Sequence analysis of the 5′ third of glycoprotein C gene of South American bovine herpesviruses 1 and 5

C.K. Traesel[1], L.M. Bernardes[1], F.R. Spilki[2], R. Weiblen[1] and E.F. Flores[1]

[1]Setor de Virologia, Departamento de Medicina Veterinária Preventiva, Universidade Federal de Santa Maria, Santa Maria, RS, Brasil
[2]Laboratório de Microbiologia Molecular, Universidade Feevale, Novo Hamburgo, RS, Brasil

Abstract

Bovine herpesviruses 1 (BoHV-1) and 5 (BoHV-5) share high genetic and antigenic similarities, but exhibit marked differences in tissue tropism and neurovirulence. The amino-terminal region of glycoprotein C (gC), which is markedly different in each of the viruses, is involved in virus binding to cellular receptors and in interactions with the immune system. This study investigated the genetic and antigenic differences of the 5′ region of the gC (5′ gC) gene (amino-terminal) of South American BoHV-1 (n = 19) and BoHV-5 (n = 25) isolates. Sequence alignments of 374 nucleotides (104 amino acids) revealed mean similarity levels of 97.3 and 94.2% among BoHV-1 gC (gC1), respectively, 96.8 and 95.6% among BoHV-5 gC (gC5), and 62 and 53.3% between gC1 and gC5. Differences included the absence of 40 amino acid residues (27 encompassing predicted linear epitopes) scattered throughout 5′ gC1 compared to 5′ gC5. Virus neutralizing assays testing BoHV-1 and BoHV-5 antisera against each isolate revealed a high degree of cross-neutralization between the viruses, yet some isolates were neutralized at very low titers by heterologous sera, and a few BoHV-5 isolates reacted weakly with either sera. The virus neutralization differences observed within the same viral species, and more pronounced between BoHV-1 and BoHV-5, likely reflect sequence differences in neutralizing epitopes. These results demonstrate that the 5′ gC region is well conserved within each viral species but is divergent between BoHV-1 and BoHV-5, likely contributing to their biological and antigenic differences.

Key words: gC amino-terminal; BoHV; Genetic diversity; Epitope prediction; Virus neutralization

Introduction

Bovine herpesviruses type 1 (BoHV-1) and 5 (BoHV-5) are important alphaherpesviruses of cattle, belonging to the family *Herpesviridae*, genus *Varicellovirus* (1). BoHV-1 is distributed worldwide – with the exception of some European countries that have eradicated the infection – and is associated with a variety of clinical manifestations including respiratory, genital disease, abortions (2,3), and rarely, neurological disease (4,5). This virus induces immune suppression, which initiates the bovine respiratory disease complex (6). BoHV-5 is the major agent of meningoencephalitis, a severe and often fatal disease primarily affecting calves and occurring predominantly in South America, especially Brazil and Argentina (3,5,7). BoHV-5 has occasionally been isolated from bull semen and other clinical conditions, including reproductive and respiratory disorders (8,9).

These viruses share many genetic and biological properties. Their double stranded DNA genomes encode approximately 70 products and present around 85% nucleotide (nt) similarity and 82% amino acid (aa) identity (10). As a consequence of the antigenic similarity, extensive serological cross-reactivity is observed between BoHV-1 and BoHV-5, posing problems for immunodiagnosis (5,11-14). Virion surface glycoproteins are major targets for the host immune system at both the humoral and cellular levels (15,16) and, as such, are particularly involved in this immunological cross-reactivity (17). These proteins are also important determinants of alphaherpesvirus tropism and pathogenesis, since they are responsible for the initial interactions with host cells by binding to cell surface receptors, attachment, fusion and entry into mammalian cells (18).

Glycoprotein C (gC), the major viral glycoprotein, is expressed at high levels in the BoHV-1 envelope and on the surface of infected cells (19,20). After post-transcriptional processing, BoHV-1 gC (gC1) is translated into a

508 aa polypeptide, whereas BoHV-5 gC (gC5) encodes 486 aa residues (10). It is a dimeric, type I transmembrane protein containing a cleavable amino-terminal (NH_2-t) signal, a highly hydrophilic glycosylated NH_2-t ectodomain, a single hydrophobic transmembrane domain, and a carboxy-terminal (COOH-t) hydrophilic region (20,21). The NH_2-t hydrophilic gC ectodomain is exposed on the surface of virions and infected cells, making it a major target for the immune system, particularly for antibodies (16,20,22). Based on competitive binding assays using gC-specific monoclonal antibodies (MAbs) and synthetic peptides, important antigenic domains have been mapped to the NH_2-t of gC, between gC1 amino acid residues 22 and 287 (16,18,23,24). Moreover, potential N-linked glycosylation sites were reported on NH_2-t gC1, located at the amino acids 93, 111, 164, and 208 (20).

Although gC1 and gC5 are 75% identical, major differences have been identified at the NH_2-t third of the protein, especially between residues 1-123 (gC1) and 1-102 (gC5) (10,25). These differences may contribute to the distinct pattern of MAb binding and to differences in cross-neutralization between BoHV-1 and BoHV-5 (17). Glycoprotein C also plays an important role in viral biology, mediating attachment of virions to heparan sulphate receptors on the surface of host cells (18). Sequences involved in receptor binding were identified in the gC NH_2-t ectodomain, in which differences might influence neurotropism, neuroinvasiveness and neurovirulence, properties that are differentially expressed in BoHV-1 and BoHV-5 (26-28).

Thus, this study was designed to analyze the 5′ region of the gC gene of a number of BoHV-1 and BoHV-5 isolates to further investigate the degree of variability of this region and possible associations between nucleotide/deduced amino acid sequences and viral phenotype.

Material and Methods

Forty-four bovine herpesvirus isolates were analyzed, including BoHV-1 and BoHV-5 field isolates obtained in Brazil, Uruguay, and Argentina from 1981 to 2010. After distinguishing BoHV-1 from BoHV-5 by differential PCR (4,29), viral DNA was amplified by PCR specific for the 5′ third of the gC gene, corresponding to the NH_2-t region of the gC protein. The 5′ gC amplicons were sequenced and the amino acid sequences were deduced for sequence comparison. In addition, a virus neutralization (VN) assay was performed using BoHV-1 and BoHV-5 specific antisera against each field isolate.

