**Short Communication**

**Intraspecific bovine herpesvirus 1 recombinants carrying glycoprotein E deletion as a vaccine marker are virulent in cattle**

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Vaccines used in control programmes of *Bovine herpesvirus 1* (BoHV-1) utilize highly attenuated BoHV-1 strains marked by a deletion of the glycoprotein E (gE) gene. Since BoHV-1 recombinants are obtained at high frequency in experimentally coinfected cattle, the consequences of recombination on the virulence of gE-negative BoHV-1 were investigated. Thus, gE-negative BoHV-1 recombinants were generated *in vitro* from several virulent BoHV-1 and one mutant BoHV-1 deleted in the gC and gE genes. Four gE-negative recombinants were tested in the natural host. All the recombinants were more virulent than the gE-negative BoHV-1 vaccine and the gC- and gE-negative parental BoHV-1. The gE-negative recombinant isolated from a BoHV-1 field strain induced the highest severe clinical score. Latency and reactivation studies showed that three of the recombinants were reexcreted. Recombination can therefore restore virulence of gE-negative BoHV-1 by introducing the gE deletion into a different virulence background.

The virulence of any given viral strain is the result of the effects of the constellation of genes carried by that strain. How these genes and their products interact with each other and interact with host proteins determine the outcome of the disease. Glycoprotein E (gE) is considered as a virulence factor of all studied members of the subfamily *Alphaherpesvirinae*. The gE-minus mutants generated hitherto from herpes simplex virus (HSV), pseudorabies virus, equine herpesvirus 1 and *Bovine herpesvirus 1* (BoHV-1) were shown to be attenuated in mice, swine, foals and calves, respectively (Jacobs et al., 1993; Dingwell et al., 1994; van Engelenburg et al., 1994; Matsumura et al., 1998). BoHV-1 gE and gI form a non-covalent-linked heterodimer in infected cells and in the virion envelope (Whitbeck et al., 1996; Yoshitake et al., 1997). *In vitro*, the analysis of mutant BoHV-1 viruses lacking gE, gI or both has shown that the complex is involved in cell-to-cell spread (Baranowski et al., 1996; Rebordosa et al., 1996; Tyborowska et al., 2000; Mahony et al., 2002; Trapp et al., 2003).

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Detection of latent BoHV-1 DNA in trigeminal ganglion is available as supplementary material and figures in JGV Online.

BoHV-1, classified as an alphaherpesvirus, is a major pathogen of cattle. Primary infection is accompanied by various clinical manifestations such as infectious bovine rhinotracheitis, infectious pustular vulvovaginitis, abortion and systemic infection (Tikoo et al., 1995; Kaashoek et al., 1996b). When an animal survives a latent infection is established in the sensory ganglia. Reactivation from latency can occur after natural stimulus exposure or corticosteroid treatment culminating in recurrent virus transmission to uninfected animals. In regards to the significant losses incurred by disease and trading restrictions, several European countries have initiated BoHV-1 control programmes based on the use of marker vaccines deleted in the gE gene. These marker vaccines, either inactivated or live attenuated, used together with a serological detection of gE-specific antibody (Ab), allow differentiation between infected and vaccinated animals (van Oirschot et al., 1997; Lehmann et al., 2002). However, all the gE-negative BoHV-1 tested hitherto have been generated from weakly virulent BoHV-1 strains (van Engelenburg et al., 1994; Chowdhury et al., 1999). Therefore, there is a concern about the virulence of ‘vaccine-like’ gE-negative BoHV-1 issued from recombination with virulent BoHV-1 strains.

Intramolecular recombination is a mechanism of genetic material exchange closely related to the alphaherpesvirus
replication cycle (Thiry et al., 2005). Previous data supported the frequent rise of recombinants in cattle after concomitant nasal infections with two BoHV-1 mutants (Schynts et al., 2003). The present study aimed at characterizing the virulence of gE-negative BoHV-1 recombinants generated in vitro from several strain backgrounds. Coinfection experiments have involved a weakly virulent BoHV-1 strain deleted in the gC- and gE-encoding genes and several wild-type BoHV-1 strains. A biological characterization led to the scoring of the BoHV-1 recombinants possessing the vaccine gE-negative phenotype (Muylkens et al., 2006). Based on this in vitro screening, four gE-negative BoHV-1 recombinants were selected for testing their virulence in the natural host.

