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Efficacy of marker vaccine candidate CP7_E2alf in piglets with maternally derived C-strain antibodies

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ABSTRACT

Marker vaccines offer the possibility to differentiate classical swine fever (CSF) infected from CSF vaccinated animals based on serology and their implementation will ensure free trade with pigs. Therefore, new generations of promising marker vaccines have been developed, among them the chimeric vaccine CP7_E2alf. However, in populations previously vaccinated with live attenuated vaccines like the C-strain, passive immunity through maternal antibodies can interfere with efficacy of CP7_E2alf vaccination. Therefore, the efficacy of CP7_E2alf was examined in piglets from sows vaccinated once intramuscularly with C-strain vaccine 4 weeks before farrowing. Thus, these piglets were vaccinated intramuscularly with CP7_E2alf at the age of 5 or 8 weeks. Subsequently, the piglets and their mock-vaccinated littermate controls were challenged 2 weeks post vaccination with highly virulent Classical swine fever virus (CSFV) strain "Koslov".

CP7_E2alf provided clinical protection upon challenge as no severe clinical signs or mortality was observed in the vaccinated piglets. Post mortem examination revealed pathological changes associated to CSFV only in the mock-vaccinated piglets. No infectious CSFV could be isolated from the tonsils of the vaccinated piglets. Two weeks after vaccination at the time of challenge, the vaccinated piglets only, had an increase in the ELISA antibody titer.

Interestingly, the maternally derived immunity in the mock-vaccinated control piglets seems to neutralize the challenge virus. Thus, the previously observed 100% mortality in naïve (negative for antibodies to CSFV) piglets infected with CSFV Koslov was reduced in the control piglets of this study to 30% for challenge at the age of 7 weeks and 50% at the age of 10 weeks, respectively.

In conclusion, CP7_E2alf proved to be effective in preventing mortality, severe clinical signs and pathological lesions in 5 or 8 weeks old piglets positive for maternal antibodies derived from sows vaccinated intramuscularly 4 weeks before farrowing with one dose of C-strain vaccine.

1. Introduction

Classical swine fever is a highly contagious, often fatal disease in pigs that can have tremendous socio-economic impact. The causative agent, *Classical swine fever virus* (CSFV), belongs to the genus *Pestivirus* of the family *Flaviviridae* [1]. It is an enveloped RNA virus closely related to *Bovine viral diarrhea virus* (BVDV) and *Border disease virus* (BDV) [2]. In the European Union (EU), outbreaks of classical swine fever (CSF) are controlled by strict sanitary measures [3]. Prophylactic vaccination is banned since 1990, but legal provision is laid for emergency vaccination under certain circumstances [4]. Discussion regarding this alternative control strategy was recently intensified both for domestic pigs and wild boar [5]. At present, only conventional modified live attenuated vaccines are used for routine vaccination in some countries outside of the EU, and in the EU as an emergency bait vaccination of affected wild boar populations. Although efficacious and safe, these vaccines do not offer the possibility to detect infection in vaccinated pigs [6] or to prove that antibodies are derived from vaccination only. The inability of a country to prove the CSF-free status by serosurveillance due to vaccination with live attenuated vaccines, leads to restriction in the pig export [7]. To overcome this problem, efficacious and safe marker vaccines with accompanying sensitive and specific diagnostic tests are needed. Two subunit marker vaccines based on baculovirus expression of the CSFV E2-glycoprotein have been registered of which only one is still

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available. Subunit vaccines can be used for prophylactic vaccination; however they have a restricted use due to the following reasons: repeated administration is needed to induce protection and they are not applicable for oral vaccination [8]. In 2004, a new chimeric marker vaccine candidate, "CP7_E2alf" was published [9]. The vaccine candidate is based on a backbone of BVDV strain "CP7" in which the E2 protein encoding sequence was replaced by the corresponding region of the CSFV strain "Alfort187". This new vaccine proved in several studies to be efficacious, safe and applicable for oral administration [10–12]. However, all of the present experiments were conducted in naïve pigs. In practice, there will be scenarios with previous vaccination using live attenuated vaccines; this may lead to the use of CP7_E2alf in animals that possess maternally derived antibodies (MDA) against CSFV. Old studies reported that the most effective time point for vaccination of domestic piglets with MDA is from 5 to 8 weeks of age [13]. For this reason, a study was conducted to define the optimal time point for protective vaccination using "CP7_E2alf" in domestic piglets with MDA obtained from C-strain vaccination of their mothers.