Viruses, cells and viral DNA extraction

Information about the viruses studied is summarized in Table 1. Virus isolation, amplification and biological cloning were performed in a Madin-Darby bovine kidney (MDBK)-derived cell line named CRIB (30). Total DNA was extracted from CRIB cells infected with each isolate using DNAzol reagent (Invitrogen, USA). The virus amplification, cloning,

quantitation and DNA extraction procedures have previously been described in detail (30).

PCR amplification

The BoHV-1 and BoHV-5 isolates present in clinical samples were initially identified by PCR, as first described by Ashbaugh et al. (29) and modified by Silva et al. (4). The viral DNA was then amplified in a second PCR targeting the 5′ region of gC (nucleotides 1-533 of gC1 and 1-559 of gC5) using specific primers for each viral type: BoHV-1 5′-CACGCGCTGAAACCAGAG-3′ (forward) and 5′-CAATCCCGGACCACGAAAG-3′ (reverse); BoHV-5 5′-TTCTGGGCCGCAAACAGC-3′ (forward) and 5′-GAAAGCACAGGACGGGAGAC-3′ (reverse). The PCR resulted in amplicons of 668 base pairs (bp) for BoHV-1 (genome AJ004801, GenBank, position 17,676-18,343) and 687 bp for BoHV-5 (genome AY261359, GenBank, position 18,492-19,178). The primers targeted the region located at the 5′ gC third, corresponding to nucleotides 1-533 (gC1) and 1-559 (gC5). All steps of the PCR amplification (primer design, PCR protocol, fragment visualization and controls) have already been described in detail (30), with minor modifications as follows. An annealing temperature of 55°C was used to amplify the 5′ gC1. For BoHV-5 isolates, a protocol was adapted using approximately 1 μg of total DNA as template in 48 μL of a mix containing ultrapure water, 25 or 50 μM of each primer, 0.4 or 0.56 mM of deoxyribonucleotides, 10% dimethylsulfoxide [6 or 8 mM of $MgCl_2$, 10% of Taq $10 \times$ buffer and 1 U of Taq DNA polymerase (Invitrogen)]. PCR conditions included a denaturation step of 5 min at 94°C, followed by 40 cycles of denaturation at 94°C for 50 s, annealing at 62°C for 55 s and a final extension at 72°C for 1 min.

Sequencing and 5′ gC sequences analysis

The PCR amplicons were purified using an Illustra™ GFX™ PCR DNA and Gel Band Purification Kit (GE Healthcare, UK), according to the manufacturer's instructions. Sequencing reactions were performed twice and in both directions in an automatic sequencer ABI-PRISM 3100 Genetic Analyzer (Applied Biosystems, USA). DNA amplicons (30-60 ng) and 4.5 pmol of forward or reverse specific 5′ gC primers of each type (described above) were used for sequencing (for more sequencing details see (Ref. 31)).

The quality of nucleotide sequences and overlapping fragments of each sequence were assembled by the Staden Package, which was submitted for comparison with reference sequences of BoHV-1.1 (strain Cooper; GenBank no. AJ004801) and BoHV-5 (SV 507/99; GenBank no. AY261359) complete genomes, using the National Center for Biotechnology Information (NCBI) database and BLAST software (http://www.ncbi.nlm.nih.gov/BLAST/). Based on nucleotide sequences, amino sequences were translated in reading frame 1 on the direct strand by Sequence Manipulation Suite software version 2 (http://www.bioinformatics.org/sms2/) and evaluated for homology comparison by

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

Virus	Classification	Clinical signs/material	Country/state	Year	GenBank ID
SV 56/90	BoHV-1	Genital/preputial lavage	BR/RS	1990	KJ143528
SV 453/93	BoHV-1	Genital/vaginal swab	BR/RS	1993	KJ143529
SV 1613/93	BoHV-1	Neurological/brain	BR/RS	1993	KJ143530
SV 265/96	BoHV-1	Respiratory/nasal swab	BR/RS	1996	KJ143531
EVI123/98	BoHV-1	Respiratory/nasal swab	BR/MS	1998	KJ143532
SV 609/03	BoHV-1	Neurological/brain	BR/RS	2003	KJ143533
SV 299/03	BoHV-1	Neurological/brain	BR/RS	2003	KJ143534
SV 47/05	BoHV-1	Neurological/brain	BR/RS	2005	KJ143535
SV 371/05	BoHV-1	Respiratory/nasal swab	BR/RS	2005	KJ143536
SV 63/06	BoHV-1	Neurological/brain	BR/RS	2006	KJ143537
SV 169/06	BoHV-1	Genital/vaginal swab	BR/RS	2006	KJ143538
SV 224/06	BoHV-1	Routine monitoring/semen	BR/SP	2006	KJ143539
SV 226/06	BoHV-1	Routine monitoring/semen	BR/SP	2006	KJ143540
SV 228/06	BoHV-1	Routine monitoring/semen	BR/SP	2006	KJ143541
SV 229/06	BoHV-1	Routine monitoring/semen	BR/SP	2006	KJ143542
SV 261/07	BoHV-1	Respiratory/nasal swab	BR/RS	2007	KJ143543
SV 167/10	BoHV-1	Neurological/brain	BR/RS	2010	KJ143544
SV 178/10	BoHV-1	Neurological/brain	BR/RS	2010	KJ143545
SV 194/10	BoHV-1	Neurological/brain	BR/RS	2010	KJ143546
Uruguay T2	BoHV-5	Neurological/brain	UY	1981	KJ143547
P 160/87	BoHV-5	Neurological/brain	BR/RJ	1987	KJ143548
EVI 88/95	BoHV-5	Neurological/brain	BR/MS	1995	KJ143549
EVI 340/96	BoHV-5	Neurological/brain	BR/MS	1996	KJ143550
EVI 345/96	BoHV-5	Neurological/brain	BR/MS	1996	KJ143551
ISO 169/96	BoHV-5	Neurological/brain	BR/SP	1996	KJ143552
A 613 (97/613)	BoHV-5	Neurological/brain	AR	1997	KJ143553
97/642	BoHV-5	Neurological/brain	AR	1997	KJ143554
SV 106/98	BoHV-5	Neurological/brain	BR/RS	1998	KJ143555
SV 507/99	BoHV-5	Neurological/brain	BR/RS	1999	KJ143556
002/00	BoHV-5	Systemic/spleen	BR/RS	2000	KJ143557
SV 190/00A	BoHV-5	Neurological/brain	BR/MS	2000	KJ143558
SV 55/02	BoHV-5	Neurological/brain	BR/RS	2002	KJ143559
SV 437/04	BoHV-5	Neurological/brain	BR/RS	2004	KJ143560
SV 198/05	BoHV-5	Neurological/brain	BR/RS	2005	KJ143561
SV 41/06	BoHV-5	Neurological/brain	BR/MS	2006	KJ143562
SV 223/06	BoHV-5	Routine monitoring/semen	BR/SP	2006	KJ143563
SV 225/06	BoHV-5	Routine monitoring/semen	BR/SP	2006	KJ143564
SV 355/06	BoHV-5	Neurological/brain	BR/RS	2006	KJ143565
SV 71/07 A	BoHV-5	Neurological/brain	BR/MS	2007	KJ143566
SV 71/07 D	BoHV-5	Neurological/brain	BR/MS	2007	KJ143567
SV 79/07	BoHV-5	Neurological/brain	BR/RS	2007	KJ143568
SV 102/07	BoHV-5	Neurological/brain	BR/RS	2007	KJ143569
SV 511/09	BoHV-5	Neurological/brain	BR/SP	2009	KJ143570
Uruguay T4	BoHV-5	Neurological/brain	UY	(-)	KJ143571

