Chimeric pestiviruses: candidates for live-attenuated classical swine fever marker vaccines

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The use of attenuated classical swine fever virus (CSFV) strains as live vaccines is no longer allowed for the control of classical swine fever in Europe, due to the inability to differentiate between infected and vaccinated animals (Differentiating Infected from Vaccinated Animals; DIVA), except as emergency vaccines or as bait vaccines for wild boars. Thus, the establishment of a DIVA vaccine(s) is of pivotal importance for the control of this infectious disease. In this study, recombinant versions of the live-attenuated vaccine strain CSFV Riems were generated by replacing parts of the E2 gene with the corresponding sequence of border disease virus strain Gifhorn. Three cDNA clones were constructed: pRiems-ABC-Gif, pRiems-A-Gif and pRiems-BC-Gif. Infectious particles were obtained from clones pRiems-ABC-Gif and pRiems-BC-Gif only, whereas transfected RNA from clone pRiems-A-Gif behaved like a replicon. Based on its ability to be differentiated in vitro from wild-type CSFV by mAbs, vRiems-ABC-Gif was assessed for immunogenicity and protection against challenge infection in pigs. Before challenge, no CSFV-specific anti-E2 antibodies could be detected with commercial E2-blocking ELISAs in vRiems-ABC-Gif-vaccinated animals, whereas vRiems-vaccinated pigs developed high titres of anti-E2 antibodies, confirming the marker properties of this vaccine candidate. After oral vaccination, only partial protection against challenge infection was observed in the vRiems-ABC-Gif vaccinees, whereas all intramuscularly vaccinated animals and all vRiems-vaccinated animals were fully protected. These experiments suggest that the strategy of exchanging specific antigenic epitopes among pestiviruses is a promising tool for the development of new CSFV marker vaccines.

INTRODUCTION

Classical swine fever (CSF) is a highly contagious, haemorrhagic disease of pigs and wild boars and is caused by the species Classical swine fever virus (CSFV), belonging to the genus Pestivirus within the family Flaviridae. This genus also contains Border disease virus (BDV) and Bovine viral diarrhea virus (BVDV). All pestiviruses are structurally and genetically closely related (Thiel et al., 2005). BVDV and BDV are able to infect ruminants and pigs, whereas CSFV infection is restricted to pigs under natural conditions (Liess & Moennig, 1990).

Pestiviruses are enveloped viruses harbouring a single-stranded, positive-sense RNA genome of about 12.3 kb, encoding a single open reading frame (ORF). This ORF is translated into a polyprotein that is processed into the mature CSFV proteins by viral and host-cell proteases (Meyers & Thiel, 1996). The structural proteins include a nucleocapsid protein and three envelope glycoproteins termed Ems, E1 and E2. Animals infected with a pestivirus develop antibodies against Ems, E2 and the non-structural protein NS3 (Wensvoort et al., 1988). Among these proteins, E2 is the most immunogenic (Donis & Dubovi, 1987). Monoclonal antibodies (mAbs) directed against E2 or Ems are used as diagnostic tools to discriminate between the three pestivirus species (Edwards et al., 1991; Wensvoort et al., 1989).

Today, outbreaks of CSF in domestic pigs still occur in many countries, causing significant economic losses in areas with intensive pig rearing (Artois et al., 2002; Edwards et al., 2000). Nevertheless, eradication programmes have been implemented and vaccination is restricted to emergency cases or is generally prohibited. The major reason to follow this policy is that although live-attenuated vaccines are highly efficacious and safe,

Supplementary tables showing plasmids used for cloning and oligonucleotides used for PCR-based plasmid construction and RT-PCR are available with the online version of this paper.

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they do not allow serological discrimination of vaccinated from infected animals. In combination with suitable diagnostic tests, DIVA (Differentiating Infected from Vaccinated Animals) vaccines could be used to monitor and control infections (van Oirschot, 2003).

Different types of DIVA vaccine have been proposed for CSFV. Non-replicating vaccines include DNA vaccines encoding the E2 gene (Hammond et al., 2001; Yu et al., 2001) and vaccines consisting of E2 peptides (Dong et al., 2002, 2005, 2006) or complete E2 proteins (Moormann et al., 2000). So far, these types have been shown to be less efficient than live vaccines and need multiple administrations for reliable protection (de Smit et al., 2000). Virus replicon particles (VRPs) contain viral genomes lacking structural protein genes (Frey et al., 2002, 2005, 2006) or complete E2 proteins (Moormann et al., 2001) and vaccines consisting of E2 peptides (Dong et al., 2002; Widjioaatmodjo et al., 2000). VRP-vaccinated animals can be distinguished from infected individuals by the absence of antibodies against the deleted protein. However, the efficacy is dependent on the administration route.

