Third generation DIVA vaccine towards classical swine fever virus. Efficacy in face of maternal immunity

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Ph.D. Thesis by Desislava Yordana Rangelova · 2013

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DTU Vet
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Indhold

PREFACE

The experimental work, on which this PhD thesis is based, has been performed during the years 2010-2012 at the Technical University of Denmark, National Veterinary Institute (DTU-Vet) - Lindholm. The project was funded by the European Community's Seventh Framework (FP/2007-2013) CSFV_goDIVA project and by the Technical University of Denmark.

The EU network of excellence "EPIZONE" (Contract no Food CT-2006-016236) provided funding for a "short term mission" with training at Anses, Ploufragan-Plouzane laboratory, Swine Virology and Immunology Unit, BP53, 22440 Ploufragan, France.

As a new area of research for me, the opportunity to be a part of a big international consortium and to be involved in all aspects of the development of a new product from testing to registration was extremely valuable. This project enabled me to learn a lot about development and production of vaccines and to get to know a large number of people working with Classical Swine Fever from across the world.

I am grateful to all of those who in different ways have helped me during this process.

In particular I would like to thank my supervisor Åse Uttenthal for always finding the time to listen and be helpful. She was always there for me to read and comment on my papers, discuss new ideas and laboratory results. Her enthusiasm and devotion to her work were very inspiring and her sense of humor made each work day easier.

I would like to thank Jens Nielsen for helping me to design the animal experiments, and with the clinical work and the autopsies. I appreciate the timely and helpful comments he provided for my papers.

I am grateful to Patricia Renson and Marie-Frederique Le Potier (Anses, France) for inviting me to their laboratory and for taking good care of me during my visit. Patricia contributed considerably to the immunological evaluation of some of the samples and the interpretation of the results. She was very kind and patient in teaching me various techniques and in showing me many beautiful places in Brittany. I had a great time at Saint–Brieuc.

The laboratory technicians at Lindholm Grith, Nethe, Frank, Pernille, Eva, Hanna, Katrine, Dorota and Lone gave me invaluable assistance.

I truly appreciate the animal keepers Marion, Henrik, Janni, Ove, Rikke and Heidi for their efforts during the four and a half months animal experiment, for the nights and weekends spent on the island.

I would like to thank Peter Risager for making the long drive enjoyable and for always being friendly and helpful.

I truly appreciate the support of my former colleagues from the Danish Medicine Agency when I was contemplating whether to start this project.

I am grateful to all of my friends Juji, Dani, Krasi, Iveta and Soraya for always being there for me.

My father is thanked for always supporting me and being interested in my education.

Most of all I would like to thank my partner, Martin, for agreeing to join me on this amazing journey. I am so grateful for your support and trust in me and for taking care of our son and the housekeeping during my absences. I am grateful for my son Nikolaj for being such a wonderful child, for helping me keep things in perspective, and for always making me smile.

Desislava Rangelova, Lindholm, January 2013

ABBREVIATIONS

APC- antigen presenting cell

APP- acute phase protein

B-cell- bone marrow- derived lymphocyte

BDV- Border disease virus

BVDV- Bovine viral diarrhea virus

CD4 - cluster of differentiation 4

CD8 - cluster of differentiation 8

CRP- C-reactive protein

CSF- Classical swine fever

CSFV- Classical swine fever virus

CS- clinical scores

C-strain- Chinese strain

CTL- cytotoxic T lymphocytes

Ct – cycle threshold

cDNA- complimentary DNA

DIVA- differentiating infection in vaccinated animals

DNA- deoxyribonucleic acid

DPC-days post challenge

DPV- days post vaccination

ELISA- enzyme-linked immunosorbent assay

EU- European Union

GMO- genetically modified organism

GPE^- - quinea-pig exaltation-negative strain

IFN- interferon

Ig- immunoglobulin

IL- interleukin

MDA- maternal derived antibdies

NPLA – neutralization peroxidase-linked assay

NTR- non-translated region

OIE- Office International des epizooties

ORF- open reading frame

PBS - phosphate buffered saline

PK-15 – pigs kidneys cells

RNA- ribonucleic acid

RT-qPCR - real time reverse transcription polymerase chain reaction

SE – standard error

SD – standard deviation

T cell - thymus-derived lymphocytes

$TCID_{50}$ - tissue culture infective dose 50%

Th- T helper cells

TNF-α – tumor necrosis factor alpha

VI- virus isolation

VNT- virus neutralization test

WBC – white blood cells

SUMMARY (English)

General purpose and objectives

Classical swine fever (CSF) is a highly contagious disease that causes huge economical losses and animal welfare concerns worldwide. Generally, vaccination is an effective and safe method to control the disease. Following vaccination the pig's immune system develops antibodies that are significant part of the protection. However, vaccination with the only live attenuated vaccines existing on the market that contain a whole CSF virus (CSFV) with reduced infectivity, leads to production of an antibody response that does not differ from the antibody response developed after infection. Thus, implementation of these vaccines in case of outbreak will not give the possibility to differentiate infection in vaccinated animals (DIVA). For countries like Denmark, which are heavily dependent upon export of pigs and pig products the use of these traditional vaccines, will hamper the ability to proof a disease free status by serosurveillance, as all vaccinated piglets will be seropositive.

This PhD-project is a part of an EU project (CSFV_goDIVA grant no 227003) that has been funded by the European Commission with a main goal to develop and test to a level of registration a new DIVA vaccine candidate. The vaccine candidate "CP7E2alf" is intended for either intramuscular vaccination of domestic pig or for bait vaccination of wild boar. In this thesis as part of the clinical testing of the injection vaccine the efficacy of "CP7E2alf" was evaluated in young piglets that were positive for maternally derived antibodies (MDA). These antibodies were obtained with colostrum from their mothers vaccinated with traditional live attenuated vaccine C-strain (Riems). The promising results concerning the safety and the efficacy of the candidate DIVA vaccine showed new opportunities for control of a possible CSF outbreak that will have reduced impact on the export.

Structure of the thesis

The thesis comprises three chapters:

Chapter 1 is an introductory review of the literature giving a brief presentation of CSF including the current knowledge concerning CSFV and the disease. Strategies for prevention and control of CSF are presented, such as the use of vaccines and the DIVA vaccines concept. A description of the interplay between vaccines and maternal immunity in the young piglet is included and the characteristics of the new marker vaccine candidate "CP7_E2alf" are presented in this part. The

PhD work is based on a large vaccination experiment which is described in the 3 manuscripts (see chapter 2). A small pilot study that involved 12 pigs vaccinated intramuscularly with one dose of C-strain vaccine was conducted at the beginning of the project. The study aimed to evaluate the humoral immune response after C-strain vaccination. The data were presented as part of a poster [1] and a graph is included in Chapter 1.

Chapter 2 describes the results generated during the PhD project, presented in three manuscripts. Manuscript 1, "Efficacy of marker vaccine candidate CP7_E2alf in piglets with maternally derived C-strain antibodies" by D. Rangelova, J. Nielsen, B. Strandbygaard, F. Koenen, S. Blome, Å. Uttenthal. Vaccine (30), 6376-6381.

This published paper describes the clinical evaluation of intramuscular vaccination with the DIVA candidate "CP7_E2alf" of piglets with C-strain MDA at 5 or 8 weeks of age. The vaccinated piglets together with their mock-vaccinated controls were challenged with highly virulent CSF strain "Koslov" two weeks post vaccination. The piglets were clinically observed for two weeks. "CP7_E2alf" proved to be effective in both age groups as it prevented mortality, morbidity and pathological lesions. The paper discusses as well the attenuation of the challenge virus in the control-mock vaccinated piglets and the relevancy of the clinical parameters followed. This animal experiment was the base for the research conducted in this thesis. The samples collected during the experiment provided material for further evaluation of the "CP7_E2alf" vaccine characteristics.

Manuscript 2, "DIVA potential of marker vaccine candidate CP7_E2alf in face of C-strain maternal immunity" by D. Rangelova, Å. Uttenthal. Vaccine - submitted.

This submitted manuscript evaluates the DIVA potential of the marker vaccine candidate in presence of C-strain MDA. The possibility for transmission with colostrums and detection of CSF E2 and E^{rns} specific antibodies was investigated. It was revealed that the candidate marker vaccine could be implemented as a DIVA vaccine in populations previously vaccinated with live attenuated vaccines as early as 5 weeks of age. No presence of specific discriminatory maternal antibodies and

[1] **A Comparative Study of CSFV Antibody Levels in Pigs Vaccinated with the Chimeric Vaccine CP7_E2GIF or a C-Strain Vaccine.** von Rosen, T; Rangelova, D.; Rasmussen, TB; Nielsen, J; Uttenthal, Å. Poster EPIZONE meeting Arnhem, Holland 2011

no interference with the DIVA concept were detected in older than 4 weeks of age piglets. Virus neutralization studies supported that E2 was transferred very efficiently from the sows to the piglets; shortly after uptake of colostrums the piglets had reached the same level of E2 antibodies as their mothers.

Manuscript 3, "Evaluation of immune modulation in sows vaccinated with C-strain and in their piglets vaccinated with new candidate marker vaccine CP7_E2alf" D. Rangelova. J. Nielsen, P. Renson, M.F. Le Potier, Å. Uttenthal.
This manuscript is prepared for journal of Veterinary Research. The manuscript evaluates the predominant activation of innate, humoral and cellular immunity in piglets positive for C-strain MDA that were vaccinated with "CP7_E2alf". Both IgG1 and IgG2 were transferred with colostrum to the piglets. The induction of IgG1 and IgG2 isotype specific antibodies was evaluated both in the mothers vaccinated intramuscularly with C-strain vaccine and in the piglets vaccinated with "CP7_E2alf". Additionally, IL-4 and IFN-γ cytokines that are related to IgG1 and IgG2 isotype specific antibodies, respectively, were evaluated in the piglets post infection. The blood kinetics of C-reactive protein as a component of the innate immune response was studied post challenge. It seems that the two vaccines exploit different mechanisms of the immune system as following intramuscular vaccination with C-strain there was a predominance induction of IgG2 and oppositely post "CP7_E2alf" vaccination mostly IgG1 was detected.

Chapter 3 includes general discussion of the obtained results, overall conclusions and future perspectives for continued research. In general it is concluded that with an optimal DIVA diagnostic tool, "CP7_E2alf" will be a very valuable additional control measure that definitely will be considered to implement in Denmark in case of outbreak.

DANSK SAMMENDRAG

Generelt formål og hensigt

Klassisk svinepest (CSF) er en meget smitsom sygdom, der har enorm betydning for økonomi og dyrevelfærd i svineproduktionen i hele verden. Generelt er vaccination en effektiv og sikker metode til at bekæmpe sygdommen. Efter vaccination udvikler grisens immunsystem antistoffer, disse er en væsentlig del af beskyttelsen. Imidlertid er den eneste kommercielle vaccine en levende afsvækket vaccine, der indeholder hel klassisk svinepestvirus (CSFV). Vaccination med denne vaccine fører til et antistof respons, som ikke kan skelnes fra antistofreaktion efter infektion. Anvendelse af disse vacciner, i tilfælde af udbrud, giver ikke mulighed for at påvise infektion blandt vaccinerede dyr (DIVA). For lande som Danmark, der har stor eksport af svin og svineprodukter, vil brugen af disse traditionelle vacciner, hindre muligheden for at påvise sygdomsfri status ved serologisk overvågning, idet alle vaccinerede grise vil være antistof positive.

Dette ph.d.-projekt er en del af CSFV_goDIVA (bevilling nr. 227003), der er finansieret af EU FP7 med det hovedmål at udvikle og afprøve en ny DIVA vaccine kandidat og føre den frem til registrering. Vaccine kandidaten "CP7E2alf" er beregnet til intramuskulær vaccination af produktions svin eller til oral ”bait” vaccination af vildsvin. Som en del af den kliniske afprøvning af injektions vaccinen er effekten af "CP7E2alf" blev evalueret hos smågrise, der var positive for maternelle antistoffer, studiet danner basis for denne afhandling. Pattegrisene fik antistoffer med råmælk fra deres mødre vaccineret med traditionelle levende svækket vaccine C-stamme (Riems). De lovende resultater vedrørende sikkerhed og effekt af kandidaten DIVA vaccinen anviser nye muligheder for kontrol af et eventuel CSF udbrud. Brug af DIVA vaccine vil have kortere indflydelse på eksporten end brug af levende vaccine.

Struktur af afhandlingen

Afhandlingen består af tre kapitler:

Kapitel 1 gennemgår litteraturen og giver en kort præsentation af CSF, herunder den aktuelle viden om CSFV og sygdommen. Strategier for forebyggelse og kontrol af CSF præsenteres, såsom brug af vacciner og DIVA vaccine konceptet. En beskrivelse af samspillet mellem vacciner og maternel immunitet i den nyfødte gris er inkluderet, og de særlige kendetegn ved den nye markørvaccine

kandidat "CP7_E2alf" præsenteres i denne del. Ph.d. arbejde er baseret på et stor vaccinations eksperiment, som er beskrevet i de 3 manuskripter (se kapitel 2). Et mindre pilotstudie, der involverede 12 fravænnings grise, vaccinerede intramuskulært med én dosis C-strain vaccine gennemførtes i starten af phd projektet. Forsøgets formål var at evaluere det humorale immunrespons efter C-strain vaccination. Data blev præsenteret som en del af en poster[2] og en graf er inkluderet i kapitel 1.

Kapitel 2 beskriver de resultater, der er genereret i løbet af ph.d.-projektet, præsenteret i tre manuskripter. Manuscript 1, "Effekten af markørvaccine kandidat CP7_E2alf i smågrise med maternelle C-stamme antistoffer" af D. Rangelova, J. Nielsen, B. Strandbygaard, F. Koenen, S. Blome, Å. Uttenthal. Vaccine (30), 6376-6381. Denne publikation beskriver den kliniske vurdering af intramuskulær vaccination med DIVA kandidat "CP7_E2alf" i 5 eller 8 ugers smågrise med maternel immunitet fra C-stammen. De vaccinerede smågrise blev, sammen med de placebo-vaccinerede kontrol grise, podet med den høj-virulente CSF-stamme "Koslov" to uger efter vaccination. Grisene blev fulgt klinisk i to uger. "CP7_E2alf" viste sig at være effektiv i både 5 og 8 ugers grise, idet den forhindrede dødelighed, sygdom og patologiske forandringer. I publikationen diskuteres endvidere reduktionen af dødeligheden efter virusbelasting i kontrol-vaccinerede grise og relevansen af de kliniske parametre, der er analyseret. Dette dyreforsøg danner grundlaget for den øvrige forskning i denne afhandling. Prøver fra forsøget analyseres og anvendes til nærmere vurdering af vaccine egenskaberne for "CP7_E2alf".

Manuscript 2, "DIVA potentiale af markørvaccine kandidat CP7_E2alf under indflydelse af maternel immunitet fra C-stammen" af D. Rangelova, Å. Uttenthal. Indsendt til Vaccine. Dette indsendte manuskript evaluerer DIVA potentialet af markør vaccinen i smågrise med maternel immunitet fra C-stamme vaccine. Både CSF E2 og Erns specifikke antistoffer kunne påvises i smågrisene efter optag af råmælk. Det påvistes, at kandidat markørvaccinen beholdt DIVA potentialet ved vaccination i populationer, der tidligere er vaccineret med levende svækkede vacciner. Smågrise kunne vaccineres fra 5 uger. Efter 4 uger blev der ikke påvist specifikke diskriminerende maternelle antistoffer i smågrisene og den maternelle immunitet gav ingen

[2] **A Comparative Study of CSFV Antibody Levels in Pigs Vaccinated with the Chimeric Vaccine CP7_E2GIF or a C-Strain Vaccine.** von Rosen, Tanya; Rangelova, Desislava Yordanova; Rasmussen, Thomas Bruun; Nielsen, Jens; Uttenthal, Åse. Poster EPIZONE møde Arnhem, Holland 2011

interferens med DIVA konceptet. Virusneutralisations undersøgelser underbyggede, at E2 meget effektivt overførtes fra søerne til pattegrisene. Kort efter optagelse af råmælk havde pattegrisene nået det samme niveau af E2-antistoffer som deres mødre.

Manuscript 3, "Evaluering af immunmodulering i søer vaccineret med C-stamme og i deres smågrise vaccineret med ny markørvaccine CP7_E2alf" D. Rangelova. J. Nielsen, P. Renson, M.F. Le Potier, Å. Uttenthal. Dette manuskript er forberedt til Journal of Veterinary Research. Manuskriptet evaluerer den medfødte immunitet og aktiveringen af humoral og cellulær immunitet hos smågrise med maternel immunitet, vaccineret med "CP7_E2alf". Både IgG1 og IgG2 overførtes med råmælk til pattegrisene. Induktion af IgG1 og IgG2 isotype specifikke antistoffer blev påvist både i søerne vaccinerede intramuskulært med C-stamme vaccine og i grisene vaccinerede med "CP7_E2alf". Desuden blev IL-4 og IFN-γ cytokiner, der er relateret til henholdsvis IgG1 og IgG2 isotype specifikke antistoffer, målt i grise efter virusbelastning. Kinetikken for C-reaktivt protein, som en komponent af den medfødte immunitet, blev undersøgt efter virusbelastning. De to vacciner benytter tilsyneladende to forskellige mekanismer i immunsystemet: efter intramuskulær vaccination med C-stammen sås overvejende induktion af IgG2, hvorimod "CP7_E2alf" vaccination især inducerede IgG1.

Kapitel 3 omfatter en generel diskussion af de opnåede resultater samt overordnede konklusioner og fremtidige perspektiver for fortsat forskning. Generelt konkluderes det, at med en optimal DIVA diagnostik, vil DIVA vaccinen "CP7_E2alf" tilføje en meget værdifuld kontrol mulighed, der vil kunne anvendes i Danmark ved udbrud af klassisk svinepest.

CHAPTER 1.

A background orientation for the work performed during the Ph.D. project is given in this part. This involves 1) a general description of Classical Swine Fever 2) a short review of the used vaccines in practice 3) introduction to DIVA vaccines 4) challenges of early life vaccinology 5) the new third generation chimeric vaccine candidate CP7_E2alf.