Brazilian states: RS: Rio Grande do Sul, SP: São Paulo, RJ: Rio de Janeiro, MS: Mato Grosso do Sul.

protein BLAST. The nucleotide and deduced amino acid sequences of each isolate, and gC of reference sequences (GenBank ID AJ004801 and AY261359) were aligned and edited with the BioEdit Sequence Alignment Editor software suite, version 7.0.5.3 (USA), using ClustalW software (Ireland). The identity matrix was also obtained using

BioEdit software (30,31).

Phylogenetic analysis

Evolutionary analysis and phylogenetic reconstruction based on 5′ gC nucleotide sequences were conducted in MEGA 5. The evolutionary history was inferred by using the Maximum Likelihood method based on the data-specific model, and the bootstrap values were calculated using 2000 replicates. The gC gene of reference sequences of BoHV-1 and BoHV-5 (GenBank ID AJ004801 and AY261359, respectively) were used in the reconstruction, as well as the pseudorabies virus (PRV) gC gene (GenBank no. AF403051) (30,31).

VN assays

Twenty BoHV-1 and 32 BoHV-5 isolates were selected for VN assays. Standard VN assays were performed in 96-well plates in duplicate. Two-fold dilutions of BoHV-1 or BoHV-5-specific antisera were incubated with 100 $TCID_{50}$ of each virus for 2 h before the addition of a suspension of CRIB cells. Plates were read after 96 h of incubation at 37°C and 5% CO_2. VN was determined as the reciprocal of the highest serum dilution that completely neutralized the virus, as shown by the absence of cytopathic effect. Antisera to BoHV-1 and BoHV-5 were produced in lambs given two intramuscular injections (21 day interval) of 10^6 $TCID_{50}$/mL of Cooper (BoHV-1) or SV507/99 (BoHV-5) virus strains. Immune sera were obtained 21 days after the second injection.

Results

Analysis of the 5′ third of gC and phylogeny

The 5′ third of the gC gene, a region corresponding to nucleotide positions 1-533 (gC1) and 1-562 (gC5), from 19 BoHV-1 and 25 BoHV-5 isolates/strains was sequenced. The length of edited sequences varied from 414 to 562 nt, corresponding to 137 to 187 aa. A consistent alignment of approximately 374 nt and 104 aa residues (starting at the first ATG) revealed a high degree of similarity among sequences within the same viral species, an average 97.3% of nucleotides and 94.2% of amino acids for BoHV-1; 96.8% of nucleotides and 95.6% of amino acids for BoHV-5 (Table 2). A sequence comparison between BoHV-1 and BoHV-5 revealed a lower degree of similarity (62% nucleotide and 53.3% amino acid). The partial alignment of amino acid sequences is shown in Figure 1.

A number of nucleotide substitutions and deletions were observed by aligning BoHV-1 and BoHV-5 sequences. Separate analysis of each species and comparing both species showed several point mutations, insertions and deletions. The alignment revealed a number of changes between both viruses species – including several deletions – that are responsible for the low similarity between BoHV-1 and BoHV-5 gC. Among BoHV-1 sequences, compared with the AJ00481 reference sequence, the isolates SV261/

Table 2. Similarity levels among the nucleotide (nt) and amino acid (aa) sequences of the amino-terminal region of glycoprotein C of isolates/strains of bovine herpesvirus 1 (BoHV-1, n = 22) and BoHV-5 (n = 25).

	BoHV-1	BoHV-5
Nt		
BoHV-1	97.3 ± 2 (91.4-100)	62 ± 1.4 (56.4-63.3)
BoHV-5	62 ± 1.4 (56.4-63.3)	96.8 ± 3.2 (86.3-100)
Aa		
BoHV-1	94.2 ± 6 (73-100)	53.3 ± 3.4 (40.3-56.7)
BoHV-5	53.3 ± 3.4 (40.3-56.7)	95.6 ± 5.7 (78.8-100)

References sequences of BoHV-1 and BoHV-5 (GenBank ID AJ004801 and AY261359, respectively) are included for comparison. Data are reported as average% ± SD (range %).

07 and SV1613/93 harbored the highest number of changes; whereas SV71/07D, compared with GenBank sequence AY26135, was the most dissimilar BoHV-5. Many nucleotide changes were reflected in amino acid substitutions. As shown in Figure 1, major differences within the same viral species included several amino acid substitutions and single or double insertions (in five 5′ gC1 and three 5′ gC5 sequences) and deletions (EVI123-98 and SV1613-93). Regarding amino substitutions within each species, exchanges of amino acids from different chemical groups were observed, as in SV261/07 positions 73, 75-76, and 80 (Figure 1). A different amino acid pattern was observed between gC1 and gC5 sequences. It included amino acid substitutions from different chemical groups (e.g., exchange of nonpolar proline in 5′ gC1 by polar threonine in 5′ gC5, position 73; Figure 1) and a 5′ gC5 deletion in a predicted epitope region (position 88; Figure 1). A notable difference was the absence of 40 aa scattered throughout the BoHV-1 sequenced fragments that were present in all BoHV-5 isolates, including anchors for predicted epitopes, 27 of which are in BoHV-5 linear epitopes. These include major deletions between residues 38-47 – which encompass a potential gC5 linear epitope – and 118-133, as shown in Figure 1.