It has been shown that genome segments from different pestiviruses can be combined, leading to recombinant viruses. These chimeric CSFVs represent a powerful approach to combining the high-protection potential of conventional live-attenuated vaccines with the DIVA principle. In contrast to VRPs, the ‘marker gene’ is not deleted, but replaced by a corresponding genome fragment from another pestivirus. Several chimeric viruses have been reported where the E2 or E′ gene was exchanged for the respective gene of a heterologous virus strain or another pestivirus (de Smit et al., 2001; Liang et al., 2003; Reimann et al., 2004; van Gennip et al., 2004). Epitopes on the E2 protein have been mapped to the three major antigenic domains, A–C, which are located at the N-terminal ectodomain (van Rijn et al., 1996). Domain A is more conserved among CSFV strains than domain B or C (Wensvoort, 1989). Sequence comparison of several E2 proteins from different pestivirus species suggests a similar overall structure. Nevertheless, E2 is the most variable among the immunogenic proteins of pestiviruses. This makes it a key candidate for the development of live marker vaccines harbouring a chimeric E2 gene that can be discriminated from wild-type CSFV by E2-antibody ELISA.

In this study, we describe the construction of chimeric CSFV Riems variants (Mayer et al., 2003) expressing E2 genes with antigenic epitopes that were replaced with the respective epitopes from BDV Gifhorn (Becher et al., 2003). Three chimeric mutants were characterized in vitro. Exchange of all three domains, A, B and C, resulted in a chimeric virus that was able to induce protection against a virulent CSFV challenge, and the serological response of vaccinated pigs could be differentiated from that of infected pigs with commercially available E2 ELISAs. Exchange of domain A only did not allow the rescue of infective progeny virus, whereas exchange of domains B and C only resulted in a virus that could not be differentiated serologically from wild-type CSFV. Here, we report the results of oral and intramuscular immunization trials with the newly constructed chimeric virus strain vRiems-ABC-Gif.

METHODS

Cells and viruses. SK-6 swine kidney cells (Kasza et al., 1972) were grown in Earle’s minimal essential medium (EMEM) supplemented with 7 % horse serum (HS) or 10 % fetal calf serum (FCS). The porcine kidney cell line PK-15 (Collection of Cell Lines in Veterinary Medicine, FLI Insel Riems, Germany) was maintained in Dulbecco’s minimal essential medium (DMEM) supplemented with 10 % FCS. SFT-R, an ovine thymus cell line (Collection of Cell Lines in Veterinary Medicine, FLI Insel Riems), was grown in EMEM supplemented with Hanks’ salts and 10 % HS. Lamb synovial membrane (LSM) cells (Institute of Veterinary Virology, University of Berne, Switzerland) were grown in DMEM supplemented with 5 % HS, 1 mM sodium pyruvate and non-essential amino acids. All cells were incubated routinely at 37 °C (5 % CO2).

CSFV Riems was derived from molecular cDNA clone pRiems-3 and highly virulent CSFV strain Eystrup from plasmid pEy-37 (Mayer et al., 2003). BDV Gifhorn was provided by the virus collection of the FLI Insel Riems. The highly virulent CSFV strain Koslov was obtained from an animal experiment. The pigs were killed 5 and 6 days post-infection (p.i.), at the time point of the highest fever peaks, and whole blood containing 1 × 106.75 TCID50 ml−1 was collected. For challenge infection, the whole-blood preparation was diluted with PBS to a titre of 0.5 × 106.75 TCID50 ml−1, and 2 ml was administered oronasally to each animal.

Bacterial strains and plasmids. Escherichia coli XL-1 Blue (Strategene) was grown routinely at 37 °C in Luria–Bertani (LB) medium supplemented with ampicillin and kanamycin at concentrations of 100 and 50 μg ml−1, respectively. Plasmids used for cloning are listed in Supplementary Table S1, available in JGV Online.

Enzymes and recombinant DNA techniques. Standard recombinant DNA techniques were performed essentially as described by Sambrook et al. (1989). Restriction endonucleases were purchased from New England Biolabs and Stratagene. For PCR, Taq DNA polymerase (Promega) and Pfu UltraPolymerase (Stratagene) were used as recommended by the manufacturers. Oligonucleotides used for the construction of chimeric clones are listed in Supplementary Table S2, available in JGV Online. DNA fragments were isolated from agarose gels by using a NucleoTrap kit (Macherey-Nagel). T4 DNA ligase was obtained from Promega. All inserts derived from PCR and ligation sites were checked by DNA sequencing according to the dideoxynucleotide chain-termination method (Sanger et al., 1977).

Construction of E2 chimeras. The CSFV/BDV chimeras displayed in Fig. 1 were constructed by using cDNA clone pRiems-3 as backbone by the overlapping PCR extension method (Horton et al., 1990). Chimeric E2 genes were generated by replacement of the antigenic domains A and/or B/C of CSFV Riems variants with the corresponding gene fragments of BDV Gifhorn.

