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An avirulent chimeric *Pestivirus* with altered cell tropism protects pigs against lethal infection with classical swine fever virus

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Abstract

A chimeric Pestivirus was constructed using an infectious cDNA clone of bovine viral diarrhea virus (BVDV) [J. Virol. 70 (1996) 8606]. After deletion of the envelope protein E2-encoding region, the respective sequence of classical swine fever virus (CSFV) strain Alfort 187 was inserted in-frame resulting in plasmid pA/CP7_E2alf. After transfection of in vitro-transcribed CP7_E2alf RNA, autonomous replication of chimeric RNA in bovine and porcine cell cultures was observed. Efficient growth of chimeric CP7_E2alf virus, however, could only be demonstrated on porcine cells, and in contrast to the parental BVDV strain CP7, CP7_E2alf only inefficiently infected and propagated in bovine cells. The virulence, immunogenicity, and "marker vaccine" properties of the generated chimeric CP7_E2alf virus were determined in an animal experiment using 27 pigs. After intramuscular inoculation of 1×10^7 $TCID_{50}$, CP7_E2alf proved to be completely avirulent, and neither viremia nor virus transmission to contact animals was observed; however, CSFV-specific neutralizing antibodies were detected from day 11 after inoculation. In addition, sera from all animals reacted positive in an E2-specific CSFV-antibody ELISA, but were negative for CSFV-E^{RNS}-specific antibodies as determined with a CSFV marker ELISA. After challenge infection with highly virulent CSFV strain Eystrup, pigs immunized with CP7_E2alf were fully protected against clinical signs of CSFV infection, viremia, and shedding of challenge virus, and almost all animals scored positive in a CSFV marker ELISA. From our results, we conclude that chimeric CP7_E2alf may not only serve as a tool for a better understanding of *Pestivirus* attachment, entry, and assembly, but also represents an innocuous and efficacious modified live CSFV "marker vaccine".

Keywords: Chimeric Pestivirus; Cell tropism; Classical swine fever; Envelope protein E2; Differentiating infected from vaccinated animals (DIVA) vaccine

Introduction

Classical swine fever (CSF) is listed within the highly contagious OIE list A diseases of the *Suidae* (Anonymous, 1996), and infection of domestic pigs as well as wild boar (*Sus scrofa*) is common. CSF is responsible for major economic losses especially in countries with an industrialized pig production (Artois et al., 2002; Edwards et al., 2000; Vandeputte and Chappuis, 1999). It is caused by an enveloped RNA virus that belongs to the genus *Pestivirus* of the *Flaviviridae* family (van Regenmortel et al., 2000). The virus is closely related to the ruminant *Pestiviruses* causing bovine viral diarrhea (BVD) and border disease (BD). Diseased animals show high fever and anorexia, and in late stage, central nervous disorders. Necroscopies often reveal hemorrhages in the mucosa and inner organs of diseased animals.

The *Pestivirus* genome consists of a single-stranded RNA of positive orientation. The RNA is approximately 12.3 kb long and encodes for one large open reading frame (ORF), which is flanked by nontranslated regions (NTR) at both ends of the genome (Collett, 1987; Rümenapf et al., 1991). The *Pestivirus* ORF is translated into one polyprotein, which is co- and posttranslationally processed into 11 or 12 mature proteins by viral and cellular proteases. The capsid protein C and the glycoproteins E^{RNS}, E1, and E2 are structural components of the virion (Rümenapf et al., 1991).

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Envelope protein E2 forms a heterodimer with E1 as well as homodimers (Weiland et al., 1990), and it may play a major role in virus attachment and entry (Donis, 1995). *Pestivirus* antibodies are directed against E^{RNS}, E2, the nonstructural protein NS3, and possibly envelope protein E1 (Donis and Dubovi, 1987; Muyldermans et al., 1993). Neutralizing activity was predominantly demonstrated for E2-specific antibodies (Donis et al., 1988), but has also been discussed for those directed against E1 and E^{RNS} (Donis, 1995; Weiland et al., 1992). On the basis of amino acid sequence analysis and determination of the N-termini, it was shown that processing of E2 is mediated by a host cell signalase. Typical hydrophobic signal sequences were identified upstream of the E2 N-terminus within E1-coding sequences, and a transmembrane anchor of about 40 hydrophobic amino acids was demonstrated at the C-terminus of E2 (Rümenapf et al., 1993).

The different *Pestivirus* species infect cells of ruminant or porcine origin. However, the highest susceptibility and the most efficient replication are observed in cells derived from the homologous animal species (Ferrari, 1985; Mengeling and Drake, 1969; Nettleton et al., 1998). Additionally, in contrast to bovine viral diarrhea virus (BVDV) and border disease virus (BVD), natural classical swine fever virus (CSFV) infections are restricted to pigs (Liess and Moennig, 1990). Nevertheless, knowledge concerning the tropism of *Pestiviruses* in vitro or in vivo is only fragmentary.

Because of the risk of CSFV transmission from the wild boar population to domestic pigs (Artois et al., 2002) and the high costs of the large scale culling associated with such outbreaks (Vandeputte and Chappuis, 1999), the development of new vaccines that allow a fast protection combined with the possibility to differentiate infected from vaccinated animals is becoming increasingly important. Furthermore, deliberate CSFV infection as an act of bioterrorism in CSFV-free countries has to be taken into consideration. Traditionally, for vaccination against CSFV infection, safe and efficacious modified live vaccines using highly attenuated CSFV strains (e.g., C-strain) have been used (Terpstra and Robijns, 1977; Terpstra and Tielen, 1976). Nevertheless, following vaccination with the conventional modified live vaccines, a serological distinction between vaccinated animals and those reconvalescent from natural infection is not possible. So-called marker or differentiating infected from vaccinated animals (DIVA) vaccines in combination with discriminating antibody assays allows differentiation of infected animals and vaccinees (van Oirschot, 1999), and recently, subunit marker vaccines based on baculovirus-expressed CSFV E2 and some discriminating ELISA tests based on the detection of E^{RNS} antibodies have been developed and used for the assessment of vaccine efficacy and DIVA properties (de Smit, 2000; Hulst et al., 1993). These studies revealed that the immune responses induced by this particular subunit vaccine were considerably less efficient compared with conventional live attenuated vaccines (Depner et al., 2001; Uttenthal et al., 2001).

Recent studies on new CSF vaccines have been facilitated by the availability of reverse genetic systems (Meyers et al., 1996b; Moormann et al., 1996; Rüggli et al., 1996) that allow the directed manipulation of *Pestivirus* genomes. Meyers et al. (1999) demonstrated that abrogating the RNase activity in E^{RNS} of CSFV led to attenuation, and pigs immunized using the attenuated E^{RNS} mutants were fully protected from lethal challenge with virulent CSFV strain Eystrup. Recently, chimeric *Pestiviruses* based on an infectious DNA copy of the CSFV vaccine strain C (van

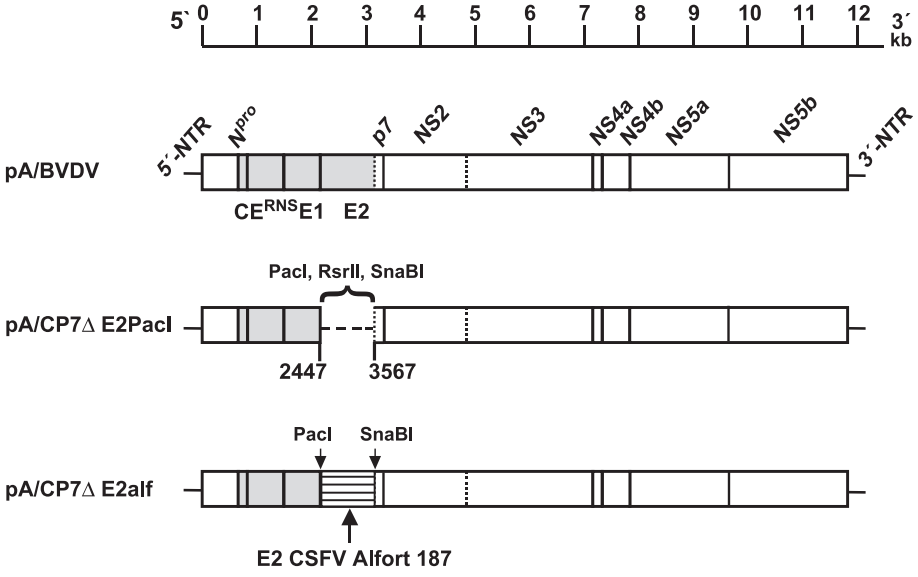

Fig. 1. Schematic representation of the engineered constructs. Filled boxes represent the BVDV structural protein region. Horizontal dotted lines show the deleted regions and numbers indicate the nucleotide position in the BVDV full-length genome. Arrows indicate restriction enzyme sites flanking the deletions. Lines at the left and right ends indicate untranslated regions. N^{pro}, autoprotease; C, capsid protein; E^{RNS}, E1, and E2, envelope proteins; p7, nonstructural protein; NS2 to NS5, nonstructural proteins; 3′-NTR and 5′-NTR, noncoding regions. The scale is given in kb.

