Available online at www.sciencedirect.com
ScienceDirect
Vaccine 25 (2007) 3391–3399
Vaccine
www.elsevier.com/locate/vaccine

CP7_E2alf: A safe and efficient marker vaccine strain for oral immunisation of wild boar against Classical swine fever virus (CSFV)

Patricia Koenig [a], Elke Lange [b], Ilona Reimann [c], Martin Beer [a,*]

[a] *Institute of Diagnostic Virology, Friedrich-Loeffler-Institut, Boddenblick 5a, 17493 Greifswald-Insel Riems, Germany*
[b] *Institute of Infectology, Friedrich-Loeffler-Institut, Boddenblick 5a, 17493 Greifswald-Insel Riems, Germany*
[c] *Institute of Molecular Biology, Friedrich-Loeffler-Institut, Boddenblick 5a, 17493 Greifswald-Insel Riems, Germany*

Received 13 September 2006; received in revised form 7 December 2006; accepted 21 December 2006
Available online 8 January 2007

Abstract

Wild boar are an important reservoir of Classical swine fever virus (CSFV) in several European countries, where most of the primary outbreaks in domestic pigs are directly related to the endemic disease situation in the wild boar population. Oral immunisation has been introduced as an additional control measure to accelerate CSF eradication in wild boar in Germany since 1993. Immunisation with an oral bait vaccine based on the conventionally attenuated live vaccine strain "C" proved to be safe and effective, but does not allow differentiation between infected and vaccinated animals. Therefore, we examined the vaccine efficacy of the recently constructed chimeric pestivirus CP7_E2alf, whose coding sequences for the major envelope protein E2 of BVDV strain CP7 are replaced by E2 of the CSFV strain Alfort187 [Reimann I, Depner K, Trapp S, Beer M. An avirulent chimeric pestivirus with altered cell tropism protects pigs against lethal infection with classical swine fever virus. Virology 2004;322(1):143–57]. Following oral immunisation of wild boar, CP7_E2alf proved to be completely avirulent. Furthermore, all vaccinees were fully protected from clinical disease after a highly virulent CSFV challenge infection. The immunised animals seroconverted within 3 weeks after vaccination for CSFV E2-specific and CSFV neutralising antibodies, whereas prior to challenge infection no antibodies against CSFV E^{rns} were detected with an appropriate CSFV-specific marker ELISA test. Thus, the BVDV backbone of CP7_E2alf enables serological and genetic differentiation from wild type CSFV infection. In conclusion, CP7_E2alf represents the first efficient and safe marker vaccine candidate for oral immunisation of wild boar against CSFV.

Keywords: Wild boar; CSFV; Oral immunisation; Attenuated marker vaccine; Differentiating infected from vaccinated animals (DIVA)

1. Introduction

Classical swine fever virus (CSFV) belongs to the genus *Pestivirus* of the Flaviviridae family [1]. CSFV is structurally and antigenetically closely related to the ruminant Bovine viral diarrhea virus (BVDV) and Border disease virus (BDV). CSFV causes a highly contagious and often fatal infection in domestic pigs and wild boar. Outbreaks in industrialized pig productions are controlled by sanitary measures and large scale culling, and cause significant economic losses [2,3].

Dependent on the strain specific virulence, disease may run either an acute, chronic or even sub-clinical course. Congenital infections may result in birth of persistently infected pigs that shed virus at high titres [4]. Pigs with chronic disease show intermittent virus excretion for considerable time spans. CSFV infection in wild boar populations is reported for various European countries and is considered to be an important virus reservoir [5–9]. In these countries, most of the primary outbreaks in domestic pigs are related to the endemic disease situation in wild boar [10,11]. Oral bait vaccination with conventional attenuated Chinese-(C−) strain live vaccines is safe and efficiently reduces CSFV prevalence by increasing herd immunity [12]. However, vaccinated animals cannot be distinguished from wild boar re-convalescent from field

* Corresponding author: Tel.: +49 38351 7 200.
E-mail address: martin.beer@fli.bund.de (M. Beer).

0264-410X/$ – see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.vaccine.2006.12.052

virus infection and epidemiological effects of the vaccination campaigns can be monitored only inefficiently and indirectly. Subunit vaccines efficiently reduce clinical disease, but are less effective in diminishing field virus shedding. So far, no, subunit vaccine preparation suitable for oral administration is available. Therefore, generation of efficacious CSF marker vaccines suitable for oral application to wild boar is urgently needed.

Serum antibodies induced by pestivirus infections are directed against the envelope proteins E2 and E^{rns}, and the non-structural protein NS3 [13,14]. Due to the high conservation of NS3 among pestiviruses and the seroprevalence of non-CSFV pestiviruses in swine, NS3 is not a suitable candidate for designing marker tests. Therefore, monoclonal antibodies directed against E2 or E^{rns} have been used to discriminate between *Pestivirus* species [15,16]. E2 is a major immunogene, which induces neutralising antibodies and therefore should be a component of efficient CSFV vaccines. Recently developed CSFV marker assays for the detection of E^{rns}-specific antibodies [16] are commercially available and discriminate between CSFV E^{rns} and E^{rns} of BVDV and BDV.

We have recently reported on construction of a chimeric full length pestivirus genome based on the cytopathogenic (cp) BVDV strain CP7. The E2 protein encoding sequence was replaced by the corresponding region of the CSFV strain Alfort187 [17]. The resulting chimeric BVDV/CSFV virus strain CP7_E2alf exhibited virus growth and cell tropism similar to the E2 donor virus CSFV Alfort187. Intramuscular inoculation of 1×10^7 50% tissue culture infective doses ($TCID_{50}$) CP7_E2alf into 27 domestic weaner pigs proved to be completely innocuous [17]. Neither viremia nor virus transmission to sentinels were detected. After challenge infection with CSFV strain Eystrup, all immunised pigs were fully protected from clinical disease, viremia and shedding of challenge virus. The animals seroconverted in the CSFV marker ELISA (Ceditest CSFV E^{rns} ELISA, Cedi Diagnostics, Lelystad, The Netherlands) until day 21 after challenge infection. Here, we report on further testing for vaccine efficacy of the chimeric BVDV/CSFV CP7_E2alf as a marker vaccine for oral immunisation of wild boar.

