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Classical swine fever: comparison of oronasal immunisation with CP7E2alf marker and C-strain vaccines in domestic pigs

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Short running title:
Classical swine fever: CP7E2alf and C-strain vaccines
Abstract

Effective oronasal vaccination against classical swine fever (CSF) is essential to achieve protection in wild boar. However the currently available live CSF vaccines, e.g. C-strain, do not allow serological differentiation between infected and vaccinated animals (DIVA). A modified live marker vaccine candidate (CP7E2alf) has been recently developed (Reimann et al., 2004). Here we report on the comparison of CP7E2alf and C-strain virus vaccines during 98 days following oronasal immunisation in domestic pigs.

C-strain vaccine virus was consistently detected in tonsils of all (n=30) animals from 3 to 77 days post vaccination (dpv) and in blood (n= 36) between 3 and 13 dpv by CSFV-specific rRT-PCR. CP7E2alf virus RNA was detected in 6 animals slaughtered between 4 and 63 dpv by a BVDV-specific rRT-PCR. The chimeric virus was not detected in blood samples.

As detected by CSFV E2-specific antibody ELISA and virus neutralisation, seroconversion first occurred at 11 dpv in the C-strain vaccinated group and between 11 and 15 dpv in the CP7E2alf vaccinated group. Serological response was still observed at 98 dpv. CP7E2alf serological response remained negative using the CSFV E\textsuperscript{ns} ELISA whereas seroconversion occurred in C-strain vaccinated group.

In conclusion, the primary replication site of CP7E2alf vaccine virus was found to be the tonsils similarly to C-strain and virulent field
strains. Persistence of CP7E2alf in the tonsils was also demonstrated up to 63 dpv. Both vaccines showed immunogenicity after oronasal administration in domestic pigs. In contrast to the C-strain, CP7E2alf vaccine allowed the use of DIVA approaches in serological tests. This study confirms CP7E2alf as a promising marker vaccine candidate for oronasal vaccination programmes to control CSF in domestic pigs and wild boar.

Keywords

Classical swine fever virus (CSFV), DIVA marker vaccine, CP7E2alf, C-strain, oronasal vaccination, diagnostic tests
Introduction

Classical swine fever (CSF) is a highly contagious and often fatal disease of domestic pigs and wild boar. Outbreaks of CSF usually cause important economic losses, and impair internal and international trade of pigs and pig products (Vandeputte and Chappuis, 1999; Edwards et al., 2000). The aetiological agent of CSF is classical swine fever virus (CSFV), a member of the Pestivirus genus of the Flaviviridae family (van Regenmortel et al., 2000).

The effectiveness of modified live vaccines, such as the Chinese strain (C-strain), has been frequently reported in domestic pigs and in wild boar (Terpstra and Robijns, 1977; Chenut et al., 1999; Kaden et al., 2000; Kaden and Lange, 2001; Dewulf et al., 2004b; Dong and Chen, 2007). Such vaccines induce protection shortly after administration. Prophylactic mass vaccination with modified live vaccines, combined with culling of infected pigs, successfully resulted in the eradication of the disease in most countries of the European Union (EU) (Vandeputte and Chappuis, 1999; Greiser-Wilke and Moennig, 2004). Currently, modified live vaccines are still in use where the disease is endemic in domestic pigs and for disease control in wild boar (von Rüden et al., 2008). Therefore, strict restrictions on the international trade in pig products are implemented from countries using vaccination (Greiser-Wilke and Moennig, 2004). The lack of serological discrimination between
naturally infected animals and animals vaccinated with a modified
live vaccine hampers disease control relying on serology (van
Oirschot, 2003; Pasick, 2004). To overcome this problem, marker
vaccines which allow differentiation of infected from vaccinated
animals (DIVA) have been developed.

The first subunit vaccine was developed in 1993 based on the
eexpression of the E2 envelope protein of the Brescia strain within the
baculovirus system (Hulst et al., 1993). Subunit vaccines induce a
DIVA serological response and reduce morbidity and mortality
following subsequent challenge with a virulent CSFV field strain
(Moormann et al., 2000). However, horizontal or vertical virus
transmission is not completely prevented when challenge occurs in
the first week post vaccination (Dewulf et al., 2001, 2004b).
Moreover, the subunit vaccines are not applicable for CSF control in
wild boar by oral immunization.

