Virulence, immunogenicity and vaccine properties of a novel chimeric pestivirus

Thomas Bruun Rasmussen, Åse Utenthal, Jens Nielsen, Klaus Depner and Martin Beer

The genus Pestivirus of the family Flaviviridae comprises Classical swine fever virus (CSFV), Bovine viral diarrhea virus (BVDV) and Border disease virus (BDV). Pestiviruses were considered to be host-specific and were therefore divided into groups differentiating between porcine, bovine and ovine viruses. However, BVDV and BDV have the potential to infect broader host spectra (Nettleton, 1990; Depner et al., 1991; Carlsson & Belak, 1994; Loken, 1995; Becher et al., 2003; Gründahl et al., 2003), whereas pigs are the only natural reservoir of CSFV. Pestiviruses are genetically and structurally related and serological cross-reactivity between all members of the genus can be demonstrated (Schirrmeyer et al., 2004). Non-CSFV pestiviruses, which normally induce a subclinical disease in pigs, can be utilized in marker vaccines against classical swine fever (CSF). Such vaccines can be obtained by construction of modified pestiviruses (Ruggli et al., 1996; Meyers et al., 1996; Moser et al., 1999; Meyer et al., 2002; Reimann et al., 2003, 2004; Liang et al., 2003; Mayer et al., 2003). We report the construction and characterization of a novel chimeric virus with E2 from a non-CSFV pestivirus, which was tested in animals to study its safety and efficacy as a vaccine candidate.

The plasmid pA/CP7_E2gif (Supplementary Fig. S1a, available in JGV Online) was constructed using pA/CP7_AE2PacI (Reimann et al., 2004), which encodes a deletion mutant of BVDV CP7 (Meyers et al., 1996). The BVDV Gifhorn E2 sequence was RT-PCR-amplified using E2-Gif-Pacl (5'-GCAATATTAAACGATTTGCCTGCTCGAGAATT-3') and E2R-Gif-SmaB (5'-GACCTACGAT-GCTGTGATTCCCCATTGCTGCTGCTGCT-3’) and cloned into pA/CP7_AE2PacI. The identity of pA/CP7_E2gif was confirmed by sequencing of the E1–p7 region. After transfection of in vitro-transcribed pA/CP7_E2gif RNA into bovine KOP-R cells, the expression of glycoproteins E<sub>ms</sub> and E2 and non-structural protein NS3 was confirmed in the cytoplasm of transfected cells using immunofluorescence assays (IFA) (Grummer et al., 2001; Reimann et al., 2003). NS3 expression and BVDV E<sub>ms</sub> expression were observed, indicating a BVDV-type pestivirus. Monoclonal antibodies specific for BDV E2 showed high expression of BDV E2 protein (Supplementary Fig. S1b), whereas cells were negative for BVDV E2 expression. Thus, chimeric virus could be differentiated from parental BVDV CP7 by using envelope protein E2 as a simple marker in IFA. Propagation of chimeric virus was not detectable after the first passage on KOP-R cells, although these cells are highly susceptible to BVDV. However, after passage on ovine SFT-R cells, chimeric virus (CP7_E2gif) could be recovered (Supplementary Fig. S1c) and, after three passages, titres of 10<sup>7</sup> TCID<sub>50</sub> ml<sup>-1</sup> were reached. The efficiency of propagation was determined to be 10-fold lower in bovine

A supplementary figure is available in JGV Online.
and porcine cells. Thus, the latter were susceptible to CP7_E2gif, but to a lower degree than ovine cells. In addition, titration in ovine cells revealed a cytopathogenic effect.

