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CONSTRUCTION AND CHARACTERIZATION OF A BOVINE HERPESVIRUS 5 MUTANT WITH A DELETION OF THE GI, GE AND US9 GENES

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ABSTRACT

Bovine herpesvirus 5 (BoHV-5) is a important cause of viral encephalitis in cattle in South America. Within the framework of developing a differential vaccine against BoHV-5, a deletion mutant was constructed based on a Brazilian BoHV-5 isolate. The target of the deletions were genes that code proteins implicated in the neurovirulence of BoHV-5, the glycoprotein I (gI), glycoprotein E (gE) and membrane protein US9. To construct the deletion mutant of BoHV-5, the flanking regions of all three genes were cloned in a prokaryotic plasmid. This deletion fragment was co-transfected with the viral DNA into bovine cells. Identification of deletion mutants was performed by immunostaining with an anti-gE monoclonal antibody. One of the gE negative viral populations found was purified, amplified and further examined by restriction endonuclesase analysis of its genomic DNA. The plaque sizes and penetration kinetics of the deletion mutant and wild type viruses were compared. The plaque sizes of the deletion mutant were significantly smaller than those of the parental strain ($p \leq 0.05$), but no statistical differences were observed in penetration kinetics. The results indicate that the gI/gE/US9 deletion mutant of BoHV-5 may have a reduced virulence in the host and is still viable enough to be a good candidate for the development of a BoHV-5 vaccine.

Key words: bovine herpesvirus 5, BoHV-5, deletion mutant, glycoprotein genes

INTRODUCTION

Bovine herpesvirus 5 (BoHV-5) is an *Alphaherpesvirus* which is responsible for a non suppurative meningoencephalitis in young bovines, occurring mostly in countries of South America, although it was first identified in Australia (12,20). BoHV-5 shares many properties with other alphaherpesviruses of the genus *Varicellovirus*, to which also bovine herpesvirus 1 (BoHV-1), suid herpesvirus 1 (SuHV-1) and varicella zoster virus (VZV) belong. BoHV-5 has a double-stranded genome of about 138 kb and has at least 70 genes. Its genome has a Unique Long region of about 110 kb and a Unique Short region of about 10 kb, which is bordered by inverted repeats of about 10 kb (9). The arrangement of the genes is essentially co-linear with the other alphaherpesviruses and it is expected that most of the functions of the encoded proteins are conserved. BoHV-5 shows the highest homology with BoHV-1 (82% on amino acid level), but BoHV-5 infections follow essentially a neurological course, different from the respiratory/reproductive symptoms caused by BoHV-1. It is presently unclear which differences between BoHV-5 and BoHV-1 are responsible for the enhanced neurovirulence of BoHV-5, although in a rabbit model several BoHV-5 genes have been found to contribute to its neurovirulence. Among these are the genes coding for glycoprotein I (gI), glycoprotein E (gE) and membrane protein US9 (4,6). Glycoproteins gI and gE are type I transmembrane proteins, and functional regions have been identified on both their extracellular and cytoplasmic domains (23,26,27). Like their

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homologues encoded by other alphaherpesviruses, the glycoproteins gI and gE of BoHV-5 form a complex and are implicated in cell-to-cell spread mechanisms. Moreover, the gE gene of SuHV-1 is found to be essential for the anterograde transport of viral glycoproteins along axons (3) and this function may be shared by BoHV-5 gE. The US9 protein is a type II transmembrane protein and has an extracellular domain of only a few amino acids and its cytoplasmic domain has a strongly conserved region. The role of the US9 protein of BoHV-5 has not been studied on the cellular level, but it probably also plays a role in the anterograde transport of viral glycoproteins along axons as has been found for the SuHV-1 US9 protein (10). Moreover, it may play a role in apoptosis of the host cell, as has been suggested for the US9 protein of BoHV-1 (19).

