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1 Title:

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

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7 Short running title:

8 Classical swine fever: CP7E2alf and C-strain vaccines

9

## 1 **Abstract**

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

10 C-strain vaccine virus was consistently detected in tonsils of all  
11 (n=30) animals from 3 to 77 days post vaccination (dpv) and in  
12 blood (n= 36) between 3 and 13 dpv by CSFV-specific rRT-PCR.  
13 CP7E2alf virus RNA was detected in 6 animals slaughtered between  
14 4 and 63 dpv by a BVDV-specific rRT-PCR. The chimeric virus was not  
15 detected in blood samples.

16 As detected by CSFV E2-specific antibody ELISA and virus  
17 neutralisation, seroconversion first occurred at 11 dpv in the C-strain  
18 vaccinated group and between 11 and 15 dpv in the CP7E2alf  
19 vaccinated group. Serological response was still observed at 98  
20 dpv. CP7E2alf serological response remained negative using the  
21 CSFV E<sub>rnS</sub> ELISA whereas seroconversion occurred in C-strain  
22 vaccinated group.

23 In conclusion, the primary replication site of CP7E2alf vaccine virus  
24 was found to be the tonsils similarly to C-strain and virulent field

1 strains. Persistence of CP7E2alf in the tonsils was also demonstrated  
2 up to 63 dpv. Both vaccines showed immunogenicity after oronasal  
3 administration in domestic pigs. In contrast to the C-strain, CP7E2alf  
4 vaccine allowed the use of DIVA approaches in serological tests.  
5 This study confirms CP7E2alf as a promising marker vaccine  
6 candidate for oronasal vaccination programmes to control CSF in  
7 domestic pigs and wild boar.

8 **Keywords**

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

11

## 1 Introduction

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

9 The effectiveness of modified live vaccines, such as the Chinese  
10 strain (C-strain), has been frequently reported in domestic pigs and  
11 in wild boar (Terpstra and Robijns, 1977; Chenut et al., 1999; Kaden  
12 et al., 2000; Kaden and Lange, 2001; Dewulf et al., 2004b; Dong and  
13 Chen, 2007). Such vaccines induce protection shortly after  
14 administration. Prophylactic mass vaccination with modified live  
15 vaccines, combined with culling of infected pigs, successfully  
16 resulted in the eradication of the disease in most countries of the  
17 European Union (EU) (Vandeputte and Chappuis, 1999; Greiser-  
18 Wilke and Moennig, 2004). Currently, modified live vaccines are still  
19 in use where the disease is endemic in domestic pigs and for  
20 disease control in wild boar (von Rden et al., 2008). Therefore, strict  
21 restrictions on the international trade in pig products are  
22 implemented from countries using vaccination (Greiser-Wilke and  
23 Moennig, 2004). The lack of serological discrimination between

1 naturally infected animals and animals vaccinated with a modified  
2 live vaccine hampers disease control relying on serology (van  
3 Oirschot, 2003; Pasick, 2004). To overcome this problem, marker  
4 vaccines which allow differentiation of infected from vaccinated  
5 animals (DIVA) have been developed.

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

16 A new generation of live marker vaccines was developed based on  
17 the construction of chimeric pestiviruses (for review see Beer et al.,  
18 2007). The E2 or E<sub>2ns</sub>-coding region of CSFV or cytopathogenic  
19 bovine viral diarrhoea virus (BVDV) strain CP7 has been replaced by  
20 that of a heterologous strain (van Gennip et al., 2000; Reimann et  
21 al., 2004; Rasmussen et al., 2007; Wehrle et al., 2007). One of these  
22 constructs, CP7E2alf, is based on the infectious full-length clone of  
23 BVDV strain CP7 in which the BVDV E2 gene is replaced by that of



1 CSFV strain Alfort/187 (Reimann et al., 2004). CP7E2alf proved to be  
2 completely safe in pigs following intramuscular inoculation. Neither  
3 viraemia, nor virus transmission to contact animals were detected.  
4 Furthermore, immunogenicity and protective effectiveness have  
5 been demonstrated after intramuscular administration in pigs  
6 (Reimann et al., 2004; Koenig et al., 2007a) and oral vaccination in  
7 wild boar (Koenig et al., 2007b; Kaden et al., 2008). Additionally, the  
8 DIVA properties of this live vaccine have been described (Reimann  
9 et al., 2004). Nevertheless additional data regarding  
10 immunogenicity after oronasal administration, dose effect and  
11 persistence over a longer period are required to improve vaccine  
12 characterisation and application efficiency.

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

21

## 1 **Materials and methods**

### 2 **Vaccine**

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

### 11 **Animals**

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

### 18 **Experimental design**

19 The study design, the administration dose and routes were similar to  
20 a previous experimental infection with a field isolate ('WIL-11722')  
21 (Tignon et al., 2008). Vaccination with CP7E2alf and C-strain were  
22 conducted separately with an interval of six months.

1 Briefly, weaner pigs were housed in a biosecure unit in agreement  
2 with EU directive 91/630/EEC and its amendments 2001/88/EC and  
3 2001/93/EC. Following a seven-day acclimatization period, the pigs  
4 were clinically inspected and blood sampled. On the first day (day  
5 0), CP7E2alf live marker vaccine was administered via the  
6 intranasal (1 ml) and the oral (1 ml) routes by means of a syringe to  
7 all animals of the first group (n=46), except two pigs kept as  
8 negative controls. The two non-vaccinated controls were  
9 slaughtered on the day of vaccination by an intravenous  
10 pentobarbital injection, followed by exsanguination. Subsequently,  
11 two randomly selected vaccinated animals were slaughtered on a  
12 daily basis between 1 and 8 dpv, at 13 and 18 dpv, and then  
13 weekly from 21 to 98 dpv.

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

#### 18 **Clinical examination and sample collection**

19 All the living animals were clinically monitored using a scoring  
20 system (Mittelholzer et al., 2000) based on the observation of ten  
21 parameters (breathing, liveliness, body tension, body shape,  
22 walking, skin, eyes, appetite, defecation and leftovers) and the  
23 rectal temperatures were recorded on a daily basis, before sample

1 collection, during the first 8 days and subsequently bi-weekly until 98  
2 dpv. Animals were considered clinically ill when their daily  
3 cumulative clinical score exceeded the value of 6. Pigs were  
4 considered febrile when rectal temperatures exceeded 40.5°C.

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

### 11 **Haematology**

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

### 18 **Virological analysis**

19 Virus isolation (VI) from the tonsil samples and from EDTA-treated  
20 blood was performed on semi-confluent monolayer of porcine  
21 kidney cells (PK15, ATCC-CCL33) (Dewulf et al., 2004a). After 48  
22 hours, the cells were fixed with isopropanol and stained with

1 polyclonal fluorescein-conjugated anti-CSFV immunoglobulins.  
2 Cultures were passaged one to three times.

3 After RNA extraction with the Viral RNA kit (Qiagen Benelux B.V.,  
4 Venlo, The Netherlands), the presence of viral RNA molecules was  
5 investigated in the tonsils and blood samples by real-time RT-PCR  
6 (rRT-PCR). The rRT-PCR described by Hoffmann et al (2005) and the  
7 commercial TaqVet CSF rRT-PCR kit (Laboratoire Service  
8 International, Lissieu, France) were used for CSFV-specific genome  
9 detection. In addition, one BVDV-specific (Letellier and Kerkhofs,  
10 2003) and one Pestivirus-specific (pan-pesti) (Hoffmann et al., 2006)  
11 rRT-PCR assays were used for CP7E2alf virus RNA detection. The rRT-  
12 PCR assays were performed using an ABI 7500 FAST machine  
13 (Applied Biosystems, Lennik, Belgium) and analyzed using the  
14 Sequence Detection Software 1.4. For CSFV-, BVDV- and pan-pesti-  
15 specific rRT-PCRs, threshold cycle (Ct) values lower than 42 were  
16 considered positive.