General Introduction

1). Description of Classical Swine Fever

Pestiviruses Taxonomy

The genus *Pestivirus* of the family *Flaviviridae* contains the four accepted species *Bovine viral diarrhea virus* (BVDV1 and 2), *Classical swine fever virus* (CSFV), and *Border disease virus* (BDV). Several new species have been suggested, such as the HoBi isolated from fetal calf serum and from cattle in Thailand and the porcine Bungowannah virus (Fig. 1) (Kirkland et al., 2007; Liu et al., 2009b; Ståhl et al., 2010).

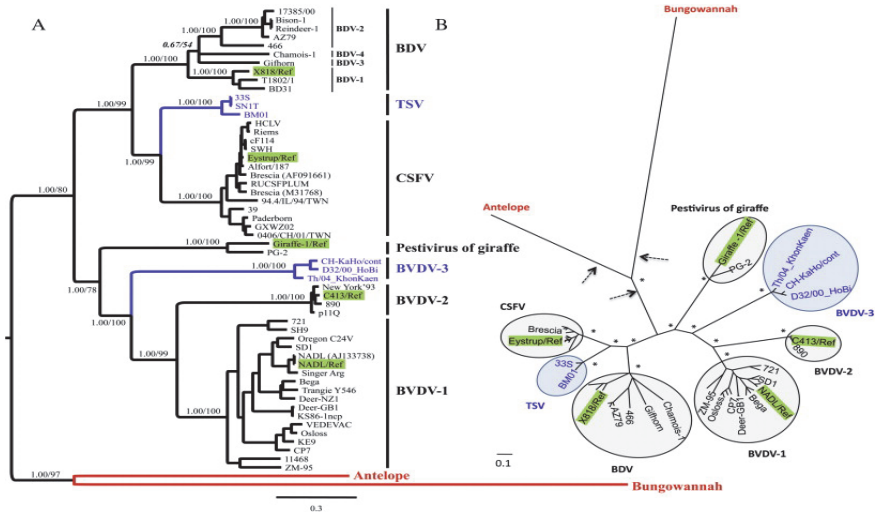


Fig.1 Phylogeny and suggested classification of pestiviruses by Maximum likelihood and Bayesian approach (Liu et al., 2009b). Only BVD I and II, BDV and CSFV are accepted species according to ICTV.

The classification of pestiviruses is mainly based on the host species. The natural host of classical swine fever (CSF) is pigs, however natural BDV infections are not restricted to sheep, and infections of pig, cattle and reindeer have been reported (Arnal et al., 2004). BVDV could infect both pigs as well as ruminants.

Classical Swine Fever Virus – Taxonomy, Structure, Immunogenicity and Stability

Based on genome sequencing, CSFVs were grouped into three or four subgroups: 1.1, 1.2, 1.3; 2.1, 2.2, 2.3; 3.1, 3.2, 3.3, and 3.4. Group 1 represents mostly the historical isolates that were circulating in Europe during the period 1920-1970 and Group 2 are responsible for the outbreaks in the 90 (Paton et al., 2000) (Fig.2). The outbreaks in the Netherlands and in the UK were caused by CSFV genotype 2.1 (Oleksiewicz et al., 2003;Graham et al., 2012). CSFV that belong to genotype 3 were mostly isolated from Asia (Sakoda et al., 1999) (Fig.2).

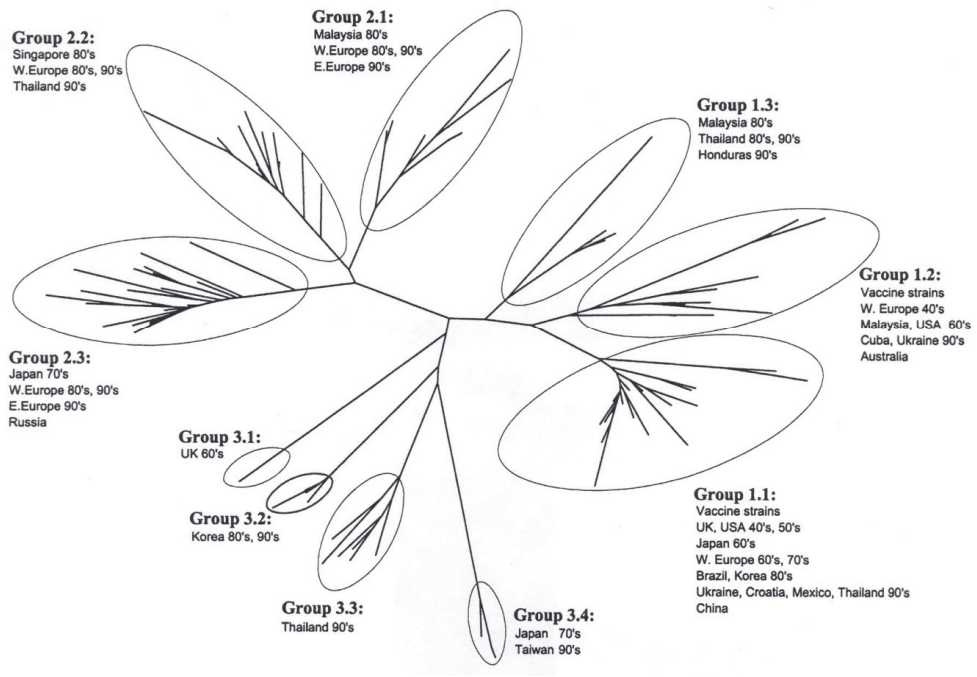


Fig.2. Phylogenetic tree obtained with sequence data from 100 diverse CSF viruses, based on analysis of 190 nt E2 sequence data-set adopted by (Paton et al., 2000).

Pestiviruses are small, enveloped positive single-stranded RNA viruses that contains one large open reading frame (ORF) flanked by non-translated regions (NTR) at the 5' and 3'genome ends (Fletcher and Jackson, 2002). These viruses possess a genome of approximately 12.3 kb encoding a single polyprotein precursor that is processed into the four structural proteins C, E^{rns}, E1 and E2 and seven to eight non-structural proteins: N^{pro}, p7, NS2, NS3/NS2-3, NS4A, NS4B, NS5A and NS5B (Fig. 3) (Meyers and Thiel, 1996).

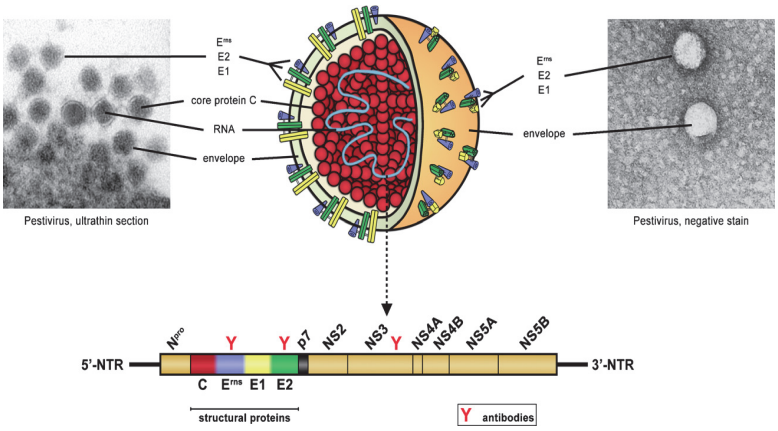


Fig.3. Schematic description of the genome organization and virion structure of CSFV (Electron microscopy: Dr. H. Granzow and Dr. F. Weiland; design: M. Joern; Friedrich- Loeffler-Institute Insel Riems) (Beer. 2007).

The protein structure of pestiviruses is of importance for their immunogenicity, stability and infectivity. The most immunogenic are the envelope structural proteins E^{rns} and E2 as they are specifically recognize by neutralizing antibodies (Paton et al., 1991). Antibodies against the NS3 protein are also induced upon infection, but despite their ability to recognize different pestiviruses, they are not able to neutralize CSFV (Greiser-Wilke et al., 1992).

The possession of a lipid envelope makes these viruses unstable to detergents and lipid solvents that easily destroy them (Moennig. 1992). Despite its envelope, CSFV virus remains infective in a relatively variable pH, in moist, excretion and fresh meat (Moennig. 2000), but is rapidly

inactivated by heat (Edwards et al., 2000). The survival of this virus is prolonged in rich of proteins environment especially in cooled or frozen products (Dahle and Liess, 1992).

Clinical Disease and Pathogenesis

CSF is included in an OIE List as it is highly contagious disease that causes major economic losses in the pig industry (Moennig et al., 2003). The main route of infection is oronasal by direct or indirect contact. Contaminated feed (swill) and transmission with semen was reported as well (Floegel. 2000). The incubation period is approximately one week, but in the field the symptoms may become evident after longer period (Laevens et al., 1999). From the few experimental studies conducted with wild boar it seems that the course of the disease in domestic pigs and wild boar is similar (Artois et al., 2002). However, there are some difficulties with the clinical detection of this disease in wild boar due to their skin pigmentation the alternations that are caused by the disease are not so obvious. Furthermore, as other wild animals they try to "hide" the symptoms of severe disease.

Depending on the virulence of the CSFV strain and the virus - host interaction several clinical forms could be observed (Paton. 2003). CSF clinical forms are very variable and this together with the extensive differential diagnosis and the usual complications by other diseases makes the detection of this disease very difficult (Moennig et al., 2003).Generally, the clinical forms of CSF can be classified as: **post natal infections**, including the subacute, acute and chronic form, **trans-placental** infections and **persistent infections** (Ganges et al., 2007).

Acute form

Post natal acute course of the disease is mostly observed in young pigs up to 12 weeks of age and last less than four weeks. The infected animals may either recover (transient infection) or die (lethal infection) (Moennig et al., 2003). This form is mainly result of infection with highly virulent CSFV and in young pigs the mortality rate may reach 100% (Lohse et al., 2011). CSFV is known to have a particular affinity for cells of the immune system (Susa et al., 1992). All leukocyte population can be deleted, but B lymphocytes are particularly sensitive. The depletion of the white blood cells (WBC) can be caused by necrosis or apoptosis and leads to severe leukopenia (Summerfield. 1998). Leukopenia is usually detected before the onset of vireamia and fever and neutralising antibodies will usually not appear before the leucopenia is overcome (Artois et al., 2002).

A usual finding during the acute form is pyrexia, usually higher than 40°C, anorexia, lethargy, depression, conjunctivitis, enlarged and discolored lymph nodes, respiratory signs and constipation followed by diarrhea, neurological signs as staggering gait and convulsion are frequently observed during this course of the disease (Moennig. 2000) (Fig.4). The typical haemorrhages of the skin are usually observed on the ear, tail, abdomen and the inner side of the legs and appear during the second week of the infection (Laevens et al., 1999) (Fig.4).

Fig.4. Typical signs of acute CSF: haemorrhagical bleedings on the ear and neurological signs (hind legs and hunched up back) (photos-Lindholm).

Similarly to the clinical picture, the pathological findings could also be variable depending on the genotype of CSFV. For example, changes such as necrotic lesions in the tonsils (Fig.5) and infraction of the spleen are often described in the literature from the 1970s when CSFV isolates belonged to genotype 1 (Mengeling and Packer, 1969). The recent genotypes of CSFV lead to changes mainly in the lymph nodes followed by necrotic lesions in the ileum and hyperaemia of the blood vessels in the brain (Uttenthal et al., 2001; Floegel-Niesmann et al., 2003).

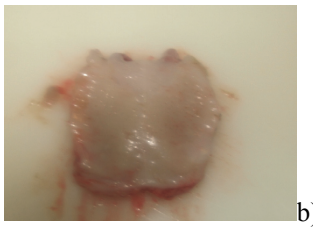
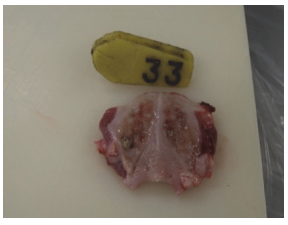
a)  b) 

Fig.5.Picture of a) tonsil obtained from not infected pig and b) tonsil of severely infected piglet no 33 from these studies with haemorrhagical bleedings and necrosis, the piglet was mock-vaccinated and challenged (own photos).

Chronic form

The chronic form of CSF always ends with death, but the animals may survive for 2 to 3 months. Although neutralizing antibodies are produced at the beginning of the infection they are present only temporarily as they are neutralized later by the virus (Depner et al., 1996). Chronic course of infection was defined when a pig was detected virus positive for more than 10 days (Uttenthal et al., 2001). With this form pathological changes are less typical and haemorrhages and other characteristic for the acute form lesions could be missing. Typical are ulcerative lesions on the ileum, ileocecal valve and the rectum (Floegel-Niesmann et al., 2003; Moennig et al., 2003). Chronically infected pigs can excrete virus continuously for a long duration without to be discovered and in this way can play a crucial role in the epidemiology of CSF disease (Weesendorp et al., 2009a).

Persistent infection

In pregnant sows, CSFV is able to cross the placenta and to infect the fetuses (Van Oirschot and Terpstra, 1977). The outcome of transplacental infection is dependent on the stage of gestation and the virulence of the virus. Early pregnancy infection results in stillbirth and mummification and infection about 50-70 days of pregnancy leads to birth of persistently infected pigs. These pigs are very important for the epidemiology of CSF disease as the animals have the virus that is excreted, but no antibodies against CSFV are present (Uttenthal et al., 2010a). The persistently infective piglets could live 2-11 months and during this time they are undetected and excrete a large amount of infective virus (Van Oirschot. 1979).

Immune Response to Classical Swine Fever Virus

The immunity against CSFV includes the three major types of resistance to infection: natural resistance, passively acquired immunity, and actively acquired immunity. Passive immunity will be reviewed separately as it is a major subject of the present thesis. Focus of this section will be mainly the active immunity (innate, cellular and humoral).

Natural resistance to CSF infection occurs on a genetic basis. It was reported a changing clinical picture and mortality after infection with the same strain CSFV in piglets of the same age but different bred (Depner. 1997). The observed different resistance could also be due to the innate immune response. The innate response is a non-specific immune response that includes phagocytosis, activation of complement system and production of inflammatory and antiviral

cytokines as well as an acute-phase protein (APP) response. The APP response is an innate reaction that follows rapidly (6-12 h) after onset of any disease (Baumann and Gauldie, 1994). The APP response involves changes in the serum concentration of different proteins, mainly as a result of alternation in their hepatic synthesis and due to the influence of IL-1, IL-6, and TNF-α (Skovgaard et al., 2009). In pigs, haptoglobin and C-reactive protein (CRP) are considered the major APPs (Heegaard et al., 1998). CRP main function is to bind to the neutrophils and to activate the clearence of damage cells by phagocytosis. Another function is the activation of the classical compliment. It was suggested that early concentration of this protein might predict the outcome of CSF infection as high concentration are detected in the infected with highly virulent strains CSFV pig (Nielsen et al., 2010). In pigs infected with CSFV maintenance of CRP concentrations may role played in the modulation of monocytes and macrophages. Enhancement of the serum CRP concentration may be observed during the late stages of CSF due to the increase number of Kupffer cells that additionally are stimulated by IL-6 and IL-1 (Sánchez-Cordón et al., 2007).

The understanding of cellular immune response is of major importance for the design of new and potent vaccines. Cellular immunity is accepted to be responsible for the early protection against CSF as the protection may precede the appearance of neutralizing antibodies while IFN-γ secreting cells are detected in the peripheral blood. It was shown a correlation between protection and production of CSF specific IFN-γ (Suradhat et al., 2001).The role of cytotoxic T lymphocytes (CTL) has been confirmed in several studies as well (Pauly. 1998; Armengol. 2002; Guzylack-Piriou et al., 2004). High and prolonged level of active CTL was reported from challenged with CSFV pigs that had very active CTL at 3 weeks post infection and that persisted up to day 74 post infection (Piriou et al., 2003). Furthermore, it was demonstrated that NS3 specific CTL are able to secrete IFN-γ (Rau et al., 2006). Very early activation of cellular immune response as 2 days post-infection might be influenced by the way of inoculating the infection. For example, a higher generation of cell-mediated immune response was reported following intranasal and oral CSFV infection than after intramuscular inoculation (Piriou et al., 2003).

Generally, neutralizing antibodies to CSFV may be detected earliest at two weeks after infection (Artois et al., 2002). Development of humoral immunity is very dependable on the virulence of CSFV. As the virus affects mainly the T and B lymphocytes, the development of virus neutralizing antibodies was shown to be spares in piglets infected with low virulent strains CSFV and non-

existent in pigs infected with moderate or high virulent strains as the piglets succumb to the infection before the onset of humoral immunity (Lohse et al., 2012). Post intramuscular administration of live attenuated vaccines seroconversion is expected to be detected after 2 weeks (Van Oirschot. 2003b). As part of this PhD project a pilot study was performed vaccinating 12 4-week-old piglets, 3 piglets were sacrificed every week, the antibody response was followed in the remaining pigs. As seen in fig 6 the first piglets were antibody positive in a whole-virus blocking ELISA at 10 to 17 days after vaccination. After 4 weeks the remaining 3 pigs were antibody positive (fig 6).

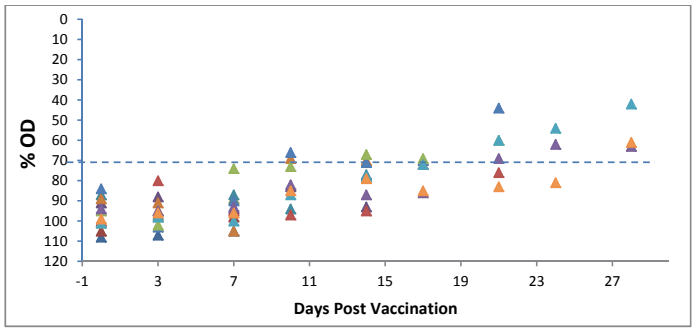


Fig.6. Results obtained by ELISA used for serosurveillance in Denmark (Have. 1984)) testing antibody response in 12 piglets vaccinated intramuscularly with C-strain (Riems) vaccine and followed for 4 weeks. At the beginning of each week 3 piglets were euthanized. Dash line represents the cut off value and all results < 70 are positive >70 are negative (own results obtained from the pilot study conducted at the beginning of the project).

Usually, humoral immunity after oral immunization appears one week later than after intramuscular vaccination (Chenut et al., 1999). B cells activation is stimulated by IL-4 which is produced by Th2 cells. This cytokine is accepted as the main indicator of antibody – mediated immunity (Paul. 1987). There is no information on how this cytokine is modulated during CSF infection.

