Inaugural-Dissertation zur Erlangung der Doktorwürde der Tierärztlichen Fakultät der Ludwig-Maximilians-Universität München

Protection against transplacental transmission of Classical swine fever virus using live marker vaccine “CP7_E2alf”

von Julia Maria Henke

aus Heidenheim an der Brenz

München 2019

Aus dem Veterinärwissenschaftlichen Department der Tierärztlichen Fakultät der Ludwig-Maximilians-Universität München

Lehrstuhl für Virologie

Arbeit angefertigt unter der Leitung von Univ.- Prof. Dr. Gerd Sutter

Angefertigt im Institut für Virusdiagnostik

des Friedrich-Loeffler-Instituts,

Bundesforschungsinstitut für Tiergesundheit, Insel Riems

Mentor: Prof. Dr. med. vet. Martin G. Beer

Gedruckt mit Genehmigung der Tierärztlichen Fakultät
der Ludwig-Maximilians-Universität München

Dekan: Univ.-Prof. Dr. Reinhard K. Straubinger, Ph.D.

Berichterstatter: Univ.- Prof. Dr. Gerd Sutter

Korreferent/en: Priv.-Doz. Dr. Wolfram Petzl

Tag der Promotion: 25. Februar 2019

Die vorliegende Arbeit wurde gemäß § 6 Abs. 2 der Promotionsordnung für die Tierärztliche Fakultät der Ludwig-Maximilians-Universität München in kumulativer Form verfasst.

Folgende wissenschaftliche Arbeiten sind in dieser Dissertationsschrift enthalten:

Blome, S., C. Staubach, J. Henke, J. Carlson, and M. Beer. 2017. **'Classical Swine Fever-An Updated Review**', erschienen in Viruses, 9, unter DOI 10.3390/v9040086 online verfügbar

Henke, J., J. Carlson, L. Zani, S. Leidenberger, T. Schwaiger, K. Schlottau, J. P. Teifke, C. Schröder, M. Beer, and S. Blome. 2018. **'Protection against transplacental transmission of moderately virulent classical swine fever virus using live marker vaccine "CP7_E2alf"'**, erschienen in Vaccine, 36: 4181-87, unter DOI 10.1016/j.vaccine.2018.06.014 online verfügbar

Weitere Arbeiten, die nicht in der Dissertationsschrift enthalten sind:

Zani L., Forth, J. H., Forth L., Nurmoja I., Leidenberger S., Henke J., Carlson J., Breidenstein C., Viltrop A., Höper D., Sauter-Louis C., Beer M. and Blome S. „**Deletion at the 5'-end of Estonian ASFV strains associated with an attenuated phenotype**" erschienen in Scientific Reports, 8: 6510, unter DOI 10.1038/s41598-018-24740-1

1 Introduction

Classical swine fever (CSF) is a highly contagious disease of swine and considered as one of the most important diseases in livestock production worldwide. Due to its tremendous economic impact on the pig industry, it is of outmost importance for all countries with considerable pig production to eradicate the disease and prevent reintroduction. The detection of the disease is notifiable to the World Organization for Animal Health (OIE) and leads to trade restrictions and strict, mandatory control measures. CSF is still present in many parts of the world despite great efforts to eradicate the disease. Eradication programs are mostly based on stamping out campaigns and vaccination programs. To this day, most of the vaccination campaigns are based on the use of conventional live attenuated vaccines. These highly efficient vaccines have been available for decades, but because of trade restrictions for vaccinated animals, it would be advisable to use marker vaccines which allow differentiation of field virus infected from vaccinated animals (DIVA principle). In this case, derogations from the restrictions are foreseen, at least in European Union legislation. Recently a new marker vaccine, “CP7_E2alf” (Suvaxyn® CSF Marker, Zoetis), has been licensed by the European Medicines Agency (EMA). This marker vaccine has been thoroughly tested in the licensing process, and while safety and efficacy against horizontal transmission was proven several times, protection against vertical transmission was not undoubtedly shown, especially with early and harsh challenge infection. In consequence, a warning was included in the summary of product characteristics. Sows should not be vaccinated, due to the risk of immunotolerant, persistently infected offspring. These piglets are the worst case scenario as they appear healthy but shed high amounts of virus while not mounting a specific immune response. Under field conditions, these animals could go unnoticed and enter the pig trade. However, highly virulent strains as used for the initial efficacy tests would rather kill the sow and the piglets than establish persistent infection. The more relevant challenge for the so-called “carrier-sow-syndrome” is the use of a moderately or even low virulent strain. To test the hypothesis that CP7_E2alf would be able to prevent vertical transmission under these conditions, a study was carried out according to OIE guidelines using the moderately virulent CSF virus (CSFV) strain “Roesrath” for challenge infection.

2 Literature review

In the framework of our recent CSF studies, an updated literature review was carried out and published in a peer-reviewed journal. To avoid unnecessary duplications, this review article is now used as background information in this thesis. Chapters on the available vaccines and the relevant legislation concerning emergency vaccination in the European Union were added to complete the scope.

2.1. Classical Swine Fever—An Updated Review

Sandra Blome [1,*], Christoph Staubach [2], Julia Henke [1], Jolene Carlson [1] and Martin Beer [1]

[1] Friedrich-Loeffler-Institut, Institute of Diagnostic Virology, Suedufer 10, 17493 Greifswald, Germany;
[2] Friedrich-Loeffler-Institut, Institute of Epidemiology, Suedufer 10, 17493 Greifswald, Germany;

Viruses 2017
DOI: 10.3390/v9040086

viruses

MDPI

Review

Classical Swine Fever—An Updated Review

Sandra Blome [1,*], Christoph Staubach [2], Julia Henke [1], Jolene Carlson [1] and Martin Beer [1]

[1] Friedrich-Loeffler-Institut, Institute of Diagnostic Virology, Suedufer 10, 17493 Greifswald, Germany; julia.henke@fli.de (J.H.); jolene.carlson@fli.de (J.C.); martin.beer@fli.de (M.B.)

[2] Friedrich-Loeffler-Institut, Institute of Epidemiology, Suedufer 10, 17493 Greifswald, Germany; christoph.staubach@fli.de

* Correspondence: sandra.blome@fli.de; Tel.: +49-38351-7-1144

Academic Editor: Linda Dixon and Simon Graham
Received: 8 March 2017; Accepted: 13 April 2017; Published: 21 April 2017

Abstract: Classical swine fever (CSF) remains one of the most important transboundary viral diseases of swine worldwide. The causative agent is CSF virus, a small, enveloped RNA virus of the genus *Pestivirus*. Based on partial sequences, three genotypes can be distinguished that do not, however, directly correlate with virulence. Depending on both virus and host factors, a wide range of clinical syndromes can be observed and thus, laboratory confirmation is mandatory. To this means, both direct and indirect methods are utilized with an increasing degree of commercialization. Both infections in domestic pigs and wild boar are of great relevance; and wild boars are a reservoir host transmitting the virus sporadically also to pig farms. Control strategies for epidemic outbreaks in free countries are mainly based on classical intervention measures; i.e., quarantine and strict culling of affected herds. In these countries, vaccination is only an emergency option. However, live vaccines are used for controlling the disease in endemically infected regions in Asia, Eastern Europe, the Americas, and some African countries. Here, we will provide a concise, updated review on virus properties, clinical signs and pathology, epidemiology, pathogenesis and immune responses, diagnosis and vaccination possibilities.

Keywords: porcine viruses; *Pestivirus*; classical swine fever; clinical signs; pathogenesis; epidemiology; diagnosis; control; vaccination; marker strategy

1. Introduction

Classical swine fever (CSF) is one of the most important viral diseases of domestic pigs and wild boar. It has tremendous impact on animal health and pig industry and is therefore notifiable to the World Organization for Animal Health (OIE) [1]. After implementation of strict control measures, several countries succeeded in eradicating CSF. Nevertheless, in most parts of the world with significant pig production, CSF is at least sporadically present. Endemicity can be assumed in several countries of South and Central America, parts of Eastern Europe and neighboring countries, as well as Asia, including India. Little is known about the African situation.

A binding legal framework exists for the surveillance and control in most countries. Integral parts of the control measures are timely and reliable diagnosis, stamping out of infected herds, establishment of restriction zones, movement restrictions, and tracing of possible contacts. Prophylactic vaccination and other treatments are often also strictly prohibited. However, in Europe, where affected wild boar populations were shown to be an important reservoir for the virus, and acted as a source for reintroduction into the domestic pig population [2,3], emergency vaccination of wild boar has been practiced to control the disease [4–7]. Emergency vaccination is also among the options to combat CSF in domestic animals. Furthermore, vaccination is still in use to reduce the disease burden in endemically affected countries.

Design of control measures and risk assessment depends on the knowledge of factors that influence disease dynamics and epidemiology. For this purpose, the presented review aims at providing an updated overview on the disease and the underlying mechanisms but also control and diagnostic options.

2. Virus Properties

2.1. Virus Organization and Replication

Classical swine fever virus (CSFV) belongs to the genus *Pestivirus* within the *Flaviviridae* family [1]. Other members of this genus are *Bovine viral diarrhea virus* 1 and 2 (BVDV-1 and -2), *Border disease virus* (BDV) and a growing number of unclassified and so-called atypical pestiviruses, from giraffe-virus over HoBi-like viruses to recently discovered Bungowannah virus and atypical porcine pestivirus [2–13].

The enveloped viral particles consist of four structural proteins, namely the core protein (C), and envelope glycoproteins E1, E2, and E^{rns} [14–18]. The core encloses the positive single-stranded RNA genome of approximately 12.3 kb [19–22] which is translated into one polyprotein. The coding region is flanked by non-translated regions (NTR) at both ends. Co- and post-translational processing of the precursor protein by viral and cellular proteases results in 13 mature proteins, the above-mentioned structural proteins and non-structural proteins N^{pro}, p7, NS2-3, NS2, NS3, NS4A, NS4B, NS5A, and NS5B. The latter have various functions in the viral replication, e.g., NS5B represents the RNA-dependent RNA polymerase [23] and NS3 acts as protease [24,25].

Virus replication takes place in the cytoplasm after receptor mediated endocytosis and does normally not lead to a cytopathic effect in cell culture (naturally occurring CSFV strains were found to be non-cytopathic) [26]. A putative receptor is the porcine complement regulatory protein cluster of differentiation (CD) 46 that was shown to play a major role in CSFV attachment, together with heparan sulfates [27]. Upon cell culture adaptation an increased usage of heparin sulfates is observed for cell-virus interaction [28]. The mutation responsible for the adaptation lies within the E^{rns} encoding region [8], namely in the C-terminus where a Ser residue is replaced by an Arg residue at amino acid 476 in the polyprotein of CSFV.

In any case, glycoproteins E2 and E^{rns} are necessary for viral attachment [9,10], and the initial contact with the host cell is mediated through the E^{rns} which interacts with glycosaminoglycans [10,11]. For receptor binding and subsequent endocytosis, the E2-E1 heterodimer is essential [12]. After fusion of the virus envelope with the endosomal membrane, the virus core is released into the cytoplasm [13–15]. Thereafter, viral RNA is released into the cytoplasm and translation takes place. The binding of ribosomes at the rough endoplasmatic reticulum is realized through an internal ribosomal entry site (IRES) at the 5′ NTR, which allows a cap-independent translation [16–18]. The processing of the resulting viral polyprotein precursor occurs with the help of viral and cellular proteases [19]. Initially, autoproteinase N^{pro} is cleaved from the polyprotein [20,21]. Subsequently, cellular proteases cleave the C-protein and E^{rns}, E1 and E2, E2 and p7 as well as NS2-3. NS2-3 is then partially processed through the autocatalytic cysteine protease activity of NS2 into NS2 and NS3. In this way NS2 generates its own C-terminal ending [22,23]. The serine protease activity of NS3 leads to the cleavage of the rest of the NS3-NS5 region [24]. While replication progresses, negative-stranded RNA is generated, which serves as template for the synthesis of the positive stranded RNA. The positive stranded RNA is then packed into the capsid [25]. Virion assembly and maturation takes place in the endoplasmatic reticulum and the Golgi apparatus after which the progeny virions bud at the cell membrane through exocytosis [26,27].

2.2. Tenacity and Virus Inactivation

The survival of CSFV under different ambient conditions varies considerably and is influenced especially by the temperature but also by the matrix in which it is found. Generally, survival times are higher under cold, moist and protein rich conditions [28]. The dependence of viral survival and temperature is well studied [29–31].

For animal disease control, survival in excretions (left in the pen or stored as slurry) and stability in meat products are crucial parameters. For CSFV in excretions, survival times were demonstrated that range from a few days at room temperature to several weeks at 5 °C [32]. If temperatures are higher than 35 °C, survival times are dramatically reduced, and inactivation occurs in hours or even minutes from temperatures above 50 °C [33]. This is an important factor when biogas plants and other industry parts are discussed. Along the same lines, Botner and Belsham [34] could show that survival of CSFV in slurry was short when heated and remained infective for weeks at cool temperature. Turner showed that complete inactivation was achieved at 60 °C for 3 min under lab conditions [35]. However, homogeneity of the mixture that is to be inactivated and thus temperature distribution is crucial [36]. For contaminated pig pens, this can mean virus survival for at least several days [37] to one month under cold winter conditions [38]. Under laboratory conditions, freeze-thawing has a negative impact on viral titers which can however be prevented by some chemical compounds such as dimethyl sulfoxide [39]. With regard to pH values, CSFV is relatively stable between pH 5 and 10. Half-lives at low pH levels are temperature dependent with mean half-lives that are more than ten-fold lower at room temperature compared to 4 °C (70 h at 4 °C compared to 5 h at 21 °C for pH 3). Overall variability is high and shows some strain dependence [40]. Another important matrix is meat or downstream products. Farez and Morley [30] report virus survival over years in meat frozen at −70 °C and of days to years in different meat products. Survival of 4.5 years in frozen meat was also reported by Edgar (reviewed in the EFSA scientific report 2009, [28]). Curing and smoking alone have little effect on the virus while higher temperatures readily inactivate the virus [31]. Survival times of more than 75 days were reported for salami [41] and over 120 days for Iberian loins or shoulders [42].

2.3. Genetic Diversity and Virulence Factors

Classical swine fever virus strains can be divided into three genotypes with three to four sub-genotypes. The most recently added sub-genotype 1.4 was only very recently described for CSFV strains from Cuba. These strains had so far been placed into sub-genotype 1.2 but formed a distinct cluster when compared based on longer genome fragments, e.g., full-length E2, N^{pro}, C, E1, and E^{rns} [43]. Further divisions that have been proposed concern sub-genotypes 2.1 and 2.3 [44–47]. However, these systems of clusters or clades were not further harmonized and did not enter routine use. The genetic diversity does not result in true serotypes and does not impact vaccine efficacy. In general, CSFV is highly stable, especially for an RNA virus [48].

Up to very recently, phylogenetic studies were mainly based on two short fragments, namely a 150 nucleotide (nt) fragment of the 5′NTR and a 190 nt fragment of the E2 encoding region [49]. Moreover, a 409 nt fragment of the region coding for the polymerase gene NS5B was employed [50]. With the advent of affordable sequencing technologies for longer fragments or even full genomes, in-detail analyses are now more often based on more than the traditional fragments. The European Union (EU) Reference Laboratory for CSF nowadays recommends using full-length E2 encoding sequences for reliable CSFV phylogenies [51]. The latter resulted, e.g., in the designation of the above-mentioned new sub-genotype 1.4. Full-length sequences are being employed for quasispecies analyses, investigation of virulence determinants but also high resolution molecular epidemiology [52–55].

