

# Evaluation of the vaccine potential of an equine herpesvirus type 1 vector expressing bovine viral diarrhoea virus structural proteins

Cristina T. Rosas,<sup>1</sup> Patricia König,<sup>2</sup> Martin Beer,<sup>2</sup> Edward J. Dubovi,<sup>1</sup> B. Karsten Tischer<sup>1</sup> and Nikolaus Osterrieder<sup>1</sup>

<sup>1</sup>Department of Microbiology and Immunology, Ithaca, NY 14853, USA

<sup>2</sup>Friedrich-Loeffler-Institut, Bundesforschungsinstitut für Tiergesundheit, Boddenblick 5A, D-17493 Insel Riems, Germany

## Correspondence

Nikolaus Osterrieder  
no34@cornell.edu

*Bovine viral diarrhoea virus* (BVDV) is an economically important pathogen of cattle that is maintained in the population by persistently infected animals. Virus infection may result in reproductive failure, respiratory disease and diarrhoea in naïve, susceptible bovines. Here, the construction and characterization of a novel vectored vaccine, which is based on the incorporation of genes encoding BVDV structural proteins (C, E<sup>ns</sup>, E1, E2) into a bacterial artificial chromosome of the equine herpesvirus type 1 (EHV-1) vaccine strain Rach, are reported. The reconstituted vectored virus, rH\_BVDV, expressed BVDV structural proteins efficiently and was indistinguishable from parental vector virus with respect to growth properties in cultured cells. Intramuscular immunization of seronegative cattle with rH\_BVDV resulted in induction of BVDV-specific serum neutralizing and ELISA antibodies. Upon experimental challenge infection of immunized calves with the heterologous BVDV strain Ib SE5508, a strong anamnestic boost of the neutralizing-antibody response was observed in all vaccinated animals. Immunized animals presented with reduced viraemia levels and decreased nasal virus shedding, and maintained higher leukocyte counts than mock-vaccinated controls.

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## INTRODUCTION

*Bovine viral diarrhoea virus* (BVDV), together with *Classical swine fever virus* of pigs and *Border disease virus* of sheep, forms the genus *Pestivirus* within the family *Flaviviridae* (Collett *et al.*, 1988a). BVDV occurs worldwide and causes significant economic losses to the cattle industry, mainly by inducing reproductive failure. In the pregnant animal, BVDV is able to cross the placenta and to establish infection of the fetus. Dependent on the viral pathotype and the stage of gestation, fetal infections can cause congenital defects, abortion or the birth of calves persistently infected (PI) with BVDV (Campbell, 2004; Dubovi, 1994; Lindberg, 2003). Non-symptomatic PI animals represent the most important source of infection for susceptible cattle because they shed large amounts of virus from mucosal surfaces throughout their lives (Fulton *et al.*, 2005; Kozasa *et al.*, 2005; Loneragan *et al.*, 2005; McClurkin *et al.*, 1984; Nobiron *et al.*, 2000; O'Connor *et al.*, 2005; Piccinini *et al.*, 2006). Acute BVDV infection often results in a significant reduction of lymphocyte, granulocyte and platelet numbers in peripheral blood, and transient leukopenia can lead to immunosuppression, making infected animals more sensitive to

secondary infections (Ellis *et al.*, 1988; Howard, 1990; Keller *et al.*, 2006; Potgieter, 1995).

Current BVDV vaccines consist of modified-live virus (MLV) preparations or contain inactivated viruses. However, neither MLV nor inactivated preparations are capable of providing complete protection against infection. BVDV MLV vaccines are susceptible to maternal antibodies and represent a potential source of immunosuppression and fortuitous *in utero* infections, which will perpetuate the infection chain in the population (Ellis *et al.*, 2001; Grooms *et al.*, 1998; Ridpath & Bolin, 1995; Van Campen *et al.*, 2000). Inactivated vaccine preparations are generally more cost-intensive and, by and large, do not provide protection from fetal infection (Fulton *et al.*, 2005; Thurmond *et al.*, 2001; Zimmer *et al.*, 2002).

The BVDV genome is a single-stranded, positive-sense RNA of 12.5 kb in length, which is translated into a single polyprotein. The polyprotein is subsequently cleaved by viral and host proteases into structural and non-structural proteins. The four structural proteins, the capsid protein (C) and the three glycoproteins (E<sup>ns</sup>, E1 and E2), cluster towards the 5' end of the genome. The non-structural proteins, NS2/NS3, NS4A, NS5A and NS5B, with the exception of the protease N<sup>pro</sup>, which is located immediately

Supplementary figures are available in JGV Online.

downstream of the 5' untranslated region, are located towards the 3' end of the genome. The p7 protein, which commonly associates with the E2 protein, is located at the junction between the structural and the non-structural regions of the viral polyprotein (Collett *et al.*, 1988a, b, 1991). Subunit vaccine preparations consisting of the viral envelope fraction were shown to confer protection in challenge-infection experiments (Carlsson *et al.*, 1991). The BVDV envelope protein E2 exhibits prominent immunogenic properties, and vaccination with a plasmid encoding E2 was partially protective against experimental challenge infection in cattle (Bruschke *et al.*, 1999; Nobiron *et al.*, 2000, 2003; Wang *et al.*, 2004).

In previous studies, equine herpesvirus type 1 (EHV-1) was proposed as a universal viral vector due to its molecular and biological properties, particularly its ability to enter cells of various host origins and the induction of protective immune responses in various non-equine species (Trapp *et al.*, 2005). EHV-1 belongs to the subfamily *Alphaherpesvirinae* and the genus *Varicellovirus* and is highly prevalent in the horse population. Virulent wild-type strains cause respiratory disease, abortion storms and neurological disorders in equines (Telford *et al.*, 1992). Cloning of herpesviruses as bacterial artificial chromosomes (BACs) has proven a reliable and efficient method to manipulate herpesviral genomes (Borst *et al.*, 2004; Brune *et al.*, 1999, 2000; Heister *et al.*, 2004; Kawaguchi & Tanaka, 2004; McGregor & Schleiss, 2001; Messerle *et al.*, 1997; Osterrieder *et al.*, 2003; Rosas *et al.*, 2006; Saeki, 2000; Wagner *et al.*, 1999, 2002). Several strains of EHV-1, including the vaccine strain RacH, have been cloned as infectious BACs by the introduction of a mini-F plasmid into the viral genome (Rudolph & Osterrieder, 2002; Rudolph *et al.*, 2002). RacH is innocuous in mice and horses and its attenuation could be attributed to a deletion of both copies of *gene 67* (*IR6*), which arose during its 256 passages on primary swine kidney cells. In addition to the deletion of *IR6*, other genomic alterations, such as truncation of the glycoprotein B, also contribute to its complete apathogenicity for a variety of species (Hubert *et al.*, 1996; Neubauer *et al.*, 1999; Osterrieder *et al.*, 1996).