A new generation of live marker vaccines was developed based on
the construction of chimeric pestiviruses (for review see Beer et al.,
2007). The E2 or E\textsuperscript{rns}-coding region of CSFV or cytopathogenic
bovine viral diarrhoea virus (BVDV) strain CP7 has been replaced by
that of a heterologous strain (van Gennip et al., 2000; Reimann et
al., 2004; Rasmussen et al., 2007; Wehrle et al., 2007). One of these
constructs, CP7E2\text{alf}, is based on the infectious full-length clone of
BVDV strain CP7 in which the BVDV E2 gene is replaced by that of
CSFV strain Alfort/187 (Reimann et al., 2004). CP7E2alf proved to be completely safe in pigs following intramuscular inoculation. Neither viraemia, nor virus transmission to contact animals were detected. Furthermore, immunogenicity and protective effectiveness have been demonstrated after intramuscular administration in pigs (Reimann et al., 2004; Koenig et al., 2007a) and oral vaccination in wild boar (Koenig et al., 2007b; Kaden et al., 2008). Additionally, the DIVA properties of this live vaccine have been described (Reimann et al., 2004). Nevertheless additional data regarding immunogenicity after oronasal administration, dose effect and persistence over a longer period are required to improve vaccine characterisation and application efficiency.

The aim of the present study was to evaluate the live marker vaccine CP7E2alf following oronasal administration to domestic pigs in comparison to the C-strain. The presence and persistence of vaccine virus was investigated in tonsils and blood samples by comparison with the C-strain. The serological response observed after oronasal vaccination was studied up to 98 days post vaccination (dpv) in the two vaccine groups and the DIVA properties of the vaccines were compared.
Materials and methods

Vaccine

The conventional C-strain vaccine (‘Riems’) was kindly provided by Dr. V. Kaden (Friedrich-Loeffler-Institut, Germany). The CP7E2alf marker vaccine (Reimann et al. (2004) was produced using Good Manufacturing Practice by Fort Dodge Veterinaria (Spain). Vaccines were diluted in saline solution to $10^{4.5}$ 50% tissue culture infective dose per ml (TCID$_{50}$/ml) and virus titre was checked by back-titration ($10^{4.48}$ and $10^{4.59}$ TCID$_{50}$/ml for CP7E2alf and C-strain viruses respectively).

Animals

For the experiment, pigs were recruited twice from the same conventional breeding farm with an interval of six months. Each group was constituted by 46 Kahyb breed pigs (Hungarian landrace-typed hybrid), two months old and weighing approximately 12 kg. These animals were free of pestivirus antibodies, as tested by standard diagnostic procedures.

Experimental design

The study design, the administration dose and routes were similar to a previous experimental infection with a field isolate (‘WIL-11722’) (Tignon et al., 2008). Vaccination with CP7E2alf and C-strain were conducted separately with an interval of six months.
Briefly, weaner pigs were housed in a biosecure unit in agreement with EU directive 91/630/EEC and its amendments 2001/88/EC and 2001/93/EC. Following a seven-day acclimatization period, the pigs were clinically inspected and blood sampled. On the first day (day 0), CP7E2alf live marker vaccine was administrated via the intranasal (1 ml) and the oral (1 ml) routes by means of a syringe to all animals of the first group (n=46), except two pigs kept as negative controls. The two non-vaccinated controls were slaughtered on the day of vaccination by an intravenous pentobarbital injection, followed by exsanguination. Subsequently, two randomly selected vaccinated animals were slaughtered on a daily basis between 1 and 8 dpv, at 13 and 18 dpv, and then weekly from 21 to 98 dpv.

Six month later, the second group of animals (n=46) was vaccinated with the C-strain modified live vaccine, except two pigs kept as negative controls. Vaccination and slaughtering protocols were conducted in the same way as previously described.

Clinical examination and sample collection

All the living animals were clinically monitored using a scoring system (Mittelholzer et al., 2000) based on the observation of ten parameters (breathing, liveliness, body tension, body shape, walking, skin, eyes, appetite, defecation and leftovers) and the rectal temperatures were recorded on a daily basis, before sample
collection, during the first 8 days and subsequently bi-weekly until 98
dpv. Animals were considered clinically ill when their daily
cumulative clinical score exceeded the value of 6. Pigs were
considered febrile when rectal temperatures exceeded 40.5°C.

Blood samples with addition of EDTA as anticoagulant and serum
samples were collected from each animal in the group during the
experiment. Samples were collected on slaughter days and
additionally at 11 and 15 dpv. At necropsy, the tonsils of the two
slaughtered animals were aseptically collected for virological
examination. The samples were stored at -80°C until analysis.