Presence of the three main immunoglobulins (IgG, IgM and IgA) was previously demonstrated in pig serum (Curtis and Bourne, 1971). IgG is the predominant class and based on gene sequence data the following subclasses were identified: IgG1, IgG2 (a & b), IgG3 and IgG4 (Furesz et al., 1998). IgG2 concentration in pig serum is 46-75% depending on the age of the pig, with higher percentage

detected in younger pigs (Bokhout et al., 1986). IgG1 is the other major immunoglobulin class in pigs. The recently conducted comparative study with live attenuated and live recombinant vaccine against CSF, showed that after oral vaccination without challenge these vaccines differ in their IgG1 and IgG2 activation (Renson et al., 2012). It was previously reported that the IgG1:IgG2 ratio in pigs is a good indicator of the adaptive immunity potentiation toward a Th1 or Th2 response (Crawley et al., 2003). Predominance secretion of isotype specific IgG1 antibodies suggests activation mainly of the humoral part of the immune response and oppositely major detection of IgG2 is related to activation of the cellular immune response (Fig.7).

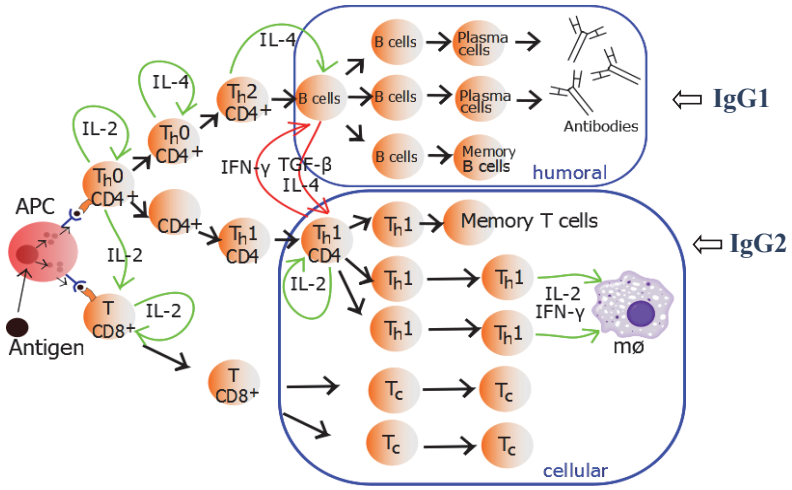


Fig.7. Schematic presentation of cellular and humoral immune response activation in relation to IgG1 or IgG2 predominant secretion (adapted by http://www.quora.com).

Infectivity of Classical Swine Fever Virus

Different factors have been discussed to influence the course of CSF infection (Dahle and Liess, 1992). Specific host-virus interactions determine disease development and clinical outcome. Host factors such as: age, genetic background and immune status, herd sanitary status as well as strain virulence are known to play significant role for the course of the disease (Lohse et al., 2012).

Characterizing virulence has been attempted in various ways: case fatality, clinical and pathological signs, characterization in cell culture or variations in the genome. Based on experimental infections it was found a correlation between clinical scores (CS) (Wood et al., 1988), fever and virulence, but no qualitative or quantitative difference was detected in the RNA replication and protein synthesis among the different strains (Mittelholzer et al., 2000). With the same study it was proposed a system for assigning a virulence based on CS: CS>15 and body temperature > 41°C as a highly virulent strain CSFV and CS of 5-15 as moderate virulent. This system is used by many laboratories and is applied to provide comparability between experiments. However, it is arguable whether this system is precise. Oppositely to the European isolates from the late 1990s that belong to genotype 2.3 and produce delay clinical signs, the historical highly virulent CSFV from genotype 1 (Koslov, Alfort, Margarita etc.) cause acute form of the disease with high mortality very shortly, generally during the first week after infection (Kaden et al., 2001b; Lohse et al., 2011; Tarradas et al., 2011). This is usually insufficient time to develop a clear clinical picture and CS >15 can be rarely reached. Thus, complex factors and parameters additionally to the CS system should be included to correctly evaluate CSFV virulence. (Floegel-Niesmann et al., 2003), introduced pathological scores as an additional parameter for detailed characterization of CSFV. The same authors, proposed modified CS schema including three other parameters such as: case fatality at 3 weeks post infection, leukocyte count between 0-14 days post infection and homologue CSF antibody titer at 14 days post infection (Floegel-Niesmann et al., 2003).

Host factor can significantly attenuate the virus and produce variable disease outcomes. For example, the described by several authors (Mittelholzer et al., 2000; Mayer. 2003; Durand et al., 2009) as highly virulent CSFV-Eystrup strain in other studies conducted with pigs from the same breed depending on the age and the sanitary status caused severe (Uttenthal et al., 2008), moderate (Rasmussen et al., 2007) or mild disease (Nielsen et al., 2010).

The role of the inoculation dose on the virulence of CSFV is a subject of discussion. In a previous study pigs were infected with high (10^6 $TCID_{50}$) or low (10^3 $TCID_{50}$) doses of highly CSFV strain Alfort and clinically no difference between the two groups pigs was observed. It was suggested that the inoculation dose is unlikely to significantly influence the virulence, thus the properties of the virus and the host were suggested to be the major factors responsible (Mittelholzer et al., 2000). Another study evaluated the effect of strain and the inoculation dose on within-pen transmission and

found that with low dose of moderate strain no infection occurred, but with moderate and high dose the outcome of the infection was the same and similar to that with high virulent strain that had the same doses (Weesendorp et al., 2009b). According to Dahle and Liess, 1995, the clinical course is dependable on the inoculation dose of CSFV. Oppositely, Depner et al., 1997 observed that if the critical infectious dose is present the infection takes its course without a significant difference.

Laboratory Diagnostic Methods for Detection of CSF

The key to CSF control during an outbreak is the early identification of CSF infected herds (Dewulf et al., 2004). Diagnostic methods for detection of CSFV are mainly divided into antigen or antibody detecting and virological or immunological methods. As all of the existing tests are not possible to be applied during an outbreak the selection of the proper assay for the exact purpose is very important. Detection of virus or viral nucleic acid in whole blood and of antibodies in serum are the methods of choice for diagnosing CSF in live pigs while detection of virus, viral nucleic acid or antigen in organ samples is most suitable when the pig is dead (OIE. 1998).

Reverse transcriptase-Polymerase chine reaction (RT-PCR) is accepted as the most powerful diagnostic tool for early diagnostic of CSF (Paton et al., 2000; Dewulf et al., 2004). After extraction from the diagnostic sample the RNA has to be transcribed to complimentary (cDNA) that then can be amplified by PCR. Evaluation of PCR or RT-PCR can be performed by agarose gel electrophoresis or by real time techniques (RT-qPCR). However, with this test is not possible to be shown real viraemia and only by virus isolation (VI) on cell culture or infectious studies in pigs can prove that the virus is alive and able to replicate. Another disadvantage of RT-PCR method is that this technique is extremely vulnerable to false negative and false positive results. False negative results can arise when the nucleic acid is degraded or when the reaction mixture contains inhibitors. Due to its high sensitivity, false positive results may arise from contamination (Greiser -Wilke et al., 2007)

The most commonly used tests for CSF antibodies are enzyme-linked immunosorbent assay (ELISA and) virus neutralization test (VNT).The VNT test is able to detect positive piglets earlier than the antibody ELISA test, but it is rather laborious and time consuming (Greiser -Wilke et al., 2007). The VNT is compulsory in the EU for confirmation of positive results obtained by CSF antibody ELISA as it can differentiate between the *Pestiviruses*.

A large comparison of diagnostic tests was performed by Dewulf er al., 2004 and the results are presented graphically in Fig. 8. In order to choose the right test it should be taken in consideration that CSFVs with different virulence may produce viraemia of variable duration and intensity (Depner et al., 1994). It has also been suggested that the sensitivity of genome detecting test (RT-PCR) may vary for different virus strains (De Smit. 2000a). Another problem is the cross reactivity that exist between CSFV and the other *Pestiviruses* (BVDV and BDV) (Wieringa-Jelsma et al., 2006). Thus, it is of importance to take into consideration the prevalence of these viruses in the pig population tested from the affected area. The use of appropriate primers and probes are necessary for the performance of specific and sensitive test.

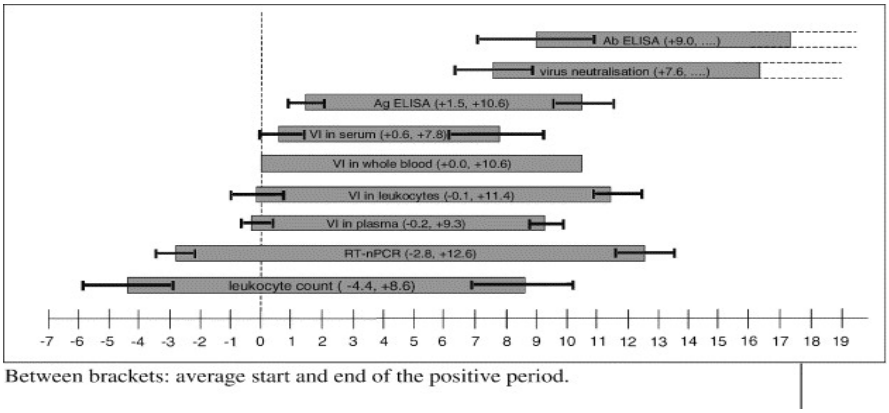


Fig.8. Graphical presentation of the average duration of the test positive period for the different diagnostic techniques in comparison to virus isolation (VI) in whole blood. Average start and end of the positive period is given in parenthesis. The zero indicates when a positive sample was detected by VI (Dewulf et al., 2004).

Strategies for prevention and control of CSF in EU

In the 1970s, CSF was endemic in European Union (EU) Member States and routine prophylactic vaccination was a commonly practiced control measure (Saatkamp et al., 2000). During the 1980s, EU aimed to eradicate CSF from the entire union. Thus, in order to ensure that the same measures for prevention and control of CSF disease are applicable for all Member States the different national policies to controls CSF were replaced by Community legislation (Anonymous. 1980). Since then, prophylactic vaccination is not allowed and in case of outbreak strict control sanitary measures are used. The following measures usually are applied: restriction in movements, establishment of a protective (of minimum 3 km) and surveillance zone (of minimum 10km) around the infection,

stamping out of the infected herd and pre-emptive culling of animals that are suspected but not proved to be infected (Terpstra and de Smith, 2000). For wild boar, bait vaccination in case of emergency is practiced in some areas (Laddomada. 2000; Kaden. 2001a).

Despite the strict control policy, complete eradication of CSF in EU is unlikely. The disease is still present in wild boar that serves as a reservoir and spreads sporadically the infection to domestic pigs (Edwards et al., 2000). The wild boar is non-selective feeder and does not rely on special food (Graves. 1984). Additionally, this species possesses a unique reproductive strategy such as: delay heat, weight-dependable fertility and reproductive season of 6-8 months (Artois et al., 2002). Due to these physiological characteristics as well as hunting practices, wild boar has dramatically increased in number and range and thus, CSF virus eradication in this population is very difficult (Griot et al., 1999) (Laddomada et al., 1994). Giving the ecological elasticity of the wild boar, hunting pressure might be very ineffective in attempting to control population densities and in this way to control CSF (Artois et al., 2002). It was revealed that oral baits are eaten mainly by older animals, but in infected population young animals are targeted, therefore to be effective this strategy needs an improvement (Laddomada. 2000; Kaden. 2001a).

In the late 90s, there were numerous outbreaks of CSF in the EU. The outbreak in the Netherlands in 1997/1998 was one of the most costly with a direct lost estimated to US $ 2 billion. The reasons for these serious consequences were mainly the late detection of the disease, the stamping-out strategies and the movement restriction. The movement restriction led to a culling of enormous number of completely healthy pigs, for example, finishing pigs that grew up and had to be killed as they could not be transported to the slaughter houses. The experience of this outbreak showed that the effectiveness of eradication of CSF is related very much to educating the people involved in the pig production business (Elbers. 2001). It highlighted the need for training the field service in disease recognition in order to increase the speed with which the infection will be diagnosed (Terpstra and de Smith, 2000). The human role in the prevention of CSF is of a significant importance especially in the new EU countries where commercial pig farms are a low percentage and most pigs are kept in traditional, family owned backyard farms for self-consumption (Blome et al., 2006). Due to the enormous cost of the many outbreaks and the ethical aspects of culling large number of healthy pigs the current policy of strict sanitary measures is evaluated. Furthermore, a strict stamping out policy without vaccination might prolong the duration of CSFV epidemics as the

shedding and the spread of the virus to other herds could exist for a long time before the infection is detected clinically and serologically (Uttenthal et al., 2001).

Thus, additionally to the described above measures, emergency vaccination as an alternative control strategy both for domestic and wild boar was recently very intensively discussed (Nigsch and Depner, 2012). Emergency vaccination is allowed in EU, but only under exceptional circumstances and following an approved emergency plan (Anonymous. 2001). However, emergency vaccination of domestic pigs was almost never implemented (Blome et al., 2012). A vaccine that is going to be applied in emergency situation should be more effective than vaccines used for prophylactic purposes. The emergency vaccine should be very potent, have to induce sufficient protection very early at 1 week post vaccination and should be able to prevent congenital infection. Like pre-emptive culling, the aim of the emergency vaccination strategy is to reduce virus spread from an infected herd. Within current legislation possible emergency vaccination strategies include: suppressive vaccination, where animals in a zone around an infected herd are vaccinated with live-attenuated vaccine and subsequently slaughtered (vaccinate-to-kill) or 2) protective vaccination, (vaccinate to live) where a marker vaccine is used (Graham et al., 2012). Effective and safe commercial vaccines are only the existing conventional modified live attenuated vaccines, but they contain complete CSF virus and due to this after vaccination it is impossible serologically to differentiate infected from vaccinated pigs. Vaccinating country is not able to prove serologically that the infection is not present what restricts their trade with pigs and pig products (Moennig. 2000). A territory can be accepted as a CSF- free if some requirements are fulfilled (OIE. 1998). There should be an absence of CSF for at least two years. One year should be passed after the last affected animal was slaughtered if the control measures were stamping out with vaccination or six months after slaughter if no vaccination was implemented. Thus, to ease the pig trade a new marker vaccine that allows detection of infection in vaccinated animals (DIVA) is needed. Furthermore, an implementation of marker vaccine would add very important information on the situation in wild boar and feral pigs as the spread of the infection can be followed in the infected population.

2). Short Review of the Existing Licensed Vaccines against Classical Swine Fever

Modified live vaccines

Vaccines can be divided into 2 large subgroups depending on their ability to replicate in the host. Killed vaccines are based on full virus suspensions that are chemically inactivated before

adjuvation. Live vaccines are attenuated so that they replicate in the host but not sufficiently to cause a disease. For CSF, killed vaccines have been used. However, these vaccines were able to prevent the clinical manifestations of the disease but not the infection. Thus, the inactivated vaccines were replaced by the modified-live attenuated vaccines (Ferrari. 1992). At present the mostly used live attenuated vaccines are based either on Chinese (C) strain, on the French cell culture adapted Thiverval strain or Japanese quinea-pig exaltation-negative strain (GPE^-). The Thiverval strain has been obtained from the virulent Alfort strain and attenuated through more than 170 serial passages at a low temperature (29-30°C). As the C-strain vaccine is produced in many companies the description below is on the registered C-strain for commercial use.

The C-strain, modified live vaccine virus is derived from genotype 1 CSFV. It induces sterile immunity (prevents replication and transmission of the challenge virus) and vaccination with this vaccine leads to very high level of protection within a week post vaccination (Van Oirschot. 2003b). The C-strain vaccine can elicit protective immune response against all CSFV genotypes (Suradhat and Damrongwatanapokin, 2003). The origin of the C strain is not exactly known. Firstly, the vaccine was attenuated by hundreds of serial passages in rabbits, but subsequently to make it cheaper and to avoid some disadvantages of using live organisms (Lorena et al., 2001), the vaccine virus that is used in Europe was adapted to growth in cell cultures (Terpstra et al., 1990). The adaptation of the vaccine to different cell cultures increased the titer of the vaccine without reducing its immunogenicity (Rivero et al., 1988). This vaccine has a U-rich insertion of 13 continuous nucleotides in the 3`non-coding region compared with that of virulent strain and it is speculated whether this insertion is involved in reducing the virulence of the vaccine (Moormann et al., 1996).

C-strain has proven to be a very safe vaccine for pigs of any age and breed. Only mild fever and neither affect on the growth nor local reactions at the site of application have been reported. The possibility for reversion to virulence is accepted as insignificant as the vaccine virus remains avirulent after 30 passages in pigs (Qiu et al., 2005). The vaccine virus primarily replicates in the tonsils (Lorena et al., 2001).

C-strain vaccine induces detectable neutralizing antibodies at 2-3 weeks post vaccination (Fig. 9), here shown by our own unpublished results following piglets after C-strain vaccination. The antibody response of the pigs was measured against the homologues virus (C-strain) as was used for

vaccination. The antibodies reach maximum levels at 4-12 weeks post vaccination and they are present at least for 6-18 months (Precausta. 1983; Terpstra and Wensvoort, 1987; Kaden et al., 2008).

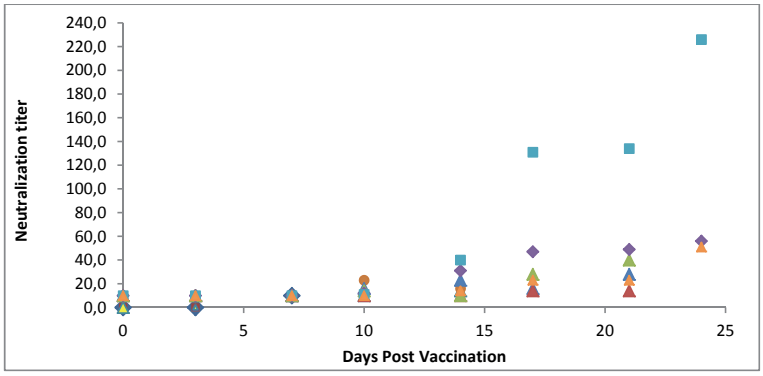


Fig.9 Results obtained by Neutralization test from 12 piglets vaccinated intramuscularly with C-strain (Riems) vaccine. The piglets were followed for 4 weeks and at the beginning of each week 3 piglets were euthanized. (Rangelova, unpublished).