In the present study, a recombinant viral vector was constructed by manipulation of a RacH BAC clone (pRacH). Shuttle mutagenesis was employed to manipulate pRacH and to insert into the EHV-1 genome a codon-optimized version of the BVDV structural gene region comprising *C*, *E<sup>ms</sup>*, *E1* and *E2*. The recombinant viral vector expressed BVDV proteins efficiently and stably. Immunization of cattle with the viral vector resulted in the induction of serum neutralizing (SN) antibodies against BVDV. Importantly, when compared with mock-vaccinated animals, immunized cattle presented with reduced levels of viraemia, shed less virus through nasal secretions and had higher leukocyte counts after experimental challenge infection with a heterologous virus strain.

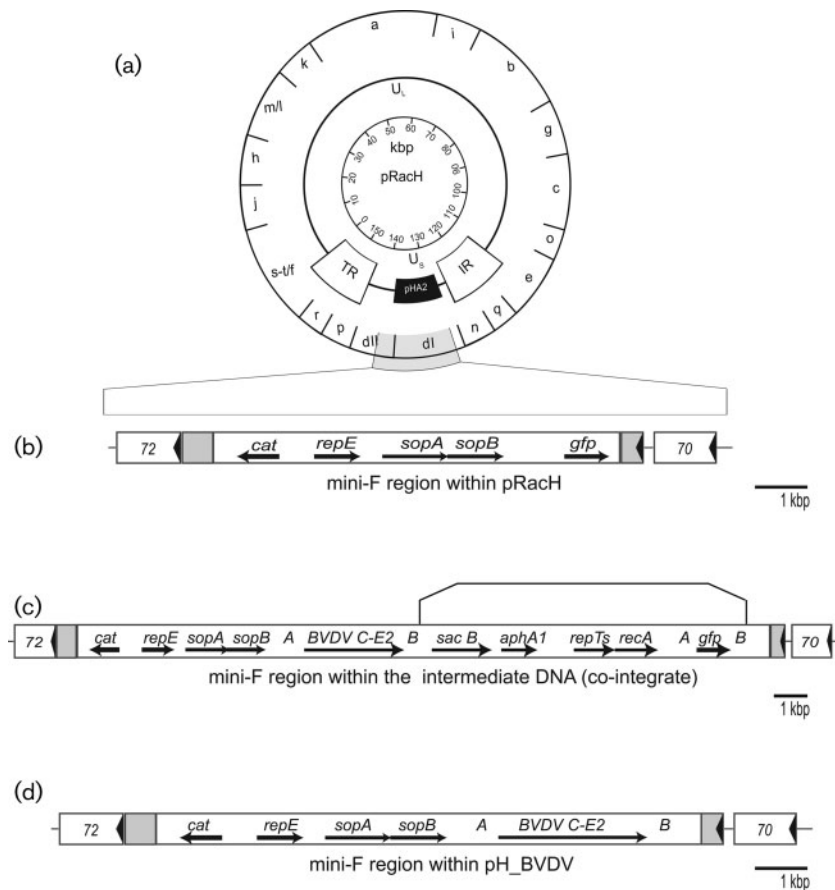
## METHODS

**Viruses and cells.** Rabbit kidney (RK13) cells were maintained in Earle's minimum essential medium (EMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and antibiotics (10 000 IU penicillin ml<sup>-1</sup>, 10 000 µg streptomycin ml<sup>-1</sup>; Mediatech, Inc.). Parental virus HΔgp2 (EHV-1 strain RacH in which *gene 71* was replaced with mini-F sequences) (Rudolph & Osterrieder, 2002) and rH\_BVDV (recombinant EHV-1 expressing BVDV structural genes) were propagated in RK13 cells. BVD viruses were grown on KOP-R, a calf oesophagopharyngeal cell line, or Madin-Darby bovine kidney (MDBK) cells (Collection of Cell Lines in Veterinary Medicine, FLI, Insel Riems, Germany).

**Bacterial strains.** *Escherichia coli* DH10B cells harbouring pRacH (Rudolph & Osterrieder, 2002) and pH\_BVDV (recombinant EHV-1 carrying BVDV genes as a mini-F-based BAC) were maintained in Luria-Bertani (LB) broth or on LB agar containing 30 µg chloramphenicol ml<sup>-1</sup>. *E. coli* DH10B cells harbouring the shuttle plasmid (pST\_BVDV) were grown in LB broth or LB agar containing 50 µg kanamycin ml<sup>-1</sup>.

**Plasmids.** The *C*, *E<sup>ms</sup>*, *E1* and *E2* (*C-E2*) synthetic open reading frame from the BVDV I isolate PT810 was released from expression plasmid pcDNA3.1 (Reimann *et al.*, 2003) and cloned into pTZ18RFr1/2, using *KpnI* and *NotI* sites, to construct pTZ\_BVDV (Fig. 1). Plasmid pTZ18RFr1/2 is a derivative of pTZ18R (Pharmacia) that contains additional flanks with homology to pRacH (nt 128709–120639 and 130428–131383, respectively). In pTZ\_BVDV, the *C-E2* region is flanked by these sequences. The *C-E2* genes and flanks were amplified from pTZ\_BVDV by using the following primers: SPBVDRv, 5'-GAGCTCCGACTCACTATAGG-3', and SPBVDFw, 5'-GAGCTCGTACGTTGCTTAAC-3'. The PCR product was cloned into the pCR2.1 TOPO vector (Invitrogen). After confirmation of the integrity of the nucleic acid sequence, the amplicon was transferred into the shuttle plasmid pST76K\_SacB (kindly provided by Dr Martin Messerle, Hannover Medical School, Hannover, Germany) via a *SacI* restriction site to generate pST\_BVDV. pST\_BVDV encodes the *recA* gene, the product of which mediates homologous recombination between circular molecules in DH10B cells. In addition to *recA*, pST\_BVDV encodes a positive-selection marker, *aphA1*, which confers resistance to kanamycin, and a negative selection marker, *sacB*, which confers sensitivity to sucrose (Borst *et al.*, 1999).