An experiment was designed to assess the virulence of these gE-negative BoHV-1 recombinants by inoculating blindly seven BoHV-1 to seven groups of four calves (Table 1). Two mock-infected calves were used as control. Four gE-negative BoHV-1 recombinants, namely rIowaΔgE, rED1ΔgE, rCineyΔgE and rCooperΔgE, were tested. The virulence of their respective wild-type strains had been assessed in vivo (Table 1). The wild-type BoHV-1 strain Iowa was used as a highly virulent comparison strain; a BoHV-1ΔAgE vaccine and BoHV-1ΔAgCAgE, the parental strain of all the recombinants, were inoculated as weakly virulent comparison strains (Table 1). The description of the BoHV-1 viruses used in this study is available as Supplementary Fig. S1 in JGV Online. The differences in the clinical, virological and serological data were tested in the form of mixed models for repeated measurements by SAS procedure (procedure MIXED) (Littell et al., 1998). The animal study was accredited by the local ethics committee (folder 115).

The serological status of calves provided prerequisite evidence that the observed clinical signs were induced by the inoculated viruses. Indeed, all the calves infected by gE-negative BoHV-1 recombinants remained seronegative to gE (data not shown). Seroconversion to gB was recorded in all the BoHV-1-infected calves. No seroconversion was observed in the mock-infected group. Calves were clinically examined and rectal temperatures were measured daily for 17 days post-infection (p.i.). All the calves inoculated with the gE-negative BoHV-1 recombinants showed clinical signs typical for BoHV-1 infection (Fig. 1a). A global score was obtained by scoring the following clinical signs: apathy, anorexia, quality and quantity of nasal discharge, cough, dyspnoea, rhinitis, lesions of nasal mucosa, ocular discharge and conjunctivitis (Goge et al., 2004). The gE-negative BoHV-1 recombinants induced higher scores than the BoHV-1ΔAgE vaccine (P<0.05) (Fig. 1a), but lower scores than the wild-type BoHV-1 (WT; P<0.001). The recombinant issued from BoHV-1 strain Ciney showed a higher virulence than the other recombinants (P<0.001) (Fig. 1a). This recombinant also induced hyperthermia above 39.5 °C, apathy and anorexia for 3 consecutive days in the four inoculated calves (Fig. 1b). At days 2 and 3, the mean temperatures of the calves infected with the CineyΔAgE recombinant were higher than the mean temperatures of the calves infected with either the BoHV-1ΔAgE vaccine or the other recombinants (P<0.01). A video-endoscope examination performed at day 3 p.i. on two calves in each group allowed investigating the lesions induced by the different viruses in the upper respiratory tract. Data are available as Supplementary Fig. S2 in JGV Online. Six of eight calves inoculated by BoHV-1 recombinants showed more severe lesions than the calves inoculated either by the parental BoHV-1ΔAgCAgE or BoHV-1ΔAgE vaccine. One calf infected by rCineyΔAgE and one infected by rED1ΔAgE exhibited lesions comparable to the lesions induced by wild-type BoHV-1.

The results presented above indicated that the gE-negative recombinants issued from virulent BoHV-1 retained the ability to induce the disease caused by BoHV-1. To determine the level of virus excretion of gE-negative BoHV-1 recombinants, nasal swabs were taken daily from each animal for 17 days p.i. The presence of BoHV-1 was detected and titrated by plaque assay on Madin-Darby bovine kidney cells (ATCC CCL-22) as described previously (Lemaire et al., 1999). BoHV-1 was isolated from the nasal swabs of all infected calves, whereas no virus was isolated from the mock-infected calves (Fig. 1c). The recombinants were excreted as the same amount as the wild-type BoHV-1 from days 1 to 5. The calves infected with the BoHV-1ΔAgE vaccine excreted less than calves infected with wild-type or recombinant BoHV-1 (P<0.001). The parental strain BoHV-1ΔAgCAgE was excreted for the shortest period