However, protection against a virulent challenge was observed during the first week post vaccination before detection of antibodies (Van Oirschot. 2003b). This early protection after vaccination with C-strain vaccine was shown to be due to the induction of cell-mediated immunity. CSFV-specific IFN-γ secreting cells were detected in pigs vaccinated with C-strain, as early as 6 days post vaccination. During the first week post vaccination no or very low level of neutralizing antibodies were detected (Suradhat et al., 2001). The onset of protection following C-strain vaccination is dependable on the administration method. It was demonstrated that the establishment of complete clinical protection after oral immunization was 10 days post vaccination (Kaden. 2001b). When C-strain vaccine was administered parenterally some protection was detected already at 2-4 days post vaccination and complete at day 7 post vaccination (Van Oirschot. 2003b; Renson et al., 2012).

Subunit marker vaccines

Subunit vaccines are inactivated vaccines, but contain only part of the virus, usually E2 protein (Uttenthal et al., 2001). The application of subunit vaccines may result in some local tissue reactions at the injected sites due to the added adjuvant, but since they are killed vaccines they are

very safe (Bouma et al., 1999; Depner et al., 2001). Two marker subunit vaccines against CSFV have been licensed, one from Bayer, Leverkusen, Germany (*BAYOVAC® CSF Marker*), the other one from Intervet, Boxmeer, The Netherlands (*PORCILIS® PESTI).* Both vaccines are based on the envelope E2 glycoprotein of the CSF virus which has been produced in insect cells by a baculovirus expression vector (Hulst et al., 1993; Hulst. 1994; Van Rijn et al., 1996). Although suitable for prophylactic vaccination, these vaccines showed insufficient efficacy when administrated in situations mirroring an emergency and thus, they are withdrawn from the market. The numerous vaccination-challenge experiments showed that the vaccines were safe, but some problems were recorded with these two subunit vaccines. As these vaccines do not replicate as live vaccines they cannot be applied for bait vaccination of wild boar. Vaccination of pregnant sows did not prevent transfer of challenge virus from the sow to the fetuses (Depner et al., 2001). It was reported that in order to achieve a sufficient efficacy, there should be a booster immunization 4 weeks after the first primer vaccination (Dewulf et al., 2000). According to Uttenthal et al. 2001, there was no sufficient protection post challenge as viraemia was still detected in some piglets challenged 21 days post vaccination, pigs challenged before 21 days after vaccination had a very low protection. Furthermore, with regard to prevention or reduction of transmission of the infective virus, it has been revealed that the majority of vaccinated in-contact pigs become infected when pigs were challenged 7 days post vaccination (Bouma. 2000). Reduction but not prevention of vertical transmission after intranasal or contact challenge with moderate - virulent strain of CSFV was demonstrated (De Smit et al., 2000b). These studies showed that E2-subunit marker vaccines are not efficacious for use as emergency vaccine. Thus, the only vaccine on the market is the live C-strain vaccine. However, as will be describe below, the disadvantage of the attenuated live vaccines is that the antibody pattern induced by these vaccines resembles that of reconvalescent animals surviving from the infection, so that vaccinated and field-virus-infected animals cannot be differentiated serologically. Thus, the control of CSFV outbreaks in domestic pigs as well as in wild boar could be significantly enhanced if a safe and efficient DIVA vaccine is available. In the next section the principal of DIVA vaccines will be described.

3). Introduction to DIVA Vaccines

The term Differentiation of Infected from Vaccinated Animals (DIVA) was introduced in 1999 (Van Oirschot. 1999). The general principal that the DIVA vaccines exploit is based on the difference of the antibodies induced after vaccination with the DIVA vaccines and on these induced

after infection with wild CSFV (Fig. 10). As the main purpose is to detect infection rather than to distinguish between vaccinated and infected animals it was suggested that "Differentiating Infection in Vaccinated Animals" might be a more accurate description (Uttenthal et al., 2010b).

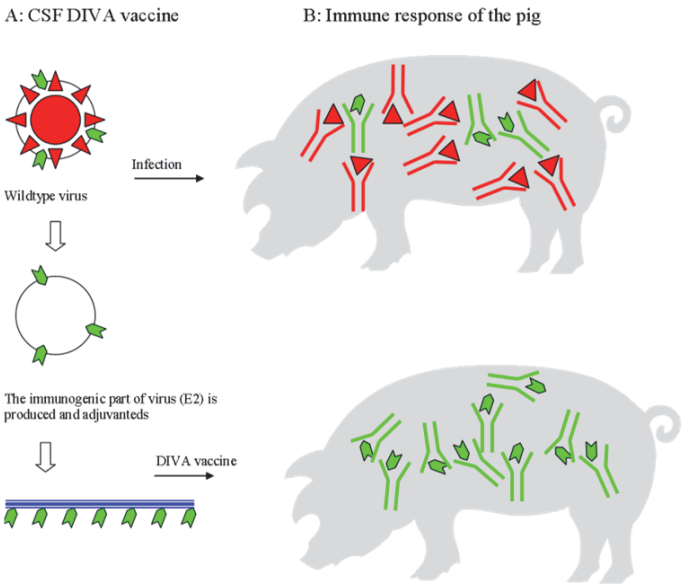


Fig. 10 Principle of vaccination with DIVA vaccine (Uttenthal. Å, with permission).

In general, there are three feasible marker design strategies:

1). "negative marker" - there is an absence of antigenic epitope, domain or protein compared with the wild-type virus

2). "extrinsic positive marker"- an extrinsic immunodominant epitope or protein is included in a potent vaccine, these vaccines are used to prove a good seroconversion to the vaccine, but they do not have DIVA potential

3). "intrinsic positive marker" - an immunogen or epitope that is from the virus itself but induce a different antibody pattern from that of the wild type.

Almost all candidate DIVA vaccines were constructed as "negative marker" vaccines and their companion diagnostic test was designed to detect antibodies against the absent in the vaccine

protein (Dong and Chen, 2007). The three proteins inducing detectable antibodies are: E2 (major immunogen, neutralizing activity), E^{rns} (low or no neutralizing activity) and NS3 (no neutralizing activity). Among *Pestiviruses* the NS3 protein has a high genetic stability and as there is a possibility for infection with BVDV and BDV viruses in pigs, detection of NS3 specific antibodies is not suitable for DIVA diagnostic (Beer. 2007). Thus, DIVA vaccines against CSFV are mainly based on the most immunogenic E2 enveloped protein (Hulst. 1994). The new chimera vaccine "CP7_E2alf" is also constructed on the principle of a "negative marker" using only E2 from CSFV.

Discriminatory Tool

Without sensitive and specific discriminatory tool a vaccine cannot be implemented as a DIVA vaccine. ELISA was the major tool that up to now has been developed as DIVA test.

Immunological DIVA tool

ELISA test for the detection of CSFV antibodies directed against E2, E^{rns} or NS3 protein are commercially available. E2-ELISAs are used in the field as conventional screening test for the detection of CSFV infection on a herd bases (European Union (EU). 2002). E^{rns} blocking ELISA test that detects specific CSFV E^{rns} antibodies was designed as accompanying discriminatory tool to the above described E2 subunit vaccines. The principle of this test is that the E^{rns} antigens expressed in baculovirus expression system and the serum of antibody positive pigs competes with several monoclonal antibodies targeting E^{rns} (Uttenthal et al., 2010b). The only commercially available E^{rns} ELISA is the PrioCHECK® CSFV test. Initially, the test had some deficiency regarding its sensitivity and specificity (Schroeder et al., 2012).

Genetic DIVA tool

The ELISA discriminatory test is possible after induction of antibodies that with CSFV are not expected before 7 days post infection. This early window phase for detection with discriminatory ELISA assay may be covered by detection of viral genome. The recently published RT-PCRs (Hoffmann et al., 2005; Hoffmann et al., 2006), permit direct differentiation between vaccinated and infected pigs. The RT-PCR assay specific for all pestiviruses (panpesti-specific) detects both, the vaccine strain and the wild CSFV (Hoffmann et al., 2005; Liu et al., 2009a). The CSFV specific RT-PCR assay detects only the infected with CSFV pigs (Hoffmann et al., 2006).

Different types of DIVA vaccines against CSFV

The next generation of marker vaccine candidates against CSF is divided in several groups and their characteristics are summarized in Table.1.

Table 1 Different types of DIVA vaccines (Beer. 2007).

Type of CSFV vaccine	Examples	Disadvantages
CSV peptide vaccines based on E2 antigen	Mono-peptide-vaccines Multi-peptide-vaccines	Fail to provide complete protection and not suitable for oral immunization
DNA vaccines	Immunization with expression plasmids containing complete or partial CSFV-E2-encodin sequence (encoding sequence of immunostimulating factors could be also included)	High doses and several applications are needed to achieve protection against mortality
Viral vectors vaccines	Expression of E2 (complete or partial) into the genome of other viruses	As with all live attenuated viruses there is a concern about recombination and reversion to virulence
Chimeric pestiviruses	-CSFV-E2-encoding sequences are inserted into a BVDV backbone -BVDV or BDV sequences are inserted into a CSFV vaccine backbone	As with all live attenuated viruses there is a concern about recombination and reversion to virulence
Trans-complemented replicons	-Packaged replicons with a deletion in the E^{rns} encoding region -Packaged replicons with a deletion in the E2-encoding region	Protection depends on the application route and the best obtained results are after dermal injection

4). Challenges of Early Life Vaccinology

One of the major challenges in vaccinology is the development of products that are able to induce protective immunity in the early life period. It is very important that biological products are designed to provide protection of the youngest individuals. However, this is a particularly challenging task given the fact that pig's immune system is not completely developed before 4 weeks of age (Povey C. 1997). Additionally to the immature porcine immune system, the presence of inhibitory concentrations of passive antibodies obtained trough colostrums from immune mothers during the early life of pigs imposes a further barrier to effective early life vaccination against CSFV in the field (Launais et al., 1978; Suradhat et al., 2007). There are different mechanisms for MDA to influence the active development of B and T- cells. Generally, administration of vaccine antigen in a host may result into the formation of antigen-antibody complexes (Fig.11). MDA bind to specific B-cell vaccine epitopes, blocking the access of the pig's own B cells to such epitopes and the level of this inhibition depends upon the vaccine antigen/MDA concentration ratio. The antigen-antibody immune complexes are further either processed by the piglet antigen presenting cells (APC) or destroyed by the reticuloendothelial system. Thus, peptide presentation at the APC leads

to priming of the cellular immunity (CD4 or CD8 T cells), despite the inhibition of the humoral immunity. However, this priming could be inhibited by high levels of MDA that could entirely neutralize the vaccine virus, which will not be able further to replicate and this will lead to significant reduction of the vaccine effectively (Siegrist. 2001).

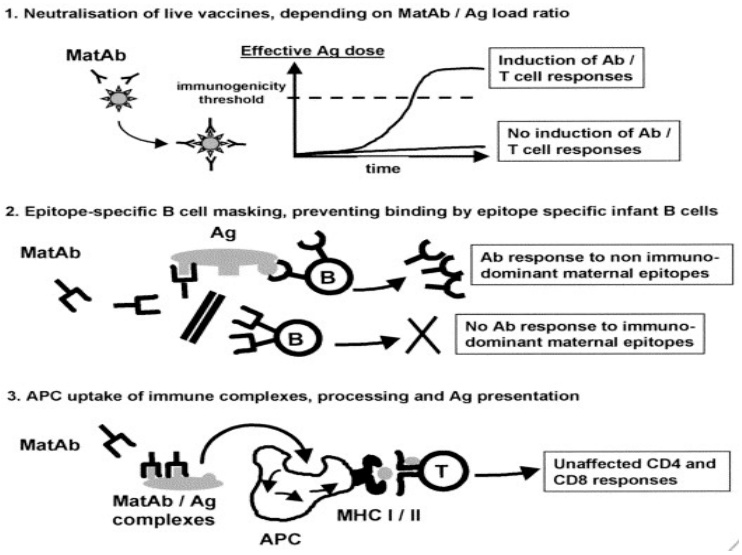


Fig.11 Influence of passively transferred antibodies on induction of vaccine response (Siegrist. 2001).

In case of CSFV outbreak, vaccination at earliest possible age is needed. However, in population previously vaccinated the presence of maternal immunity may cause some complication. MDA has been shown to affect antibody responses to vaccination against CSFV (Vandeputte et al., 2001). As mentioned above, the level of this influence is very much dependable on the MDA concentration. For CSF, it was demonstrated that the half life of MDA depends on the time point of the mother's vaccination. The earlier the mothers are vaccinated the higher are the antibody titers at the time of colostrums production that they transfer to the piglets. Fig. 12 shows the relation between sows antibody concentration and the levels of the obtained with colostrums antibodies in the present study. All piglets were antibody negative prior to uptake of colostrums but at the next sampling 3-7 days after birth the piglets had antibody titers close to what was seen in the mothers. The longest persistence of CSF MDAs was reported when sows were vaccinated before pregnancy with several doses (Launais et al., 1978; Suradhat and Damrongwatanapokin, 2003). Studies have shown that

following oral immunization MDAs are present for longer time than after intramuscular immunization (Müller et al., 2005; Suradhat et al., 2007). According to Launais et al. 1978, the most successful period for vaccination is from 5 to 8 weeks of age, as during this time the pigs still have MDAs with moderate concentrations that protect them, but do not interfere with the efficacy of the vaccine. It was demonstrated that CSF vaccine induced complete protection when the MDAs neutralization titer at the time of the vaccination was lower than 64 (Suradhat et al., 2005). This was also confirmed in our studies when using the "CP7_E2alf" vaccine (Rangelova et al., 2011).

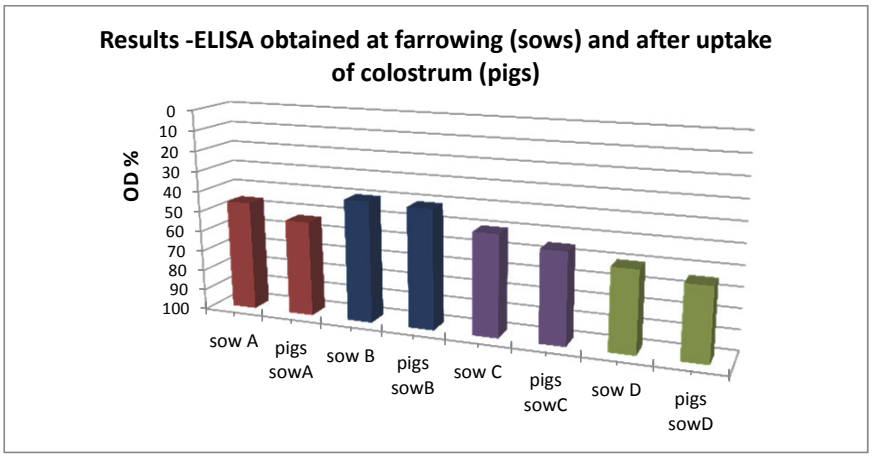


Fig.12. Results obtained by blocking in house ELISA (Have,P. 1984) and depicted as average optical density (OD) % at farrowing in four sows vaccinated intramuscularly with C-strain at four weeks before farrowing and in their piglets after uptake of colostrums. Cut off value is 70 and results ≥ 70 are negative and < 70 are positive. (Rangelova, unpublished)

Thus, vaccine type, age of the pig at vaccination, administration route, dose and presence of adjuvant are factors that influence also the early life antibody response to vaccination (Siegrist. 2007). Different strategies such as: choosing the right type vaccine, addition of adjuvant and increasing the vaccine dose could be used to enhance efficacy in presence of MDAs. However, the safety profile of a vaccine could be compromised if these strategies are not appropriately balanced. Thus, the identification of effective and safe early life strategies requires specific research approach in the search for the right vaccination schedule that will provide efficacy/safety balance.

5). The New Third Generation Chimeric Vaccine Candidate "CP7_E2alf"

The chimeric "CP7_E2alf" virus was constructed using a complimentary DNA (cDNA) clone of cytopathic BVDV type 1 strain CP7, which is avirulent for pigs. The entire E2- encoding region, of CP7 which is the most immunogenic protein in the structure of *Pestiviruses*, was replaced by that of CSFV strain Alfort 187 (Fig.13) (Reimann et al. 2004).

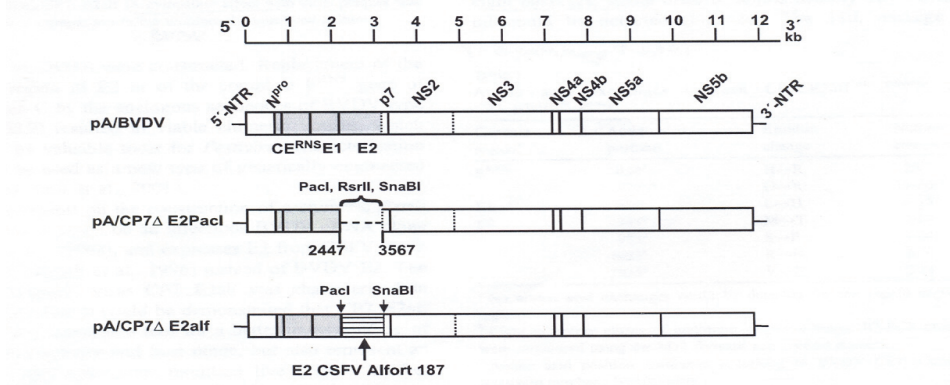


Fig.13 Schematic representation of the engineered construct of "CP7_E2alf" (Reimann et al., 2004).

Different from the conventional vaccines, marker vaccines must fulfill both the general demands (safety, efficacy, clinical protection, etc.) and special requirement (induction of characteristic antibody pattern). Moreover, as these vaccines are intended for emergency vaccination there is a higher demand for very fast onset of solid immunity.

Safety and Efficacy of "CP7_E2alf"

The safety and the efficacy of "CP7_E2alf after intramuscular or oral administration were addressed in several published studies (Reimann et al., 2004; Koenig et al., 2007; Leifer et al., 2009; Tignon et al., 2010; Blome et al., 2012; Gabriel et al., 2012; Rangelova et al., 2012). In the frameworks of these studies, the safety was tested in different age categories ranging from approximately 1-8 months. The pigs were observed for 14 to 98 days periods. None of the animals showed CSFV-associated clinical signs or pathological lesions, fever or leucopenia, even after triple vaccination. Moreover, no local reactions at the site of injection were observed in any of these experiments.