For the PCR fragment comprising the 5′ border of domain A, primers SigE2L3 and PGiF-A5′-rev were used; the 3′ part was amplified with primers PGiF-A3′-for and P7R2. The required portion of the BDV- Gifhorn E2 gene was amplified by the oligonucleotide pair PGiF-A5′-for/PGiF-A3′-rev. Plasmids pRiems-3 and pGEM-Gif-E2, respectively, served as template. The purified PCR fragments were used as partially
overlapping templates for a second PCR using primers SigE2L3 and P7R2. The resulting fragment was subsequently digested with Spel and SmaI and cloned into plasmid pSK-Riems_SpeBam, yielding plasmid pSK-Riems-A-Gif. Plasmid pSK-Riems-BC-Gif was constructed as described above using primer pairs SigE2L3/PGif-BC5-rev, PGf-B5I-for/P7R2 and PGf-B5I-for/PGif-B5I-rev, respectively, and plasmid pSK-Riems-ABC-Gif was established with oligonucleotides SigE2L3/PGif-BC5-rev, PGf-A3-for/P7R2 and PGf-B5I-for/PGif-A3I-rev.

To obtain full-length cDNA clones, two further cloning steps were required. The chimeric pSK-Riems subclones were cleaved with restriction enzymes Spel and NcoI and ligated into plasmid pACNR-5’Riems-Sal/Bam. Insertion of the SalI/BamHI digestion products from p5’Riems subclones into the SalI/BamHI-cut backbone fragment from pACNR-3’Riems-Bam/Xho resulted in full-length constructs pRiems-A-Gif, pRiems-BC-Gif and pRiems-ABC-Gif.

**Rescue of recombinant viruses and virus replication kinetics.**

_in vitro_ transcription, transfection of SK-6 cells and virus rescue were performed as described previously (Mendez et al., 1998; Ruggli et al., 1996). Virus stocks were prepared by passaging the virus once on SK-6 cells, obtaining a titre of about 10^6 TCID50 ml^-1. Virus replication kinetics were described as described previously (Mittelholzer et al., 2000).

**Immunohistochemistry and immunofluorescence.** For the detection of CSFV proteins in infected cells, the E2-specific mAb HC/TC26 (Greiser-Wilke et al., 1990) and NS3-specific mAb C16 (Greiser-Wilke et al., 1992) or a CSFV mAb mix (Hy24/6-7, Hy24/23-2, Hy24/10-6-4 and Hy4c/6-32-16; Kosmidou et al., 1995) were used. For differentiation purposes, mAbs used in the Ceditest CSFV2.0 (Cedex Diagnostics), HerdChek CSFV/Ab (IDEXX Laboratories) and CHEKIT-CSF-sero (Bommeli Diagnostics) antibody-blocking ELISAs were kindly provided by the kit manufacturers. Bound primary mAb was detected by a secondary horseradish peroxidase-labelled rabbit anti-mouse conjugate (DAKO) or Alexa Fluor 488-conjugated F(ab’)2 fragments of goat anti-mouse IgG (Molecular Probes). Fixation of cells and staining were performed as described elsewhere (Ahrens et al., 2000; Kaden et al., 1999b; Mittelholzer et al., 1997).

**Virus titration.** Virus titrations were carried out by end-point dilution of clarified cell lysates in SK-6, LSM or SFT-R cells. Titres were determined 48–72 h p.i. by immunohistochemistry or immunofluorescence using mAb C16.

**Vaccination and challenge experiments.** In a first experiment, the protection potential of orally administered chimeric CSFV/BDV vaccine vRiems-ABC-Gif was evaluated in comparison to vRiems. Eight 13-week-old specific-pathogen-free pigs (Edelweinbreed) were divided into three groups of three or two animals. The first group of three pigs (A) was vaccinated with virus from clone pRiems-ABC-Gif and the second group (B; three pigs) was immunized with Riems virus derived from the infectious cDNA clone pRiems-3. All pigs were inoculated orally with 10 ml clarified cell lysate containing 1 x 10^7 TCID50 recombinant virus. The third group (control group C; two pigs) was mock-immunized with 10 ml lysate from uninfected SK-6 cells. Before challenge, the three groups were kept separately. On day 23 post-vaccination (p.vacc.), oronasal challenge infection was carried out by slowly instilling 1 x 10^7 TCID50 of the highly virulent CSFV strain Eyrstrup. Body temperature and clinical scores were monitored daily according to a modified scoring scheme (Greiser-Wilke, 2006). Blood samples for serum preparation were collected the day before vaccination and twice a week until death of the animals. In addition, 1 ml EDTA blood samples from the ear vein were collected at days 0, 2, 6 and 9 p.vacc., and daily after challenge infection, to determine viraemia and leukocyte numbers.

In a second animal trial, the efficacy of intramuscularly administered pRiems-ABC-Gif was evaluated. A group of five pigs (11 weeks old) was inoculated intramuscularly with 2 ml pRiems-ABC-Gif containing 1 x 10^4 TCID50 ml^-1. After immunization, one animal was placed into the group as a sentinel in order to detect vaccine-virus excretion. Two pigs served as mock-vaccinated controls. On day 28 p.vacc., all animals were challenged oronasally with 1 x 10^5.75 TCID50 of the highly virulent CSFV strain Kloslov. Samples were collected 0, 7, 14, 21 and 28 days p.vacc., as well as 4, 7, 10 and 21 days (day of necropsy) post-challenge (p.chall.). Except at 4 days p.chall. (blood samples only), blood and serum samples were collected on all bleeding days. After challenge infection, nasal swabs were taken to monitor challenge-virus excretion.