<table>
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<th>Inoculated BoHV-1</th>
<th>Virus characteristic</th>
<th>Virulence of the wild-type strain</th>
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<tr>
<td>rCineyΔgE</td>
<td>–</td>
<td>–Ciney</td>
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<tr>
<td>rED1ΔgE</td>
<td>Recombinant between BoHV-1ΔAgCAgE and</td>
<td>–ED1</td>
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<td>rIowaΔgE</td>
<td>–</td>
<td>–Iowa</td>
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<tr>
<td>rCooperΔgE</td>
<td>–</td>
<td>–Cooper</td>
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<td>BoHV-1 vaccine</td>
<td>–</td>
<td>Vaccine ΔgE/US9</td>
</tr>
<tr>
<td>BoHV-1ΔAgCAgE</td>
<td>–</td>
<td>Parental ΔgCAgE</td>
</tr>
<tr>
<td>Iowa</td>
<td>–</td>
<td>Parental wild type</td>
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Shedding of the gE-negative BoHV-1 recombinants was not reduced at the peak of excretion. This massive virus excretion contrasts with virus shedding recorded with gE-negative BoHV-1 mutants analysed hitherto, LamDgE, and CooperDgE were excreted in 100-fold smaller amounts (Kaashoek et al., 1996a, 1998; Chowdhury et al., 1999). With regards to the CooperDgE mutant, the presence of the β-gal gene under the control of a strong promoter from human cytomegalovirus (HCMV) could affect viral cycle regulation and therefore progeny virus production. In our study, the gE-negative recombinant Cooper, which was devoid of the β-gal gene and the HCMV promoter, was excreted similarly as wild-type BoHV-1. The phenotype of the gE-negative recombinants was confirmed positive for gC expression and negative for gE expression by double immunofluorescence staining (Schynts et al., 2001) (data not shown). The virus identity was further confirmed by HindIII restriction analysis using the disappearance of

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**Fig. 1.** Mean clinical scores (a) mean rectal temperatures (b) and mean BoHV-1 excretion titres (c) recorded during primary infection and reactivation of seven groups of calves infected by the four gE-negative recombinants, the highly virulent BoHV-1 Iowa, the BoHV-1ΔgE vaccine and the BoHV-1ΔgCΔgE. (a) To obtain a global score, the evaluated clinical signs included apathy, anorexia, quality and quantity of nasal discharge, cough, dyspnoea, rhinitis, lesions of nasal mucosa, ocular discharge and conjunctivitis. (c) BoHV-1 excretion titres were expressed as log_{10} p.f.u. per 100 mg nasal secretions.
the K fragment as evidence of the gE deletion (data provided as Supplementary Fig. S3 available in JGV Online).

In order to investigate the latency-reactivation properties of gE-negative BoHV-1 recombinants, calves were injected intravenously with sodium orthophosphate of dexamethasone (Rapidexon) at 0.1 mg kg\(^{-1}\) body weight from days 90 to 95 p.i. A slight increase in the rectal temperature was recorded in all the inoculated calves post-reactivation treatment (PRT) (Fig. 1b). Serous and/or mucous nasal discharges and lesions in the nasal mucosa were observed in all inoculated calves within days 2–13 PRT (Fig. 1a). All the calves inoculated with the wild-type BoHV-1 reexcreted at days 5–8 PRT, and some calves of the groups infected with rCiney\(\Delta\)gE, rED1\(\Delta\)gE and rCooper\(\Delta\)gE reexcreted from days 7 to 14 PRT (Fig. 1c). There was a marked temporal delay between the reexcretion periods of the recombinants and the wild-type BoHV-1 (Fig. 1c). No virus was isolated from the mock-infected calves, nor from any calf infected with rIowa\(\Delta\)gE, BoHV-1\(\Delta\)gC\(\Delta\)gE or BoHV-1\(\Delta\)gE vaccine (Fig. 1c). The reexcretion of some of the gE-negative recombinants indicates that these recombinants are able to establish latency and could disseminate the infection under reactivation conditions. Calves were euthanized at days 112, 113 and 114. Trigeminal ganglia were dissected within 2 h after death and immediately stored at -80°C. Latent BoHV-1 DNA was detected and characterized by PCR as described in Supplementary material available in JGV Online. In the presence of trigeminal ganglion extracts, no cell culture presented cytopathic effect. This prerequisite allowed exclusion of any positive result through PCR due to infectious replication competent BoHV-1. The electrophoretic pattern of PCR products is available as Supplementary Fig. S4 in JGV Online. The calves infected by BoHV-1\(\Delta\)gC\(\Delta\)gE were positive for gD amplicon but negative for gC and gE amplicons, showing the establishment of latency of this strain, even if no virus was recovered PRT. All calves infected by recombinants gave positive results in gC and gD amplification and no signal in gE amplification. This demonstrated that all the gE-negative BoHV-1 recombinants established latency. Finally, all the calves infected by the wild-type BoHV-1 were positive in the three BoHV-1-specific PCR assays.