Shuttle mutagenesis was employed to insert the BVDV *C-E2* sequences in lieu of the *gfp* gene within the mini-F locus of pRacH (Borst *et al.*, 1999; Messerle *et al.*, 1997; O'Connor *et al.*, 1989). Electrocompetent *E. coli* DH10B cells harbouring pRacH were transformed with 200 ng pST\_BVDV. Electroporation was performed in 0.1 cm cuvettes (1250 V, 200 Ω, 25 µF). Upon electroporation, cells were grown for a short period of time at 37 °C to induce expression of the *recA* gene, which is under the control of a heat-inducible promoter (*repT*). Cells were then plated onto LB agar containing chloramphenicol (30 µg ml<sup>-1</sup>) and kanamycin (50 µg ml<sup>-1</sup>) and incubated overnight at 42 °C to select for integration of the shuttle plasmid into pRacH via recombination of one of the homology flanks. DNA from kanamycin- and chloramphenicol-resistant colonies was isolated by using alkaline lysis (Sambrook *et al.*, 1989), digested with *Bam*HI and *Eco*RI and analysed by 0.8% agarose-gel electrophoresis. Positive clones were grown in LB medium containing chloramphenicol for 4 h at 37 °C, 10% (w/v) sucrose was added to the medium and cultures were incubated for an additional 4–8 h. Serial 10-fold dilutions were grown overnight at 30 °C on LB agar plates containing chloramphenicol and 10% sucrose. The latter procedure selected for loss of *sacB* sequences of the shuttle plasmid via a second recombination mediated through one of the flanks. Larger individual colonies were replica-plated onto LB agar plates containing either chloramphenicol alone or kanamycin and



**Fig. 1.** Schematic illustration of the construction of the pH\_BVDV vaccine vector from pRach. (a) EHV-1 genome organization in the infectious BAC clone (pRach). (b) Detailed organization of the unique short region of pRach harbouring the introduced mini-F sequences instead of *gene 71*, which were targeted for mutagenesis. (c, d) Depiction of the kanamycin-resistant intermediate and the resolved co-integrate, pH\_BVDV, respectively.

chloramphenicol. DNA was isolated from colonies that were kanamycin-sensitive but chloramphenicol-resistant, and restriction patterns were analysed as described above. After agarose-gel electrophoresis, restriction digests were analysed further by Southern blotting (Sambrook *et al.*, 1989) using a digoxigenin-labelled probe (Roche) that hybridized specifically with BVDV *C-E2* sequences.

#### Indirect immunofluorescence assay (IFA) and Western blot.

Monoclonal antibody (mAb) A8 (Rudolph & Osterrieder, 2002), directed against EHV-1 glycoprotein M (gM), was used to detect EHV-1 infection, whilst mAb 15C5 (Corapi *et al.*, 1990a), directed against E<sup>rms</sup>, was used for routine screening of transgene expression. For IFA, RK13 cells were seeded in six-well plates and infected with either HAgp2 or rH\_BVDV virus at an m.o.i. of 0.0001. One hour post-infection (p.i.), medium was removed and infected cells were overlaid with 0.25% methylcellulose in EMEM/10% FBS. At 48 h p.i., cells were fixed with 90% ice-cold acetone for 10 min at  $-20^{\circ}\text{C}$ . After rehydration with PBS, non-specific binding sites on cells were blocked by using PBS/0.5% BSA for 30 min at room temperature. Cells were incubated with the 15C5 or A8 antibody at a 1:50 dilution for 30 min at room temperature and washed extensively with PBS before the secondary antibody [Alexa 488-conjugated goat anti-mouse IgG antibody (Molecular Probes)] was added (30 min at room temperature). After thorough washing, plaques were inspected by using an inverted fluorescence microscope (Zeiss Axiovert 25).

For Western blot analysis, pellets of infected primary bovine testicle cells were resuspended in radioimmunoprecipitation assay (RIPA) buffer (20 mM Tris, pH 7.5; 150 mM NaCl; 1% Nonidet P-40; 0.5% sodium deoxycholate; 1 mM EDTA; 0.1% SDS) and protease inhibitor cocktail (Roche). Sample buffer with  $\beta$ -mercaptoethanol ( $\beta$ -ME)

[0.15 M Tris/HCl, pH 6.8; 1.2% SDS; 0.3% glycerol; 0.15%  $\beta$ -ME; 0.018% (w/v) bromophenol blue] or without  $\beta$ -ME was added and samples were subjected to SDS-PAGE (12% gel) to separate the proteins. mAbs 4E1 (against BVDV E2, undiluted) or A8 (1:100 dilution) were used to detect proteins after transfer to nitrocellulose sheets (Schleicher & Schuell Biosciences, Inc.) by the semi-dry method (Kyhse-Andersen, 1984; Sambrook *et al.*, 1989).

#### Plaque-area measurements and single-step growth kinetics.

Plaque areas on RK13 cells were measured after infection of cells seeded in a six-well plate at an m.o.i. of 0.0001 and overlay with EMEM/FBS containing 0.25% methylcellulose at 1 h p.i. At 3 days p.i., plaques were analysed by IFA using anti-EHV-1 gM mAb A8. Fifty plaques were photographed and mean plaque areas were determined by using the ImageJ software (<http://rsb.info.nih.gov/ij/>). Values were compared with HAgp2 plaque areas, which were set to 100%. Mean percentages of plaque areas and SD were determined from at least three independent experiments. Single-step growth kinetics were done by infection of  $1 \times 10^5$  RK13 cells at an m.o.i. of 3. Virus was allowed to attach for 1 h at  $4^{\circ}\text{C}$ , followed by a penetration period of 1.5 h at  $37^{\circ}\text{C}$ . At 0, 8, 16, 20 and 24 h p.i., supernatants and cells were harvested separately, and cell-associated and extracellular viral titres were determined by plating onto RK13 cells that were then overlaid with EMEM/10% FBS/0.25% methylcellulose. At 3 days p.i., cells were fixed with 10% formalin in PBS, stained with 0.3% crystal violet and plaques were counted. Single-step growth curves were computed for three independent experiments.

