BRAZILIAN JOURNAL
OF MEDICAL AND BIOLOGICAL RESEARCH
www.bjournal.com.br

ISSN 0100-879X
Volume 43 (02) 124-225 February 2010

BIOMEDICAL SCIENCES
AND
CLINICAL INVESTIGATION

Braz J Med Biol Res, February 2010, Volume 43(2) 217-224

Construction and growth properties of bovine herpesvirus type 5 recombinants defective in the glycoprotein E or thymidine kinase gene or both

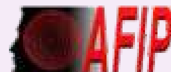
M.C.S. Brum, R. Weiblen, E.F. Flores and S.I. Chowdhury

The Brazilian Journal of Medical and Biological Research is partially financed by

Ministério
da Ciência e Tecnologia

Ministério
da Educação

Institutional Sponsors



Brazilian Journal of Medical and Biological Research (2010) 43: 217-224
ISSN 0100-879X

Construction and growth properties of bovine herpesvirus type 5 recombinants defective in the glycoprotein E or thymidine kinase gene or both

M.C.S. Brum[1], R. Weiblen[1], E.F. Flores[1] and S.I. Chowdhury[2]

[1]Setor de Virologia, Departamento de Microbiologia e Parasitologia, Departamento de Medicina Veterinária Preventiva, Universidade Federal de Santa Maria, Santa Maria, RS, Brasil
[2]Department of Diagnostic Medicine and Pathobiology, College of Veterinary Medicine, Kansas State University, Manhattan, KS, USA

Abstract

Bovine herpesvirus type 5 (BoHV-5) is an important pathogen of cattle in South America. We describe here the construction and characterization of deletion mutants defective in the glycoprotein E (gE) or thymidine kinase (TK) gene or both (gE/TK) from a highly neurovirulent and well-characterized Brazilian BoHV-5 strain (SV507/99). A gE-deleted recombinant virus (BoHV-5 gEΔ) was first generated in which the entire gE open reading frame was replaced with a chimeric green fluorescent protein gene. A TK-deleted recombinant virus (BoHV-5 TKΔ) was then generated in which most of the TK open reading frame sequences were deleted and replaced with a chimeric β-galactosidase gene. Subsequently, using the BoHV-5 gEΔ virus as backbone**,** a double gene-deleted (TK plus gE) BoHV-5 recombinant (BoHV-5 gE/TKΔ) was generated. The deletion of the gE and TK genes was confirmed by immunoblotting and PCR, respectively. In Madin Darby bovine kidney (MDBK) cells, the mutants lacking gE (BoHV-5 gEΔ) and TK + gE (BoHV-5 gE/TKΔ) produced small plaques while the TK-deleted BoHV-5 produced wild-type-sized plaques. The growth kinetics and virus yields in MDBK cells for all three recombinants (BoHV-5 gEΔ, BoHV-5 TKΔ and BoHV-5 gE/TKΔ) were similar to those of the parental virus. It is our belief that the dual gene-deleted recombinant (BoHV-5 gE/TKΔ) produced on the background of a highly neurovirulent Brazilian BoHV-5 strain may have potential application in a vaccine against BoHV-5.

Key words: Bovine herpesvirus 5; Glycoprotein E; Thymidine kinase; Deletion mutants; Vaccine candidate; Recombinant herpesvirus

Introduction

Bovine herpesvirus type 5 (BoHV-5) is a neurovirulent alpha-herpesvirus associated with necrotizing meningoencephalitis in cattle (1). BoHV-5 infection has been occasionally described in several countries including Australia, the United States and European countries, while the disease is more prevalent in South American countries such as Brazil and Argentina (2-5). BoHV-5 is closely related to another important alpha-herpesvirus of cattle, bovine herpesvirus type 1 (BoHV-1), the agent of infectious bovine rhinotracheitis and infectious pustular vulvovaginitis/balanoposthitis, also associated with abortion (1).

BoHV-5 and BoHV-1 are closely related viruses belonging to the family Herpesviridae, subfamily Alphaherpesvirinae, genus *Varicellavirus* (6,7). The BoHV-5 genome consists of a linear double-stranded DNA molecule of approximately 138 kb, encodes at least 70 gene products and shares 82% identity with the BoHV-1 genome at the protein level (7). Based on the requirement for replication in cell culture, the genes of alpha-herpesviruses have been classified into two groups, essential and non-essential (6,8). Alpha-herpesviruses contain approximately 12 virus-encoded envelope proteins. These proteins are important for the virus life cycle in cells and for pathogenesis in their respective natural hosts (6,8). The envelope glycoprotein E (gE) is important for the neuroinvasion and neurovirulence of BoHV-5 (9). The virally coded enzyme thymidine kinase (TK) is involved in the metabolism of deoxyribonucleotides in neuronal cells and is important for the neurovirulence

Correspondence: E.F. Flores, DMVP/CCR/UFSM, 97105-900 Santa Maria, RS, Brasil. Fax: +55-55-3220-8034.
E-mail: flores@ccr.ufsm.br

Received May 13, 2009. Accepted October 23, 2009. Available online November 6, 2009. Published February 1, 2010.

of alpha-herpesvirus (10,11). Interestingly, the gE and TK genes are not required for alpha-herpesviruses replication in tissue culture. This has prompted the targeting of the gE and TK genes for attenuating deletions (10,12).