**Analysis of blood samples.** In the first animal trial (oral immunization), EDTA blood was diluted in Türk’s solution (Merck), and peripheral blood leukocytes (PBLs) were counted in a Neubauer’s counting chamber. Monitoring of viraemia was done as described previously (Frey et al., 2006). Viraemia was also determined by TaqMan RT-PCR (Applied Biosystems) as described previously (Hofmann, 2003).

The sera were analysed for E2-specific antibodies by an in-house indirect ELISA (CSFI-ELISA; Moser et al., 1996) and commercially available E2-blocking ELISAs (CHEKIT-CSF-sero, Bommeli Diagnostics; HerdChek CSFV/Ab, IDEXX Laboratories). For detection of E3-specific antibodies, the CSFV-ELISA (Bommeli Diagnostics) was used. CSFV-specific neutralizing antibodies were determined in a neutralizing peroxidase-linked assay (NPLA) (Terpstra et al., 1984). Similarly, NPLA was performed to determine the titre of BDV-neutralizing antibodies by using serial twofold dilutions of serum mixed with an equal volume of EMEM containing 100 TCID50 BDV strain Gifhorn. After 3 days, cells were stained with anti-NS3-specific mAb C16 by immunohistochemistry.

In the second experiment (intramuscular immunization), buffy coat (Bc) was prepared as described by Kaden et al. (1999a) and used to inoculate PK-15 cells. Leukocyte counts were determined by using a CELL-DYN 3700 counter (Abbott). Cell cultures were inoculated with 200 μl Bc. All sera were tested for neutralizing antibodies as described by Kaden et al. (2001). In addition, all sera were tested with the commercial E2-blocking ELISAs mentioned above.

**Analysis of organ samples and nasal swabs.** Organ samples and nasal swabs were prepared and used for virus isolation on PK15-cells as described by Ahrens et al. (2000).
RESULTS

Construction and in vitro characterization of chimeric CSFV E2 mutants

Three different chimeric full-length recombinants were constructed by using cDNA clone pRiems-3 as backbone. Based on the proposed antigenic structure of the E2 protein (van Rijn et al., 1996), different domains were replaced by the respective regions from BDV Gifhorn (Fig. 1). In construct pRiems-ABC-Gif, aa 693–864, representing the antigenic domains A and B/C, were exchanged, whereas in pRiems-A-Gif and pRiems-BC-Gif, only domains A (aa 801–864) or B/C (aa 693–746), respectively, were replaced by the corresponding amino acids from BDV Gifhorn.

In vitro-transcribed RNA from the three chimeric clones was transfected into SK-6 cells to generate recombinant viruses. Immunostaining of the transfected cells indicated that all RNAs were replication-competent. The specific infectivities of the RNAs as determined by infectious-centre assay were 1 × 10^4.6 focus-forming units (f.f.u.) (µg RNA)^−1 for pRiems-ABC-Gif, 1 × 10^5.1 f.f.u. (µg RNA)^−1 for pRiems-A-Gif, 1 × 10^5.1 f.f.u. (µg RNA)^−1 for pRiems-BC-Gif and 1 × 10^5.2 f.f.u. (µg RNA)^−1 for pRiems-3, respectively. The cDNA-derived progeny viruses vRiems-ABC-Gif, vRiems-BC-Gif and vRiems-3 were harvested from freeze-thawed supernatants of transfected SK-6 cells. The respective titres of the infectious particles were 1 × 10^4.7 TCID_{50} ml^−1 for vRiems-ABC-Gif and 1 × 10^4.3 TCID_{50} ml^−1 for vRiems-BC-Gif, whereas the parent strain vRiems-3 yielded a titre of 1 × 10^4.1 TCID_{50} ml^−1. Interestingly, in extracts of SK-6 cells electroporated with RNA from pRiems-A-Gif, no progeny virus could be detected, indicating that infectious-particle formation was impaired in this chimeras. This result was consistent with the findings in the infectious-centre assay, where transfection of pRiems-A-Gif merely resulted in single stained cells, whereas in the wild type and the other constructs, foci of several positive cells were found.

One-step growth kinetics of vRiems-3, vRiems-ABC-Gif and vRiems-BC-Gif in SK-6 cells showed that the two mutants exhibited very similar growth characteristics. Both mutants had a lag phase that was shortened by about 3 h compared with that of the parent virus. In addition, the final titres of the two chimeras were about 10-fold higher than those of vRiems-3 after 49 h. To compare the growth rates of clone-derived and SK-6-adapted virus, the viruses were passaged ten times in SK-6 cells. The titres remained stable for the wild type and vRiems-ABC-Gif chimaera after passage 1, whereas vRiems-BC-Gif required four passages to reach a peak-titre plateau. The two chimeras replicated to similar peak titres of approximately 1 × 10^6.5 TCID_{50} ml^−1, which was about 10-fold higher than the parental strain.