At present no vaccine is available for BoHV-5. Cross protection against BoHV-5 by a BoHV-1 vaccine has been found, for a less virulent BoHV-5 strain (2), but BoHV-1 vaccines probably can't protect against more virulent BoHV-5 strains (Spilki FR, unpublished results). A modified live vaccine of BoHV-5 should be sufficiently attenuated and be released from its neurovirulence to be safe. Therefore, it has been decided to construct a mutant of BoHV-5 from which the genes coding for glycoproteins gI and gE and for the US9 protein are deleted.

MATERIALS AND METHODS

Virus strain and cells

Madin-Darby bovine kidney cells (MDBK, ATCC-CCL 22) and Embryonic bovine tracheal cells (Ebtr) were grown in Minimal Essential Medium (MEM) supplemented with 5 to 10% of foetal bovine serum (FBS) and antibiotics. Cell cultures were grown at 37ºC in a 5% CO_2 incubator. The EVI 88/95 isolate was used as the BoHV-5 parental strain and it was isolated from a meningoencephalitis outbreak in the central region of Brazil in 1995.

BoHV-5 viral DNA extraction and cloning

Sub-confluent Ebtr cells grown in 900 cm^2 roller bottles were infected with the EVI 88/95 strain of BoHV-5 virus at a multiplicity of infection of 0.1 to 1. Approximately 36 h after infection, when typical herpesvirus cytopathic effect (CPE) was evident in about 90% of the cells, the supernatant was removed, clarified at 5 000 x g for 20 min and ultracentrifuged at 100 000 x g for two h at 4ºC. The viral pellet was resuspended in TE pH 7.4 (10mM Tris, 1mM EDTA) and treated with sodium dodecyl sulfate (SDS) and proteinase K (final concentrations of 1% and 100 µg/ml, respectively) for 1 h at 37ºC. The viral DNA was extracted with equilibrated phenol, precipitated with ethanol, resuspended in TE pH 7.4 and stored at 4ºC.

The genomic DNA of EVI 88/95 strain was digested with *Bam*HI and the obtained fragments were cloned randomly in pBR322, using standard methods (13). To construct the gI/gE/US9 deletion fragment, the DNA fragments bordering the gI/gE/US9 locus were subcloned into the pCR Blunt plasmid (Invitrogen), using standard methods (24).

Co-transfection of deletion fragment with wild type BoHV-5 DNA

A BoHV-5 subclone, which contained the gI/gE/US9 deletion fragment, was linearized at the *Hind* III site, located in the plasmid, to allow the recombination process to take place. One µg of linearized plasmid and 2 µg of BoHV-5 genomic DNA of EVI 88/95 strain were co-transfected into monolayers of Ebtr cells using the calcium phosphate method (13). The Ebtr cells were seeded on 6 well plates one day prior to the co-transfection at a concentration of 10^6 cells per well. The tissue culture medium was refreshed 4 h before the transfection. One ml of precipitate was then used for each well containing the cells. Four h after the co-transfection, the medium was replaced and the cells were treated for 1 min with 15% glycerol in 1 x medium. After washing the cells two times with PBS, medium was added and the transfected cells were further incubated for 24 h. One out of six wells was covered with semi-solid medium (1 x medium with 1% agarose) to count the number of plaques 3 days after co-transfection. When at least seventeen plaques per well were obtained, the co-transfection was considered successful.

Isolation and molecular characterization of the BoHV-5 gI/gE/US9$^-$ deletion mutant

Co-transfected cells of 5 wells and their medium were freeze/thawed and collected in a 50 ml tube. After clarification for 20 minutes at 500 x g, the supernatant was used to infect new monolayers of Ebtr cells on 6-wells plates and semi-solid medium was overlaid. After the appearance of CPE, isolated viral plaques were picked and used to infect monolayers of Ebtr cells on a 96 wells plate. When CPE was visible, cells were fixated and an immunoperoxidase monolayer assay (IPMA) (16) was performed using a monoclonal antibody against gE (Mab 51) and an additional antibody against gC (MAb 14) (22). Viruses in wells, with clear CPE and without any staining with anti-gE Mab 51 were considered to be deletion mutant candidates. Supernatants of such wells were used to infect new cell monolayers and one candidate, named EVI 88/95 gI/gE/US9$^-$ was submitted to three more rounds of plaque purification under semi-solid medium.