The distribution of genotypes shows a distinct geographical pattern [50,56]: Whereas isolates belonging to group 3 seem to occur solely in Asia, all European CSFV isolates of the 1990s and later belonged to one of the subgroups within group 2 (2.1, 2.2, or 2.3) [45,51,57–64] and were clearly distinct from former CSF reference viruses, which belong to group 1 [50,65]. On the global scale, the most prevalent genotype over the last decades was undoubtedly genotype 2 [66]. However, all field isolates from the American continent belong to genotype 1 with only 1.1 strains from Argentina, Brazil, Colombia, and Mexico; 1.3 strains from Honduras and Guatemala; and the above-mentioned sub-genotype 1.4 strains from Cuba [43,67–69]. Little is known about the CSF situation in Africa and the Middle East. Exceptions are the 2005 outbreak in South Africa and the 2009 outbreak in Israel that were both caused by 2.1 CSFV strains [70,71]. Reports from India are increasingly detailed and

demonstrate that sub-genotypes 1.1, 2.1, and 2.2 are co-circulating [72–79]. This changes the historical situation where genotype 1.1 strains predominated. From Nepal, strains of sub-genotype 2.2 were reported [80]. The situation in China is characterized by high variability of strains that belong mainly to sub-genotypes 1.1, 2.1, 2.2, and 2.3 [81–84]. Taiwan is also experiencing a change in sub-genotypes. It seems that the historical 3.4 strains are replaced by the Chinese 2.1 strains [85]. However, Taiwanese reports include all the above-mentioned sub-genotypes [85–87]. Sub-genotype 2.1 and 2.2 strains are also reported from Laos [88,89]. From Korea, strains of sub-genotypes 3.2 and 2.1 were reported [44], and, for Japan, indications exist that genotype 3 is found [90]. Generally, endemicity is accompanied or driven by strains of moderate or low virulence. These strains have been found in several regions with long-term circulation of CSFV (e.g., Cuba [91]), and mathematical models have shown that these strains may represent the viral optimum for long-term persistence [92]. An overview of the genotype distribution is provided in Figure 1.

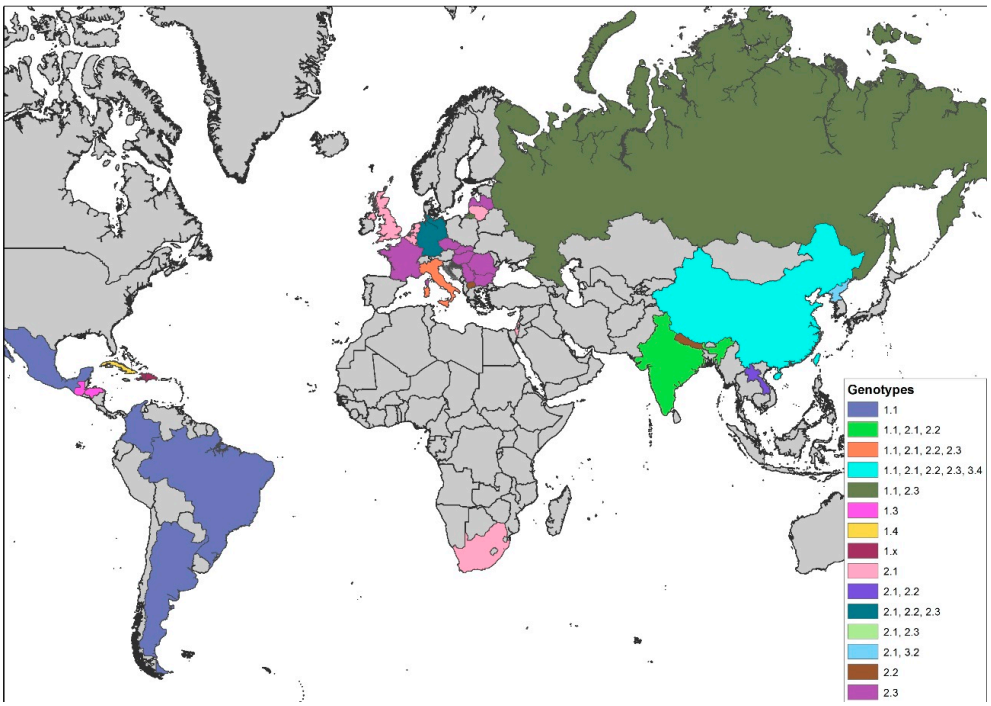


Figure 1. Global distribution of classical swine fever virus (CSFV) sub-genotypes (map based on Global Administrative Areas (GADM database 2.8; November 2015)).

European CSFV sequences were collected and made available through the semi-public CSF-database (DB) at the EU and OIE reference laboratory for CSF in Hannover, Germany [49,93–95]. Since the Institute of Virology became European Reference Laboratory for CSF more than 30 years ago, the virus isolates involved in European outbreaks but also other accessible sequence data were collected and stored. The database includes the above-mentioned fragments and also partial NS5B, full E2, and full-length CSFV sequences. It also allows automated typing and retrieval of sequences for in-detail analyses [95].

The search for virulence markers indicated a role of the N^{pro} [96], the E2 [97], the ribonuclease activity and dimerization of the E^{rns} [98,99], and NS4B [100]. Furthermore, glycosylation of structural proteins was shown to affect virulence [101–105]. However, these determinants are still far from being understood and do not seem to be transferrable among strains. Even the direct comparison of vaccine strains and their virulent ancestors did not reveal clear pattern [100,106]. Investigations into the role of quasispecies composition did not lead to the establishment of a clear correlation between variability and virulence [52]. There were also no predictors for different disease courses found [107].

3. Clinical Signs and Pathomorphological Lesions

Classical swine fever can be divided into the following forms of the disease: an acute (transient or lethal), a chronic and a persistent course, which usually requires infection during pregnancy [65]. In general, the same clinical signs are seen in both domestic pigs and wild boar, and show up after an incubation period of four to seven (seldom 10) days after the infection. The progression is dependent on strain virulence, host responses, and secondary infections and may vary considerably. However, infection of young pigs (weaners) with a moderately virulent CSFV strain may serve as an example for the acute disease course: During the first two weeks upon infection, the acute phase is characterized by unspecific (often referred to as "atypical") clinical signs like high fever, anorexia, gastrointestinal symptoms, general weakness, and conjunctivitis [108]. Around two to four weeks after infection neurological signs can occur including incoordination, paresis, paralysis and convulsions. At the same time, skin hemorrhages or cyanosis can appear in different locations of the body such as the ears, limbs, and ventral abdomen. These late signs are the textbook cases and are therefore referred to as "typical" CSF signs. Examples of clinical signs can be found in Figure 2.

In acute-lethal courses, death usually occurs 2–4 weeks after CSFV infection. Mortality can reach up to 100% from 10 to 30 days depending on the age of the animal and the virulence of the virus strain [65,109–111]. Due to the immunosuppressive character of CSF infection, severe respiratory and gastrointestinal secondary infections can complicate the disease course and overlay the CSF signs. This is particularly important for clinical diagnosis. Infections with highly virulent CSFV strains such as "Margarita" or "Koslov" (the ones that are often used for vaccine testing) show a less age-dependent clinical course and may result in 100% mortality in all age classes of animals and severe neurological signs within 7 to 10 days (see, e.g., [112]).

Chronic course occurs when an infected pig is not able to mount an adequate immune response. In general, only non-specific clinical signs are observed in infected animals like remittent fever, depression, wasting and diffuse dermatitis (see Figure 3). It is acknowledged opinion that all chronically infected animals will eventually die. However, they can live for month in which they constantly shed high amounts of virus. Affected animals may develop antibodies that are in some cases only intermittently present and do not effect viral clearance. This, together with persistent infection, can play a role especially for affected wild boar populations [113–115], but also in endemically affected regions with constant virus circulation. Host rather than viral factors seem to play a role for the establishment of chronic infection [107].

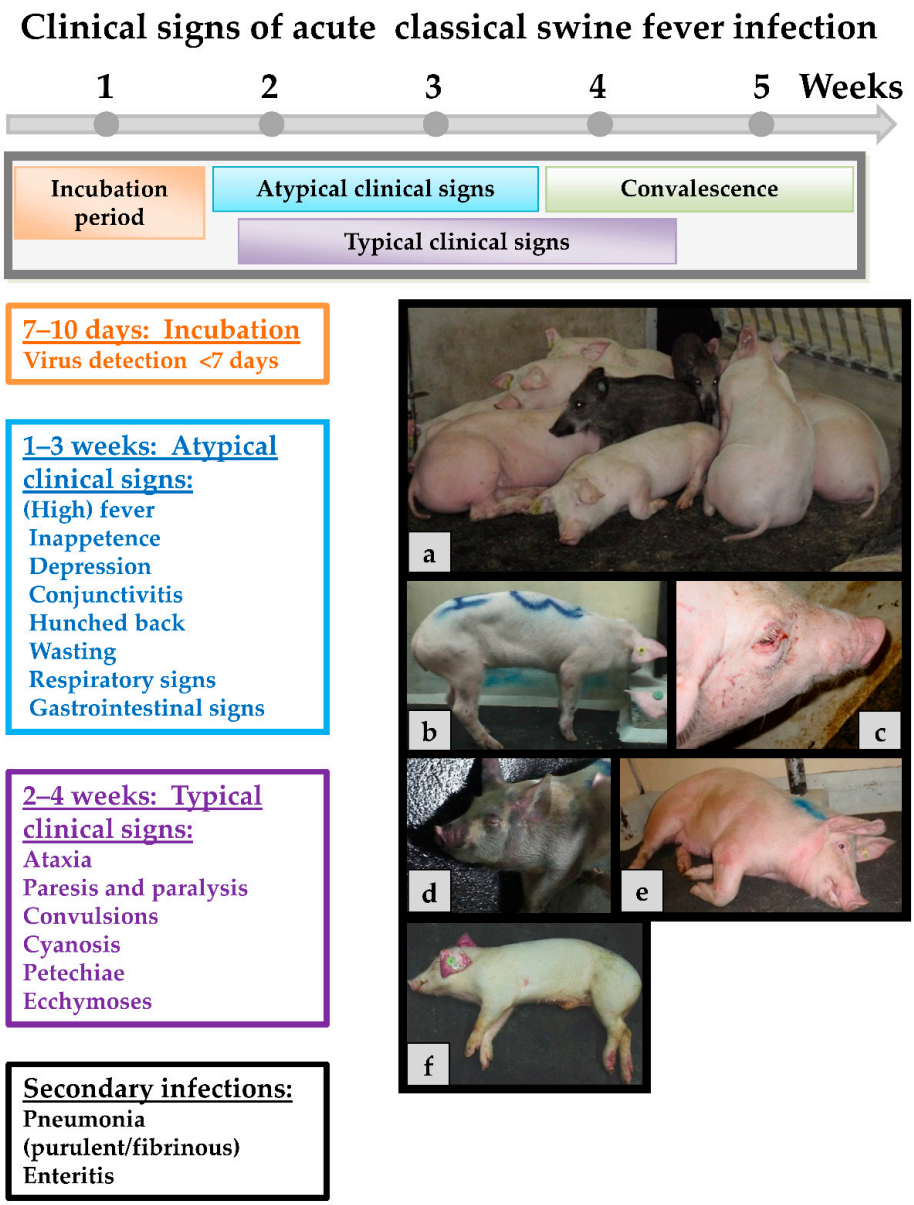


Figure 2. Acute CSFV infection with moderately virulent strains. The incubation period in most cases is from 7 to 10 days. Atypical clinical signs range from one to two weeks. Typical clinical signs occur around 2 to 4 weeks. The convalescent period is from 3 to 4 weeks. Death can occur as late as five weeks post-infection. (**a**) Swine are huddling, 10–15 days post-infection; (**b**) swine are presenting with hunched back; (**c**) severe conjunctivitis; (**d**) severe cyanosis of the skin around the face, ears, and limbs; (**e**) neurological signs, swine was unable to stand; and (**f**) dead swine with classic cyanosis of the ears.

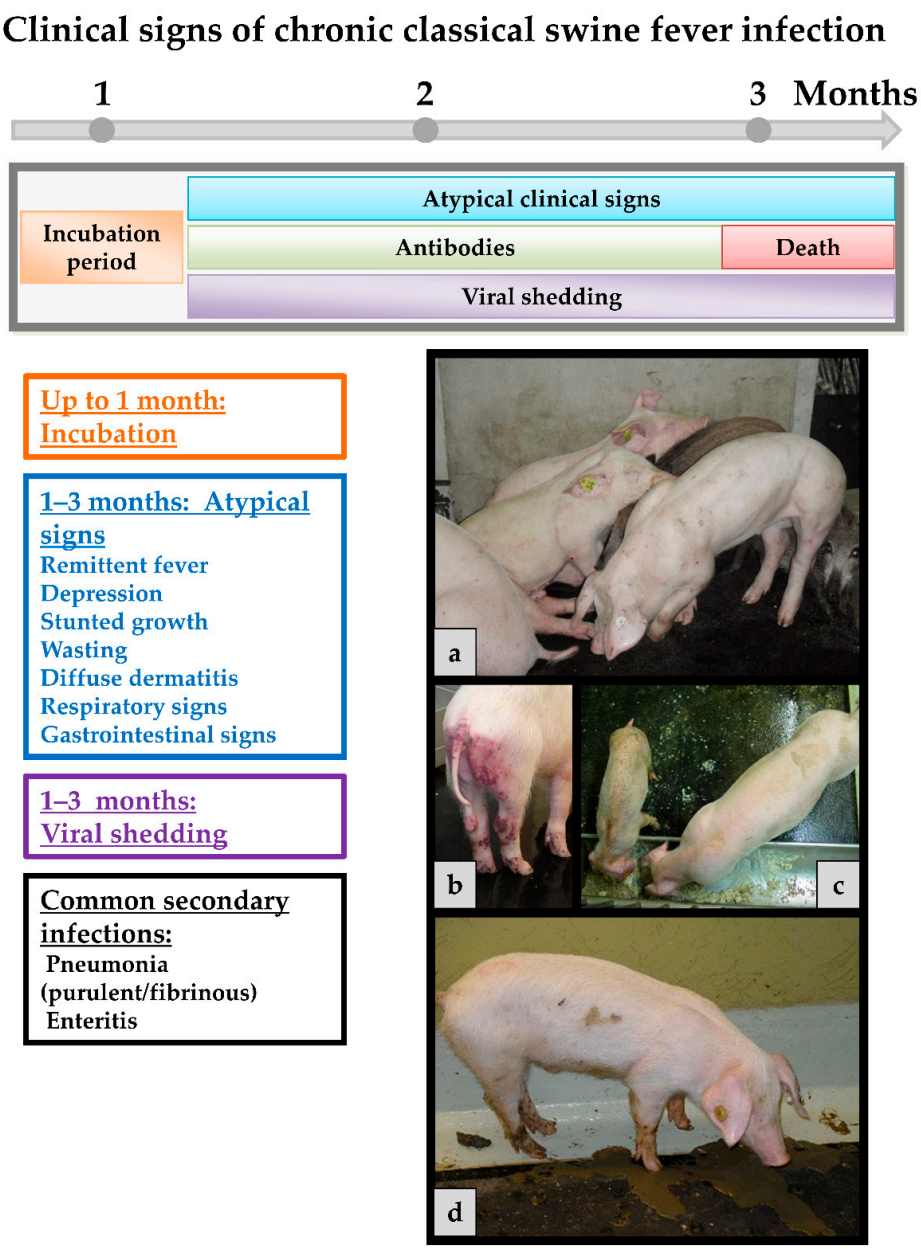


Figure 3. Chronic CSFV infection. The incubation period is the same as with the acute course. However, it may take up to a month until they are truly recognized. Atypical clinical signs can be present throughout and until death, occurring up to three months or even later after the infection. Antibodies can be detected at low levels after two weeks or later but do usually not persist. Viral shedding is observed from about four days post infection till the death of the animal. (**a**) Pigs are depressed, hunched over, and anorexic; (**b**) pig with petechial bleedings and ecchymosis in the anogenital region; (**c**) stunted and wasting pig beside a normally developed one of the same age; and (**d**) pig with diarrhea, shedding high viral loads until death.