Most of the vaccines marketed in South America contain inactivated BoHV-1 strains, which are not effective with respect to the cell immune response (13,14), and lack a serological marker, which is an important criterion for vaccination with differential vaccines (15). Some European countries have successfully eradicated BoHV-1 infection based on differential vaccines (12). Reports indicate that modified live BoHV-1 vaccines including the gE-deleted marker vaccines do not efficiently protect against BoHV-5 infections (13,14). Therefore, a better vaccine candidate for the protection against BoHV-5 infection is needed.

In the present study, we describe the construction and *in vitro* characterization of three deletion mutants of the extremely neurovirulent Brazilian BoHV-5 strain SV507/99 (7,16). The recombinant viruses are defective in the genes encoding the gE, TK and both genes (gE plus TK). This is the first description of the deletion of the BoHV-5 TK gene. Since the TK gene is necessary for efficient virus replication in the central nervous system (CNS), it may represent an attractive target for attenuation. Thus, we propose that these BoHV-5 recombinant viruses may be useful for future BoHV-5 pathogenesis studies and vaccine development in South American countries.

Material and Methods

Virus strain and cells

The Brazilian BoHV-5 strain SV507/99 was used as the parental virus to construct the recombinant viruses. This BoHV-5 strain is highly neurovirulent and has been isolated from a cow that died from neurological infection in southern Brazil. The entire genome of this virus has been sequenced (7). The virus was propagated in Madin Darby bovine kidney cells (MDBK, ATCC-22) maintained in Dulbecco's modified Eagle's medium (Invitrogen, USA), supplemented with 10% inactivated and γ-irradiated fetal bovine serum (HyClone, USA) and 100 U/mL penicillin and 100 µg/mL streptomycin (Invitrogen).

Virus DNA extraction

Extraction of viral DNA was performed essentially as described by Chowdhury et al. (17). Briefly, MDBK cells were inoculated with SV507/99 at a multiplicity of infection (moi) of 0.01. When the cytopathic effect reached about 90% of the monolayer, the supernatant was collected and clarified by low speed centrifugation. The virus was pelleted by ultracentrifugation for 2 h at 112,500 *g* in a 30% sucrose cushion. The viral pellet was resuspended in 1X TE (10 mM Tris-HCl, 5 mM EDTA, pH 8.0) and digested in 1% SDS containing 500 µg/mL proteinase K (Sigma, USA) at 56°C for 1 h. Following digestion, viral DNA was extracted with phenol:chloroform:isoamyl alcohol (25:24:1), followed by ether extraction and ethanol precipitation according to routine protocols.

Construction of BoHV-5 gE and TK deletion plasmids

The construction of gE-deletion plasmid pBHV-5 gEΔ enhanced green fluorescent protein gene (eGFP) containing the deletion of the entire gE open reading frame (ORF) coding region and expressing the eGFP has been described elsewhere (18). To engineer a TK transfer plasmid, the TK upstream and downstream sequences were amplified by long PCR using the XL-long PCR kit (Applied Biosystems, Foster City, CA, USA). The TK upstream sequence was amplified using a pair of primers (5TKup forward 5'-ATGAgGaTCCGCTCGTCCGGCACGAAGAC-3' and 5TKup reverse 5'-ATCCgAaTTcAGGCGTCCGTGGGG AACATC-3'), resulting in a product of 1108 bp, which incorporated *Bam*HI/*Eco*RI sites in the 5' and 3' product ends, respectively. The TK downstream sequence was amplified using a pair of primers (5TKdn forward 5'-CGAaAgCtT GTTTGCGGCGCTCAAGTGC-3' and 5TKdn reverse 5'-GGCGGTaCcCGCAAAGTCGACGTGAAC-3') resulting in a product of 702 bp, which incorporated the *Hin*dIII/*Kpn*I sites in the 5' and 3' ends, respectively. The PCR products were digested with *Bam*HI/*Eco*RI or *Hin*dIII/*Kpn*I and cloned into an appropriately digested pBlueScript KS (+) vector (Stratagene, USA), resulting in the plasmids pBoHV-5 TK up and pBHV-5 TK down, respectively. To assemble the TK transfer plasmid, the pBoHV-5 TK down was digested with *Hin*dIII/*Kpn*I and a ~700-bp fragment was gel purified and cloned into pBoHV-5 TK up previously digested with the same enzymes, resulting in the plasmid pBoHV-5 TKΔ. In this plasmid a 593-bp long segment of the TK ORF coding sequences specifying amino acids 67 to 258 of TK is deleted and the deletion site is flanked by the *Eco*RI and *Hin*dIII restriction sites suitable for beta gal gene insertion (see below). TK is flanked by UL 24 and gH, two essential genes (Figure 1). The start codon of the UL24 and the TK start codon overlap partially (GenBank Accession No. NC005261). Therefore, the promoter sequence of UL24 is located within the aminoterminal third of the TK coding region. Similarly, the promoter sequence of gH is predicted to be within the carboxyterminal third of TK. To protect these two essential genes, the aminoterminal and carboxyterminal third of the TK coding sequences were not deleted. To insert the β-galactosidase (β-gal) cassette into pBoHV-5 TKΔ, a 4529-bp fragment containing the CMV promoter, the β-gal coding sequence and the SV40 poly A site was isolated from pCMV β-gal (Stratagene, USA) after *Hin*dIII/*Eco*RI digestion, and cloned into the *Hin*dIII/*Eco*RI-digested pBoHV-5 TKΔ plasmid, resulting in pBoHV-5 TKΔ β-gal.