#### Animal experiments

**Mice.** Three-week-old female BALB/c mice (Harlan) were allocated randomly to four groups of four mice each and immunized twice in

a 3 week interval. Group I was primed and booster-immunized intranasally (i.n.) with  $1 \times 10^5$  p.f.u. rH\_BVDV. For group II, the same schedule and antigen were used, but applications were intramuscular (i.m.). Group III was first primed i.m. with 25 µg plasmid DNA (pTZ\_BVDV) and then booster-vaccinated i.m. with  $1 \times 10^5$  p.f.u. rH\_BVDV. Group IV was immunized according to the group I schedule and route, but using parental HΔgp2 virus. All mice were bled at days 7, 14, 35 and 42. Serum was collected by centrifugation and standard serum microneutralization assays were performed (Corapi *et al.*, 1990b).

**Cattle.** Female Simmental calves aged 3–6 months were purchased from farms free of bovine herpesvirus type 1 (BHV-1) and BVDV. Animals were randomized to two groups of four animals each. Prior to vaccination, absence of antibodies against BVDV was confirmed by SN and ELISA testing (see below). The animals were housed in separate stalls in the high-containment unit of the FLI Insel Riems. After 3 weeks quarantine, calves were vaccinated i.m. with  $10^{7.0}$  TCID<sub>50</sub> rH\_BVDV or mock-vaccinated with cell-culture supernatant. Booster immunization was done 27 days later. Twenty-five days after the second vaccination, all animals were challenge-infected with  $10^{6.5}$  TCID<sub>50</sub> of the virulent BVDV Ib strain SE5508; 1 ml challenge virus was applied into each nostril by using a nebulizer. After vaccination, calves were examined for adverse reactions. The animals were monitored daily for clinical responses for 2 weeks after vaccinations and challenge infection. Clinical investigation included evaluation of pyrexia and intestinal or respiratory distress, such as nasal or ocular discharge, sneezing, coughing, diarrhoea, depression and reduction of feed uptake.

Nasal swabs were collected daily for a period of 10 days after vaccination and for 14 days after challenge infection, using cotton swabs (Mast Diagnostica). Swabs were submerged in 1.2 ml cell-culture medium supplemented with 200 U penicillin ml<sup>-1</sup>, 200 µg streptomycin ml<sup>-1</sup> and 5 µg amphotericin B ml<sup>-1</sup> (Sigma-Aldrich). Swab fluid (100 µl) was inoculated into susceptible cells in four replicates. Vaccine virus shedding was evaluated on RK13 cultures and BVDV excretion on MDBK cells. After an incubation period of 5 days, the cultures, as well as two consecutive passages of the supernatants, were screened macroscopically for EHV-1 replication or examined microscopically after indirect immunofluorescent staining for BVDV.

Calves were bled daily for 14 days after vaccination–challenge infection. For leukocyte isolation, blood (4 ml) was collected into EDTA-containing tubes and incubated for 10 min at ambient temperature with a 2.5-fold excess of erythrocyte lysis buffer (155 mM NH<sub>4</sub>Cl; 10 mM KHCO<sub>3</sub>; 1 mM EDTA, pH 7.4). After extensive washing with PBS, purified blood leukocytes were resuspended in 1 ml PBS. One hundred microlitres of the cell suspension was inoculated as described above. Differential blood counts and numbers of circulating blood cells were determined from EDTA/blood by using an automated counter (CELL-DYN 3700 haematology analyser; Abbott). Blood cells were characterized by size, complexity and depolarization capacity.

BVDV-specific antibody responses were determined at weekly intervals. Three replicates of 2-fold dilutions of heat-inactivated (30 min, 56 °C) serum samples were incubated with 100 TCID<sub>50</sub> of the BVDV type Ib challenge virus SE5508 or of the BVDV type II strain US890 (2/4) in microtitre plates (2 h, 37 °C). After addition of  $5 \times 10^4$  KOP-R cells per well, the plates were incubated for 6 days and scored for virus replication after indirect immunofluorescence staining. Titres were expressed as the reciprocal of the highest dilution that resulted in 50% neutralization. To exclude vector-induced neutralizing cross-reactivity against BHV-1, neutralization tests with BHV-1 strain Schönböken were performed as described above.

Besides SN testing, serum samples were analysed for BVDV-specific antibodies by using commercially available ELISA tests (BAR VAC BVDV Diagnostikum, Boehringer; HerdChek BVDV antibody test kit, Idexx) according to the manufacturers' instructions.

**Statistical analysis.** Statistical analyses were performed by using SAS for Macintosh (SAS 6.12 TS051). Student's *t*-tests were performed to determine differences in plaque areas between recombinant and parental viruses. The maximum neutralizing-antibody titres achieved in vaccinated mice were reported for day 35 and compared with those for the control group by using a non-parametric Kruskal–Wallis test. Virus shedding and viraemia data were analysed by using multivariate analysis of variance (MANOVA).

## RESULTS

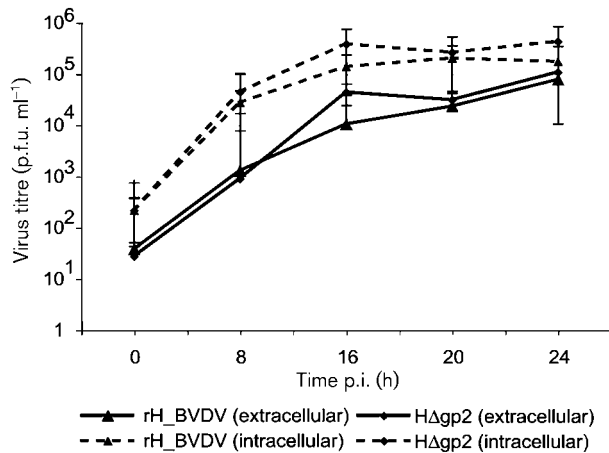
### Construction of recombinant EHV-1/BVDV virus (rH\_BVDV)