The efficacy studies used for the challenge infections the highly virulent CSFV strains. These strains were reported to cause severe clinical signs and 100% mortality in naïve piglets during the

first week post infection (Mittelholzer et al., 2000). It was shown that intramuscular or oral vaccination with "CP7_E2alf" resulted in full clinical protection when challenge was performed very shortly after vaccination, which is prerequisite for the use as an emergency vaccine. Already 1 week after intramuscular vaccination was demonstrated complete prevention of morbidity, even with 1:10 diluted vaccine (Leifer et al., 2009). Although, an early protection against highly virulent strain CSFV after oral vaccination is a difficult task to achieve due to the late onset of humoral immunity (Greiser -Wilke et al., 2007), "CP7_E2alf" performed even better than the "gold-standard" C-strain "Riems" when the piglets were challenged 14 days post vaccination (Leifer et al., 2009).The same tendency was observed with oral "CP7_E2alf" vaccination that resulted in 100% complete protection 14 days post challenge while with C-strain only 83% of the pigs were protected (Blome et al., 2012).

Concerning prevention of viraemia, no sterile immunity was induced after oral vaccination with "CP7_E2alf" as it seems that a limited replication of the challenged virus occurred based on the real-time PCR (RT-PCR) results and on the positive virus isolation from the blood sample of one vaccinated pig (Blome et al., 2012). More studies from the same group reported complete lack of virus isolation from nasal swabs and leukocytes and only limited genome detection in intramuscularly vaccinated pigs (Reimann et al., 2004; Koenig et al., 2007; Leifer et al., 2009; Gabriel et al., 2012). The absence of live virus is very promising for the future use of this vaccine.

Antibody titers elicited by one shot intramuscular and oral vaccination with "CP7_E2alf" demonstrated to be stable for at least six months as with the exception of one orally vaccinated pig, protection against mortality and clinical signs was proven (Gabriel et al., 2012).

Transmission of challenge virus to contact animals

Full protection against transmission of infection virus to in-contact domestic pigs and wild boar was shown when the challenge was carried out 21 days post oral vaccination (Koenig et al., 2007; Blome et al., 2012). In the same study, challenge at 14 days post vaccination resulted in clinically apparent CSF and 100% mortality between 15 and 22 day post challenge. Intramuscular vaccination seems to provide very early protection against transmission. When challenge was given as early as 1 week post vaccination no infectious virus was detected in the vaccinated animals (Leifer et al., 2009), however to prove the lack of excretion of challenge virus contact animals should be included.

DIVA potential

E^{rns} differentiation

Being a marker vaccine candidate, "CP7_E2alf" was evaluated for its DIVA potential. This vaccine allows a limited replication, which was proven to be insufficient for transmission of the challenge virus and is a very useful property of "CP7_E2alf "in relation to its DIVA potential. Earliest seroconversion was reported at day 7 post challenge in wild boar orally vaccination (Koenig et al., 2007). Generally, seroconversion to the challenge virus E^{rns} following both oral and intramuscular vaccination was reported at day 10 post infection and at day 21 all tested pigs were positive (Reimann et al., 2004; Koenig et al., 2007; Leifer et al., 2009). DIVA potential of "CP7_E2alf" was also demonstrated in domestic pigs positive for maternal C-strain antibodies (Manuscript 2 in this thesis). No seroconversion to the vaccine E^{rns} specific antibodies was shown even after triple oral vaccination (Blome et al., 2012)

Genetic differentiation

The two described above RT-PCRs (Hoffmann et al., 2005; Hoffmann et al., 2006), seems to be a promising accompanying to "CP7_E2alf" discriminatory tool as it permits direct differentiation at early time point between "CP7_E2alf" and CSFV when it was targeted the CSFV 5'UTR region (Tignon et al., 2010).

Ecotoxicity

As this vaccine is genetically modified organism (GMO) and it is a live attenuated vaccine some concerns were expressed about its release in the environment. The risk for the environment linked to the use of "CP7_E2alf"however, seems negligible as this chimeric virus proved to be completely avirulent in vaccinated target and non-target species (König et al., 2011) even in very high repeated dose."CP7_E2alf" is constructed on DIVA principle and there is a validated ELISA method to differentiate this chimeric virus from the wild type CSF and BVD viruses. Thus, tracing the virus in the environment is possible by easy and reliable methods. Possibility for genetic recombination between the vaccine BVDV with other field BVDVs (Dong and Chen, 2007) is very low as there was observed only limited viral replication of "CP7_E2alf" in the target species and incidents of BVDV infections in pigs and wild boar are very rare.

Project Aims

This general literature review aimed to give an overview of CSF as a disease and the existing strategies for controlling an outbreak as well as a possible implementation of new measures in the fight against this disease. Vaccination with effective and safe DIVA vaccine could be a solution to confine an outbreak and to ease the meat trade. However, in many countries C-strain vaccine is used either for prophylactic vaccination or for emergency baits vaccination and interference of MDA with the efficacy of a new DIVA vaccine is very possible.

Thus, the general aims of this project have been:

- Evaluation of the possibility for implementation of a new live chimeric marker vaccine in population previously vaccinated with modified live vaccine.
- Investigation of efficacy of chimeric marker vaccine "CP7_E2alf" in preventing clinical sings and mortality in 5 or 8 weeks old piglets positive for C-strain MDA.
- Evaluation of DIVA potential of "CP7_E2alf" in piglets with MDA.
- Investigation of the immune modulation post vaccination with C-strain live attenuated vaccine and with "CP7_E2alf.

CHAPTER 2

Manuscript 1

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

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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.

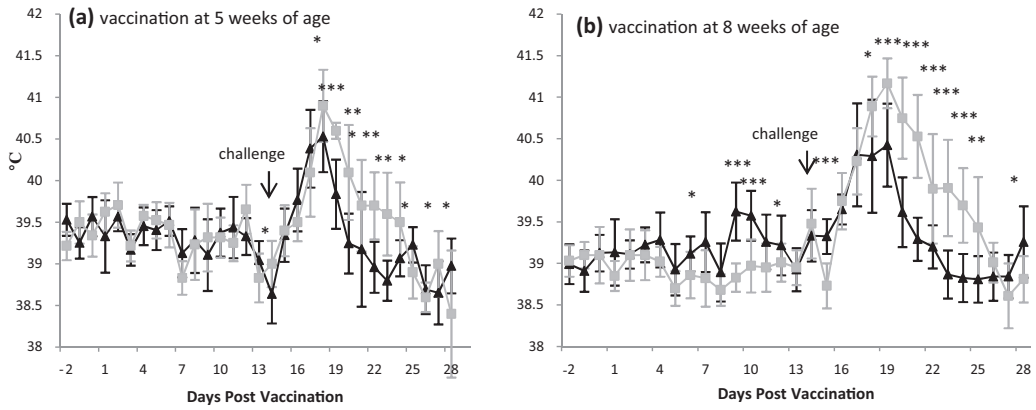

Fig. 1. Development of body temperature in: (a) vaccinated at 5 weeks of age and challenged at 7 weeks of age V5 (▲–▲) and control mock-vaccinated at 5 weeks of age and challenged at 7 weeks of age C5 (■-■) and in (b) vaccinated at 8 weeks of age and challenged at 10 weeks of age V8 (▲–▲) and control 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 $^{*}p < 0.01$; $^{**}p < 0.001$; $^{***}p < 0.0001$.

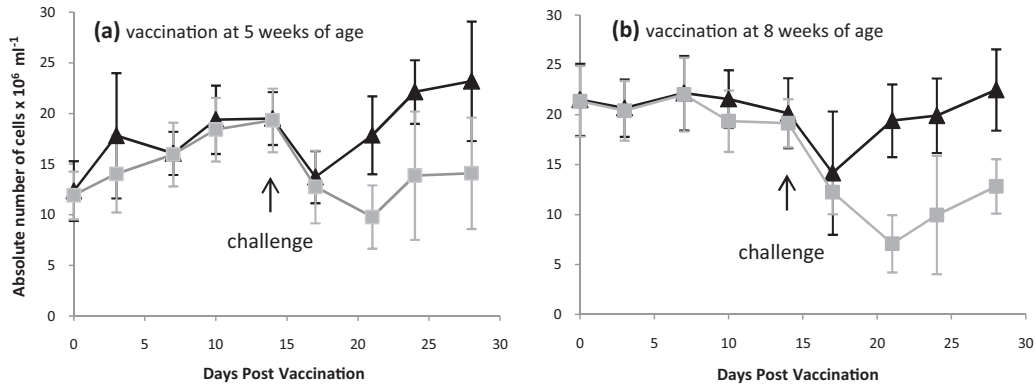

Fig. 2. Total white blood cell (WBC) counts in: (a) vaccinated at 5 weeks of age piglets and challenged at 7 weeks of age V5 (▲–▲) and controls C5 mock-vaccinated at 5 weeks of age and challenged at 7 weeks of age (■-■) and in (b) vaccinated at 8 weeks of age and challenged at 10 weeks of age V8 (▲–▲)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

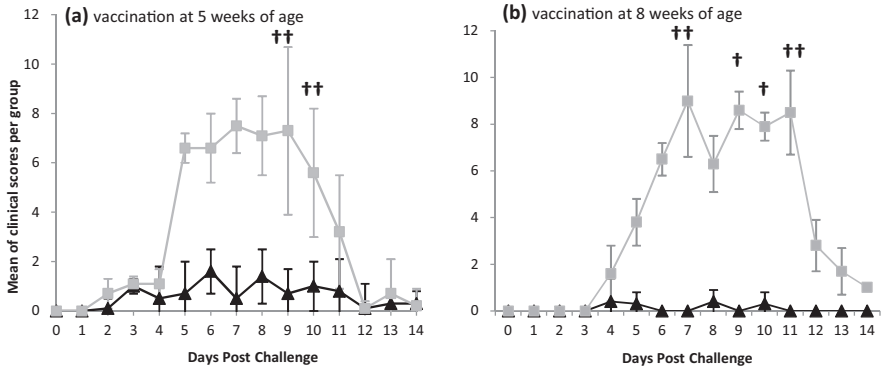

Fig. 3. Clinical scores (CS) recorded post challenge. CS in (a) vaccinated at 5 weeks of age and challenged at 7 weeks of age V5 (▲–▲) and control mock-vaccinated at 5 weeks of age and challenged at 7 weeks of age C5 (■-■), and in (b) vaccinated at 8 weeks of age and challenged at 10 weeks of age V8 (▲–▲) 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

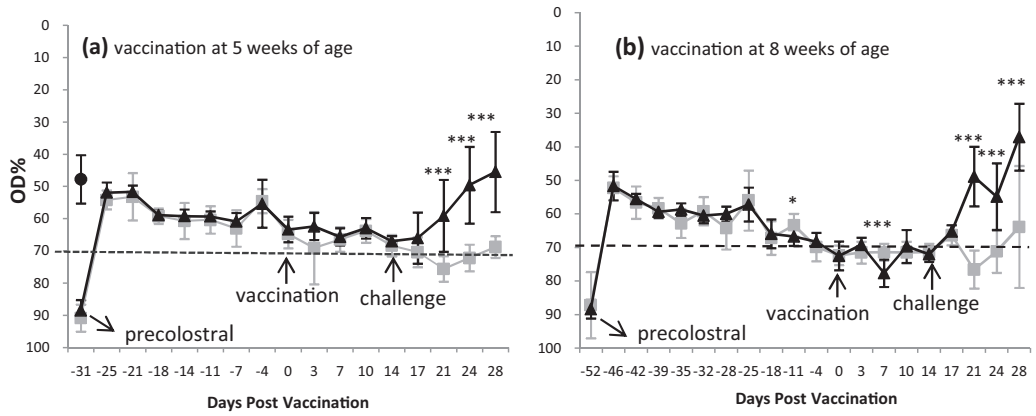

Fig. 4. Results obtained by blocking ELISA and depicted as average optical density (OD) % with a cut-off value of 70 (dashed line). Results ≥70 are negative for CSFV antibodies and results <70 are positive for CSFV antibodies. Graph (a) shows vaccinated at 5 weeks of age and challenged at 7 weeks of 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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Manuscript 2

DIVA potential of marker vaccine candidate CP7_E2alf in face of C-strain maternal immunity

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DIVA potential of marker vaccine candidate CP7_E2alf in face of C-strain maternal immunity

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Abstract

Implementation of vaccination in emergency situation with marker vaccine against Classical Swine Fever (CSF) together with a sensitive and specific discriminatory test will give the possibility of tracing residual infections and ease the pig trade restrictions. CP7_E2alf is a promising CSF live chimeric marker vaccine candidate that use as a backbone Bovine Viral Diarrhea Virus (BVDV) in which E2 protein is replaced by E2 protein of Classical Swine Fever Virus (CSFV). The present study aimed to evaluate the differentiation of infection in vaccinated animals (DIVA) potential of CP7_E2alf in piglets from sows vaccinated once intramuscularly with live attenuated C-strain vaccine. At 5 or 8 weeks of age these piglets were vaccinated intramuscularly with CP7_E2alf. After two weeks the vaccinated piglets and their mock-vaccinated controls were challenged and euthanized 14 days later. Five piglets from each group and each sow were analyzed.

Both E^{rns} and E2 specific antibodies were transferred with colostrums. Priocheck® CSFV E^{rns} ELISA detected maternal E^{rns} antibodies up to 4 weeks of age only in 7 out of 18 tested piglets. As expected, after vaccination until challenge no E^{rns} specific antibodies were detected. All 20 challenged piglets, except one vaccinated at 8 weeks of age, seroconverted to E^{rns} at day 10 post challenge. In conclusion, the new marker candidate CP7_E2alf could be applied as a DIVA vaccine in piglets positive for C-strain maternal antibodies when the piglets are vaccinated intramuscularly at 5 or 8 weeks of age.

Keywords
Classical swine fever, DIVA diagnostics, Pestivirus, emergency vaccination, maternal immunity, Erns antibodies.

1.Introduction

Classical Swine Fever Virus (CSFV) is a positive stranded enveloped RNA virus that together with *Bovine* Viral Diarrhea Virus (BVDV) and *Border Disease Virus* (BDV) belongs to the genus *Pestivirus* within the family *Flaviviridae* [1]. Outbreaks of CSF in the European Union (EU) countries with an industrialized pig production result in enormous economic losses due to the current control strategy including culling of infected animals. An alternative to this strategy is an emergency vaccination. However, live attenuated vaccines that are used until present do not allow a differentiation of infection in vaccinated animal (DIVA) [2]. A novel efficacious and safe marker vaccine that follows the DIVA concept may overcome this problem. Accompanying DIVA test that detects infected pigs in a vaccinated population will allow declaring infection free status and shortening of the export restriction period. Therefore, without reliable sensitive and specific discriminatory test marker vaccines will not be applicable during an emergency field situation. CSF humoral immunity relays mainly on antibodies directed against E2 and E^{rns} nonstructural proteins [3]. Based on these characteristics a marker vaccine candidate "CP7_E2alf" was constructed [4]. Here, E2 protein encoding sequence in this vaccine was deleted in BVDV strain "CP7"backbone and replaced by the same protein of the CSFV strain "Alfort187". The corresponding discriminatory ELISA test detects antibodies specific against the CSFV E^{rns} glycoprotein and antibody positive pigs are regarded as CSFV infected. Problems might appear when this discriminatory test is implemented in population previously vaccinated with conventional live attenuated vaccines. Firstly, E^{rns} antibodies could be passively transferred from mothers to their piglets that will be tested positive in absence of infection. Secondly, maternal antibodies (MA) may neutralize even highly virulent CSFV as previously described [5]. Uptake of MAs might prevent induction of active antibodies against E^{rns} protein, later, when the MAs decrease, no specific E^{rns} antibodies will be produced. Additionally the delayed humoral immunity may also impair Erns response post CSFV infection [6].

These scenarios are possible in European endemic areas where C-strain vaccine baits oral vaccination is used for wild boar in case of outbreak [7]. Some EU candidate countries currently use

conventional vaccination with local live attenuated vaccine variants, but would like to cease vaccination as soon as possible [8]. For these countries an intermediate step could be implementation of vaccination with CP7_E2alf. Therefore, MA due to the practice of routine vaccination to prevent circulation of CSFV might interfere with the sensitivity of the discriminatory ELISA test [9].

Thus, the present study evaluated the DIVA potential of CP7_E2alf in piglets with C-strain maternal antibodies that were vaccinated at 5 or 8 weeks of age. The DIVA concept that implies the possibility to detect infected animals within a vaccinated population was tested when the piglets were infected two weeks post vaccination.

2. Materials and methods

2.1. Animals trials, sample collection and clinical examination

Animal trial, sample procedure and clinical examination were performed as described previously [5]. Briefly, pregnant sows were vaccinated intramuscularly with one dose of live attenuated C-strain vaccine four weeks before farrowing. At 4 weeks of age, the piglets were weaned and divided into four groups representing offspring of each sow. Group V5 and V8 were vaccinated intramuscularly with 1ml of CP7_E2alf at age of 5 or 8 weeks, respectively. The control groups C5 and C8 were mock-vaccinated at the same time points as their corresponding groups. All groups were challenged with highly virulent strain "Koslov" two weeks post vaccination and euthanized 14 days post challenge.

Serum samples were obtained from the four sows before vaccination, at day 14 post vaccination, at farrowing, 14 days post farrowing and at euthanization. Five piglets were analyzed from each group, they were sampled prior to uptake of colostrums and once a week until vaccination. Post vaccination the blood samples were collected twice a week to be tested for development of neutralization antibodies, E2 and E^{rns} specific antibodies.

2.2. Antibody analyses

Serum samples were examined for neutralizing antibodies to CSFV through a direct virus neutralization peroxidise-linked assay (NPLA) [10], with some modifications. In brief, two fold dilutions in triplicate of heat - inactivated serum starting from 1:10 were incubated for 1hour against 100 infective units homologous C-strain vaccine virus (for the samples obtained from vaccinated and mock-vaccinated piglets) or the reference CSFV Alfort 187 strain (for the samples obtained from vaccinated piglets only). To increase the titer of the C-strain vaccine virus the virus was propagated four times in SFT cells. After incubation, PK-15 cells were added and the plates were incubated 3 days. The cell-monolayers were fixed and stained as previously described [11] and viral titers were calculated [12].

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 [13] after RNA extraction using the QIAamp RNA Blood mini kit (Qiagen).

The presence of CSFV E2-specific antibodies was tested using HerdChek® CSFV Ab ELISA (IDEXX Laboratories, Hoofddorp, The Netherlands). Priocheck® CSFV E^{rns} ELISA kits were kindly provided by Prionics Lelystad BV, Lelystad, The Netherlands. Both tests were performed according to the manufacturer's instructions.