When infection occurs during pregnancy, the virus can also infect the fetus in the womb due to its ability to pass the placental barrier which in turn might lead to persistent infection in the piglets. While the sows often show only mild clinical signs, an infection depending on the stage of gestation, leads to absorption or mummification of the fetuses and to abortions or stillbirth [114,116–123]. When infected between days 50 and 70 of pregnancy, an immunotolerance phenomenon can be induced and persistently infected offspring are born. The problem is that those piglets seem to be healthy and survive for several months but die due to the so-called late onset form of CSF. During that period

they shed high viral loads which are sufficient for transmission. Recent studies discuss that persisting infection can also be induced when infecting newborn piglets within the first eight hours of life or even 48 h after birth [124,125]. This was shown to impact on the efficacy of vaccines and may complicate control in endemically affected countries.

The pathological findings (Figure 4) depend on the course of the viral infection. In the acute course of CSF, pathology often reveals enlarged lymph nodes, hemorrhages and petechiae on serosal and mucosal surfaces of different organs such as the, lungs, kidneys, intestines and urinary bladder. Tonsillitis, necrotic ulcers in the intestines, lesions in the lymphoreticular system, and non-purulent encephalitis can be observed [126] Splenic infarctions can occur and are considered pathognomic for CSF [127]. Infected piglets develop leukopenia, thrombocytopenia and immunosuppression, which increases the risk for secondary infections and thus to diseases of the gastrointestinal and respiratory system [128]. In the chronic form, pathological lesions include atrophy of the thymus, depletion of the lymphoid organs, necrosis and ulceration of the small intestine, colon, and ileocecal valve. It is important to consider that these clinical signs and pathological lesions should be considered as differentials for a number of swine pathogens. These unspecific clinical signs and lesions can vary among animals depending on host factors and the virulence of the CSFV strain. Often, the age, breed and immune status play a role in the outcome of the disease [65,108,129].

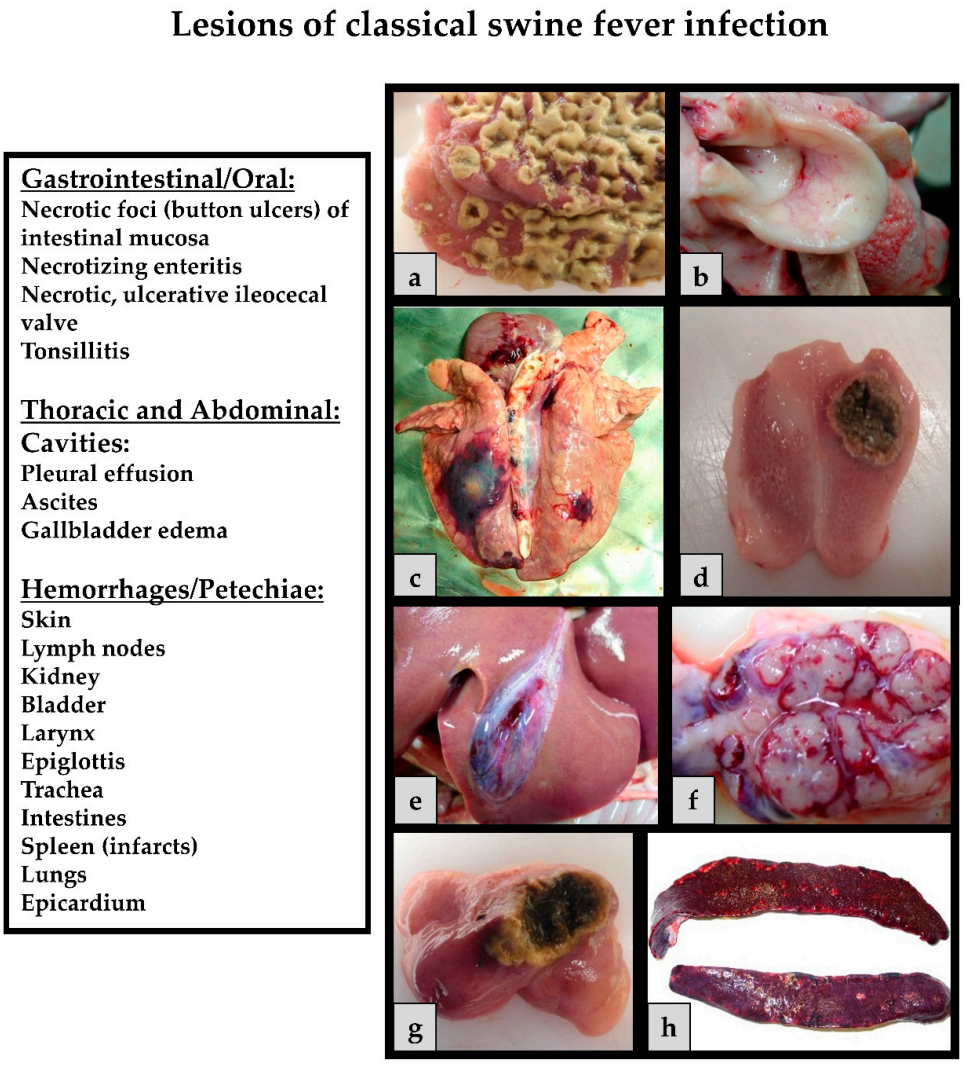


Figure 4. CSF related lesions: (**a**) Diphtheroid-necrotizing enteritis; (**b**) hemorrhages on the epiglottis; (**c**) severe secondary infections of the lung (*Actinobacillus pleuropneumoniae*); (**d**) necrotic tonsillitis with

an ulcer; (**e**) gallbladder edema; (**f**) hemorrhagic lymph node; (**g**) necrotizing ileocecal valve; and (**h**) splenic infarcts.

4. Pathogenesis and Immune Responses

As mentioned above, clinical signs of CSFV infections can vary considerably from peracute deaths to unapparent courses depending on virulence of the virus strain involved and different (partly unknown) host factors [65]. Unspecific clinical signs predominate, and differentiation from several other infectious diseases of swine is only possibly based on laboratory diagnosis. Acute-lethal forms can be viral hemorrhagic fever-like with severe thrombocytopenia, pulmonary edema, petechial bleedings, and increased vascular leakage [130]. Cytokine involvement is discussed for many lesions observed in acute CSF [131].

Infection with CSFV is followed by primary replication in the tonsils and subsequently spread to surrounding lymphoid tissues [132]. The virus reaches the regional lymph nodes through lymphatic vessels. Here further replication takes place and the virus is spread via blood to secondary replication sites such as spleen, bone marrow, and visceral lymph nodes [133–135]. Apoptotic reactions as well as phagocytic and secretory activation can be observed in several macrophage populations [136–144]. These activated macrophages seem to play a crucial role in (immuno-)pathogenesis while direct damage by the virus could be almost excluded for many lesions occurring in the course of CSFV infection. Moreover, dendritic cells are targeted and disturbance of the interferon system contributes to the pathogenesis [136–140]. There seems to be a correlation between high interferon (IFN)-α in the serum and disease severity and virulence of the strain involved [140,141]. High IFN-α concentrations are found as early as two days post infection, prior to the onset of clinical symptoms [112]. These findings are confirmed by microarray analyses of peripheral blood monocytic cells derived from CSFV-infected pigs [142].

Especially in the acute-lethal course, CSF is accompanied by severe lymphopenia and resulting immunosuppression as well as granulocytopenia [143–146]. Moreover, a marked thrombocytopenia starts very early after infection [147–149]. The mechanisms leading to this platelet decrease are not yet understood but disseminated intravascular coagulation (DIC), degeneration of megakaryocytes, bone marrow lesions, and accelerated deterioration have been discussed [130]. In addition, massive activation and subsequent phagocytosis of platelets has been discussed as an etiological factor [147] while DIC related correlates were not observed upon infection with a genotype 2.3 CSFV strain [150]. At least in vitro, endothelial cells are also activated and expression levels of pro-inflammatory and pro-coagulatory factors are increased [151]. The pathogenic mechanism involved in hemorrhagic lesions include damage of endothelial cells, causal involvement of thrombocytopenia (and DIC), erythrodiapedesis, and capillary vasodilatation and increased permeability [146,148,149,152,153]. However, several factors remain unclear and studies with different strains have given conflicting results.

Despite the immunopathogenesis of most CSF-related lesions, pigs recovering from CSFV infection mount an effective immune response with E2-specific antibodies detectable after 10–14 days. The E2 antibodies are able to neutralize CSFV in vitro and induce protective immune responses [154,155]. These antibodies and protection against re-infection persist probably livelong. In addition to E2, antibodies are raised against the E^{rns} and the non-structural protein NS3 [156,157]. Immunization with live attenuated CSFV can be efficient as early as 3–5 days post vaccination [158–160]. Thus, protection is possible without neutralizing antibodies and even before specific T-cell responses can be seen. Despite the fact that this very early protection is far from being understood, IFN-γ secreting T-cells seem to play a role [161–163].

5. Epidemiology

Susceptible hosts are different members of the *Suidae* family, particularly domestic pigs (*Sus scrofa domesticus*) and European wild boar (*Sus scrofa scrofa*) [113,164]. Moreover, the susceptibility of common warthogs (*Phacochoerus africanus*) and bushpigs (*Potamochoerus larvatus*) was recently demonstrated [165].

Classical swine fever virus can be transmitted both horizontally and vertically. Horizontal transmission takes places through direct or indirect contact between infected and susceptible pigs. Important indirect routes include feeding of virus contaminated garbage/swill and mechanical transmission via contact to humans or agricultural and veterinary equipment [127]. Aerogenic transmission was reported under experimental conditions [166–168], and it can probably play a role for within herd transmission [169].

Upon contact, infection usually occurs through the oronasal route, or less frequently via conjunctiva, mucus membranes, skin abrasions, insemination, and the use of contaminated instruments [170–173]. Infected pigs show high-titer viremia and shed virus at least from the beginning of clinical disease until death or specific antibodies have developed. The main excretion routes are by saliva, lacrimal secretions, urine, feces, and semen [127,135,173]. As mentioned above, chronically infected pigs shed the virus continuously or intermittently until death [65]. Vertical transmission from pregnant sows to fetuses is possible throughout all stages of gestation and can lead to persistently infected offspring (see above).

Classical swine fever affected wild boar populations can serve as reservoir of the virus and present a constant risk for domestic pigs. Fritzemeier et al. [2] could show that almost 60% of the primary CSF outbreaks in Germany between 1993 and 1998 were linked to infected wild boar. This link was particularly important for holdings with low biosecurity or problems in biosafety management.

Over the last decades, a decreasing virulence was observed for the CSFV strains involved in many outbreaks among wild boar and domestic pigs. In Europe, the most prevalent genotype 2.3 strains showed moderate virulence with a highly age-dependent clinical picture and rather unspecific clinical pictures in older animals (see above). These strains showed potential to establish endemicity in affected wild boar populations rather than showing the self-limiting behavior of the historical highly virulent CSFV strains. It was discussed whether these strains are somewhat the ideally adapted variants of CSFV for long-term perpetuation in wildlife [92].

In endemically affected countries with official but imperfect vaccination, circulation of less virulent CSFV strains is often masked by partial protection. In combination with management and biosecurity issues (swill feeding, contacts, shared equipment), the virus is maintained over prolonged periods in the domestic pig population.

6. Diagnosis

Rapid and reliable diagnosis is of utmost importance for the timely implementation of control measures against CSF. On the international level, laboratory methods as well as sampling and shipping guidelines can be found in the OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals and the respective EU Diagnostic Manual (European Commission Decision 2002/106/EC).

For CSFV, primary detection is performed using well established real-time reverse transcription polymerase chain reaction (RT-qPCR) systems [174–183], of which many are available commercially. Recently, field applicable RT-PCRs [184] but also alternatives have been designed such as loop-mediated isothermal amplification (LAMP) assays [185–190], primer-probe energy transfer RT-qPCR [191,192] or recently insulated isothermal RT-qPCR [193]. Moreover, CSFV can be isolated on different permanent cell lines such as porcine kidney cell lines PK15 or SK6 (Technical Annex to Commission Decision 2002/106/EC). In addition, detection of antigen on fixed cryosections of tissues is possible using fluorescence antibody or immune-peroxidase assays [194,195]. The available antigen ELISAs are recommended for the use with herd-based testing only. While the sensitivity of panpesti-specific assays (based on the E^{rns}) is usually at least comparable with virus isolation, most CSF specific assays lack sensitivity [196]. Serological screening can be performed using different commercially available E2 antibody enzyme-linked immunosorbent assays (ELISAs). In addition, neutralization assays allow, to a certain extent, differentiation of pestivirus antibodies and are used for confirmation [197].

Reliable DIVA (differentiation of infected and vaccinated animals) assays are needed when using DIVA vaccines. Commercially available tests that can accompany both E2 subunit vaccines and

chimeric vaccines such as "CP7_E2alf", target the detection of antibodies directed against glycoprotein E^{rns} [196,198,199]. Recently, additional diagnostic tests have been developed. One is a double-antigen ELISA format that was recently commercialized [200], another is an ELISA with a screening and a confirmation part [201]: Moreover, a microsphere immunoassay was also developed as a confirmatory test [202].

Due to the increased sensitivity of diagnostic tools (especially RT-qPCR), vaccine virus detections are quite common in oral vaccination campaigns of wild boar and vaccination programs of domestic pigs. For this reason, different RT-qPCR systems have been developed and tested, these allow differentiation between vaccine and field viruses (genetic DIVA) [203–208].

Sampling can be the bottleneck of swine fever diagnosis, especially in the case of wild boar, but also in remote areas. For this reason, alternative sampling strategies and sample matrices have been tested for CSF (often combined with African swine fever sampling) especially for wildlife specimens and under rural conditions [209–212]. However, most of them are not yet in routine use and need further validation.

