

## **IN VITRO CHARACTERIZATION OF GE NEGATIVE BOVINE HERPESVIRUS TYPES 1.1 (BHV-1.1) AND 1.2A (BHV-1.2A)**

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Submitted: January 06, 2003; Approved: July 05, 2004

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### **ABSTRACT**

This study aimed the *in vitro* growth characterization of a previously constructed Brazilian bovine herpesvirus 1.2a with a deletion in the glycoprotein E gene (BHV-1.2a gE<sup>-</sup>). The plaque sizes, penetration and growth kinetics of the Brazilian BHV-1.2a gE<sup>-</sup> were studied and compared with the parental virus, as well as with a BHV-1.1 gE<sup>-</sup> recombinant derived from an European BHV-1.1 strain. No statistical differences were observed between the gE<sup>-</sup> recombinants and the respective parental viruses penetration assays were performed. When single step growth curves were studied, no statistical differences were observed between gE<sup>-</sup> and parental viruses. However, it was observed that both gE<sup>-</sup> viruses were excreted from cells in significantly higher titres at 11 hours post infection in comparison with parental viruses. No statistical differences were observed when plaque sizes of parental viruses or gE<sup>-</sup> viruses we analyzed separately in each cell type. However, both gE<sup>-</sup> recombinants displayed a significantly reduced plaque areas on three different cell cultures, in comparison with parental viruses, indicating that the lack of gE had the same effect on both BHV-1 subtypes, manifested by a restricted cell-to-cell spread in infected cells.

**Key words:** infectious bovine rhinotracheitis/infectious pustular vulvovaginitis, differential vaccine, *in vitro* growth characteristics, gE<sup>-</sup> recombinant, BHV-1.2a, bovine herpesviruses

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### **INTRODUCTION**

Bovine herpesvirus 1 (BHV-1), a major pathogen of cattle, is the agent of infectious bovine rhinotracheitis/infectious pustular vulvovaginitis virus (IBR/IPV). The virus is associated to a number of other clinical syndromes, including pustular balanopostitis, abortion, infertility and conjunctivitis (7). BHV-1 strains can be subdivided into three distinct genotypes, BHV-1.1, BHV-1.2a and BHV-1.2b (11). Genotypes 1.1 and 1.2a are related with a more severe disease, while genotype 1.2b is less virulent (11).

The BHV-1 genome encodes several glycoproteins which are expressed on both the viral envelope and infected cell

membranes. These glycoproteins participate in different functions, such as viral attachment and entry, cell-to-cell spread or host immune response evasion (14). The glycoprotein E (gE) gene is located in the unique short (U<sub>S</sub>) region of the BHV-1 genome, consisting of a 2300 bp long fragment and encoding a glycoprotein of about 575 aminoacids (18). Although this glycoprotein is conserved among other members of the *Herpesviridae*, the role of gE in the *in vitro* growth characteristics may vary in function of the virus species and the host cell (1,10). In herpes simplex virus type 1 (HSV-1) and varicella-zoster virus (VZV), gE can be found noncovalently linked to gI, forming an Fc binding site for immunoglobulins, which has been proposed as a mechanism to protect virus-infected cells from lysis by the

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immune system (12). The gE complex in HSV-1 and VZV is also important in the cell-to-cell spread of the virus on different cell types (1,3,4,5,10). In BHV-1.1, the Fc binding function of gE has not been demonstrated (17,18). However, BHV-1.1 gE forms a complex with gI and is important for cell-to-cell spread (2,15). In comparison to wild type viruses, gE negative (gE<sup>-</sup>) recombinants produce smaller plaque sizes *in vitro*, although the growth kinetics or penetration process of the virus seem not to be dependent on the presence of gE (2,13). In addition, BHV-1.1 gE<sup>-</sup> recombinants showed decreased virulence in experimentally inoculated (2,8,15). Based on this reduced virulence, these recombinant viruses have been used in the development of vaccines against BHV-1 infections (2,15).