2. Materials and methods

2.1. Animals and vaccination trials

Four pregnant sows were purchased at approximately 86 days of gestation from a commercial Danish farm. The sows were tested negative for CSFV and BVDV. Four weeks before farrowing the sows were vaccinated intramuscularly using live attenuated Riemser® C-strain vaccine (Riemser Schweinepestvakzine, Riemser Arzneimittel AG, Germany) according to the manufacturer's instructions. At farrowing piglets were numbered as follows: sow A piglets 1–14; sow B piglets 21–33; sow C piglets 41–57; sow D piglets 61–76. During the first two weeks, eleven piglets were excluded from the study due to general health problems. At 4 weeks of age, the piglets were divided into four groups representing offspring of each sow. Group V5 included 13 piglets, while groups V8, C5 and C8 included 12 piglets.

The CP7_E2alf pilot vaccine used for vaccination of the piglets was produced by Pfizer Olot S.L.U (Spain). The vaccine was diluted in sterile solution prior to administration based on pre-existing potency data [14].

Animals of groups V5 and V8 were intramuscularly vaccinated with 1 ml of CP7_E2alf at 5 and 8 weeks of age, respectively. Control groups C5 and C8 received 1 ml of vaccine diluent at the same time points. All animals were challenged two weeks after treatment. The four groups were kept in separate high-containment units and observed daily for symptoms associated with CSF for a period of 2 weeks post challenge. At the end of this period, the animals were euthanized and pathological examinations were performed. Piglets that developed severe symptoms associated with CSF before the end of the 2 weeks were euthanized due to welfare reasons.

The experiment was conducted in the animal facilities at Technical University of Denmark, National Veterinary Institute, Lindholm Denmark (DTU-Vet) in accordance with the requirements of the Danish Animal Experiments Inspectorate (License 2008/561-1540).

2.2. Virus

The challenge virus (highly virulent CSFV strain "Koslov") was provided by the Friedrich-Loeffler-Institute (FLI, Germany). The challenge material was diluted with Phosphate buffered saline (PBS) to the theoretical titer (obtained from previous experiments) of $10^{5.5}$ $TCID_{50}$ per ml [14]. After back titrations, the virus had a titer of $10^{5.9}$ $TCID_{50}$ per ml for the first challenge trial of groups V5 and C5 and $10^{6.8}$ $TCID_{50}$ per ml for the second challenge trial of group V8 and C8, respectively. The challenge material was administered intranasally at a dose of 2 ml per piglet.

2.3. Sample collection

EDTA blood and serum samples were collected from the sows on days 0 and 14 post vaccination, at farrowing, 2 weeks post farrowing, and at the day of euthanasia. Piglets were sampled prior to uptake of colostrum and once a week until vaccination. For practicability reasons, the piglets with even and uneven numbers were blood sampled at different days of the week. After vaccination, blood samples were collected twice a week from all piglets. At euthanasia, tonsils, spleen, kidney and mesenterial lymph nodes were collected. All samples were stored at −40 °C until analysis.

2.4. Clinical examination

Following challenge, clinical signs were recorded daily according to the protocol by Mittelholzer et al. [15]. Cumulative clinical scores (CS) were calculated over time for individual pigs and for the respective groups. In addition, rectal body temperature was measured on a daily base from 2 days before vaccination until 14 days post challenge (dpc). A body temperature of >40.0 °C for at least two consecutive days was recorded as fever.

2.5. Sample analysis

Total white blood cell (WBC) counts were carried out on EDTA blood using a semi-automated Animal Blood Counter (Vet abc™, ABX, Montpellier, France). Physiological reference values were defined as follows: WBC $10–22 \times 10^6$ ml^{-1} and platelets $200–800 \times 10^6$ ml^{-1}.

CSFV was isolated from tissue homogenates on PK-15 cells as previously described [16], titrations were performed on triplicates.

The serum samples were tested for the presence of CSFV antibodies by an in-house blocking ELISA [17].