The phylogenetic tree reconstruction based on nucleotide sequences yielded two major clusters, allowing for the clear differentiation between BoHV-1 (n = 19) and BoHV-5 (n = 25) (Figure 2). Subtype differentiation was not possible by this sequence analysis.

VN assays

The results of VN assays demonstrated a high degree of cross-neutralization between BoHV-1 and BoHV-5. The isolates from both viral types were neutralized by homologous and heterologous antisera, in variable titers (Figure 3) Most BoHV-1 (Figure 3A) and BoHV-5 (Figure 3B) isolates were neutralized at the highest titers by the homologous antiserum (≥16); a few were neutralized at similar titers by both antisera (ranging from 8 to 32). Thus, the differences in
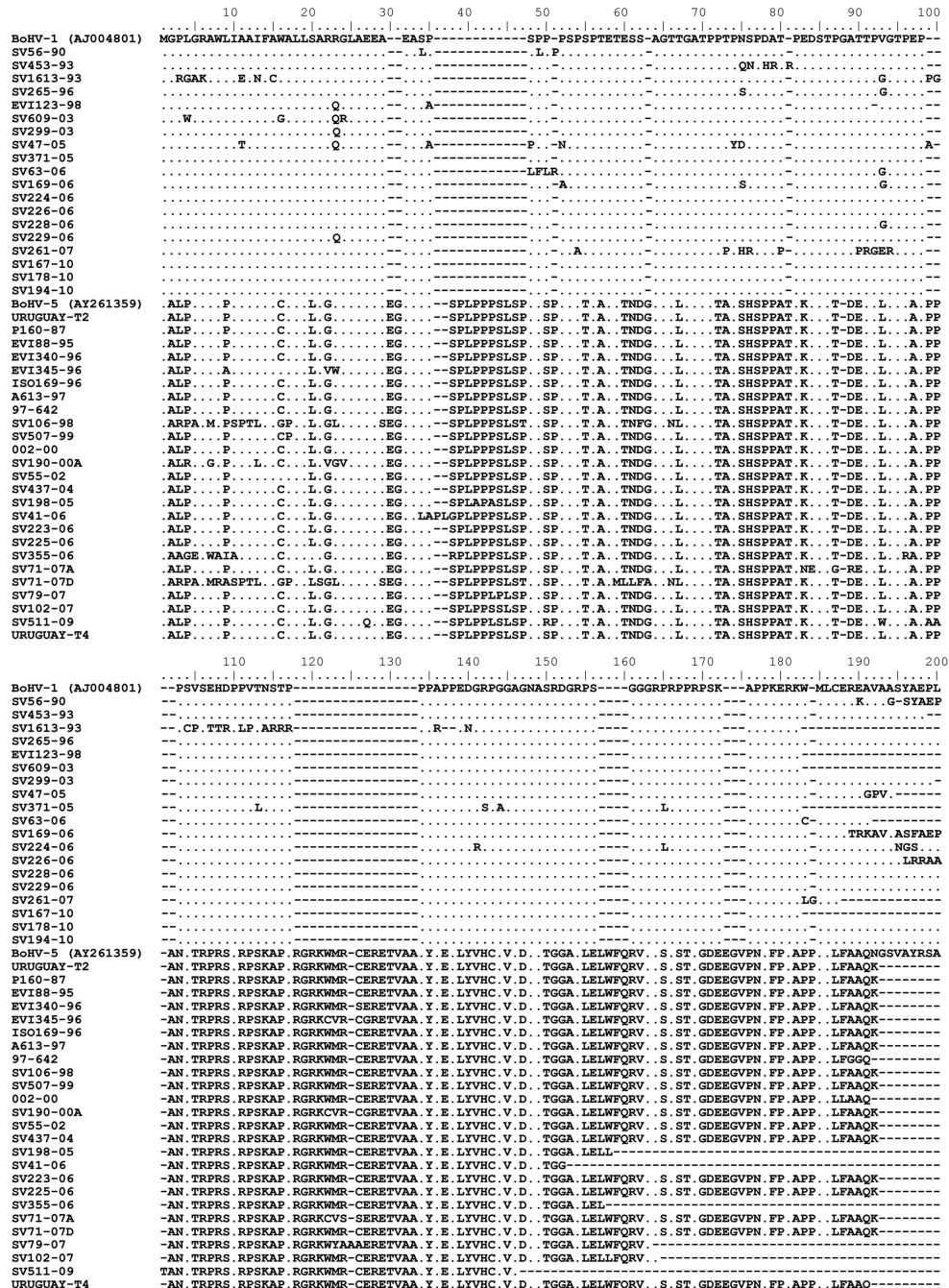
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               10        20        30        40        50        60        70        80        90       100
            ...|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
BoHV-1 (AJ004801) MGPLGRAWLIAAIFAWALLSARRGLAEEA--EASP------------SPP-PSPSPTETESS-AGTTGATPPTPNSPDAT-PEDSTPGATTPVGTPEP--
SV56-90        ...........................--.L.------------.L.P.................-........-...................--
SV453-93       ...........................--...------------.-.................-........-.................--
SV1613-93      ..RGAK....E.N.C.............--...------------.-.................-........-.QN.HR.R............--
SV265-96       ...........................--...------------.-.................-........-...............G....PG
EVI123-98      ...........................--...A-----------.-.................-........S....-..........-....--
SV609-03       ...W.........G.....--.......................-........-...................--
SV299-03       .............QR....--..............................-........-...................--
SV47-05        ...........Q.......--...A------------P..-N.........-........YD....-..........A-
SV371-05       ...........T....Q......--...A------------P..-N.........-........YD....-...........A-
SV63-06        ...........................--...------------.-.................-........-...................--
SV169-06       ...........................--...------------LFLR..............-........-.................G....--
SV224-06       ...........................--...------------.-A...............-........S....-.............G....--
SV226-06       ...........................--...------------.-.................-........-...................--
SV228-06       ...........................--...------------.-.................-........-.................G....--
SV229-06       ............Q..............--...------------.-.................-........-...................--
SV261-07       ...........................--...------------.-..A.............-........P.HR...P-........PRGER...--
SV167-10       ...........................--...------------.-.................-........-...................--
SV178-10       ...........................--...------------.-.................-........-...................--