Earlier studies demonstrated that the cell specificity of pestiviruses is determined by the origin of the E2 protein (Liang et al., 2003). We therefore compared growth properties on the porcine cell line SK-6 and ovine cell lines SFT-R and LSM. As expected, the wild-type strains vRiems-3 and BDV Gifhorn grew to higher titres on cells derived from their respective natural host. However, both chimeras showed growth characteristics similar to those of their CSFV parent strain vRiems-3, i.e. higher titres on SK-6 than on SFT-R or LSM cells (data not shown).

In vitro differentiation of the CSFV/BDV hybrids

An important objective of this study was the development of a chimeric live marker vaccine that allows application of commercially available diagnostic tests as serological differentiation tools. Three blocking ELISAs based on mAbs directed against the CSFV E2 protein (anti-E2 mAbs) were evaluated in this study. In order to assess the CSFV specificity of the mAbs used in these test kits, monolayers of SK-6 cells were infected with different chimeric pestiviruses. CSFV vRiems-3 and BDV Gifhorn served as controls. Detection of viral E2 protein was performed by immunohistochemistry with the kit-derived mAbs and mAb HC/TC26. As expected, cells infected with vRiems showed a specific staining pattern with all tested anti-CSFV E2 mAbs, whereas no specific labelling was detected in BDV Gifhorn-infected cells. Construct vRiems-BC-Gif showed the same staining pattern as the CSFV wild type. However, none of the four anti-CSFV-E2 mAbs stained chimera vRiems-ABC-Gif-infected cells specifically; hence, this hybrid could be differentiated from its parent strain. As pRiems-A-Gif behaved like a VRP, this construct was not tested. The presence of viral proteins in all infected cells was confirmed with mAb C16, directed against the NS3 protein of pestiviruses (data not shown).

vRiems-ABC-Gif exhibited the desired in vitro properties and was subsequently evaluated in two vaccination experiments (oral and intramuscular) for its protection from a challenge infection with highly virulent CSFV and its marker property.

Experiment 1: oral immunization of pigs with vRiems-ABC-Gif

Two groups of three pigs each were immunized by the oral route with vRiems-ABC-Gif (group A, animals 826, 827 and 830) or vRiems (group B, animals 831–833). Two additional animals were mock-immunized with SK-6 cell lysate (group C, animals 834 and 835). After vaccination, none of the animals showed any clinical signs of disease or fever, and virus isolation from EDTA blood was negative for all animals (Fig. 2). After challenge infection, all animals of group B and two animals of group A (827 and 830) remained clinically healthy and did not show any CSFV-specific symptoms. Unexpectedly, animals 827 and 830 developed severe transient leukaemia from days 2 to 4 p.chall., whereas PBL counts of vRiems-vaccinated animals remained at physiological levels. Three to four days after challenge, the pigs of group C and animal 826 from group A developed fever of up to 42 °C. The course of
disease was severe and, based on the clinical scores, it was not possible to distinguish the vRiems-ABC-Gif-vaccinated animal 826 from the mock-vaccinated control group. All diseased pigs showed a dramatic drop in PBL counts, high fever and clinical scores up to 15, and had to be euthanized 8 days p.chall. (Fig. 3a, b). The diseased animals were viraemic as early as 2 or 3 days p.chall. and consistently thereafter until death, whereas the vRiems-vaccinated animals and vRiems-ABC-Gif-vaccinated pig 830 did not show viraemia throughout the experiment (Fig. 2). A transient viraemia was detected for animal 827 at days 4 and 5 p.chall., although no clinical signs were observed. These data were further corroborated by TaqMan RT-PCR of RNA isolated from EDTA blood (Fig. 2). EDTA blood samples collected from the diseased animals showed increasing amounts of viral RNA until death, whereas only low amounts of viral RNA were transiently detected in blood from pig 827.

By using the E2 antibody-specific indirect CSFi-ELISA, a serological response was first detected for vRiems-vaccinated animals 1 day before challenge (22 days p.vacc.). At this time point, no CSFV E2-specific antibodies were detected in group A or C. vRiems-ABC-Gif-vaccinated animals 827 and 830 developed CSFV-specific antibodies as early as 7 days p.chall., indicating a booster effect (Table 1). In order to determine the neutralizing activity of the antibodies induced in the vaccinated animals, NPLA for BDV and CSFV was carried out. Antibodies detected at 22 days p.vacc. in group B neutralized CSF infectivity, and titres increased towards the end of the trial (Fig. 4a). Cross-reactivity for BDV was only found at titres &gt;1:200 for CSFV antibodies (Fig. 4b). Animals 827 and 830 of group A had already developed high titres of BDV-neutralizing antibodies by 22 days p.vacc. (Fig. 4b). The serum of pig 830 also neutralized CSFV at this time point, but not the serum of animal 827 (Fig. 4a). These results indicated that vRiems-ABC-Gif is able to induce a strong immune response. However, the protection upon vaccination was mainly BDV-specific. As expected, none of the unprotected pigs produced detectable amounts of CSFV- or BDV-neutralizing antibodies.