Detection of viral RNA was performed on serum samples using the CP7_E2alf and CSFV specific real-time reverse transcription polymerase chain reaction (RT-qPCR) assays described by Rasmussen et al. [18] after RNA extraction using the QIAamp RNA Blood mini kit (Qiagen).

2.6. Statistical analysis

Statistical analysis was performed using GraphPad in Stat version 3.00 (GraphPad Software, San Diego, CA). Student's *t*-test was used to examine the significance of differences between vaccinated and mock-vaccinated control groups when it was relevant. Differences were considered statistically significant with a probability of $p \leq 0.05$.

3. Results

3.1. Clinical, hematological and pathological findings

3.1.1. Vaccination at 5 weeks of age and challenge at 7 weeks of age

Following vaccination, neither fever nor other side effects were observed in the piglets vaccinated at 5 weeks of age (group V5). Upon challenge infection, none of the piglets developed severe CSF symptoms, but slight depression, increased temperatures (Fig. 1a) as well as transient WBC and platelet count decreases were observed around 3 dpc in almost all animals of group V5 (Fig. 2a). From 6 dpc onwards, all piglets had physiological temperatures again, but four piglets remained depressed.

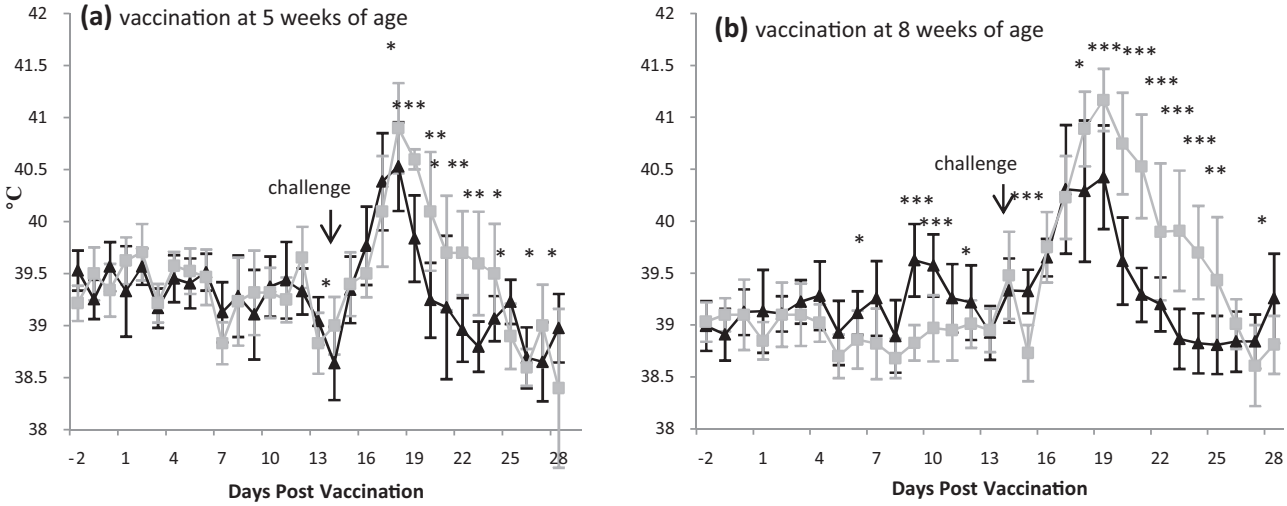

Fig. 1. Development of body temperature in: (a) vaccinated at 5 weeks of age and challenged at 7 weeks of age V5 (▲–▲) and control mock-vaccinated at 5 weeks of age and challenged at 7 weeks of age C5 (■-■) and in (b) vaccinated at 8 weeks of age and challenged at 10 weeks of age V8 (▲–▲) and control mock-vaccinated at 8 weeks of age and challenged at 10 weeks of age C8 (■-■). Each symbol represents the mean ± SD. The significant difference in the body temperatures between vaccinated and control groups were indicated with $^{*}p < 0.01$; $^{**}p < 0.001$; $^{***}p < 0.0001$.