A Brazilian gE<sup>-</sup> BHV-1.2a virus was previously constructed aiming the development of a vaccine strain (6). The proper *in vitro* and *in vivo* characterization of this virus will allow us to access its potential as a vaccinal virus. In the present study, the *in vitro* growth characteristics of the Brazilian BHV-1.2a gE<sup>-</sup> were studied and compared with the parental virus, as well as with a BHV-1.1 gE<sup>-</sup> recombinant, derived from an European BHV-1.1 strain.

## MATERIALS AND METHODS

### Cells and viruses

The wild type BHV-1 strain SV265 (SV265 wt), was initially isolated from a calf with respiratory disease during an outbreak of infectious bovine rhinotracheitis (IBR) in São Borja, Rio Grande do Sul, Brazil. The virus was used for the construction of the BHV-1.2a gE<sup>-</sup> negative virus (265 gE<sup>-</sup>) as described elsewhere (6).

The parental Lam strain (Lam wt), a BHV-1.1 strain, isolated from an animal with IBR in The Netherlands, and the Lam gE<sup>-</sup> recombinant, were described previously (15). All viruses were multiplied in Madin Darby Bovine Kidney (MDBK), embryonic bovine trachea (Ebtr) or calf testis (CT) cells. Cell cultures were kept in Eagle's minimal essential medium (EMEM) supplemented with 5% to 10% fetal calf serum, 2 mM glutamine and antibiotics (100 IU/ml penicillin and 100 mg/ml streptomycin). When appropriate, one percent low melting point agarose (Sigma) was added to EMEM to obtain the semi-solid medium.

### Plaque assays

Confluent MDBK, Ebtr and CT monolayers prepared on 6 well plates were infected with 50 p.f.u. of the appropriate virus. After two hours of adsorption at 37°C, the inoculum was removed and the cells overlaid with semi-solid medium. Four days post inoculation (p.i.) the cells were covered with fixative solution (10% formalin, 1% crystal violet) for at least 4 hours. The agarose was removed and monolayers dried at 37°C overnight. The diameter of at least 50 viral plaques was measured in each cell type.

### Penetration kinetics

Penetration was assessed by allowing approximately 500 p.f.u. of the appropriate virus to adsorb on either MDBK or Ebtr monolayers for 1 hour at 37°C. At different time intervals (5, 10, 20, 30, 45, 60 and 120 minutes) p.i., the inoculum was removed and the residual extracellular virus inactivated by a brief wash with a low pH buffer (1 M sodium citrate, pH 3.0). The cells were then overlaid with fresh 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 then fixed with 10% formalin 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.

### Multiplication kinetics

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 37°C 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 Spearman and Kärber (9) and expressed as the log<sub>10</sub> tissue culture infectious doses per 50 ml (TCID<sub>50</sub>/50 µl).

### Statistical analysis

Statistical analysis was performed using the Student *t*-test and the analysis of variance (ANOVA).