Generation of recombinant viruses

BoHV-5 gEΔ was generated as described elsewhere (17). After 48-72 h, green fluorescent viral plaques were

visualized directly under UV light with an epifluorescence microscope, picked and plaque purified three times. Expanded clones were subsequently analyzed for the presence of gE by Western blotting. To generate a BoHV-5 TKΔ virus, the linearized pBoHV-5 TKΔ β-gal plasmid was co-transfected with a wild-type viral DNA virus into MDBK cells as described above. Recombinant viruses expressing β-gal were screened using the Bluo-Gal agar overlay assay (Invitrogen) (19), blue plaques were picked, purified three times and further analyzed for the deletion of the TK gene-specific sequence by PCR. To generate a double deletion BoHV-5 gE/TKΔ recombinant virus, the full length BoHV-5 gEΔ virus DNA was co-transfected with the linearized pBoHV-5 TKΔ β-gal plasmid into MDBK cells. Recombinant viruses expressing β-gal were identified and plaque purified as described above.

SDS-PAGE and immunoblotting

Confluent MDBK cells were mock-infected or infected with wild-type BoHV-5 or BoHV-5 gEΔ at a moi of 1. Twelve hours after infection the cells were scraped, the pellet was harvested by low speed centrifugation at 4°C and resuspended (10% w/v) in extraction buffer (0.15 M NaCl, 10 mM Tris, pH 8.0, 10 mM EDTA, 1.0% Nonidet P-40, 0.5% sodium deoxycholate, 1.0 mM PMSF and 2 µg aprotinin per mL), frozen at -80°C and thawed on ice. Triton X-100 (1%) was added and the preparation was incubated on ice for 30 min. The suspension was centrifuged at 13,000 *g* for 10 min at 4°C and the supernatant was saved as the protein extract and used for electrophoresis. The protein extracted (20 µL) was diluted in 2X sample buffer, boiled for 5 min and subjected to 15% SDS-PAGE. Immunoblotting analysis was performed using a rabbit anti-BHV-5 gE polyclonal antibody as primary antibody (18).

TK-specific PCR

To confirm the deletion of an internal segment of the BoHV-5 TK coding region, PCR using a pair of primers that anneal and amplify the deleted region was performed. Total DNA from mock-infected MDBK cells and from wild-type BoHV-5, BoHV-5 TKΔ β-gal and BoHV-5 gE/TKΔ infected cells was extracted by proteinase K digestion and phenol/chloroform extraction as described above. PCR was carried out in a 50 µL volume containing 1X PCR buffer, 0.2 mM dNTPs, 0.4 µM of each primer (5TKdel forward 5'-GACGTCGTGACCCTCGTGTTTG-3' and 5TKdel reverse 5'-TAGGAAGGCGCACGTGTTCG-3'), 1.25 U Taq polymerase, 1.5 mM $MgCl_2$, 5% DMSO, and 0.5 µg DNA as template. The PCR conditions consisted of initial denaturation at 94°C for 5 min followed by 35 cycles at 94°C for 45 s, 56°C for 30 s, and 72°C for 45 s, and a final extension of 10 min at 72°C. Five microliters of each reaction was electrophoresed on 1.5% agarose gel and stained with ethidium bromide.

Viral growth curve and plaque morphology

Viral growth properties were examined by one-step growth curve and plaque assays. Cultures of MDBK cells (32 T-25 cm^2 flasks) were infected with either wild-type or recombinant viruses at a moi of 5. Virus titers were

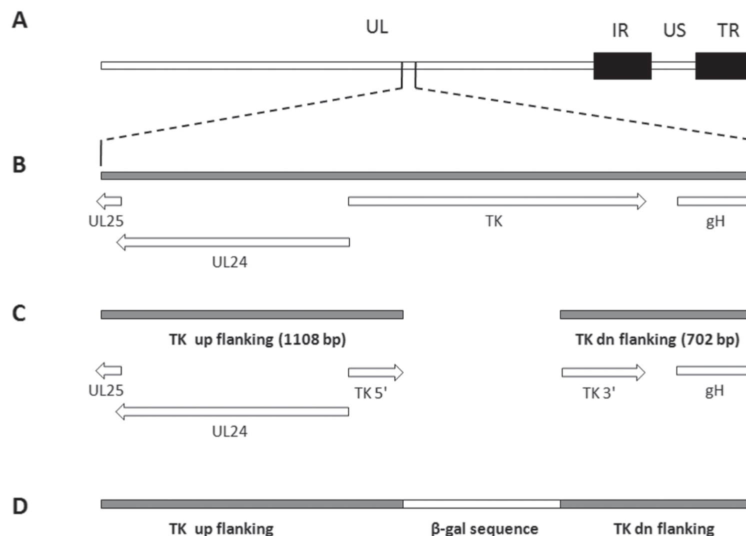


Figure 1. Schematic representation of the construction of the BoHV-5 thymidine kinase (TK)-deleted fragment. *A*, The complete BoHV-5 genome; UL (unique long), US (unique short), IR (internal repeat), and TR (terminal repeat) regions. *B*, BoHV-5 TK gene flanking region. *C*, PCR-amplified fragment of the TK flanking region (TK up and dn = upstream and downstream, respectively) using the primers described in Material and Methods. *D*, Deletion of part (539 bp) of the TK coding region and insertion of the β-galactosidase (β-gal) reporter gene.