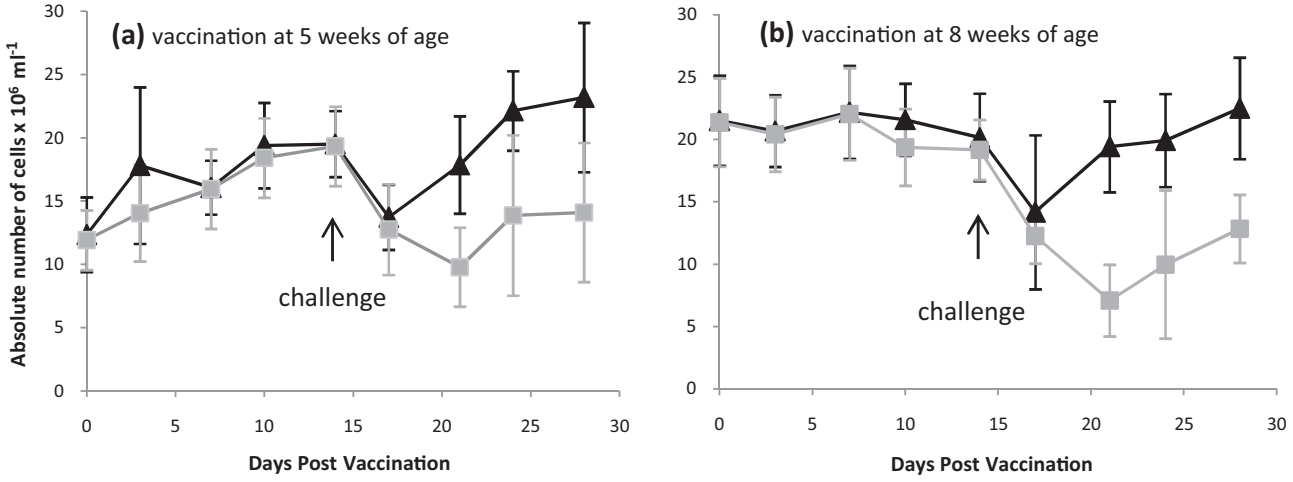

Fig. 2. Total white blood cell (WBC) counts in: (a) vaccinated at 5 weeks of age piglets and challenged at 7 weeks of age V5 (▲–▲) and controls C5 mock-vaccinated at 5 weeks of age and challenged at 7 weeks of age (■-■) and in (b) vaccinated at 8 weeks of age and challenged at 10 weeks of age V8 (▲–▲) and controls mock-vaccinated at 8 weeks of age and challenged at 10 weeks of age C8 (■-■). Each symbol represents the mean ± SD.