The animal experiment was conducted over a 10 week period. Twenty BVDV-negative 5-to-6-week-old Danish Landrace pigs were randomly assigned to four groups (Table 1). Vaccination with CP7_E2gif was performed as outlined in Table 1. Before challenge, 'clones' were separated from 'contacts' to prevent transmission of inoculum. CSFV Eystrup (10⁵ TCID₅₀ per pig) was deposited orally onto the tonsils of 'clones' and 'challenge controls'. After 1 day, 'clones' were moved back to the pen with the 'contacts'. All animals were monitored daily for their general health status and clinical signs of disease, including examination of skin for discoloration or petechial bleedings. Rectal temperatures were measured daily. Vaccinated pigs did not display any clinical signs of disease or any increase of body temperature in response to CP7_E2gif (Fig. 1c). In 'challenge controls', the first clinical signs of disease were observed on post-infection day (PID) 3, when three out of five pigs showed increased body temperatures (Fig. 1d). A marked increase in body temperature (mean of 41°C) was observed 4 days after challenge in all five pigs. Fever was transient and body temperatures declined from PID 10 on, except for pig 18, which had elevated body temperature up to PID 13. Clinical signs of disease, including lethargy, diarrhoea, unsteady gait, seizures and apathy, were observed from PID 7–10. Three of the vaccinated pigs responded to the challenge infection by a slight transient increase in body temperature (Fig. 1c). In addition, two pigs were lethargic with reduced appetite on PID 4, only. Among the sentinel 'contacts' as well as in 'negative controls' none of the animals displayed any signs of disease, including changes in body temperature (Fig. 1a, b).

Quantitative RT-PCR targeting the 5′-NTR region was used for detection of viral RNA from CP7_E2gif or CSFV Eystrup. Primers CSFV6 and CSFV7 (Uttenthal et al., 2003) and a CSFV-Taqman probe (5′-FAM-TTGGATGGCTGAAGCCCTGAGTAC-TAMRA-3′) were used for detection of CSFV. For CP7_E2gif, CSFV6 and CSFV7 were used in combination with a BVDV-Taqman probe (5′-FAM-TTGGATGGCTGAAGCCCTGAGTAC-TAMRA-3′). Extraction of nucleic acids was performed as described previously (Uttenthal et al., 2003). Five microlitres of RNA was used as template for the Quantitect Probe RT-PCR kit (Qiagen) in 25 μl reactions containing primers (500 nM each) and Taqman probe (400 nM). Reactions were cycled in a MX4000 (Stratagene) using 50 °C for 30 min, 95 °C for 15 min, 55 cycles of 94 °C for 15 s and 60 °C for 60 s. Fluorescence data were obtained to assign a cycle threshold value (Cₜ) to each sample. The relative quantity of viral RNA (the viral load) in each sample was expressed as 50 minus Cₜ without recalculating to genome equivalents. Serum samples and nasal swabs, obtained during the first two weeks after vaccination, were tested for the presence of chimeric RNA; all samples were negative. Furthermore, attempts to isolate CP7_E2gif on SFT-R cells from serum were negative. Serum was tested for the presence of CSFV RNA after challenge infection as a measure of viraemia. Relative quantification of CSFV RNA in 'challenge controls' showed viral RNA in serum from PID 3–10 (Fig. 2a). Virus isolation was performed to confirm the presence of infectious CSFV in serum and positive isolation of CSFV was restricted to PID 4–7 only. Thus, viraemia measured by quantitative RT-PCR was detectable 1 day earlier and 3 days after recovery of infectious virus. Furthermore, the viremic period coincided with the fever observed in 'challenge controls' (Figs 2a, 1d). Shedding of virus was investigated by testing nasal swabs for CSFV RNA. Two 'challenge controls' were positive on PID 3 and three animals were positive from PID 7. Subsequently, shedding of CSFV declined and CSFV RNA was not detectable from PID 14 on. CSFV RNA was also detected in serum after challenge in 'clones' (Fig. 2b). However, the viral loads were approximately 12 Cₜ values lower, indicating a 1000-fold lower viraemia in these pigs compared to 'challenge controls' (Fig. 2a, b). No virus was isolated from the serum of 'clones'. In addition, only two of the 'clones' (pig 12 and pig 13) tested positive for CSFV RNA in nasal swabs on PID 10 and 7, respectively. Two 'challenge controls' were killed on PID 9 and 15 to analyse for viral load of CSFV in various tissues. CSFV RNA was detected in tonsils and lymphoid tissue from pig 19 (killed on PID 9), with the highest viral load in the tonsils. In pig 18.