### 17 **Serological analysis**

18 The CSFV E2 and E<sup>rns</sup> antibody responses were investigated with the  
19 HerdChek CSFV ELISA (IDEXX Europe B.V., Schiphol-Rijk, The  
20 Netherlands) and PrioCHECK CSFV E<sup>rns</sup> ELISA (kindly provided by  
21 Prionics A.G., Zurich, Switzerland) respectively. Values obtained in  
22 the ELISAs were expressed as blocking percentages. Blocking  
23 percentages between 30 and 40% in the E2 ELISA were considered

1 as doubtful while those above 40% were considered positive.  
2 Blocking percentages in the E<sup>rns</sup> ELISA were considered as positive  
3 when above 50%. The neutralising response was determined in the  
4 virus neutralisation test (VNT) according to the OIE manual  
5 (Anonymous, 2004) using two viral strains: Alfort/187 and CP7E2alf.  
6 For VNT, 50% neutralising doses (ND<sub>50</sub>) were expressed as Log<sub>10</sub>-  
7 transformed values of neutralising antibody titres, with values  
8 greater than one regarded as positive.

### 9 **Statistical analysis**

10 The data were presented as medians with either the corresponding  
11 range of values or the 25<sup>th</sup> and 75<sup>th</sup> percentiles. The temperature,  
12 haematological and serological data (E2 and E<sup>rns</sup> ELISA blocking  
13 percentage, virus neutralising titres) collected after vaccination with  
14 CP7E2alf and C-strain were subject to statistical test for difference  
15 between groups. The two vaccines groups were considered as two  
16 independent populations. The data were compared per day after  
17 linear ranking with the Wilcoxon-Mann-Whitney test for  
18 nonparametric samples (XLstat, Addinsoft). The null hypothesis was  
19 considered rejected at  $P \leq 0.05$ .

20

## 1 **Results**

### 2 **Clinical examination**

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

### 18 **Haematology**

19 The initial median PBL population before infection was  $20.5 \times 10^3$   
20 cells/ $\mu$ l (with range from 15.7 to  $25.5 \times 10^3$ ) and  $20.7 \times 10^3$  cells/ $\mu$ l  
21 (with range from 17.8 to  $28.3 \times 10^3$ ) for the C-strain and CP7E2alf  
22 groups, respectively (Figure 2). Leucopaenia was not observed in  
23 either vaccinated group. PBL populations of both groups were

1 significantly distinct at 4 ( $P \leq 0.01$ ), 5 ( $P \leq 0.05$ ) and 7 dpv ( $P \leq 0.01$ ), as  
2 well as at 28 ( $P \leq 0.001$ ) and 63 dpv ( $P \leq 0.01$ ) by the Wilcoxon-Mann-  
3 Whitney test.

#### 4 **Vaccine virus detection in sequential tonsil samples**

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

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

#### 19 **Vaccine virus detection in sequentially collected blood samples**

20 The VI and rRT-PCR assays revealed no viraemia in blood samples  
21 from the CP7E2alf vaccinated group (Table 2). In contrast, viraemia  
22 started between 3 and 8 dpv in the C-strain group, as detected by



1 VI. The majority of the animals (14 pigs) were viraemic for only one  
2 day but some others for up to three days (2 animals) (data not  
3 shown). C-strain vaccine RNA was detected by CSFV-specific rRT-  
4 PCRs in blood samples collected between 3 and 11 dpv with a  
5 mean duration of two days (data not shown). Four additional rRT-  
6 PCR positive samples were detected up to 42 dpv in the C-strain  
7 vaccine group.

#### 8 **CSFV E2-specific antibody response**

9 Seroconversion to CSFV E2-specific antigen was first observed on  
10 day 15 dpv (2 out of 26 animals) in the CP7E2alf group and on day  
11 11 dpv (19 out of 28 animals) in the C-strain group (Figures 3A and  
12 3B). At 42 dpv all animals but one had seroconverted in the  
13 CP7E2alf group within the range of 61.24 to 92.64 E2 blocking  
14 percents. Decrease of the median value observed after 77 dpv is  
15 mainly imputed to random slaughtering of strongly E2 positive  
16 animals. After 70 dpv, blocking percentages above 80% were  
17 detected in sera from 5 of the 8 remaining animals while at 91 dpv  
18 in only one of the 4 remaining animals. Decrease of antibody titre  
19 after 77 dpv was only observed in two animals, with the weakest  
20 positive reactions, which were slaughtered at 91 and 98 dpv.

21 In the C-strain group, all animals presented seroconversion at 18  
22 dpv within the range of 40.95 to 74.66 %. The E2 blocking  
23 percentage values of the two groups were significantly different

1 from 11 to 91 dpv with  $P \leq 0.001$  from 11 to 70 dpv,  $P \leq 0.01$  at 77 and  
2 84 dpv and  $P \leq 0.05$  at 91 dpv. Individual variations in the E2-specific  
3 antibody response, noticed by high range of E2 blocking  
4 percentages, were observed in the CP7E2alf group with some  
5 animals exhibiting delayed, low intensity or transient seroconversion.

#### 6 **CSFV E<sup>rns</sup>-specific antibody response**

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

#### 22 **VNT response**

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

13 Using the homologous strain in the VNT (CP7E2alf), the onset of  
14 neutralising antibodies was observed earlier, at 11 dpv, in three out  
15 of the 27 CP7E2alf-vaccinated animals (Figure 5). Most of the  
16 animals (22/26 animals) presented neutralising antibodies at 18 dpv  
17 within the range of positivity from 0.70 to 2.20 ND<sub>50</sub>. The last animal  
18 seroconverted at 49 dpv. The median ND<sub>50</sub> value increased from 11  
19 dpv until 56 dpv and then decreased up to 98 dpv. At 77 dpv, 5  
20 animals of the 8 remaining had VNT titres above 2.00 ND<sub>50</sub> while at  
21 91 dpv only one VNT positive animal remained. Reduction of VNT  
22 titres after 77 dpv was observed in the three low positive animals  
23 slaughtered at 91 and 98 dpv. VNT values obtained with Alfort/187  
24 and CP7E2alf in the CP7E2alf group were significantly different

1 between 13 and 54 dpv ( $P \leq 0.001$ ), at 63 dpv ( $P \leq 0.01$ ) and between  
2 70 and 91 ( $P \leq 0.05$ ). As previously described for the E2 ELISA,  
3 individual variations were observed in the VNT response to  
4 vaccination in the CP7E2alf group. Some animals had a delayed  
5 seroconversion occurring later than 35 dpv. Others seroconverted  
6 with low neutralising titres while transient seroconversion was also  
7 observed.

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

13 VNT values obtained with Alfort/187 in the two vaccination groups  
14 were significantly different from 11 to 91 dpv, with  $P \leq 0.001$  from 11 to  
15 77 dpv,  $P \leq 0.01$  at 84 dpv and  $P \leq 0.05$  at 91 dpv. Comparing the  
16 mean VN titres obtained in the CP7E2alf group with the homologous  
17 strain and in the C-strain group with Alfort/187, significant  
18 differences were observed at 11 dpv ( $P \leq 0.05$ ), between 21 and 28  
19 dpv ( $P \leq 0.05$ ) and at 84 dpv ( $P \leq 0.01$ ).