To identify the borders of the deletion at the genomic level, the DNA of EVI 88/95 gI/gE/US9$^-$ virus was extracted and submitted to nucleotide sequence analysis. To characterize the gI bordering fragment the following primers were designed: P1: 5' cctcgcgatcgctttgctagtgc 3' and P2: 5' gcgagcgcgcggacgcggc 3'. These primers amplify a region between nucleotides 120721 and 122530 of the BoHV-5 genome (accession number AY261359). To analyze the US9 bordering fragment the following primers were used: P3: 5' cgccatcctggcgctagagc3' and P4: 5' gacgactacgaggtcgactacg 3'. These primers amplify a region

between nucleotides 126243 and 127760. The amplified fragments were directly submitted to the nucleotide sequencing using a Beckman CEQ™ 8000 eight-channel capillary DNA Analysis System (Beckman Coulter, Fullerton, CA).

***In vitro* growth characteristics**

The plaque size assay was performed as described previously (11). Briefly, confluent MDBK monolayers prepared on 6-wells plates were infected with 50 p.f.u. of either the EVI 88/95 gI/gE/US9$^-$ or the EVI 88/95 strain and grown under semisolid medium for five days. The monolayers were fixated, stained and the diameter of at least 50 viral plaques of each virus were measured.

Penetration kinetics was assessed by allowing approximately 500 p.f.u. of the EVI 88/95 gI/gE/US9$^-$ or the EVI 88/95 strain to adsorb on either MDBK or Ebtr monolayers for 1 h at 37ºC. At different time intervals (5, 3, 20, 30, 45, 60 and 120 min) p.i., the inoculum was removed and cells were overlaid with semi-solid medium and incubated for four days. In a control experiment, additional cell monolayers were infected with 500 p.f.u. of all viruses, without low pH buffer treatment. Monolayers were fixated, stained and viral plaques were counted at each time interval. The degree of penetration was calculated by comparison with the plaque count of the control experiment (set at 100% penetration). All tests were done in triplicate.

One step growth curves were assessed following infection of preformed MDBK cell monolayers at a m.o.i. of 10. Adsorption was allowed for 1 hour at 37ºC before the inoculum was removed and extracellular virus inactivated with 1 M sodium citrate (pH 3.0). The monolayers were then washed with fresh medium and incubated for different intervals (3, 5, 7, 9, 11, 13, 16, 24, 36 and 48 hours p.i.). After the incubation period, the supernatants were harvested and assayed for virus. All experiments were performed in triplicate. Virus titres were calculated according to the method of Spearmann and Kärber (25) and expressed as the $\log_{10}$ tissue culture infectious doses per 50 µl ($TCID_{50}$/50 µl).

Statistical analysis

The statistical analysis was carried out using ANOVA, comparing the mean results of plaque sizes and titres for each group. P values ≤ 0.05 were considered to be statistically significant. The calculation was made using Data Supplemental Analysis for Excel (Microsoft Office XP, Microsoft, USA).

RESULTS

Cloning of the US region of BoHV-5 strain EVI 88/95

EVI 88/95 genomic DNA was digested with *Bam*HI and the obtained fragments were cloned randomly in pBR322. The obtained clones were analysed by restriction enzyme digestioon. To identify the *Bam*HI fragment covering the Unique Short (US) region, all clones with inserts of about 14 kb to 17 kb were analysed further. Based on published BoHV-5 maps the *Bam*HI fragment covering the US region is the *Bam*HI C fragment, but this could either be 16.35 kb (1) or 14.6 kb (9) depending on the BoHV-5 strain analysed. Based on the positions of the *Stu*I sites bordering the US region (9) the 16.4 kb *Bam*HI fragment in the clone named pAC41 was identified as the EVI 88/95 fragment that covered the US region.