Serological monitoring was performed on blood samples collected weekly from each animal. BoHV-1-neutralizing antibodies (NAb) titres were measured in one large-scale assay where the BoHV-1 strain Iowa has been used (Lemaire et al., 2000). No effect of the viral strain on the NAb titre was evidenced by performing cross neutralization assay on two sera per group with four BoHV-1 strains: Iowa wt, Lam wt, Lam\(\Delta\)gE and Lam\(\Delta\)gC. The titres of BoHV-1 NAb (Fig. 2) showed that all the BoHV-1-inoculated groups reached a plateau at day 21 p.i. except for the BoHV-1\(\Delta\)gE vaccine (plateau at day 28 p.i.). The mean Ab titre of wild-type was higher than titres obtained in the groups infected with the different gE-negative BoHV-1 recombinants \(P<0.001\). The Ab titres remained at slightly decreased levels until reactivation. After reactivation stimulus, the Ab titres fell before rising again from days 98 to 112 p.i. (Fig. 2). The strongest stimulation of the humoral immune response was observed in the four reexcreting groups of calves, likely due to this reexcretion.

This study reports the first description of a virulent gE-negative alphaherpesvirus generated by recombination. This study shows that attenuation with a single attenuating marker deletion may not be sufficient. The virulence of gE-negative BoHV-1 recombinants demonstrated in this study is clearly in opposition with the pronounced attenuation observed after the inoculation of calves with genetically modified gE-negative BoHV-1 (van Engelenburg et al., 1994; Kaashoek et al., 1996a, 1998; Chowdhury et al., 1999). In a view of straightforward approach to design new generation vaccines, these gE-negative BoHV-1 mutants were constructed from weakly virulent BoHV-1 strains, namely Lam (van Engelenburg et al., 1994) and Cooper (Chowdhury et al., 1999).
et al., 1999). However, in our study, three gE-negative recombinants originated from two virulent wild-type BoHV-1, namely ED-1 and Cinney, and one from a highly virulent BoHV-1, namely Iowa (Kaashoek et al., 1996b; Lemaire et al., 1999). Because virulence determinants are highly interdependent, recombination is susceptible to restore the loss of virulence due to the absence of one virulence factor (gE) by implementing this deletion character in another strain’s background.

The generated recombinants tested here were characterized on short genome stretches. Immunofluorescence staining and restriction endonuclease analysis showed that the gC ORF was acquired from the wild-type BoHV-1 used in each coinfection situation, while the gE ORF deletion was inherited from the parental gC–gE-negative BoHV-1. It cannot be ruled out that some recombinants arose from multiple crossing over events, as described previously in both HSV-1 and HSV-2 (Brown et al., 1992). Because the distance between the two deletion markers used in this study does not allow us to explore the recombination along the entire genome, some recombinants could not have been detected. Nevertheless, our data support the hypothesis that a single gE deletion is not sufficient to provide an avirulent phenotype.

In conclusion, we have demonstrated the virulence of BoHV-1 recombinants having acquired the gE deletion vaccine marker. Latency and reactivation monitoring suggest that these recombinants are susceptible to perpetuation in the cattle population but further experiments are needed to study their dissemination after reactivation. The present study contributes to the assessment of the consequences of recombination between vaccine and field strains of BoHV-1. The next step will be the investigation of the rise and spread of virulent gE-negative BoHV-1 recombinants in natural populations.

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