2. Materials and methods

2.1. Cell culture and virus propagation

Cells and viruses were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% BVDV-free foetal bovine serum at 37 °C in a humidified atmosphere containing 5% CO_2. CSFV strain Alfort187 and CP7_E2alf were propagated on PK15 cells (porcine kidney cell line 15; ATCC, Wesel, Germany), BVDV strain CP7 was grown on KOP-R cultures (bovine pharyngeal cell line; Collection of Cell Lines in Veterinary Medicine, FLI, Insel Riems, Germany). CSFV strains Alfort187 and Koslov were obtained from the National Reference Laboratory for CSF (FLI, Insel Riems, Germany). Cytopathogenic BVDV strain CP7 was reconstituted after transfection of RNA transcribed from the infectious cDNA clone pA/BVDV [18]. Virus titres were determined by end point titrations. Cells seeded in microtitration plates were infected with 10-fold serial dilutions of clarified supernatants. The titres expressed in $TCID_{50}$ per milliliter were obtained by immunofluorescence staining of the cultures with the monoclonal antibody (mAb) C16 directed against the pestiviral protein NS3 (kindly provided by the Institute of Virology, TiHo, Hannover, Germany) and an Alexa Fluor®488 conjugated $F(ab')_2$ fragment of goat anti-mouse IgG (Molecular Probes, Leiden, The Netherlands). Standard immunofluorescence analyses using a fluorescence microscope (IX51, Olympus, Hamburg, Germany) were performed as previously described [19]. Virus preparations were tested for the absence of BVDV and mycoplasma.

2.2. Experimental design

All animals were tested for absence of pestiviruses and antibodies specific for CSFV and ruminant pestiviruses before the onset of the experiment. Prior to infection or sample collection the wild boar had to be anaesthetized with 1 mg/kg weight tiletamine and zolazepam (Zoletil®, Virbac, France), which is recommended for the sedation of many species of wild mammals [20]. The 6.3×10^6 $TCID_{50}$ CP7_E2alf in a volume of 2 ml were orally instilled by syringe to three wild boar aged 8 months (day 0). A non-vaccinated animal was kept as a sentinel to indicate possible vaccine virus shedding. Additional domestic pigs were included as challenge controls because no age-matched wild boar were available for the experiment. Twenty-eight days after immunisation all animals, including the non-immunised contact boar and two domestic weaner pigs, were orally challenged with 1×10^6 $TCID_{50}$ of CSFV strain Koslov in an 1 ml volume. The challenge virus preparation was obtained from whole blood of an agonally diseased swine after experimental Koslov infection. For 10 days after immunisation and 14 days after challenge infection, the animals were monitored daily for clinical signs and rectal temperatures, the latter with exception of the control boar, which could not be handled without anaesthesia. Clinical signs were cumulatively scored from slight (1 point) to severe (3 points). General health, feed uptake, nervous disorders and ocular, respiratory or gastrointestinal discharge was evaluated. Nasal virus shedding and leukocyte counts from EDTA blood were also examined. To evaluate the antibody responses, serum samples were collected in weekly intervals. Four weeks after challenge infection, the surviving animals were euthanized and necropsy was performed.

2.3. Sample collection and virological analyses

At weekly intervals after immunisation and at 5, 7, 9, 13 and 21 days after challenge infection blood samples and

nasal swabs were collected. Differential blood counts were determined from EDTA-blood using a cell-dyn® 3700 haematology analyser (Abbott). Nasal swab plugs were submerged in 1.2 ml cell culture medium containing 200 U/ml penicillin, 200 μg/ml streptomycin, 20 μg/ml gentamicin and 5 μg/ml Amphotericin B. Leukocytes were prepared from whole blood samples after erythrocyte lysis with buffered ammonium chloride solution (155 mM NH_4Cl, 10 mM $KHCO_3$, 1 mM EDTA/pH 7.4). Blood samples containing 1×10^7 leukocytes were incubated with a three-fold excess of lysis buffer (10 min, 4 °C). After sedimentation, leukocytes were washed twice with phosphate buffered saline (PBS) and finally resuspended in 1 ml PBS. Viremia or virus excretion was determined by inoculation of 3×10^6 leukocytes or 100 μl nasal swab fluid on PK15 cells in 2–4 replicates. After 6 days indirect immunofluorescence staining with the pestivirus NS3-specific mAb C16 was performed. In case of a first negative result, supernatants were twice sub-cultured. After RNA preparation (RNeasy mini, Qiagen) from nasal swabs and purified leukocytes, viral loads were quantified by real-time reverse transcriptase-polymerase chain reaction (RT-PCR). A pestivirus specific primer pair (324/326, [21]) and a FAM-labelled probe [22] enabled detection of a 288 base pair fragment located in the 5′-non-coding region (NCR) of both CP7_E2alf and CSFV strain Koslov. Reverse transcription was performed for 30 min at 50 °C (QuantiTect® probe RT-PCR kit, Qiagen, Hilden, Germany). DNA was amplified after denaturation (15 min, 95 °C) at 42 cycles of 15 s at 94 °C, 30 s at 57 °C and 20 s at 60 °C with an iCycler 170–8740 (Bio-Rad, München, Germany). PCR fragments could be quantified by using control samples with defined copy numbers [23].

2.4. Serological examinations

Serum samples were tested for CSFV-E2- and E^{rns}-specific antibodies with commercial ELISA assays. CSFV-E2-specific antibody response was monitored with an E2 blocking ELISA (Classical Swine Fever Antibody Test Kit, Idexx Laboratories, Maine, USA). A CSFV marker ELISA (Ceditest CSFV-E^{rns}, Cedi Diagnostics, Lelystad, The Netherlands) permitted survey of reactivity for CSFV-E^{rns}-specific antibodies. ELISA testing was performed according to the instructions given by the manufacturers. Serum neutralisation titres for CSFV and BVDV were determined according to the OIE manual [24]. Replicates of two-fold dilutions of heat-inactivated (30 min, 56 °C) serum samples (50 μl) were incubated with 100 $TCID_{50}$/50 μl CSFV strain Alfort 187, the vaccine virus CP7_E2alf or the parental BVDV CP7 in microtitration plates (2 h, 37 °C). After addition of 5×10^4 PK15 or 1×10^5 KOP-R cells and incubation for 6 days, cultures were subjected to immunofluorescence staining with the pestivirus NS3-specific mAb C16. Titres were expressed as $\log_2$ of the reciprocal of dilutions that caused 50% neutralisation (ND_{50}).