Following immunization and challenge infection, titres of ‘marker antibodies’ were determined by using the two CSFV E2-specific antibody-blocking ELISAs whose mAbs had been applied earlier for in vitro differentiation (Table 1). In the HerdChek CSFV/Ab-blocking ELISA, all pigs were negative before challenge. Seven days p.chall., two of the vRiems-vaccinated animals (832 and 833) scored positive, whereas pig 831 displayed slowly increasing titres slightly below the cut-off level. The two surviving animals of group A also showed CSFV E2-specific antibodies, albeit with a delay of 11 days compared with vRiems-vaccinated animals. This late seroconversion was probably due to the challenge infection. As expected, none of the clinically diseased animals developed anti-E2 antibodies. Analogous results were obtained in the CHEKIT-CSF-sero ELISA, i.e. pig 832 was positive even before challenge (22 days p.vacc.), whereas animal 833 was first positive 4 days p.chall., followed by pig 831 on day 11 p.chall. The vRiems-ABC-Gif-vaccinated animals showed anti-E2 antibodies from 11 days p.chall. onwards. These results were in agreement with the data obtained with the CSFi ELISA.

As the E^{em} epitopes were not affected in the chimeric mutants, the anti-E^{em} immune response was also studied.

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**Fig. 2.** Detection of viral RNA in EDTA blood by TaqMan quantitative RT-PCR. The results are expressed as 50–C_{t} to obtain a positive correlation between C_{t} value and amount of detected RNA. Black bars, vRiems-ABC-Gif; grey bars, vRiems; light-grey bars, mock-vaccinated control. Hatched boxes represent samples that scored positive in virus isolation on SK-6 cell culture.
by detecting E-specific antibodies using the CHEKIT-CSF-marker ELISA. One day before challenge, pigs 830 (group A) and 833 (group B) scored positive, whereas anti-E antibodies were detected in the remaining animals of group B 4 days p.chall., indicating seroconversion due to vaccination for all four pigs. In animal 827, however, E-specific antibodies were detected only from 11 days p.chall. (Table 1). The peak \( A_{405} \) values obtained were comparable among animals 830–833, but the \( A_{405} \) value for animal 827 remained lower.

**Experiment 2: intramuscular immunization of pigs with vRiems-ABC-Gif**

A group of five pigs (1–5) was immunized intramuscularly with vRiems-ABC-Gif. In order to detect shedding of vaccine virus, one sentinel pig was kept together with the vaccinees. Two additional animals (22 and 23) were used as controls for the challenge infection. Twenty-eight days p.vacc., all vaccinated animals and both controls were challenged oronasally with CSFV Koslov.

After vaccination, all pigs remained healthy and none of the animals showed any clinical signs (data not shown). In addition, until 28 days p.vacc., all animals of experiment 2 scored negative in three commercially available CSFV E2-antibody ELISAs (Fig. 5), but four of the five vaccinated animals developed low CSFV-neutralizing antibody titres of 1:5 to 1:10 (Fig. 4c). The sentinel pig remained seronegative.

Following challenge infection, all vRiems-ABC-Gif-vaccinated pigs remained healthy, whereas the mock-vaccinated controls showed severe clinical symptoms and had to be euthanized 9 and 18 days p.chall. Both controls showed fever of up to 41.5 °C (Fig. 3c) and severe leukopenia (Fig. 3d). In contrast, the mean body temperature of the five vaccinated pigs remained below 40 °C, and only a moderate decrease of the mean leukocyte counts could be detected at 4 days p.chall. (Fig. 3c, d). Twenty-one days p.chall., all vaccinated animals scored clearly positive in three different CSFV E2-antibody ELISAs (Fig. 5) and the CSFV-neutralizing antibodies reached titres of 1:906 to 1:7249 (Fig. 4c).

High titres of CSFV strain Koslov were detected in blood leukocytes, organ samples and nasal swabs of both control animals. In contrast, neither viraemia nor virus shedding was found in samples from the vaccinated pigs. Furthermore, no CSFV was isolated from the organ samples taken at 21 days p.chall. (data not shown).
DISCUSSION

In order to differentiate the antibody patterns of vaccinated and infected animals, the use of marker vaccines is of pivotal importance in livestock husbandry. So far, several live chimeric pestiviruses have been constructed where the glycoproteins E2 and Erns were exchanged either partially or entirely between BVDV and CSFV (de Smit et al., 2001; Liang et al., 2003; Reimann et al., 2004; van Gennip et al., 2000, 2002) or between BVDV and BDV (Rasmussen et al., 2007). Pigs vaccinated with these chimeras were protected from challenge infection to a varying degree. However, in these studies, either the animals being protected reliably from challenge were vaccinated repeatedly and by the parenteral route, or the vaccines were based on non-CSFV parent viruses. The use of BVDV or BDV as backbone for constructing chimeric viruses might lead to vaccination failure if the animals had previously been infected with BVDV or BDV. In our study, we present a novel live-attenuated marker-vaccine candidate suitable for oral administration, based on the CSFV Riems vaccine strain. This allows its use both in domestic pigs and in wild boar (as a bait vaccine).