<table>
<thead>
<tr>
<th>Experimental groups</th>
<th>Pig no.</th>
<th>Vaccination* (PID – 28)</th>
<th>Challenge† (PID 0)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative controls</td>
<td>1–5</td>
<td>Eagle’s</td>
<td>Eagle’s</td>
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<tr>
<td>Contacts</td>
<td>6–10</td>
<td>Eagle’s</td>
<td>Eagle’s</td>
</tr>
<tr>
<td>Clones</td>
<td>11–15</td>
<td>CP7_E2gif</td>
<td>CSFV Eystrup</td>
</tr>
<tr>
<td>Challenge controls</td>
<td>16–20</td>
<td>Eagle’s</td>
<td>CSFV Eystrup</td>
</tr>
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*Intramuscular injection with either CP7_E2gif (10⁷.15 TCID₅₀ per pig) or Eagle’s medium.
†Challenge infection with either CSFV Eystrup (10⁵ TCID₅₀ per pig) or mock challenge with Eagle’s medium.
Fig. 1. Body temperatures of (a) 'negative controls', (b) 'contacts', (c) 'clones' and (d) 'challenge controls'. The dotted line indicates fever, defined as body temperature above 40 °C.

Fig. 2. (a, b) Detection of CSFV RNA in serum from 'challenge controls' (pigs 16–20) and 'clones' (pigs 11–15) after challenge infection. Viral loads in serum are displayed as arbitrary units, defined as 50 minus the observed Cₜ value. (c, d) Neutralizing antibodies in serum from 'clones' and 'challenge controls'. Pigs 18 and 19 were killed on PID 15 and 9, respectively.
(killed on PID 15), only the tonsils displayed detectable levels of viral RNA. CSFV was isolated from lymphoid tissues and tonsils from pig 19, whereas all tissues analysed from pig 18 were negative for virus isolation. CSFV was not detected in the tonsils of any other pigs, either by quantitative RT-PCR or virus isolation. Attempts to detect CSFV in other organs were not carried out, since tonsils are considered to be the most suitable tissue for CSFV isolation (Anonymous, 2002).

Virus neutralization test was performed on serum from all pigs following vaccination and challenge. The vaccinated ‘clones’ developed neutralizing antibody titres 10–14 days after vaccination. Following the challenge, a 10-fold increase in antibody titres was observed, which remained constant until the end of the experiment (Fig. 2c). Induction of neutralizing antibodies was not observed in ‘contacts’ or ‘negative controls’. After PID 14, three of the ‘challenge controls’ became antibody-positive. Serum samples were similarly tested for the presence of CSFV neutralizing antibodies (Fig. 2d). On PID 7, seroconversion was observed in all ‘clones’. In ‘challenge controls’, seroconversion was observed 3 days later. Antibody titres against CSFV were not observed in ‘contacts’ or ‘negative controls’. The onset of CSFV neutralizing antibodies in ‘clones’ and ‘challenge controls’ coincided with observed clearance of virus in both groups.

In this study, a novel chimeric clone was constructed. This infectious chimeric clone resembles a clone containing E2 from CSFV Alfort (Reimann et al., 2004), with the exception that the E2 gene is derived from BDV Gifhorn. This pestivirus was isolated from pigs, multiplies in these animals without causing clinical symptoms or immunosuppression and induces a strong antibody response (Schirrmeyer et al., 2002; Depner & Schirrmeyer, 2002; Uttenthal et al., 2004). The nucleotide sequence of the BDV Gifhorn genome differs considerably from those of the classical BDV isolates and is classified between the genetic groups of the typical BDVs and CSFV (Schirrmeyer et al., 2002; Becher et al., 2003). Therefore, the BDV Gifhorn strain was considered to constitute a good candidate for the construction of a chimeric virus that would be able to infect and replicate in pigs. The chimeric virus was rescued from ovine cells and could easily be differentiated from the parent BVDV clone by using the structural E2 protein as a marker in IFA and by RT-PCR using primers for the BDV and BVDV parts. CP7_E2gif showed a cell tropism indicating that introduction of the BDV Gifhorn E2 changed the cell preference from bovine to ovine cells. However, bovine and porcine cells were also susceptible to CP7_E2gif, but to a markedly lower degree.