20

## 1 **Discussion**

2 Vaccine virus distribution after CP7E2alf vaccination has previously  
3 been described up to 42 dpv (Reimann et al., 2004; Koenig et al.,  
4 2007a) but data for longer persistence in the tissues and blood are  
5 lacking. Similarly, although the persistence of serological response  
6 induced by C-strain vaccine has already been established for  
7 several years (Kaden and Lange, 2001; van Oirschot, 2003), it has  
8 not been monitored for more than 42 days after CP7E2alf  
9 vaccination (Koenig et al., 2007a). Therefore, the presence of  
10 CP7E2alf and C-strain virus in the tonsils and blood and the host  
11 serological response were investigated during 98 dpv in this study to  
12 determine the vaccine virus persistence in tonsils and blood and  
13 further evaluate the duration of the CP7E2alf serological response.  
14 As reported in the present study, the chimeric CP7E2alf virus was  
15 present in the tonsils early after oronasal vaccination. This result  
16 confirms other observations done after CP7E2alf intramuscular  
17 administration (Reimann et al., 2004; Koenig et al., 2007a). More  
18 importantly, the time point of detection in the tonsils was similar for  
19 CP7E2alf and C-strain which confirms previous reports (Lorena et al.,  
20 2001; Kaden et al., 2004). This not only suggests that the tonsils are  
21 the initial replication site for CP7E2alf, as already described for C-  
22 strain vaccine virus and for virulent CSFV strains (Mittelholzer et al.,  
23 2000; Tignon et al., 2008), but further confirms the CSFV-like

1 behaviour of the CP7E2alf chimera (Reimann et al., 2004). The  
2 specific design of our animal experiment allowed for the first time  
3 the evaluation of the persistence of CP7E2alf vaccine for an  
4 extended period. Hereby, it could be demonstrated that CP7E2alf  
5 vaccine virus persisted in the tonsils for a similar length to C-strain, up  
6 to 63 dpv and more than 77 dpv respectively.

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

17 The serological response observed after oronasal C-strain  
18 vaccination was consistent with early onset and intensity  
19 established by Kaden and Lange (2001). In contrast, a slightly  
20 delayed and reduced response with high variability between  
21 animals was observed in the CP7E2alf group that has not been  
22 described previously (Reimann et al., 2004; Koenig et al., 2007a,  
23 2007b; Kaden et al., 2008). In those studies, domestic pigs or wild  
24 boars were vaccinated intramuscularly with  $10^{5.3}$  to  $10^7$  TCID<sub>50</sub>/ml or

1 orally with  $10^{5.75}$  to  $10^{6.5}$  TCID<sub>50</sub>/ml of CP7E2alf marker vaccine. This  
2 suggests that the reduced vaccine dose combined with oronasal  
3 delivery may be the determining factors for our results (Reimann et  
4 al., 2004; Koenig et al., 2007a, 2007b; Kaden et al., 2008). This is  
5 supported by a recent report of dose effect on the onset and  
6 intensity of the serological response after CP7E2alf vaccination  
7 (Leifer et al., 2008). Notwithstanding the delayed serological  
8 response, our data showed that neutralising and total antibody  
9 titres remained stable up to 77 dpv. At that time, the observed  
10 decrease of the mean serological response could mostly be  
11 explained by elimination of animals with higher serological response  
12 during the random slaughtering procedure. However, antibodies  
13 persisted to the end of the experiment, at least in one of the two  
14 remaining animals. Nonetheless, the present study demonstrated  
15 the immunogenic potential of CP7E2alf despite a suboptimal  
16 vaccine dose, such as it may occur where there is incomplete bait  
17 uptake by animals or where vaccine titre is reduced in the field or  
18 during the production process (Kaden et al., 2008). Moreover,  
19 variations here observed in VNT titres using different CSFV VNT strains  
20 also indicated that cross-neutralisation of CP7E2alf with CSFV was  
21 influenced by the antigenicity of the BVDV backbone of the  
22 chimera and the homology with E2 (Dekker et al., 1995; van Rijn et  
23 al., 1997), thus adjusting the conclusion of Koenig et al. (2007b) that  
24 E2 is the major or even the only pestiviral protein that induces

1 relevant amounts of neutralising antibodies. Therefore, the cross-  
2 reactivity of the marker vaccine with CSFV genotypes  
3 representative of field strains should be further evaluated.

4 It was not the purpose of the present study to directly evaluate the  
5 protective capacity of CP7E2alf, as this has already been  
6 demonstrated elsewhere (Reimann et al., 2004; Koenig et al.,  
7 2007b). The protective role of neutralising antibodies has been  
8 established elsewhere (Terpstra and Wensvoort, 1988; Suradhat et  
9 al., 2001). If it is generally accepted that vaccinated pigs with  
10 active neutralising antibody titres higher than 1.50 ND<sub>50</sub> are  
11 protected against challenge (Terpstra and Wensvoort, 1988; review  
12 in Granges et al., 2007 and in Suradhat et al., 2007), the VNT titres  
13 observed in some of the CP7E2alf vaccinated animals of this  
14 experiment may not be conclusive based on this minimal protective  
15 VNT value. However, protection after vaccination has even been  
16 observed in the absence of neutralising antibodies (reviewed by  
17 Ganges et al., 2007 and Suradhat et al., 2007), indicating that other  
18 aspects of the immune response may also be involved.  
19 Nevertheless, the late seroconversion and reduced VNT titres,  
20 obtained after vaccination with suboptimal dose, as well as  
21 differences in cross-reactivity observed in VNT, should be further  
22 investigated in regard to the protection, as well as the importance  
23 of cellular components in the immune response against CSFV  
24 infection.



1 The lack of DIVA properties remains the major drawback of C-strain  
2 vaccination. Genetic differentiation between the chimera CP7E2alf  
3 and CSFV strains can be easily achieved with RT-PCRs or rRT-PCRs  
4 targeting non-homologous regions (Vilcek and Belak, 1997;  
5 Hoffmann et al., 2005; Haegeman et al., 2006; Beer et al., 2007). This  
6 is clearly demonstrated in the present study where CSFV-specific  
7 molecular detection methods (rRT-PCRs) targeting the CSFV 5'UTR  
8 region did not detect the CP7E2alf vaccine in tonsils and blood  
9 while BVDV-specific rRT-PCR did. By contrast, those rRT-PCR tests are  
10 not discriminatory for C-strain vaccine and other CSFV strains  
11 (Hoffmann et al., 2005; Tignon et al. 2008). Although certain RT-PCR  
12 and rRT-PCRs have been developed to differentiate between C-  
13 strain and certain CSFV genotypes (Pan et al., 2008; Zhao et al.,  
14 2008), none is currently available which is able to differentiate C-  
15 strain from all CSFV genotypes.

16 In view of the construction of the chimeric vaccine virus,  
17 characterised by the presence of CSFV E2 but not CSFV E<sup>ns</sup> gene  
18 (Reimann et al., 2004), the CP7E2alf allows a serological  
19 differentiation from wild-type CSFV. The latter can be clearly seen in  
20 this study as the CP7E2alf vaccinated animals did not seroconvert  
21 for CSFV E<sup>ns</sup> but did for CSFV E2, confirming previous studies  
22 (Reimann et al., 2004; Koenig et al., 2007a, 2007b). As expected, no  
23 serological DIVA was possible for C-strain vaccine, as it induced  
24 CSFV-specific E2, E<sup>ns</sup> and neutralising antibodies, in accordance

1 with previous descriptions (Kaden and Lange, 2001; Kaden et al.,  
2 2004) and similarly to the serological picture observed after infection  
3 with mildly to moderately virulent strains (Mittelholzer et al., 2000).