determined by plaque assays at 0, 2, 4, 6, 18, 21, 24, and 27 h post-infection and the results are reported as plaque forming units (PFU/mL). To compare plaque morphology produced by the recombinant viruses with that of the wild-type SV507/99, confluent monolayers of MDBK cells on six-well plates were infected individually with approximately 100 PFU/well of each recombinant virus and overlaid with 1.6% carboxymethyl cellulose. At 48 h post-infection, cells were fixed with 10% formaldehyde, washed and stained with 0.35% crystal violet.

Results

Construction of a BoHV-5 gE-deleted mutant

To construct a BoHV-5 gE-deleted recombinant virus, the gE transfer plasmid pBoHV-5 gEΔ eGFP constructed earlier (18) was used in a recombination experiment. In this plasmid the entire gE ORF is replaced with the GFP gene. Recombination efficiency based on fluorescence-positive plaques was approximately 1% (7 GFP-positive plaques for approximately each 500 PFUs). The eGFP-positive fluorescent plaques were plaque purified three times and further analyzed by immunoblotting for the lack of gE expression. One of the seven gE-deleted recombinants was selected at random and used for subsequent studies (Figure 2).

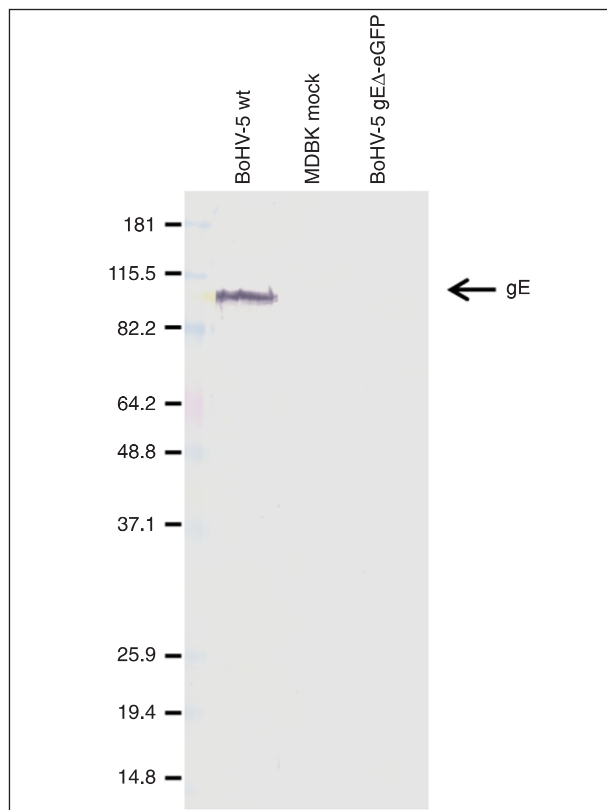


Figure 2. Immunoblotting analysis of BoHV-5 glycoprotein E (gE). Madin Darby bovine kidney (MDBK) cells were inoculated with BoHV-5 wild-type (wt), BoHV-5 gEΔ-eGFP (enhanced green fluorescent protein) or mock infected and submitted to protein extraction, SDS-PAGE electrophoresis and immunoblotting with a rabbit polyclonal anti-gE antibody.

Construction of a BoHV-5 TK deletion mutant

To generate a BoHV-5 TK defective virus, a deletion plasmid pBoHV-5 TKΔ β-gal containing the 5' and 3' flanking regions of the TK gene and β-gal gene marker was constructed (Figure 1). TK gene is flanked by UL24 and gH and a central 593-bp long segment (corresponding to amino acids 67 to 258 of TK protein) was deleted. Subsequently, a chimeric β-gal gene was inserted at the TK deletion locus. The TK deletion plasmid pBoHV-5 TKΔ β-gal was then used for transfection with full-length BoHV-5 genomic DNA. The recombination efficiency in this case was approximately 0.2% (1 blue plaque/500 plaques). Two β-gal-positive recombinant virus isolates were plaque purified and analyzed by TK-specific PCR (Figure 3).