To generate recombinant infectious DNA of EHV-1 strain RacH harbouring BVDV structural genes, shuttle mutagenesis of EHV-1 BAC clone pRacH was employed. In the first round of RecA-mediated recombination, integration of the shuttle plasmid pST\_BVDV into pRacH resulted in the formation of an intermediate molecule (co-integrate) (Fig. 1). The co-integrate harboured, in addition to the synthetic *C–E2* sequences from BVDV, a gene that confers resistance to the antibiotic kanamycin, *aphA1*. Correct integration was first assessed by the appearance of kanamycin-resistant colonies and demonstrated further by analyses of the DNA restriction-fragment patterns (see Supplementary Fig. S1, available in JGV Online). Under proper conditions, a second round of RecA-mediated recombination resulted in the removal of shuttle sequences and maintenance of the BVDV sequences (Fig. 1). Resolution of the co-integrate was assessed by replica plating and identification of kanamycin-sensitive colonies. Maintenance of BVDV sequences was also confirmed by analyses of expected alterations in the restriction pattern and Southern blotting using a probe that spanned the BVDV synthetic *C–E2* sequences (Supplementary Fig. S1). From the analyses, we concluded that the mutant BAC, pH\_BVDV, harboured correctly inserted, synthetic *C–E2* sequences from BVDV strain Pt810 under the control of the human cytomegalovirus immediate-early promoter (*P<sub>HCMV</sub>*).

### *In vitro* characterization of rH\_BVDV

To reconstitute recombinant viruses, pH\_BVDV or pRacH DNA was transfected into RK13 cells by calcium phosphate precipitation. The recombinant viruses were characterized *in vitro* by determining plaque diameters and growth rates. There was no significant difference observed between the plaque diameters of the parental virus HΔgp2 and the rH\_BVDV virus (data not shown). In addition, both viruses exhibited comparable growth kinetics during a 24 h period, with respect to both the cell-associated and extracellular virus titres (Fig. 2).

Expression of BVDV antigens by EHV-1 was assessed by IFA and Western blotting. RK13 cells were infected at an m.o.i.

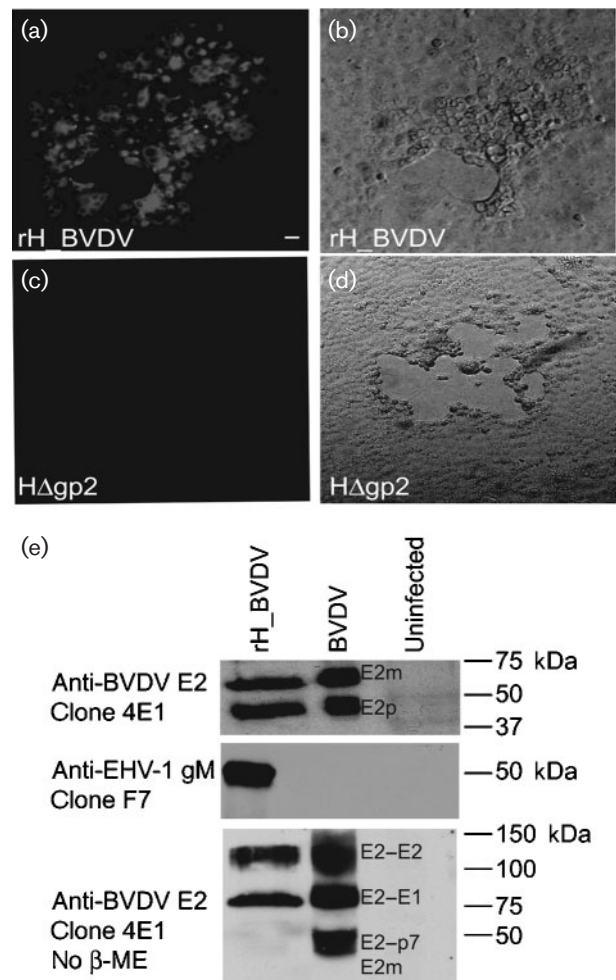


**Fig. 2.** *In vitro* growth properties of rH\_BVDV are comparable to those of the parental HΔgp2 virus. For single-step growth kinetics, RK13 cells were infected at an m.o.i. of 3, and virus supernatant (extracellular) and cellular (cell-associated) fractions were collected at the indicated time points. Viral titres were determined by plaque assay. These results are representative of three independent experiments. Error bars represent SD.

of 0.0001 and, at 3 days p.i., plaques were immunostained with the anti-BVDV E<sup>rns</sup> antibody mAb 15C5. Only in plaques induced by rH\_BVDV, but not in those of parental HΔgp2, was expression of the BVDV E<sup>rns</sup> envelope protein detected (Fig. 3a–d). Importantly, after ten consecutive passages of the recombinant virus in RK13 cells, expression of E<sup>rns</sup> and of E2 (data not shown) was observed for all plaques formed by rH\_BVDV, which indicated stable integration and expression of the transgene in the vectored virus. To confirm authentic processing of BVDV proteins upon expression by recombinant EHV-1, Western blot analyses under reducing and non-reducing conditions were performed. Expression of BVDV E2 protein as the unglycosylated precursor (41 kDa) and the mature, glycosylated protein (53 kDa) was confirmed by this technique. Under non-reducing conditions, E2–E1 dimers (75 kDa) and E2–E2 dimers (100 kDa) were readily detected, indicating proper processing of the BVDV structural proteins (Fig. 3e). In summary, the protein analyses demonstrated that the structural proteins of BVDV, E<sup>rns</sup>, E1 and E2, were expressed by the recombinant rH\_BVDV virus and that processing, as well as homo- and heterodimer formation, in rH\_BVDV-infected cells were comparable to those in cells infected with BVDV strain Singer (Fig. 3e).