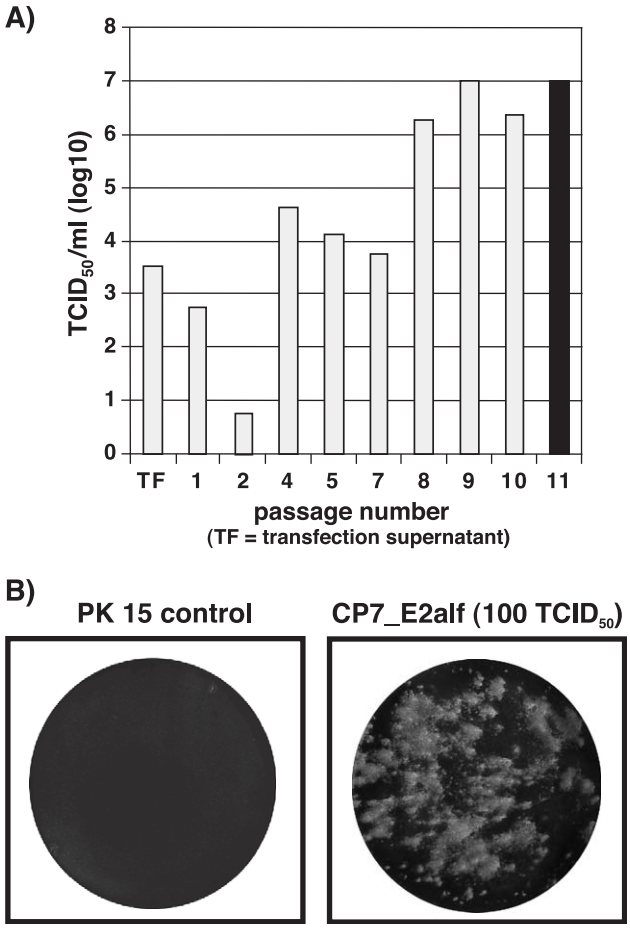

Fig. 2. (A) Passage history of chimeric Pestivirus CP7_E2alf using PK15 cells and whole freeze/thaw lysates. Endpoint titers are given as $TCID_{50}$/ml. First titers are from transfection supernatant (TF) collected at 24 h post-transfection. (B) Crystal violet staining of PK15 cells at 96 h after infection with 100 $TCID_{50}$ CP7_E2alf. A cytopathic effect with virus plaques was clearly visible in comparison to the uninfected control cell culture.

Gennip et al., 2000) were constructed. Replacement of the antigenic region of E2 or of the complete E^{RNS} gene of CSFV strain C by the analogous sequences of BVDV type II strain 5250 resulted in viable chimeric viruses, which proved to be valuable tools for *Pestivirus* characterization and could be used as a new type of genetically engineered vaccine (de Smit et al., 2001).

Here we report on the construction of a chimeric *Pestivirus*, which is based on an infectious BVDV cDNA clone (Meyers et al., 1996a), and expresses E2 from CSFV strain Alfort 187 (Rüggli et al., 1996) instead of BVDV E2. The resulting chimeric virus CP7_E2alf was characterized in vitro and in vivo. It could be demonstrated that CP7_E2alf may not only serve as a tool for a better understanding of *Pestivirus* infectivity and host range, but also represent an innocuous and efficacious modified live CSFV "marker vaccine". In comparison to other chimeric *Pestiviruses*, our BVDV/CSFV chimera CP7_E2alf exhibits unique features, especially regarding replication and cell tropism in vitro and in vivo.

Results

Cloning and recovery of the chimeric CP7_E2alf virus

A chimeric BVDV/CSFV full-length clone was constructed, in which the entire E2-coding region of cytopathogenic BVDV strain CP7 was replaced by that of CSFV strain Alfort 187 (Fig. 1). RNA transcribed from either pA/CP7_ΔE2p7 or pA/CP7_ΔE2PacI (Fig. 1) was sufficient to replicate after transfection in both KOP-R and PK15 cells (data not shown). For recovery of chimeric *CP7_E2alf* virus, RNA transcribed from the plasmid pA/CP7_E2alf was transfected into KOP-R or PK15 cells. At 24 h after transfection, CP7_E2alf virus progeny could be recovered from transfection supernatants of KOP-R and PK15 cells. RNA replication and translation of NS3, E2, and E^{RNS} were observed in both cell types (data not shown). The specific infectivity of in vitro-transcribed RNA was determined after transfection of 1×10^7 PK15, MDBK, or KOP-R cells with 1 μg RNA. On average, at 24 h post-transfection, RNA transcribed in vitro from CP7_E2alf yielded $10^{2.65}$ $TCID_{50}$/μg in PK15 cells, $10^{1.375}$ $TCID_{50}$/μg in MDBK cells, and $10^{1.75}$ $TCID_{50}$/μg in KOP-R cells. At 12 h post-transfection, we observed 2.5×10^5 fluorescing PK15 cells/μg, 0.5×10^5 fluorescing MDBK cells/μg, and 1.25×10^5 fluorescing KOP-R cells/μg. However, efficient propagation of CP7_E2alf was restricted to PK15 cells, and CP7_E2alf was serially passaged on PK15 cells using lysates obtained after freeze–thawing. Virus titers of every PK15 passage were determined by end point titrations. During the first three passages, the titer of CP7_E2alf declined from about $10^{3.5}$ $TCID_{50}$/ml to $<10^1$ $TCID_{50}$/ml, but increased between passages 4 and 8 to more than 1×10^6 $TCID_{50}$/ml. After eight passages, stable titers of approximately 10^7 $TCID_{50}$/ml could be detected (Fig. 2a). The 11th passage of

Table 1
Amino acid exchanges between $CP7\_E2alf^{1st\ passage}$ and $CP7\_E2alf^{11th\ passage}$

Genome region[a]	Amino acid position	Residue change	Number of clones/total[b]
E^{RNS}	450[c]	H→R	2/8
	479[c,d]	G→R	19/20
E1	686[c]	L→H	9/16
E2	783[e]	M→T	1/10
	973[e]	S→P	7/14
	1023[e]	R→H	2/14
	1035[e]	V→E	2/14

[a] No amino acid exchanges could be detected for the capsid encoding region.
[b] Eight to twenty clones of minimum of two different RT-PCR-products were sequenced using the M13 forward and reverse primers.
[c] Amino acid position numbered according to BVDV CP7 (GenBank accession number , BVU63479).
[d] The exchange at amino acid position 479 could be confirmed in a consensus sequence using direct sequencing.
[e] Amino acid position numbered according to CSFV Alfort 187 (GenBank accession number , X87939).

CP7_E2alf (CP7_E2alf^{11th}) was used for the in vitro characterization and for the immunization experiments. Infection of PK15 cells with CP7_E2alf resulted in a cytopathogenic effect, which developed at 4–6 days after inoculation, dependent on the amount of virus used for infection (Fig. 2b).

Transfection experiments with in vitro-transcribed CP7_E2alf RNA performed with PK15 cells at 35, 37, and 39 °C did not reveal significant differences in the obtained virus titers (data not shown).

Sequence comparison of the 1st and the 11th passage of CP7_E2alf

To determine whether sequence changes occurred in the region encoding the structural proteins (capsid, E^{RNS},