Haematology

The total number and differentiation of peripheral blood leucocytes
(PBLs) were determined in EDTA-treated blood samples by using a
Sysmex E-4000 automatic haemocytometer (Toa Medical
Electronics, Kobe, Japan). Counts below 10,000 leukocytes/µl were
considered as leucopaenia. From 0 until 70 dpv, data were
collected from 10 animals and later from the remaining animals.

Virological analysis

Virus isolation (VI) from the tonsil samples and from EDTA-treated
blood was performed on semi-confluent monolayer of porcine
kidney cells (PK15, ATCC-CCL33) (Dewulf et al., 2004a). After 48
hours, the cells were fixed with isopropanol and stained with
polyclonal fluorescein-conjugated anti-CSFV immunoglobulins. Cultures were passaged one to three times.

After RNA extraction with the Viral RNA kit (Qiagen Benelux B.V., Venlo, The Netherlands), the presence of viral RNA molecules was investigated in the tonsils and blood samples by real-time RT-PCR (rRT-PCR). The rRT-PCR described by Hoffmann et al (2005) and the commercial TaqVet CSF rRT-PCR kit (Laboratoire Service International, Lissieu, France) were used for CSFV-specific genome detection. In addition, one BVDV-specific (Letellier and Kerkhofs, 2003) and one Pestivirus-specific (pan-pesti) (Hoffmann et al., 2006) rRT-PCR assays were used for CP7E2 alf virus RNA detection. The rRT-PCR assays were performed using an ABI 7500 FAST machine (Applied Biosystems, Lennik, Belgium) and analyzed using the Sequence Detection Software 1.4. For CSFV-, BVDV- and pan-pesti-specific rRT-PCRs, threshold cycle (Ct) values lower than 42 were considered positive.

Serological analysis

The CSFV E2 and E\text{rs} antibody responses were investigated with the HerdChek CSFV ELISA (IDEXX Europe B.V., Schiphol-Rijk, The Netherlands) and PrioCHECK CSFV E\text{rs} ELISA (kindly provided by Prionics A.G., Zurich, Switzerland) respectively. Values obtained in the ELISAs were expressed as blocking percentages. Blocking percentages between 30 and 40% in the E2 ELISA were considered
as doubtful while those above 40% were considered positive. Blocking percentages in the E\textsubscript{ns} ELISA were considered as positive when above 50%. The neutralising response was determined in the virus neutralisation test (VNT) according to the OIE manual (Anonymous, 2004) using two viral strains: Alfort/187 and CP7E2alf. For VNT, 50% neutralising doses (ND\textsubscript{50}) were expressed as Log\textsubscript{10}\textsuperscript{-} transformed values of neutralising antibody titres, with values greater than one regarded as positive.

**Statistical analysis**

The data were presented as medians with either the corresponding range of values or the 25\textsuperscript{th} and 75\textsuperscript{th} percentiles. The temperature, haematological and serological data (E2 and E\textsubscript{ns} ELISA blocking percentage, virus neutralising titres) collected after vaccination with CP7E2alf and C-strain were subject to statistical test for difference between groups. The two vaccines groups were considered as two independent populations. The data were compared per day after linear ranking with the Wilcoxon-Mann-Whitney test for nonparametric samples (XLstat, Addinsoft). The null hypothesis was considered rejected at P≤0.05.
Results

Clinical examination

Clinical disease was not recorded in the two groups vaccinated with the CP7E2alf and C-strain using the referred scoring system. The maximal daily cumulative clinical score per animal observed was 2 and 5 in CP7E2alf and C-strain groups respectively. Median rectal temperatures remained below 40.5°C during the 98 dpv (Figure 1). Between 2 and 21 dpv, 16 febrile animals with rectal temperatures below 42°C were recorded in the CP7E2alf group. Fever generally disappeared after one to five days. During this time, 12 transiently febrile animals, with fever during one to two days, were observed in the C-strain group. In the two vaccine groups, fever could not be linked with an increased clinical scoring. Temperatures of both groups were statistically distinct using the Wilcoxon-Mann-Whitney test at 3, 7 to 10, 12 to 28, 49 to 67, 74 to 77 and 84 to 91 dpv with $P \leq 0.001$ from 8 to 10, 25, 49 and 56 dpv, with $P \leq 0.01$ at 7, 18 and 67 to 84 dpv and $P \leq 0.05$ at 3, 21, 28, 60 to 63 and 91 dpv.

Haematology

The initial median PBL population before infection was 20.5 x 10³ cells/µl (with range from 15.7 to 25.5 x 10³) and 20.7 x 10³ cells/µl (with range from 17.8 to 28.3 x 10³) for the C-strain and CP7E2alf groups, respectively (Figure 2). Leucopaenia was not observed in either vaccinated group. PBL populations of both groups were
significantly distinct at 4 (P≤0.01), 5 (P≤0.05) and 7 dpv (P≤0.01), as well as at 28 (P≤0.001) and 63 dpv (P≤0.01) by the Wilcoxon-Mann-Whitney test.