### Serological responses induced in rH\_BVDV-immunized cattle prior to experimental challenge with a virulent strain of BVDV

In order to determine whether immunization with rH\_BVDV was able to induce production of SN antibodies, preliminary vaccination studies were done in mice to



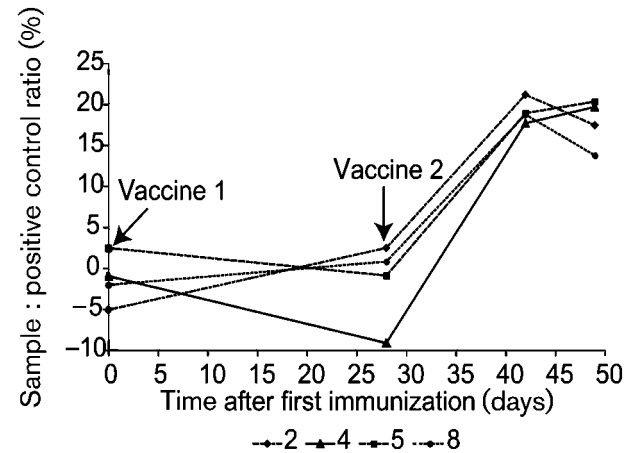
**Fig. 3.** Expression of BVDV antigens after infection with rH\_BVDV or parental HΔgp2 virus. RK13 cells were infected with rH\_BVDV (a, b) or HΔgp2 (c, d) at an m.o.i. of 0.0001. Plaques were stained with anti-BVDV E2 mAb 5C15. Bound antibodies were detected with anti-mouse IgG–Alexa 488. Plaques in (a) and (c) were visualized with an inverted fluorescence microscope (Zeiss Axiovert 25). Plaques in (b) and (d) were visualized under a light microscope. Bar, 100 μm. (e) Primary bovine testicle cells were infected with rH\_BVDV or BVDV Singer. Cell lysates were prepared from each sample once all infected cells displayed cytopathic effect. Cell lysates were analysed by SDS-PAGE (10% gel) using a mAb against BVDV E2 (clone 4E1), and a mAb against EHV-1 gM (clone F7) as a control. The secondary antibody was a goat anti-mouse horseradish peroxidase-conjugated antibody (Sigma). Western blots were done under reducing (upper panels) or under non-reducing (without addition of β-ME to the sample buffer; lower panel) conditions. The BVDV E2 monomer (E2m), the non-glycosylated precursor (E2p), the non-processed form (E2–p7) and the E2 dimeric forms (E2–E1 and E2–E2) are labelled.

determine the ideal route of immunization, and subsequently applied to calves to determine the onset of antibody production following vaccination with rH\_BVDV. In the

initial study, all rH\_BVDV-immunized mice developed SN antibodies against the heterologous BVDV strain Singer, regardless of the route of immunization (see Supplementary Fig. S2, available in JGV Online). Preliminary testing of the immunogenicity of rH\_BVDV on two calves had indicated induction of ELISA and neutralizing antibodies against the homologous strain, Pt810, as early as day 28 following two applications of the vaccine (data not shown). In the second cattle experiment, four calves were immunized twice with rH\_BVDV in a 4 week interval, whilst four animals were mock-vaccinated with cell-culture supernatant. All immunized animals developed SN antibodies against EHV-1, but none of the immunized animals shed EHV-1 in nasal secretions or became viraemic, as determined by virus isolation from nasal swabs and purified blood leukocytes. Cross-reactivity with BHV-1 in SN assays and ELISA was not observed. Also, none of the immunized animals developed any clinical disease upon immunization, as monitored by rectal body temperatures, nasal discharge and assessment of coughing/sneezing. In addition, lymphopenia, which is characteristic of BVDV infection, was not observed following vaccination (data not shown). Importantly, all of the immunized animals developed SN antibodies against heterologous BVDVI and BVDVII strains (Table 1) and seroconverted, as demonstrated by an indirect BVDV ELISA, whereas all animals in the control group remained seronegative (Fig. 4).

### Immunization of calves with rH\_BVDV results in partial protection of immunized animals in an experimental challenge with BVDV

On day 52 of the trial, 25 days after the second immunization, calves in both groups were challenged i.n. with the heterologous BVDV 1b strain SE5508. Three days after challenge, all animals displayed reduced leukocyte counts; however, rH\_BVDV-immunized animals recovered from the leukopenia faster and had higher leukocyte counts than control animals at 8, 9 and 10 days p.i. (MANOVA,  $P < 0.05$ ). In addition, rH\_BVDV-immunized animals shed significantly less virus (MANOVA,  $P < 0.05$ ) and exhibited lower levels of viraemia (MANOVA,  $P < 0.01$ ) (Fig. 5). By 9 days p.i., no virus could be isolated from nasal swabs of immunized animals and, by 10 days p.i., no virus



**Fig. 4.** Immunization of cattle with rH\_BVDV induces anti-BVDV specific antibodies. Two groups of four animals were vaccinated i.m. twice in a 27 day interval with  $10^{7.0}$  TCID<sub>50</sub> rH\_BVDV or mock-vaccinated with cell-culture supernatant. Relative amounts of BVDV-specific antibodies detected by blocking ELISA in the sera of vaccinated animals at the indicated days post-vaccination are shown.

could be isolated from the blood. In contrast, control calves continued to shed virus until day 12 p.i. and remained viraemic until 11 days p.i. (Fig. 5).

Whilst immunized animals had readily detectable SN antibody activity against BVDV 1b SE5508 at 14 days after the second vaccination (day 42 of the trial), SN activity was not detectable in non-immunized animals until 13 days after challenge infection (day 65 of the trial). Most importantly, we observed a marked anamnestic boost with consistently higher SN activities against both BVDV 1b SE5508 and BVDV II US890 after challenge in immunized animals compared with control animals (Fig. 6). Finally, at days 23 and 32 post-challenge infection, anti-NS3 antibodies were found at considerably higher concentrations in the sera of non-immunized animals compared with immunized animals, indicating that a general reduction in challenge virus replication was induced by vaccination with the EHV-1 recombinant in comparison to the mock-immunized animals (see Supplementary Fig. S3, available in JGV Online). From the results of the vaccination–challenge infection experiment, we concluded that a partially protective immune response against BVDV could be induced in the natural host by using EHV-1 as a vector delivering structural genes of this bovine pestivirus.