SV194-10       ...........................--...------------.-.................-........-...................--
BoHV-5 (AY261359) .ALP....P......C...L.G.......EG....--SPLPPPSLSP..SP...T.A..TNDG...L....TA.SHSPPAT.K...T-DE..L...A.PP
URUGUAY-T2     .ALP....P......C...L.G.......EG....--SPLPPPSLSP..SP...T.A..TNDG...L....TA.SHSPPAT.K...T-DE..L...A.PP
P160-87        .ALP....P......C...L.G.......EG....--SPLPPPSLSP..SP...T.A..TNDG...L....TA.SHSPPAT.K...T-DE..L...A.PP
EVI88-95       .ALP....P......C...L.G.......EG....--SPLPPPSLSP..SP...T.A..TNDG...L....TA.SHSPPAT.K...T-DE..L...A.PP
EVI340-96      .ALP....P......C...L.G.......EG....--SPLPPPSLSP..SP...T.A..TNDG...L....TA.SHSPPAT.K...T-DE..L...A.PP
EVI345-96      .ALP....A........L.VW.......EG....--SPLPPPSLSP..SP...T.A..TNDG...L....TA.SHSPPAT.K...T-DE..L...A.PP
ISO169-96      .ALP....P......C...L.G.......EG....--SPLPPPSLSP..SP...T.A..TNDG...L....TA.SHSPPAT.K...T-DE..L...A.PP
A613-97        .ALP....P......C...L.G.......EG....--SPLPPPSLSP..SP...T.A..TNDG...L....TA.SHSPPAT.K...T-DE..L...A.PP
97-642         .ALP....P......C...L.G.......EG....--SPLPPPSLSP..SP...T.A..TNDG...L....TA.SHSPPAT.K...T-DE..L...A.PP
SV106-98       .ARPA.M.PSPTL..GP..L.GL.....SEG....--SPLPPPSLST..SP...T.A..TNFG..NL....TA.SHSPPAT.K...T-DE..L...A.PP
SV507-99       .ALP.......CP..L.G.......EG....--SPLPPPSLSP..SP...T.A..TNDG...L....TA.SHSPPAT.K...T-DE..L...A.PP
002-00         .ALP....P......C...L.G.......EG....--SPLPPPSLSP..SP...T.A..TNDG...L....TA.SHSPPAT.K...T-DE..L...A.PP
SV190-00A      .ALR..G.P....L...C...L.VGV.....EG....--SPLPPPSLSP..SP...T.A..TNDG...L....TA.SHSPPAT.K...T-DE..L...A.PP
SV55-02        .ALP....P........L.G.......EG....--SPLPPPSLSP..SP...T.A..TNDG...L....TA.SHSPPAT.K...T-DE..L...A.PP
SV437-04       .ALP....P......C...L.G.......EG....--SPLPPPSLSP..SP...T.A..TNDG...L....TA.SHSPPAT.K...T-DE..L...A.PP
SV198-05       .ALP....P......C...L.G.......EG....--SPLAPASLSP..SP...T.A..TNDG...L....TA.SHSPPAT.K...T-DE..L...A.PP
SV41-06        .ALP....P......C...L.G.......EG..LAPLGPLPPPSLSP..SP...T.A..TNDG...L....TA.SHSPPAT.K...T-DE..L...A.PP
SV223-06       .ALP....P......C...L.G.......EG....--SPLPPPSLSP..SP...T.A..TNDG...L....TA.SHSPPAT.K...T-DE..L...A.PP
SV225-06       .ALP....P......C...L.G.......EG....--SPLPPPSLSP..SP...T.A..TNDG...L....TA.SHSPPAT.K...T-DE..L...A.PP
SV355-06       .AAGE.WAIA.....C.....G.......EG....--RPLPPPSLSP..SP...T.A..TNDG...L....TA.SHSPPAT.K...T-DE..L...RA.PP
SV71-07A       .ALP....P......C...L.G.......EG....--SPLPPPSLSP..SP...T.A..TNDG...L....TA.SHSPPAT.NE..G-RE..L...A.PP
SV71-07D       .ARPA.MRASPTL..GP..LSGL.....SEG....--SPLPPPSLSP..SP...T.A..MLLFA..NL....TA.SHSPPAT.K...T-DE..L...A.PP
SV79-07        .ALP....P...C..L.G.......EG....--SPLPPLPLSP..SP...T.A..TNDG...L....TA.SHSPPAT.K...T-DE..L...A.PP
SV102-07       .ALP....P......C...L.G.......EG....--SPLPPSSLSP..SP...T.A..TNDG...L....TA.SHSPPAT.K...T-DE..L...A.PP
SV511-09       .ALP....P......C...L.G.......Q..EG....--SPLPPLSLSP..RP...T.A..TNDG...L....TA.SHSPPAT.K...T-DE..W...A.AA
URUGUAY-T4     .ALP....P......C...L.G.......EG....--SPLPPPSLSP..SP...T.A..TNDG...L....TA.SHSPPAT.K...T-DE..L...A.PP