4 Vaccine virus detection in sequential tonsil samples

The CP7E2alf virus was only isolated on cell culture from the tonsils of two animals at 4 and at 5 dpv, respectively (Table 1). The marker vaccine virus genome was detected in the tonsils of four more animals at 7, 18, 21 and 63 dpv by BVDV-specific rRT-PCR. However, CP7E2alf vaccine genome was not detected in the tonsils by the pan-pesti-specific rRT-PCR, nor could the vaccine virus be detected in the tonsils by the two CSFV-specific rRT-PCRs.

The C-strain vaccine virus was isolated from the tonsils collected between 3 and 13 dpv except for one animal at 3 dpv and another at 8 dpv. CSFV-specific rRT-PCRs detected the vaccine virus genome in the tonsils from 3 until 77 dpv with only one of the two collected samples being positive at 3, 63, 70 and 77 dpv. At the end of the experiment (98 dpv) the C-strain vaccine virus was still present in one of the two remaining animals.

5 Vaccine virus detection in sequentially collected blood samples

The VI and rRT-PCR assays revealed no viraemia in blood samples from the CP7E2alf vaccinated group (Table 2). In contrast, viraemia started between 3 and 8 dpv in the C-strain group, as detected by
VI. The majority of the animals (14 pigs) were viraemic for only one day but some others for up to three days (2 animals) (data not shown). C-strain vaccine RNA was detected by CSFV-specific rRT-PCRs in blood samples collected between 3 and 11 dpv with a mean duration of two days (data not shown). Four additional rRT-PCR positive samples were detected up to 42 dpv in the C-strain vaccine group.

CSFV E2-specific antibody response

Seroconversion to CSFV E2-specific antigen was first observed on day 15 dpv (2 out of 26 animals) in the CP7E2alf group and on day 11 dpv (19 out of 28 animals) in the C-strain group (Figures 3A and 3B). At 42 dpv all animals but one had seroconverted in the CP7E2alf group within the range of 61.24 to 92.64 E2 blocking percents. Decrease of the median value observed after 77 dpv is mainly imputed to random slaughtering of strongly E2 positive animals. After 70 dpv, blocking percentages above 80% were detected in sera from 5 of the 8 remaining animals while at 91 dpv in only one of the 4 remaining animals. Decrease of antibody titre after 77 dpv was only observed in two animals, with the weakest positive reactions, which were slaughtered at 91 and 98 dpv.

In the C-strain group, all animals presented seroconversion at 18 dpv within the range of 40.95 to 74.66 %. The E2 blocking percentage values of the two groups were significantly different
from 11 to 91 dpv with $P \leq 0.001$ from 11 to 70 dpv, $P \leq 0.01$ at 77 and 84 dpv and $P \leq 0.05$ at 91 dpv. Individual variations in the E2-specific antibody response, noticed by high range of E2 blocking percentages, were observed in the CP7E2alf group with some animals exhibiting delayed, low intensity or transient seroconversion.

**CSFV E$^{ms}$-specific antibody response**

E$^{ms}$ blocking percentages in the C-strain and CP7E2alf groups were significantly different between 28 and 91 dpv, with $P \leq 0.05$ at 28, 84 and 91 dpv, $P \leq 0.01$ from 63 to 77 dpv and $P \leq 0.001$ from 35 to 56 dpv. No animal seroconverted in the CP7E2alf group and the E$^{ms}$ blocking percentages remained negative until 98 dpv (Figures 4A and 4B). Seroconversion to the CSFV E$^{ms}$-specific antigen was first observed on 15 dpv (3 out of 26 animals) in the C-strain group within the range of positivity from 52.88 to 60.27 E$^{ms}$ blocking percents. At 35 dpv, 11 of the 20 remaining animals of the C-strain group were E$^{ms}$ positive for E$^{ms}$ (range of positivity from 50.24 to 75.73%). Proportion of E$^{ms}$ positive animals increased until 91 dpv when all the remaining animals presented seroconversion within the range of positivity from 51.78 to 87.31%). Individual variations in the E$^{ms}$ response were observed among animals in the C-strain group, with some transient positive responses.