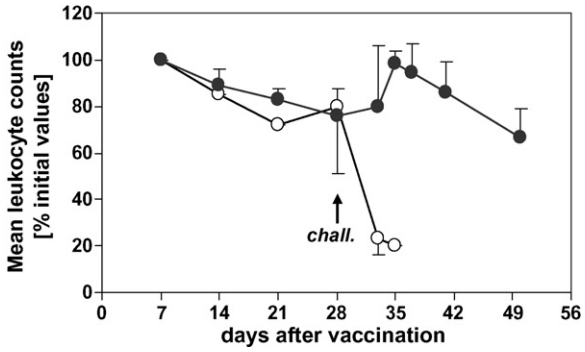

Fig. 1. Mean leukocyte counts. Blood leukocytes were counted after immunisation with CP7_E2alf and after challenge infection (chall.) with CSFV strain Koslov. Animals were immunised at day 0 and infected at day 28. Mean values of the CP7_E2alf group (closed circles; $n = 3$), and of the control group (open circles; $n = 3$) are indicated as percentage of the initial values. Differential blood counts were determined from EDTA-blood using a cell-dyn® 3700 haematology analyser (Abbott). Standard deviations are shown as error bars.

3. Results

3.1. Vaccination

In order to test for in vivo replication competence, attenuation and induction of protective immunity against CSFV, we inoculated wild boar with the chimeric pestivirus CP7_E2alf. Following oral application of 6.3×10^6 $TCID_{50}$ CP7_E2alf per animal no adverse reactions occurred. Over an observation period of 4 weeks after vaccination, neither clinical disease nor elevated body temperatures were observed (data not shown). Reduction of leukocyte counts constitutes a sensitive marker of an acute CSFV infection. Therefore, we determined at weekly intervals after immunisation differential leukocyte counts from EDTA-blood using an automated haematology analyser. The wild boar exhibited an inconspicuous pattern of white blood cell counts (Fig. 1).

To characterise the safety profile of CP7_E2alf, we examined nasal excretion of the vaccine virus and cell-bound viremia in purified white blood cells. Virus isolation by co-cultivation of 3×10^6 purified leukocytes on PK15 cells and subsequent staining with the pestivirus NS3-specific mAb C16 gave likewise negative results (Table 1). These findings were in accordance with data obtained from highly sensitive, pestivirus-specific real-time RT-PCR investigations (Table 2). CP7_E2alf could not be re-isolated after inoculation of nasal swab fluid on porcine kidney cells (Table 1) and no viral genome equivalents could be amplified from nasal swabs by real-time RT-PCR (Table 2). The control boar, which was kept in contact after vaccination, remained seronegative for CSFV-specific antibodies (Fig. 2 and 3). In conclusion, neither CP7_E2alf viremia nor nasal shedding could be detected after oral application.

However, all animals showed a strong antibody response and seroconverted for CSFV-E2-specific antibodies by days

Table 1
Virus isolation was performed by inoculation of (A) nasal swab fluid or (B) 3×10^6 blood leukocytes on PK15 cells

(A) Virus isolation from nasal swabs following immunisation or challenge infection

Animal					Days after challenge infection (CSFV Koslov)					
No.	Ear tag				0	5	7	9	13	21
		Days after immunisation with CP7_E2alf								
		7	14	21	28	33	35	37	41	50
Group										
Controls										
1	2057	0/2[a]	0/2	0/2	0/2	**4/4**	n.d.	n.d.	0/4*	✞
5	0019	n.d.	n.d.	n.d.	0/2	**4/4**	**4/4**	✞	✞	✞
6	0020	n.d.	n.d.	n.d.	0/2	**4/4**	**4/4**	✞	✞	✞
CP7_E2alf										
2	2058	0/2	0/2	0/2	0/2	0/4	0/4	0/4	0/4	0/4
3	2059	0/2	0/2	0/2	0/2	0/4	0/4	0/4	0/4	0/4
4	2060	0/2	0/2	0/2	0/2	0/4	0/4	0/4	0/4	0/4

(B) Virus isolation from blood leukocytes following immunisation or challenge infection

Group										
Controls										
1	2057	0/2[a]	0/2	0/2	0/2	**2/2**	n.d.	n.d.	✞	✞
5	0019	n.d.	n.d.	n.d.	0/2	**2/2**	**2/2**	✞	✞	✞
6	0020	n.d.	n.d.	n.d.	0/2	**2/2**	**2/2**	✞	✞	✞
CP7_E2alf										
2	2058	0/2	0/2	0/2	0/2	0/4	0/4	0/4	0/4	0/4
3	2059	0/2	0/2	0/2	0/2	0/4	0/4	0/4	0/4	0/4
4	2060	0/2	0/2	0/2	0/2	0/4	0/4	0/4	0/4	0/4

After 6 days virus replication was verified by immunofluorescence staining. A first negative result was confirmed after two further blind passages of the supernatants. n.d., not done; (✞), animals died or were euthanized due to severe clinical symptoms; (*), animal died at 12 days after challenge infection, sample was collected *post-mortem*.

[a] Number of positive inoculations/number of total inoculations. Positive inoculations are printed in bold.

14–21 after vaccination as determined by a commercial E2-blocking ELISA assay (Fig. 2A). Serum antibody titres scored clearly negative in the E^{rns} marker ELISA test (Fig. 2B). Neutralising serum activity directed against the parental BVDV strain CP7 did not exceed titres of 1:1.6 (0.7 $\log_2$ ND_{50}) after immunisation (Fig. 3A). CP7_E2alf neutralising antibodies were detected at 14 days after vaccination. At the day of challenge infection, titres ranged from 1:203 to 1:256 (7.7–8 $\log_2$ ND_{50}) (Fig. 3B). CSFV neutralising titres were determined for the CSFV strain Alfort187 (Fig. 3C). In general, corresponding neutralising activities were observed against the homologous vaccine virus and CSFV Alfort with values between 1:79 and 1:645 (6.3–9.3 $\log_2$ ND_{50}). Taken together, a pronounced humoral response was observed after a single oral immunisation with CP7_E2alf. The non-vaccinated wild boar remained seronegative for E2 specific as well as for CSFV neutralising antibodies.