7. Vaccination

Highly efficacious and safe live-attenuated CSF vaccines have existed for decades [160]. The underlying virus strains (e.g., the C-strain of CSFV, the Lapinized Philippines Coronel, the Thiverval or the Japanese guinea-pig exaltation negative GPE strain) were attenuated through serial passages in animals (rabbits) or cell culture. These vaccines have been implemented in mandatory control programs that led, together with strict hygiene measures, to the eradication of CSF from several regions of the world [213]. At this time, they are still in use in several Asian countries including China [84], countries of South and Central America, Trans-Caucasian Countries, and Eastern Europe (see Table 1). In 2016, 22 countries officially reported mandatory vaccination campaigns (OIE WAHIS [214]).

Table 1. CSF vaccination: Countries that reported official vaccination campaigns through World Organization for Animal Health (OIE) in 2016 (their last reported outbreaks are presented in brackets; no reports for some countries since 2005) (WAHIS Interface [214]).

Country	Last reported CSF outbreak
Albania	no reports
Armenia	2006
Azerbaijan	no reports
Belarus	no reports
Bosnia and Herzegovina	2007
Bulgaria (wb)	2009 wb
China	2015
Colombia	2016
Cuba	2016
Dominican Republic	2016
Ecuador	2016
Macedonia	2008
Georgia	no reports
Hong Kong	2005
Madagascar	2016
Moldova	(no reports)
Mongolia	2016
Myanmar	2015

Table 1. *Cont.*

Peru	2016
Philippines	2016
Russia	2016
Ukraine	2015

Wb: Wild boar

In addition, these vaccines were also adapted to a bait format for oral immunization of wild boar [6,215,216] and were recently explored for the vaccination of domestic pigs under backyard conditions [217–219]. While these vaccines usually have outstanding virtues in terms of onset, spectrum and duration of immunity [158,220–223], the main drawback is the lack of a serological marker concept [160] that would allow differentiation of field virus infected from vaccinated animals (DIVA concept). This is usually less important in endemically affected countries where prophylactic vaccination is carried out to reduce the disease burden and to ensure product safety. In general, there are also no legal obligations to use a certain type of vaccine for an emergency vaccination scenario. However, due to the trade restrictions that are imposed on pigs vaccinated with conventional live attenuated vaccines, only DIVA vaccines are considered a feasible option for domestic pigs [224]. Up to very recently, only E2 subunit marker (DIVA) vaccines were available on the market (at present, one E2 marker vaccine is commercially available, Porcilis® Pesti, MSD Animal Health, Unterschleißheim, Germany). These vaccines are safe and were shown to provide clinical protection and limit the spread of CSF [225–235]. However, they show drawbacks especially in terms of early protection [160,236] and protection against transplacental transmission [237]. Due to these problems, emergency vaccination was hardly implemented in domestic pigs (one exception being Romania). Several research groups have therefore sought to develop a next-generation marker vaccine candidate that would ideally answer all demands with regard to safety, efficacy, DIVA potential, and marketability [238]. Among the concepts that have been investigated are different vector vaccines based on vaccinia virus, pseudorabies virus or adenoviruses. Other vaccine designs include recombinant attenuated vaccines with chimeric constructs, subunit vaccines based on different expression systems, and RNA/DNA vaccines (recently reviewed by Blome et al., [239]. In 2014, the European Medicines Agency (EMA) licensed one of the chimeric marker vaccine candidates, "CP7_E2alf", after extensive testing in the framework of an EU-funded research project [159,240–257]. This new marker vaccine is still under investigation and could be a powerful tool for both emergency vaccination of domestic pigs and also wild boar.

Oral emergency vaccination of wild boar with baits has proven to be a potent tool to control the disease in wildlife and to safeguard domestic pigs [3]. For this purpose, the above-mentioned C-strain formulations have been used in several European countries including Germany and France. To further optimize the strategy, a DIVA vaccine such as "CP7_E2alf" could be used. The latter was already tested for use in wild boar under both laboratory and field conditions and could be a medium term option [241,246,251].

Conflicts of Interest: The authors were involved in the design and testing of some of the vaccines and received third party funds to carry out the studies (industry funding and EU framework programs FP6 and FP7 under grant agreement numbers 227003 CP-FP and SSPE-CT-2003-501559). No other conflicts of interest exist.

Reference

1. Edwards, S.; Fukusho, A.; Lefevre, P.C.; Lipowski, A.; Pejsak, Z.; Roehe, P.; Westergaard, J. Classical swine fever: The global situation. *Vet. Microbiol.* **2000**, *73*, 103–119.
2. Fritzemeier, J.; Teuffert, J.; Greiser-Wilke, I.; Staubach, C.; Schlüter, H.; Moennig, V. Epidemiology of classical swine fever in germany in the 1990s. *Vet. Microbiol.* **2000**, *77*, 29–41.
3. Rossi, S.; Staubach, C.; Blome, S.; Guberti, V.; Thulke, H.H.; Vos, A.; Koenen, F.; Le Potier, M.F. Controlling of csfv in european wild boar using oral vaccination: A review. *Front. Microbiol.* **2015**, *6*, 1141.

4. Rossi, S.; Pol, F.; Forot, B.; Masse-Provin, N.; Rigaux, S.; Bronner, A.; Le Potier, M.F. Preventive vaccination contributes to control classical swine fever in wild boar (*Sus scrofa* sp.). *Vet. Microbiol.* **2010**, *142*, 99–107.
5. von Rüden, S.; Staubach, C.; Kaden, V.; Hess, R.G.; Blicke, J.; Kühne, S.; Sonnenburg, J.; Fröhlich, A.; Teuffert, J.; Moennig, V. Retrospective analysis of the oral immunisation of wild boar populations against classical swine fever virus (csfv) in region Eifel of Rhineland-Palatinate. *Vet. Microbiol.* **2008**, *132*, 29–38.
6. Kaden, V.; Heyne, H.; Kiupel, H.; Letz, W.; Kern, B.; Lemmer, U.; Gossger, K.; Rothe, A.; Böhme, H.; Tyrpe, P. Oral immunisation of wild boar against classical swine fever: Concluding analysis of the recent field trials in Germany. *Berl. Munch. Tierarztl. Wochenschr.* **2002**, *115*, 179–185.
7. Blome, S.; Gabriel, C.; Staubach, C.; Leifer, I.; Strebelow, G.; Beer, M. Genetic differentiation of infected from vaccinated animals after implementation of an emergency vaccination strategy against classical swine fever in wild boar. *Vet. Microbiol.* **2011**, *153*, 373–376.
8. Hulst, M.M.; van Gennip, H.G.; Moormann, R.J. Passage of classical swine fever virus in cultured swine kidney cells selects virus variants that bind to heparan sulfate due to a single amino acid change in envelope protein E^{rns}. *J. Virol.* **2000**, *74*, 9553–9561.
9. Weiland, E.; Ahl, R.; Stark, R.; Weiland, F.; Thiel, H.J. A second envelope glycoprotein mediates neutralization of a pestivirus, hog cholera virus. *J. Virol.* **1992**, *66*, 3677–3682.
10. Iqbal, M.; Flick-Smith, H.; McCauley, J.W. Interactions of bovine viral diarrhoea virus glycoprotein E^{rns} with cell surface glycosaminoglycans. *J. Gen. Virol.* **2000**, *81*, 451–459.
11. Hulst, M.M.; van Gennip, H.G.; Vlot, A.C.; Schooten, E.; de Smit, A.J.; Moormann, R.J. Interaction of classical swine fever virus with membrane-associated heparan sulfate: Role for virus replication in vivo and virulence. *J. Virol.* **2001**, *75*, 9585–9595.
12. Wang, Z.; Nie, Y.; Wang, P.; Ding, M.; Deng, H. Characterization of classical swine fever virus entry by using pseudotyped viruses: E1 and E2 are sufficient to mediate viral entry. *Virology* **2004**, *330*, 332–341.
13. Donis, R.O. Molecular biology of bovine viral diarrhea virus and its interactions with the host. *Vet. Clin. N. Am. Food Anim. Pract.* **1995**, *11*, 393–423.
14. Krey, T.; Thiel, H.J.; Rümenapf, T. Acid-resistant bovine pestivirus requires activation for ph-triggered fusion during entry. *J. Virol.* **2005**, *79*, 4191–4200.
15. Lecot, S.; Belouzard, S.; Dubuisson, J.; Rouille, Y. Bovine viral diarrhea virus entry is dependent on clathrin-mediated endocytosis. *J. Virol.* **2005**, *79*, 10826–10829.
16. Rijnbrand, R.; van der Straaten, T.; van Rijn, P.A.; Spaan, W.J.; Bredenbeek, P.J. Internal entry of ribosomes is directed by the 5' noncoding region of classical swine fever virus and is dependent on the presence of an RNA pseudoknot upstream of the initiation codon. *J. Virol.* **1997**, *71*, 451–457.
17. Pestova, T.V.; Hellen, C.U. Internal initiation of translation of bovine viral diarrhea virus RNA. *Virology* **1999**, *258*, 249–256.
18. Poole, T.L.; Wang, C.; Popp, R.A.; Potgieter, L.N.; Siddiqui, A.; Collett, M.S. Pestivirus translation initiation occurs by internal ribosome entry. *Virology* **1995**, *206*, 750–754.
19. Rümenapf, T.; Unger, G.; Strauss, J.H.; Thiel, H.J. Processing of the envelope glycoproteins of pestiviruses. *J. Virol.* **1993**, *67*, 3288–3294.
20. Stark, R.; Meyers, G.; Rümenapf, T.; Thiel, H.J. Processing of pestivirus polyprotein: Cleavage site between autoprotease and nucleocapsid protein of classical swine fever virus. *J. Virol.* **1993**, *67*, 7088–7095.
21. Wiskerchen, M.; Belzer, S.K.; Collett, M.S. Pestivirus gene expression: The first protein product of the bovine viral diarrhea virus large open reading frame, p20, possesses proteolytic activity. *J. Virol.* **1991**, *65*, 4508–4514.
22. Lackner, T.; Müller, A.; Pankraz, A.; Becher, P.; Thiel, H.J.; Gorbalenya, A.E.; Tautz, N. Temporal modulation of an autoprotease is crucial for replication and pathogenicity of an rna virus. *J. Virol.* **2004**, *78*, 10765–10775.
23. Lackner, T.; Thiel, H.J.; Tautz, N. Dissection of a viral autoprotease elucidates a function of a cellular chaperone in proteolysis. *Proc. Natl. Acad. Sci. USA* **2006**, *103*, 1510–1515.
24. Tautz, N.; Elbers, K.; Stoll, D.; Meyers, G.; Thiel, H.J. Serine protease of pestiviruses: Determination of cleavage sites. *J. Virol.* **1997**, *71*, 5415–5422.
25. Gong, Y.; Trowbridge, R.; Macnaughton, T.B.; Westaway, E.G.; Shannon, A.D.; Gowans, E.J. Characterization of RNA synthesis during a one-step growth curve and of the replication mechanism of bovine viral diarrhoea virus. *J. Gen. Virol.* **1996**, *77*, 2729–2736.

26. Gray, E.W.; Nettleton, P.F. The ultrastructure of cell cultures infected with border disease and bovine virus diarrhoea viruses. *J. Gen. Virol.* **1987**, *68*, 2339–2346.
27. Ohmann, H.B. Electron microscopy of bovine virus diarrhoea virus. *Rev. Sci. Tech.* **1990**, *9*, 61–73.
28. Scientific report submitted to EFSA prepared by Kramer, M., Staubach, C., Koenen, F., Haegeman, A., Pol, F., Le Potier, M.F. Greiser-Wilke, I. *On Classical Swine Fever* **2009,** pp 1–92.
29. Wijnker, J.J.; Depner, K.R.; Berends, B.R. Inactivation of classical swine fever virus in porcine casing preserved in salt. *Int. J. Food Microbiol.* **2008**, *128*, 411–413.
30. Farez, S.; Morley, R.S. Potential animal health hazards of pork and pork products. *Rev. Sci. Tech.* **1997**, *16*, 65–78.
31. Edwards, S. Survival and inactivation of classical swine fever virus. *Vet. Microbiol.* **2000**, *73*, 175–181.
32. Weesendorp, E.; Stegeman, A.; Loeffen, W.L. Survival of classical swine fever virus at various temperatures in faeces and urine derived from experimentally infected pigs. *Vet. Microbiol.* **2008**, *132*, 249–259.
33. Haas, B.; Ahl, R.; Böhm, R.; Strauch, D. Inactivation of viruses in liquid manure. *Rev. Sci. Tech.* **1995**, *14*, 435–445.
34. Botner, A.; Belsham, G.J. Virus survival in slurry: Analysis of the stability of foot-and-mouth disease, classical swine fever, bovine viral diarrhoea and swine influenza viruses. *Vet. Microbiol.* **2012**, *157*, 41–49.
35. Turner, C.; Williams, S.M.; Cumby, T.R. The inactivation of foot and mouth disease, aujeszky's disease and classical swine fever viruses in pig slurry. *J. Appl. Microbiol.* **2000**, *89*, 760–767.
36. Gale, P. Risks to farm animals from pathogens in composted catering waste containing meat. *Vet. Rec.* **2004**, *155*, 77–82.
37. Artois, M.; Depner, K.R.; Guberti, V.; Hars, J.; Rossi, S.; Rutili, D. Classical swine fever (hog cholera) in wild boar in Europe. *Rev. Sci. Tech.* **2002**, *21*, 287–303.
38. Harkness, J.W. Classical swine fever and its diagnosis: A current view. *Vet. Rec.* **1985**, *116*, 288–293.
39. Tessler, J.; Stewart, W.C.; Kresse, J.I. Stabilization of hog cholera virus by dimethyl sulfoxide. *Can. J. Comp. Med.* **1975**, *39*, 472–473.
40. Depner, K.; Bauer, T.; Liess, B. Thermal and pH stability of pestiviruses. *Rev Sci Tech* **1992**, *11*, 885–893.
41. Panina, G.F.; Civardi, A.; Cordioli, P.; Massirio, I.; Scatozza, F.; Baldini, P.; Palmia, F. Survival of hog cholera virus (HCV) in sausage meat products (italian salami). *Int. J. Food Microbiol.* **1992**, *17*, 19–25.
42. Mebus, C.; House, C.; Gonzalvo, F.R.; Pineda, J.; Tapiador, J.; Pire, J.; Bergada, J.; Yedloutschnig, R.; Sahu, S.; Becerra, V. Survival of foot-and-mouth disease, african swine fever, and hog cholera viruses in spanish serrano cured hams and Iberian cured hams, shoulders and loins. *Food Microbiol.* **1993**, *10*, 133–143.
43. Postel, A.; Schmeiser, S.; Perera, C.L.; Rodriguez, L.J.; Frias-Lepoureau, M.T.; Becher, P. Classical swine fever virus isolates from Cuba form a new subgenotype 1.4. *Vet. Microbiol.* **2013**, *161*, 334–338.
44. Cha, S.H.; Choi, E.J.; Park, J.H.; Yoon, S.R.; Kwon, J.H.; Yoon, K.J.; Song, J.Y. Phylogenetic characterization of classical swine fever viruses isolated in Korea between 1988 and 2003. *Virus Res.* **2007**, *126*, 256–261.
45. Blome, S.; Grotha, I.; Moennig, V.; Greiser-Wilke, I. Classical swine fever virus in South-Eastern Europe—Retrospective analysis of the disease situation and molecular epidemiology. *Vet. Microbiol.* **2010**, *146*, 276–284.
46. Chen, N.; Hu, H.; Zhang, Z.; Shuai, J.; Jiang, L.; Fang, W. Genetic diversity of the envelope glycoprotein E2 of classical swine fever virus: Recent isolates branched away from historical and vaccine strains. *Vet. Microbiol.* **2008**, *127*, 286–299.
47. Jiang, D.L.; Liu, G.H.; Gong, W.J.; Li, R.C.; Hu, Y.F.; Tu, C.; Yu, X.L. Complete genome sequences of classical swine fever virus isolates belonging to a new subgenotype, 2.1c, from Hunan province, China. *Genome Announc.* **2013**, *28*, e00080.
48. Vanderhallen, H.; Mittelholzer, C.; Hofmann, M.A.; Koenen, F. Classical swine fever virus is genetically stable in vitro and in vivo. *Arch. Virol.* **1999**, *144*, 1669–1677.
49. Greiser-Wilke, I.; Dreier, S.; Haas, L.; Zimmermann, B. Genetic typing of classical swine fever viruses—A review. *Dtsch. Tierarztl. Wochenschr.* **2006**, *113*, 134–138.
50. Paton, D.J.; McGoldrick, A.; Greiser-Wilke, I.; Parchariyanon, S.; Song, J.Y.; Liou, P.P.; Stadejek, T.; Lowings, J.P.; Bjorklund, H.; Belak, S. Genetic typing of classical swine fever virus. *Vet. Microbiol.* **2000**, *73*, 137–157.
51. Postel, A.; Schmeiser, S.; Bernau, J.; Meindl-Boehmer, A.; Pridotkas, G.; Dirbakova, Z.; Mojzis, M.; Becher, P. Improved strategy for phylogenetic analysis of classical swine fever virus based on full-length E2 encoding sequences. *Vet. Res.* **2012**, *43*, 50.

