary events and genetic distance (Nei & Kumar 2000). However the results obtained in each analysis by different methods were very similar, and only relevant data are shown (Fig. 2 and 3). Apparently, no association was found between phylogenetic distance and temporal and/or geographical distribution. Almost all analyzed isolates had been already classified as such by gC-based studies (Esteves et al. 2008, Traesel et al. 2013). The isolate ISO97/45, a BoHV-5 recovered from neurological disease, contains the most dissimilar gD5 and grouped separately within the BoHV-5 cluster in both nt and aa tree. This separation and grouping with "non-a/non-b" isolates was already observed (Esteves et al. 2008, Traesel et al. 2013) and ISO97/45 was previously classified as a "non-a/non-b" BoHV-5 subtype (D'Arce et al. 2002).

The main difference in the 3' gD region phylogenetic trees, based on nt and that based on aa sequences, was observed in SV169/06. This genital isolate grouped separately within the BoHV-1 branch only in the aa tree, due to the lowest aa identity comparing with other

gD1. By comparing gD1 and gD5, SV169/06 and ISO97/45 presented the lowest aa identity level (58.2%), the most dissimilar within each specie. Another genital isolate should be addressed - SV453/93, which presented a markedly distinct aa/nt pattern at the 3' region of gC (Traesel et al. 2013). However, these differences on SV453/93 could not be seen in the 3' region of gD, which presented 100% of aa identity with the reference sequence in this analysis. Previously studies were able to distinguish between BoHV-1.1 and 1.2 subtypes (Esteves et al. 2008, Ros & Belak 1999, Traesel et al. 2013). This was not possible in the present study. It is known that some isolates may escape the classical classification of BoHV-1.1 (respiratory/reproductive signs) and BoHV-1.2 (genital disease) (Traesel et al. 2013). However, our data do not allow inferences about associations between BoHV-1 subtypes phylogenetic classification and clinical manifestation/phenotype.

This work was initially designed to study genetic differences in respiratory or genital BoHV-1 (n=7), neurological BoHV-5 (n=7) and neurological BoHV-1 (n=7) isolates, and possibly associate these differences with the clinical presentation. The participation of gD in the pathogenesis of neurological disease has been described for HSV-1 (Izumi & Stevens 1990). These authors studied two HSV-1 strains with significant difference in neuroinvasiveness and concluded that HSV-1 gD is responsible for this phenotypic difference *in vivo*, and that gD amino acid 84 is critical to this process. Another study with intertypic gD-exchange mutants with BoHV-1- and BoHV-5-backbones showed that gD5 confers an extended cellular host range and provide a virulent phenotype to BoHV-1 in mice. However, only a virus with BoHV-5-backbone, independent of the gD-type, was detected in the brain by immunohistology (Gabev et al. 2010). Thus, these authors concluded that gD5 may be considered as a virulence factor but it does not contribute to the invasion of the brain. Despite the role of gD in the neuropathogenesis, the changes at the 3' region of gD of BoHV-1 and BoHV-5 found herein seem not to suffice to explain the neurovirulent/non-neurovirulent phenotypes, as all BoHV-1 isolates/strains (respiratory, genital and neurological) grouped together in the same cluster. More studies are needed to clarify this aspect, based on other glycoproteins and, perhaps, the 5' region of gD, as the binding to cell receptors appears to occur at the gD amino-terminal region (Spear & Longnecker 2003).

The major differences between gD1 and gD5 seem to be in the COOH-t region, located within 283 to 354 aa of gD5 (Abdelmagid et al. 1995). This data corroborate with another study that reports major divergences in a glicin-rich ectodomain closer to the transmembrane region, between gD5 residues 280 and 330 (Gabev et al. 2010). The 3' region of gD analyzed here included the cited locations (Abdelmagid et al. 1995, Gabev et al. 2010)

and the differences are reflected in the levels of similarity of nt and aa calculated between BoHV-1 and BoHV-5: 73.7% and 64.1% on average, respectively. However, there is no agreement about the similarity level in the literature. It was reported that predicted aa sequences of gD1 and gD5 were 98% identical (Delhon et al. 2003) and, later, a study revealed only 79.9% aa identity (Gabev et al. 2010). In this analysis of 21 isolates/strains restricted to the 3' region of gD, where are located the major changes, the aa identity level was even lower (64.1%). It is interesting to note that the similarity based on nt averaged higher than aa identity in most cases, suggesting that are many *non-synonimous* mutations (those which result in aa substitution). The COOH-t region can contribute to the protein-protein interactions and residues 245 to 320 were reported to be important for proper processing and transport of gD to the cell surface (Tikoo et al. 1993).