**VNT response**
In the VNT with the Alfort/187 strain, neutralising antibodies were first
detected 13 days after CP7E2alf vaccination, in one of the 28
remaining animals (Figures 5A and B). Most of the animals
seroconverted between 18 and 35 dpv, with the last one at 49 dpv.
At that time, the range of positive values extended from 1.00 to 2.51
ND<sub>50</sub>. Decrease of the median value observed from 77 dpv is mainly
due to random slaughtering of strongly VNT positive animals. At 77
dpv, 4 animals of the 8 remaining had VNT titres above 2.00 ND<sub>50</sub>
while one was negative. At 91 dpv, the negative animal was still
present along with one strongly positive pig. Reduction in the VNT
titre after 77 dpv was only observed for one positive animal
slaughtered at 84 dpv.

Using the homologous strain in the VNT (CP7E2alf), the onset of
neutralising antibodies was observed earlier, at 11 dpv, in three out
of the 27 CP7E2alf-vaccinated animals (Figure 5). Most of the
animals (22/26 animals) presented neutralising antibodies at 18 dpv
within the range of positivity from 0.70 to 2.20 ND<sub>50</sub>. The last animal
seroconverted at 49 dpv. The median ND<sub>50</sub> value increased from 11
dpv until 56 dpv and then decreased up to 98 dpv. At 77 dpv, 5
animals of the 8 remaining had VNT titres above 2.00 ND<sub>50</sub> while at
91 dpv only one VNT positive animal remained. Reduction of VNT
titres after 77 dpv was observed in the three low positive animals
slaughtered at 91 and 98 dpv. VNT values obtained with Alfort/187
and CP7E2alf in the CP7E2alf group were significantly different
between 13 and 54 dpv (P ≤ 0.001), at 63 dpv (P ≤ 0.01) and between 70 and 91 (P ≤ 0.05). As previously described for the E2 ELISA, individual variations were observed in the VNT response to vaccination in the CP7E2alf group. Some animals had a delayed seroconversion occurring later than 35 dpv. Others seroconverted with low neutralising titres while transient seroconversion was also observed.

In the C-strain group, neutralising antibodies were observed from 11 dpv in 7 out of 28 animals (Figure 5). At 18 dpv, neutralising antibodies were detected in all the animals of the C-strain group within the range of positivity from 1.00 to 2.20 ND$_{50}$. The median ND$_{50}$ value increased from 11 dpv until 98 dpv.

VNT values obtained with Alfort/187 in the two vaccination groups were significantly different from 11 to 91 dpv, with P ≤ 0.001 from 11 to 77 dpv, P ≤ 0.01 at 84 dpv and P ≤ 0.05 at 91 dpv. Comparing the mean VN titres obtained in the CP7E2alf group with the homologous strain and in the C-strain group with Alfort/187, significant differences were observed at 11 dpv (P ≤ 0.05), between 21 and 28 dpv (P ≤ 0.05) and at 84 dpv (P ≤ 0.01).
Discussion

Vaccine virus distribution after CP7E2alf vaccination has previously been described up to 42 dpv (Reimann et al., 2004; Koenig et al., 2007a) but data for longer persistence in the tissues and blood are lacking. Similarly, although the persistence of serological response induced by C-strain vaccine has already been established for several years (Kaden and Lange, 2001; van Oirschot, 2003), it has not been monitored for more than 42 days after CP7E2alf vaccination (Koenig et al., 2007a). Therefore, the presence of CP7E2alf and C-strain virus in the tonsils and blood and the host serological response were investigated during 98 dpv in this study to determine the vaccine virus persistence in tonsils and blood and further evaluate the duration of the CP7E2alf serological response.

As reported in the present study, the chimeric CP7E2alf virus was present in the tonsils early after oronasal vaccination. This result confirms other observations done after CP7E2alf intramuscular administration (Reimann et al., 2004; Koenig et al., 2007a). More importantly, the time point of detection in the tonsils was similar for CP7E2alf and C-strain which confirms previous reports (Lorena et al., 2001; Kaden et al., 2004). This not only suggests that the tonsils are the initial replication site for CP7E2alf, as already described for C-strain vaccine virus and for virulent CSFV strains (Mittelholzer et al., 2000; Tignon et al., 2008), but further confirms the CSFV-like
behaviour of the CP7E2alf chimera (Reimann et al., 2004). The specific design of our animal experiment allowed for the first time the evaluation of the persistence of CP7E2alf vaccine for an extended period. Hereby, it could be demonstrated that CP7E2alf vaccine virus persisted in the tonsils for a similar length to C-strain, up to 63 dpv and more than 77 dpv respectively.