52. Töpfer, A.; Höper, D.; Blome, S.; Beer, M.; Beerenwinkel, N.; Ruggli, N.; Leifer, I. Sequencing approach to analyze the role of quasispecies for classical swine fever. *Virology* **2013**, *438*, 14–19.
53. Leifer, I.; Hoeper, D.; Blome, S.; Beer, M.; Ruggli, N. Clustering of classical swine fever virus isolates by codon pair bias. *BMC Research Notes* **2011**, *4*, 521.
54. Goller, K.V.; Gabriel, C.; Dimna, M.L.; Potier, M.F.; Rossi, S.; Staubach, C.; Merboth, M.; Beer, M.; Blome, S. Evolution and molecular epidemiology of classical swine fever virus during a multi-annual outbreak amongst european wild boar. *J. Gen. Virol.* **2016**, *97*, 639–645.
55. Fahnoe, U.; Pedersen, A.G.; Risager, P.C.; Nielsen, J.; Belsham, G.J.; Höper, D.; Beer, M.; Rasmussen, T.B. Rescue of the highly virulent classical swine fever virus strain "Koslov" from cloned cDNA and first insights into genome variations relevant for virulence. *Virology* **2014**, *468–470*, 379–387.
56. Björklund, H.; Lowings, P.; Stadejek, T.; Vilcek, S.; Greiser-Wilke, I.; Paton, D.; Belak, S. Phylogenetic comparison and molecular epidemiology of classical swine fever virus. *Virus Genes* **1999**, *19*, 189–195.
57. Greiser-Wilke, I.; Fritzemeier, J.; Koenen, F.; Vanderhallen, H.; Rutili, D.; De Mia, G.M.; Romero, L.; Rosell, R.; Sanchez-Vizcaino, J.M.; San Gabriel, A. Molecular epidemiology of a large classical swine fever epidemic in the European Union in 1997–1998. *Vet. Microbiol.* **2000**, *77*, 17–27.
58. Jemersic, L.; Greiser-Wilke, I.; Barlic-Maganja, D.; Lojkic, M.; Madic, J.; Terzic, S.; Grom, J. Genetic typing of recent classical swine fever virus isolates from Croatia. *Vet. Microbiol.* **2003**, *96*, 25–33.
59. Bartak, P.; Greiser-Wilke, I. Genetic typing of classical swine fever virus isolates from the territory of the Czech Republic. *Vet. Microbiol.* **2000**, *77*, 59–70.
60. Wonnemann, H.; Floegel-Niesmann, G.; Moennig, V.; Greiser-Wilke, I. Genetic typing of German isolates of classical swine fever virus. *Dtsch. Tierarztl. Wochenschr.* **2001**, *108*, 252–256.
61. Biagetti, M.; Greiser-Wilke, I.; Rutili, D. Molecular epidemiology of classical swine fever in Italy. *Vet. Microbiol.* **2001**, *83*, 205–215.
62. Pol, F.; Rossi, S.; Mesplede, A.; Kuntz-Simon, G.; Le Potier, M.F. Two outbreaks of classical swine fever in wild boar in France. *Vet. Rec.* **2008**, *162*, 811–816.
63. Leifer, I.; Hoffmann, B.; Höper, D.; Bruun Rasmussen, T.; Blome, S.; Strebelow, G.; Höreth-Böntgen, D.; Staubach, C.; Beer, M. Molecular epidemiology of current classical swine fever virus isolates of wild boar in germany. *J. Gen. Virol.* **2010**, *91*, 2687–2697.
64. Simon, G.; Le Dimna, M.; Le Potier, M.F.; Pol, F. Molecular tracing of classical swine fever viruses isolated from wild boars and pigs in France from 2002 to 2011. *Vet. Microbiol.* **2013**, *166*, 631–638.
65. Moennig, V.; Floegel-Niesmann, G.; Greiser-Wilke, I. Clinical signs and epidemiology of classical swine fever: A review of new knowledge. *Vet. J.* **2003**, *165*, 11–20.
66. Beer, M.; Goller, K.V.; Staubach, C.; Blome, S. Genetic variability and distribution of classical swine fever virus. *Anim. Health Res. Rev.* **2015**, *16*, 33–39.
67. Pereda, A.J.; Greiser-Wilke, I.; Schmitt, B.; Rincon, M.A.; Mogollon, J.D.; Sabogal, Z.Y.; Lora, A.M.; Sanguinetti, H.; Piccone, M.E. Phylogenetic analysis of classical swine fever virus (CSFV) field isolates from outbreaks in south and central America. *Virus. Res.* **2005**, *110*, 111–118.
68. Diaz de Arce, H.; Nunez, J.I.; Ganges, L.; Barreras, M.; Teresa Frias, M.; Sobrino, F. Molecular epidemiology of classical swine fever in Cuba. *Virus. Res.* **1999**, *64*, 61–67.
69. de Arce, H.D.; Ganges, L.; Barrera, M.; Naranjo, D.; Sobrino, F.; Frias, M.T.; Nunez, J.I. Origin and evolution of viruses causing classical swine fever in Cuba. *Virus. Res.* **2005**, *112*, 123–131.
70. Sandvik, T.; Crooke, H.; Drew, T.W.; Blome, S.; Greiser-Wilke, I.; Moennig, V.; Gous, T.A.; Gers, S.; Kitching, J.A.; Buhrmann, G.; et al. Classical swine fever in South Africa after 87 years' absence. *Vet. Rec.* **2005**, *157*, 267.
71. David, D.; Edri, N.; Yakobson, B.A.; Bombarov, V.; King, R.; Davidson, I.; Pozzi, P.; Hadani, Y.; Bellaiche, M.; Schmeiser, S.; et al. Emergence of classical swine fever virus in Israel in 2009. *Vet. J.* **2011**, *190*, e146–e149.
72. Barman, N.N.; Bora, D.P.; Khatoon, E.; Mandal, S.; Rakshit, A.; Rajbongshi, G.; Depner, K.; Chakraborty, A.; Kumar, S. Classical swine fever in wild hog: Report of its prevalence in northeast India. *Transbound. Emerg. Dis.* **2014**, *63*, 540–547.
73. Roychoudhury, P.; Sarma, D.K.; Rajkhowa, S.; Munir, M.; Kuchipudi, S.V. Predominance of genotype 1.1 and emergence of genotype 2.2 classical swine fever viruses in north-eastern region of India. *Transbound. Emerg. Dis.* **2014**, *61, Suppl. 1*, 69–77.

74. Patil, S.S.; Hemadri, D.; Shankar, B.P.; Raghavendra, A.G.; Veeresh, H.; Sindhoora, B.; Chandan, S.; Sreekala, K.; Gajendragad, M.R.; Prabhudas, K. Genetic typing of recent classical swine fever isolates from India. *Vet. Microbiol.* **2010**, *141*, 367–373.
75. Patil, S.S.; Hemadri, D.; Veeresh, H.; Sreekala, K.; Gajendragad, M.R.; Prabhudas, K. Phylogenetic analysis of NS5B gene of classical swine fever virus isolates indicates plausible Chinese origin of Indian subgroup 2.2 viruses. *Virus Genes* **2012,** *44*, 104–108.
76. Rajkhowa, T.K.; Hauhnar, L.; Lalrohlua, I.; Mohanarao, G.J. Emergence of 2.1. Subgenotype of classical swine fever virus in pig population of India in 2011. *Vet. Q.* **2014**, *34*, 224–228.
77. Desai, G.S.; Sharma, A.; Kataria, R.S.; Barman, N.N.; Tiwari, A.K. 5' UTR-based phylogenetic analysis of classical swine fever virus isolates from India. *Acta Virol.* **2010**, *54*, 79–82.
78. Sarma, D.K.; Mishra, N.; Vilcek, S.; Rajukumar, K.; Behera, S.P.; Nema, R.K.; Dubey, P.; Dubey, S.C. Phylogenetic analysis of recent classical swine fever virus (CSFV) isolates from Assam, India. *Comp. Immunol. Microbiol. Infect. Dis.* **2011**, *34*, 11–15.
79. Nandi, S.; Muthuchelvan, D.; Ahuja, A.; Bisht, S.; Chander, V.; Pandey, A.B.; Singh, R.K. Prevalence of classical swine fever virus in India: A 6-year study (2004–2010). *Transbound. Emerg. Dis.* **2011**, *58*, 461–463.
80. Postel, A.; Jha, V.C.; Schmeiser, S.; Becher, P. First molecular identification and characterization of classical swine fever virus isolates from Nepal. *Arch. Virol.* **2013**, *158*, 207–210.
81. An, T.Q.; Peng, J.M.; Tian, Z.J.; Zhao, H.Y.; Li, N.; Liu, Y.M.; Chen, J.Z.; Leng, C.L.; Sun, Y.; Chang, D.; et al. Pseudorabies virus variant in Bartha-K61-vaccinated pigs, China, 2012. *Emerg. Infect. Dis.* **2013**, *19*, 1749–1755.
82. Afshar, A.; Dulac, G.C.; Dubuc, C.; Howard, T.H. Comparative evaluation of the fluorescent antibody test and microtiter immunoperoxidase assay for detection of bovine viral diarrhea virus from bull semen. *Can. J. Vet. Res.* **1991**, *55*, 91–93.
83. Luo, T.R.; Liao, S.H.; Wu, X.S.; Feng, L.; Yuan, Z.X.; Li, H.; Liang, J.J.; Meng, X.M.; Zhang, H.Y. Phylogenetic analysis of the *E2* gene of classical swine fever virus from the Guangxi province of southern China. *Virus Genes* **2011**, *42*, 347–354.
84. Luo, Y.; Li, S.; Sun, Y.; Qiu, H.J. Classical swine fever in China: A minireview. *Vet. Microbiol.* **2014**, *172*, 1–6.
85. Pan, C.H.; Jong, M.H.; Huang, T.S.; Liu, H.F.; Lin, S.Y.; Lai, S.S. Phylogenetic analysis of classical swine fever virus in Taiwan. *Arch. Virol.* **2005**, *150*, 1101–1119.
86. Deng, M.C.; Huang, C.C.; Huang, T.S.; Chang, C.Y.; Lin, Y.J.; Chien, M.S.; Jong, M.H. Phylogenetic analysis of classical swine fever virus isolated from Taiwan. *Vet. Microbiol.* **2005**, *106*, 187–1893.
87. Lin, Y.J.; Chien, M.S.; Deng, M.C.; Huang, C.C. Complete sequence of a subgroup 3.4 strain of classical swine fever virus from Taiwan. *Virus Genes* **2007**, *35*, 737–744.
88. Blacksell, S.D.; Khounsy, S.; Boyle, D.B.; Gleeson, L.J.; Westbury, H.A.; Mackenzie, J.S. Genetic typing of classical swine fever viruses from Lao PDR by analysis of the 5' non-coding region. *Virus Genes* **2005**, *31*, 349–355.
89. Blacksell, S.D.; Khounsy, S.; Boyle, D.B.; Greiser-Wilke, I.; Gleeson, L.J.; Westbury, H.A.; Mackenzie, J.S. Phylogenetic analysis of the *E2* gene of classical swine fever viruses from Lao PDR. *Virus Res.* **2004**, *104*, 87–92.
90. Sakoda, Y.; Ozawa, S.; Damrongwatanapokin, S.; Sato, M.; Ishikawa, K.; Fukusho, A. Genetic heterogeneity of porcine and ruminant pestiviruses mainly isolated in Japan. *Vet. Microbiol.* **1999**, *65*, 75–86.
91. Coronado, L.; Liniger, M.; Munoz-Gonzalez, S.; Postel, A.; Perez, L.J.; Perez-Simo, M.; Perera, C.L.; Frias-Lepoureau, M.T.; Rosell, R.; Grundhoff, A.; et al. Novel poly-uridine insertion in the 3' UTR and E2 amino acid substitutions in a low virulent classical swine fever virus. *Vet. Microbiol.* **2017**, *201*, 103–112.
92. Lange, M.; Kramer-Schadt, S.; Blome, S.; Beer, M.; Thulke, H.H. Disease severity declines over time after a wild boar population has been affected by classical swine fever--legend or actual epidemiological process? *Prev Vet. Med.* **2012**, *106*, 185–195.
93. Dreier, S.; Zimmermann, B.; Moennig, V.; Greiser-Wilke, I. A sequence database allowing automated genotyping of classical swine fever virus isolates. *J. Virol. Methods* **2007**, *140*, 95–99.
94. Greiser-Wilke, I.; Zimmermann, B.; Fritzemeier, J.; Floegel, G.; Moennig, V. Structure and presentation of a world wide web database of CSF virus isolates held at the EU reference laboratory. *Vet. Microbiol.* **2000**, *73*, 131–136.