CONCLUSION

The phylogenetic analysis of the 3' region of gD gene of typical BoHV-1 and BoHV-5 isolates/strains, as well as BoHV-1 isolated from neurological disease, provided a clear differentiation between viral species. The similarity levels revealed a high conserved region within the same viral specie, and a less conserved region between BoHV-1 and BoHV-5. However, the genetic differences observed at the 3' region of gD seem not to be associated with the neurovirulent phenotype.

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Table 1. Bovine herpesvirus 1 (BoHV-1) and BoHV-5 isolates/strains included in the present study

Virus	Classification	Clinical sings/material	Country/state	Year
Cooper	BoHV-1	Respiratory	US	(-)
SV 265/96	BoHV-1	Respiratory/nasal swab	BR/RS	1996
EVI 123/98	BoHV-1	Respiratory/nasal swab	BR/RS	1998
SV 261/07	BoHV-1	Respiratory/nasal swab	BR/RS	2007
SV 56/90	BoHV-1	Genital/preputial lavage	BR/RS	1990
SV 453/93	BoHV-1	Genital/vaginal swab	BR/RS	1993
SV 169/06	BoHV-1	Genital/vaginal swab	BR/RS	2006
SV 1613/93	BoHV-1	Neurological/brain	BR/RS	1993
SV 609/03	BoHV-1	Neurological/brain	BR/RS	2003
SV 47/05	BoHV-1	Neurological/brain	BR/RS	2005
SV 63/06	BoHV-1	Neurological/brain	BR/RS	2006
SV 167/10	BoHV-1	Neurological/brain	BR/RS	2010
SV 178/10	BoHV-1	Neurological/brain	BR/RS	2010
SV 194/10	BoHV-1	Neurological/brain	BR/RS	2010
EVI 88/95	BoHV-5	Neurological/brain	BR/MS	1995
EVI 345/96	BoHV-5	Neurological/brain	BR/MS	1996
A 613 (97/613)	BoHV-5	Neurological/brain	AR	1997
ISO 97/45	BoHV-5	Neurological/brain	BR/RJ	1997
SV 507/99	BoHV-5	Neurological/brain	BR/RS	1999
SV 198/05	BoHV-5	Neurological/brain	BR/RS	2005
SV 387/12	BoHV-5	Neurological/brain	BR/RS	2012

US – United States of America; (-) – no information; BR – Brazil; RS – Rio Grande do Sul; MS – Mato Grosso do Sul; AR – Argentina; RJ – Rio de Janeiro.

Table 2. Similarity levels among the nucleotide (nt) and amino acid (aa) sequences of the 3' region of glycoprotein D gene/open reading frame of isolates/strains of bovine herpesvirus 1 (BoHV-1, n=14) and BoHV-5 (n=7). Reference sequences of BoHV-1 and BoHV-5 (GenBank ID AJ004801 and AY261359, respectively) were included for comparison. The results are shown as average similarity % ± standard deviation (lower – higher similarity %)

		BoHV-1	BoHV-5
nt	BoHV-1	98.3 ± 1.8 (93.8 – 100)	73.7 ± 0.8 (70.9 – 74.5)
	BoHV-5	73.7 ± 0.8 (70.9 – 74.5)	97.8 ± 2.4 (92.5 – 100)
aa	BoHV-1	98.3 ± 2.4 (92.2 – 100)	64.1 ± 1.6 (58.2 – 66)
	BoHV-5	64.1 ± 1.6 (58.2 – 66)	95.8 ± 4.8 (84.4 – 100)