#### 4 **Conclusion**

5 In conclusion, the initial replication of CP7E2alf and C-strain  
6 vaccines occurred in the tonsils, which is similar to the wild virus. The  
7 marker vaccine induced an immune response observed up to 98  
8 days after oronasal administration in domestic pigs and allowed the  
9 use of serological DIVA tests combining CSFV-specific E2 and E<sup>ns</sup>  
10 antibody detection. This study confirms the suitability of CP7E2alf as  
11 a interesting and practical marker vaccine candidate for the oral  
12 vaccination programmes to control classical swine fever in  
13 domestic pigs and wild boar.

14

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11

12   **Conflict of Interest**

13   None

14

## 1   **References**

- 2   Anonymous, 2004, OIE Manual of Standards for Diagnostic Tests and  
3       Vaccines. List A and B Diseases of Mammals, Birds and Bees, 3rd  
4       Edition, Paris, 244– 257 pp.
- 5   Beer, M., Reimann, I., Hoffmann, B., Depner, K., 2007, Novel marker  
6       vaccines against classical swine fever. *Vaccine* 25, 5665-5670.
- 7   Chenut, G., Saintilan, A.F., Burger, C., Rosenthal, F., Cruciere, C., Picard,  
8       M., Bruyere, V., Albina, E., 1999, Oral immunisation of swine with a  
9       classical swine fever vaccine (Chinese strain) and transmission  
10      studies in rabbits and sheep. *Vet Microbiol* 64, 265-276.
- 11   Dekker, A., Wensvoort, G., Terpstra, C., 1995, Six antigenic groups within  
12      the genus pestivirus as identified by cross neutralization assays. *Vet*  
13      *Microbiol* 47, 317-329.
- 14   Dewulf, J., Koenen, F., Mintiens, K., Denis, P., Ribbens, S., de Kruif, A., 2004a,  
15      Analytical performance of several classical swine fever laboratory  
16      diagnostic techniques on live animals for detection of infection. *J*  
17      *Virology Methods* 119, 137-143.
- 18   Dewulf, J., Laevens, H., Koenen, F., Mintiens, K., de Kruif, A., 2001, An E2  
19      sub-unit marker vaccine does not prevent horizontal or vertical  
20      transmission of classical swine fever virus. *Vaccine* 20, 86-91.
- 21   Dewulf, J., Laevens, H., Koenen, F., Mintiens, K., de Kruif, A., 2004b, Efficacy  
22      of E2-sub-unit marker and C-strain vaccines in reducing horizontal  
23      transmission of classical swine fever virus in weaner pigs. *Prev Vet*  
24      *Med* 65, 121-133.
- 25   Dong, X.N., Chen, Y.H., 2007, Marker vaccine strategies and candidate  
26      CSFV marker vaccines. *Vaccine* 25, 205-230.

- 1 Edwards, S., Fukusho, A., Lefevre, P.C., Lipowski, A., Pejsak, Z., Roehe, P.,  
2 Westergaard, J., 2000, Classical swine fever: the global situation.  
3 Vet Microbiol 73, 103-119.
- 4 Ganges, L., Núñez, J.I., Sobrino, F., Borrego, B., Fernández-Borges, N., Frías-  
5 Lepoureau, M.T., Rodríguez, F., 2008, Recent advances in the  
6 development of recombinant vaccines against classical swine  
7 fever virus: cellular responses also play a role in protection. Vet J  
8 177, 169-177.
- 9 Greiser-Wilke, I., Moennig, V., 2004, Vaccination against classical swine  
10 fever virus: limitations and new strategies. Anim Health Res Rev 5,  
11 223-226.
- 12 Haegeman, A., Dewulf, J., Vrancken, R., Tignon, M., Ribbens, S., Koenen,  
13 F., 2006, Characterisation of the discrepancy between PCR and  
14 virus isolation in relation to classical swine fever virus detection. J  
15 Virol Methods 136, 44-50.
- 16 Hoffmann, B., Beer, M., Schelp, C., Schirmeier, H., Depner, K., 2005,  
17 Validation of a real-time RT-PCR assay for sensitive and specific  
18 detection of classical swine fever. J Virol Methods 130, 36-44.
- 19 Hoffmann, B., Depner, K., Schirmeier, H., Beer, M., 2006, A universal  
20 heterologous internal control system for duplex real-time RT-PCR  
21 assays used in a detection system for pestiviruses. J Virol Methods  
22 136, 200-209.
- 23 Hulst, M.M., Westra, D.F., Wensvoort, G., Moormann, R.J., 1993,  
24 Glycoprotein E1 of hog cholera virus expressed in insect cells  
25 protects swine from hog cholera. J Virol 67, 5435-5442.

- 1 Kaden, V., Lange, B., 2001, Oral immunisation against classical swine fever  
2 (CSF): onset and duration of immunity. *Vet Microbiol* 82, 301-310.
- 3 Kaden, V., Lange, B., Faust, A., 2008, Oral vaccination against classical  
4 swine fever with a chimeric Pestivirus : comparative investigations of  
5 liquid and lyophilized virus. *Eur J Wildl Res* 54, 237-244
- 6 Kaden, V., Lange, E., Fischer, U., Strebelow, G., 2000, Oral immunisation of  
7 wild boar against classical swine fever: evaluation of the first field  
8 study in Germany. *Vet Microbiol* 73, 239-252.
- 9 Kaden, V., Lange, E., Riebe, R., Lange, B., 2004, Classical swine fever virus  
10 Strain 'C'. How long is it detectable after oral vaccination? *J Vet*  
11 *Med B Infect Dis Vet Public Health* 51, 260-262.
- 12 Koenig, P., Hoffmann, B., Depner, K.R., Reimann, I., Teifke, J.P., Beer, M.,  
13 2007a, Detection of classical swine fever vaccine virus in blood and  
14 tissue samples of pigs vaccinated either with a conventional C-  
15 strain vaccine or a modified live marker vaccine. *Vet Microbiol* 120,  
16 343-351.
- 17 Koenig, P., Lange, E., Reimann, I., Beer, M., 2007b, CP7\_E2alf: a safe and  
18 efficient marker vaccine strain for oral immunisation of wild boar  
19 against Classical swine fever virus (CSFV). *Vaccine* 25, 3391-3399.
- 20 Leifer, I., Lange, E., Reimann, I., Plana, J., Hofmann, M., Beer, M., 2008. First  
21 efficacy data of the GMP-produced modified live marker vaccine  
22 prototype CP7\_E2alf. In: Annual Meeting of National Swine Fever  
23 Laboratories, Hannover, Germany, May 5-7, 2008.
- 24 Letellier, C., Kerkhofs, P., 2003, Real-time PCR for simultaneous detection  
25 and genotyping of bovine viral diarrhoea virus. *J Virol Methods* 114,  
26 21-27.