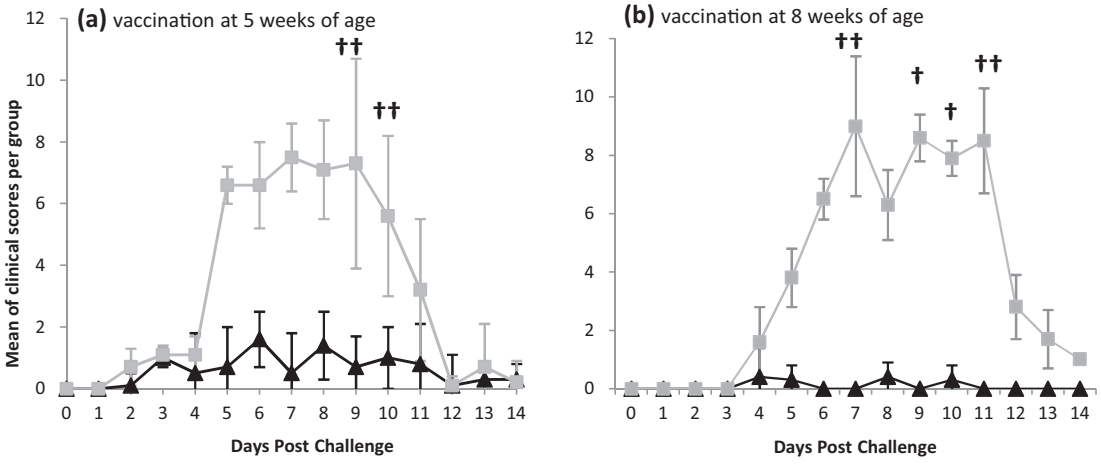

Fig. 3. Clinical scores (CS) recorded post challenge. CS in (a) vaccinated at 5 weeks of age and challenged at 7 weeks of age V5 (▲–▲) and control mock-vaccinated at 5 weeks of age and challenged at 7 weeks of age C5 (■-■), and in (b) vaccinated at 8 weeks of age and challenged at 10 weeks of age V8 (▲–▲) and control mock-vaccinated and challenged at 10 weeks of age C8 (■-■). Each symbol represents the mean ± SD. The † symbol stands for euthanasia and the clinical scores obtained from the euthanized animals at the euthanization day are included in the data for the indicated time point.

Table 1
Results obtained from control Group C5 (mock-vaccinated at 5 weeks of age and challenged at 7 weeks of age) and control Group C8 (mock-vaccinated at 8 weeks of age and challenged at 10 weeks of age) at euthanization. Earlier days of euthanization and positive results for virus isolations are indicated in bold.

Group	Pig #	Euthanization day	Body temperature at euthanization	Clinical scores at euthanization	Virus isolation from tonsils	WBC ($\times 10^6$ ml^{-1}) at euthanization	Platelets ($\times 10^6$ ml^{-1}) at euthanization	RNA viral load in serum (Ct value)
C5	3	14	39.0	0	**Positive**	19.6	808	Negative
	7	14	38.6	0	**Positive**	13.6	375	Negative
	13	14	37.8	0	**Positive**	10.5	385	Negative
	24	14	39.0	2	**Positive**	2.3	62	Ct 37
	29	**11**	38.9	8	Negative	20	654	Negative
	33	**11**	38.3	7	Negative	6.5	173	Negative
	46	14	38.3	0	**Positive**	19.5	860	Negative
	50	14	39.6	0	**Positive**	14.8	819	Ct 45
	56	**10**	39.6	8	**Positive**	8.3	91	Ct 47
	64	14	37.8	0	**Positive**	19.4	747	Negative
	69	**10**	40.8	13	Negative	3.7	37	Ct 35
	74	14	37.6	0	**Positive**	12.9	472	Negative
C8	4	14	39.2	3	Negative	10.6	573	Negative
	9	14	39.1	1	Negative	18	804	Negative
	14	**7**	40.9	14	**Positive**	6.6	79	Ct 24
	25	14	38.5	1	Negative	14	536	Negative
	30	**7**	41.6	12	**Positive**	4.4	127	Ct 22
	41	**11**	38.3	12	Negative	6.4	170	Negative
	47	14	39.1	1	Negative	10.5	319	Negative
	52	14	38.8	1	Negative	12	750	Negative
	61	14	38.8	3	Negative	12.5	657	Negative
	65	**10**	40.6	9	**Positive**	6.4	28	Ct 27
	71	**11**	39.9	9	Negative	4	39	Ct 36
	76	**9**	41.2	9	**Positive**	3	103	Ct 27

In contrast, several mock-vaccinated piglets developed fever (Fig. 1), leucopenia (Fig. 2) and severe clinical symptoms including diarrhea, ataxia, and convulsions (Fig. 3). Four piglets of this group had to be euthanized prior to the end of the trial (Table 1).

3.1.2. Vaccination at 8 weeks of age and challenge at 10 weeks of age

The vaccinated piglets from group V8 did not exhibit adverse reactions post vaccination. On day 3 pc three piglets from group V8 had elevated temperatures and slight transient decreases of their WBC counts. Similarly to group V5, on day 6 pc all of the piglets were back to normal. Slight depression and reduced appetite was observed in two piglets on day 4 pc. These mild symptoms were observed up to day 10 pc. None of the piglets from group V8 displayed signs of disease during the last four days of the experiment.

The mock-vaccinated piglets from group C8 were severely affected post challenge and six piglets were euthanized before the termination of the experiment. Fever started from day 2 pc and 6 piglets had to be euthanized day 7–11 pc (Table 1). The maximum mean CS of 9 was reached at day 7 pc (Fig. 3b).