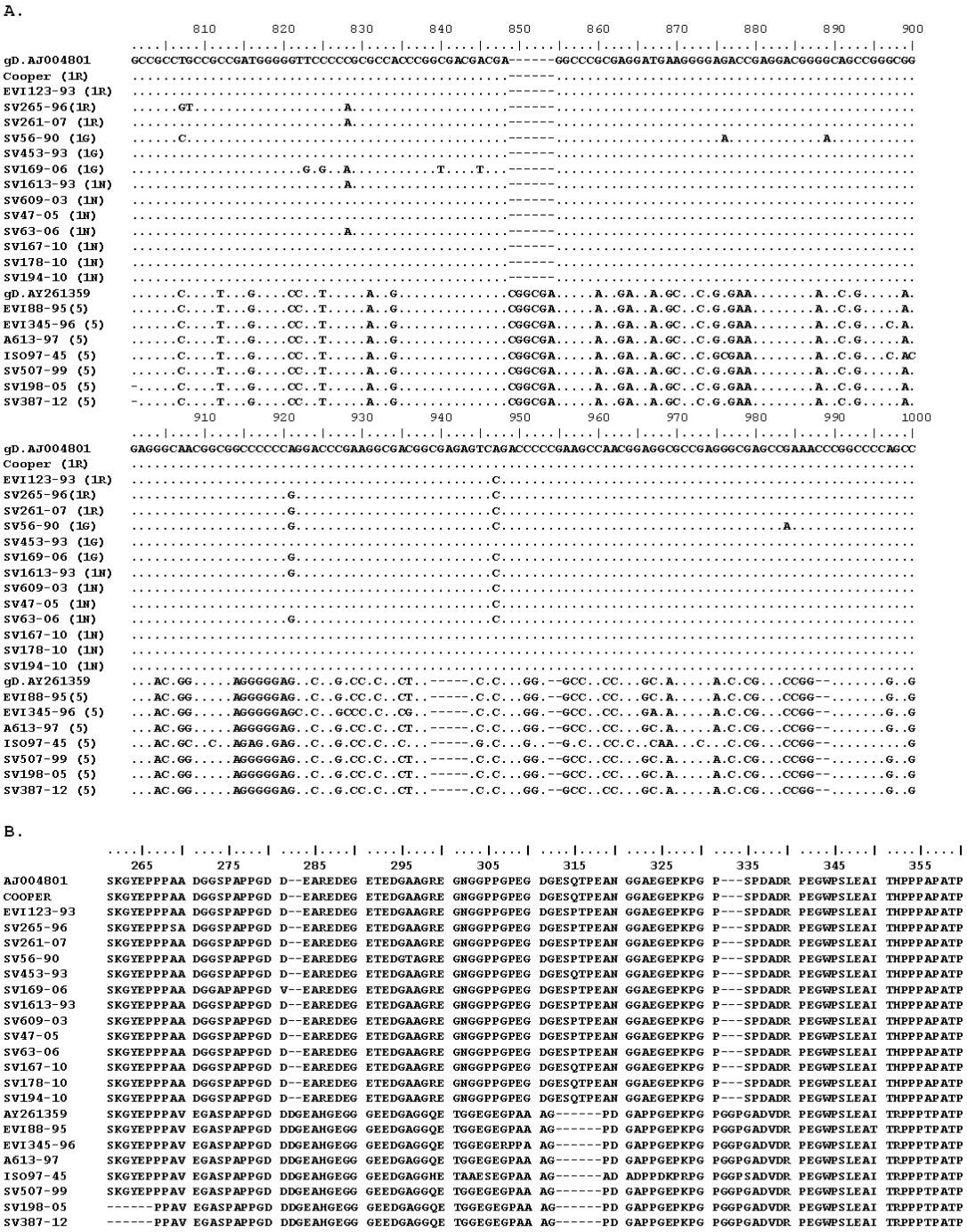

Fig.1. Nucleotide (A) and deduced amino acid (B) sequences alignment of the 3'region of glycoprotein D (gD) gene of bovine herpesvirus 1 (BoHV-1, n=14) from respiratory (1R), genital (1G) and neurological (1N) disease, and BoHV-5 (n=7) from neurological disease (5N). References sequences of BoHV-1 and BoHV-5 (GenBank ID AJ004801 and AY261359) were included for comparison. The gD open reading frame position is shown above.