- 1 Lorena, J., Barlic-Maganja, D., Lojkic, M., Madic, J., Grom, J., Cac, Z., Roic,  
2 B., Terzic, S., Lojkic, I., Polancec, D., Cajavec, S., 2001, Classical  
3 swine fever virus (C strain) distribution in organ samples of  
4 inoculated piglets. *Vet Microbiol* 81, 1-8.
- 5 Mittelholzer, C., Moser, C., Tratschin, J.D., Hofmann, M.A., 2000, Analysis of  
6 classical swine fever virus replication kinetics allows differentiation  
7 of highly virulent from avirulent strains. *Vet Microbiol* 74, 293-308.
- 8 Moormann, R.J., Bouma, A., Kramps, J.A., Terpstra, C., De Smit, H.J., 2000,  
9 Development of a classical swine fever subunit marker vaccine and  
10 companion diagnostic test. *Vet Microbiol* 73, 209-219.
- 11 Pasick, J., 2004, Application of DIVA vaccines and their companion  
12 diagnostic tests to foreign animal disease eradication. *Anim Health*  
13 *Res Rev* 5, 257-262.
- 14 Pan, C., Jong, M., Huang, Y., Huang, T., Chao, P., Lai, S., 2008, Rapid  
15 detection and differentiation of wild-type and three attenuated  
16 lapinized vaccine strains of Classical swine fever virus by reverse  
17 transcription polymerase chain reaction. *Journal of veterinary*  
18 *diagnostic investigation* 20, 448-456.
- 19 Rasmussen, T.B., Uttenthal, A., Reimann, I., Nielsen, J., Depner, K., Beer, M.,  
20 2007, Virulence, immunogenicity and vaccine properties of a novel  
21 chimeric pestivirus. *J Gen Virol* 88, 481-486.
- 22 Reimann, I., Depner, K., Trapp, S., Beer, M., 2004, An avirulent chimeric  
23 Pestivirus with altered cell tropism protects pigs against lethal  
24 infection with classical swine fever virus. *Virology* 322, 143-157.

- 1 Suradhat, S., Damrongwatanapokin, S., Thanawongnuwech, R., 2007,  
2 Factors critical for successful vaccination against classical swine  
3 fever in endemic areas. *Vet Microbiol* 119, 1-9.
- 4 Suradhat, S., Intrakamhaeng, M., Damrongwatanapokin, S., 2001, The  
5 correlation of virus-specific interferon-gamma production and  
6 protection against classical swine fever virus infection. *Vet Immunol*  
7 *Immunopathol* 83, 177-189.
- 8 Terpstra, C., Robijns, K.G., 1977, Experience with regional vaccination  
9 against swine fever in enzootic areas for limited periods using C-  
10 strain virus. *Tijdschr Diergeneeskd* 102, 106-112.
- 11 Terpstra, C. and Wensvoort, G., 1988, The protective value of vaccine-  
12 induced neutralising antibody titres in swine fever. *Vet Microbiol* 16,  
13 123-128.
- 14 Tignon, M., Kulcsár, G., Belák, K., Haegeman, A., Barna, T., Fábíán, K.,  
15 Lévai, R., Farsang, A., Van der Stede, Y., Vrancken, R., F., K., 2008,  
16 Application of a Commercial Real-time RT-PCR Assay for  
17 Surveillance of Classical Swine Fever: Evaluation by Testing  
18 Sequential Tissue and Blood Samples. *The Open Veterinary Science*  
19 *Journal* 2, 104-110.
- 20 van Gennip, H.G., van Rijn, P.A., Widjojoatmodjo, M.N., de Smit, A.J.,  
21 Moormann, R.J., 2000, Chimeric classical swine fever viruses  
22 containing envelope protein E(RNS) or E2 of bovine viral diarrhoea  
23 virus protect pigs against challenge with CSFV and induce a  
24 distinguishable antibody response. *Vaccine* 19, 447-459.
- 25 van Oirschot, J.T., 2003, Vaccinology of classical swine fever: from lab to  
26 field. *Vet Microbiol* 96, 367-384.



- 1 van Regenmortel, M.H.V., Fauquet, C.M., Bishop, D.H.L., Carstens, E.B.,  
2 Estes, M.K., Lemon, S.M., Maniloff, J., Mayo, M.A., McGeoch, D.J.,  
3 Pringle, C.R., R.B., W., 2000, Virus Taxonomy: Classification and  
4 Nomenclature of Viruses. Seventh Report of the International  
5 Committee on Taxonomy of Viruses. Academic Press London.
- 6 van Rijn, P.A., van Gennip, H.G., Leendertse, C.H., Brusckhe, C.J., Paton,  
7 D.J., Moormann, R.J., van Oirschot, J.T., 1997, Subdivision of the  
8 pestivirus genus based on envelope glycoprotein E2. *Virology* 237,  
9 337-348.
- 10 Vandeputte, J., Chappuis, G., 1999, Classical swine fever: the European  
11 experience and a guide for infected areas. *Rev Sci Tech* 18, 638-  
12 647.
- 13 von Rüden, S., Staubach, C., Kaden, V., Hess, R.G., Blicke, J., Kühne, S.,  
14 Sonnenburg, J., Fröhlich, A., Teuffert, J., Moennig, V., 2008,  
15 Retrospective analysis of the oral immunisation of wild boar  
16 populations against classical swine fever virus (CSFV) in region Eifel  
17 of Rhineland-Palatinate. *Vet Microbiol* 132, 29-38.
- 18 Vilcek, S., Belak, S., 1997, Organization and diversity of the 3'-noncoding  
19 region of classical swine fever virus genome. *Virus Genes* 15, 181-  
20 186.
- 21 Wehrle, F., Renzullo, S., Faust, A., Beer, M., Kaden, V., Hofmann, M.A., 2007,  
22 Chimeric pestiviruses: candidates for live-attenuated classical swine  
23 fever marker vaccines. *J Gen Virol* 88, 2247-2258.
- 24 Zhao, J.J., Cheng, D., Li, N., Sun, Y., Shi, Z., Zhu, Q.H., Tu, C., Tong, G.Z., Qiu,  
25 H.J., 2008, Evaluation of a multiplex real-time RT-PCR for

- 1 quantitative and differential detection of wild-type viruses and C-
- 2 strain vaccine of Classical swine fever virus. Vet Microbiol 126, 1-10.
- 3

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- 1 Table 1: Detection of CP7E2alf and C-strain vaccine viruses in the tonsils of
- 2 slaughtered animals of CP7E2alf and C-strain vaccine groups by virus
- 3 isolation and rRT-PCR assays between 0 to 98 days post administration

Detection of CP7E2alf and C-strain vaccine viruses in the tonsils by virus isolation and rRT-PCR assays									
dpv	CP7E2alf vaccinated group					C-strain vaccinated group			
	Animals	rRT-PCRs (Ct values*)				Animals	rRT-PCRs (Ct values*)		
		VI	CSFV (a,b)	BVDV (c)	pan-pesti (d)		VI	CSFV (a)	CSFV (b)
0	#1	-	-	-	-	#1	-	-	-
	#2	-	-	-	-	#2	-	-	-
1	#3	-	-	-	-	#3	-	-	-
	#4	-	-	-	-	#4	-	-	-
2	#5	-	-	-	-	#5	-	-	-
	#6	-	-	-	-	#6	-	-	-
3	#7	-	-	-	-	#7	-	-	-
	#8	-	-	-	-	#8	+	40.4	-
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
6	#13	-	-	-	-	#13	+	33.9	33.6
	#14	-	-	-	-	#14	+	32.2	32.4
7	#15	-	-	33.6	-	#15	+	31	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	41
28	#25	-	-	-	-	#25	-	38.4	39.4
	#26	-	-	-	-	#26	-	32	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	-