While none of the vaccinated piglets showed thrombocytopenia throughout the trial, all but one of the animals that had to be euthanized prior to the end of the trial showed marked reduction of platelet counts (Table 1). Over time, cumulative CS of 120, 18, 488 and 567 were calculated for groups V5, V8, C5 (challenged at 7 weeks of age) and C8 (challenged at 10 weeks of age), respectively, showing that the control piglets challenged at 10 weeks of age were most severely affected.

The post mortem examination of the two vaccinated groups (V5 and V8) revealed no pathological changes associated with CSF. In contrast, typical lesions for CSF such as; petechiae in the tonsils, spleen and kidney, and hyperemia in the mesenterial lymph nodes were observed in seven piglets from C5 and eight piglets from C8, respectively.

3.2. Antibody detection

At farrowing, all sows were tested positive for CSFV antibodies (Fig. 4). The piglets that were blood sampled prior to uptake of colostrum were CSFV antibodies negative. At the next sampling 3 days later, all piglets were seropositive. Antibody decrease was detected until challenge in all groups and around week 5 of age the antibody levels fell below the threshold. After challenge the antibody levels increased in the vaccinated piglets whereas the antibody levels of the mock-vaccinated piglets remained close to threshold.

3.3. Virus isolation from tonsils

In the control groups, infectious virus was isolated from the tonsils of nine piglets from control group C5 and four piglets from control group C8 (Table 1). No CSFV was isolated from the tonsils of the vaccinated piglets.

3.4. Viral RNA detection in serum

Based on the RT-qPCR, viral BVDV RNA could not be detected in the vaccinated piglets (data not shown). On day 3 pc, a low CSF viral load of mean cycle threshold (Ct) 36 was detected in the serum samples from all piglets in both vaccinated groups V5 and V8 (Table 2).

Table 2
Virus RNA detection by RT-qPCR using CSFV probe in serum samples collected post challenge. The Ct values are expressed as the mean of all positive animals.

Group	3 dpc	7 dpc	10 dpc	14 dpc
V5 (vaccinated at 5 and challenged at 7 weeks of age)	13/13 Ct 36	7/13 Ct 40	1/13 Ct 38	1/13 Ct 38
C5 (mock-vaccinated at 5 and challenged at 7 weeks of age)	12/12 Ct 35	12/12 Ct 36	4/12 Ct 39	2/8 Ct 41
V8 (vaccinated at 8 and challenged at 10 weeks of age)	12/12 Ct 36	3/12 Ct 36	2/12 Ct 40	1/12 Ct 43
C8 (mock-vaccinated at 8 and challenged at 10 weeks of age)	12/12 Ct 35	12/12 Ct 30	4/9 Ct 38	0/6