95. Postel, A.; Schmeiser, S.; Zimmermann, B.; Becher, P. The European classical swine fever virus database: Blueprint for a pathogen-specific sequence database with integrated sequence analysis tools. *Viruses* **2016**, *8*, 302.
96. Mayer, D.; Hofmann, M.A.; Tratschin, J.D. Attenuation of classical swine fever virus by deletion of the viral N^{pro} gene. *Vaccine* **2004**, *22*, 317–328.
97. Risatti, G.R.; Borca, M.V.; Kutish, G.F.; Lu, Z.; Holinka, L.G.; French, R.A.; Tulman, E.R.; Rock, D.L. The E2 glycoprotein of classical swine fever virus is a virulence determinant in swine. *J. Virol.* **2005**, *79*, 3787–3796.
98. Tews, B.A.; Schurmann, E.M.; Meyers, G. Mutation of cysteine 171 of pestivirus E^{rns} RNase prevents homodimer formation and leads to attenuation of classical swine fever virus. *J. Virol.* **2009**, *83*, 4823–4834.
99. Meyers, G.; Saalmüller, A.; Büttner, M. Mutations abrogating the RNAse activity in glycoprotein E^{rns} of the pestivirus classical swine fever virus lead to virus attenuation. *J. Virol.* **1999**, *73*, 10224–10235.
100. Tamura, T.; Sakoda, Y.; Yoshino, F.; Nomura, T.; Yamamoto, N.; Sato, Y.; Okamatsu, M.; Ruggli, N.; Kida, H. Selection of classical swine fever virus with enhanced pathogenicity reveals synergistic virulence determinants in E2 and NS4B. *J. Virol.* **2012**, *86*, 8602–8613.
101. Risatti, G.R.; Holinka, L.G.; Fernandez Sainz, I.; Carrillo, C.; Kutish, G.F.; Lu, Z.; Zhu, J.; Rock, D.L.; Borca, M.V. Mutations in the carboxyl terminal region of E2 glycoprotein of classical swine fever virus are responsible for viral attenuation in swine. *Virology* **2007**, *364*, 371–382.
102. Risatti, G.R.; Holinka, L.G.; Fernandez Sainz, I.; Carrillo, C.; Lu, Z.; Borca, M.V. *N*-linked glycosylation status of classical swine fever virus strain brescia E2 glycoprotein influences virulence in swine. *J. Virol.* **2007**, *81*, 924–933.
103. Sainz, I.F.; Holinka, L.G.; Lu, Z.; Risatti, G.R.; Borca, M.V. Removal of a *N*-linked glycosylation site of classical swine fever virus strain Brescia E^{rns} glycoprotein affects virulence in swine. *Virology* **2008**, *370*, 122–129.
104. Tang, F.; Pan, Z.; Zhang, C. The selection pressure analysis of classical swine fever virus envelope protein genes E^{rns} and E2. *Virus Res.* **2008**, *131*, 132–135.
105. Wu, Z.; Wang, Q.; Feng, Q.; Liu, Y.; Teng, J.; Yu, A.C.; Chen, J. Correlation of the virulence of CSFV with evolutionary patterns of E2 glycoprotein. *Front. Biosci. (Elite Ed.)* **2010**, *2*, 204–220.
106. Ishikawa, K.; Nagai, H.; Katayama, K.; Tsutsui, M.; Tanabayashi, K.; Takeuchi, K.; Hishiyama, M.; Saitoh, A.; Takagi, M.; Gotoh, K., *et al.* Comparison of the entire nucleotide and deduced amino acid sequences of the attenuated hog cholera vaccine strain GPE- and the wild-type parental strain ALD. *Arch. Virol.* **1995**, *140*, 1385–1391.
107. Jenckel, M.; Blome, S.; Beer, M.; Höper, D. Quasispecies composition and diversity do not reveal any predictors for chronic classical swine fever virus infection. *Arch. Virol.* **2017**, *162*, 775–786.
108. Petrov, A.; Blohm, U.; Beer, M.; Pietschmann, J.; Blome, S. Comparative analyses of host responses upon infection with moderately virulent classical swine fever virus in domestic pigs and wild boar. *Virol. J.* **2014**, *11*, 134.
109. Bunzenthal, C. Determination of the virulence of classical swine fever virus isolates [Bestimmung der Virulenz von Virusisolaten der Klassischen Schweinepest]. PhD thesis, University of Veterinary Medicine Hannover, Foundation, Hannover, 2003.
110. Floegel-Niesmann, G.; Blome, S.; Gerss-Dülmer, H.; Bunzenthal, C.; Moennig, V. Virulence of classical swine fever virus isolates from Europe and other areas during 1996 until 2007. *Vet. Microbiol.* **2009**, *139*, 165–169.
111. Floegel-Niesmann, G.; Bunzenthal, C.; Fischer, S.; Moennig, V. Virulence of recent and former classical swine fever virus isolates evaluated by their clinical and pathological signs. *J. Vet. Med. B Infect. Dis. Vet. Public Health* **2003**, *50*, 214–220.
112. Tarradas, J.; de la Torre, M.E.; Rosell, R.; Perez, L.J.; Pujols, J.; Munoz, M.; Munoz, I.; Munoz, S.; Abad, X.; Domingo, M., et al. The impact of CSFV on the immune response to control infection. *Virus Res.* **2014**, *185*, 82–91.
113. Depner, K.R.; Müller, A.; Gruber, A.; Rodriguez, A.; Bickhardt, K.; Liess, B. Classical swine fever in wild boar (*Sus scrofa*)—Experimental infections and viral persistence. *Dtsch. Tierarztl. Wochenschr.* **1995**, *102*, 381–384.
114. Kaden, V.; Steyer, H.; Schnabel, J.; Bruer, W. Classical swine fever (CSF) in wild boar: The role of the transplacental infection in the perpetuation of CSF. *J. Vet. Med. B Infect. Dis. Vet. Public Health* **2005**, *52*, 161–164.

115. Kern, B.; Depner, K.R.; Letz, W.; Rott, M.; Thalheim, S.; Nitschke, B.; Plagemann, R.; Liess, B. Incidence of classical swine fever (CSF) in wild boar in a densely populated area indicating CSF virus persistence as a mechanism for virus perpetuation. *Zentralbl. Veterinarmed. B* **1999**, *46*, 63–67.
116. von Benten, K.; Trautwein, G.; Richter-Reichhelm, H.B.; Liess, B.; Frey, H.R. Experimental transplacental transmission of hog cholera virus in pigs. III. Histopathological findings in the fetus. *Zentralbl. Veterinarmed. B* **1980**, *27*, 714–724.
117. Stewart, W.C.; Carbrey, E.A.; Kresse, J.I. Transplacental hog cholera infection in susceptible sows. *Am. J. Vet. Res.* **1973**, *34*, 637–640.
118. Stewart, W.C.; Carbrey, E.A.; Kresse, J.I. Transplacental hog cholera infection in immune sows. *Am. J. Vet. Res.* **1972**, *33*, 791–798.
119. Richter-Reichhelm, H.B.; Trautwein, G.; von Benten, K.; Liess, B.; Frey, H.R. Experimental transplacental transmission of hog cholera virus in pigs. II. Immunopathological findings in the fetus. *Zentralbl. Veterinarmed. B* **1980**, *27*, 243–252.
120. Overby, E.; Eskildsen, M. *Transplacental Infection in Susceptible Gilts after Inoculation with: I. Lapinized Swine Fever Vaccine, II. Bovine Viral Diarrhoea Virus Strains*; Commission of the European Communities, DG Scientific and Technical Information and Information Management: Luxembourg, 1977; Vol. EUR 5904.
121. Meyer, H.; Liess, B.; Frey, H.R.; Hermanns, W.; Trautwein, G. Experimental transplacental transmission of hog cholera virus in pigs. IV. Virological and serological studies in newborn piglets. *Zentralbl. Veterinarmed. B* **1981**, *28*, 659–668.
122. Hermanns, W.; Trautwein, G.; Meyer, H.; Liess, B. Experimental transplacental transmission of hog cholera virus in pigs. V. Immunopathological findings in newborn pigs. *Zentralbl. Veterinarmed. B* **1981**, *28*, 669–683.
123. Frey, H.R.; Liess, B.; Richter-Reichhelm, H.B.; von Benten, K.; Trautwein, G. Experimental transplacental transmission of hog cholera virus in pigs. I. Virological and serological studies. *Zentralbl. Veterinarmed. B* **1980**, *27*, 154–164.
124. Cabezon, O.; Colom-Cadena, A.; Munoz-Gonzalez, S.; Perez-Simo, M.; Bohorquez, J.A.; Rosell, R.; Marco, I.; Domingo, M.; Lavin, S.; Ganges, L. Post-natal persistent infection with classical swine fever virus in wild boar: A strategy for viral maintenance? *Transbound. Emerg. Dis.* **2017**, *64*, 651–655.
125. Munoz-Gonzalez, S.; Perez-Simo, M.; Munoz, M.; Bohorquez, J.A.; Rosell, R.; Summerfield, A.; Domingo, M.; Ruggli, N.; Ganges, L. Efficacy of a live attenuated vaccine in classical swine fever virus postnatally persistently infected pigs. *Vet. Res.* **2015**, *46*, 78.
126. Gomez-Villamandos, J.C.; Garcia de Leaniz, I.; Nunez, A.; Salguero, F.J.; Ruiz-Villamor, E.; Romero-Trevejo, J.L.; Sanchez-Cordon, P.J. Neuropathologic study of experimental classical swine fever. *Vet. Pathol.* **2006**, *43*, 530–540.
127. van Oirschot, J.T. *Classial swine fever (hog cholera)*. 8th ed.; Straw B.E., D.A., S., Mengelin, W.L. and Taylor, D. J., Ed. Iowa State University Press: Ames, IA, USA, 1999; pp. 159–172.
128. Depner, K.R.; Lange, E.; Pontrakulpipat, S.; Fichtner, D. Does porcine reproductive and respiratory syndrome virus potentiate classical swine fever virus infection in weaner pigs? *Zentralbl. Veterinarmed. B* **1999**, *46*, 485–491.
129. Kaden, V.; Ziegler, U.; Lange, E.; Dedek, J. Classical swine fever virus: Clinical, virological, serological and hematological findings after infection of domestic pigs and wild boars with the field isolate "Spante" originating from wild boar. *Berl. Munch. Tierarztl. Wochenschr.* **2000**, *113*, 412–416.
130. Gomez-Villamandos, J.C.; Carrasco, L.; Bautista, M.J.; Sierra, M.A.; Quezada, M.; Hervas, J.; Chacon Mde, L.; Ruiz-Villamor, E.; Salguero, F.J.; Sonchez-Cordon, P.J.; et al. African swine fever and classical swine fever: A review of the pathogenesis. *Dtsch. Tierarztl. Wochenschr.* **2003**, *110*, 165–169.
131. Lange, A.; Blome, S.; Moennig, V.; Greiser-Wilke, I. Pathogenesis of classical swine fever—Similarities to viral haemorrhagic fevers: A review. *Berl. Munch. Tierarztl. Wochenschr.* **2011**, *124*, 36–47.
132. Liess, B. Pathogenesis and epidemiology of hog cholera. *Ann Rech Vet.* **1987**, *18*, 139–145.
133. Dunne, H.W. *Hog Cholera*, 3rd ed.; Dunne, H.W., Ed.; The Iowa State University Press: Ames, IA, USA, 1970; Vol. Diseases of Swine, pp. 177–239.
134. Ressang, A.A. Studies on the pathogenesis of hog cholera. II. Virus distribution in tissue and the morphology of the immune response. *Zentralbl. Veterinarmed. B* **1973**, *20*, 272–288.
135. Ressang, A.A. Studies on the pathogenesis of hog cholera. I. Demonstration of hog cholera virus subsequent to oral exposure. *Zentralbl. Veterinarmed. B* **1973**, *20*, 256–271.

136. Bauhofer, O.; Summerfield, A.; McCullough, K.C.; Ruggli, N. Role of double-stranded RNA and N^{pro} of classical swine fever virus in the activation of monocyte-derived dendritic cells. *Virology* **2005**, *343*, 93–105.
137. Carrasco, C.P.; Rigden, R.C.; Vincent, I.E.; Balmelli, C.; Ceppi, M.; Bauhofer, O.; Tache, V.; Hjertner, B.; McNeilly, F.; van Gennip, H.G.; et al. Interaction of classical swine fever virus with dendritic cells. *J. Gen. Virol.* **2004**, *85*, 1633–1641.
138. Fiebach, A.R.; Guzylack-Piriou, L.; Python, S.; Summerfield, A.; Ruggli, N. Classical swine fever virus N^{pro} limits type I interferon induction in plasmacytoid dendritic cells by interacting with interferon regulatory factor 7. *J. Virol.* **2011**, *85*, 8002–8011.
139. Jamin, A.; Gorin, S.; Cariolet, R.; Le Potier, M.F.; Kuntz-Simon, G. Classical swine fever virus induces activation of plasmacytoid and conventional dendritic cells in tonsil, blood, and spleen of infected pigs. *Vet. Res.* **2008**, *39*, 7.
140. Summerfield, A.; Ruggli, N. Immune responses against classical swine fever virus: Between ignorance and lunacy. *Front. Vet. Sci.* **2015**, *2*, 10.
141. Summerfield, A.; Alves, M.; Ruggli, N.; de Bruin, M.G.; McCullough, K.C. High IFN-α responses associated with depletion of lymphocytes and natural IFN-producing cells during classical swine fever. *J. Interferon Cytokine Res.* **2006**, *26*, 248–255.
142. Renson, P.; Blanchard, Y.; Le Dimna, M.; Felix, H.; Cariolet, R.; Jestin, A.; Le Potier, M.F. Acute induction of cell death-related IFN stimulated genes (ISG) differentiates highly from moderately virulent CSFV strains. *Vet. Res.* **2010**, *41*, 7.
143. Pauly, T.; König, M.; Thiel, H.J.; Saalmüller, A. Infection with classical swine fever virus: Effects on phenotype and immune responsiveness of porcine T lymphocytes. *J. Gen. Virol.* **1998**, *79*, 31–40.
144. Summerfield, A.; Knoetig, S.M.; Tschudin, R.; McCullough, K.C. Pathogenesis of granulocytopenia and bone marrow atrophy during classical swine fever involves apoptosis and necrosis of uninfected cells. *Virology* **2000**, *272*, 50–60.
145. Susa, M.; König, M.; Saalmüller, A.; Reddehase, M.J.; Thiel, H.J. Pathogenesis of classical swine fever: B-lymphocyte deficiency caused by hog cholera virus. *J. Virol.* **1992**, *66*, 1171–1175.
146. Trautwein, G. Classical swine fever and related infections. In *Pathology and pathogenesis of the disease.*, Martinus Nijhoff: Boston, MA, USA, 1988; pp. 27–54.
147. Bautista, M.J.; Ruiz-Villamor, E.; Salguero, F.J.; Sanchez-Cordon, P.J.; Carrasco, L.; Gomez-Villamandos, J.C. Early platelet aggregation as a cause of thrombocytopenia in classical swine fever. *Vet. Pathol.* **2002**, *39*, 84–91.
148. Heene, D.; Hoffmann-Fezer, G.; Müller-Berghaus, G.; Hoffmann, R.; Weiss, E.; Lasch, H.G. Coagulation disorders in acute hog cholera. *Beitr. Pathol.* **1971**, *144*, 259–271.
149. Weiss, E.; Teredesai, A.; Hoffmann, R.; Hoffmann-Fezer, G. Volume distribution and ultrastructure of platelets in acute hog cholera. *Thromb. Diath. Haemorrh.* **1973**, *30*, 371–380.
150. Blome, S.; Meindl-Böhmer, A.; Nowak, G.; Moennig, V. Disseminated intravascular coagulation does not play a major role in the pathogenesis of classical swine fever. *Vet. Microbiol.* **2013**, *162*, 360–368.
151. Bensaude, E.; Turner, J.L.; Wakeley, P.R.; Sweetman, D.A.; Pardieu, C.; Drew, T.W.; Wileman, T.; Powell, P.P. Classical swine fever virus induces proinflammatory cytokines and tissue factor expression and inhibits apoptosis and interferon synthesis during the establishment of long-term infection of porcine vascular endothelial cells. *J. Gen. Virol.* **2004**, *85*, 1029–1037.
152. Gomez-Villamandos, J.C.; Ruiz-Villamor, E.; Bautista, M.J.; Quezada, M.; Sanchez, C.P.; Salguero, F.J.; Sierra, M.A. Pathogenesis of classical swine fever: Renal haemorrhages and erythrodiapedesis. *J. Comp. Pathol.* **2000**, *123*, 47–54.
153. Hoffmann, R.; Hoffmann-Fezer, G.; Kimeto, B.; Weiss, E. Microthrombi as morphological evidence of consumption coagulopathy in acute hog cholera. *Zentralbl. Veterinarmed. B* **1971**, *18*, 710–718.
154. Hulst, M.M.; Westra, D.F.; Wensvoort, G.; Moormann, R.J. Glycoprotein E1 of hog cholera virus expressed in insect cells protects swine from hog cholera. *J. Virol.* **1993**, *67*, 5435–5442.
155. Rümenapf, T.; Stark, R.; Meyers, G.; Thiel, H.J. Structural proteins of hog cholera virus expressed by vaccinia virus: Further characterization and induction of protective immunity. *J. Virol.* **1991**, *65*, 589–597.
156. König, M.; Lengsfeld, T.; Pauly, T.; Stark, R.; Thiel, H.J. Classical swine fever virus: Independent induction of protective immunity by two structural glycoproteins. *J. Virol.* **1995**, *69*, 6479–6486.
157. Paton, D.J.; Ibata, G.; Edwards, S.; Wensvoort, G. An ELISA detecting antibody to conserved pestivirus epitopes. *J. Virol. Methods* **1991**, *31*, 315–324.