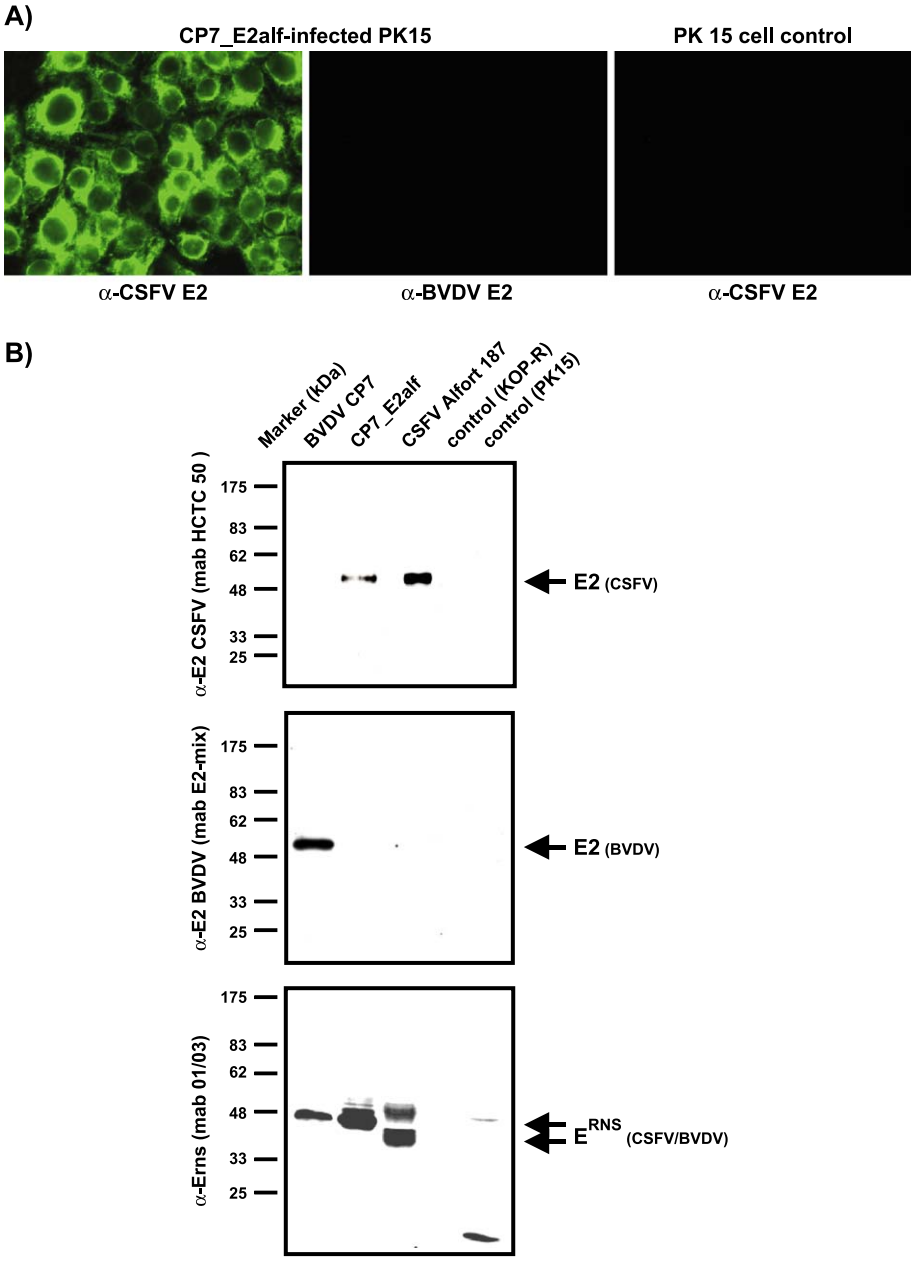

Fig. 3. (A) IF analysis of PK15 cells infected with CP7_E2alf. The cells were stained using CSFV E2 (HC34) and BVDV E2 (CA3)-specific mab. The infected cell culture was IF-positive for the donor CSFV-E2, but negative for parental BVDV E2. Control cells remained negative. (B) Western blot analysis of infected KOP-R and PK15 cells using BVDV-E2 (CA3/WB215) and CSFV-E2 (HC/TC 50)-specific mab as well as the panpesti anti-E^{RNS} mab 01–03. Lysates of KOP-R and PK15 cells were subjected to SDS-Page (12% acrylamide) and subsequent protein transfer to a nitrocellulose membrane. CSFV E2 of the expected size could be detected in lysates of CP7_E2alf and CSFV Alfort 187-infected cell cultures. BVDV E2 was exclusively detected in lysates of BVDV CP7-infected KOP cells. E^{RNS} protein could be detected in all infected cell lysates; however, there was an identical single protein band for CP7_E2alf and BVDV CP7, which were different from the band pattern detected in the CSFV Alfort 187 lysate. Control KOP-R and PK15 cell lysates were negative for E2 or E^{RNS} proteins. Sizes are indicated in kilodaltons.

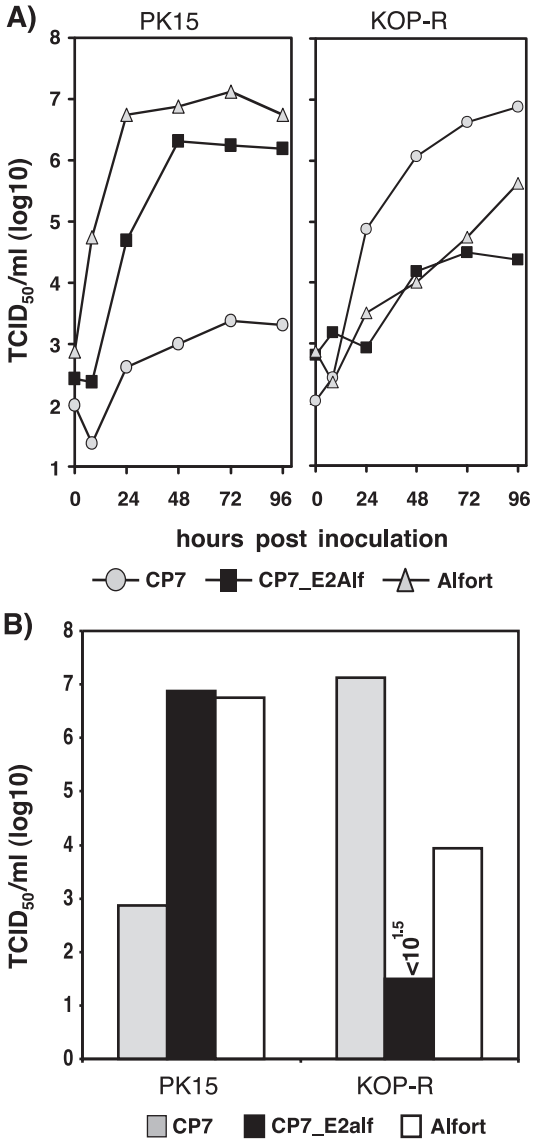

Fig. 4. (A) Multistep growth curves of chimeric CP7_E2alf, parental BVDV CP7, and donor virus Alfort 187 in KOP-R and PK15 cells. Confluent monolayers of PK15 or KOP-R cells were infected at a multiplicity of infection (m.o.i.) of 1 and total freeze/thaw lysates were titrated at 0, 8, 24, 48, 72, and 96 h after inoculation. Virus titers are given as $TCID_{50}$/ml in log 10 steps. (B) Plating efficiency of CP7_E2alf (11th passage on PK15 cells), CP7, and Alfort 187 using porcine (PK15) and bovine (KOP-R) cell cultures. Virus stocks were titrated in log 10 dilutions on confluent monolayers of PK15 and KOP-R cells. Plating efficiency of the different cell types was calculated as $TCID_{50}$/ml. There was no CP7_E2alf detectable after inoculation of KOP-R cells with 0.1 ml of a 1:10 diluted virus suspension (plating efficiency $< 10^{1.5}$ $TCID_{50}$/ml). In contrast, a high plating efficiency could be determined for CP7_E2alf after inoculation of PK15 cells ($10^{6.875}$ $TCID_{50}$/ml).

E1, and E2) of CP7_E2alf during serial passage on PK15 cells, the entire region encoding the structural proteins of CP7_E2alf of the 1st and 11th passage was amplified by using RT-PCR, and cloned into the pGEM-T Easy Vector System (Promega). Eight to twenty different clones from a minimum of two independent RT-PCRs were selected and sequenced (Table 1). In 19 of 20 clones from CP7_E2alf$^{11th\ passage}$, an exchange at amino acid position 479 (numbered according to BVDV CP7), where a glycine was changed into an arginine, could be detected (Table 1). Other amino acid changes in the E^{RNS}, E1, or E2 encoding regions of the genome were detected in only 10–55% of the sequenced plasmid clones (Table 1). No amino acid changes could be detected in the capsid encoding region (Table 1). The 479 Gly to Arg exchange is of special interest because of the fact that amino acid position 479 of CP7 and CP7_E2alf corresponds to amino acid position 476 of CSFV, which was demonstrated to play a key role in

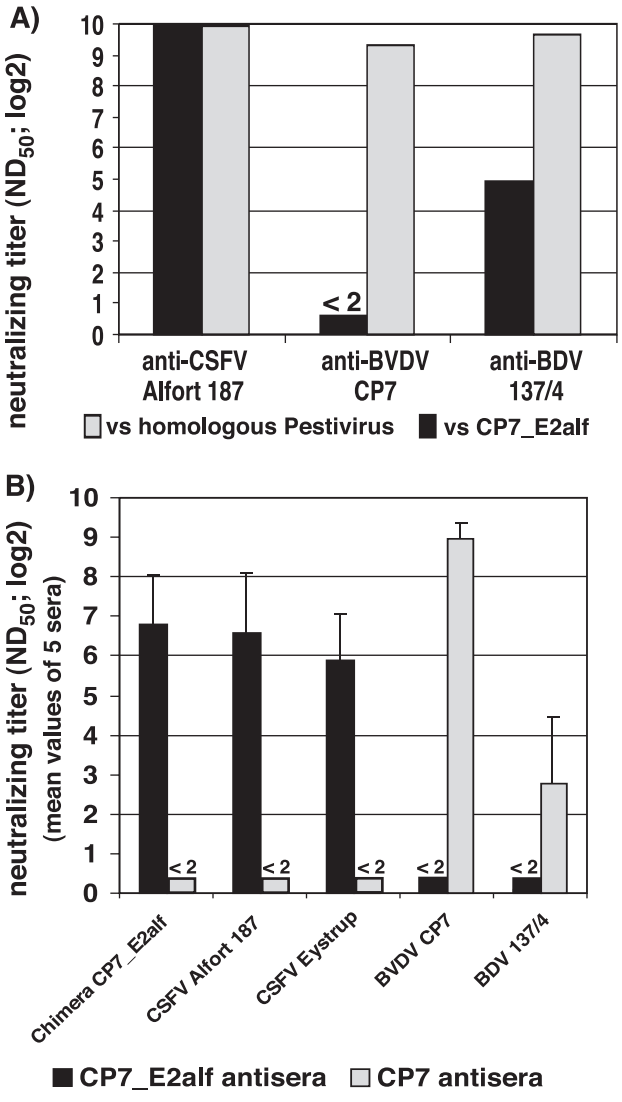

Fig. 5. (A) Cross-neutralization of 100 $TCID_{50}$ CP7_E2alf versus porcine antisera directed against CSFV strain Alfort 187, BVDV strain CP7, and BDV strain 137/4. A high neutralizing antibody titer of 1:960 could be detected for the anti-CSFV serum only. (B) Cross-neutralization of different Pestiviruses in comparison to chimeric CP7_E2alf using CP7_E2alf ($n = 5$) and CP7 ($n = 5$)-specific pig sera. CP7_E2alf-specific antisera exclusively neutralized CSF viruses and the BVDV/CSFV chimera. In contrast, neutralizing antibody titers against BVDV CP7 and to a lower extent to BDV 137/4 were detectable with CP7-specific pig sera only.

the interaction with membrane-associated heparan sulfate (Hulst et al., 2000). Using direct sequencing of the different RT-PCR products with specific primers, only the 479 Gly to Arg exchange was detected in the consensus sequence.