3.2. Challenge infection

To evaluate protective immunity and vaccination efficacy, the vaccinees as well as the non-vaccinated control animals were orally infected with a dose of 1×10^6 $TCID_{50}$ of the highly virulent CSFV strain Koslov at 28 days after immunisation. At 5, 7, 9, 13 and 21 days after challenge infection, the animals were investigated for viremia and nasal virus excretion. Serological antibody responses were monitored in weekly intervals. Over a period of 14 days, the pigs were examined for symptoms of a virulent CSFV infection such as ocular discharge, coughing, diarrhoea, depression and nervous disorder. The immunised wild boar were protected from disease and exhibited no clinical signs beside minor depression for 1 day (Fig. 4A). Onset of clinical disease was observed at 4 days after challenge infection in the control animals. Initially the non-vaccinated animals showed reduced feed consumption, than became anorectic and severely depressed. After development of nervous disorders the weaners were euthanized (8 day post-challenge [p. chall.]). A slightly delayed progression of the disease was observed in the wild boar. After exhibition of anorexia, neurological symptoms and severe depression the animal died at 12 days after infection with CSFV strain Koslov.

Acute CSFV infection is characterized by febrile disease appearing 2–4 days after infection. In the controls, temperatures increased at 4 days after challenge infection (>40 °C). Until day 8, when the animals had to be euthanized, body temperatures exceeded 41 °C with maximum values of 42 °C (Fig. 4B). In contrast, none of the vaccinated wild boar

Table 2
Viral loads of (A) nasal excretions and (B) blood leukocytes were determined by real-time reverse transcriptase-polymerase chain reaction (rRT-PCR) with pestivirus-specific primers (primer pair 324/326 [21])

(A) rRT-PCR from nasal swabs following immunisation or challenge infection

Animal		Days after challenge infection (CSVV Koslov)								
No.	Ear tag				0	5	7	9	13	21
		Days after immunisation with CP7_E2alf								
		7	14	21	28	33	35	37	41	50
Group										
Controls										
1	2057	neg.	neg.	neg.	neg.	**7.1×10^4**	n.d.	n.d.	**$7.3 \times 10^{5*}$**	✞
5	0019	n.d.	n.d.	n.d.	neg.	**9.8×10^6**	**4.6×10^9**	✞	✞	✞
6	0020	n.d.	n.d.	n.d.	neg.	**7.7×10^6**	**1.1×10^8**	✞	✞	✞
CP7_E2alf										
2	2058	neg.	neg.	neg.	neg.	neg.	neg.	neg.	neg.	neg.
3	2059	neg.	neg.	neg.	neg.	neg.	neg.	neg.	neg.	neg.
4	2060	neg.	neg.	neg.	neg.	neg.	neg.	neg.	neg.	neg.

(B) rRT-PCR from blood leukocytes following immunisation or challenge fection

Animal		Days after challenge infection (CSFV Koslov)								
No.	Ear tag				0	5	7	9	13	21
		Days after immunisation with CP7_E2alf								
		7	14	21	28	33	35	37	41	50
Group										
Controls										
1	2057	neg.	neg.	neg.	neg.	**2.7×10^6**	n.d.	n.d.	✞	✞
5	0019	n.d.	n.d.	n.d.	neg.	**1.6×10^8**	**2.1×10^9**	✞	✞	✞
6	0020	n.d.	n.d.	n.d.	neg.	**6.7×10^5**	**4.0×10^8**	✞	✞	✞
CP7_E2alf										
2	2058	neg.	neg.	neg.	neg.	neg.	neg.	neg.	neg.	neg.
3	2059	neg.	neg.	neg.	neg.	neg.	neg.	neg.	neg.	neg.
4	2060	neg.	neg.	neg.	neg.	neg.	neg.	neg.	neg.	neg.

Titres are indicated as viral genome equivalents (vge) per milliliter and printed in bold. neg., negative; n.d., not done; (✞), animals died or were euthanized because of severe clinical symptoms. (*), animal died at 12 days after challenge infection, sample was collected *post-mortem*.

exhibited elevation of body temperatures or actually fever. Necropsy after euthanasia or death of the controls revealed prominent gross lesions typical for CSF like mucosal and visceral petechiations, necrotizing tonsillitis, fibrinonecrotic colitis, hemorrhages of lymph nodes and fibrinous bronchopneumonia. In the vaccinees, no evidence of CSFV infection was detected at necropsy 4 weeks after challenge infection.

Between days 4 and 7 p. chall., the control animals showed severe leukopenia with leukocyte counts of less than 20% of the initial values, while in the CP7_E2alf group no significant reduction of the mean leukocyte counts was observed (Fig. 1). One of the immunised animals showed a slight decrease of white blood cells by 18% at 5 days p. chall. with already increased counts after two further days.

Nasal secretions were examined for shedding of challenge virus by inoculation of swab fluid on PK15 cultures. Pre-existing neutralising antibodies are known to counteract isolation of infectious virus in cell culture. Therefore, all samples were additionally investigated by real-time RT-PCR. All control weaners excreted challenge virus from day 5 after challenge until death (Table 1). The control boar was sampled only at 5 days p. chall., when viral loads at high titres were detected. Seven days later, the animal was found dead. Post-mortem analysis revealed a high load of viral genomes, whereas no infectious virus could be re-isolated. Shedding of 7.7×10^6 to 4.6×10^9 viral genome equivalents (vge) per milliliter swab fluid was ascertained by quantitative real-time RT-PCR investigations for the control weaners (Table 2). The wild boar excreted virus to a lower extent with a maximum of 7.3×10^5 vge per milliliter. In the vaccinees, no nasal virus excretion was detected by virus isolation in cell culture as well as by RT-PCR examinations.