	#34	-	-	-	-	#34	-	39.2	40.3
63	#35	-	-	36.3	-	#35	-	-	-
	#36	-	-	-	-	#36	-	41.2	41
70	#37	-	-	-	-	#37	-	-	-
	#38	-	-	-	-	#38	-	-	41.2
77	#39	-	-	-	-	#39	-	-	-
	#40	-	-	-	-	#40	-	-	41.1
84	#41	-	-	-	-	#41	-	-	-
	#42	-	-	-	-	#42	-	-	-
91	#43	-	-	-	-	#43	-	-	-
	#44	-	-	-	-	#44	-	-	-
98	#45	-	-	-	-	#45	-	-	-
	#46	-	-	-	-	#46	-	-	40.4

- 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  $\geq 42$  were considered
- 4 negative
- 5

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

Detection of CP7E2alf and C-strain vaccine viruses in blood samples by virus isolation and rRT-PCR assays							
dpv	Size of groups (N)	CP7E2alf vaccinated group			C-strain vaccinated group		
		Number of VI-positive	Number of rRT-PCR-positive		Number of VI-positive	Number of rRT-PCR-positive	
			CSFV (b)	BVDV (c)		CSFV (b)	mean Ct (SD)
0	46	0	0	0	0	0	-
1	30	0	0	0	0	0	-
2	28	0	0	0	0	0	-
3	26	0	0	0	2	2	39.9 (0.1)
4	24	0	0	0	0	3	39.9 (1.2)
5	22	0	0	0	9	9	38.7 (1.8)
6	20	0	0	0	5	5	37.9 (1.1)
7	18	0	0	0	1	6	39.4 (1.9)
8	16	0	0	0	1	4	38.8 (1.1)
11	28	0	0	0	0	5	39.8 (1.1)
13	28	0	0	0	0	1	41.4 (*)
15	26	0	0	0	0	0	-
18	26	0	0	0	0	0	-
21	24	0	0	0	0	0	-
28	22	0	0	0	0	1	39.9 (*)
35	20	0	0	0	0	1	41.6 (*)
42	18	0	0	0	0	1	34.2 (*)
49	16	0	0	0	0	0	-
56	14	0	0	0	0	0	-
63	12	0	0	0	0	0	-
70	10	0	0	0	0	0	-
77	8	0	0	0	0	0	-
84	6	0	0	0	0	0	-
91	4	0	0	0	0	0	-
98	2	0	0	0	0	0	-

5 VI: virus isolation, b: Hoffmann et al., 2005, c: Letellier et al., 2003, Ct:  
 6 threshold cycle, SD: Standard deviation, - : Ct negative ( $\geq 42$ ), (\*): no SD  
 7 due to only one positive sample

8

1 Figure 1: Evolution of rectal temperatures (lines) and clinical scores  
2 (vertical bars), expressed as median and 25<sup>th</sup> and 75<sup>th</sup> percentiles, in  
3 groups of pigs after oronasal administration of CP7E2alf (black  
4 circle) and C-strain vaccines (white bar and white square). The  
5 median of clinical scores remains null for CP7E2alf vaccinated  
6 group. Dash line indicates the fever limit (40.5°C)

7

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

15

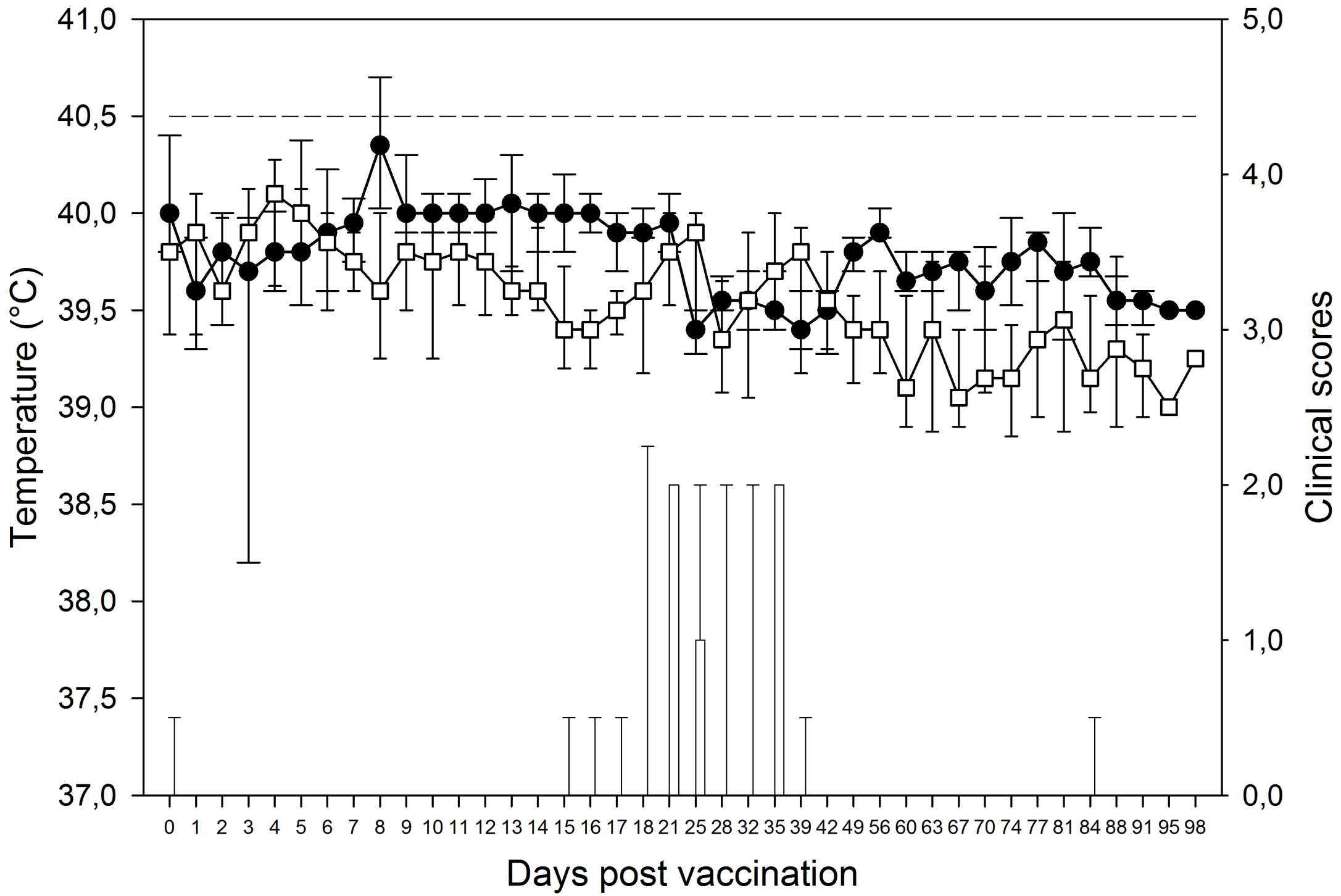
16 Figure 3: Proportion of CSFV E2 antibody positive animals (A) and  
17 range of blocking E2 antibody percentages (B) for positive animals,  
18 expressed as median and 25<sup>th</sup> and 75<sup>th</sup> percentiles, after oronasal  
19 administration of CP7E2alf (black bar and black circle) and C-strain  
20 vaccines (white bar and white square). Dash line indicates the cut-  
21 off value for E2 blocking percentage (40%). Asterisks indicate values  
22 significantly different between groups with  $P \leq 0.05$  (\*),  $P \leq 0.01$  (\*\*) and  
23  $P \leq 0.001$  (\*\*\*)