Construction of a gI/gE/US9 deletion fragment

To construct a recombination fragment that could be used to delete the gI/gE/US9 loci from the genome of BoHV-5 strain EVI 88/95, both an upstream and a downstream fragment had to be cloned (Fig. 1C). The upstream fragment that was chosen was the 1.8 kb *Nru*I fragment that starts 149 nt upstream of the end of the gG coding region, covers the complete gD coding region and ends at the start of the gI coding region (5,8). The downstream fragment that was chosen was 1.6 kb *Not*I fragment that starts at the end of the US9 coding region, covers the partial US1.67 gene and ends in the terminal repeat region (5). Both fragments were isolated from the pAC41 clone and subcloned into pCR-Blunt next to each other in the same orientation as found in the original 16.4 *Bam*HI fragment. (Fig. 1B). The upstream fragment covering the complete gD coding region was only separated by a small part of the multiple cloning site of pCR-Blunt (about 30 nucleotides between the blunt site to the *Not*I site) from the downstream fragment covering the partial US1.67 gene and part of the terminal repeat region.

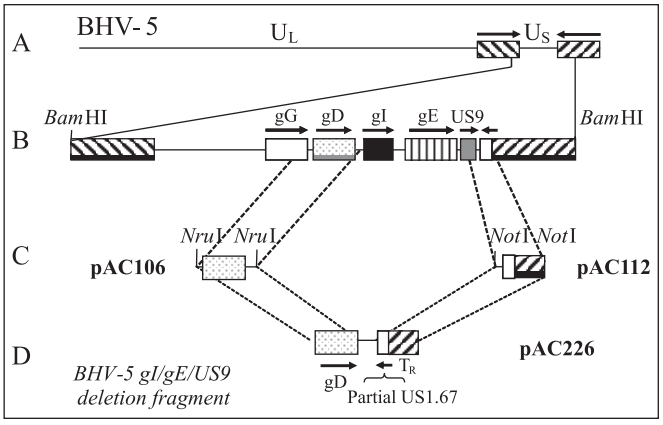

Figure 1. Diagram of the construction of the deletion fragment used to produce the BoHV-5 EVI 88/95 gI/gE/US9$^-$. A indicates the structure of the BoHV-5 genome. The Unique Long (UL), the Unique Short (US) regions and the terminal and internal repeats (TR and IR) are indicated. The *Bam*HI C fragment is indicated in B. The 1.8 kb *Nru*I and 1.6 kb *Not*I fragments were cloned separately (C) and then together (D) to be used as a deletion fragment for the co-transfection.

Co-transfection, isolation and analysis of the BoHV-5 gI/gE/US9$^-$ recombinant virus

In order to generate a gI/gE/US9 deletion mutant of BoHV-5, plasmid pAC226 was linearized with *Hind*III, 722 nucleotides upstream to the insert, site localized in the vector, and co-transfected with genomic DNA of wild type EVI 88/95. After co-transfection, about 100 primary plaques were found, a transfection efficiency considered sufficient to allow the formation of recombinants. After seeding the supernatant of the freeze/thawed primary plaque culture, 4 out of 290 plaques could be isolated that failed to react with anti-gE Mab 51. The viruses in the supernatants of these 4 gE negative plaques were three times plaque purified and were tested again with anti-gE Mab 51. All four showed clear CPE and remained Mab51 negative (data not shown). One of these four gE negative viral populations was named BoHV-5 gI/gE/US9$^-$ and multiplied on MDBK cells. The genomic DNA of the BoHV-5 gI/gE/US9$^-$ was isolated and digested with *Bam*HI to compare the restriction pattern with the DNA of the EVI 88/95 virus. As expected, the original 16.4 kb *Bam*HI fragment was absent in the pattern of the gI/gE/US9 mutant and a new *Bam*HI fragment of 12.8 kb appeared (Fig. 2). This is consistent with a 3.6 kb deletion covering the gI/gE/US9 loci. No other gross rearrangements, other than the intended deletion were observed.