## RESULTS

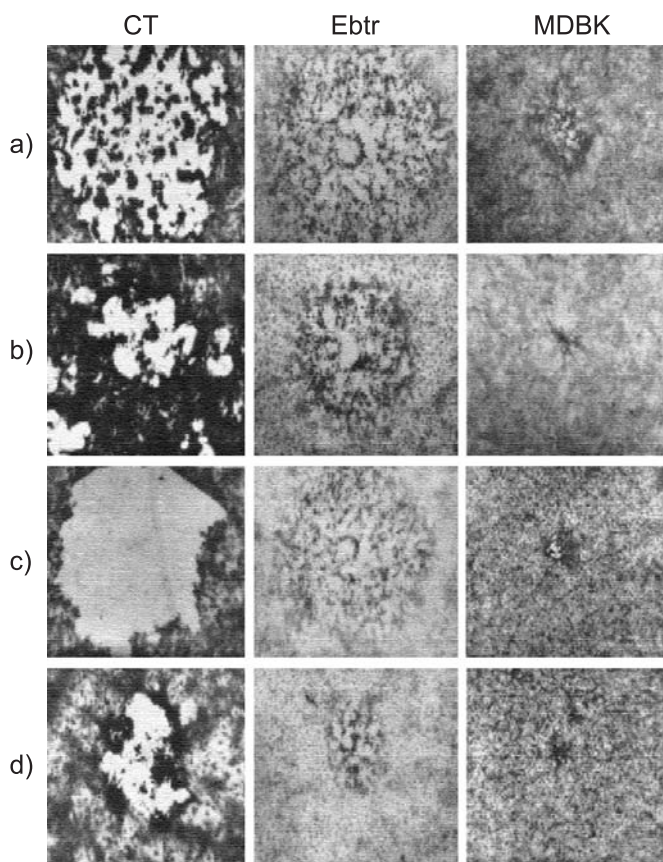
### Plaque Size

The results of the plaque size analysis are shown in Table 1. The mean plaque areas formed by both gE<sup>-</sup> recombinants were strikingly reduced when compared to their respective parental viruses, in all three cell types. The reduction in virus plaque diameters was statistically significant for both gE<sup>-</sup> viruses on all three cell types (p<0.001). Both parental viruses produced similar plaque sizes in each cell type. In addition, mean plaque diameters obtained from both gE<sup>-</sup> recombinants in each cell type were very similar. The largest viral plaques were evident on CT cells, regardless of the virus strain under study (p<0.001). On the other hand, the smallest plaques were invariably found on MDBK cells (p<0.001; Table 1). Fig. 1 shows the plaque morphology of both gE<sup>-</sup> recombinants and the respective parental viruses in all three cell types.

**Table 1.** Plaque diameters of parental (SV265, Lam) and gE<sup>-</sup> recombinants (265 gE<sup>-</sup>, Lam gE<sup>-</sup>) on three cell types (CT, Ebtr and MDBK).

Virus	Plaque diameter (mm) <sup>a</sup>		
	CT	Ebtr	MDBK
SV265	0.25 (0.04)	(0.004) 0.07	0.16 (0.04)
265 gE <sup>-</sup>	0.13 (0.015)	(0.02) 0.018	0,06 (0.006)
Lam	0.26 (0.02)	(0.034) 0.09	0.13 (0.03)
Lam gE <sup>-</sup>	0.15 (0.02)	(0.03) 0.018	0.07 (0.008)

<sup>a</sup> Plaques were measured (average of 50 plaques/measure) 4 days after infection of cultures. Numbers in brackets refer to standard deviation.



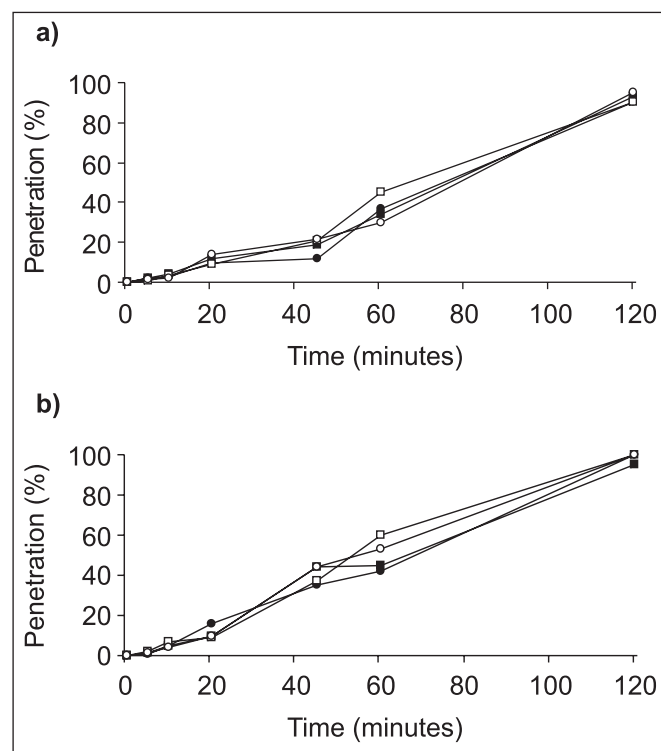
**Figure 1.** Plaque morphology of SV265 (a), 265 gE<sup>-</sup> (b), Lam (c) and Lam gE<sup>-</sup> (d) produced on CT (column 1), Ebtr (column 2) and MDBK (column 3). Magnification 50X.