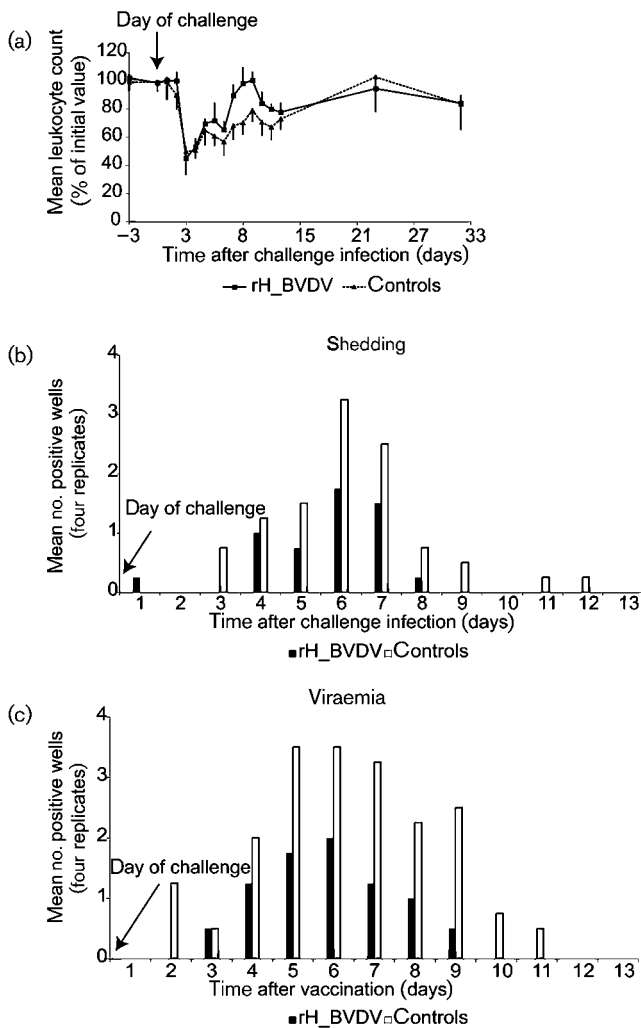
## DISCUSSION

In this study, we describe the generation and characterization of a recombinant EHV-1 virus harbouring the BVDV structural genes, *C*, *E<sup>ns</sup>*, *E1* and *E2* (*C–E2*). For generation of the recombinant virus, we inserted synthetic, codon-optimized BVDV *C–E2* sequences (Reimann *et al.*, 2003)

**Table 1.** Geometric means of neutralizing-antibody titres prior to challenge infection

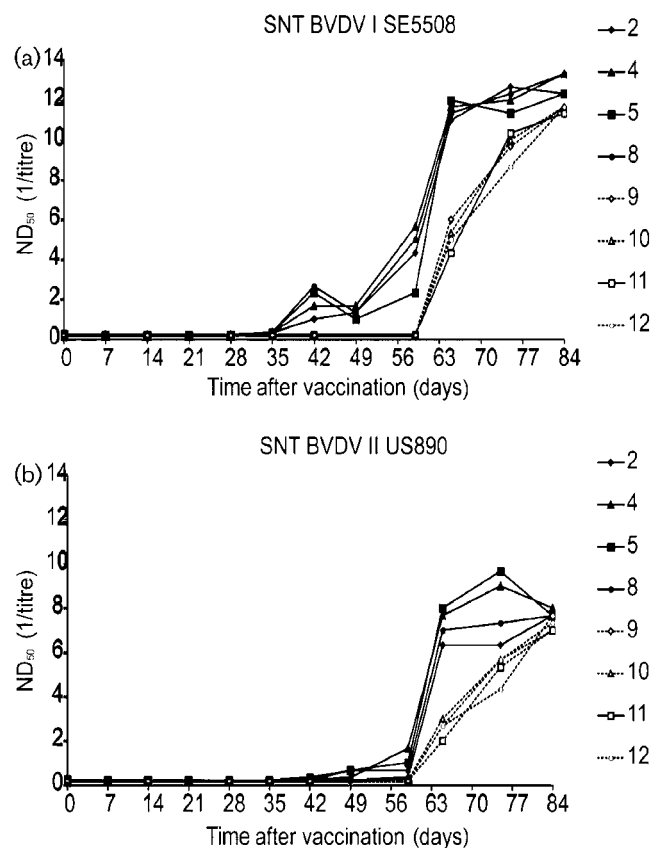
Neutralizing-antibody titres ( $\log_2$ ) were determined against BVDV 1b SE5508 and BVDV II US890.

BVDV type	Mean SN titre on day:							
	0	7	14	21	28	35	42	49
SE5508	0.2	0.2	0.2	0.2	0.2	0.33	1.78	1.31
US890	0.2	0.2	0.2	0.2	0.2	0.2	0.23	0.41



**Fig. 5.** Immunization with rH\_BVDV confers protection against challenge infection with the virulent BVDV 1b strain SE5508. Twenty-five days after the second vaccination, all calves were challenged i.n. with  $10^{6.5}$  TCID<sub>50</sub> of the virulent BVDV 1b strain SE5508. (a) Mean leukocyte counts as determined daily for rH\_BVDV-vaccinated and mock-vaccinated calves for 7 days after challenge infection and on days 23 and 28 after challenge infection. (b) Virus shedding as determined daily from nasal swabs for 14 days following challenge infection. Swab fluids (100  $\mu$ l) were inoculated in quadruplicate into susceptible cells. (c) Viraemia as measured daily from purified blood leukocytes for 14 days after challenge infection. Cell suspension (100  $\mu$ l) was added in quadruplicate to KOP-R cultures to determine cell-associated viraemia. Virus replication was scored after indirect immunofluorescence staining in (b) and (c).

into an infectious clone of EHV-1 MLV vaccine strain RaCh by using homologous, RecA-mediated recombination. BVDV-specific immune responses were induced in cattle following immunization with the recombinant virus, and immunized animals exhibited reduced viraemia, shed less virus and retained higher leukocyte counts following i.n. challenge with a heterologous BVDV strain.



**Fig. 6.** Vaccination of cattle with rH\_BVDV results in induction of serum neutralizing antibodies against both BVDV 1b SE5508 and BVDV II US890. Calves were bled at weekly intervals for analyses of BVDV-specific antibody responses. Triplicates of twofold dilutions of heat-inactivated serum samples were incubated with 100 TCID<sub>50</sub> of the BVDV type 1b challenge virus SE5508 (a) or of the BVDV type II strain US890 (b). Virus replication was scored after indirect immunofluorescence staining and titres ( $\log_2$ ) are expressed as reciprocals of the highest dilution that caused 50% neutralization.