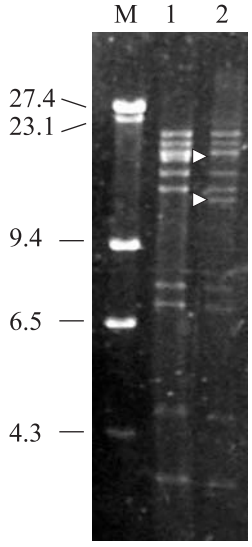

Figure 2. Restriction endonuclease analysis of viral DNA (EVI 88/95, first lane and EVI 88/95 gI/gE/US9$^-$, second lane) digested with *Bam*HI. The arrows indicate the loss of the 16.4 kb *Bam*HI fragment and the appearance of a new 12.8 kb *Bam*HI fragment in the deletion mutant. M indicates the DNA marker Lambda *Hind*III. The sizes of the bands are expressed in kilo base pairs (kb).

The alignment of the nucleotide sequence obtained from the EVI88/95 gI/gE/US9$^-$ showed that the right border of the gI upstream region has 98% homology with the end of the gD gene and stops just before the gI start codon (data not shown), indicating that the deletion started at the expected position. The sequence data of the US9 downstream region has 100% homology with the end of US9 gene and with part of the US1.67 gene (data not shown), indicating the deletion of almost all the US9 gene and the presence of part of the US1.67 gene, as expected, in the viral genome.

The immunoperoxidase assay performed with two monoclonal antibodies on cells infected with either the wild type or the EVI88/95 gI/gE/US9$^-$ revealed that both viruses expressed gC, while only the wild type was positive for gE (Fig. 3).

***In vitro* growth characteristics**

The mean plaque size of the EVI 88/95 gI/gE/US9$^-$ after 5 days post-infection was 0.19 (+/- 0.08 mm), while the parental strain had a mean plaque size of 0.54 (+/- 0.06 mm). The mean plaque size of EVI 88/95 gI/gE/US9$^-$ was only 35% of the size of the wild type virus, a difference that was considered statistically significant and indicates that the triple deletion mutant is impaired in its cell-to-cell spread.

The penetration kinetics of EVI 88/95 gI/gE/US9$^-$ on both MDBK cells and Ebtr cells were essentially the same as the parent strain (Fig. 4). Both the triple deletion mutant and the wild type strain could penetrate these cell types to a level of about 60% at 60 min post infection and reached a level of about 95% of penetration at 2 h post infection. The differences found between the mean penetration percentages were statistically not significant. These data suggest that the proteins encoded by the three genes (gI, gE and US9) do not play an important role in the penetration of the host cells tested.

The one step growth curves of EVI 88/95 and EVI 88/95 gI/gE/US9$^-$ are shown in Fig. 5. The multiplication kinetics of both viruses were undistinguishable. In both cases, viral progeny was first detected at 7 hours p.i. and the maximum viral titres were reached at 36 hours p.i. when infectious titres were very similar (between 10^6 and 10^7 $TCID_{50}$).

DISCUSSION

The EVI 88/95 open reading frames of the genes encoding glycoproteins gI, gE and membrane protein US9 could be identified by comparison with published nucleotide sequences amino acid sequences of the gI, gE and US9 homologues of related alphaherpesviruses. This allowed us to design a deletion that encompasses all three reading frames and to introduce this deletion into the genome of BoHV-5 strain EVI 88/95. However, a full analysis of the cis-regulatory sequences and a detailed transcription map of the region in which these