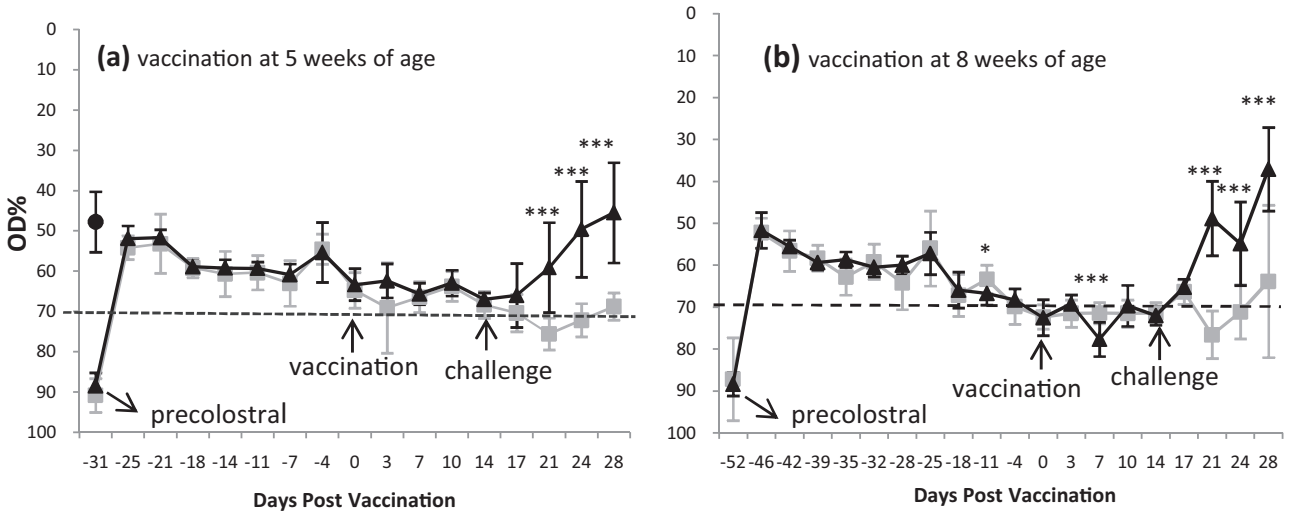

Fig. 4. Results obtained by blocking ELISA and depicted as average optical density (OD) % with a cut-off value of 70 (dashed line). Results ≥70 are negative for CSFV antibodies and results <70 are positive for CSFV antibodies. Graph (a) shows vaccinated at 5 weeks of age and challenged at 7 weeks of age V5 (▲–▲), control mock-vaccinated at 5 weeks of age and challenged at 7 weeks of age C5 (■-■) and sows at farrowing (●). Graph (b) shows vaccinated at 8 weeks of age and challenged at 10 weeks of age V8 (▲–▲) and control mock-vaccinated at 8 weeks of age and challenged at 10 weeks of age C8 (■-■). Each symbol represents the mean ± SD. The significant difference in the OD% between vaccinated and control groups were indicated with $^{*}p < 0.01$; $^{**}p < 0.001$; $^{***}p < 0.0001$.

On day 7 pc viral RNA was detected in the serum samples of seven piglets from group V5 with a mean Ct of 40, and in three piglets from group V8 with a mean Ct of 36. At day 14 pc, only one pig in both groups V5 and V8 was tested positive for CSF RNA.

Viral RNA was detected in the serum samples of all piglets from the control groups C5 and C8 on day 3 and 7 pc. Two of the eight remaining pigs from group C5 were tested positive with very low viral load of 41 at day 14 pc. No viral RNA was detected in the remaining piglets from group C8 at the end of the experiment.

4. Discussion

The economy of many countries depends on the export of pigs and pig products, and free trade relies on guarantees based on sero-surveillance. Therefore, development of a marker vaccine that will allow the detection of infection in vaccinated pigs is of paramount importance. In several comparative studies the chimeric vaccine "CP7_E2alf" was chosen as the final marker candidate to be brought to registration [19]. The efficacy of this vaccine candidate was tested only in naïve animals. However, the humoral immunity in endemic areas will be based on MDA, thus we analyzed the efficacy of a live chimeric marker vaccine "CP7_E2alf" in piglets from C-strain vaccinated sows when the piglets were 5 or 8 weeks old. The outcome of challenge infection with highly virulent CSFV strain "Koslov" was compared at an age of 7 and 10 weeks with and without a prior CP7_E2alf vaccination.

The maternal immunity was transferred very efficiently as all piglets after colostrums ingestion were antibody positive at a level close to what was seen for the sows. Following vaccination an increase in the antibody levels at 2 weeks post vaccination coincided with the time of challenge. The significant difference observed post vaccination between the antibody levels of the vaccinated and the mock-vaccinated animals showed that the MDA at 5 or 8 weeks of age did not neutralize the vaccine virus. The experiment used piglets from domestic sows. In wild boar population the MDA level is expected to be higher [20] as sows give birth to fewer piglets and there is a repeated vaccination in a presence of acute infection. Thus, the outcome of vaccination with CP7_E2alf in wild boar piglets might be different.

Challenge of unprotected piglets at 7 weeks of age with highly virulent CSFV strain "Koslov" is expected to result in high virus levels in serum, severe disease, and almost 100% mortality within 2 weeks [21]. In this study, both vaccinated and mock-vaccinated piglets reacted with an increased body temperature. The mock-vaccinated pigs had increased CS, and low CSFV RNA loads corresponding to viral loads detected after infection with moderately or low virulent strains were detected in the sera of all tested animals at days 3 and 7 pc. The course of the infection was very mild compared to earlier studies in naïve piglets and the percent of mortality decreased from 100% [22] to 50% when the piglets were challenged at 10 weeks of age and to 30% when challenged at 7 weeks of age, respectively. Based on the back titrations a higher dose of infectious material was used for group C8/V8 than for C5/V5. This is considered to be of minor importance, as the titer of the infectious material used for both challenge trials were higher than the titer reported to cause 100% mortality [22].