158. Graham, S.P.; Everett, H.E.; Haines, F.J.; Johns, H.L.; Sosan, O.A.; Salguero, F.J.; Clifford, D.J.; Steinbach, F.; Drew, T.W.; Crooke, H.R. Challenge of pigs with classical swine fever viruses after C-strain vaccination reveals remarkably rapid protection and insights into early immunity. *PLoS One* **2012**, *7*, e29310.
159. Renson, P.; Le Dimna, M.; Keranflech, A.; Cariolet, R.; Koenen, F.; Le Potier, M.F. CP7_E2alf oral vaccination confers partial protection against early classical swine fever virus challenge and interferes with pathogeny-related cytokine responses. *Vet. Res.* **2013**, *44*, 9.
160. van Oirschot, J.T. Vaccinology of classical swine fever: From lab to field. *Vet. Microbiol.* **2003**, *96*, 367–384.
161. Graham, S.P.; Haines, F.J.; Johns, H.L.; Sosan, O.; La Rocca, S.A.; Lamp, B.; Rümenapf, T.; Everett, H.E.; Crooke, H.R. Characterisation of vaccine-induced, broadly cross-reactive IFN-γ secreting T cell responses that correlate with rapid protection against classical swine fever virus. *Vaccine* **2012**, *30*, 2742–2748.
162. Tarradas, J.; Argilaguet, J.M.; Rosell, R.; Nofrarias, M.; Crisci, E.; Cordoba, L.; Perez-Martin, E.; Diaz, I.; Rodriguez, F.; Domingo, M., *et al.* Interferon-γ induction correlates with protection by DNA vaccine expressing E2 glycoprotein against classical swine fever virus infection in domestic pigs. *Vet. Microbiol.* **2010**, *142*, 51–58.
163. Ganges, L.; Barrera, M.; Nunez, J.I.; Blanco, I.; Frias, M.T.; Rodriguez, F.; Sobrino, F. A DNA vaccine expressing the E2 protein of classical swine fever virus elicits T cell responses that can prime for rapid antibody production and confer total protection upon viral challenge. *Vaccine* **2005**, *23*, 3741–3752.
164. Blacksell, S.D.; Khounsy, S.; Van Aken, D.; Gleeson, L.J.; Westbury, H.A. Comparative susceptibility of indigenous and improved pig breeds to classical swine fever virus infection: Practical and epidemiological implications in a subsistence-based, developing country setting. *Trop. Anim. Health Prod.* **2006**, *38*, 467–474.
165. Everett, H.; Crooke, H.; Gurrala, R.; Dwarka, R.; Kim, J.; Botha, B.; Lubisi, A.; Pardini, A.; Gers, S.; Vosloo, W., *et al.* Experimental infection of common warthogs (*Phacochoerus africanus*) and bushpigs (*Potamochoerus larvatus*) with classical swine fever virus. I: Susceptibility and transmission. *Transbound. Emerg. Dis.* **2011**.
166. Terpstra, C. Epizootiology of swine fever. *Vet. Q.* **1987**, *9 Suppl. 1*, 50S-60S.
167. Weesendorp, E.; Landman, W.J.; Stegeman, A.; Loeffen, W.L. Detection and quantification of classical swine fever virus in air samples originating from infected pigs and experimentally produced aerosols. *Vet. Microbiol.* **2008**, *127*, 50–62.
168. Weesendorp, E.; Stegeman, A.; Loeffen, W.L. Quantification of classical swine fever virus in aerosols originating from pigs infected with strains of high, moderate or low virulence. *Vet. Microbiol.* **2009**, *135*, 222–230.
169. Laevens, H.; Koenen, F.; Deluyker, H.; de Kruif, A. Experimental infection of slaughter pigs with classical swine fever virus: Transmission of the virus, course of the disease and antibody response. *Vet. Rec.* **1999**, *145*, 243–248.
170. de Smit, A.J.; Bouma, A.; Terpstra, C.; van Oirschot, J.T. Transmission of classical swine fever virus by artificial insemination. *Vet. Microbiol.* **1999**, *67*, 239–249.
171. Floegel, G.; Wehrend, A.; Depner, K.R.; Fritzemeier, J.; Waberski, D.; Moennig, V. Detection of classical swine fever virus in semen of infected boars. *Vet. Microbiol.* **2000**, *77*, 109–116.
172. Moennig, V.; Greiser-Wilke, I. Classical swine fever virus. In *Encyclopedia of Virology*; Mahy B. W. J.; van Regenmortel, M.H.V., Eds. Elsevier: Amsterdam, The Netherlands, 2008; pp. 525–532.
173. Pasick, J. Classical swine fever. In *Foreign Animal Diseases*, 7th ed.; Brown, C., Torres, A., Eds.; Committee on Foreign and Emerging Diseases of the United States Animal Health Association, Boca Publications Group: Boca Raton, USA, 2008; pp. 197–205.
174. Hoffmann, B.; Beer, M.; Reid, S.M.; Mertens, P.; Oura, C.A.; van Rijn, P.A.; Slomka, M.J.; Banks, J.; Brown, I.H.; Alexander, D.J.; et al. A review of RT-PCR technologies used in veterinary virology and disease control: Sensitive and specific diagnosis of five livestock diseases notifiable to the World Organisation for Animal Health. *Vet. Microbiol.* **2009**, *139*, 1–23.
175. Hoffmann, B.; Beer, M.; Schelp, C.; Schirrmeier, H.; Depner, K. Validation of a real-time RT-PCR assay for sensitive and specific detection of classical swine fever. *J. Virol. Methods* **2005**, *130*, 36–44.
176. Hoffmann, B.; Blome, S.; Bonilauri, P.; Fernandez-Pinero, J.; Greiser-Wilke, I.; Haegeman, A.; Isaksson, M.; Koenen, F.; Leblanc, N.; Leifer, I.; et al. Classical swine fever virus detection: Results of a real-time reverse transcription polymerase chain reaction ring trial conducted in the framework of the European network of excellence for epizootic disease diagnosis and control. *J. Vet. Diagn. Investig.* **2011**, *23*, 999–1004.

177. Le Dimna, M.; Vrancken, R.; Koenen, F.; Bougeard, S.; Mesplede, A.; Hutet, E.; Kuntz-Simon, G.; Le Potier, M.F. Validation of two commercial real-time RT-PCR kits for rapid and specific diagnosis of classical swine fever virus. *J. Virol. Methods* **2008**, *147*, 136–142.
178. Le Potier, M.F.; Le Dimna, M.; Kuntz-Simon, G.; Bougeard, S.; Mesplede, A. Validation of a real-time RT-PCR assay for rapid and specific diagnosis of classical swine fever virus. *Dev. Biol. (Basel)* **2006**, *126*, 179–186; discusssion 326–177.
179. Leifer, I.; Blome, S.; Beer, M.; Hoffmann, B. Development of a highly sensitive real-time RT-PCR protocol for the detection of classical swine fever virus independent of the 5' untranslated region. *J. Virol. Methods* **2011**, *171*, 314–317.
180. McGoldrick, A.; Lowings, J.P.; Ibata, G.; Sands, J.J.; Belak, S.; Paton, D.J. A novel approach to the detection of classical swine fever virus by RT-PCR with a fluorogenic probe (TaqMan). *J. Virol. Methods* **1998**, *72*, 125–135.
181. Paton, D.J.; McGoldrick, A.; Belak, S.; Mittelholzer, C.; Koenen, F.; Vanderhallen, H.; Biagetti, M.; De Mia, G.M.; Stadejek, T.; Hofmann, M.A.; et al. Classical swine fever virus: A ring test to evaluate RT-PCR detection methods. *Vet. Microbiol.* **2000**, *73*, 159–174.
182. Paton, D.J.; McGoldrick, A.; Bensaude, E.; Belak, S.; Mittelholzer, C.; Koenen, F.; Vanderhallen, H.; Greiser-Wilke, I.; Scheibner, H.; Stadejek, T.; et al. Classical swine fever virus: A second ring test to evaluate RT-PCR detection methods. *Vet. Microbiol.* **2000**, *77*, 71–81.
183. Belak, S. Experiences of an oie collaborating centre in molecular diagnosis of transboundary animal diseases: A review. *Dev. Biol. (Basel)* **2007**, *128*, 103–112.
184. Liu, L.; Luo, Y.; Accensi, F.; Ganges, L.; Rodriguez, F.; Shan, H.; Stahl, K.; Qiu, H.J.; Belak, S. Pre-clinical evaluation of a real-time PCR assay on a portable instrument as a possible field diagnostic tool: Experiences from the testing of clinical samples for African and classical swine fever viruses. *Transbound. Emerg. Dis.* **2016**.
185. Chen, H.T.; Zhang, J.; Ma, L.N.; Ma, Y.P.; Ding, Y.Z.; Liu, X.T.; Chen, L.; Ma, L.Q.; Zhang, Y.G.; Liu, Y.S. Rapid pre-clinical detection of classical swine fever by reverse transcription loop-mediated isothermal amplification. *Mol. Cell. Probes* **2009**, *23*, 71–74.
186. Chen, L.; Fan, X.Z.; Wang, Q.; Xu, L.; Zhao, Q.Z.; Zhou, Y.C.; Liu, J.; Tang, B.; Zou, X.Q. A novel RT-LAMP assay for rapid and simple detection of classical swine fever virus. *Virol. Sin.* **2010**, *25*, 59–64.
187. Chowdry, V.K.; Luo, Y.; Widen, F.; Qiu, H.J.; Shan, H.; Belak, S.; Liu, L. Development of a loop-mediated isothermal amplification assay combined with a lateral flow dipstick for rapid and simple detection of classical swine fever virus in the field. *J. Virol. Methods* **2014**, *197*, 14–18.
188. Yin, S.; Shang, Y.; Zhou, G.; Tian, H.; Liu, Y.; Cai, X.; Liu, X. Development and evaluation of rapid detection of classical swine fever virus by reverse transcription loop-mediated isothermal amplification (RT-LAMP). *J. Biotechnol.* **2010**, *146*, 147–150.
189. Zhang, X.J.; Han, Q.Y.; Sun, Y.; Belak, S.; Liu, L.; Qiu, H.J. Development of a loop-mediated isothermal amplification for visual detection of the HCLV vaccine against classical swine fever in China. *J. Virol. Methods* **2011**, *171*, 200–205.
190. Zhang, X.J.; Sun, Y.; Liu, L.; Belak, S.; Qiu, H.J. Validation of a loop-mediated isothermal amplification assay for visualised detection of wild-type classical swine fever virus. *J. Virol. Methods* **2010**, *167*, 74–78.
191. Liu, L.; Xia, H.; Belak, S.; Widen, F. Development of a primer-probe energy transfer real-time PCR assay for improved detection of classical swine fever virus. *J. Virol. Methods* **2009**, *160*, 69–73.
192. Zhang, X.J.; Xia, H.; Everett, H.; Sosan, O.; Crooke, H.; Belak, S.; Widen, F.; Qiu, H.J.; Liu, L. Evaluation of a primer-probe energy transfer real-time PCR assay for detection of classical swine fever virus. *J. Virol. Methods* **2010**, *168*, 259–261.
193. Lung, O.; Pasick, J.; Fisher, M.; Buchanan, C.; Erickson, A.; Ambagala, A. Insulated isothermal reverse transcriptase PCR (iiRT-PCR) for rapid and sensitive detection of classical swine fever virus. *Transbound. Emerg. Dis.* **2016,** *63*, e395–402.
194. Turner, L.W.; Brown, L.N.; Carbrey, E.A.; Mengeling, W.L.; Perella, D.H.; Solorzano, R.F. Recommended minimum standards for the isolation and identification of hog cholera by the fluorescent antibody-cell culture technique. *Proc. Annu. Meet. U. S. Anim. Health Assoc.* **1968**, *72*, 444–447.
195. de Smit, A.J.; Eble, P.L.; de Kluijver, E.P.; Bloemraad, M.; Bouma, A. Laboratory experience during the classical swine fever virus epizootic in the Netherlands in 1997–1998. *Vet. Microbiol.* **2000**, *73*, 197–208.