               110       120       130       140       150       160       170       180       190       200
            ...|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
BoHV-1 (AJ004801) --PSVSEHDPPVTNSTP---------------PPAPPEDGRPGGAGNASRDGRPS----GGGRPRPPRPSK---APPKERKW-MLCEREAVAASYAEPL
SV56-90        --...............----------------..............----...........---.........---........-.......K...G-SYAEP
SV453-93       --...............----------------..............----...........---.........---........-...........--
SV1613-93      --.CP.TTR.LP.ARRR----------------.R--.N.............----...........---.........---........------------------
SV265-96       --...............----------------..............----...........---.........---........-...........--
EVI123-98      --...............----------------..............----...........---.........---........------------------
SV609-03       --...............----------------..............----...........---.........---........-...........--
SV299-03       --...............----------------..............----...........---.........---........-...........-
SV47-05        --...............----------------..............----...........---.........---........-.....GPV.------
SV371-05       --.........L.....----------------.......S.A.....----...L.......---.........---........------------------
SV63-06        --...............----------------..............----...........---........C-......----------
SV169-06       --...............----------------..............----...........---........-...TRKAV.ASFAEP
SV224-06       --...............----------------.......R.......----...L.......---.........---........-.....NGS...
SV226-06       --...............----------------..............----...........---.........---........-.......LRRAA
SV228-06       --...............----------------..............----...........---.........---........------------------
SV229-06       --...............----------------..............----...........---.........---........-.-----------
SV261-07       --...............----------------..............----...........---.........---.....LG..------------------
SV167-10       --...............----------------..............----...........---.........---........------------------
SV178-10       --...............----------------..............----...........---.........---........-...........--
SV194-10       --...............----------------..............----...........---.........---........-...........--
BoHV-5 (AY261359) -AN.TRPRS.RPSKAP.RGRKWMR-CERETVAA.Y.E.LYVHC.V.D...TGGA.LELWFQRV...S.ST.GDEEGVPN.FP.APP..LFAAQNGSVAYRSA
URUGUAY-T2     -AN.TRPRS.RPSKAP.RGRKWMR-CERETVAA.Y.E.LYVHC.V.D...TGGA.LELWFQRV...S.ST.GDEEGVPN.FP.APP..LFAAQK--------
P160-87        -AN.TRPRS.RPSKAP.RGRKWMR-CERETVAA.Y.E.LYVHC.V.D...TGGA.LELWFQRV...S.ST.GDEEGVPN.FP.APP..LFAAQK--------
EVI88-95       -AN.TRPRS.RPSKAP.RGRKWMR-CERETVAA.Y.E.LYVHC.V.D...TGGA.LELWFQRV...S.ST.GDEEGVPN.FP.APP..LFAAQK--------
EVI340-96      -AN.TRPRS.RPSKAP.RGRKWMR-SERETVAA.Y.E.LYVHC.V.D...TGGA.LELWFQRV...S.ST.GDEEGVPN.FP.APP..LFAAQK--------
EVI345-96      -AN.TRPRS.RPSKAP.RGRKCVR-CGRETVAA.Y.E.LYVHC.V.D...TGGA.LELWFQRV...S.ST.GDEEGVPN.FP.APP..LFAAQK--------
ISO169-96      -AN.TRPRS.RPSKAP.RGRKWMR-CERETVAA.Y.E.LYVHC.V.D...TGGA.LELWFQRV...S.ST.GDEEGVPN.FP.APP..LFAAQK--------
A613-97        -AN.TRPRS.RPSKAP.RGRKWMR-CERETVAA.Y.E.LYVHC.V.D...TGGA.LELWFQRV...S.ST.GDEEGVPN.FP.APP..LFAAQK--------
97-642         -AN.TRPRS.RPSKAP.RGRKWMR-CERETVAA.Y.E.LYVHC.V.D...TGGA.LELWFQRV...S.ST.GDEEGVPN.FP.APP..LFGGQ--------
SV106-98       -AN.TRPRS.RPSKAP.RGRKWMR-CERETVAA.Y.E.LYVHC.V.D...TGGA.LELWFQRV...S.ST.GDEEGVPN.FP.APP..LFAAQK--------
SV507-99       -AN.TRPRS.RPSKAP.RGRKWMR-SERETVAA.Y.E.LYVHC.V.D...TGGA.LELWFQRV...S.ST.GDEEGVPN.FP.APP..LFAAQK--------
002-00         -AN.TRPRS.RPSKAP.RGRKWMR-CERETVAA.Y.E.LYVHC.V.D...TGGA.LELWFQRV...S.ST.GDEEGVPN.FP.APP..LLAAQ---------
SV190-00A      -AN.TRPRS.RPSKAP.RGRKCVR-CGRETVAA.Y.E.LYVHC.V.D...TGGA.LELWFQRV...S.ST.GDEEGVPN.FP.APP..LFAAQK--------
SV55-02        -AN.TRPRS.RPSKAP.RGRKWMR-CERETVAA.Y.E.LYVHC.V.D...TGGA.LELWFQRV...S.ST.GDEEGVPN.FP.APP..LFAAQK--------
SV437-04       -AN.TRPRS.RPSKAP.RGRKWMR-CERETVAA.Y.E.LYVHC.V.D...TGGA.LELWFQRV...S.ST.GDEEGVPN.FP.APP..LFAAQK--------
SV198-05       -AN.TRPRS.RPSKAP.RGRKWMR-CERETVAA.Y.E.LYVHC.V.D...TGGA.LELL---------------------------------------
SV41-06        -AN.TRPRS.RPSKAP.RGRKWMR-CERETVAA.Y.E.LYVHC.V.D...TGG------------------------------------------------
SV223-06       -AN.TRPRS.RPSKAP.RGRKWMR-CERETVAA.Y.E.LYVHC.V.D...TGGA.LELWFQRV...S.ST.GDEEGVPN.FP.APP..LFAAQK--------
SV225-06       -AN.TRPRS.RPSKAP.RGRKWMR-CERETVAA.Y.E.LYVHC.V.D...TGGA.LELWFQRV...S.ST.GDEEGVPN.FP.APP..LFAAQK--------
SV355-06       -AN.TRPRS.RPSKAP.RGRKWMR-CERETVAA.Y.E.LYVHC.V.D...TGGA.LEL--------------------------------------------
SV71-07A       -AN.TRPRS.RPSKAP.RGRKCVS-SERETVAA.Y.E.LYVHC.V.D...TGGA.LELWFQRV...S.ST.GDEEGVPN.FP.APP..LFAAQK--------
SV71-07D       -AN.TRPRS.RPSKAP.RGRKWMR-CERETVAA.Y.E.LYVHC.V.D...TGGA.LELWFQRV...S.ST.GDEEGVPN.FP.APP..LFAAQK--------
SV79-07        -AN.TRPRS.RPSKAP.RGRKWYAAAERETVAA.Y.E.LYVHC.V.D...TGGA.LELWFQRV.--------------------------------------
SV102-07       -AN.TRPRS.RPSKAP.RGRKWMR-CERETVAA.Y.E.LYVHC.V.D...TGGA.LELLFQRV.--------------------------------------
SV511-09       TAN.TRPRS.RPSKAP.RGRKWMR-CERETVAA.Y.E.LYVHC.V.----------------------------------------------------
URUGUAY-T4     -AN.TRPRS.RPSKAP.RGRKWMR-CERETVAA.Y.E.LYVHC.V.D...TGGA.LELWFQRV...S.ST.GDEEGVPN.FP.APP..LFAAQ---------
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Figure 1. Deduced amino acid sequence alignment of the 5′ third of glycoprotein C gene of bovine herpesvirus 1 (BoHV-1, n = 19) and BoHV-5 (n = 25). Reference sequences of BoHV-1 and BoHV-5 (GenBank ID AJ004801 and AY261359) are included for comparison.