According to Mittelholzer et al. [15], CSs and body temperatures of highly virulent CSFVs are defined as: CS >15 and fever >41.0 °C. Although 10 control piglets in our study were severely affected and succumbed to the infection only one pig reached CS of 14 and temperature rarely exceeded 41.0 °C. Similar tendency of mortality with low CS were observed in pigs challenged with highly virulent strain "Margarita" that also belongs to genotype 1 [23]. Possibly, the parameters used in the present study will be more precise for strains of genotype 2.3 that show a prolong disease course with multisystemic signs.

A decrease in the platelets was observed at day 3 pc, and severe depletion (below 100×10^6 cells ml^{-1}) in this population was observed from day 7 pc, which is later than previously reported in naïve animals [24]. With the exception of one piglet that was euthanized with mild clinical signs, thrombocytopenia was observed only in the piglets that were euthanized earlier with severe clinical signs. This is in correlation with previous findings that thrombocytopenia is not a characteristic hematological parameter in piglets with mild CSF [25].

Interestingly, many control piglets survived after challenge and seemed to recover from infection. According to the planning all piglets were euthanized 2 weeks after challenge, so true recovery could not be shown. Decreases in the CS in both control groups were observed from day 7 pc. Normal level of the WBC at day 14 pc was detected in all except one pig. No fever was recorded in these pigs at the end of the experiment. The more severe clinical picture

and the higher fatality in the piglets challenged at 10 weeks of age compared to the piglets challenged at 7 weeks of age was probably due to the lower level of MDA in these piglets.

The higher MDA level in group C5 represents a possible explanation for the contrasting results obtained by virus isolation from the tonsils of the piglets from this group. Although, CSFV was isolated from the tonsils of all of the remained eight mock-vaccinated piglets of group C5 no clinical signs were shown by these piglets at the end of the experiment. Oppositely, three of the four piglets euthanized earlier with high CS were tested negative for virus isolation from their tonsils. No correlation was found between virus isolation from tonsils and detection of viral genome in the serum of these piglets. In group C8, viral genome in serum and infectious virus in the tonsils was detected in the earlier euthanized piglets only. Probably, the MDA in group C5 neutralized the virus and prevented spreading. These piglets may become severely affected later when the MDA decrease and may have experienced a chronic infection. These findings support that the immune status contribute significantly to the clinical course of CSF [26].

Hyperthermia was observed in several animals in both vaccinated groups, but they were not accompanied by clinical signs other than slight depression and lack of appetite. The mild clinical picture correlated with the negative virus isolation from tonsils and the lack of pathological lesions in the vaccinated piglets. Possibility for transmission of the challenge virus from the vaccinated piglets seemed negligible as very low quantities of viral genome were detected at day 3 pc. In the vaccinated groups the number of positive tested pigs decreased at day 7 and at day 14 pc only two vaccinated piglets remained positive.

The higher cumulative CS for group V5 compared to group V8 was due to the more frequently observed depression. Porcine immune system is fully matured at the age of four weeks [27], less than five weeks of age the maternal immunity will protect and interfere with vaccination [13].

5. Conclusions

CP7_E2alf proved to be effective in 5 and 8 weeks old piglets with MDA from sows vaccinated once intramuscularly with C-strain 4 weeks before farrowing. The vaccine prevented mortality, severe clinical signs and pathological lesions when the piglets were challenged with highly virulent CSFV strain, two weeks post vaccination. Although maternal immunity in some of the mock-vaccinated piglets proved to be sufficient to prevent mortality it did not adversely affect the successful vaccination with CP7_E2alf. The vaccine was safe in piglets of 5 and 8 weeks of age positive for C-strain MDA as no side effects or fever was observed post vaccination. When comparing the efficacy of CP7_E2alf in 5 and in 8 weeks-of-age piglets no major differences were found. Considering the remarkable protection by the maternal immunity up to 7 weeks-of-age it is advised to vaccinate domestic piglets in C-strain vaccinated areas at 5 weeks of age.

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