We investigated the properties of CP7_E2gif in animals. Safety and efficacy of the chimeric virus as a live vaccine were studied. CP7_E2gif proved to be avirulent after vaccination with a high dose (> 10⁷ TCID₅₀ per pig). By analysing sequential samples of serum and nasal swabs, a detailed picture of viral load and virus shedding was obtained from challenged animals. CP7_E2gif was not detected at any time point in serum or nasal swabs of the vaccinated animals by quantitative RT-PCR or by virus isolation, indicating that CP7_E2gif has a low potential for causing viraemia. These results were consistent with the inability to isolate viruses from pigs vaccinated with BVDV containing the E2 gene from CSFV Alfort in an analogous study (Reimann et al., 2004). This BVDV/CSFV virus displayed growth characteristics comparable to those of wild-type CSFV Alfort. The E2 glycoprotein carries epitopes for virus receptors (Hulst & Moormann, 1997) and accordingly the E2 of CSFV Alfort would have been expected to be more permissive for infection in pigs than the E2 of BDV Gifhorn. However, CP7_E2gif did replicate in porcine cells, indicating that these can be infected, although the capacity to spread in pigs is obviously strongly reduced. These results are also consistent with the finding that the parent viruses, BVDV CP7 and BDV Gifhorn, can multiply in experimentally infected pigs (Reimann et al., 2004; Uttenthal et al., 2004). In addition, CP7_E2gif was capable of inducing a humoral immune response, since detectable neutralizing antibodies were induced in all ‘clones’ after vaccination (Fig. 2c, d). The sentinel ‘contacts’ remained uninfected, although these pigs were housed together with the ‘clones’. This finding substantiates the low probability of transmission of CP7_E2gif from vaccinated pigs to other animals.

CSFV strain Eystrup is a highly virulent strain (Mittelholzer et al., 2000; Mayer et al., 2003), which induces severe acute disease with high fever and mortality. In this study, the Eystrup strain was shown to be of moderate virulence, although distinct clinical signs of disease were observed in ‘challenge controls’. However, severe CSF symptoms, such as skin haemorrhages or profound neurological signs and increased mortality, were not observed. The signs of CSFV infection in vaccinated pigs were much weaker than those observed in the ‘challenge controls’. This observation, together with the fact that CSFV was not isolated from the vaccinated animals, shows that vaccination with CP7_E2gif severely limited the multiplication of CSFV during subsequent infection and caused a considerably attenuated course of disease.

The chimeric virus used in this study was tested as a live vaccine candidate. Accordingly, the safety of the vaccine requires strong attention. A vaccine should not itself cause disease or damage in the animal and transmission of vaccine virus from vaccinated animals to other animals should be limited or completely prevented. The results presented in this work demonstrate that CP7_E2gif is avirulent in pigs and does not, under our experimental conditions, affect the pigs. Furthermore, the results show that horizontal transmission of CP7_E2gif to sentinels did not occur. Other possible transmission routes, such as vertical transmission from pregnant sows to fetuses, need to be addressed in additional experiments. Also, possible spread of CP7_E2gif to and between ovine or bovine species remains to be investigated. Another safety issue associated
with live vaccines is that they may revert to virulence during multiplication in animals. Our CP7_E2gif is derived from two non-CSF pestiviruses that are capable of infecting pigs, but without causing clinical signs (Reimann et al., 2004; Uttenthal et al., 2004). Since neither of the parent viruses are virulent in pigs, reversion of CP7_E2gif to a virulent virus is considered to be highly unlikely. However, consecutive animal passages are needed to elucidate this.

An important advantage of the use of chimeric viruses as live vaccines resides in their capacity to be manipulated to achieve the characteristics desired for safe and efficacious marker-vaccines. For marker-vaccine development, the ability of the non-CSF pestiviruses to infect pigs can be advantageous for inducing an efficient immune response against CSF. Furthermore, commercial assays for the detection of CSFV E2-specific antibodies normally do not cross-react with BDV E2-specific antibodies. Therefore, these highly sensitive and CSFV-specific assays could be used as part of a DIVA (Differentiating Infected from Vaccinated Animals) strategy together with CP7_E2gif.

In conclusion, the present study showed that pigs vaccinated with CP7_E2gif developed protective immunity against challenge infection with CSFV Eystrup. However, the challenge virus was of moderate virulence and therefore it remains to be shown that vaccination with the chimeric virus can protect pigs against lethal infections with highly pathogenic strains of CSFV.

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References