The viraemia observed after intramuscular CP7E2alf vaccination by Koenig et al. (2007a) was not confirmed on blood samples collected in this study. A different administration route and/or the nature of the used samples (blood instead of purified leucocytes) may explain this result. However, transient viraemia was detected in the C-strain group, as previously described (Lorena et al., 2001; Koenig et al., 2007a). Nevertheless, the number of viraemic animals detected by VI and rRT-PCR, as well as the duration of viraemia, was lower than those observed after infection with a virulent strain (‘WIL-11722’) in similar experimental conditions (Tignon et al., 2008).

The serological response observed after oronasal C-strain vaccination was consistent with early onset and intensity established by Kaden and Lange (2001). In contrast, a slightly delayed and reduced response with high variability between animals was observed in the CP7E2alf group that has not been described previously (Reimann et al., 2004; Koenig et al., 2007a, 2007b; Kaden et al., 2008). In those studies, domestic pigs or wild boars were vaccinated intramuscularly with $10^{5.3}$ to $10^7$ TCID$_{50}$/ml or
orally with $10^{5.75}$ to $10^{6.5}$ TCI$D_{50}$/ml of CP7E2alf marker vaccine. This suggests that the reduced vaccine dose combined with oronasal delivery may be the determining factors for our results (Reimann et al., 2004; Koenig et al., 2007a, 2007b; Kaden et al., 2008). This is supported by a recent report of dose effect on the onset and intensity of the serological response after CP7E2alf vaccination (Leifer et al., 2008). Notwithstanding the delayed serological response, our data showed that neutralising and total antibody titres remained stable up to 77 dpv. At that time, the observed decrease of the mean serological response could mostly be explained by elimination of animals with higher serological response during the random slaughtering procedure. However, antibodies persisted to the end of the experiment, at least in one of the two remaining animals. Nonetheless, the present study demonstrated the immunogenic potential of CP7E2alf despite a suboptimal vaccine dose, such as it may occur where there is incomplete bait uptake by animals or where vaccine titre is reduced in the field or during the production process (Kaden et al., 2008). Moreover, variations here observed in VNT titres using different CSFV VNT strains also indicated that cross-neutralisation of CP7E2alf with CSFV was influenced by the antigenicity of the BVDV backbone of the chimera and the homology with E2 (Dekker et al., 1995; van Rijn et al., 1997), thus adjusting the conclusion of Koenig et al. (2007b) that E2 is the major or even the only pestiviral protein that induces
relevant amounts of neutralising antibodies. Therefore, the cross-reactivity of the marker vaccine with CSFV genotypes representative of field strains should be further evaluated.

It was not the purpose of the present study to directly evaluate the protective capacity of CP7E2alf, as this has already been demonstrated elsewhere (Reimann et al., 2004; Koenig et al., 2007b). The protective role of neutralising antibodies has been established elsewhere (Terpstra and Wensvoort, 1988; Suradhat et al., 2001). If it is generally accepted that vaccinated pigs with active neutralising antibody titres higher than 1.50 ND$_{50}$ are protected against challenge (Terpstra and Wensvoort, 1988; review in Granges et al., 2007 and in Suradhat et al., 2007), the VNT titres observed in some of the CP7E2alf vaccinated animals of this experiment may not be conclusive based on this minimal protective VNT value. However, protection after vaccination has even been observed in the absence of neutralising antibodies (reviewed by Ganges et al., 2007 and Suradhat et al., 2007), indicating that other aspects of the immune response may also be involved. Nevertheless, the late seroconversion and reduced VNT titres, obtained after vaccination with suboptimal dose, as well as differences in cross-reactivity observed in VNT, should be further investigated in regard to the protection, as well as the importance of cellular components in the immune response against CSFV infection.
The lack of DIVA properties remains the major drawback of C-strain vaccination. Genetic differentiation between the chimera CP7E2alf and CSFV strains can be easily achieved with RT-PCRs or rRT-PCRs targeting non-homologous regions (Vilcek and Belak, 1997; Hoffmann et al., 2005; Haegeman et al., 2006; Beer et al., 2007). This is clearly demonstrated in the present study where CSFV-specific molecular detection methods (rRT-PCRs) targeting the CSFV 5'UTR region did not detect the CP7E2alf vaccine in tonsils and blood while BVDV-specific rRT-PCR did. By contrast, those rRT-PCR tests are not discriminatory for C-strain vaccine and other CSVF strains (Hoffmann et al., 2005; Tignon et al. 2008). Although certain RT-PCR and rRT-PCRs have been developed to differentiate between C-strain and certain CSFV genotypes (Pan et al., 2008; Zhao et al., 2008), none is currently available which is able to differentiate C-strain from all CSFV genotypes.