In addition, no challenge virus could be isolated in cell culture from 3×10^6 blood leukocytes from any of the CP7_E2alf vaccinated animals between 5 and 21 days after challenge infection (Table 1) and also detection of viral genomes by real-time RT-PCR remained negative (Table 2). In the controls, cell-bound viremia was verified at 5 and 7 days after challenge by co-cultivation and RT-PCR. Viral loads determined by real-time RT-PCR-quantification exceeded 1×10^6 vge per milliliter whole blood and peaked to 1×10^9 copies.

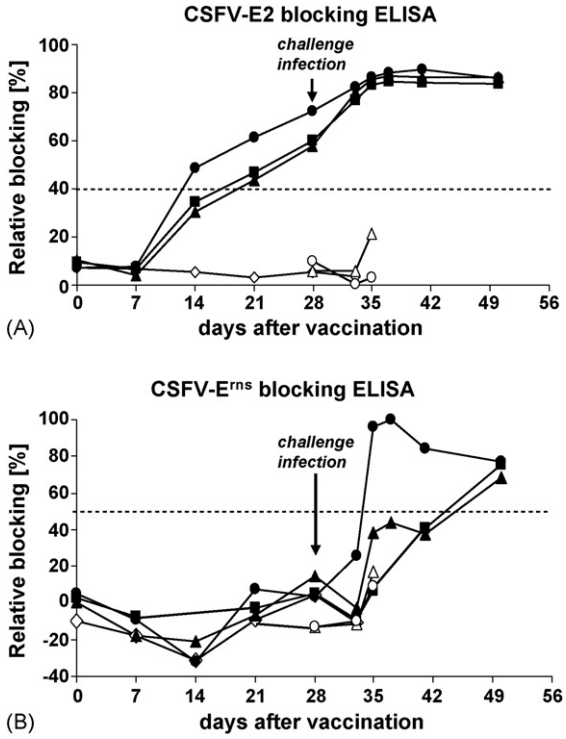

Fig. 2. ELISA antibody development after vaccination and challenge infection. Animals were immunised with CP7_E2alf at day 0 and infected with CSFV strain Koslov at day 28. ELISA values of the vaccinees (closed symbols) and the controls (open symbols) are given in blocking percentages. Threshold values are indicated by dotted lines. (A) E2-specific antibodies were determined with the HerdChek CSFV Ab antibody test kit (Idexx Laboratories, Maine, USA). (B) Marker properties were specified with the commercially available Ceditest CSFV E^{rns} ELISA system (Cedi Diagnostics, Lelystad, The Netherlands).

Monitoring of the humoral immune reactivity after challenge infection revealed a strong anamnestic response for CSFV E2-specific and CSFV neutralising antibodies in the CP7_E2alf-immunised animals. Mean titres of antibodies neutralising CSFV Alfort187 raised to 1:20,939 (Fig. 3C). Neutralisation of the parental BVDV CP7 did not exceed titres of 1:16 (Fig. 3A).

Prior to the challenge infection, the immunised wild boar remained negative for CSFV E^{rns}-specific antibodies as determined by a commercial E^{rns} marker ELISA (Fig. 2B). Between 7 and 21 days after inoculation with wild type CSFV, the vaccinees seroconverted for CSFV E^{rns}-specific antibodies and scored clearly positive with a mean blocking value of 74%. Thus, all animals were recognised as field virus infected. The control animals remained seronegative for CSFV over the whole observation period as determined by ELISA testing.

These findings clearly demonstrate, that a single oral inoculation with CP7_E2alf completely protected wild boar against a challenge infection with the highly virulent CSFV strain Koslov. Moreover, the vaccinated boar could be serologically discriminated from animals infected with wild type CSFV by appropriate ELISA testing.

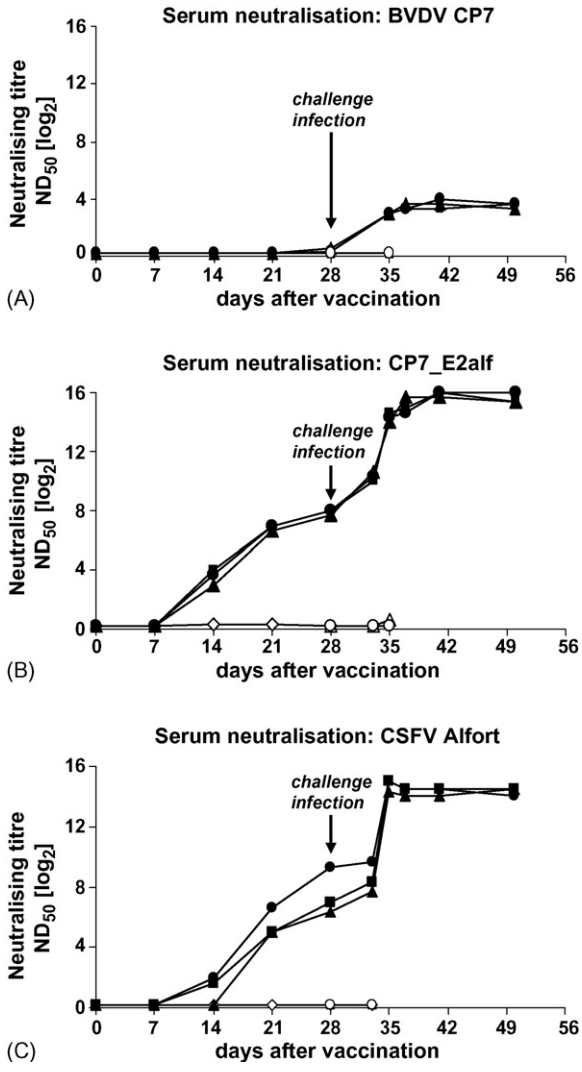

Fig. 3. Serum neutralisation responses against BVDV CP7 (A), the chimeric pestivirus CP7_E2alf (B), and CSFV strain Alfort187 (C) after vaccination and challenge infection were determined. Animals were vaccinated at day 0 and challenged with CSFV strain Koslov at day 28 (closed symbols). Sera of the control animals (open symbols) remained negative until 1 week after challenge (1/3 animals: 0.7 ND_{50}). The domestic weaners were only sampled after challenge infection (day 28).