Construction of a BoHV-5 gE and TK double deletion mutant

In a third recombination experiment, a BoHV-5 double deletion mutant (TK + gE deleted) was generated. For this, the BoHV-5 gEΔ viral DNA was co-transfected with pBoHV-5 TKΔ β-gal DNA in MDBK cells. Recombination efficiency in this experiment was approximately 4% (18 blue plaques/500 PFU). One recombinant TK plus gE-deleted virus was analyzed by PCR (Figure 3) and DNA sequencing for TK deletion and by immunoblotting as described for gE

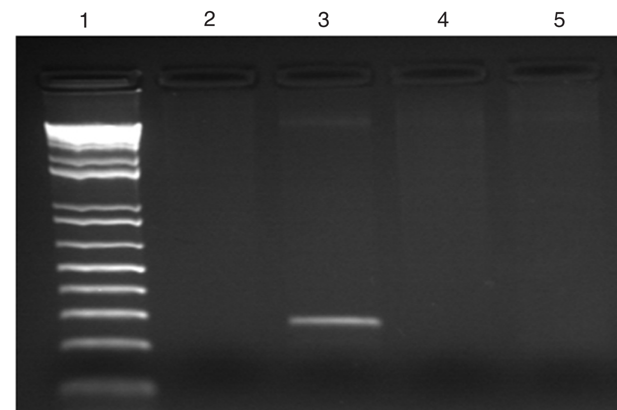


Figure 3. PCR amplification of the BoHV-5 TK gene internal region (285 bp). Madin Darby bovine kidney cells were mock infected or inoculated with wild-type (wt) or recombinant viruses and total DNA was extracted and submitted to PCR amplification of the BoHV-5 TK gene. A positive reaction indicates the presence of the entire TK gene. Five microliters of each reaction was resolved on 1.5% agarose gel and stained with ethidium bromide. *Lane 1*, Molecular weight marker; *lane 2*, MDBK mock; *lane 3*, BoHV-5 wt; *lane 4*, BoHV-5 TKΔ, and *lane 5*, BoHV-5 gE/TKΔ.

deletion (data not shown).

Characterization of BoHV-5 gE, TK and gE + TK deletion mutants

The *in vitro* characteristics of the recombinants BoHV-5 gEΔ, BoHV-5 TKΔ and BoHV-5 gE/TKΔ were studied and compared with the parental wild-type BoHV-5. All selected recombinant viruses were readily amplified to prepare virus stock with titers above 10^7 PFU/mL and used in subsequent experiments. The relative plaque sizes produced by BoHV-5 TKΔ recombinants were morphologically similar to those produced by wild-type BoHV-5. However, plaques produced by BoHV-5 gEΔ and BoHV-5 gE/TKΔ were on average smaller than those produced by wild-type virus and by BoHV-5 TKΔ (Figure 4). The single step growth of the deleted recombinants and wild-type BoHV-5 viruses was assayed on MDBK cells and the results are shown in Figure 5. Even though the gE-deleted and gE + TK-deleted recombinants produced smaller plaques, all three recombinant viruses (BoHV-5 gEΔ, BoHV-5 TKΔ and BoHV-5 gE/TKΔ) replicated with similar kinetics and virus yield when compared with the wild-type BoHV-5 virus.

The recombinant viruses selected can be used in pathogenesis studies and for vaccine development. In this respect, viral stability is very important and was determined by five serial passages in cell culture following identification of the respective reporter gene and PCR of the deleted gene segment. After five serial passages, all reporter genes were present in the respective viruses and could not be amplified by an internal deleted gene segment (data no shown), demonstrating the stability of the recombinants.

Discussion

We report here the construction and *in vitro* growth properties of BoHV-5 recombinants defective in gE or TK or in both genes. The construction of recombinants was achieved by homologous recombination in MDBK cells after co-transfection of viral DNA and plasmids containing partial or entire gene deletions. The gE and TK genes were chosen for deletion because they are non-essential for viral replication in tissue culture and their products have been associated with neurovirulence in other alpha-herpesviruses (11,19). Moreover, gE deletion has been useful as the antigenic marker in vaccines to allow serologic differentiation of vaccinated from naturally infected animals (15). The deleted gene sequences were replaced with reporter genes to facilitate the identification of recombinant viruses. The recombinant viruses retained their ability to replicate in tissue culture and, therefore, may be potentially used for pathogenesis studies and for vaccine development as well.

Homologous recombination in cell culture was the methodology used to construct the presented viruses. In this methodology, viral DNA is co-transfected with a plasmid containing the flanking sequence of the deleted target

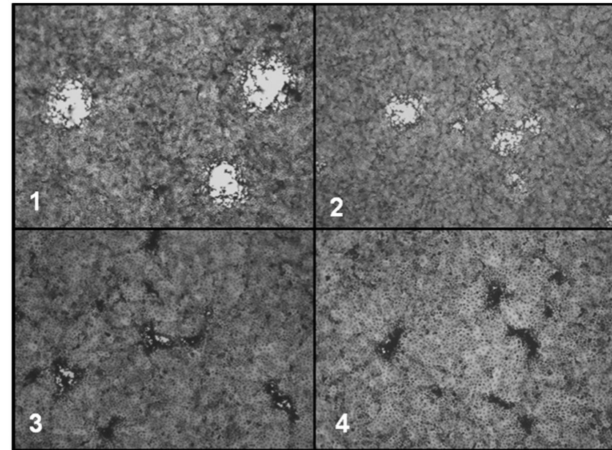


Figure 4. Plaque morphology. Madin Darby bovine kidney cells were inoculated with each recombinant and wild-type (wt) viruses and overlaid with 1.6% carboxymethyl cellulose. At 48 h post-infection, cell monolayers were fixed with 10% buffered formalin and stained with 0.35% crystal violet. 1) BoHV-5 wt; 2), BoHV-5 TKΔ; 3) BoHV-5 gEΔ; 4) BoHV-5 gE/TKΔ (magnification 400X).