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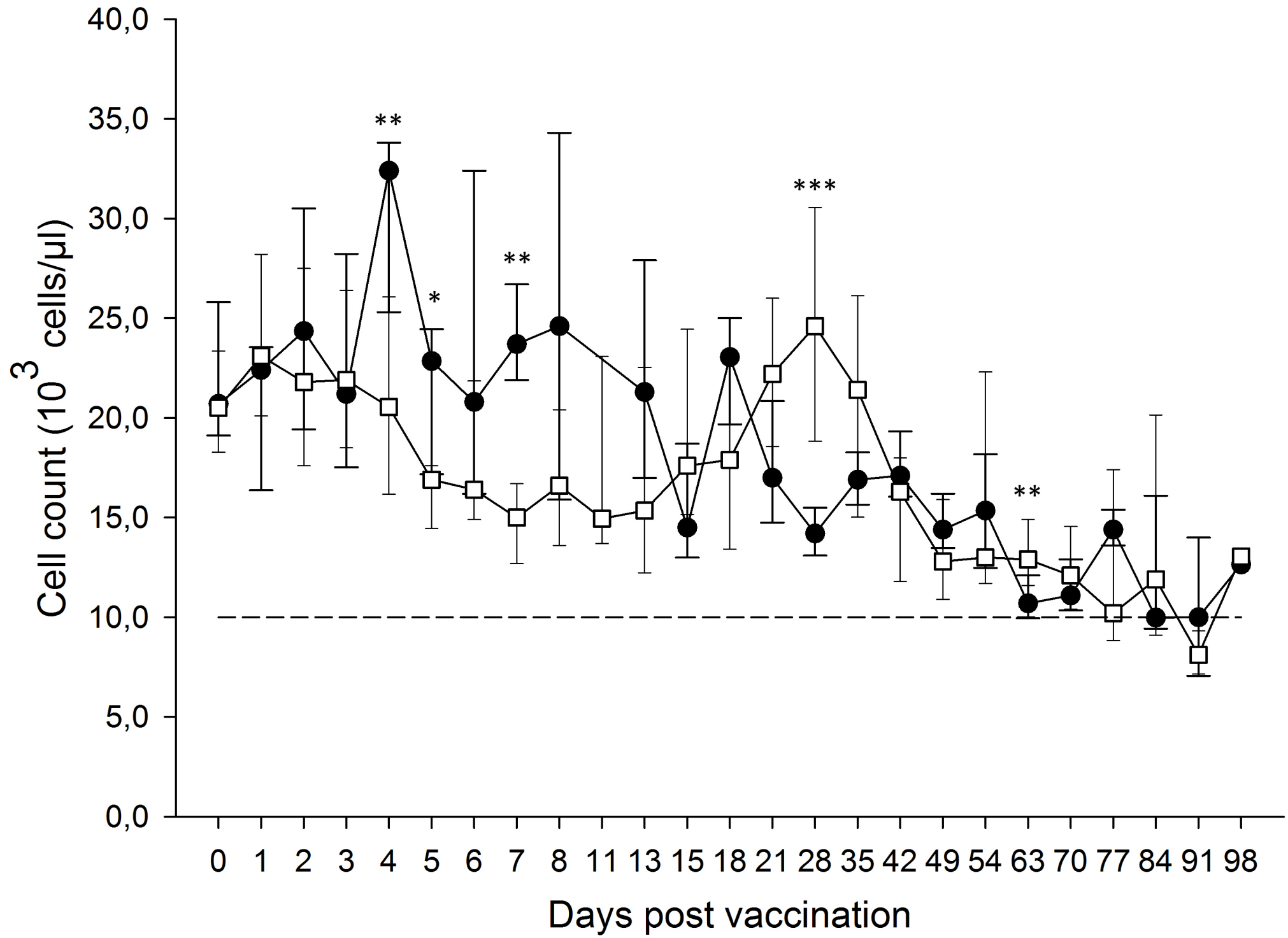
2 Figure 4: Proportion of CSFV E<sup>ns</sup> antibody positive animals (A) and  
3 range of blocking E<sup>ns</sup> antibody percentages (B) for positive animals,  
4 expressed as median and 25<sup>th</sup> and 75<sup>th</sup> percentiles, after oronasal  
5 administration of CP7E2alf and C-strain vaccines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