Characterization of the chimeric CP7_E2alf virus

The chimeric CP7_E2alf virus could be differentiated from the parental BVDV strain CP7 by using the E2 protein as a marker in IF and Western blotting (Fig. 3). Combining the data of IF staining and Western blot analyses, the chimeric CP7_E2alf virus displayed the expected phenotype, which was different from the parental BVDV strain CP7 and the donor CSFV strain Alfort 187. Proteins of the chimeric virus reacted with the CSFV E2-specific mab in both IF and Western blots, but not with the BVDV E2 mab (Figs. 3a, b). In addition, the Western blot analyses of CP7_E2alf resulted in an E2 protein that was indistinguishable in size from the E2 of CSFV Alfort 187. The E^{RNS} proteins of CP7 and CP7_E2alf had an identical molecular weight of approximately 48 kDa, although CSFV Alfort 187 E^{RNS} showed the characteristic doublet pattern (Rümenapf et al., 1991) (Fig. 3b). Furthermore, by using Western blot analyses under nonreducing conditions, E2-homodimers as well as chimeric E1–E2 heterodimers could be detected for CP7_E2alf (data not shown).

Growth kinetics of the chimeric virus and both the donor CSFV Alfort 187 and the parental BVDV CP7 were performed in bovine KOP-R and porcine PK15 cells (Fig. 4a). The multistep growth curve of Alfort 187 and CP7_E2alf indicated a similar growth of both viruses in PK15 cells with virus titers of more than 10^6 $TCID_{50}$/ml at 96 h post-infection (p.i.). However, a reduced growth rate with approximately threefold less maximum virus titers at 96 h p.i. was determined for CP7_E2alf compared to Alfort 187 in PK15 cells. In the same experiment, BVDV strain CP7 was shown to replicate very inefficiently in PK15 cells, and reached about 1000-fold less maximum end point titers compared to CSFV Alfort 187 and CP7_E2alf (Fig. 4a). Vice versa, CP7 reached titers of more than 10^6 $TCID_{50}$/ml on bovine KOP-R cells at 96 h p.i., although CP7_E2alf did not replicate efficiently in KOP-R cells yielding more than 300-fold reduced end point titers (Fig. 4a). Replication of CSFV Alfort 187 in KOP-R cells was marked delayed and reduced compared to CP7; nevertheless, end point titers at 96 h p.i. were almost 20-fold higher than those of CP7_E2alf. From these experiments, we concluded that porcine PK15 cells supported efficient growth of chimeric CP7_E2alf, but not of parental CP7 (Fig. 4a).

In addition, monolayers of KOP-R or PK15 cells were infected with different dilutions of CP7_E2alf or CP7 to determine the plating efficiency of both viruses on these different cell types (Fig. 4b). It was demonstrated that CP7_E2alf could easily infect PK15 cells (plating efficiency: $10^{6.875}$ $TCID_{50}$/ml), although only single KOP-R cells were infected even with the 1:10-diluted virus suspension (plating efficiency: $<10^{1.5}$). Titration of CP7 resulted in a more than 10 000-fold reduced plating effi-

Table 2
Virus isolation from blood leukocytes following immunization or challenge infection

	Animal	Days post immunization (dpi)							Days post challenge infection (dpc)							
		0	2	4	7	9	11	14	0	2	4	7	9	11	14	28
		0	2	4	7	9	11	14	28	30	32	35	37	39	42	56
CP7_E2alf	2181	0[a]	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	2182	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	2183	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	2184	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	2185	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
CP7	2191	0	0	0	0	0	0	0	0	0	2[b]	0	0	0	0	0
	2192	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0
	2193	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0
	2194	0	0	2[c]	0	0	0	0	0	1	2	1	0	0	0	0
	2195	0	0	1	0	0	0	0	0	2	2	0	0	0	0	0
	2196	-	-	-	-	-	-	-	0	1	2	2	2	2	‡[d]	-
controls	2197	-	-	-	-	-	-	-	0	1	2	2	2	0	0	0
	2198	-	-	-	-	-	-	-	0	2	2	2	2	2	‡	-
	2199	-	-	-	-	-	-	-	0	0	2	2	0	0	0	0
	2200	-	-	-	-	-	-	-	0	0	2	2	2	0	0	0
	2201	-	-	-	-	-	-	-	0	1	2	2	2	0	0	0
	2202	-	-	-	-	-	-	-	0	0	2	2	2	‡	-	-

[a]Number of positive inoculations (1×10^6 leukocytes per inoculation, two inoculations per sample; no positive samples = white, one positive sample = light gray, two positive samples = dark gray).
[b]Virus isolation of CSFV strain Eystrup on PK15 cells.
[c]Virus isolation of BVDV strain CP7 on KOP-R cells.
[d]Animals were euthanized because of severe clinical symptoms.

ciency on PK15 as compared with KOP-R cells, although the plating efficiency of the Alfort 187 virus on KOP-R cells was more than 500-fold lower than that detected on PK15 cells (Fig. 4b).

In cross-neutralization assays with antisera specific for one certain *Pestivirus* species (anti-CSFV, anti-BVDV, and anti-BDV), and with CP7_E2alf- and CP7-specific pig sera collected at day 28 after immunization (sera from five animals), a characteristic reaction pattern for CP7_E2alf and the corresponding anti-CP7_E2alf serum was observed (Figs. 5a, b). The CSFV-specific antiserum was able to neutralize 100 $TCID_{50}$ of CP7_E2alf with neutralization titers of 1:960, although a serum containing high titers of BVDV or BVD antibodies failed to do so. Using pig sera obtained 4 weeks after immunization with CP7 or CP7_E2alf, the chimeric CP7_E2alf as well as CSFV strains Alfort 187 and Eystrup were readily neutralized by the CP7_E2alf antiserum. In contrast, there was no detectable neutralizing effect of CP7_E2alf antisera versus BVDV strain CP7 or BDV strain 137/4 (Fig. 5b). The CP7-specific antisera were capable of neutralizing parental BVDV strain CP7, but failed to neutralize the chimera as well as both tested CSFV strains, whereas a weak neutralizing effect against the BDV strain 137/4 could be determined (Fig. 5b).

Immunization and challenge infection of pigs

After immunization of pigs with the various viruses, the general condition of the animals was good, irrespective of the inoculum, and no clinical signs, leukopenia, or fever were observed (data not shown). Virus isolation from blood leukocytes after immunization was negative for all animals in the *CP7_E2alf group*. In contrast, BVDV CP7 could be isolated from two animals in the *CP7 group* (Table 2). Serological screening with a CP7- or Alfort 187-specific neutralization assay demonstrated that none of the CP7_E2alf contact animals seroconverted, although one animal in the *CP7 contact group* had a neutralizing antibody titer of 1:40 against BVDV CP7 (Table 3). In conclusion, neither a CP7_E2alf viremia nor shedding of the chimeric virus to the contact animals could be observed. In contrast, BVDV CP7 viremia was detectable in two of the immunized animals, and serological data provided evidence that CP7 spread to one of the contact animals (Tables 2 and 3). Using a CSFV-specific blocking ELISA for the detection of E2-antibodies, all CP7_E2alf-immunized animals developed positive signals with a mean value of more than 65% at day 28 post-immunization (p. imm.; Fig. 6a). A first serological reaction could be detected at day 11 p. imm. for the *CP7_E2alf group* (Fig. 6a). No CSFV E2-specific antibodies were detected in any of the sera from animals in the *CP7 group* (Fig. 6a). By using a CP7- or Alfort 187-specific neutralization assay, homologous antibody titers of more than 1:10 could be detected in most of the CP7- and CP7_E2alf-immunized animals at day 11 p. imm. At day 28 p. imm., mean titers of homologous neutralizing antibodies were determined, which ranged from 1:100 for the *CP7_E2alf* to 1:500 for the *CP7 group* (Fig. 6b, Table 3). At day 28 p. imm., none of the animals in the *CP7_E2alf group* had detectable CP7-specific antibodies and no Alfort 187-specific antibodies could be detected either in sera of CP7-immunized pigs (Table 3).