2.3 Statistical analysis

Statistical tests were 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 $p \leq 0.05$ (statistical significance is indicated in the figures).

3. Results

3.1. Kinetics of maternal immunity

The sows were tested positive for neutralizing antibodies two weeks post vaccination but no viral genome was detected at this time point. At farrowing the mean antibody titer was 195 and the

similar titer of 145 was detected in the one-week-old piglets after ingestion of colosrum (Fig.1). Before uptake of colostrum all piglets were antibody negative in all tests. The neutralization antibody titer of the sows was increasing up to euthanization. From week 2 of age the neutralization antibody titers of the piglets decreased and at 5 weeks of age all piglets had titer below 64. At 10 weeks of age 4 piglets still tested positive in very low titers. At farrowing, all four sows were seroconverted to E2-specific antibodies. Stable increase in the percentages of inhibition values in the four sows were observed from day 49 post vaccination until euthanization. No clear CSFV E^{rns} specific antibody response was detected in the sows post C-strain vaccination; however, at farrowing and 14 days later, two of the sows had an increased inhibition percent close to threshold. In the piglets, neither E^{rns} nor E2 specific antibodies were detected prior to uptake of colostrum (Fig.2). After ingestion of colostrum, week one 4 out of 18 tested piglets were positive for E^{rns} specific antibodies and at week 4 the positive piglets were only two. From week 5 of age until week 3 post vaccination all piglets remained E^{rns} antibody negative. E2 specific antibodies were detected in all of the tested piglets after uptake of colostrum and these antibodies were present in 2 out of 20 piglets up to six weeks of age.

3.2. Neutralization response post vaccination and challenge

Neutralization antibody response to vaccination in the piglets vaccinated at 5 weeks of age (V5) was first detected at day 14 post vaccination (Fig.3 a). The piglets vaccinated at 8 weeks of age (V8) had the highest titer of neutralizing antibodies post vaccination (Fig.3 b). The antibody levels in group V8 increased from day 7 after vaccination. Slightly higher antibody titers against CSF strain Alfort than against C-strain vaccine virus were detected for both groups V5 and V8, respectively.

Increase in the neutralization antibody titer was detected 7 days post challenge in the remaining piglets from group C5 only (Fig.3 a). No increase in the antibody response was detected in the remaining piglets from group C8 until the termination of the experiment (Fig.3 b).

3.3. DIVA potential in piglets with maternal C-strain antibodies

At the time of vaccination the youngest piglets (V5 and C5) had, as expected, higher levels of E2 antibodies than those vaccinated at 8 weeks (V8 and C8). After vaccination a significant difference

in the E2 specific response between vaccinated and mock-vaccinated piglets occurred. Both vaccinated groups seroconverted at day 21 post vaccination whereas only one mock-vaccinated pig was tested positive at euthanization (Fig.4 a & b).

The first post vaccination response to CSFV specific E^{rns} antibodies appeared at day 3 post challenge in one piglet from group V8. With the exception of one pig from group V8 all vaccinated and mock-vaccinated piglets had seroconverted to E^{rns} at day 10 post challenge (Fig. 5 a & b).

4. Discussion

CP7_E2alf vaccine proved to be efficacious and safe [14], but without a specific and sensitive accompanying discriminatory tool it would be not feasible as a DIVA vaccine. Priocheck® CSFV E^{rns} ELISA is the only commercially available test that is specific for CSF and is suitable as discriminatory tool after vaccination with CP7_E2alf. In the present study, the DIVA potential of this ELISA test was evaluated in piglets with C-strain maternal antibodies vaccinated intramuscularly with CP7_E2alf at 5 or 8 weeks of age.

Although, all tested piglets were positive for neutralizing antibodies at 5 weeks of age and 4 out of 12 piglets still had MA at 10 weeks, no piglets were tested positive for CSFV specific E^{rns} antibodies from 5 weeks of age up to challenge. Thus, no interference of maternal C-strain antibodies with the DIVA potential of CP7_E2alf in domestic piglets older than 4 weeks of age was observed.

None of the vaccinated animals were positive for E^{rns} CSFV specific antibodies prior challenge. Already at day 3 and 7 post challenge one and three piglets, respectively, were tested E^{rns} positive. As antibody response to CSFV does not appear earlier than 7 days post infection and there was a significant difference between the vaccinated group V8 and the mock-vaccinated C8, we assume that the Erns positive results were boosted by a cross reactivity with the vaccine BVDV E^{rns} proteins. As was mentioned above, the piglets of V8 had a lower MA levels at the time of vaccination than the piglets of V5 group and due to this also responded with a higher neutralization titer after vaccination. E^{rns} proteins are highly conserved among *Pestiviruses* and correlation between false positive for E^{rns} antibodies results and high neutralization antibody titer was previously reported [15]. In the present study the addition of a vaccinated-not-challenged group

would have been of use. Positive CSFV E^{rns} results in this a group would confirm the cross reactivity to the vaccine BVDV E^{rns} proteins. The recently published confirmatory ELISA test [15] might be also a solution in future analyses.

According to the Diagnostic Manual [16], serum samples, obtained 21 days post infection, must be detected by ELISA tests. In the present study, all except one piglet from the vaccinated group V8 seroconverted to E^{rns} specific antibodies before day 10 post challenge. The piglet that did not seroconvert was euthanized as planned at the termination of the experiment on day 14 post challenge and it was not possible to prove later seroconversion.

Interestingly, no correlation between the results obtained from the sows by neutralization test and E^{rns} specific ELISA were observed. No clear E^{rns} specific antibodies seroconversion was detected in the sows, but at farrowing, two sows showed an inhibition percent close to the cut off value of 40 % (29% and 35%). The piglets that tested E^{rns} positive after uptake of colostrum were offspring of these sows. However, for all sows high levels of neutralizing antibodies and E2 antibodies were detected until euthanization. Previous study has also shown that vaccination with C-strain do not produce strong E^{rns} ELISA antibodies [2, 17]. Possible difference between C-strain and CSFV genotype used for validation of these tests was proposed as an explanation. The very limited replication of C-strain vaccine virus might be another reason [6, 14, 18]. In the present study, no CSF viral genome was detected in serum samples obtained by the sows after vaccination.

The correlation between the positive results obtained from the sows by NPLA and E2 specific ELISA supports the major role of E2 specific antibodies in the development of immunological response to CSF. Also after vaccination of the piglets, a clear increase of the neutralization antibodies and E2 inhibition percent was observed in the vaccinated piglets only. This confirmed that CP7_E2alf vaccine virus overcome the maternal C-strain antibodies and induced active immunity. As expected, these results consolidated the inability of NPLA to be applied as a confirmatory test after vaccination with CP7_E2alf, due to the CSFV E2 gene in this chimera [15]. Because of the lower MA, the piglets vaccinated at 8 weeks of age responded earlier to challenge infection with higher neutralization titer than the piglets vaccinated at 5 weeks of age. Despite the fact that C-strain and strain Alfort used for the NPLA belong to genotype 1, higher neutralization

antibody titers after vaccination with CP7_E2alf were obtained when the test was performed against homologous strain Alfort.

Although, there was no increase of the antibody titer in the mock-vaccinated group C8 and a slight increase in group C5, the mortality was significantly reduced in both groups. After challenge, the detected low titer in the survived mock-vaccinated piglets was able to protect them from the challenge infection with high dose of CSF virulent strain Koslov [5]. The results showed that a lower neutralization antibody titer than previously reported of ≥25 [19] resulted in adequate protection of some piglets. This finding is in agreement with previous study that revealed no relation of MA to survival [20] and suggests that besides humoral immunity, other individual complex mechanisms were involved.

Conclusion

In domestic pig populations previously vaccinated intramuscularly with C-strain vaccine a discriminatory test could be implemented as early as 5 weeks of age. No interference of maternal immunity with the DIVA potential of CP7_E2alf is expected when domestic piglets are vaccinated intramuscularly at the age of 5 or 8 weeks and challenged 2 weeks later.

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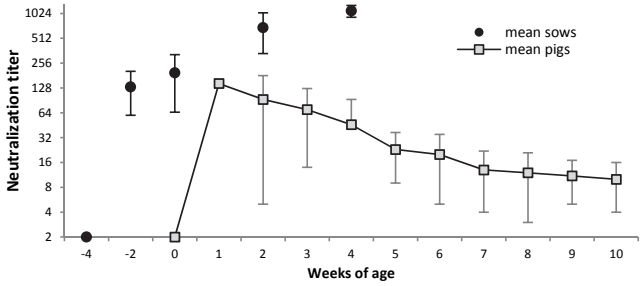

Fig.1. Kinetics of CSF maternally derived C-strain antibodies tested in the piglets by neutralization test, mean values of 12 nonvaccinated piglets (group C8), the black symbols represents the mean antibody titers of the C-strain vaccinated mothers. Each symbol represents the mean ± SD.

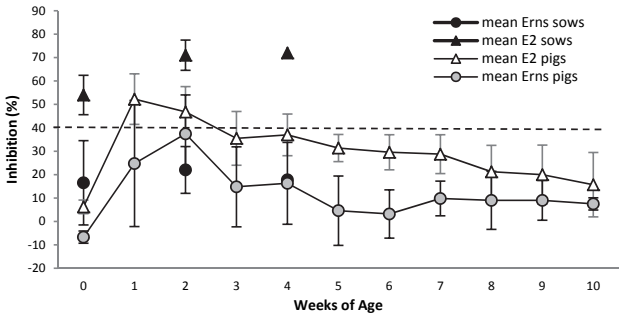

Fig.2. E2 and E^{rns} specific maternal antibodies kinetics tested by blocking ELISA in piglets from C-strain vaccinated sows. The dashed line marks the cut-off. Each symbol represents the mean ± SD.

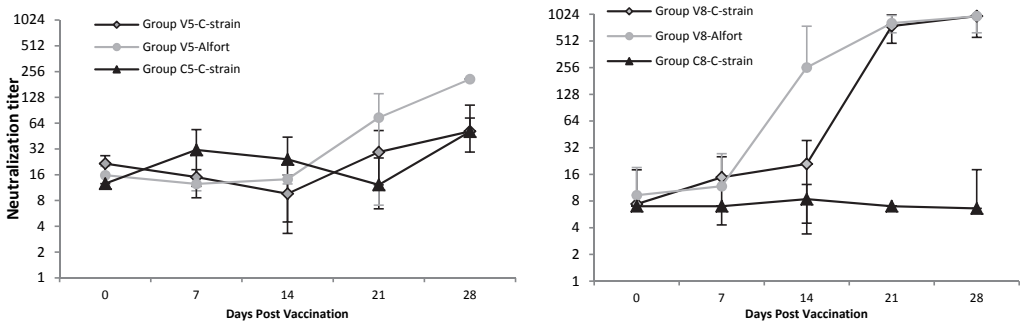

Fig.3. Neutralizing antibody response post vaccination against CSF C-strain vaccine virus (for the vaccinated pigs) or strain CSFV Alfort/187 (for both vaccinated and control pigs). Graph (a) shows group C5 and V5-vaccinated at 5 weeks of age and challenged at 7 weeks of age. Graph (b) shows group C8 andV8-vaccinated at 8 weeks of age and challenged at 10 weeks of age. Each symbol represents the mean ± SD.

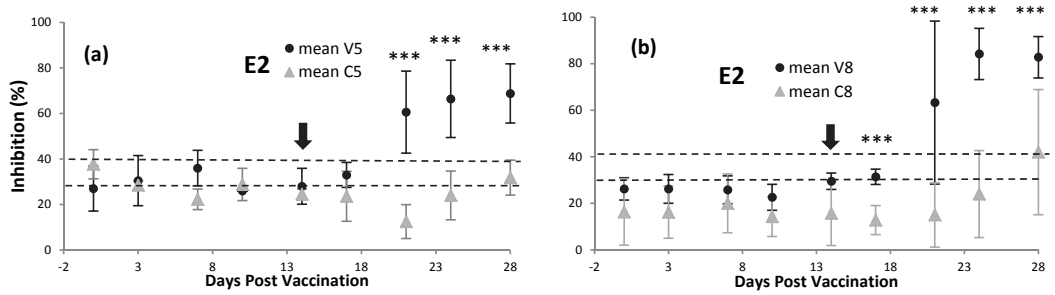

Fig.4.Response to CSFV E2 specific antibodies in (a) group V5 (vaccinated at 5 weeks of age) and C5 (mock-vaccinated at 5 weeks of age) and (b) group V8 (vaccinated at 8 weeks of age) and C8 (mock-vaccinated at 8 weeks of age). The dashed line marks the positive and the doubtful results according to the manufacturer. Black arrows mark the challenge, 14 days after vaccination. Each symbol represents the mean ± SD. (***)values significantly different between groups with $p \leq 0.001$.

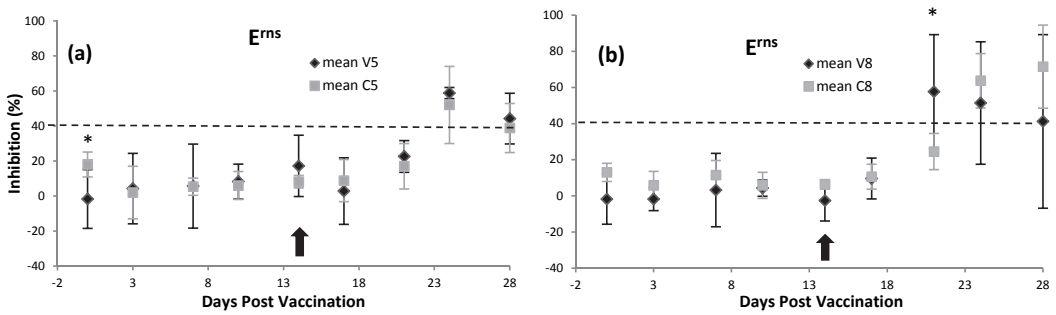

Fig.5. Response to CSFV E^{rns} specific antibodies in (a) group V5 (vaccinated at 5 weeks of age) and C5 (mock-vaccinated at 5 weeks of age) and (b) group V8 (vaccinated at 8 weeks of age) and C8 (mock-vaccinated at 8 weeks of age). The dashed line marks the cut-off. Black arrows mark the challenge infection time point. Each symbol represent the mean ± SD. (*)values significantly different between groups with $p \leq 0.05$.

Manuscript 3

Evaluation of immune modulation in sows vaccinated with C-strain and in their piglets vaccinated with new candidate marker vaccine CP7_E2alf

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Prepared for Veterinary Research

Evaluation of immune modulation in sows vaccinated with C-strain and in their piglets vaccinated with new candidate marker vaccine CP7_E2alf

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Abstract

Generally, humoral immunity has been accepted to be the major protective mechanism against classical swine fever (CSF). Recently, however the role of cellular immunity in the defense against this infection has been revealed as well. Evaluation of the predominant part of the immune system activated after vaccination will aid the development of more effective vaccines. In this experiment, we studied the kinetics and the modulation of the immune system in sows vaccinated intramuscularly with C-strain and in their piglets vaccinated with a new live marker vaccine CP7_E2alf. The piglets were vaccinated or mock-vaccinated intramuscularly at 5 or 8 weeks of age and challenged two weeks later.

Bias towards IgG1 isotype antibodies over IgG2 suggests predominant activation of Th2 cells (humoral immunity) than Th1 cells (cellular immunity).Thus, we evaluated the induction of CSFV-specific IgG1 and IgG2 responses and their relation to Th1 and Th2 cytokines (IL-4 and IFN-γ, respectively). Modulation of C-reactive acute phase protein (CRP) by CP7_E2alf vaccine was also investigated. C-strain vaccination of sows seems to activate predominantly the cellular immunity (Th1) that probably was transferred to the offspring and might be the reason for the reduced mortality in the mock-vaccinated control. Oppositely, the predominant elevation of IgG1 isotype specific antibodies following vaccination with CP7_E2alf indicates a possible activation of the humoral part of the immunity (Th2). No IL-4 was detected and a low concentration of IFN-γ was present in all groups except the vaccinated at 8 weeks of age V8. The lack of IFN-γ in the presence of high level of IgG1antibodies detected in V8 supports the hypothesis that intramuscular vaccination with CP7_E2alf activates mainly the humolar part of the immune system. The detected decrease in the CRP secretion by CP7_E2alf vaccine and the maternal immunity confirmed the possibility to utilize this protein as a tool for early prediction of CSF infection outcome.

1. Introduction

Classical swine fever virus (CSFV) is a small enveloped positive-stranded RNA virus classified in the genus *Pestiviruses* family *Flaviviridae* [1]. It is one of the most important viral infectious diseases of domestic pigs as an outbreak leads to substantial economical losses as well as to culling of enormous number of completely healthy pigs [2]. Highly virulent strains of CSFV cause an acute form of the disease, characterized by high fever, hemorrhagic lesions and high mortality [3]. The virus can efficiently evade and compromise the host immune system, causing lymphoid organ atrophy, with thrombocytopenia, granulocytopenia, and severe lymphopenia, particularly in B cells, due to apoptosis [4]. Protection is mainly associated with the humoral immune response, but neutralizing antibodies to CSFV usually appear 2 weeks post vaccination [5]. Thus, it is suggested that mechanisms as cellular immunity are involved during the early protection [6]. Cell-mediated immunity (CMI) is known to have both an effector and regulatory role on the immune system and it is believed to be essential for immunity against intracellular pathogens, including viruses. Evaluation of specific cytokine release that is known to influence or directly relate to humoral or cellular immunity is a way to investigate the part of the immune response that is predominantly activated after vaccination or infection and is responsible for providing protection. The role of cytokines in regulation and modulation of the immune response has been widely studied since the Th1/Th2 paradigm was postulated. This paradigm suggests that after priming T helper cells are polarized in two distinct subsets (Th1 or Th2) that can be distinguished by their cytokine expression [7]. Thus, IL-12 and INF-γ (Type 1 cytokines) are considered to be key cytokines in Th1 profiles and serves as indicators of a predominance of cell-mediated responses, whereas IL-4 (Type 2 cytokine) participates in Th2 polarization and dominance of humoral response [8]. In all animal species studied, immunoglobulin (Ig) G isotype expression is controlled by Type 1 and Type 2 cytokines. In pigs, it was reported that a high IgG1:IgG2 ratio (IgG1>IgG2) was a response to Type 2 (IL-4) cytokines and the inverse was to Type 1 (INF-γ) [9]. Recently, Graham et al. [10] proved the role of INF-γ response induced by intramuscular vaccination with the conventional live attenuated C-strain vaccine in early protection induced against CSFV challenge. Previously, Suradhat et al. [11] revealed that cellular immunity was also induced by C-strain vaccination in piglets from sows vaccinated with C-strain. Although, this vaccine is very effective and safe it is not possible to distinguish serologically between vaccinated and infected piglets [5]. The new marker candidate CP7_E2alf is a chimera vaccine that proved to be safe, efficacious and suitable as a

DIVA vaccine [12]. However, there is no knowledge about the modulations of the immune system by CP7_E2alf vaccination and consequent CSFV infection in piglets with maternal C-strain antibodies.