VN titers between homologous *vs* heterologous serum-virus pairs ranged from none (identical titers) to 4 serum dilutions (BoHV-1 SV47/05), with most differences being of 1 dilution. Low antibody titers (≤4) to heterologous serum were observed for some isolates, and in 9% of isolates (EVI340/96, SV198/05, SV71/07A, SV102/07), low titers against both sera were observed. These results confirm extensive serological cross-neutralization between BoHV-1 and BoHV-5, yet indicate the existence of antigenic differences. Some of these differences probably encompass epitopes involved in
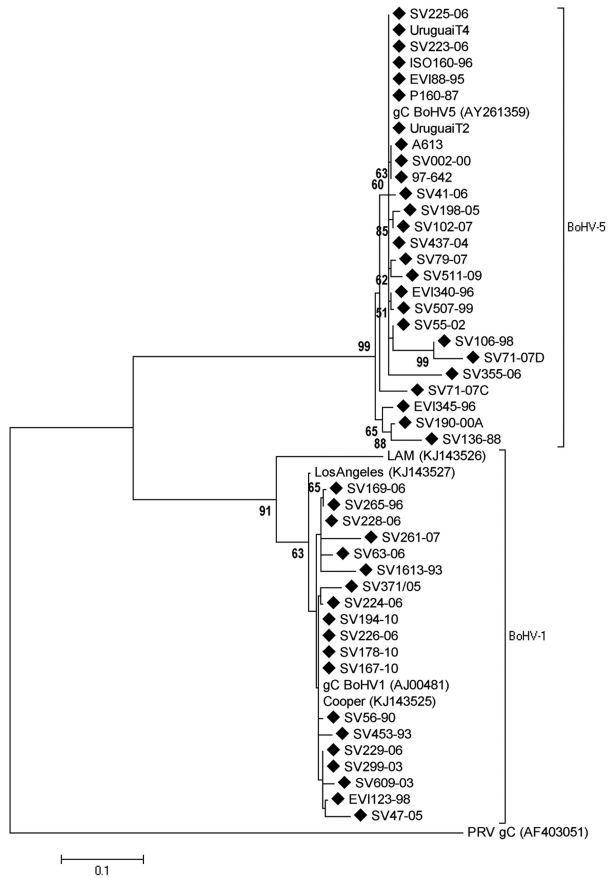
Figure 2. Phylogenetic tree based on the maximum likelihood method for the nucleotide sequence of the 5′ third of glycoprotein C (gC) gene of 19 bovine herpesvirus 1 (BoHV-1) and 25 BoHV-5 isolates from South America (black diamond; 310 positions). Reference strain sequences are included in the analysis. Only bootstrap values higher than 50% of 2000 replicates are shown.

data will contribute to the overall knowledge of this glycoprotein, which plays important roles in viral biology, interacts with the immune system, and represents major target antigens for immunodiagnostic tests and immunization strategies.

This study confirmed the high degree of 5′ gC similarity among isolates of each viral species and a low similarity between BoHV-1 and BoHV-5. The high gC conservation allows for phylogenetic differentiation of BoHV-1 and BoHV-5 (Figure 2). The main differences between gC1 and gC5 are located within the NH_2-t third of the protein, notably at amino acid residues 1-123 of gC1 and 1-102 of gC5 (10,25). Our data extend these observations, showing that 5′ gC1 and 5′ gC5 differ substantially throughout the sequenced region, displaying a nucleotide similarity of 62% and amino acid similarity of 53.3%, slightly higher than the 45% nucleotide similarity found by Hecht et al. (32). The differences included several substitutions of amino acid residues from different chemical groups (Figure 1), which may result in differences in amino acid function (33). In contrast with previous data reporting a deletion of 35 amino acid residues in gC5 (10), our data showed the absence of about 40 residues throughout 5′ gC1 compared with 5′ gC5. Sequence differences were even observed between gC1 and gC5 predicted epitopes (33).

The differences observed between 5′ gC1 and 5′ gC5 sequences/epitopes may affect their antigenic/serological properties and may explain most antigenic differences detected in VN assays. Glycoprotein C is the major BoHV surface protein and is involved in attachment to cellular receptors and interactions with the immune system, acting as a major target for neutralizing antibodies (16,18,23,26), especially the exposed gC NH_2-t ectodomain. Differences in the antigenic structures of gC1 and gC5 were previously demonstrated using a gC-specific MAb panel and assays of virus-neutralizing MAbs (17). Those findings confirmed that the gCs of each virus were very dissimilar and there was no cross-reactivity/neutralization by monospecific bovine antisera raised against the heterologous virus. Thus, the 5′ gC sequence differences likely explain the higher titers against homologous serum observed in this study, as well as some antigenic variation among isolates of the same species. In spite of important sequence differences in 5′ gC, VN assays revealed a high level of cross-neutralization between BoHV-1 and BoHV-5. It is reasonable to speculate that some cross-neutralization observed between BoHV-1 and BoHV-5 was due to the conserved epitopes in gC, in addition to neutralizing antibodies against other envelope glycoproteins (e.g., gB and gD), which are well conserved between BoHV-1 and BoHV-5 and also harbor neutralizing epitopes (10,24).