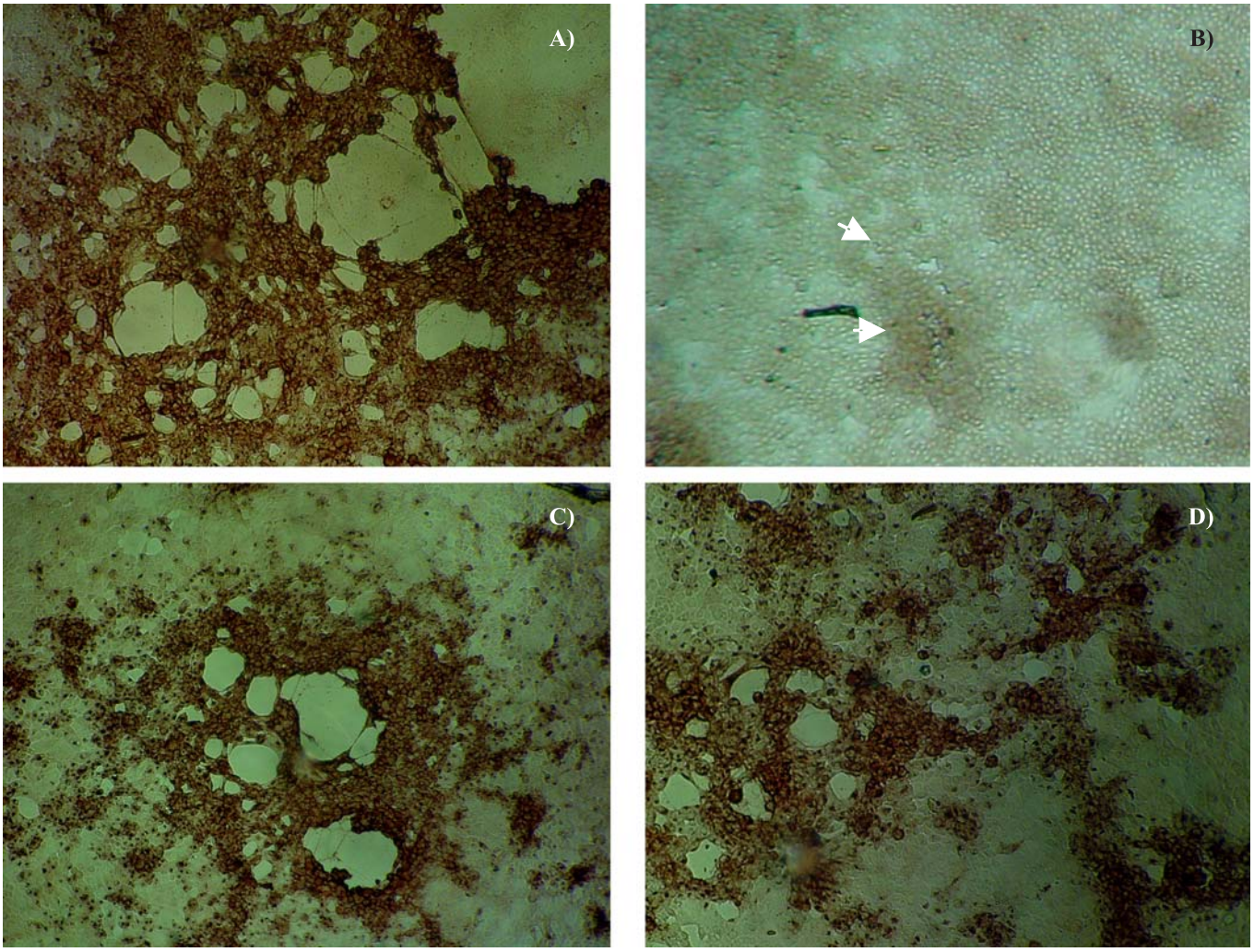

Figure 3. Immunoperoxidase monolayer assay on MDBK cells infected with EVI 88/95 (A and C) and EVI 88/95 gI/gE/US9$^-$ (B and D) viruses using the anti-gE MAb 51 (A and B) and the anti-gC MAb 14 (C and D). Cells expressing gE and gC are stained in red. The arrows in B show unstained cells infected with the EVI 88/95 gI/gE/US9$^-$.