After intramuscular challenge with the highly virulent CSFV strain Eystrup at 28 days after immunization, the health status of the pigs previously inoculated with CP7_E2alf as well as that of the contact animals within the *CP7_E2alf group* remained completely undisturbed, and neither fever nor leukopenia were detectable in any of the

Table 3
Serology post-immunization and challenge infection

Group	Animal #	Antibody[a] (ND_{50}, vs. Alfort/vs. CP7)			Marker ELISA (%)
		0 dpi[b]	28 dpi[c]	49–59 dpi	53 dpi[d]
CP7_E2alf	2181	<2/<2	240/<2	5120/40	44
	2182	<2/<2	120/<2	10240/30	72
	2183	<2/<2	240/<2	10240/<2	53
	2184	<2/<2	20/<2	10240/7	96
	2185	<2/<2	60/<2	10240/40	76
	Mean[e]	<2/<2	96/<2	8914/13	68
Contact[f]	2176	<2/<2	<2/<2	<2/<2	0
CP7_E2alf	2177	<2/<2	<2/<2	<2/<2	21
	2178	<2/<2	<2/<2	<2/<2	12
	2179	<2/<2	<2/<2	<2/<2	7
	2180	<2/<2	<2/<2	<2/<2	13
		<2/<2	<2/<2	<2/<2	11
CP7	2191	<2/<2	<2/640	960/>640	81
	2192	<2/<2	<2/640	2560/>640	98
	2193	<2/<2	<2/480	3840/>640	68
	2194	<2/<2	<2/480	1920/>640	99
	2195	<2/<2	<2/320	640/>640	72
		<2/<2	<2/497	1633/>640	84
Contact CP7	2186	<2/<2	<2/<2	7/<2	6
	2187	<2/<2	<2/<2	5/<2	10
	2188	<2/<2	<2/<2	120/<2	100
	2189	<2/<2	<2/40	<2/60	9
	2190	<2/<2	<2/<2	7/<2	14
		<2/<2	<2/2	8/2	28
Controls	2196	<2/<2	<2/<2	‡[g]	‡
	2197	<2/<2	<2/<2	320/5	79
	2198	<2/<2	<2/<2	‡	‡
	2199	<2/<2	<2/<2	640/<2	99
	2200	<2/<2	<2/<2	640/<2	91
	2201	<2/<2	<2/<2	320/30	99
	2202	<2/<2	<2/<2	‡	‡
		<2/<2	<2/<2	453/3	92

[a] Neutralizing antibody titer (ND_{50}) against CSFV strain Alfort 187 and BVDV strain CP7.
[b] Days post-immunization with chimeric CP7_E2alf or parental strain CP7.
[c] Day of challenge infection with CSFV strain *Eystrup*.
[d] E^{RNS} blocking ELISA values in % at day 53 p. imm. (day 25 after challenge).
[e] Geometric mean values of the neutralizing antibody titers.
[f] Contact animals ($n = 5$) were housed in close contact to the immunized animals (CP7_E2alf and CP7) during the whole experiment.
[g] Animals were euthanized before day 49 post-immunization and did not develop detectable neutralizing antibodies.

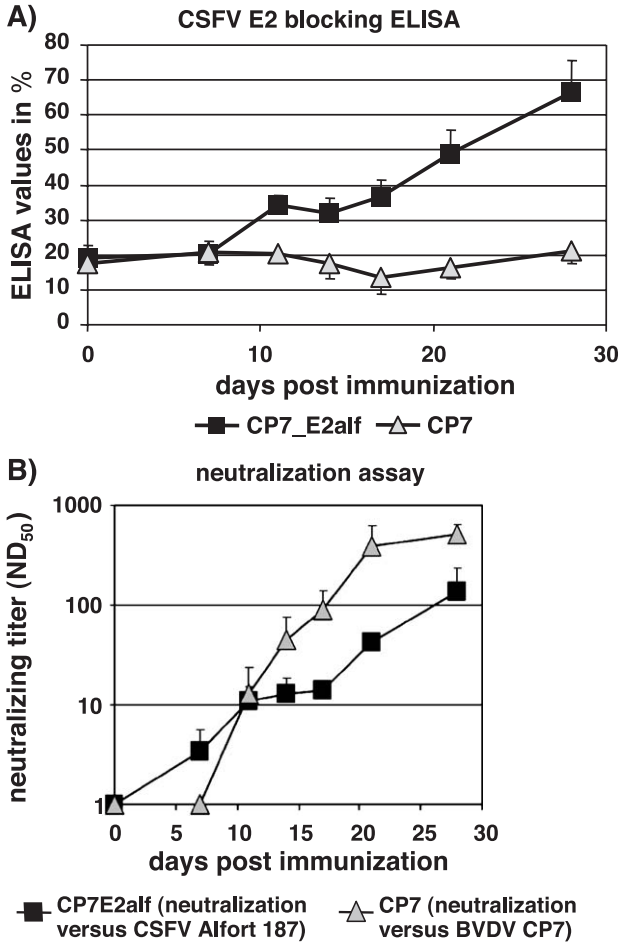

Fig. 6. (A) CSFV E2-specific ELISA antibodies after immunization with CP7_E2alf or CP7. ELISA values are given in blocking percentages. Samples with more than 30% blocking are scored positive. The mean values ($n = 5$) of the CP7_E2alf and the CP7 group are given, and standard deviations are shown as error bars. The mean values of sera of the CP7 group were negative until challenge infection, although the mean value of the CP7_E2alf group scored positive with about 35% at day 11 p. imm. and more than 60% at the day of challenge infection. (B) Homologous neutralizing antibody titers after immunization with CP7_E2alf or CP7. Mean values ($n = 5$) of the neutralizing titers of the CP7_E2alf and the CP7 group are given. Standard deviations are shown as error bars. The homologous neutralizing antibody titers versus CP7_E2alf were determined using Alfort 187. The mean values (arithmetic mean) of CP7_E2alf-immunized animals reached homologous titers of about 1:100 at day 28 p. imm., while the *CP7 group* animals had a mean neutralizing titer of about 1:500 at the same time point.

animals (Figs. 7a, b). No challenge virus could be re-isolated from 2×10^6 blood leukocytes of any of the CP7_E2alf-immunized animals between day 0 and 28 p. chall. (Table 2). In addition, none of the CP7_E2alf contact animals developed antibodies against CSFV or BVDV (Table 3), suggesting that no virus shedding occurred or shedding of challenge virus was too low to result in infection of sentinel animals.

In contrast, all five pigs inoculated with BVDV strain CP7 showed a rise of body temperature (>40 °C) and significant leukopenia between days 3 and 9 after challenge infection (Figs. 7a, b). Challenge virus could be isolated from all animals of the CP7 group at different days p.i. (Table 2). Uptake of feed was reduced during days 2 and 7 p. chall.; nevertheless, all five pigs fully recovered. One of the contact animals in the *CP7 group* showed a rise in body temperature between days 9 and 13 p. chall. (animal No. 2188), and CSFV challenge virus could be re-isolated from blood leukocytes of animal No. 2188 (data not shown). Four of the CP7 contact animals had CSFV-specific antibody titers ranging between 1:5 and 1:120. One animal remained CSFV seronegative, but showed a CP7-specific titer of 1:60 (Table 3).

All of the control pigs became anorectic and severely depressed after CSFV inoculation. Typical CSF symptoms

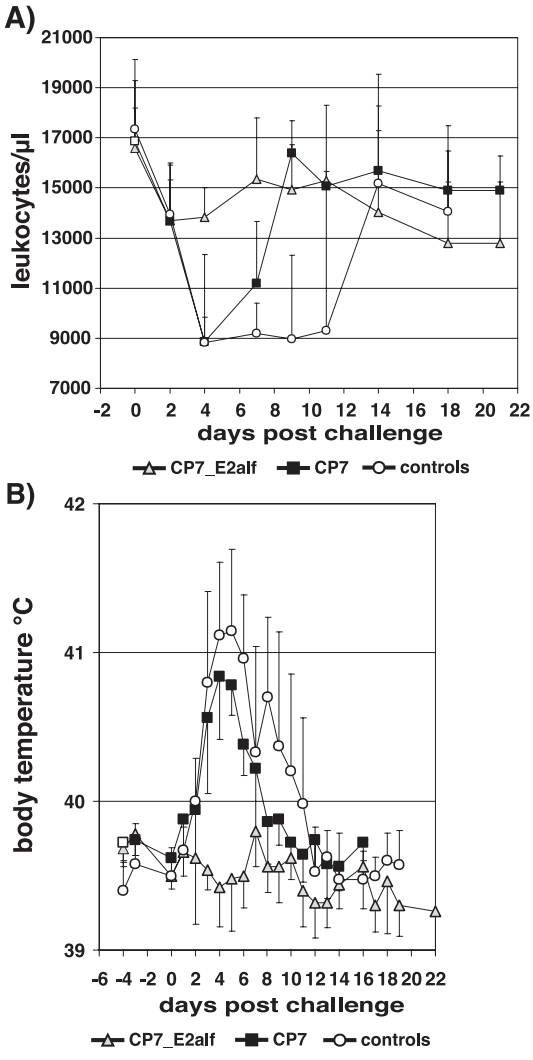

Fig. 7. (A) Leukopenia after challenge infection with highly virulent CSFV strain Eystrup. Mean values of the leukocyte counts of the *CP7_E2alf group* ($n = 5$), the *CP7 group* ($n = 5$), and the *control group* ($n = 7$) are given. Standard deviations are shown as error bars. (B) Body temperature after challenge infection with highly virulent CSFV strain Eystrup. Mean values of the rectal body temperature of the *CP7_E2alf group* ($n = 5$), the *CP7 group* ($n = 5$), and the *control group* ($n = 7$) are given. Standard deviations are shown as error bars.

started 3–4 days after challenge and lasted between 8 and 14 days. Uptake of feed was reduced during this period. A severe leukopenia between days 4 and 14 p. chall. (Fig. 7a), and fever with maximum body temperatures of more than 41.5 °C lasting for several days was observed (Fig. 7b). Challenge virus could be re-isolated from blood leukocytes of the control animals between days 2 and 11 after challenge infection (Table 2). Three pigs were moribund (body temperature > 41.5 °C, diarrhea, central nervous disorders) and had to be euthanized on days 10 and 14 after challenge, respectively. These animals did not develop any detectable neutralizing antibodies against CSFV. The remaining pigs recovered completely and CSFV-specific antibody titers between 1:320 and 1:640 were determined (Table 3).