In view of the construction of the chimeric vaccine virus, characterised by the presence of CSFV E2 but not CSFV E\textsuperscript{rns} gene (Reimann et al., 2004), the CP7E2alf allows a serological differentiation from wild-type CSFV. The latter can be clearly seen in this study as the CP7E2alf vaccinated animals did not seroconvert for CSFV E\textsuperscript{rns} but did for CSFV E2, confirming previous studies (Reimann et al., 2004; Koenig et al., 2007a, 2007b). As expected, no serological DIVA was possible for C-strain vaccine, as it induced CSFV-specific E2, E\textsuperscript{rns} and neutralising antibodies, in accordance
with previous descriptions (Kaden and Lange, 2001; Kaden et al., 2004) and similarly to the serological picture observed after infection with mildly to moderately virulent strains (Mittelholzer et al., 2000).

**Conclusion**

In conclusion, the initial replication of CP7E2alf and C-strain vaccines occurred in the tonsils, which is similar to the wild virus. The marker vaccine induced an immune response observed up to 98 days after oronasal administration in domestic pigs and allowed the use of serological DIVA tests combining CSFV-specific E2 and E\textsubscript{rms} antibody detection. This study confirms the suitability of CP7E2alf as a interesting and practical marker vaccine candidate for the oral vaccination programmes to control classical swine fever in domestic pigs and wild boar.
Acknowledgements

We would like to thank Prof. Sándor Belák and Dr Michael O’Connor for suggestions, discussions, and critical comments. We are also grateful to R. Debaugnies, M.-L. Denne, F. Jebbari and C. Thoraval at the VAR and the laboratory team at the DVMP for technical support and we address a special thanks to the technicians at the DVMP for excellent animal care. This study was funded by the European Union in the 6FP project CSFVACCINE & WILDBOAR (SSP1-501599). The animal experiments were conducted with the approval of the VAR and DVMP ethical committees.

Conflict of Interest

None
References


Terpstra, C., Robijns, K.G., 1977, Experience with regional vaccination against swine fever in enzootic areas for limited periods using C-strain virus. Tijdschr Diergeneeskde 102, 106-112.


quantitative and differential detection of wild-type viruses and C-strain vaccine of Classical swine fever virus. Vet Microbiol 126, 1-10.
Table 1: Detection of CP7E2alf and C-strain vaccine viruses in the tonsils of slaughtered animals of CP7E2alf and C-strain vaccine groups by virus isolation and rRT-PCR assays between 0 to 98 days post administration

| dpv | Animals | VI | rRT-PCRs (Ct values*) | | dpv | Animals | VI | rRT-PCRs (Ct values*) |
|-----|---------|----|-----------------------| | | | | | |
|     |         |    | CSFV (a,b) | BVDV (c) | pan-pesti (d) | | | CSFV (a) | CSFV (b) |
| 0   | #1      | -  | - | - | | #1   | -  | - | - | |
|     | #2      | -  | - | - | | #2   | -  | - | - | |
| 1   | #3      | -  | - | - | | #3   | -  | - | - | |
|     | #4      | -  | - | - | | #4   | -  | - | - | |
| 2   | #5      | -  | - | - | | #5   | -  | - | - | |
|     | #6      | -  | - | - | | #6   | -  | - | - | |
| 3   | #7      | -  | - | - | | #7   | -  | - | - | |
|     | #8      | -  | - | - | | #8   | -  | - | - | |
| 4   | #9      | +  | - | 35.8 | - | #9   | +  | 34.9 | 36.1 | |
|     | #10     | -  | - | - | | #10  | +  | 32.7 | 33.2 | |
| 5   | #11     | -  | - | - | | #11  | +  | 38.1 | 35.6 | |
|     | #12     | +  | - | 33.8 | - | #12  | +  | 33.1 | 32.0 | |
| 6   | #13     | -  | - | - | | #13  | +  | 33.9 | 33.6 | |
|     | #14     | -  | - | - | | #14  | +  | 32.2 | 32.4 | |
| 7   | #15     | -  | - | 35.6 | - | #15  | +  | 31.0 | 30.6 | |
|     | #16     | -  | - | - | | #16  | +  | 36.3 | 37.6 | |
| 8   | #17     | -  | - | - | | #17  | +  | 33.2 | 33.4 | |
|     | #18     | -  | - | - | | #18  | -  | 35.4 | 36.2 | |
| 13  | #19     | -  | - | - | | #19  | +  | 36.6 | 36.8 | |
|     | #20     | -  | - | - | | #20  | +  | 37.1 | 38.3 | |
| 18  | #21     | -  | - | - | | #21  | -  | 35.7 | 36.9 | |
|     | #22     | -  | - | 37.2 | - | #22  | -  | 39.5 | 39.2 | |
| 21  | #23     | -  | - | - | | #23  | -  | 38.4 | 39.2 | |
|     | #24     | -  | - | 34.7 | - | #24  | -  | 37.0 | 41.0 | |
| 28  | #25     | -  | - | - | | #25  | -  | 38.4 | 39.4 | |
|     | #26     | -  | - | - | | #26  | -  | 32.0 | 30.7 | |
| 35  | #27     | -  | - | - | | #27  | -  | 39.8 | - | |
|     | #28     | -  | - | - | | #28  | -  | 39.8 | 37.9 | |
| 42  | #29     | -  | - | - | | #29  | -  | - | 40.6 | |
|     | #30     | -  | - | - | | #30  | -  | - | 41.3 | |
| 49  | #31     | -  | - | - | | #31  | -  | 39.6 | 40.3 | |
|     | #32     | -  | - | - | | #32  | -  | 40.8 | - | |
| 56  | #33     | -  | - | - | | #33  | -  | 41.2 | - | |