The current experiment aimed to study the activation of early specific immunity induced against CSF, based on IgG1 and IgG2 antibodies levels in piglets from C-strain vaccinated mothers, intramuscularly vaccinated at 5 or 8 weeks of age with the new CP7_E2alf marker vaccine and then challenged two weeks post vaccination. The duration of maternal antibodies as well as their kinetics after vaccination with CP7_E2alf and consequent challenge was assessed. In order to support the predominance of one of the two parts of the specific immunity (humoral or cellular) post infection, the release of Type 1 (IFN-γ) and Type 2 (IL-4) cytokines was analyzed. Furthermore, to better understand the protective role of specific immunity in the piglets post infection, the release of C-reactive protein (CRP), as an indicator of CSFV infection outcome was studied as well.

2. Materials and methods

2.1. Animals trials, sample collection and clinical examination

Animal trial, sample procedure and clinical examination were performed as described [13]. Briefly, the piglets of four sows vaccinated with C-strain vaccine were divided at 4 weeks of age into four groups. Group V5 and V8 were vaccinated intramuscularly at 5 or 8 weeks of age, respectively, with a new marker vaccine CP7_E2alf and their control groups C5 and C8 were mock-vaccinated. The piglets were challenged two weeks post vaccination, and euthanized 14 days post challenge. The groups were selected, so that piglets from all sows were represented in each group. From the two control mock-vaccinated groups were included piglets that developed severe clinical signs of CSF and were euthanized before the termination of the experiment. The piglets from the two vaccinated groups did not show clinical signs, so they were randomly selected. Serum samples from the four sows were tested at farrowing and at euthanization. Piglets were blood sampled prior to uptake of colostrums and once a week until vaccination. Post vaccination the blood samples were collected twice a week.

2.2. Sample analysis

CSFV-specific IgG1and IgG2 antibodies were measured in serum by indirect ELISA as described before [14]. Briefly, CSFV infected cell extract, prepared using a detergent solution containing 2% Octyl beta, 1 D glucopyranoside (OGP), was applied to Nunc Maxisorp microplates overnight at room temperature and blocked for 1 h at room temperature with 10% FBS-2% milk in Phosphate buffered saline (PBS). Thereafter, sera were incubated for 1 h at 37°C with either mouse anti-porcine IgG1 antibody diluted at 1/250 (for detection of specific IgG1 antibodies) or mouse anti-porcine IgG2 antibody diluted at 1/100. Anti-mouse IgG HRP-conjugated secondary antibody was added at a dilution of 1/250 (DAKO) for 30 min at 37°C and the peroxidase activity was measured using the TMB substrate (Sigma-Aldrich) at 450 nm. The values obtained for tested sera were compared to negative control and to high positive control sera from either unvaccinated or C-strain vaccinated piglets from unvaccinated sows.

Furthermore, porcine cytokines were measured in serum post challenge using ELISA kits from Invitrogen (Life Technologies, Carlsbad, USA) for IFN-γ and IL-4 according to the manufacturer's instructions and the concentrations in the sera were determined from a standard curve. The level of C-reactive protein (CRP) in serum obtained from the piglets post challenge was determined using an indirect ELISA as previously described [15].

2.3 Statistical tests

The variation for a vaccinated group compared to the respective control group was analyzed by Student's *t*-test using GraphPad in Stat version 3.00 (GraphPad Software, San Diego, CA). Differences were considered statistically significant with a probability of $p \leq 0.05$.

3. Results

3.1. Isotype specific antibody response post C-strain intramuscular vaccination in sows and evaluation of the longevity of these maternal antibodies in piglets

Similar levels of IgG2 and IgG1 were detected in the four sows at 4 weeks post vaccination, but at euthanization IgG2 was the predominant isotype (Fig.1). In the piglets, both IgG1 and IgG2 CSF specific antibodies were present after uptake of colostrum (Fig.1). Higher levels of IgG2 than of IgG1 CSF specific maternal antibodies were detected during the tested period with both isotypes

peaking week 1 and declining untill 10 weeks. The ratio of IgG1 over IgG2 was calculated to obtain more precise information on the specific immunity as results < 1 indicate Th1 cellular immunity and results > 1 indicate Th2 humoral immunity. Early protection provided by maternal immunity in piglets from C-strain vaccinated mothers revealed to be mainly to Th1 cellular immunity as the IgG1/IgG2 ratio was calculated < 1(Fig. 1b). Similarly to the neutralizing antibodies (Manuscript 2), the levels of the CSF specific IgG1 and IgG2 antibodies started to decrease from week 2 of age.

3.2. Immune response modulation post CP7_E2alf intramuscular vaccination

For both groups of unvaccinated piglets (C5 and C8), an increase of CSFV-specific antibody levels was not observed, neither for IgG1 nor IgG2, post-challenge (Fig.2). At 7 weeks or 10 weeks of age, when the challenge was administered, maternal isotype specific antibodies were detected at very low levels in piglets (Fig.1). However, a stimulation of IgG1 production was observed from 21 dpv (i.e. 7 days post-challenge) for both groups of CP7_E2alf vaccinated piglets (V5 and V8) (Fig.2a). The strongest IgG1 response was detected in the piglets vaccinated at 8 weeks of age (V8). No significant IgG2 production was stimulated until the end of the experiment for both vaccinated groups (Fig.2b)

No significant elevation of the IgG2 specific antibody levels was detected post vaccination in the four groups (Fig.2 b). The raise of IgG1 response started from day 21 post vaccination in the piglets from group V8 (vaccinated at 8 weeks of age) and group V5 (vaccinated at 5 weeks of age). In the two vaccinated groups, the IgG1 levels were increasing until the termination of the experiment. The strongest IgG1 response was detected in the piglets vaccinated at 8 weeks of age (V8). In contrast to the vaccinated groups, the IgG1 level in the mock-vaccinated piglets started to decrease from day 17 post vaccination and continued until the end of the experiment (Fig. 2 a).

Due to the single stimulation of IgG1 isotype induced by CP7_E2alf vaccination, the IgG1:IgG2 ratio indicates a balance towards Th2 humoral response (Fig.2 c). However, no Il-4 was detected post challenge in any of the four groups (data not shown). Low concentrations of IFN-γ were found in a few piglets mainly from the mock-vaccinated group C5 (Fig.3). Two piglets from V5 and one piglet from C8 were tested positive for IFN-γ at day 3 and day 7 post challenge, respectively. This cytokine was not detected in the vaccinated at 8 weeks of age piglets.

The duration of CRP over-production induced by challenge infection was significantly reduced at day 10 post challenge in the vaccinated group V8 compared to the mock-vaccinated group C8 (Fig. 4 b). At day 3 post challenge, the piglets vaccinated at 5 weeks of age had significantly higher concentration of CRP than their control mock-vaccinated group. The two groups (V5 and C5) did not have significantly different levels of CRP from day 7 post challenge until euthanization (Fig. 4 a).

4. Discussion

The role of the cellular immunity in protection against CSF infection has been revealed [16,]. The early protection that this part of the immune system provides against CSFV is especially important in confining spread of the infection in situation of emergency. In the European Union, marker vaccines may be implemented in a case of outbreak, thus, the capacity of a new marker vaccine CP7_E2alf to polarize the adaptive immunity towards Th1or Th2 response is important to investigate.

With the present study, we aimed to evaluate the polarization of the immune response against CSFV in positive for C-strain MDA piglets that were vaccinated with CP7_E2alf at 5 or 8 weeks of age and challenged 2 weeks later. Although, similar levels of IgG1 and IgG2 isotype antibodies were detected in the sows at farrowing, at the end of the experiment in the sows and after uptake of colostrums in the piglets the level of IgG2 was significantly higher than that of IgG1.The predominance of IgG2 isotype antibodies transferred to the piglets with colostrum is in agreement with the previously reported IgG2 concentration of up to 75% in young piglets [17]. The over-time elevation of IgG2 concentration detected in the sows suggests a long time activation of Th1 cells post C-strain vaccination.

Oppositely to the predominant IgG2 elevation detected post administration of C-strain vaccine, the CP7_E2alf vaccination led to increase mainly of IgG1 isotype specific antibody levels. It can be argued that the observed difference in the outcome of the two vaccinations was due to challenge infection post CP7_E2alf vaccination. However, a possible influence of the challenge infection and the maternal immunity on the IgG1 response post CP7_E2alf vaccination might be excluded as a significant difference in the IgG1 levels was detected between the vaccinated and the mock-vaccinated piglets.

Predominant activation of Th1 cells post C-strain vaccination and a possible consequent transfer with the colostrum mainly of maternal Th1 cells and probably the related cytokines might be the reason for the observed reduced mortality in presence of very low or insufficient levels of neutralization antibodies in the mock-vaccinated piglets (Manuscript 2). However, this theory has to be supported by further investigation of the colostrum's components and the correlation between their presence in the piglets and the provided protection.

IFN-γ appears to serve as a good marker for anti - CSFV cell mediated response and its relation to IgG2 production in pigs was proven [18, 19]. Thus, it was relevant with the present study to analyze IFN-γ release in piglets post challenge. No clear correlation was found between protection and detection of IFN-γ in serum samples from the mock-vaccinated piglets. IFN-γ in low concentration was present both in the surviving piglets and in the piglets that succumbed to the challenge infection. However, there was a correlation between virus replication or high virus levels and detection of IFN-γ in serum. CSFV was isolated from the tonsils of all mock-vaccinated piglets tested positive for IFN-γ (pig 3, 13 and 30) [13]. Virus genome was only detected at day 3 post challenge in the two piglets from V5 group that were tested positive for IFN-γ at the same time point. These results revealed that at an early time point, CSFV infection might enhance the induction of IFN-γ.

The lack of IFN-γ secreted in serum samples obtained post challenge from the vaccinated at 8 weeks of age piglets (V8) correlated with the absence of IgG2 antibody increase in these piglets during the same period and is probably due to the very fast activation of the humoral immunity. From the two vaccinated groups, the piglets of V8 responded earlier with higher levels of IgG1specific antibodies. Thus, the lack of IFN-γ in the presence of high level of IgG1antibodies indicates that intramuscular vaccination with CP7_E2alf activates mainly the humolar part of the immune system.

As IL-4 is a type 2 cytokine that stimulates B cells activation and immunoglobulin - class switching, correlation of results obtained for this cytokine and the above presented findings could confirm the predominant activation of Th2 cells post CP7_E2alf intramuscular vaccination. However, the negative results obtained for secreted cytokine in presence of very clear antibody response [13], suggest as previously reported [8] a low sensitivity of the utilized ELISA test in serum samples.

The acute-phase response is an early nonspecific protective mechanism against pathogens that precedes the specific immune defense and declines shortly after removal of the triggering factor [20]. In pigs, CRP is considered a major acute-phase protein that quickly reacts and exhibits a moderate increase [15]. In the present study the level of CRP post challenge infection with highly virulent CSFV strain was generally lower than previously reported [21, 22]. The attenuation of the challenge virus by the maternal immunity as we reported previously [13] is a probable explanation for the reduced secretion of CRP in serum. The finding supports the proposed hypothesis [22] that the early kinetics of CRP in blood may constitute an additional tool to predict the clinical outcome of CSF infection. In a compliance with this hypothesis is also the finding that the highest level of this protein was detected in the piglets from group C8 that were most severely affected by the challenge infection.

In conclusion, our results suggest that the two studied vaccines (C-strain and CP7_E2alf) exploited different mechanisms of the immune system. C-strain vaccination seems to activate predominantly the cellular immunity that probably was transferred to the offspring and might be the reason for the reduced mortality in the mock-vaccinated control piglets. Oppositely, the predominant elevation of IgG1 isotype specific antibodies following vaccination with CP7_E2alf indicates activation mainly of the humoral part of the acquired immunity. The detected attenuation of the CRP secretion by CP7_E2alf vaccine and the maternal immunity confirmed the possibility to utilize this protein as a tool for early prediction of CSF infection outcome.

Acknowledgments

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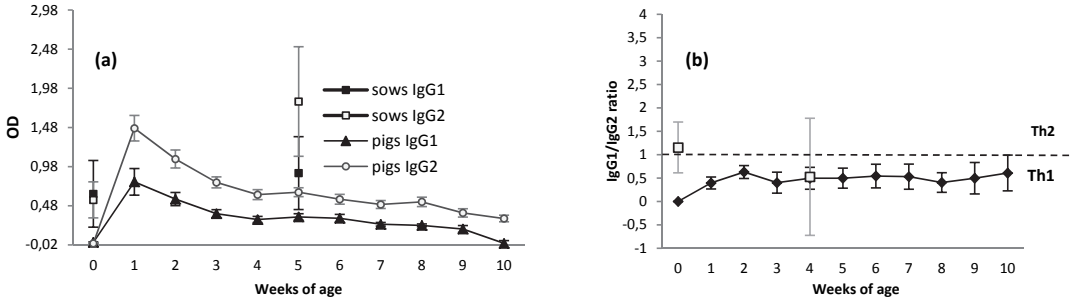


Fig.1. (a) Analysis of isotype specific maternal IgG1 and IgG2 antibodies kinetics tested by ELISA in piglets obtained from sows vaccinated with CSFV C-strain vaccine, each symbol for the piglets represents the mean ± SE (not equal number of samples were tested at each time point) and for the sows ±SD. (b) The IgG1/IgG2 ratio, each symbol for the piglets represent the mean ± SE and for the sows ±SD. There was a signifficant difference of $p<0.0001$between IgG1 and IGg2 at each time point tested.

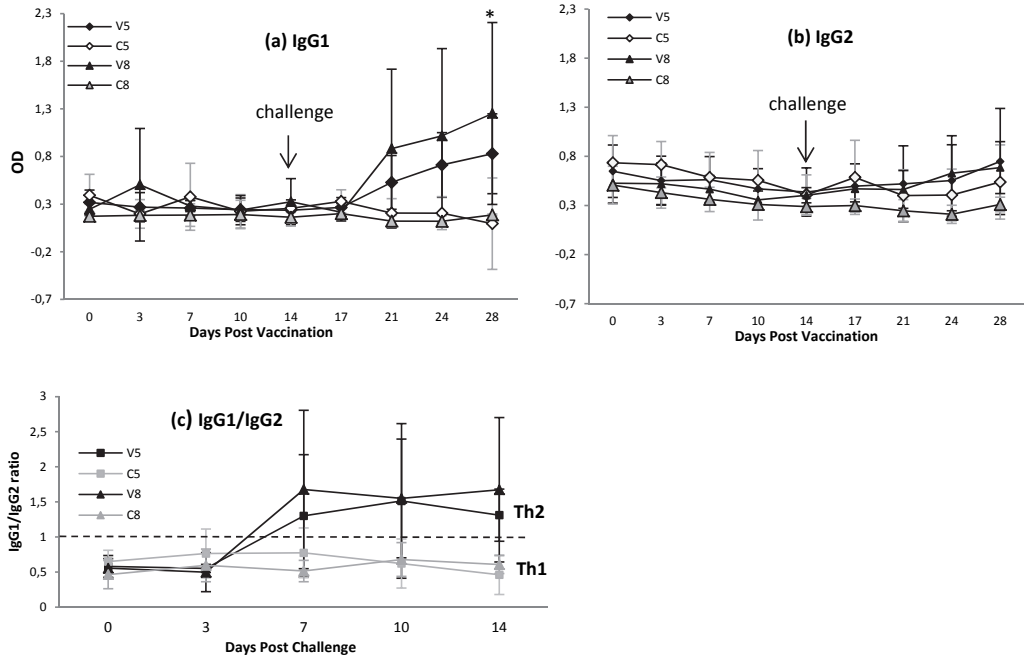


Fig.2. Analysis of IgG1 and IgG2 towards CSFV of piglets following CSFV challenge. Vaccinated piglets are shown in solid symbols and mock vaccinated in grey symbols. Serum samples were analysed for CSFV-specific IgG1(a) or CSFV-specific IgG2 (b) . The IgG1/IgG2 ration post challenge (c) is based on the results from Fig 2a and 2b. Each symbol represents the mean ± SD. The significant differences between the CRP level in the vaccinated and mock-vaccianted control groups were indicated with *$p<0.01$.

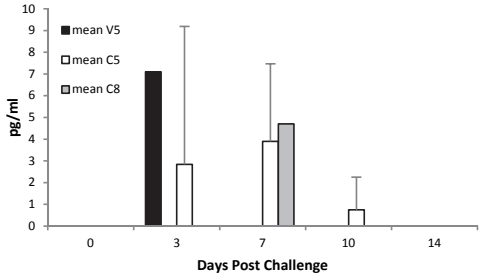


Fig. 3. Analysis of the secreted IFN-γ in serum of piglets group V5, C5 and C8; no IFN-γ was detected in the V8 group. Each symbol represents the mean +SD.