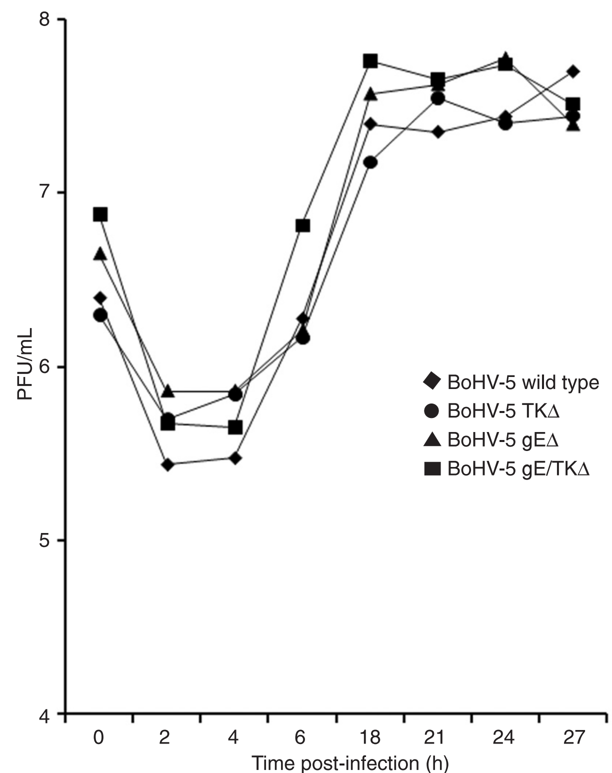


Figure 5. One-step viral growth curve. Madin Darby bovine kidney cells were inoculated with the recombinant or wild-type virus as described in Material and Methods. At different times post-infection cells were frozen at -80ºC and the viral progeny was quantified. The virus titers are reported as log_{10} plaque forming units (PFU/mL).

gene, following selection of recombinant viruses (20). Recombination efficiency ranged from 0.2 to ~4%, and the identification and purification of recombinant viruses were facilitated by the use of reporter genes such as GFP and β-gal (19,20). This methodology has been adapted to several herpesviruses and has the advantage of allowing the introduction of mutations in the desired region of the genome. Other methodologies, such as classical chemical selection or, more recently, bacterial artificial chromosome, can be used to introduce mutations or to manipulate the viral genome (20-22). Classical chemical selection is a random process that introduces mutations in a specific region and possibly in other segments of the genome (21). The bacterial artificial chromosome technology is available for several herpesviruses, but is not yet available for BoHV-5 (20).

BoHV-5 infection has been associated with neurological disease, which is usually fatal for young calves (1,23). However, the mechanisms of pathogenesis leading to the development of clinical disease are not completely understood. Viral pathogenesis involves virus replication in the nasal mucosa, axonal transport to the brain, virus replication and dissemination within the CNS, and the development of non-suppurative meningoencephalitis (24,25). As demonstrated for BoHV-5 and other neurotropic alpha-herpesviruses, gE and TK gene products are important and necessary for virus dissemination and replication, respectively, in neural tissues (9,11,19,26). Thus, our recombinants defective in gE, TK or both, constructed out of a highly virulent BoHV-5 strain, may be used to elucidate different mechanisms involved in BoHV-5 neuropathogenesis. These two genes are important for virus dissemination (gE) and replication in neuronal cells (TK), and therefore the mutants can be used to evaluate these two parameters in the CNS during the acute phase, latency establishment, and reactivation.

The herpesvirus envelope glycoproteins play important roles in the viral replication cycle and in the pathogenesis and the interactions of the virus with the immune system (10,23,27). BoHV-5 gE is present in the viral envelope forming a non-covalent heterodimer complex with glycoprotein I (gI), which is required for gE-gI maturation, cell-to-cell spread, neuroinvasiveness, and neurovirulence in calves and rabbits (9,28,29). The BoHV-5 gE coding sequence is located in the unique short (US) sequence of the viral genome and is flanked upstream and downstream by gI and US9 genes, respectively (7,9). The methodology described here for gE deletion removed most of the coding sequence, keeping the intergenic regions and the last three amino acids of the gE coding sequence. This strategy was designed to preserve the integrity and functionality of the flanking genes (18). The ability to replicate in tissue culture was unchanged in both recombinants lacking gE, confirming that BoHV-5 gE is not essential for viral replication *in vitro* (9). On the other hand, both viruses produced slightly smaller plaques in cell monolayers, probably reflecting a reduced ability in cell-to-cell dissemination (9). It has been demonstrated *in vivo* that gE from bovine herpesvirus type 1 and other alpha-herpesviruses is associated with the anterograde transport of virus in neuronal cells, directly affecting the neuroinvasiveness (9,11). Subsequent studies will determine whether the recombinant viruses lacking gE would retain their ability to replicate *in vivo*, a condition absolutely required for their potential use as live virus vaccines.

Like other alpha-herpesviruses, BoHV-5 replicates initially in epithelial cells and subsequently in neuronal cells and encodes its own TK (30). Thymidine kinase is an enzyme involved in the metabolism of deoxyribonucleotides in non-replicating cells such as neurons (26,30). This enzyme is not necessary for virus replication in epithelial cells or in cell culture, but it is required absolutely for virus replication in neurons and for efficient reactivation from latency (10,26). PRV, BoHV-1 and HSV defective in the TK gene product display reduced virulence and relative inability to reactivate from latent infection (11,26). As BoHV-5 neuropathogenesis is associated with the ability of the virus to replicate and disseminate within the brain, it may be assumed that functional TK is necessary for the full expression of virulence *in vivo*.