**Penetration kinetics**

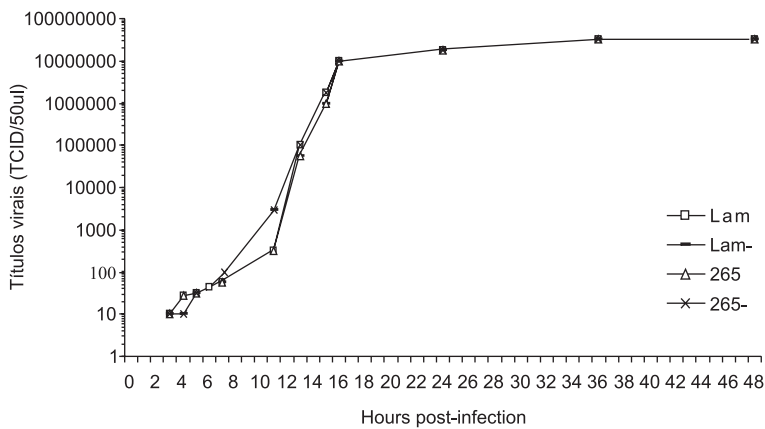
Plaque production was first visualized after 20 minutes of adsorption on both MDBK and Ebtr cells by both gE<sup>-</sup> and wild type viruses (Fig. 2). An increase in penetration rates was observed from 45 minutes p.i. when the two gE<sup>-</sup> recombinants and parental viruses were analyzed on MDBK or Ebtr cultures. However, both Lam wt and Lam gE<sup>-</sup> showed a significantly larger number of viral plaques at 45 and 60 minutes p.i. when compared to SV265 wt and 265 gE<sup>-</sup>. All four viruses produced the maximum number of plaques at about 120 minutes p.i. No significant differences were observed when gE<sup>-</sup> recombinants were compared to their respective parental viruses on both cell cultures.

**Multiplication kinetics**

The one step growth curves of SV265, 265 gE<sup>-</sup>, Lam wt and Lam gE<sup>-</sup> are shown in Fig. 3. The multiplication kinetics of 265 gE<sup>-</sup> was undistinguishable from its parental strain. Similarly, no significant differences were found between the multiplication kinetics of Lam gE<sup>-</sup> and Lam wt. In all cases, viral progeny was first detected at 7 hours p.i. About 10 times more infectious



**Figure 2.** Penetration kinetics of SV265 wt, 265 gE<sup>-</sup> (a), Lam wt and Lam gE<sup>-</sup> (b). Wild type viruses in MDBK and Ebtr are represented by: (●) and (■), respectively. gE<sup>-</sup> viruses in MDBK and Ebtr are represented by: (○) and (□), respectively. Time is expressed in minutes p.i.



**Figure 3.** Growth kinetics of Lam wt (□), Lam gE<sup>-</sup> (×), SV265 wt (△) and 265 gE<sup>-</sup> (-) in MDBK cells. Virus titres in TCID<sub>50</sub>/50µl, are expressed as the reciprocal of virus titres in log<sub>10</sub>.

virus was detected in the supernatants harvested from cells infected with gE<sup>-</sup> viruses than with parental viruses. However, all viruses reached maximum viral titres 36 hours p.i., when infectious titres for both gE<sup>-</sup> and wild type were very similar (between 10<sup>6</sup> and 10<sup>7</sup> TCID<sub>50</sub>).

## DISCUSSION

Glycoprotein E gene of alphaherpesviruses can be deleted from the viral genome with no major implications in viral replication. As the deletion of gE can be accompanied by a significant reduction in virulence, (2,15,16), as part of a study to develop a vaccine against BHV-1 infections, it was intended to examine some *in vitro* properties of a Brazilian BHV-1.2a gE<sup>-</sup>. In this study, the *in vitro* properties of two gE<sup>-</sup> recombinants of BHV-1 (BHV-1.1 and BHV1.2a) and the respective parental viruses were examined.