Differentiating infected from immunized animals

Following immunization and challenge infection, "marker antibodies" were determined using a CSFV-specific E^{RNS} antibody blocking ELISA. In all pigs, the E^{RNS} ELISA was negative before but positive after challenge infection with CSFV strain Eystrup (Table 3). After immunization and before challenge infection, the mean ELISA values of the *CP7_E2alf group* clearly were lower than 10% (Fig. 8). After challenge infection, the mean blocking values of the control and the *CP7 groups* scored positive with values of more than 50% starting between days 11 and 14 p. chall., where seven out of nine animals were positive. In contrast, four out of five sera from the CP7_E2alf group scored negative at the same time point. At day 25 p. chall., the mean E^{RNS} ELISA value of the control animals (92%) was higher than the ELISA percentages of the CP7 group (84%), which in turn were higher than those of the animals of the CP7_E2alf group (68%; Fig. 8, Table 3). The contact animals remained negative until the end of the experiment, except for one animal, which was clearly positive for CSFV-specific E^{RNS} antibodies (no. 2188; 100%, Table 3). These results indicate that the CP7_E2alf-immunized pigs could be differentiated from CSFV-infected animals and seroconverted with CSFV-E^{RNS}-specific antibodies after a CSFV challenge infection.

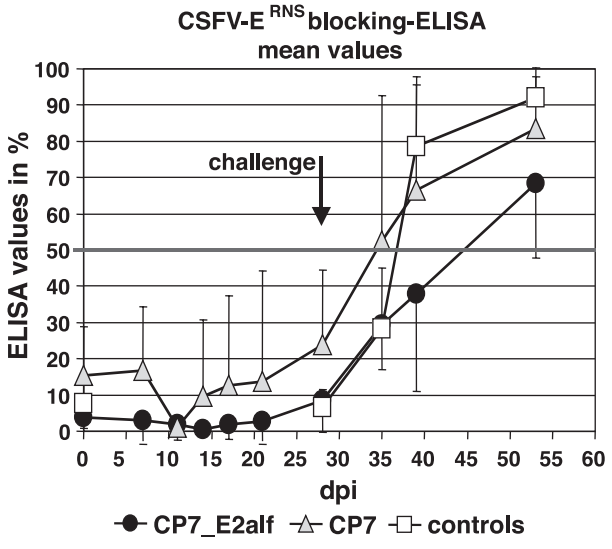

Fig. 8. Blocking ELISA analysis using a commercially available ELISA system (Ceditest CSFV-ERNS; Cedi-Diagnostics, Netherlands). Mean values of the E^{RNS} blocking percentages of the *CP7_E2alf group* ($n = 5$), the *CP7 group* ($n = 5$), and the *control group* ($n = 7$) are given. A serum is scored positive with a percentage of more than 50% following the manufacturers instructions. Standard deviations in percent are included as error bars.

Discussion

In this study, we describe the construction of a viable BVDV/CSFV chimeric *Pestivirus*, in which BVDV E2 sequences were replaced with E2 sequences of CSFV strain Alfort 187. Previously, several viable chimeric *Pestiviruses* have been constructed, including some with exchanges in the E2-coding region. Sequences encoding for the N-terminal part of E2 or full-length E^{RNS} from BVDV II were inserted into an infectious CSFV genome (de Smit et al., 2001; van Gennip et al., 2000), or border disease virus E2 replaced E2 of an infectious BVDV cDNA clone (Liang et al., 2003). Expression of the here inserted CSFV E2 by BVDV CP7 was detected using Western blot analysis and indirect immunofluorescence; thus, the cytopathogenic chimeric CP7_E2alf could be easily distinguished from parental BVDV CP7 and donor CSFV strain Alfort 187 using monoclonal antibodies.

Concerning replication and cell tropism, the generated chimeric *Pestiviruses* reported so far displayed varying phenotypes in vitro. The CSFV/BVDV chimera expressing the N-terminal portion of BVDV II-derived E2 in a CSFV background grew to virus titers, which were reduced not more than 10-fold compared to wild-type CSFV in porcine kidney cells (van Gennip et al., 2000). It was therefore concluded that the replacement of the antigenic region of E2 did not change cellular tropism of C-strain-based CSF viruses for porcine SK6 and bovine FBE cells. In contrast, insertion of a sequence encoding full-length BDV E2 into the BVDV genome resulted in a chimeric virus that exhibited growth properties very similar to those of the parental BDV that donated the E2 gene (Liang et al., 2003). In one step growth kinetics, the end point titers of the BVDV/BDV chimera after 24 h were reduced approximately 100-fold in bovine MDBK cells when compared to the parental BVDV NADL. Nevertheless, all the reported chimeric viruses could be easily propagated and further passaged with the cell system used for the parental *Pestivirus*. The BVDV/CSFV chimeric CP7_E2alf virus reported here, however, could not be efficiently propagated using bovine KOP-R cells, which usually yield the best titers for parental BVDV CP7. Following RNA transfection, virus replication and protein expression could be demonstrated, and recombinant chimeric virus was detectable in the

transfection supernatant after 24 h. The infectivity/μg of the in vitro-transcribed CP7_E2alf RNA was comparable to that reported previously for infectious BVDV cDNA clones (Kümmerer and Meyers, 2000). However, the highest virus titers could be obtained with PK15 cells, and further propagation after transfection in KOP-R cells failed. In addition, after passaging of the virus on porcine PK15 cells, which are suitable for the propagation of the E2 donor CSFV Alfort 187, the chimeric CP7_E2alf could be propagated very efficiently. Nevertheless, in the first passages, the titers decreased or remained low until passage eight, when stable virus titers of more than 10^6 $TCID_{50}$/ml could be determined. Multistep growth analyses of the 11th PK15 passage revealed that CP7_E2alf replicates in KOP-R cells with marked reduced end point titers compared to those of BVDV CP7. In contrast, titers of chimeric CP7_E2alf in PK15 cells were reduced only about threefold compared to those of CSFV Alfort and almost 1000-fold higher than those of BVDV CP7 on PK15 cells. Furthermore, plating efficiency of CP7_E2alf was extremely reduced on KOP-R cells in comparison to those determined on PK15 cells. In conclusion, the reported exchange of BVDV E2 by CSFV E2 in the CP7 genome resulted in a marked change of in vitro host range of the chimeric virus compared to the parental BVDV, and very closely resembled that of E2 donor strain CSFV Alfort 187.

The chimeric CP7_E2alf virus had to be passaged several times on PK15 cells until stable and high virus titers were reached. At passage 11, nucleotide sequence comparisons of the entire structural genes (capsid, E^{RNS}, E1, and E2) revealed a predominant amino acid exchange at amino acid position 479 (according to the BVDV CP7 sequence), with the uncharged amino acid glycine being exchanged by positively charged arginine. Amino acid position 479 in the BVDV genome corresponds to position 476 in the CSFV E^{RNS}, for which an identical exchange from an uncharged residue (serine) to arginine was reported during adaptation of CSFV strain Brescia to SK6 cells (Hulst et al., 2000). In the case of CSFV Brescia, virus variants carrying this mutation were able to attach to the surface of cells by the interaction with membrane-associated heparan sulfate thus resulting in enhanced virus replication in vitro (Hulst et al., 2000, 2001). For Sindbis virus, similar adaptations to the use of heparan sulfate as an attachment receptor have been reported, and the magnitude of binding was shown to be positively correlated with the attenuation of these viruses (Klimstra et al., 1998). However, a role of the described E^{RNS} mutation for the avirulence of CP7_E2alf is unlikely, because it was clearly shown that the similar adaptive mutation had no effect on the virulence of CSFV strain Brescia (Hulst et al., 2001). However, it may be speculated that the described mutation of CP7_E2alf enabled a more efficient growth of CP7_E2alf in porcine PK15 cells. Experiments specifically addressing this hypothesis are currently ongoing.

The CP7_E2alf virus was then characterized by cross-neutralization assays with antisera specific for different *Pestivirus* species. In these experiments, CP7_E2alf reacted in a way that was indistinguishable from the E2 donor strain Alfort 187, in that it was efficiently neutralized by a CSFV-specific serum, but only marginally by sera with high titers of BDV or BVDV type I neutralizing antibodies. Vice versa, a CP7_E2alf-specific antiserum efficiently neutralized different CSFV strains like Alfort 187 or Eystrup, but did not have any effect on BVDV or BDV infection (neutralization titer < 1:2). These results strongly support the hypothesis that E2 might be the major or even the only Pestiviral protein, able to induce detectable amounts of neutralizing antibodies.