genes are located are still missing. This is especially important with respect to the effects of the deletion on the neighboring genes: the glycoprotein D gene located upstream of the deletion and the BICP 4 gene located downstream. It is very likely that cis-regulatory sequences of one or both neighboring genes have been affected by the deletion. A similar case has been described for BoHV-1, where a polyadenylation signal is located between gI and gE, which is used to process gI transcripts but which is also used for the processing of glycoprotein D transcripts (17). Given the high homology between the BoHV-5 and BoHV-1, this may also be the case for the transcripts of gD of BoHV-5. It is not clear where the polyadenylation signals for BICP4 transcripts are located, but they may as well be located within the deleted region. Both the gD and the BICP4 genes are essential for viral replication and therefore it is unlikely that their expression is seriously affected. Nevertheless, the deletion of gI, gE and US9 may have modified their levels of expression and consequently the viability of the deletion mutant. In case of a double deletion of gI and gE of BoHV-1, significantly lower titres were found *in vivo* compared to the parental strain (14). The BoHV-5 deletion mutant that was selected was studied using restriction enzyme analysis and partial sequencing of the borders of the deletion and no obvious changes in its genome other than the intended deletion of the gI, gE and US9 encoding region could be observed. However, this analysis couldn't exclude unintended additional changes, like small insertions or deletions or nucleotide substitutions.

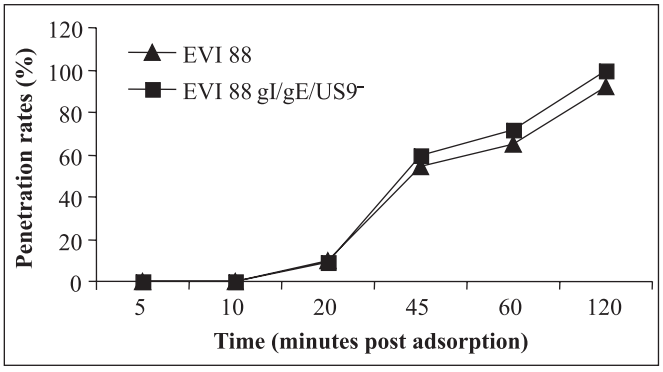

Figure 4. Comparison of the penetration kinetics of wild type strain EVI 88/95 and deletion mutant EVI 88/95 gI/gE/US9- in MDBK cells.

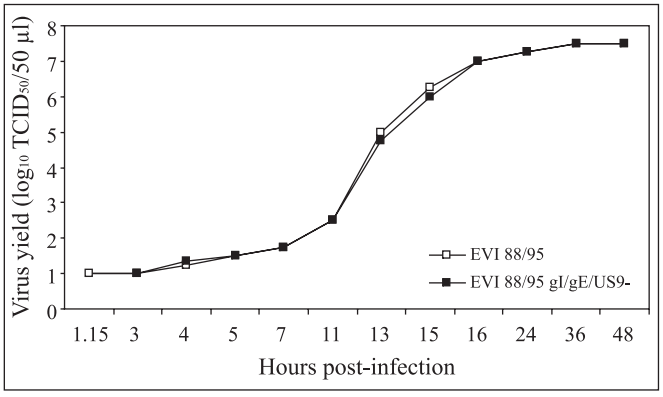

Figure 5. Growth kinetics of EVI 88/95 (□) and EVI 88/95 gI/gE/US9- (■) in MDBK cells. Virus titres in TCID50/50 ml, are expressed as the reciprocal of virus titres in log10.