In this study, the plaque sizes produced by gE<sup>-</sup> recombinants and their parental viruses were measured on Ebtr, MDBK and CT cells. There was a significant decrease in the mean diameters of viral plaques produced by both gE<sup>-</sup> recombinants, in comparison with their respective parental viruses, on all three cell types tested. The reduction in plaque size was at the order of 50 to 70%, depending on the virus and cell type. In addition, both gE<sup>-</sup> recombinants showed similar reduction in plaque sizes on the three cell types analyzed. These results indicate that both gE<sup>-</sup> negative viruses were severely impaired in direct cell-to-cell spread, as already described for other gE<sup>-</sup> herpesviruses (1,2,13).

To investigate the entry of the viruses into cells, two different cell types were used, Ebtr and MDBK. No statistically significant differences were observed between the penetration kinetics of parental and gE<sup>-</sup> recombinants, which indicates that gE does

not play an important role in virion penetration of both BHV-1 subtypes.

To compare the multiplication efficiency of the gE<sup>-</sup> and parental viruses, virus production on cells infected at a high m.o.i. was evaluated. Although no significant differences were found in the final virus titres obtained, the gE<sup>-</sup> recombinants were released in higher titres from infected cells at 11 hours p.i. This effect might be due to the difficulty of gE<sup>-</sup> viruses to spread directly from cell to cell. Therefore, more virions might have the chance to reach the apical cell membrane, being released in higher titres at early stages of infection.

In conclusion, cell-to-cell spread was the most important *in vitro* property affected by the absence of gE in both BHV-1 subtypes. This *in vitro* growth characteristic is probably related to the *in vivo* viral pathogenicity, as herpesviruses which have an impaired cell-to-cell spread show a decreased virulence when inoculated in animals (2,8,15). Experiments are in progress to access the *in vivo* pathogenicity of the Brazilian BHV-1.2a gE<sup>-</sup> in order to examine its potential as a vaccine virus.

## ACKNOWLEDGEMENTS

F.R.S is a CNPq MSc fellow. P.M.R. is a CNPq 1C research fellow. This work was supported by PRONEX, CNPq, FAPERGS and IRFA Biotecnologia Industrial Ltda.

## RESUMO

### Caracterização *in vitro* de herpes vírus bovino tipos 1.1 (BHV-1.1) e 1.2a (BHV-1.2a) gE<sup>-</sup> negativos

O presente estudo teve como objetivo a caracterização das propriedades de crescimento *in vitro* de uma amostra brasileira de herpesvírus bovino tipo 1.2a que apresenta uma deleção no gene que codifica a glicoproteína E (BHV-1.2a gE<sup>-</sup>). Os tamanhos de placa, cinética de penetração e cinética de multiplicação do vírus BHV-1.2a gE<sup>-</sup> foram estudados e comparados com o vírus parental, bem como com um vírus BHV-1.1 gE<sup>-</sup> recombinante, o qual é derivado de uma amostra europeia de BHV-1.1. Em termos de cinética de penetração, não foram observadas diferenças significativas quando comparados os vírus gE<sup>-</sup> com os parentais. A determinação da cinética de multiplicação não demonstrou diferenças significativas entre os quatro vírus estudados. Foi entretanto observado que 11 horas pós infecção os dois vírus gE<sup>-</sup> foram excretados das células em títulos significativamente maiores do que os vírus parentais. Não foram observadas diferenças significativas quando comparados os diâmetros de placas formadas pelos dois vírus parentais. Da mesma forma, os diâmetros de placas dos vírus gE<sup>-</sup> foram semelhantes nos

três tipos celulares estudados. Entretanto, a comparação dos diâmetros de placas entre os vírus gE<sup>-</sup> e os parentais mostrou uma redução significativa das placas dos vírus gE<sup>-</sup> em todos os tipos celulares. Esta característica indica que a falta da gE teve o mesmo efeito em ambos os subtipos de BHV-1, representado por uma disseminação viral célula-célula reduzida.

**Palavras-chave:** rinotraqueíte infecciosa bovina/vulvovaginite pustular infecciosa; vacinas diferenciais; características de crescimento *in vitro*; BHV-1.2a, vírus gE negativos, herpesvírus bovinos

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