Upon inoculations of pigs, CP7_E2alf proved to be completely apathogenic and did not spread to contact animals. No virus was isolated from the infected animals, and none of the sentinel pigs seroconverted, indicating that CP7_E2alf has a very low potential for viremia and virus shedding. In contrast, parental BVDV strain CP7 was re-isolated from two of the CP7-immunized animals at day 4 p. imm., and one contact animal seroconverted with CP7 neutralizing antibodies. The reduced replication of CP7_E2alf in pigs was consistent with the reduced mean neutralizing antibody titer of the animals immunized with the chimeric virus, which was fourfold lower than that of pigs inoculated with parental BVDV strain CP7. Therefore, the in vivo growth properties seem to conflict to a certain degree with the replication of chimeric CP7_E2alf and parental CP7 in cultured cells. Insertion of CSFV E2 into the BVDV background may enable the virus to efficiently replicate in epitheloid PK15 cells, but impairs virus replication in cells, which are important for in vivo virus shedding and spread. It is tempting to speculate that the acquired E^{RNS} mutation of CP7_E2alf, which is possibly responsible for a better heparan sulfate binding, may compensate for the loss of a specific CSFV-E2 function in cell culture but not in the animal. It is known from in vitro assays using bovine and porcine cell lines that E2 is the major partner for receptor binding and attachment, with CD46 as a binding partner (T. Rümenapf, personal communication), and recombinant E2 is able to block Pestivirus infection in cultured cells (Hulst and Moormann, 1997). The in vivo role of E2 concerning the interaction with specific target cells, for example, leukocytes, however, is not well understood. It has been shown that the E1–E2 heterodimer may play an important role for a fully functional E2 (Rümenapf et al., 1993). Therefore, in the light of the data presented here, a certain degree of incompatibility of BVDV E^{RNS}-E1 and CSFV E2 is likely, and the chimeric E1–E2 heterodimer of CP7_E2alf may be rendered less effective. Contrary to our findings, the recently reported chimeric *Pestivirus* mutants, in which BVDV II E2 or E^{RNS} was inserted into a CSFV background, induced a detectable viremia and were transmitted to the contact animals (de Smit et al., 2001). In conclusion, CP7_E2alf is highly attenuated in pigs, although a clear in vitro adaptation to porcine kidney cells was demonstrated. A restoration of

virulence after serial animal passage is highly unlikely, because (i) the parental CP7 virus was shown to be avirulent for pigs, (ii) no virus was shed to contact animals, (iii) cytopathogenicity of CP7_E2alf leads to a self-limitation, and (iv) the inserted CSFV E2 is obviously not a major determinant of pathogenicity. Interestingly, the CSFV vaccine virus C-strain also efficiently replicates in porcine kidney cells in vitro, but was demonstrated to be apathogenic for pigs inducing only limited viremia or virus spread, which could be detected only in a fraction of inoculated animals (Lorena et al., 2001; Terpstra et al., 1990). For a better understanding of the in vivo cell tropism of the chimeric virus, further in vitro infection studies with primary porcine cells and blood leukocytes are in progress, and additional chimeric viruses have been constructed.

After challenge infection with highly virulent CSFV strain Eystrup, all animals of the *CP7_E2alf group* were completely protected, although all control animals developed severe CSF, and three animals had to be euthanized. CP7-immunized animals exhibited an intermediate protection status after challenge infection. All animals developed clinical signs like fever or leukopenia, but to a lower extent than the naive control animals, and the animals in this group recovered completely. However, challenge virus was transmitted to four of the five contact animals. Titer differences in the CP7 contact animals at day 56 p. chall. support the conclusion that one initial contact infection subsequently caused seroconversion of three additional contact animals. Protection of BVDV-immunized pigs against clinical signs of CSF was reported previously and is probably cell-mediated, as no neutralizing antibodies were detectable (this report) (Leforban et al., 1992; Overby, 1973; Simonyi and Biro, 1967). In summary, CP7_E2alf immunization proved to be as efficient and safe as the attenuated CSFV C-strain (Bironi et al., 1987; de Smit et al., 2001). In addition, we conclude that E2 is the major immunogen for an effective protection against highly virulent CSFV. In contrast to baculovirus-expressed CSFV E2 (Uttenthal et al., 2001), a single application was sufficient to achieve a complete protection at day 28 p. imm. and neutralizing antibodies could be observed as early as 11 days p. imm. Therefore, in analogy to studies with the conventional C-strain vaccine (Bironi et al., 1987; Kaden et al., 2001) or a replication-competent CSFV/BVDV chimera (de Smit et al., 2001), protection at an earlier time point after immunization may be possible.

Lastly, the results we obtained have shown that the CP7_E2alf-induced E^{RNS}-antibody pattern can be used for discrimination between vaccinated and infected animals. At day 25 p. chall., four pigs in the *CP7_E2alf group* were clearly positive for E^{RNS} antibodies, and one animal reacted doubtful. Because of the fact that the highest E^{RNS} antibody responses in our experiment could be detected in the *control group*, followed by the partially protected *CP7 group*, we hypothesize that the one animal with a weak E^{RNS} antibody response in the *CP7_E2alf group* was so well protected by the vaccine that replication of the challenge virus was reduced to an extent that no detectable E^{RNS}-specific humoral immune response was mounted. Besides the presented possibility for serological differentiation, the described BVDV/CSFV chimera has the advantage that the genetic BVDV background can be used also for a direct differentiation of the recombinant virus strain from wild-type CSFV and CSFV vaccine strains by using real time RT-PCR targeting regions of the Pestiviral genome that allow species differentiation (McGoldrick et al., 1998, 1999).

The first BVDV/CSFV chimeric *Pestivirus* described in this study had unique in vitro and in vivo characteristics. It may prove not only useful for the understanding of Pestiviral cell tropism but also represents the basis for the development of genetically engineered safe and effective CSFV live marker vaccines. In the future, comprehensive experiments will be performed to collect additional data on virus replication as well as on the longevity of immunity, vaccine efficiency after oral application, the protective dosages, block of transplacental infection, and virus spread.

Material and methods

Virus and cells

Porcine kidney cell line 15 (PK15; ATCC), Madin Darby bovine kidney cells (MDBK, RIE261, CCLV), and KOP-R cells (RIE244, CCLV), a diploid bovine esophageal cell line, were obtained from the collection of cell lines in veterinary medicine at the Federal Research Center of Virus Diseases of Animals, Insel Riems (CCLV). Cells were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% BVDV-free fetal bovine serum (FBS). KOP-R cells were selected due to their susceptibility to BVDV infection and their superior suitability for BVDV propagation (Reimann et al., 2003).

CSFV strain Alfort 187 as well as the BDV strain 137/4 were obtained from the National Reference Laboratory for CSF (Federal Research Centre for Virus Diseases of Animals, Insel Riems, Germany). Highly virulent CSFV strain Eystrup was provided by Martin Hofman (IVI, Mittelhäusern, Switzerland). Cytopathogenic BVDV strain CP7 was reconstituted after transfection of RNA transcribed from the infectious cDNA clone pA/BVDV (Meyers et al., 1996a) into KOP-R cells.

Monoclonal antibodies, antisera, ELISA, and neutralization assays

For the detection of BVDV and CSFV proteins, the monoclonal antibodies (mab) *WB210* (anti-E^{RNS} BVDV, CVL Weybridge), *01–03* (anti-E^{RNS} panpesti, kindly provided by Christian Schelp, Intervet BV), *WB215* (anti-E2 BVDV, CVL Weybridge), *CA3* (anti-E2 BVDV, Institute for Virology, TiHo, Hannover), HC/TC50 (anti-E2 CSFV, In-

stitute for Virology), *HC34* (anti-E2 CSFV, Institute for Virology), mab-mix *WB103/105* (anti-NS3 panpesti, CVL Weybridge), and *C16* (anti-NS3 panpesti, Institute for Virology) were used (Edwards et al., 1988, 1991; Peters et al., 1986). Standard immunofluorescence (IF) analysis using a fluorescence-activated cell sorter (FACS; Becton Dickinson) or a fluorescence microscope (Olympus) were performed as previously described (Beer et al., 1997; Grummer et al., 2001).

Chimeric CP7_E2alf was tested in neutralization assays with porcine hyperimmune sera directed against BDV, BVDV, or CSFV, which were collected from experimentally infected pigs (provided by the National Reference Laboratory for CSF, Insel Riems, Germany). Homologous neutralization titers of the sera ranged from 1:640 to 1:960 neutralizing dosage 50% (ND_{50}). In addition, sera from five pigs sampled at day 28 post-infection with CP7_E2alf or CP7, respectively, were used for cross-neutralization experiments. Porcine sera free of *Pestivirus*-specific antibodies served as controls. Standard neutralization assays using inactivated sera were performed according to the OIE manual (Anonymous, 1996) as previously described (Depner et al., 2001). Neutralization titers were determined as neutralizing dosage 50%. In addition, serum or plasma samples were tested for the presence of antibodies against CSFV E2 and E^{RNS} with the commercialized ELISA assays HerdCheck CSFV AB (IDEXX) and Ceditest CSFV-ERNS (Cedi-Diagnostics). Both test systems were used according to the instructions given by the manufacturers.

Western blot analysis

Infected or uninfected KOP or PK15 cells (1.5×10^6) were lysed by adding 300 μl of lysis buffer [1% Triton X-100, 20 mM sodium phosphate (pH 7.1), 150 mM NaCl, 2 mM EDTA]. The cell lysate was cleared of cell debris by low speed centrifugation at 10 000 × *g* for 5 min. Proteins in 20 μl of lysate were separated by 12% sodium-dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE), and subsequently transferred onto a nitrocellulose membrane (Schleicher and Schüll). The membrane was incubated for 1 h at room temperature with anti-E^{RNS} mab 01–03 (1:500), anti-E2 CSFV mab HC/TC50 (1:300), or anti-E2 mab BVDV CA3/WB215 (1:100) in TBST buffer (20 mM Tris–HCl, pH 7.5; 150 mM NaCl, 0.1% Tween 20). After washing with TBST buffer, the membrane was incubated at room temperature for 1 h with an anti-mouse immunoglobulin G (IgG) conjugated to horseradish peroxidase (Dianova; 1:30 000). Following washing, the membrane was incubated with SuperSignal^R chemiluminescent detection reagent (Pierce) and exposed to an X-ray film (Kodak).