The BoHV-5 TK coding sequence is located in the unique long (UL) region of the viral genome and is flanked by UL24 (upstream) and gH (downstream) genes (7,30,31). Adjacent TK and UL24 share the first two bases of the initiation codon in opposite directions, and probably their regulatory regions are located in the coding sequence of the neighbor gene (7). Glycoprotein gH (UL22) is an essential gene for BoHV-1, HSV, PRV and likely also for BoHV-5 (31). In the case of BoHV-1, some regulatory regions of gH are located at the third end of the TK coding sequence (19). Thus, our strategy to produce a deleted TK BoHV-5 was designed to remove an intermediate part from its coding sequence possibly affecting the catalytic sites. As expected, both viruses lacking part of TK sequence showed no alterations in plaque morphology or kinetics of replication. The same phenotype was observed when a similar deletion strategy was used for BoHV-1 (19).

Neurological diseases by alpha-herpesviruses in cattle have been described in many parts of the world but the incidence is notably higher in Argentina and Brazil (2,4). In these countries, BoHV-1-based vaccines have been increasingly used in the last decades, and only a few vaccines have been formulated that contain both BoHV-1 and BoHV-5 strains. BoHV-1 and -5 show extensive serological cross-reactivity so that they cannot be distinguished by routine virus neutralization tests (32). In addition, it has been demonstrated that a BoHV-1 vaccine induced partial protection against a BoHV-5 virulent challenge in calves (13,14). Thus, the recombinants BoHV-5 gEΔ, TKΔ and gE/TKΔ described herein represent potential vaccine candidates to prevent BoHV-1 and -5 associated diseases in South American countries.

Control and eradication programs of BoHV-1 and PRV

infections based on inactivated or modified-live marker vaccines have been successfully conducted in some European countries and in the United States (15). The use of a virus vaccine containing an antigenic marker allows the differentiation between vaccinated and infected animals by using an appropriate serological test. Most BoHV-1 and PRV marker vaccines contain a deletion in the gE gene (as the antigenic marker), combined or not with a TK deletion (for attenuation) (15,27). Thus, our double deletion mutant would fulfill the major requirements for a herpesvirus modified-live vaccine and therefore represents an attractive strain for vaccine development. *In vivo* studies in calves are underway in order to ascertain its attenuation, immunogenicity and the ability to confer protection upon homologous (BoHV-5) and heterologous (BoHV-1) challenge.

Acknowledgments

Research supported by MCT/CNPq/CAPES/FINEP (Pronex em Virologia Veterinária 215/96). E.F. Flores (#301666/04-0) and R. Weiblen (#301339/04-0) are recipients of CNPq fellowships. M.C.S. Brum is the recipient of doctoral scholarships from CNPq and CAPES (PDEE/UFSM 3307/06-9).