The E2 and E\textsuperscript{ns} genes are divergent among pestiviruses, and antibodies directed against the respective proteins allow the discrimination of the different pestivirus species. As the A and B/C domains of the E2 protein represent the most variable regions within this immunologically important viral protein, we constructed chimeric E2 genes composed of sequences from CSFV Riems and BDV Gifhorn. BDV Gifhorn was chosen (and preferred to BVDV) because its

### Table 1. Serological response after vaccination with vRiems or vRiems-ABC-Gif, followed by challenge with highly virulent CSFV Eystrup

ELISA results: –, negative; ?, doubtful; +, positive; ND, not done.

<table>
<thead>
<tr>
<th>Group (vaccine)</th>
<th>Assay</th>
<th>Animal</th>
<th>Response on indicated day post-challenge</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>-25</td>
<td>-7</td>
</tr>
<tr>
<td>A (vRiems-ABC-Gif)</td>
<td>E2 ELISA (CSFi)</td>
<td>826</td>
<td>-</td>
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<tr>
<td></td>
<td>E2 ELISA (HerdChek)</td>
<td>827</td>
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<tr>
<td>B (vRiems)</td>
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<tr>
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<tr>
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<td>E\textsuperscript{ns} ELISA (CSF-marker)</td>
<td>835</td>
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*No data, as the pigs were killed beforehand.*
E2 sequence is more similar to that of CSFV than to that of BVDV, making it more likely that the resulting chimeric E2 protein will fold in a correct way, yet it can be differentiated from CSFV by using the mAbs provided in the commercial CSFV E2-antibody ELISAs. Therefore, the resulting E2 proteins were expected to induce an antibody response in immunized pigs that is distinguishable from that induced by wild-type CSFV infection, but is fully protective after a single vaccine dose.

vRiems-ABC-Gif and vRiems-BC-Gif, the two CSFV chimeras where domains A and B/C or B/C only were replaced, yielded infectious progeny virus. In determinations of growth kinetics on SK-6 cells, the chimeras showed a shortened lag phase and the peak titres were about 10-fold higher than those of CSFV vRiems. Furthermore, the two chimeras replicated more efficiently on SK-6 cell cultures used for the propagation of their parental strain vRiems than on the ovine cell lines SFT-R or LSM, which is in contrast to earlier findings (Liang et al., 2003). As E2 is supposed to be a determinant of host-cell specificity, our data indicate that the relevant structural elements that are responsible for the host-cell tropism are located on the C-terminal moiety of the protein. This conclusion is in agreement with data published by van Gennip et al. (2000). Passage of the chimeric viruses on SK-6 cells revealed that maximal titres were already reached for vRiems-ABC-Gif after only one passage, which was comparable to the wild type. vRiems-BC-Gif, in contrast, required four passages. Wang et al. (2004) suggested that the formation of E1–E2 heterodimers is a prerequisite for efficient cell entry of the viral particles. This heterodimerization most probably involves the ectodomains of E1 and E2 and/or particular cysteine residues. Thus, it is conceivable that exchange of the almost-complete ectodomain with a closely related domain that has a similar conformation can replace the original function. On the other hand, exchange of a smaller part could interfere with proper folding of the ectodomain (see also data obtained with vRiems-A-Gif).

RNA transcribed from vRiems-A-Gif, the CSFV chimera harbouring BDV Gifhorn E2 domain A only, was replication-competent, but no progeny virus was generated. Furthermore, in contrast to deletion-based VRPs, this construct could not be packaged in cell line SK-6(E2), stably expressing glycoprotein E2 of CSFV Alfort/187.
The orally vRiems-ABC-Gif-vaccinated animals showed an inconsistent degree of protection after challenge infection. Although two pigs (827 and 830) remained clinically healthy, they developed transient leukopenia, although this was less pronounced than in the animals of the mock-immunized group. Unexpectedly, pig 826 came down with severe CSF symptoms and had to be killed 8 days p.chall. The clinical picture of this pig was similar to that of the mock-immunized control group. The virological data were also varying, i.e. pig 826 showed increasing virus load until death, whereas pig 827 was viraemic for 2 days only, and the challenge virus was never detected in the blood of animal 830. The leukopenia and viraemia findings were supported by serological data. At the time point of challenge, pigs 826 and 827 showed no detectable CSFV-neutralizing antibodies, whereas animal 830 scored positive. Remarkably, when compared with CSFV vRiems, both surviving vRiems-ABC-Gif-vaccinated pigs developed high titres of BDV-neutralizing antibodies. This outcome indicated that the prevention of disease in vRiems-ABC-Gif-vaccinated animals was mainly a consequence of cross-neutralization of CSFV challenge virus by the BDV-specific E2 antibodies, and was not due to neutralizing antibodies directed against the remaining parts of E2 or any other CSFV proteins. We conclude that chimera vRiems-ABC-Gif represents a vaccine that is primarily protective against BDV. Furthermore, in this trial, a (low) dose, known to be protective for the conventional C-strain vaccine (Kaden et al., 2006), was used. Our results indicate that this dose might not be high enough for complete protection upon oral vaccination in the case of vRiems-ABC-Gif. This is further supported by a recent additional oral vaccine trial, where vRiems-ABC-Gif led to complete protection of the vaccinated pigs and wild boars when it was used at a 50 times higher dose (unpublished data). Our results are also in agreement with data of others (Maurer et al., 2005; van Gennip et al., 2002), who found that at least antigenic domain A was required for a protective immune response. vRiems-ABC-Gif-vaccinated animal 826, which showed a course of disease similar to that in the mock-vaccinated animals, did not produce neutralizing antibodies at all. This result, together with the partial protection of pig 827 from viraemia, does not allow us to conclude whether the inconsistent results obtained after oral immunization are due to a less efficient immunogenicity of the chimeric E2 protein or to an impaired replication capacity of the chimeric viruses compared with the conventional Riems vaccine strain in the host target cells, requiring a higher vaccine dose to be fully protective.