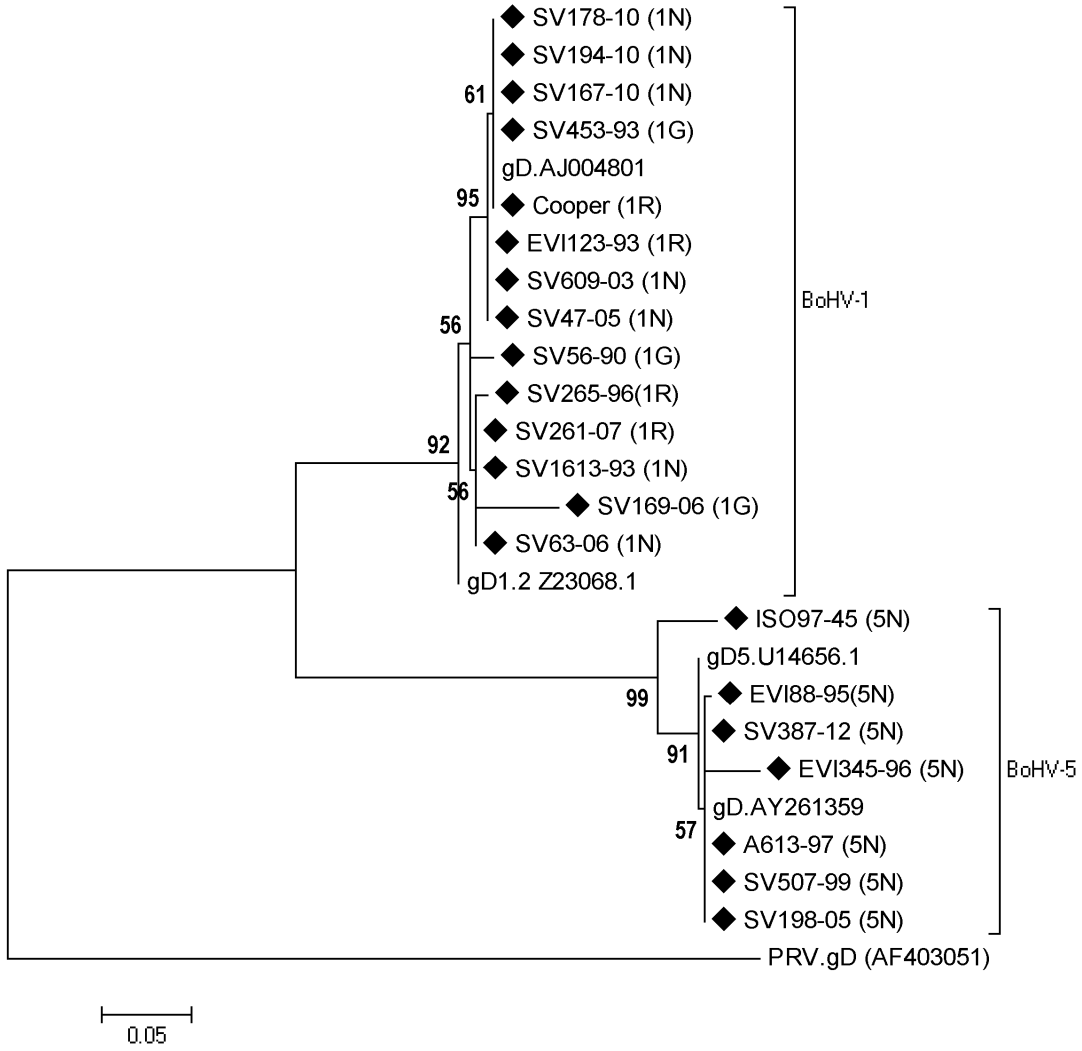

Fig.2. Phylogenetic tree based on Maximum Likelihood method for the nucleotide sequence of the 3' region of glycoprotein D (gD) gene of 14 bovine herpesvirus 1 (BoHV-1) isolates/strains from respiratory (1R), genital (1G) and neurological (1N) disease, and seven BoHV-5 isolates from neurological disease (5N) (♦; 310 positions). Only bootstraps values higher than 50% of 2000 replicates are shown.