4. Discussion

Oral immunisation of wild boar may serve as an effective instrument for CSF control. Wild boar vaccination programmes aim at reducing the number of susceptible animals below the threshold density for transmission of the infection [25,26]. Increase of herd immunity and subsequent reduction of field virus incidence are important tools to contain CSF in infected populations and to keep the virus out of healthy populations [27]. As a result of the field trials in Germany, it can be concluded that triple oral bait vaccination successfully supported CSF prevention and control measures [12,26]. The conventional C-strain vaccine induces a strong, long-lasting immunity but exhibits no discriminatory properties

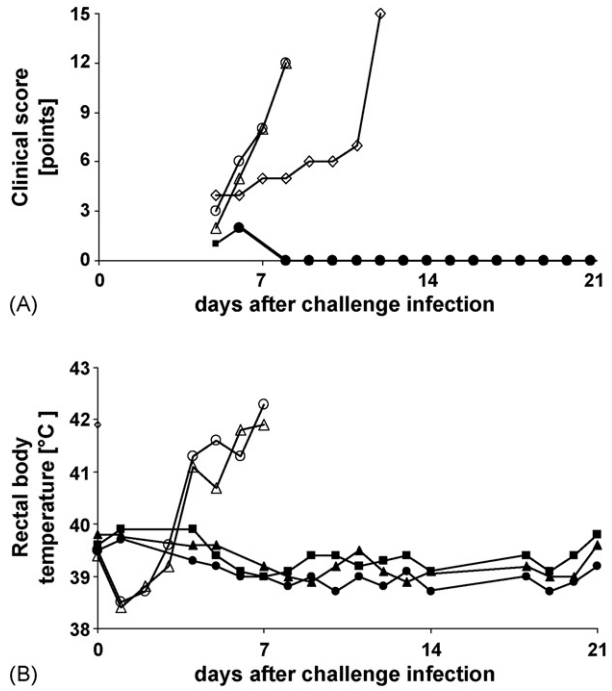

Fig. 4. (A). Evaluation of clinical responses after challenge infection. Clinical signs as depression, reduced feed consumption, diarrhoea, dyspnoe and neurological disorders of the non-vaccinated controls (open symbols) and of the vaccinated wild boar (closed symbols) were monitored daily and scored from low grade (1 point) to severe (3 points). A cumulative maximum score of 15 points was calculated for lack of feed intake, for severe neurological, respiratory and gastrointestinal disorders, succumbing and death. (B). Body temperature curves after challenge infection with CSFV strain Koslov. Rectal temperatures of the non-vaccinated controls (open symbols) and of the vaccinated animals (closed symbols) were recorded daily until 3 weeks after challenge infection or until the animals had to be euthanized. Survey of the control boar was omitted because the animal could only be handled under anaesthesia.

from field virus infection [28]. Therefore, direct epidemiological surveillance in vaccination areas is not possible and actual circulation of field-virus can only be estimated. Efficacious vaccines for immunisation of wild boar should permit serological differentiation of infected from vaccinated animals and combine suitability for oral application, induction of early protection, as well as prevention of horizontal and vertical virus spreading.

In this study, we investigated the efficacy and safety of the modified life vaccine candidate CP7_E2alf on oral vaccination of wild boar. Our recombinant vaccine candidate CP7_E2alf is based on an infectious cDNA clone of the cytopathogenic BVDV strain CP7 [18]. The BVDV backbone enables serological differentiation from wild type CSFV infection using a commercial CSFV E^{rns} blocking ELISA. In this study, suitability for oral vaccination was evaluated in wild boar. The vaccine dose was orally instilled by syringe in a volume of 2 ml corresponding to the content of one bait. Following a high dose application of CP7_E2alf, neither adverse side-effects nor alteration of the general health condition was recorded. In conclusion, CP7_E2alf proved to be innocuous in the wild boar after oral inoculation. Thus, attenuation observed for preliminary animal experiments in domestic pigs [17] correlated to apathogenicity also in the wild species.

Nasal swab samples and purified leukocytes were analysed for vaccine virus loads in cell culture and by highly sensitive real-time RT-PCR investigations. Under the applied experimental conditions, neither nasal shedding of the vaccine nor viremia was detected by virus isolation. Amplification of CP7_E2alf genomes on swab and blood samples was not successful. In addition, no horizontal transmission of the vaccine virus to the wild boar in contact was recorded. Due to the small number of animals no final conclusions concerning virus transmission can be drawn. But it should be considered that in previous animal experiments no seroconversion ($n = 7$) was observed in contact pigs ([17]; unpublished data). These data indicate that the chimera is highly attenuated in its in vivo replication efficacy. A further major safety aspect concerning vertical transmission of CP7_E2alf to susceptible foetuses is currently under investigation.

As early as 2 weeks after a single oral application of our chimeric vaccine CSFV-neutralising antibodies were detectable. At challenge infection, 50%-neutralising titres ranged from 1:79 to 1:645. Neutralising antibodies play a major role in defence against the disease. Titres of about 1:50 predict a stable protection against CSF infection [29]. Cross neutralisation assays support the hypothesis that the envelope protein E2 is the major or even the only pestiviral protein that induces relevant amounts of neutralising antibodies. CP7_E2alf specific antisera efficiently neutralised different CSFV strains, but neutralisation of the chimera and different CSFV strains by sera with high titres of BVDV specific antibodies was kept to a minimum [17]. We therefore speculate that a previous BVDV infection of pigs may only marginally interfere with the protective efficacy of our vaccine candidate. However, immunisation/challenge experiments using animals with a past BVDV infection have to be performed in the future.

The CP7_E2alf vaccinated wild boar seroconverted within 3 weeks for CSFV E2-specific antibodies as tested with commercial E2 blocking ELISA assays. These data implicate that surveillance of vaccination can be readily monitored by established serological tests. Furthermore, the animals remained seronegative in a commercial CSFV E^{rns} marker assay. Vaccination with CP7_E2alf induced an antibody response, which was clearly distinguishable from CSF field virus infection.