In contrast to the results obtained after oral immunization, intramuscular vaccination with vRiems-ABC-Gif was highly efficacious. All vaccinated pigs remained healthy after immunization and challenge infection. In this trial, a higher vaccine dose (compared with experiment 1) was chosen in order to determine whether vRiems-ABC-Gif is able to principally induce a fully protective immunity. Four of the five pigs developed CSFV-specific neutralizing-

![Fig. 5. Mean percentage inhibition values obtained with commercial E2-blocking ELISAs after parenteral vaccination and challenge infection. Error bars indicate SD.](http://vir.sgmjournals.org)
antibody titres after vaccination, and neither viraemia nor virus shedding was detected following challenge infection. Interestingly, the animal without a detectable CSFV neutralizing-antibody titre was also protected completely, corroborating the partial protection in the first animal trials, as well as the hypothesis that cross-reactivity and T-cell immunity play an important role in protection against CSFV challenge infection. Furthermore, the second animal trial indicates that, after parenteral application of vRiems-ABC-Gif, replication and immunogenicity were enhanced in comparison to oral administration. A possible explanation could be that, after oral immunization, local infection is a prerequisite for a further replication in the host and, if the in vivo infectivity of virus vRiems-ABC-Gif is reduced, an insufficient immune response might be a possible consequence of oral immunization. Future experiments will show whether the minimal protective dose required for vRiems-ABC-Gif is higher after oral than after intramuscular immunization.

vRiems-ABC-Gif-vaccinated pigs seroconverted for E2-specific antibodies with a distinct lag phase compared with vRiems-vaccinated pigs after challenge infection with CSFV, indicating a booster effect by the challenge virus. Hence, vRiems-ABC-Gif represents a promising candidate for a DIVA vaccine. However, multiple vaccination of animals is required to prove the continued differentiation potential before challenge infection. Moreover, anti-Erns antibodies were detected around the day of challenge in all vRiems-vaccinated pigs, as well as vRiems-ABC-Gif-vaccinated pig 830, indicating that vRiems-ABC-Gif is able to trigger an efficient immune response.

Several vaccination trials have been carried out previously using the molecular clone-derived CSFV strain vEy-37 (Frey et al., 2006; Maurer et al., 2005), which was shown to be as virulent as its parent strain Eystrup (Mayer et al., 2003). However, recent data indicated that Eystrup might not always be as highly virulent (Rasmussen et al., 2007; A. Uttenthal, personal communication). Furthermore, after experiment 1 had been performed, it was decided between all of the project partners performing animal trials to use the same challenge virus stock for all future experiments, in order to allow a more direct comparison of the results obtained by the different partners. Hence, a virus stock was produced by collecting and pooling EDTA blood of three weaner pigs inoculated with CSFV Koslov that were sacrificed 10 days p.i. (V. Kaden, unpublished data). This blood was distributed to the different project partners and was also used for experiment 2 described in the present study.

In order to evaluate the efficacy of the vRiems-ABC-Gif candidate vaccine in a more detailed way, additional animal experiments are currently in progress, using higher titres of vaccine virus or a second (booster) vaccination. Preliminary data indeed indicate that vRiems-ABC-Gif is fully protective upon oral vaccination for domestic pigs, as well as wild boars, when >10^6 TCID_{50} per animal is used (data not shown). Other currently ongoing studies aim at optimizing our first generation of chimeric CSFV/BDV vaccines by minimizing the exchanged parts of E2 in order to improve the immunogenic properties and the replication capacity of the chimeric viruses, yet still maintaining their differentiation capacity.

ACKNOWLEDGEMENTS

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REFERENCES


Chimeric CSFV/BDV live marker vaccines