In vitro transcription and electroporation

In vitro transcription of linearized full-length cDNA constructs was performed by T7 RiboMax Large Scale RNA Production System (Promega) according to the manufacturer's instructions. The amount of RNA was estimated by ethidium bromide staining following agarose gel electrophoresis. For transfections, 1×10^7 PK15 or KOP-R cells were detached using a trypsin solution, washed with PBS, mixed with 1–5 μg of in vitro-synthesized RNA and transfected (two pulses at 850 V, 25 μF, 156 Ω) using an *EasyjecT Plus* (EquiBio) electroporation unit.

Polymerase chain reaction and sequencing

For polymerase chain reaction (PCR), a PTC-200 thermal cycler (MJ Research, Inc.) was used. DNA-based amplification was done by Expand High Fidelity PCR System (Roche Molecular Biochemicals) according to the supplier's protocol. Primers for generation of the respective constructs are summarized in Table 4. For RT-PCR, total RNA of virus-infected cells was extracted using the TRIZOL reagent (Gibco-Life Technologies). cDNA was amplified from the RNA template by using reverse transcriptase (AMV; Promega) and sequence-specific primers (Table 4). RNA for RT-PCR experiments was treated with RNase-free DNases and the RT step was omitted to detect contamination with cDNA. The synthesized cDNA was amplified with a thermostable Taq Polymerase (Promega) and resulting PCR products were cloned into the pGEM-T Easy Vector System (Promega), or directly sequenced. For sequence comparison of the region encoding the structural proteins of CP7_E2alf$^{1st\ passage}$ and CP7_E2alf$^{11th\ passage}$, several fragments of the whole region were cloned from a minimum of two different RT-PCR products, and 8–20 different clones per fragment were sequenced. In addition, the RT-PCR fragments were directly

Table 4
PCR primers used for plasmid construction

Primer	Sequence (5′ to 3′)[a]	Genomic region (nucleotides)
E2Alf_*PacI*	*GCA*TTAATTAA**C**CAGCTAGCCTGCAAGGAAGATT	2441–2462 (+ sense)[b]
E2AlfR_*SnaBI*	*GACC*TACGTAACCAGCGGCGAGTTGTTCTGTT	3538–3559 (− sense)[b]
p7_*PacI*	*CAAG*GGTACC**CA**TTAATTAACGGTCCTACGTAGTCCAGTATGGGGCAGGTGA	3567–3586 (+ sense)[c]
p7R	GCTCTAGGTACCCCTGGGCA	3785–3804 (− sense)[c]

[a] Restriction enzyme sites are underlined, overlaps to facilitate restriction enzyme digestion are in italics and additional nucleotides for in-frame ligation are in bold.
[b] Nucleotide position in CSFV Alfort 187 sequence.
[c] Nucleotide position in BVDV CP7 sequence.

sequenced and a consensus sequence of the analyzed virus population was determined.

Sequencing was carried out using a Thermo Sequenase Cycle Sequencing Kit (Amersham Biosciences). Nucleotide sequences were read with a LI-COR automatic sequencer (MWG Biotech) and analyzed using the Wisconsin software package version 9.1 (Genetics Computer Group, USA). For sequencing of cloned DNA, M13 forward and reverse primers as well as sequence-specific primers were used (MWG Biotech).

Plasmid construction

The chimeric BVDV/CSFV clone shown in Fig. 1 was constructed based on the full-length cDNA clone pA/BVDV (kindly provided by Gregor Meyers, Federal Research Centre for Virus Diseases of Animals, Tübingen, Germany). BVDV-specific restriction sites are indicated by superscript numbers corresponding to their positions on the genome of BVDV strain CP7, from which pA/BVDV (pA/CP7) was derived.

pA/CP7_ΔE2p7: Plasmid pA/BVDV was cleaved with *KpnI*^{2447,3797} and religated, which resulted in an in-frame deletion of E2 and p7 as shown previously (Reimann et al., 2003).

pA/CP7_ΔE2PacI: In order to repair the p7 coding region and insert a small polylinker (*Pac*I, *Rsr*II, *Sna*BI), a PCR fragment was amplified using the primers p7-*PacI* and p7R-*KpnI* (Table 4, Fig. 1). The PCR fragment was cleaved with *KpnI* and cloned into the *KpnI*^{2447} site of plasmid CP7ΔE2p7.

pA/CP7_E2alf: Plasmid pA/CP7_ΔE2*PacI* was digested with *PacI* and *SnaBI*. For the purpose of generating a CSFV-E2-expressing replicon, an RT-PCR-fragment was amplified by using total cellular RNA of PK15 cells infected with CSFV strain Alfort 187 and primers E2-Alfort-*PacI* and E2R-Alfort-R-*SnaBI* (Table 4, Fig. 1). The PCR fragment was digested with *PacI* and *SnaBI*, and subsequently ligated in-frame into plasmid pA/CP7_ΔE2*PacI* (Fig. 1).

Recovery of Pestivirus from plasmid constructs and plaque assay

For virus recovery, in vitro-transcribed RNA (1–5 μg) was transfected into PK15 or KOP-R cells by electroporation. Cell culture supernatants or complete cell lysates after freezing/thawing were harvested at 24 h to 120 h *post-transfection* (p. t.), and titrated using KOP-R or PK15 cells. At the day of supernatant collection, virus replication was monitored by IF staining. Virus preparations were further passaged using PK15 or KOP-R cells, and all virus titers were determined as $TCID_{50}$. In all cases, the whole virus population was passaged to avoid negative selection that would be caused by biologically cloned virus. To detect a possible temperature sensitivity, transfections of CP7_E2alf RNA into PK15 cells were also incubated at 35, 37, and 39 °C.

For the determination of both fluorescent cells/μg electroporated RNA and infectious units per μg of RNA 1×10^7 PK15, MDBK and KOP-R cells were transfected in duplicate with 1 μg RNA. At 12 h post-transfection, all cell cultures were IF-stained and the percentage of NS3-expressing cells was detected by FACS-analysis. At 24 h post-transfection, supernatants of the transfected cell cultures were titrated using PK15 cells.

In a standard plaque assay, cytopathogenicity of CP7_E2alf was determined. PK15 cells were inoculated with 100 $TCID_{50}$ of CP7_E2alf, incubated for 1 h at 37 °C, washed with PBS^-, and subsequently overlayed with methyl cellulose (0.6% in DMEM). After 96 h incubation at 37 °C, the overlay was removed and the cells were stained with crystal violet. Crystal violet staining was performed after cells were washed once with PBS^-, fixed for 10 min with 5% formaldehyde, and washed again with water. For staining, the cells were incubated for 10 min with 1% (w/v) crystal violet in 50% ethanol.

Multistep growth and plating efficiency

KOP-R cells or PK15 cells were infected with the various viruses at a multiplicity of infection (m.o.i.) of 1 and incubated for 0, 8, 24, 48, 72, and 96 h before harvesting by freeze–thawing. Virus titers in $TCID_{50}$/ml were determined by end point titration. CSFV Alfort 187 and chimeric CP7_E2alf were titrated using PK15 cells; BVDV CP7 was titrated in KOP-R cells.

For calculation of plating efficiency, the different virus preparations were adjusted to a titer of 10^7 $TCID_{50}$/ml in the cell system, where the highest titers had been determined. Subsequently, viruses were inoculated in KOP-R cells and PK15 cells by limiting dilution (log 10 steps from 10^{-1} to 10^{-8}; eight wells per dilution step) using 0.1 ml of the diluted virus suspension per well in a 96 well plate. Infection was detected by IF staining at 4 days post-inoculation. Plating efficiency of the various viruses was determined as $TCID_{50}$/ml on the different cell types.

Animal experiments

Animals

Twenty-seven 6-week-old crossbred weanling pigs weighing approximately 12 kg each and free of neutralizing antibodies against *Pestiviruses* were randomly divided into two groups of 10 animals and one group of 7 animals. Five pigs out of one group with 10 pigs were inoculated with CP7_E2alf virus derived from infectious chimeric cDNA clone pA/CP7_E2alf and challenged 28 days later with virulent CSFV strain Eystrup (*CP7_E2alf group*). The remaining five pigs from the group were kept as contact animals. The inoculation procedure for the second group with 10 pigs (*CP7 group*) was analogous to that of the *CP7_E2alf group*, differing only in the virus used for the first inoculation. In this group, BVDV strain CP7 derived from infectious

cDNA clone pA/BVDV (pA/CP7) was used instead of CP7_E2alf. In both groups, the contact animals were kept apart from the inoculated pigs during the first three days after inoculation with CP7_E2alf or CP7, respectively. The third group (*control group*) was mock-immunized, and also challenged with 1×10^8 $TCID_{50}$ of CSFV strain Eystrup at 28 days after inoculation. All pigs were inoculated intramuscularly (i.m.) with 2 ml of cell culture supernatant containing either 1×10^7 $TCID_{50}$ of CP7_E2alf or 1×10^7 $TCID_{50}$ of CP7, respectively. The contact controls were left unchallenged and housed in the same room with the respective vaccination group.

During the course of the experiment, pigs were monitored daily for clinical signs of CSF. Blood samples for virological, serological, and hematological examinations were taken by venipuncture in weekly intervals before and after primary inoculation or challenge, respectively. Moribund animals were euthanized and necropsied, as were the healthy animals 9 weeks after the start of the experiment.

Virus was isolated from blood samples by using cocultivation of 1×10^6 blood leukocytes with PK15 or KOP-R cells for 5–6 days in duplicate. After an incubation period of 5–6 days, the cells were analyzed for NS3 expression by IF.

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