RecA-mediated shuttle mutagenesis was carried out in *E. coli* strain DH10B, which is deficient in homologous recombination unless the RecA protein is provided *in trans*. The inability of DH10B cells to perform recombination ensures the stability of the BAC plasmids. A major advantage of BAC mutagenesis over conventional recombination in eukaryotic cells is that reconstitution of the infectious clone results only in recombinant virus, which is free of potential contamination with parental virus, and that the starting material for vaccine production is essentially monoclonal. Reconstitution of the recombinant virus generated here resulted in a recombinant vector in which expression of the BVDV transgene was observed in all virus plaques examined. Additionally, by using infectious EHV-1 clones and RecA-mediated recombination, we were able to overcome two major hurdles on the way to the development of efficient viral vectors, i.e. transient and/or low-level gene

expression and low virus titres (Hendrie & Russell, 2005; Miletic *et al.*, 1999; Wolff, 2002). The recombinant EHV-1–BVDV virus showed plaque sizes and growth kinetics that were comparable to those of the parental virus. The parental recombinant virus HΔgp2, despite the deletion of *gene 71*, which encodes a major immunomodulatory glycoprotein (gp2), was able to replicate to high titres and exhibited growth kinetics comparable to those of RacH (Rudolph & Osterrieder, 2002; this report). Most importantly, stable expression of the codon-optimized, synthetic BVDV sequences was observed even after ten passages. From the cell-culture and antigen-detection studies, we concluded that insertion of the BVDV in the selected locus was stable and did not alter the replication of EHV-1 *in vitro*.

Previous studies have demonstrated that induction of even low titres of neutralizing antibodies protects cattle against BVDV infection, reduces virus replication and thus prevents virus transmission (Bolin & Ridpath, 1996; Howard *et al.*, 1989). We found that immunization of cattle with recombinant EHV-1 expressing BVDV structural proteins resulted in the induction of neutralizing antibodies against heterologous BVDV strains in the absence of an adjuvant. Whilst at the time of challenge infection, immunized cattle had relatively low levels of neutralizing antibodies, these titres increased rapidly upon challenge infection. The booster effect after challenge infection in immunized calves resulted in consistently higher antibody levels than in non-immunized animals, which suggests a robust, mostly humorally biased, T-helper memory response. In contrast, induction of anti-NS3 antibodies upon experimental challenge infection with BVDV has been associated with increasing levels of virus replication (Beer *et al.*, 2000). Calves immunized with recombinant EHV-1 expressing BVDV antigens exhibited reduced levels of NS3 antibodies after challenge infection compared with the mock-vaccinated controls. Moreover, at days 8–10 post-challenge, mock-immunized animals still had reduced leukocyte counts and also exhibited high levels of anti-NS3 antibodies. At this time point, however, animals immunized with rH\_BVDV presented with comparatively low levels of viraemia and were shedding significantly less virus than controls. Taken together, our results suggest that immunization with recombinant EHV-1 reduces virus replication, resulting in lower levels of viraemia and reduced viral shedding that are concomitant with an accelerated rebound of leukocyte counts in peripheral blood.

MLV vaccines, including MLV vectors, are considered potent inducers of immunological responses because they generate antigens endogenously and exogenously. Recent reports have shown that BVDV MLV vaccines are capable of inducing complete or partial protection against virulent BVDV strains. In one study, complete protection was achieved after challenge infection with a heterologous strain of BVDV in calves vaccinated with a MLV BVDV type 1 vaccine (Kelling *et al.*, 2005). In another study, a commercially available MLV vaccine using colostrum

containing BVDV antibodies as an adjuvant was reported to increase leukocyte numbers significantly on days 6–8 post-challenge infection and to induce neutralizing antibodies at titres 5- to 8-fold higher than those presented in this study (Zimmerman *et al.*, 2006). However, these studies did not address the high risk of vaccinating pregnant cows with BVDV MLV (Ellis *et al.*, 2001; Grooms *et al.*, 1998; Ridpath & Bolin, 1995; Van Campen *et al.*, 2000). Other reports involving application of adjuvanted inactivated vaccines have achieved partial protection and induced neutralizing-antibody titres that were 100- to 2000-fold higher than those presented in this study, but those titres were raised against homologous strains (Beer *et al.*, 2000; DesCoteaux *et al.*, 2003). Reports on the protective effect of inactivated vaccines against heterologous BVDV strains and in the absence of adjuvant effects have not increased leukocyte numbers significantly and have shown only limited protection (Hamers *et al.*, 2003). Additionally, the susceptibility of BVDV MLV and inactivated vaccines to maternal antibodies against BVDV was not addressed in most of these studies. Whilst we did not address the safety of recombinant EHV-1 vaccine in pregnant cows, we speculate that a RacH-based vaccine will not induced adverse effects in pregnant cows or young calves, as the vaccine has been demonstrated extensively to be safe in pregnant mares and young foals (Purdy *et al.*, 1978).

Previous reports have identified EHV-1 isolates in non-equid ruminants, perhaps an indication of EHV-1 as a possible pathogen of bovines (Chowdhury *et al.*, 1988). Immunization of cattle with the recombinant EHV-1, based on the EHV-1 MLV strain RacH, whilst not causing detectable clinical signs, resulted in the induction of neutralizing antibodies against EHV-1. Importantly, cross-reactivity with BHV-1, a major pathogen of cattle, was not observed. This result is consistent with an earlier report that showed that anti-EHV-1 antibodies did not cross-react with human herpesviruses of any of the three subfamilies (Trapp *et al.*, 2005), which is critical if distinction of serological responses induced by vaccination or natural infection of bovines with BHV-1 is an issue. The lack of cross-reactivity of anti-EHV-1 neutralizing antibodies with BHV-1 may suggest – by inference – that anti-BHV-1 antibodies would not neutralize the vector and, as such, allow vaccination of BHV-1-positive cattle. As such, it will avoid a common complication of using vectored vaccines, i.e. anti-vector immunities in previously vaccinated or infected animals (Dudek & Knipe, 2006).

The study presented here shows that recombinant EHV-1 can induce protective immune responses against BVDV in immunized animals. Future studies will concentrate on the expansion and improvement of the delivery of the engineered vaccine to cattle using various prime–boost regimens, such as priming with a DNA vaccine and boosting with the rH\_BVDV construct, as well as the testing of the safety and efficacy in pregnant animals and bovines that have encountered infection or vaccination with BHV-1.

These future studies will be designed to increase vaccine efficacy and avoid potential problems with respect to vector immunity, as outlined above.

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