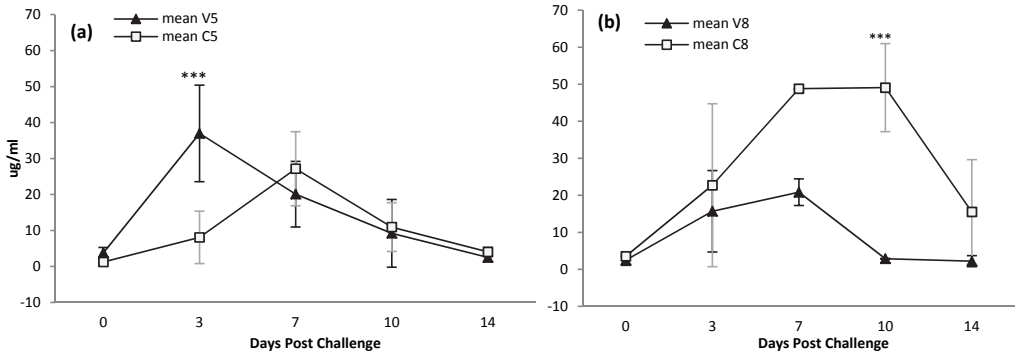
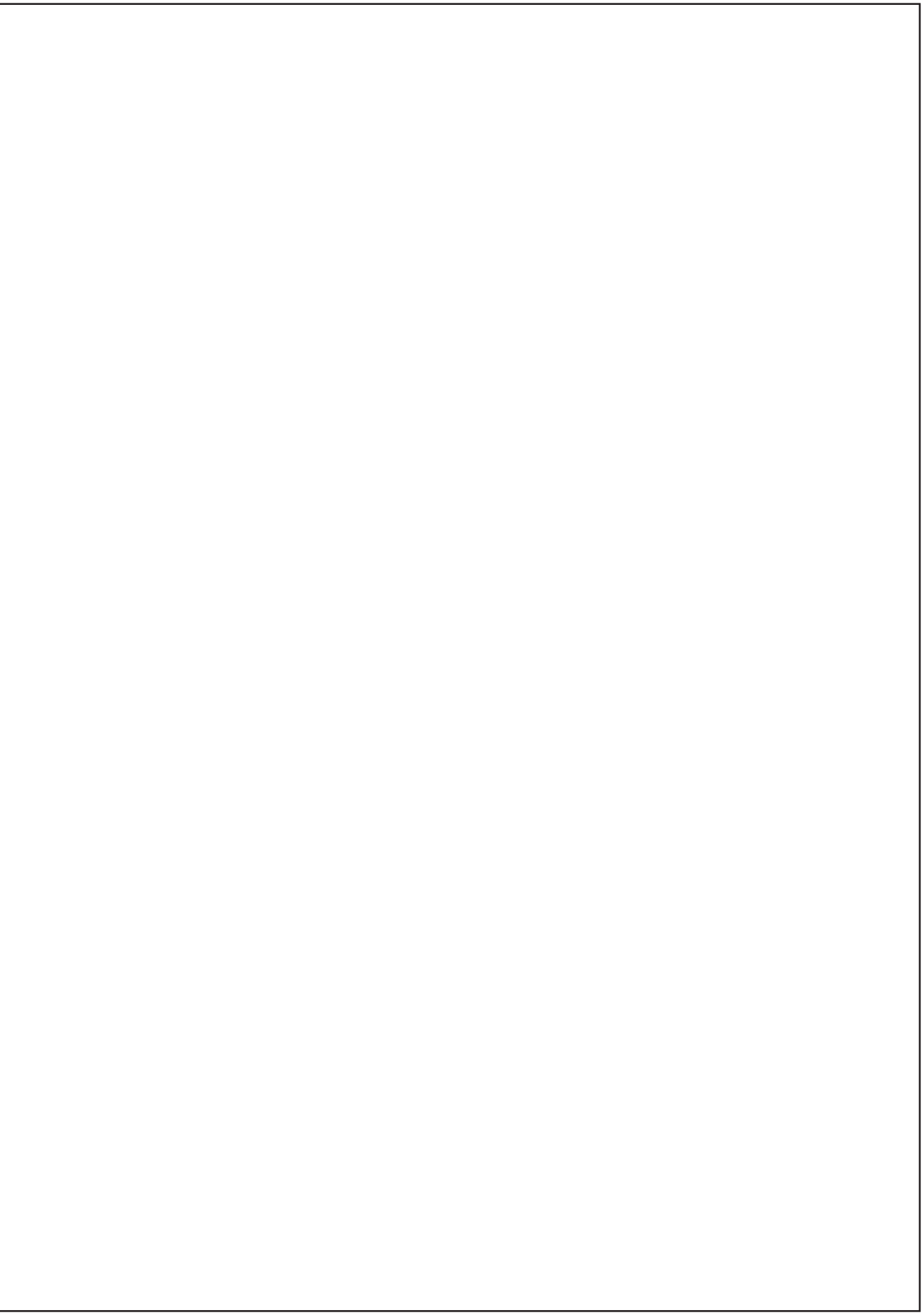
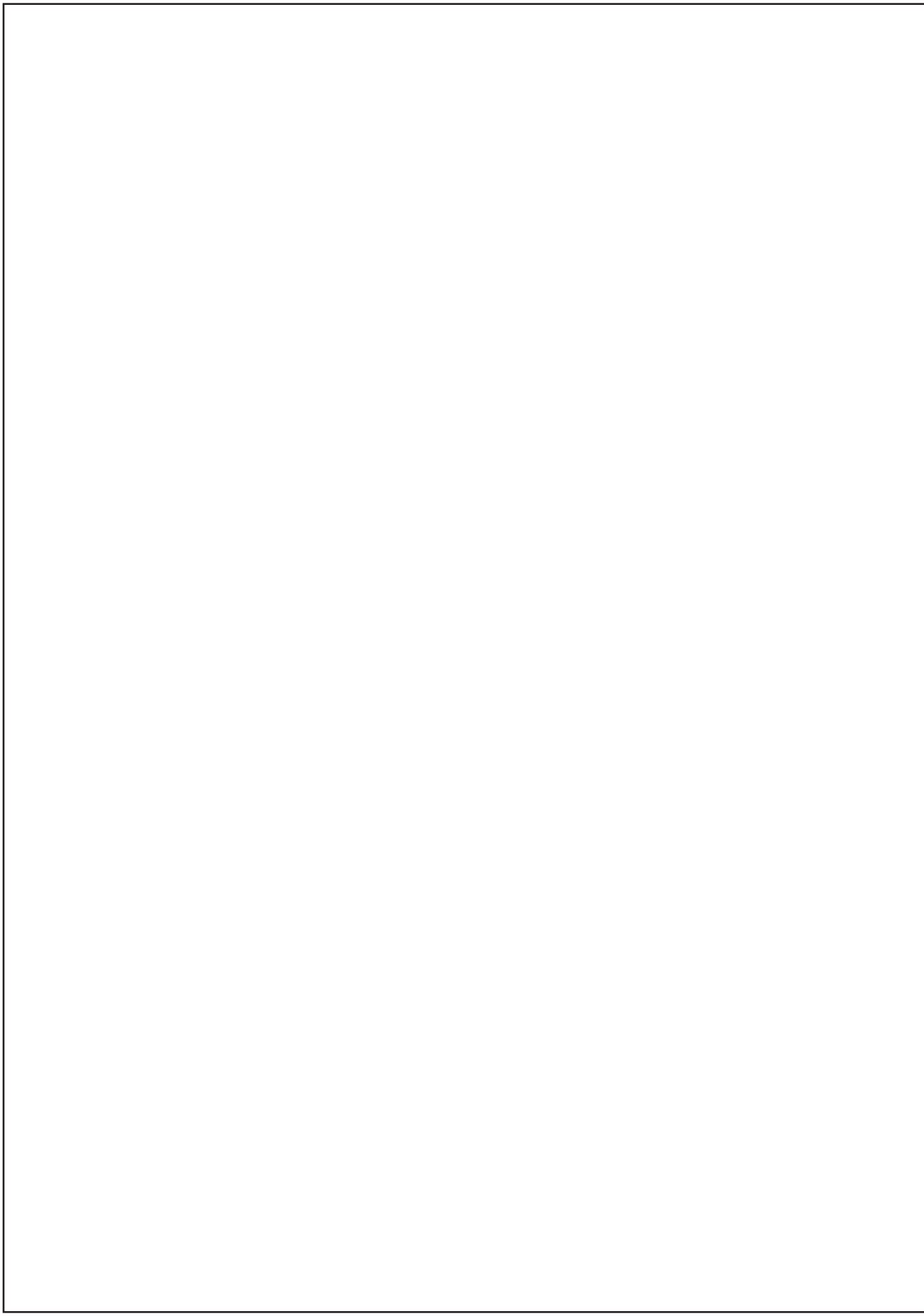


Fig.4. Kinetics of C-reactive protein (CRP) concentration in serum of mock-vaccinated (grey symbol) or CP7_E2alf vaccinated (black symbol) piglets following CSFV challenge. Each symbol represents the mean ± SD. The significant differences between the CRP level in the vaccinated and mock-vacciantед control groups were indicated with ***p<0.0001.





CHAPTER 3.

Discussion, Conclusion and Future Perspectives

The studies presented in this thesis focus on the efficacy of a new marker vaccine candidate "CP7_E2alf" in presence of MDA. This vaccine is the first live DIVA vaccine, and its potential for vaccination in naïve animals has been evaluated by other groups previously. At present, the live C-strain vaccine is used widely in both domestic pigs and wild boar to prevent CSF. The "CP7_E2alf" will replace the C-strain. Therefore, the efficacy and the DIVA diagnostic potential of "CP7_E2alf" were investigated in piglets from C-strain vaccinated mothers. Additionally, immune modulation in these pigs and their mothers was evaluated. A small pilot study that analyzed the onset of humoral immune response in pigs vaccinated intramuscularly with one dose of C-strain vaccine was conducted at the beginning of the project.

The first manuscript described the clinical evaluation of "CP7_E2alf" efficacy in vaccinated at 5 or 8 weeks of age piglets and in their mock-vaccinated controls. The piglets were obtained from sows vaccinated intramuscularly at 4 weeks before farrowing with one dose of live attenuated C-strain vaccine. The challenge virus used for the infection of the vaccinated piglets was the highly virulent strain "Koslov". Post challenge, the protocol proposed by Mittelholzer et al., 2000 was followed in order to evaluate the progress of the infection. The results revealed that "CP7_E2alf" provided clinical protection as no severe clinical signs or mortality was observed in the vaccinated piglets. The DIVA potential of the new vaccine and the possibility for transfer of C-strain maternal derived E^{rns} and E2 specific antibody was evaluated in the same piglets by E^{rns} and E2 specific antibody ELISA test. MDA kinetics was assessed as well. The results presented in the second manuscript demonstrated that "CP7_E2alf" could be implemented as a DIVA vaccine in population previously vaccinated with the modified live attenuated C-strain vaccine. Maternal E^{rns} specific antibodies were transferred to some of the piglets and were detected up to four weeks of age. Immune modulation after vaccination of the sows and the piglets with regard to IgG1 and IgG2 isotype specific antibodies was evaluated. Additionally, the related to IgG1 and IgG2 isotype specific antibodies cytokines were analyzed in the piglets post challenge. The results suggest that C-strain and "CP7_E2alf" exploit different mechanisms of the immune system in providing protection.

The mortality rate in the mock-vaccinated piglets was significantly reduced compared to previous studies (Mittelholzer et al., 2000; Lohse et al., 2011) and there was a clear difference between the piglets challenged at 7 or at 10 weeks of age. We first suggested that the MDAs level at the time of challenge might be an explanation for the significant attenuation of the infectious virus. Surprisingly, when the serum samples were analyzed with neutralization test the results showed no difference in the neutralization titer obtained from the piglets that survived and these that succumbed to the infection. Furthermore, the neutralization titer was lower than previously claimed to be necessary for protection (Terpstra and Wensvoort, 1987). Thus, the attenuation of the infectious virus probably was due to a complex of virus-host factors. The neutralization of "Koslov" strain by low titers of MDAs might be explained with the close phylogeny as both "Koslov" and the vaccine C-strain belong to genotype 1 (Bartak and Greiser-Wilke, 2000). Additionally, the involvement of other than humoral part of the immune system host factors could play a significant role in the removal of the virus. Transfer of functional protective colostral antigen-specific T-cell from vaccinated sows to their piglets was previously reported (Bandrick et al., 2008). The third manuscript demonstrated detection of significantly higher concentration of IgG2 compared to IgG1 isotype specific antibodies in the sows what suggests predominant activation of the cellular immune response post C-strain vaccination. Thus, a transfer of protective cellular mediated immunity to their piglets is very likely.

CSF vaccine virus is known to target mainly the tonsils and the challenge virus of this study showed the same pattern. Interestingly, some of the mock vaccinated piglets challenged at 7 weeks of age had the virus in the tonsils, but did not show clinical signs. A possible development of a chronic infection in these piglets due to the MDA that prevented morbidity could be an explanation. In a case of outbreak, these piglets are a potential risk for long undetected transmission of CSFV.

We can discuss the relevancy of the used protocol and CS (Mittelholzer et al., 2000) in regard to the challenge virus. The mock-vaccinated animals that succumbed to the infection had low CS and their temperature rarely exceeded 41.0°C, which is expected for infections with highly virulent strains CSFV. It is not clear whether the reason is a possible attenuation of the infectious virus by the maternal immunity or an acute course of the disease that cause mortality before onset of clinical signs. Mortality with low CSs after challenge with another highly virulent strain that belongs also to genotype 1 was reported previously (Tarradas et al., 2011). Thus, more relevant for these CSFV strains might be the proposed by Floegel-Niesmann et al. 2003 modified CS schema that includes

some other parameters such as: case fatality, pathological scores, leukocyte count and homologue CSF antibody titer. In agreement with this schema, our observations revealed clear correlation between white blood cells count and the virulence of the CSFV. Furthermore, some additional hematological and immunological parameters could be included when CSF virulence is evaluated in vivo. Mark reduction of the platelets counts were detected in all piglets that succumbed to the challenge infection. Based on the detected correlation of CRP concentration in blood and the outcome of the infection, early kinetics of CRP could be another supplementary parameter.

Despite the variable disease course in the control-mock vaccinated animals no interference of maternal immunity with DIVA concept in these animals was observed and seroconversion to E^{rns} specific antibodies was detected in all piglets from day 10 post challenge. The very early seroconversion at day 3 post challenge of one vaccinated piglet indicated a possible cross reactivity with the vaccine BVDV E^{rns} specific antibodies. This possibility is supported by the statistically significant difference between these results and the results obtained by the corresponding control mock-vaccinated group.

The transfer of maternal immunity seemed to show discrepancy between the results obtained from the sows by NLPA assay and E^{rns} ELISA test. This low sensitivity of the E^{rns} ELISA test with serum samples from C-strain vaccinated pigs was reported previously and the lack of vaccine viral replication is likely the explanation. However, when analyzing the E^{rns} specific antibodies that the piglets had received from the sows, they had a higher inhibition percent than their mothers.

Future Perspectives

Results of the studies presented here suggest a number of possibilities for future research as outlined below.

The efficacy of "CP7_E2alf" vaccine in face of maternal immunity in domestic piglets intramuscularly vaccinated was evaluated. As this vaccine is intended also for baits vaccination of wild boar future studies could be performed in this target species. The persistence of MDA in the piglets depends on the level of antibodies in the individual sow and the amount of colostrums they obtain from their mothers. Wild boar give birth to fewer piglets than domestic sows, thus with fewer piglets to feed it is probable that the outcome of vaccination study in wild boar will be different. Oral immunization activates different parts of the immune system, what could additionally alter the successful vaccination observed in our study.

The protective ability of the MDA may differ with the challenge virus used. A future study could test as well the protection ability of MDA from C-strain in piglets infected with highly virulent strains of genotype 2 or 3. CSFVs that belong to genotype 2 are responsible for the latest outbreaks of CSFV in Europe and strains from genotype 3 are wildly spread in Asia, where routine prophylactic vaccination with C-strain is performed.

Half life of MDA depends on the time point of sow's vaccination and on the infection pressure in the population, thus different vaccination schedules of the sows could be performed to mirror field situation with piglets having variable neutralization antibodies titers.

Addition of a vaccinated not challenged group could be valuable in providing conformation of the DIVA potential of "CP7_E2alf". In order to evaluate the marker vaccine's efficacy in case of emergency the challenge infection could be performed one week after vaccination. This new marker is going to be used in a case of outbreak and its ability to prevent transmission of the infectious virus is of significant importance. Our results showed that the vaccine is able to reduce the challenge viral genome, but a contact not infected group is needed to support this observation. Longer post challenge period could be very beneficial. At the end of the animal experiment, the survived infection mock-vaccinated piglets did not show severe clinical signs, fever or their white blood cell counts come to physiological levels. Thus, observation of these piglets for more than two weeks will give us the possibility to confirm their true recovery.

It will be very interesting to study the efficacy of CP7_E2alf in piglets younger than 5 weeks. According to the literature, when younger piglets were vaccinated with C-strain clear interference of the MDA with the vaccine efficacy was observed. However, as it seems that the two vaccines exploit different mechanisms of the immune system, the outcome of CP7_E2alf in younger piglets could be quite different.

The potential role of the cellular part of the immune system in the protection against CSF needs further evaluation. Transfer of activated maternal cellular and innate immunity components with colostrums and milk will give us a possibility to better understand the factors involved in early life vaccinology. An animal study conducted at CVI Netherlands tested the efficacy of "CP7E2alf" in piglets from "CP7E2alf" vaccinated mothers and oppositely to our findings all of the piglets succumbed to the challenge infection (personal correspondence, CSFV_goDIVA). Thus, a future

collaboration between the two research groups to study the possible reason for the obtained results could be very beneficial.

Further investigation of immune modulation after C-strain and "CP7_E2alf" vaccination could be also of interest. This research should include additional Th1 and Th2 cytokines. We tested IL4 and IFN-γ concentration in serum by ELISA test and found only a few IFN-γ and no IL4, but a recently published study (Renson et al., 2012) assessed the gene expression of these cytokines in blood cells by real time RT-PCR.

Conclusions

The studies performed in this thesis leads to several conclusions.

Studies within the CSFV_goDIVA project has shown that "CP7E2alf" is a promising marker vaccine that can replace the current modified live attenuated C-strain vaccine. Previous studies have been conducted in naïve piglets. Here it is shown that the successful vaccination with the new marker vaccine was not affected by the C-strain MDA as all vaccinated piglets were protected against morbidity, mortality and pathological lesions.

"CP7_E2alf" can be implemented as a marker vaccine in populations previously vaccinated with C-strain when the piglets have reached 5 weeks of age, as no interference of the MDA and the vaccine with the DIVA potential at this age was observed. The MDA concentrations at 7 and 10 weeks of age were insufficient to neutralize the challenge virus and to prevent seroconversion to E^{rns} specific antibodies.

Pig producers are in need of a vaccine against CSFV with good efficacy and DIVA ability. The "CP7_E2alf" seems to have all these potentials and with the ability to overcome maternal immunity it looks even more promising. The vaccine is primarily intended for emergency use and further studies are still needed before the vaccine can be used also in countries with large pig export. The collaboration between a large group of research partners and a commercial partner in this EU project has been a great leap forward.

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