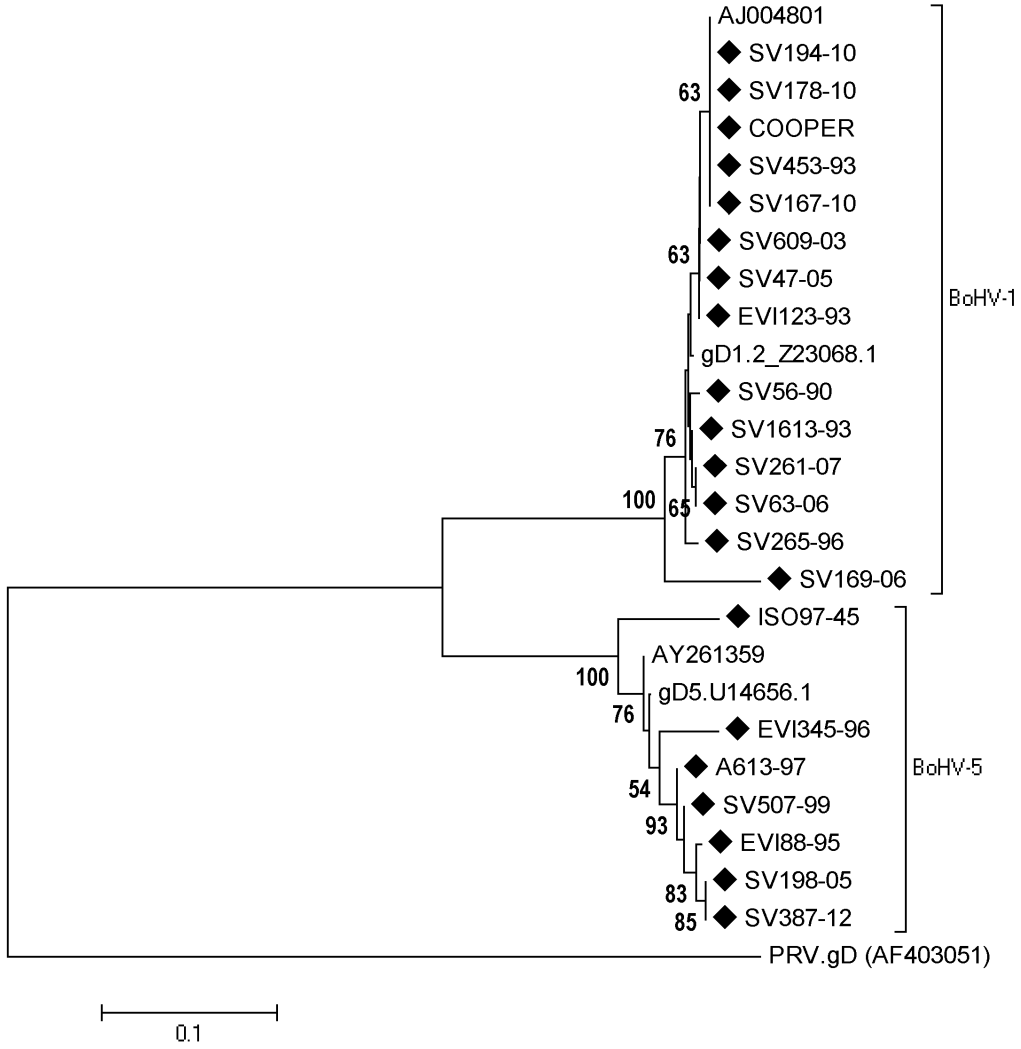

Fig.3. Phylogenetic analysis estimated by Neighbor-joining method based on amino acid sequences of the 3' region of glycoprotein D (gD) open reading frame of 14 bovine herpesvirus 1 (BoHV-1) isolates/strains and seven BoHV-5 isolates (♦; 555 positions). The evolutionary distances were computed using the p-distance method and are in the units of the number of amino acid differences per site. Only bootstraps values higher than 50% of 2000 replicates are shown.