Several chimeric CSFV/BVDV viruses have been constructed as live CSF marker vaccines [13,30]. The CSFV E2 or E^{rns} genes were exchanged with the homologous genes of BVDV, creating antigenically distinguishable chimera. The published studies were so far restricted to intramuscular or intranasal application and no data are available on vaccine virus shedding. Seroconversion for CSF specific antibodies in commercial ELISA tests was obtained only after 2–3 applications and furthermore, some animals remained negative even after multiple applications [30]. These findings were also

observed for CSF replicon vaccines [31,32]. After a single immunisation no CSFV specific antibodies could be detected independently from the route of application (intramuscular, intradermal, oronasal, nasal). Based on the finding that CSFV replicon particles lacking the complete E2 gene were able to mediate partial protection, it was under discussion that E^{rns} rather than E2 is crucial for the induction of mucosal immunity [32]. However, our results suggest that CSFV E2 is the major immunogen also for induction of a protective mucosal immune response.

As reported so far, all modified live vaccine candidates like CSFV replicons [31–33], CSFV/BVDV chimera [13,31] and viral vectors that express structural proteins of CSFV [34] required multiple or very high dosed administration to induce clinical protection. However, in some of the vaccinated animals pyrexia or leukopenia was observed upon challenge infection. Our modified live vaccine virus CP7_E2alf mediated complete protection from a highly virulent CSF challenge infection even after a single application. Except for a minor 1-day lasting depression, no clinical signs were observed. The vaccinees were protected from fever with body temperatures at pre-challenge levels. In contrast, the control pigs diseased severely and died or had to be euthanized due to high grade neurological disorders. Leukocyte counts of the vaccinees remained stable and neither challenge virus viremia nor shedding was detectable. Samples were tested negative for challenge virus by co-cultivation in cell culture and also by real-time RT-PCR investigations. Especially in the presence of neutralising antibodies, detection of viral genomes was described to be more sensitive than conventional virus isolation [35]. We have already shown for domestic weaners that a single intramuscular immunisation with CP7_E2alf induced complete clinical protection and prevented virus shedding [17]. Non-vaccinated contact animals neither seroconverted for CSFV nor exhibited signs of disease. Therefore, we speculate that CP7_E2alf may be highly effective in reduction or prevention of CSF field virus transmission after oral immunisation of wild boar.

After challenge infection all wild boar seroconverted for CSFV E^{rns} and were recognised as field virus infected. However, for E2 based marker vaccination using E2 subunit vaccines, complemented replicons or chimeric viruses a lack of sensitivity of the corresponding E^{rns} marker ELISAs is under discussion. Additionally, it was observed that comprehensive protection by the vaccine hampered challenge virus replication. Thus, low levels of the E^{rns} protein might be insufficient to induce a detectable antibody response [13,36]. We are currently addressing animal trials with higher numbers of animals for statistical evaluation of the E^{rns} response. Our data collected so far underline the suitability of the E^{rns} marker ELISA on the herd level and indicate that only in exceptional cases infected pigs may not react clearly positive [17]. In addition, the genetic background of our BVDV/CSFV chimera can be used for differentiation of the recombinant virus and CSF field and vaccine strains using suitable discriminatory RT-PCR assays [23,37].

In conclusion, the chimeric BVDV/CSFV CP7_E2alf represents the first efficient and safe marker vaccine candidate suitable for oral immunisation of wild boar against CSFV. Protective vaccine dosage, duration of the immunity and foetal protection remain to be studied in further animal experiments.

Acknowledgements

The studies were financially supported by the European Union (project “CSFV Vaccine & Wild Boar”, SSPE-CT-2003-501599). We thank Brigitte Dannenfeld for excellent technical assistance.

References

[1] Fauquet CM, Mayo MA, Maniloff U, Desselberger U, Ball LA. Virus Taxonomy, VIIIth Report of the International Commitee on Taxonomy of Viruses. Elsevier/Academic Press; 2005.
[2] Edwards S, Fukusho A, Lefevre PC, Lipowski A, Pejsak Z, Roehe P, et al. Classical swine fever: the global situation. Vet Microbiol 2000;73(2–3):103–19.
[3] Vandeputte J, Chappuis G. Classical swine fever: the European experience and a guide for infected areas. Rev Sci Tech 1999;18(3):638–47.
[4] Depner KR, Muller A, Gruber A, Rodriguez A, Bickhardt K, Liess B. Classical swine fever in wild boar (Sus scrofa)-experimental infections and viral persistence. Dtsch Tierarztl Wochenschr 1995;102(10):381–4.
[5] Laddomada A, Patta C, Oggiano A, Caccia A, Ruiu A, Cossu P, et al. Epidemiology of classical swine fever in Sardinia: a serological survey of wild boar and comparison with African swine fever. Vet Rec 1994;134(8):183–7.
[6] Leforban Y, Cariolet R. Characterization and pathogenicity for pigs of a hog cholera virus strain isolated from wild boars. Ann Rech Vet 1992;23(1):93–100.
[7] Biagetti M, Greiser-Wilke I, Rutili D. Molecular epidemiology of classical swine fever in Italy. Vet Microbiol 2001;83(3):205–15.
[8] Hergarten G, Hurter KP, Hess RG. Detection of infection with classical swine fever virus in wild boar: a comparison of different laboratory diagnostic methods. Dtsch Tierarztl Wochenschr 2001;108(2):51–4.
[9] Zupancic Z, Jukic B, Lojkic M, Cac Z, Jemersic L, Staresina V. Prevalence of antibodies to classical swine fever, Aujeszky’s disease, porcine reproductive and respiratory syndrome, and bovine viral diarrhoea viruses in wild boars in Croatia. J Vet Med B Infect Dis Vet Public Health 2002;49(5):253–6.
[10] Fritzemeier J, Moenning V, Staubach C, Teuffert J, Thulke H, Schlüter H. Investigation on occurrence, spread and control of classical swine fever (CSF) in Germany. Report on research project 96HS022: Commission Decision 96/553/EC. Commission European Community, VI/6311/98-EN; 1998.
[11] Artois M, Depner KR, Guberti V, Hars J, Rossi S, Rutili D. Classical swine fever (hog cholera) in wild boar in Europe. Rev Sci Tech 2002;21(2):287–303.
[12] Kaden V, Renner C, Rothe A, Lange E, Hanel A, Gossger K. Evaluation of the oral immunisation of wild boar against classical swine fever in Baden-Wurttemberg. Berl Munch Tierarztl Wochenschr 2003;116(9–10):362–7.
[13] de Smit AJ, Bouma A, van Gennip HG, de Kluijver EP, Moormann RJ. Chimeric (marker) C-strain viruses induce clinical protection against virulent classical swine fever virus (CSFV) and reduce transmission of CSFV between vaccinated pigs. Vaccine 2001;19(11–12):1467–76.