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1 VI: virus isolation, Ct: threshold cycle, a: TaqVet CSFV rRT-PCR kit, b:

2 Hoffmann et al., 2005, c: Letellier et al., 2003, d: Hoffmann et al.,

3 2006, -: negative, +: positive, *: Ct values ≥42 were considered

4 negative
Table 2: Detection of CP7E2alf and C-strain vaccine viruses in blood samples collected from animals of CP7E2alf and C-strain vaccine groups by virus isolation and rRT-PCR assays between 0 to 98 days post administration

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VI: virus isolation, b: Hoffmann et al., 2005, c: Letellier et al., 2003. Ct: threshold cycle, SD: Standard deviation, - : Ct negative (≥42), (*) : no SD due to only one positive sample
Figure 1: Evolution of rectal temperatures (lines) and clinical scores (vertical bars), expressed as median and 25\textsuperscript{th} and 75\textsuperscript{th} percentiles, in groups of pigs after oronasal administration of CP7E2alf (black circle) and C-strain vaccines (white bar and white square). The median of clinical scores remains null for CP7E2alf vaccinated group. Dash line indicates the fever limit (40.5°C).

Figure 2: Evolution of peripheral blood leucocyte (PBL) population counts, expressed as median and 25\textsuperscript{th} and 75\textsuperscript{th} percentiles, in groups of pigs after oronasal administration of CP7E2alf (black circle) and C-strain vaccines (white square). Dash line indicates the leucopaenia limit (10,000 leukocytes/µl). Asterisks indicate values significantly different between groups with $P \leq 0.05$ (*), $P \leq 0.01$ (**) and $P \leq 0.001$ (***)

Figure 3: Proportion of CSFV E2 antibody positive animals (A) and range of blocking E2 antibody percentages (B) for positive animals, expressed as median and 25\textsuperscript{th} and 75\textsuperscript{th} percentiles, after oronasal administration of CP7E2alf (black bar and black circle) and C-strain vaccines (white bar and white square). Dash line indicates the cut-off value for E2 blocking percentage (40%). Asterisks indicate values significantly different between groups with $P \leq 0.05$ (*), $P \leq 0.01$ (**) and $P \leq 0.001$ (***)
Figure 4: Proportion of CSFV E<sub>ns</sub> antibody positive animals (A) and range of blocking E<sub>ns</sub> antibody percentages (B) for positive animals, expressed as median and 25<sup>th</sup> and 75<sup>th</sup> percentiles, after oronasal administration of CP7E2alf and C-strain vaccines (white bar and white square). Proportion of E<sub>ns</sub> seroconverting animals in the CP7E2alf vaccinated group remains null. Dash line indicates the cut-off value for E<sub>ns</sub> blocking percentage (50%). Asterisks indicate values significantly different between groups with P ≤ 0.05 (*), P ≤ 0.01 (**), and P ≤ 0.001 (***)

Figure 5: Proportion of animals positive in VNT (A) and range of neutralising titres (B) for positive animals after oronasal administration of CP7E2alf or C-strain vaccines, expressed as median and 25<sup>th</sup> and 75<sup>th</sup> percentiles. VNT titres were obtained in CP7E2alf vaccine group with Alfort/187 (gray bar and gray circle) CP7E2alf (black bar and black circle) as VNT strain and in C-strain vaccine group with Alfort/187 strain (white bar and white square). Dash line indicates the cut-off value for VNT (1 ND<sub>50</sub>).