References

1. Studdert MJ. A brief review of studies of bovine and equine herpesviruses. *Aust Vet J* 1989; 66: 401-402.
2. Carrillo BJ, Ambrogi A, Schudel AA, Vazquez M, Dahme E, Pospischil A. Meningoencephalitis caused by IBR virus in calves in Argentina. *Zentralbl Veterinarmed B* 1983; 30: 327-332.
3. d'Offay JM, Ely RW, Baldwin CA, Whitenack DL, Stair EL, Collins JK. Diagnosis of encephalitic bovine herpesvirus type 5 (BHV-5) infection in cattle: virus isolation and immunohistochemical detection of antigen in formalin-fixed bovine brain tissues. *J Vet Diagn Invest* 1995; 7: 247-251.
4. Rissi DR, Pierezan F, Silva MS, Flores EF, de Barros CS. Neurological disease in cattle in southern Brazil associated with bovine herpesvirus infection. *J Vet Diagn Invest* 2008; 20: 346-349.
5. Engels M, Giuliani C, Wild P, Beck TM, Loepfe E, Wyler R. The genome of bovine herpesvirus 1 (BHV-1) strains exhibiting a neuropathogenic potential compared to known BHV-1 strains by restriction site mapping and cross-hybridization. *Virus Res* 1986; 6: 57-73.
6. Roizmann B, Desrosiers RC, Fleckenstein B, Lopez C, Minson AC, Studdert MJ. The family Herpesviridae: an update. The Herpesvirus Study Group of the International Committee on Taxonomy of Viruses. *Arch Virol* 1992; 123: 425-449.
7. Delhon G, Moraes MP, Lu Z, Afonso CL, Flores EF, Weiblen R, et al. Genome of bovine herpesvirus 5. *J Virol* 2003; 77: 10339-10347.
8. Schwyzer M, Ackermann M. Molecular virology of ruminant herpesviruses. *Vet Microbiol* 1996; 53: 17-29.
9. Chowdhury SI, Lee BJ, Ozkul A, Weiss ML. Bovine herpesvirus 5 glycoprotein E is important for neuroinvasiveness and neurovirulence in the olfactory pathway of the rabbit. *J Virol* 2000; 74: 2094-2106.
10. Tikoo SK, Campos M, Babiuk LA. Bovine herpesvirus 1 (BHV-1): biology, pathogenesis, and control. *Adv Virus Res* 1995; 45: 191-223.
11. Enquist LW, Husak PJ, Banfield BW, Smith GA. Infection and spread of alphaherpesviruses in the nervous system. *Adv Virus Res* 1998; 51: 237-347.
12. van Oirschot JT, Kaashoek MJ, Rijsewijk FA, Stegeman JA. The use of marker vaccines in eradication of herpesviruses. *J Biotechnol* 1996; 44: 75-81.
13. Del Medico Zajac MP, Puntel M, Zamorano PI, Sadir AM, Romera SA. BHV-1 vaccine induces cross-protection against BHV-5 disease in cattle. *Res Vet Sci* 2006; 81: 327-334.
14. Silva AD, Spilki FR, Franco AC, Esteves PA, Hubner SO, Driemeier D, et al. Vaccination with a gE-negative bovine herpesvirus type 1 vaccine confers insufficient protection to a bovine herpesvirus type 5 challenge. *Vaccine* 2006; 24: 3313-3320.
15. van Oirschot JT. Diva vaccines that reduce virus transmission. *J Biotechnol* 1999; 73: 195-205.
16. Vogel FS, Caron L, Flores EF, Weiblen R, Winkelmann ER, Mayer SV, et al. Distribution of bovine herpesvirus type 5 DNA in the central nervous systems of latently, experimentally infected calves. *J Clin Microbiol* 2003; 41: 4512-4520.
17. Chowdhury SI, Ross CS, Lee BJ, Hall V, Chu HJ. Construction and characterization of a glycoprotein E gene-deleted bovine herpesvirus type 1 recombinant. *Am J Vet Res* 1999; 60: 227-232.
18. Al-Mubarak A, Simon J, Coats C, Okemba JD, Burton MD, Chowdhury SI. Glycoprotein E (gE) specified by bovine herpesvirus type 5 (BHV-5) enables trans-neuronal virus spread and neurovirulence without being a structural component of enveloped virions. *Virology* 2007; 365: 398-409.
19. Chowdhury SI. Construction and characterization of an attenuated bovine herpesvirus type 1 (BHV-1) recombinant virus. *Vet Microbiol* 1996; 52: 13-23.
20. Brune W, Messerle M, Koszinowski UH. Forward with BACs: new tools for herpesvirus genomics. *Trends Genet* 2000; 16: 254-259.
21. Wagner M, Ruzsics Z, Koszinowski UH. Herpesvirus genetics has come of age. *Trends Microbiol* 2002; 10: 318-324.
22. Liu ZF, Brum MC, Doster A, Jones C, Chowdhury SI. A bovine herpesvirus type 1 mutant virus specifying a carboxyl-terminal truncation of glycoprotein E is defective in anterograde neuronal transport in rabbits and calves. *J Virol* 2008; 82: 7432-7442.
23. Engels M, Ackermann M. Pathogenesis of ruminant herpesvirus infections. *Vet Microbiol* 1996; 53: 3-15.
24. Lee BJ, Weiss ML, Mosier D, Chowdhury SI. Spread of bovine herpesvirus type 5 (BHV-5) in the rabbit brain after intranasal inoculation. *J Neurovirol* 1999; 5: 474-484.
25. Perez SE, Bretschneider G, Leunda MR, Osorio EA, Flores EF, Odeon AC. Primary infection, latency, and reactivation of bovine herpesvirus type 5 in the bovine nervous system. *Vet Pathol* 2002; 39: 437-444.

26. Whetstone CA, Miller JM, Seal BS, Bello LJ, Lawrence WC. Latency and reactivation of a thymidine kinase-negative bovine herpesvirus 1 deletion mutant. *Arch Virol* 1992; 122: 207-214.
27. van Drunen Littel-van den Hurk. Cell-mediated immune responses induced by BHV-1: rational vaccine design. *Expert Rev Vaccines* 2007; 6: 369-380.
28. Al-Mubarak A, Zhou Y, Chowdhury SI. A glycine-rich bovine herpesvirus 5 (BHV-5) gE-specific epitope within the ectodomain is important for BHV-5 neurovirulence. *J Virol* 2004; 78: 4806-4816.
29. Hubner SO, Oliveira AP, Franco AC, Esteves PA, Silva AD, Spilki FR, et al. Experimental infection of calves with a gI, gE, US9 negative bovine herpesvirus type 5. *Comp Immunol Microbiol Infect Dis* 2005; 28: 187-196.
30. Smith GA, Young PL, Mattick JS. Nucleotide and amino acid sequence analysis of the thymidine kinase gene of a bovine encephalitis herpesvirus. *Arch Virol* 1991; 119: 199-210.
31. Meyer G, Bare O, Thiry E. Identification and characterization of bovine herpesvirus type 5 glycoprotein H gene and gene products. *J Gen Virol* 1999; 80 (Part 11): 2849-2859.
32. Bratanich AC, Sardi SI, Smitsaart EN, Schudel AA. Comparative studies of BHV-1 variants by *in vivo-in vitro* tests. *Zentralbl Veterinarmed B* 1991; 38: 41-48.