4 CONSIDERAÇÕES FINAIS

Os herpesvírus bovino 1 (BoHV-1) e 5 (BoHV-5) são importantes patógenos de bovinos e estão associados com perdas econômicas, no Brasil e em outros países. A biologia desses agentes é muito semelhante, além de compatilharem características genéticas e antigências. No entanto, pequenas diferenças genômicas existentes entre eles possivelmente estejam associadas com funções biológicas, como o distinto potencial neurovirulento. O presente estudo teve como objetivo realizar análises genotípicas e filogenéticas em isolados de BoHV-1 e BoHV-5, utilizando como base os genes das glicoproteínas C (gC) e D (gD), procurando identificar as diferenças genéticas determinantes para classificação desses vírus. Ainda, foram analisados aspectos relacionados à neurovirulência diferencial entre BoHV-1 e BoHV-5, utilizando amostras de BoHV-1 isoladas do encéfalo de bovinos com doença neurológica.

Os isolados de doença respiratória e genital são historicamente classificados como BoHV-1.1 e BoHV-1.2, respectivamente, e amostras provenientes de bovinos com doença neurológica, como BoHV-5. No entanto, essa classificação clássica não parece ser definitiva e mutuamente excludente. À medida que mais estudos moleculares vem sendo realizados, associando diferenças genéticas e apresentação clínica, mais isolados são identificados divergindo dessa classificação clássica. Isolados de doença respiratória aqui apresentados foram classificados como BoHV-1.2 e de doença genital como BoHV-1.1. A associação do BoHV-1 com doença neurológica já havia sido descrita em alguns estudos, no entanto, o seu envolvimento era considerado ocasional e atípico, e a neurovirulência, uma característica diferencial do BoHV-5. Os resultados aqui apresentados confirmam que a associação do BoHV-1 com doença neurológica é mais frequente do que anteriormente relatada, com muitos isolados recentes sendo classificados como tal.

A suspeita inicial que impulsionou o estudo era de que o envolvimento de isolados de BoHV-1 com doença neurológica poderia ser explicado por diferenças genéticas em relação aos demais BoHV-1. No entanto, existe também a possibilidade de que outras condições específicas como fatores do hospedeiro, associadas ou não a divergências genotípicas, poderiam permitir uma replicação mais eficiente e o transporte para o SNC, levando à apresentação do fenótipo neurovirulento. Ainda, essas diferenças genéticas podem estar localizadas em regiões associadas à neurovirulência ainda não estudadas nesses isolados BoHV-1 neurológicos. Dessa forma, os resultados obtidos nesta tese demonstram que o

BoHV-1 e o BoHV-5 podem estar envolvidos em apresentações clínicas diferentes das tipicamente reportadas, chamando a atenção também para possíveis falhas na identificação e classificação de isolados de BoHV no diagnóstico de rotina para essas enfermidades.

Em contrapartida, diferenças genéticas encontradas sem associação aparente com mudanças fenotípicas também puderam ser observadas, como na região da gC do isolado SV 453/93, e merecem ser melhor pesquisadas, tanto em nível molecular quanto por meio de inoculação experimental. Assim, as regiões 3' dos genes da gC e gD aqui analisadas, correspondentes à região carboxi-terminal das glicoproteínas, são conservadas e permitem a diferenciação entre as espécies virais, podendo ser utilizadas na classificação filogenética. A associação não consistente entre os subtipos de BoHV-1 e sinais clínicos sugere que essa diferenciação pode ser dependente da região genômica analisada, e que não é possível atribuir uma classificação definitiva com base na origem clínica dos isolados.

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