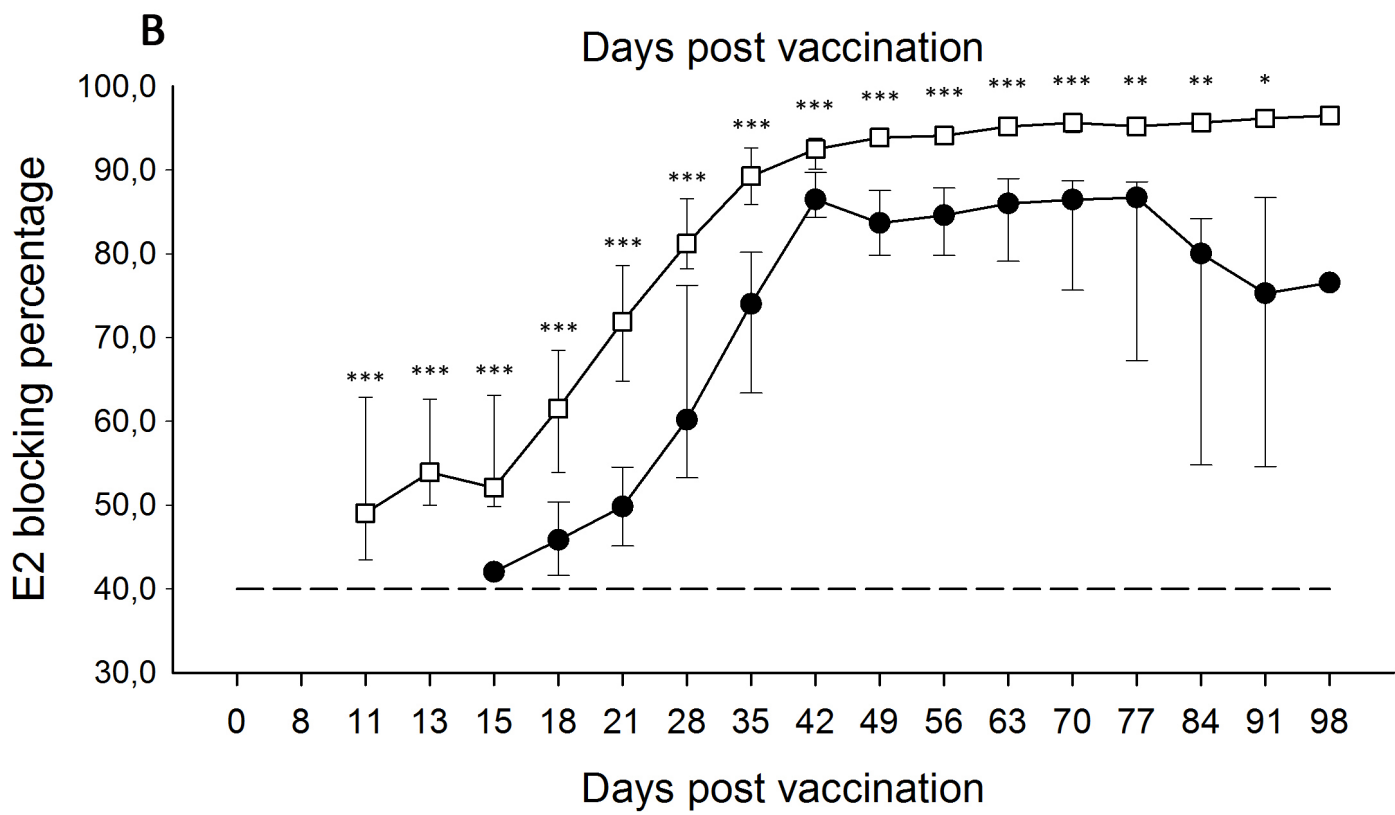
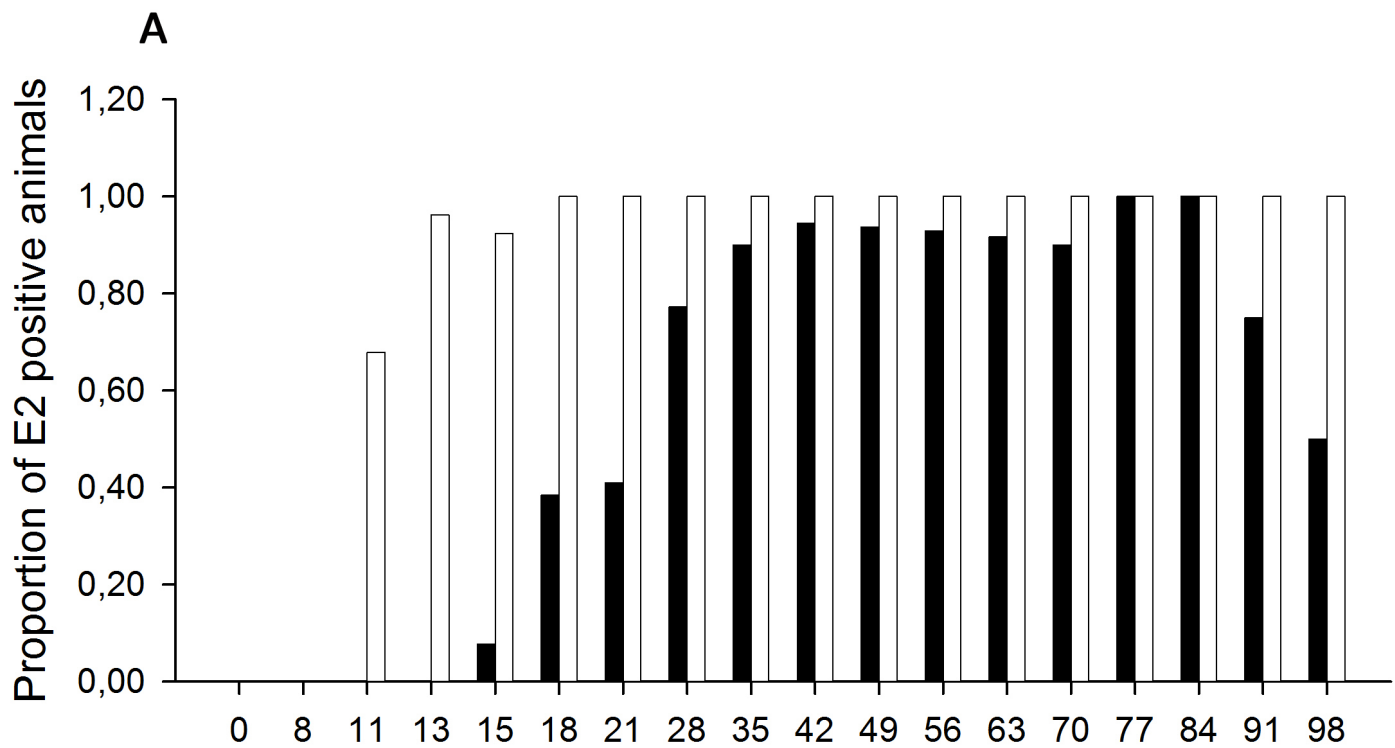
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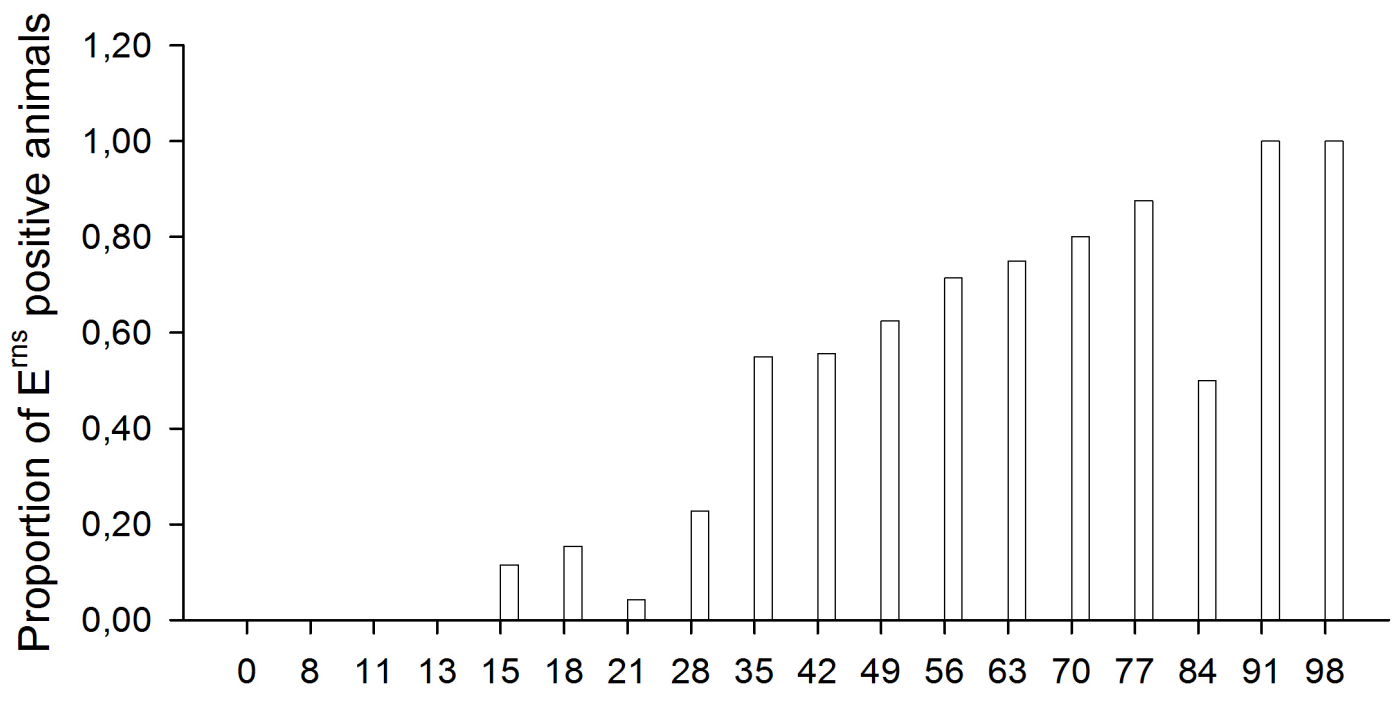
12 Figure 5: Proportion of animals positive in VNT (A) and range of  
13 neutralising titres (B) for positive animals after oronasal  
14 administration of CP7E2alf or C-strain vaccines, expressed as  
15 median and 25<sup>th</sup> and 75<sup>th</sup> percentiles. VNT titres were obtained in  
16 CP7E2alf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









**A****B**