For an attenuated live vaccine, safety as well as efficacy are important. Consequently, the triple deletion mutant should have lost its virulence, but could still be viable enough to be able to induce immunity to protect against a challenge with a wild type BoHV-5 strain. The *in vitro* properties of the triple deletion mutant indicate that it is attenuated: the small plaque phenotype of the triple deletion mutant suggests that the mutant is attenuated *in vivo*. The glycoproteins gI and gE of BoHV-5 as well as the homologous gI and gE proteins of related alphaherpesviruses are implicated in cell-to-cell spread mechanisms (6, 21). Consequently, the small plaque phenotype in monolayers of bovine cells under semi-solid medium growth conditions was expected. gI or gE deletion mutants, or gI/gE double deletion mutants of related alphaherpesviruses all have a small plaque phenotype and the mutants tested *in vivo* showed an attenuated phenotype. Whether the US9 deletion contributed to the small plaque phenotype is not clear. A BoHV-1 US9 deletion mutant does not have a small plaque phenotype (11) and the role of the US9 protein may be more clear in neuronal cell-to-cell spread along axons (10). In case of BoHV-1, deletion of the gE gene alone already attenuated the virus sufficiently to be safe in young calves (7). The multiplication and penetration kinetics of the triple deletion mutant are unaffected when compared to the wild type. These results indicate that none of the deleted genes are important either for the virus penetration or replication in MDBK cells, which was already shown for gI, gE and US9 homologues in other alphaherpesviruses (4,11) and suggest that the deletion mutant is viable enough to be used as a candidate for a viral vaccine.

Whether the BoHV-5 triple deletion is sufficiently safe and efficacious as a vaccine has still to be confirmed *in vivo*. In case this virus is still too virulent, additional mutations that lower its virulence, without affecting immunity triggering, or with enhancing its immunization properties have to be introduced. One option is the additional deletion of the virus-host shut off gene (vhs) that is known to down regulate the expression of MHC class I molecules (15).

The deletion of the gI and gE genes has the additional advantage that the triple deletion mutant can be used as a marker vaccine. Marker vaccines allow the serological differentiation between vaccinated and infected animals and are a useful tool in a control program. For BoHV-1 and SuHV-1 successful control programs are in progress based on the use of gE, or gI/gE deletion mutants (28,18). In case of BoHV-5, serological studies are complicated by cross reaction with BoHV-1 positive sera. This may be solved by the use of tests that are specific for antibodies against the BoHV-5 gI/gE complex or by the simultaneous control of BoHV-1.

In conclusion, a BoHV-5 gI/gE/US9- triple deletion mutant was constructed that may be used in vaccines to control BoHV-5 infections and reduce the incidence of viral induced encephalitis in cattle.

RESUMO

Construção e caracterização de um mutante herpesvírus bovino 5 com uma deleção nos genes gI, gE e US9

O herpesvírus bovino 5 (BoHV-5) é uma causa importante de encefalite viral em bovinos na América do Sul. Buscando o desenvolvimento de uma vacina diferencial contra o BoHV-5, um mutante deletado foi construído com base em um isolado brasileiro deste vírus. O alvo das deleções foram genes que codificam proteínas implicadas na neurovirulência do BoHV-5, a glicoproteína I (gI), a glicoproteína E (gE) e a proteína de membrana US9. Para construir o mutante deletado de BoHV-5, as regiões flanqueadoras dos três genes foram clonadas em um plasmídeo procarioto. Este fragmento de deleção foi co-

transfectado com o DNA viral em células de bovinos. A identificação dos mutantes deletados foi feita por meio da técnica de imunoperoxidase com um anticorpo monoclonal anti-gE. Uma das populacões virais gE negativas encontradas foi purificada, amplificada e seu genoma foi examinado por análise de restrição enzimática. Os tamanhos de placas virais e taxas de penetração do vírus mutante foram determinados e comparados com os do vírus selvagem. As placas virais do vírus mutante deletado foram significativamente menores do que as do vírus selvagem ($p \leq 0,05$), mas não foram encontradas diferenças significativas quando comparadas as taxas de penetração dos dois vírus. Estes resultados indicam que o vírus mutante deletado gI/gE/US9 de BoHV-5 pode apresentar virulência reduzida e é viável o suficiente para ser um bom candidato para o desenvolvimento de uma vacina contra o BoHV-5.

Palavras chave: herpesvírus bovino 5, BoHV-5, mutantes deletados, genes de glicoproteínas

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