[14] Rau H, Revets H, Balmelli C, McCullough KC, Summerfield A. Immunological properties of recombinant classical swine fever virus NS3 protein in vitro and in vivo. Vet Res 2006;37(1):155–68.
[15] van Rijn PA, van Gennip HG, Moormann RJ. An experimental marker vaccine and accompanying serological diagnostic test both based on envelope glycoprotein E2 of classical swine fever virus (CSFV). Vaccine 1999;17(5):433–40.
[16] Floegel-Niesmann G. Classical swine fever (CSF) marker vaccine. Trial III. Evaluation of discriminatory ELISAs. Vet Microbiol 2001;83(2):121–36.
[17] Reimann I, Depner K, Trapp S, Beer M. An avirulent chimeric pestivirus with altered cell tropism protects pigs against lethal infection with classical swine fever virus. Virology 2004;322(1):143–57.
[18] Meyers G, Tautz N, Becher P, Thiel HJ, Kummerer BM. Recovery of cytopathogenic and noncytopathogenic bovine viral diarrhea viruses from cDNA constructs. J Virol 1996;70(12):8606–13.
[19] Grummer B, Beer M, Liebler-Tenorio E, Greiser-Wilke I. Localization of viral proteins in cells infected with bovine viral diarrhoea virus. J Gen Virol 2001;82:2597–605.
[20] Fowler M. Restraint and handling of wild and domestic animals. 2nd ed. USA: Iowa State Univ Press; 1995.
[21] Vilcek S, Herring AJ, Herring JA, Nettleton PF, Lowings JP, Paton DJ. Pestiviruses isolated from pigs, cattle and sheep can be allocated into at least three genogroups using polymerase chain reaction and restriction endonuclease analysis. Arch Virol 1994;136(3–4):309–23.
[22] Gaede W, Reiting R, Schirrmeier H, Depner KR, Beer M. Detection and species-specific differentiation of pestiviruses using real-time RT-PCR. Berl Munch Tierarztl Wochenschr 2005;118(3–4):113–20.
[23] Hoffmann B, Beer M, Schelp C, Schirrmeier H, Depner K. Validation of a real-time RT-PCR assay for sensitive and specific detection of classical swine fever. J Virol Methods 2005;130:36–44.
[24] Anonymous. OIE manual of standards for diagnostic tests and vaccines. List A and B diseases of mammals, birds and bees. 5th ed. Paris: Off Int Epiz; 2004, ISBN 92-9044-622-6.
[25] Laddomada A. Incidence and control of CSF in wild boar in Europe. Vet Microbiol 2000;73(2–3):121–30.
[26] Kaden V, Heyne H, Kiupel H, Letz W, Kern B, Lemmer U, et al. Oral immunisation of wild boar against classical swine fever: concluding analysis of the recent field trials in Germany. Berl Munch Tierarztl Wochenschr 2002;115(5–6):179–85.
[27] European Commission. Classical swine fever in wild boar. Scientific Committée on Animal Health and Animal Welfare; 1999.
[28] Kaden V, Lange B. Oral immunisation against classical swine fever (CSF): onset and duration of immunity. Vet Microbiol 2001;82(4):301–10.
[29] Terpstra C, Wensvoort G. The protective value of vaccine-induced neutralising antibody titres in swine fever. Vet Microbiol 1988; 16(2):123–8.
[30] van Gennip HG, van Rijn PA, Widjojoatmodjo MN, de Smit AJ, Moormann RJ. Chimeric classical swine fever viruses containing envelope protein E(RNS) or E2 of bovine viral diarrhoea virus protect pigs against challenge with CSFV and induce a distinguishable antibody response. Vaccine 2000;19(4–5):447–59.
[31] van Gennip HG, Bouma A, van Rijn PA, Widjojoatmodjo MN, Moormann RJ. Experimental non-transmissible marker vaccines for classical swine fever (CSF) by trans-complementation of E(rns) or E2 of CSFV. Vaccine 2002;20(11–12):1544–56.
[32] Maurer R, Stettler P, Ruggli N, Hofmann MA, Tratschin JD. Oronasal vaccination with classical swine fever virus (CSFV) replicon particles with either partial or complete deletion of the E2 gene induces partial protection against lethal challenge with highly virulent CSFV. Vaccine 2005;23(25):3318–28.
[33] Widjojoatmodjo MN, van Gennip HG, Bouma A, van Rijn PA, Moormann RJ. Classical swine fever virus E(rns) deletion mutants: trans-complementation and potential use as nontransmissible, modified, live-attenuated marker vaccines. J Virol 2000;74(7):2973–80.
[34] Hammond JM, Jansen ES, Morrissy CJ, Hodgson AL, Johnson MA. Protection of pigs against 'in contact' challenge with classical swine fever following oral or subcutaneous vaccination with a recombinant porcine adenovirus. Virus Res 2003;97(2):151–7.
[35] Haegeman A, Dewulf J, Vrancken R, Tignon M, Ribbens S, Koenen F. Characterisation of the discrepancy between PCR and virus isolation in relation to classical swine fever virus detection. J Virol Methods 2006;136:44–50.
[36] de Smit AJ, Bouma A, de Kluijver EP, Terpstra C, Moormann RJ. Prevention of transplacental transmission of moderate-virulent classical swine fever virus after single or double vaccination with an E2 subunit vaccine. Vet Q 2000;22(3):150–3.
[37] McGoldrick A, Bensaude E, Ibata G, Sharp G, Paton DJ. Closed one-tube reverse transcription nested polymerase chain reaction for the detection of pestiviral RNA with fluorescent probes. J Virol Methods 1999;79(1):85–95.