The antigenic similarity shared by these viruses (as shown here by VN assays) frequently hamper serological differentiation and might potentially interfere with control/eradication efforts (13,14). For many years, this antigenic relationship was a problem for virus identification, diagnosis and epidemiological studies because it hindered
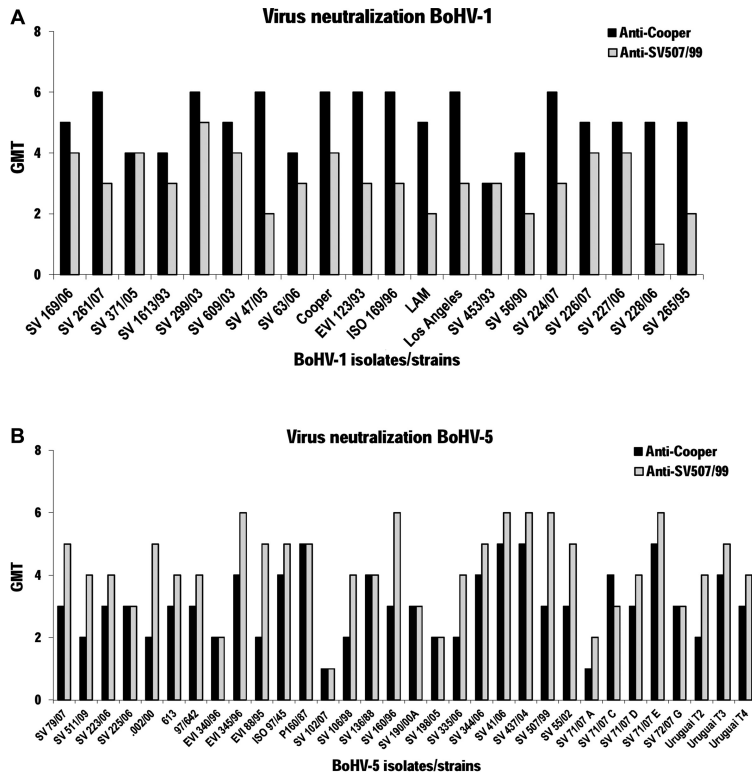
virus neutralization as demonstrated by the variable degree of virus neutralization by either BoHV-1 or BoHV-5 antisera.

Discussion

Bovine herpesviruses 1 and 5 are important pathogens of cattle, having either a virtually global distribution (BoHV-1) or a high prevalence in South America (BoHV-5). However, in spite of their importance and the critical role of gC in virus biology and its implications for diagnostic and immunization success, limited information is available on BoHV-1/BoHV-5 5′ gC. Only 5 BoHV-1 and 3 BoHV-5 sequences covering residues 1-174 are available in GenBank. The lack of more consistent information on this gene product is due in part to technical difficulties in amplifying and sequencing this genomic region. Our data provides consistent 5′ gC sequence information on 44 isolates of both viral types. These

Figure 3. Virus neutralization geometrical mean titers (GMT) of 20 isolates/strains of bovine herpesvirus 1 (BoHV-1) against homologous (Cooper strain) and heterologous (SV507/99 strain) antisera are demonstrated (*A*). *B*, GMT of 32 BoHV-5 isolates/strains against homologous (SV507/99) and heterologous (Cooper) antisera.

differentiating these agents by routine immunodiagnostic tests (12,34). Interestingly, differences in 5′ gC of BoHV-1 and BoHV-5 may be further exploited for differential diagnosis. Due to its low similarity, 5′ gC has been used to differentiate BoHV-1 and BoHV-5 by molecular and antigenic methods. This is a target region for differential PCR, which can distinguish between BoHV-1 and BoHV-5 by variation in amplicon length (4,29,35). These viruses can also be differentiated by distinct patterns of MAb recognition (23,36). In spite of the genetic variability, 5′ gC may serve as an additional target genomic region for phylogenetic differentiation of BoHV-1 and BoHV-5 (Figure 2).

On the other hand, the antigenic similarity between BoHV-1 and BoHV-5 results in cross-protection, to a variable degree, by vaccines against either virus and may benefit control programs based on vaccination (11,14,37). In this sense, our results showed that all isolates cross-reacted extensively *in vitro*, the majority producing moderate to high neutralizing titers against the homologous and heterologous sera. However, low antibody titers to heterologous serum were observed for some isolates and, four of them (BoHV-5) were neutralized poorly by both sera. These results suggest that, in addition to antigenic variability, the susceptibility to neutralizing antibodies may vary among isolates.

Glycoprotein C of alphaherpesviruses is involved in receptor binding and virus attachment to the cell surface, thus assisting in the determination of tissue-cell tropism (18). Thus, the sequence differences between gC1 and gC5

shown in this study may contribute, in part, to the differences in tropism and virulence possessed by BoHV-1 and BoHV-5. The main phenotypic difference between these viruses *in vivo* is neurovirulence, a property more frequently associated with BoHV-5 (4,38). In this sense, it has been shown that differences in the quality of the heparin binding of gC1 and gC5 would affect neurotropism and neurovirulence (26,27). Nonetheless, gC seems not to be the sole determinant of the BoHV-5 neurovirulence phenotype, but is important for high levels of virus replication in the central nervous system and for full expression of virulence in rabbits (25-27). The binding to host cell receptor occurs at the gC NH_2-t domain and apparently involves residues172-323 (20,28). Although this specific region has not been sequenced in the present study, it is conceivable that the marked differences observed between 5′ gC1 and 5′ gC5 would somehow influence protein folding and tridimensional structure (changing binding sites) and, perhaps, influence binding to cell receptors. Results from the homologous human herpesvirus (HHV) gC indicated that binding of this protein to the cell surface involves more than a simple interaction with heparin, and showed that its NH_2-t (residues 33-123) is important for efficient attachment to cells (39).

The 5′ region of gC gene sequences are very dissimilar between BoHV-1 and BoHV-5, but well conserved within the same viral species. In addition to the sequence differences in this region, high serologic cross-reactivity is observed between BoHV-1 and BoHV-5 isolates. However, a few

BoHV-5 isolates reacted weakly to either sera, which suggests a need for reevaluating immunization protocols with a single viral species. This study adds new information to the limited available genetic database on the 5′ gC gene and contributes more consistent information on the molecular and antigenic characteristics of BoHV-1 and BoHV-5.

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