196. Blome, S.; Meindl-Böhmer, A.; Loeffen, W.; Thuer, B.; Moennig, V. Assessment of classical swine fever diagnostics and vaccine performance. *Rev. Sci. Tech.* **2006**, *25*, 1025–1038.
197. Greiser-Wilke, I.; Blome, S.; Moennig, V. Diagnostic methods for detection of classical swine fever virus--status quo and new developments. *Vaccine* **2007**, *25*, 5524–5530.
198. Floegel-Niesmann, G. Marker vaccines and companion diagnostic tests for classical swine fever. *Dev. Biol. (Basel)* **2003**, *114*, 185–191.
199. Floegel-Niesmann, G. Classical swine fever (CSF) marker vaccine. Trial III. Evaluation of discriminatory ELISAs. *Vet. Microbiol.* **2001**, *83*, 121–136.
200. Meyer, D.; Fritsche, S.; Luo, Y.; Engemann, C.; Blome, S.; Beyerbach, M.; Chang, C.Y.; Qiu, H.J.; Becher, P.; Postel, A. The double-antigen ELISA concept for early detection of E^{rns} -specific classical swine fever virus antibodies and application as an accompanying test for differentiation of infected from marker vaccinated animals. *Transbound. Emerg. Dis.* **2017**.
201. Aebischer, A.; Müller, M.; Hofmann, M.A. Two newly developed E^{rns}-based ELISAs allow the differentiation of classical swine fever virus-infected from marker-vaccinated animals and the discrimination of pestivirus antibodies. *Vet. Microbiol.* **2013**, *161*, 274–285.
202. Xia, H.; Harimoorthy, R.; Vijayaraghavan, B.; Blome, S.; Widen, F.; Beer, M.; Belak, S.; Liu, L. Differentiation of classical swine fever virus infection from CP7_E2alf marker vaccination by a multiplex microsphere immunoassay. *Clin. Vaccine. Immunol.* **2015**, *22*, 65–71.
203. Huang, Y.L.; Pang, V.F.; Pan, C.H.; Chen, T.H.; Jong, M.H.; Huang, T.S.; Jeng, C.R. Development of a reverse transcription multiplex real-time PCR for the detection and genotyping of classical swine fever virus. *J. Virol. Methods* **2009**, *160*, 111–118.
204. Leifer, I.; Depner, K.; Blome, S.; Le Potier, M.F.; Le Dimna, M.; Beer, M.; Hoffmann, B. Differentiation of C-strain "Riems" or CP7_E2alf vaccinated animals from animals infected by classical swine fever virus field strains using real-time RT-PCR. *J. Virol. Methods* **2009**, *158*, 114–122.
205. Li, Y.; Zhao, J.J.; Li, N.; Shi, Z.; Cheng, D.; Zhu, Q.H.; Tu, C.; Tong, G.Z.; Qiu, H.J. A multiplex nested RT-PCR for the detection and differentiation of wild-type viruses from C-strain vaccine of classical swine fever virus. *J. Virol. Methods* **2007**, *143*, 16–22.
206. Liu, L.; Hoffmann, B.; Baule, C.; Beer, M.; Belak, S.; Widen, F. Two real-time RT-PCR assays of classical swine fever virus, developed for the genetic differentiation of naturally infected from vaccinated wild boars. *J. Virol. Methods* **2009**, *159*, 131–133.
207. Widen, F.; Everett, H.; Blome, S.; Fernandez Pinero, J.; Uttenthal, A.; Cortey, M.; von Rosen, T.; Tignon, M.; Liu, L. Comparison of two real-time RT-PCR assays for differentiation of C-strain vaccinated from classical swine fever infected pigs and wild boars. *Res. Vet. Sci.* **2014**, *97*, 455–457.
208. Zhao, J.J.; Cheng, D.; Li, N.; Sun, Y.; Shi, Z.; Zhu, Q.H.; Tu, C.; Tong, G.Z.; Qiu, H.J. Evaluation of a multiplex real-time RT-PCR for quantitative and differential detection of wild-type viruses and C-strain vaccine of classical swine fever virus. *Vet. Microbiol.* **2008**, *126*, 1–10.
209. Dietze, K.; Tucakov, A.; Engel, T.; Wirtz, S.; Depner, K.; Globig, A.; Kammerer, R.; Mouchantat, S. Rope-based oral fluid sampling for early detection of classical swine fever in domestic pigs at group level. *BMC Vet. Res.* **2017**, *13*, 5.
210. Michaud, V.; Gil, P.; Kwiatek, O.; Prome, S.; Dixon, L.; Romero, L.; Le Potier, M.F.; Arias, M.; Couacy-Hymann, E.; Roger, F.; et al. Long-term storage at tropical temperature of dried-blood filter papers for detection and genotyping of RNA and DNA viruses by direct PCR. *J. Virol. Methods* **2007**, *146*, 257–265.
211. Mouchantat, S.; Globig, A.; Böhle, W.; Petrov, A.; Strebelow, H.G.; Mettenleiter, T.C.; Depner, K. Novel rope-based sampling of classical swine fever shedding in a group of wild boar showing low contagiosity upon experimental infection with a classical swine fever field strain of genotype 2.3. *Vet. Microbiol.* **2014**, *170*, 425–429.
212. Prickett, J.R.; Zimmerman, J.J. The development of oral fluid-based diagnostics and applications in veterinary medicine. *Anim. Health Res. Rev.* **2010**, *11*, 207–216.
213. Greiser-Wilke, I.; Moennig, V. Vaccination against classical swine fever virus: Limitations and new strategies. *Anim. Health Res. Rev.* **2004**, *5*, 223–226.
214. World animal health information database (WAHIS) interface. Available online: http://www.oie.int/wahis_2/public/wahid.php/Diseasecontrol/measures (accessed 19 April 2017),
215. Kaden, V.; Lange, E.; Fischer, U.; Strebelow, G. Oral immunisation of wild boar against classical swine fever: Evaluation of the first field study in Germany. *Vet. Microbiol.* **2000**, *73*, 239–252.

216. Kaden, V.; Lange, E.; Küster, H.; Müller, T.; Lange, B. An update on safety studies on the attenuated "RIEMSER Schweinepestoralvakzine" for vaccination of wild boar against classical swine fever. *Vet. Microbiol.* **2010**, *143*, 133–138.
217. Milicevic, V.; Dietze, K.; Plavsic, B.; Tikvicki, M.; Pinto, J.; Depner, K. Oral vaccination of backyard pigs against classical swine fever. *Vet. Microbiol.* **2012**, *225*, 167–171.
218. Dietze, K.; Milicevic, V.; Depner, K. Prospects of improved classical swine fever control in backyard pigs through oral vaccination. *Berl. Munch. Tierarztl. Wochenschr.* **2013**, *126*, 476–480.
219. Monger, V.R.; Stegeman, J.A.; Dukpa, K.; Gurung, R.B.; Loeffen, W.L. Evaluation of oral bait vaccine efficacy against classical swine fever in village backyard pig farms in Bhutan. *Transbound. Emerg. Dis.* **2016**, *63*, e211-e218.
220. Dahle, J.; Liess, B. Assessment of safety and protective value of a cell culture modified strain "C" vaccine of hog cholera/classical swine fever virus. *Berl. Munch. Tierarztl. Wochenschr.* **1995**, *108*, 20–25.
221. Kaden, V.; Riebe, B. Classical swine fever (CSF): A historical review of research and vaccine production on the Isle of Riems. *Berl. Munch. Tierarztl. Wochenschr.* **2001**, *114*, 246–251.
222. Terpstra, C.; Woortmeyer, R.; Barteling, S.J. Development and properties of a cell culture produced vaccine for hog cholera based on the Chinese strain. *Dtsch. Tierarztl. Wochenschr.* **1990**, *97*, 77–79.
223. Ferrari, M. A tissue culture vaccine with lapinized Chinese (LC) strain of hog cholera virus (HCV). *Comp. Immunol. Microbiol. Infect. Dis.* **1992**, *15*, 221–228.
224. Blome, S.; Gabriel, C.; Beer, M. Possibilities and limitations in veterinary vaccine development using the example of classical swine fever. *Berl. Munch. Tierarztl. Wochenschr.* **2013**, *126*, 481–490.
225. Ahrens, U.; Kaden, V.; Drexler, C.; Visser, N. Efficacy of the classical swine fever (CSF) marker vaccine Porcilis Pesti in pregnant sows. *Vet. Microbiol.* **2000**, *77*, 83–97.
226. Bouma, A.; De Smit, A.J.; De Jong, M.C.; De Kluijver, E.P.; Moormann, R.J. Determination of the onset of the herd-immunity induced by the E2 sub-unit vaccine against classical swine fever virus. *Vaccine* **2000**, *18*, 1374–1381.
227. Bouma, A.; de Smit, A.J.; de Kluijver, E.P.; Terpstra, C.; Moormann, R.J. Efficacy and stability of a subunit vaccine based on glycoprotein E2 of classical swine fever virus. *Vet. Microbiol.* **1999**, *66*, 101–114.
228. de Smit, A.J.; Bouma, A.; de Kluijver, E.P.; Terpstra, C.; Moormann, R.J. Duration of the protection of an E2 subunit marker vaccine against classical swine fever after a single vaccination. *Vet. Microbiol.* **2001**, *78*, 307–317.
229. de Smit, A.J.; Bouma, A.; de Kluijver, E.P.; Terpstra, C.; Moormann, R.J. Prevention of transplacental transmission of moderate-virulent classical swine fever virus after single or double vaccination with an E2 subunit vaccine. *Vet. Q.* **2000**, *22*, 150–153.
230. Dewulf, J.; Laevens, H.; Koenen, F.; Vanderhallen, H.; Mintiens, K.; Deluyker, H.; de Kruif, A. An experimental infection with classical swine fever in E2 sub-unit marker-vaccine vaccinated and in non-vaccinated pigs. *Vaccine* **2000**, *19*, 475–482.
231. Klinkenberg, D.; Moormann, R.J.; de Smit, A.J.; Bouma, A.; de Jong, M.C. Influence of maternal antibodies on efficacy of a subunit vaccine: Transmission of classical swine fever virus between pigs vaccinated at 2 weeks of age. *Vaccine* **2002**, *20*, 3005–3013.
232. Lipowski, A.; Drexler, C.; Pejsak, Z. Safety and efficacy of a classical swine fever subunit vaccine in pregnant sows and their offspring. *Vet. Microbiol.* **2000**, *77*, 99–108.
233. Moormann, R.J.; Bouma, A.; Kramps, J.A.; Terpstra, C.; De Smit, H.J. Development of a classical swine fever subunit marker vaccine and companion diagnostic test. *Vet. Microbiol.* **2000**, *73*, 209–219.
234. van Aarle, P. Suitability of an E2 subunit vaccine of classical swine fever in combination with the E^{rns}-marker-test for eradication through vaccination. *Dev. Biol. (Basel)* **2003**, *114*, 193–200.
235. van Oirschot, J.T. DIVA vaccines that reduce virus transmission. *J. Biotechnol.* **1999**, *73*, 195–205.
236. van Oirschot, J.T. Emergency vaccination against classical swine fever. *Dev. Biol. (Basel)* **2003**, *114*, 259–267.
237. Depner, K.R.; Bouma, A.; Koenen, F.; Klinkenberg, D.; Lange, E.; de Smit, H.; Vanderhallen, H. Classical swine fever (CSF) marker vaccine. Trial II. Challenge study in pregnant sows. *Vet. Microbiol.* **2001**, *83*, 107–120.
238. Beer, M.; Reimann, I.; Hoffmann, B.; Depner, K. Novel marker vaccines against classical swine fever. *Vaccine* **2007**, *25*, 5665–5670.
239. Blome, S.; Moss, C.; Reimann, I.; König, P.; Beer, M. Classical swine fever vaccines-state-of-the-art. *Vet. Microbiol.* **2017,** *201–204*, 10.1016/j.vetmic.2017.01.001.

240. Blome, S.; Aebischer, A.; Lange, E.; Hofmann, M.; Leifer, I.; Loeffen, W.; Koenen, F.; Beer, M. Comparative evaluation of live marker vaccine candidates "CP7_E2alf" and "flc11" along with C-strain "Riems" after oral vaccination. *Vet. Microbiol.* **2012,** *158*, 42–59.
241. Blome, S.; Gabriel, C.; Schmeiser, S.; Meyer, D.; Meindl-Böhmer, A.; Koenen, F.; Beer, M. Efficacy of marker vaccine candidate CP7_E2alf against challenge with classical swine fever virus isolates of different genotypes. *Vet. Microbiol.* **2014**, *169*, 8–17.
242. Dräger, C.; Petrov, A.; Beer, M.; Teifke, J.P.; Blome, S. Classical swine fever virus marker vaccine strain CP7_E2alf: Shedding and dissemination studies in boars. *Vaccine* **2015**, *33*, 3100–3103.
243. Dräger, C.; Schröder, C.; König, P.; Tegtmeyer, B.; Beer, M.; Blome, S. Efficacy of Suvaxyn CSF marker (CP7_E2alf) in the presence of pre-existing pestiviral antibodies against bovine viral diarrhea virus type 1. *Vaccine* **2016**, in press.
244. Eble, P.L.; Geurts, Y.; Quak, S.; Moonen-Leusen, H.W.; Blome, S.; Hofmann, M.A.; Koenen, F.; Beer, M.; Loeffen, W.L. Efficacy of chimeric pestivirus vaccine candidates against classical swine fever: Protection and DIVA characteristics. *Vet. Microbiol.* **2012**, *162*, 437–146.
245. Eble, P.L.; Quak, S.; Geurts, Y.; Moonen-Leusen, H.W.; Loeffen, W.L. Efficacy of csf vaccine CP7_E2alf in piglets with maternally derived antibodies. *Vet. Microbiol.* **2014**, *174*, 27–38.
246. Feliziani, F.; Blome, S.; Petrini, S.; Giammarioli, M.; Iscaro, C.; Severi, G.; Convito, L.; Pietschmann, J.; Beer, M.; De Mia, G.M. First assessment of classical swine fever marker vaccine candidate CP7_E2alf for oral immunization of wild boar under field conditions. *Vaccine* **2014**, *32*, 2050–2055.
247. Gabriel, C.; Blome, S.; Urniza, A.; Juanola, S.; Koenen, F.; Beer, M. Towards licensing of CP7_E2alf as marker vaccine against classical swine fever-duration of immunity. *Vaccine* **2012**, *30*, 2928–2936.
248. Goller, K.V.; Dräger, C.; Höper, D.; Beer, M.; Blome, S. Classical swine fever virus marker vaccine strain CP7_E2alf: Genetic stability in vitro and in vivo. *Arch. Virol.* **2015**, *160*, 3121–3125.
249. König, P.; Blome, S.; Gabriel, C.; Reimann, I.; Beer, M. Innocuousness and safety of classical swine fever marker vaccine candidate CP7_E2alf in non-target and target species. *Vaccine* **2011**, *30*, 5–8.
250. König, P.; Hoffmann, B.; Depner, K.R.; Reimann, I.; Teifke, J.P.; Beer, M. Detection of classical swine fever vaccine virus in blood and tissue samples of pigs vaccinated either with a conventional C-strain vaccine or a modified live marker vaccine. *Vet. Microbiol.* **2007**, *120*, 343–351.
251. König, P.; Lange, E.; Reimann, I.; Beer, M. CP7_E2alf: A safe and efficient marker vaccine strain for oral immunisation of wild boar against classical swine fever virus (CSFV). *Vaccine* **2007**, *25*, 3391–3399.
252. Leifer, I.; Lange, E.; Reimann, I.; Blome, S.; Juanola, S.; Duran, J.P.; Beer, M. Modified live marker vaccine candidate CP7_E2alf provides early onset of protection against lethal challenge infection with classical swine fever virus after both intramuscular and oral immunization. *Vaccine* **2009**, *27*, 6522–6529.
253. Levai, R.; Barna, T.; Fabian, K.; Blome, S.; Belak, K.; Balint, A.; Koenen, F.; Kulcsar, G.; Farsang, A. Pre-registration efficacy study of a novel marker vaccine against classical swine fever on maternally derived antibody negative (MDA-) target animals. *Biologicals* **2015**, *14*, 1045–1056.
254. Rangelova, D.; Nielsen, J.; Strandbygaard, B.; Koenen, F.; Blome, S.; Uttenthal, A. Efficacy of marker vaccine candidate CP7_E2alf in piglets with maternally derived C-strain antibodies. *Vaccine* **2012**, *30*, 6376–6381.
255. Reimann, I.; Depner, K.; Trapp, S.; Beer, M. An avirulent chimeric *Pestivirus* with altered cell tropism protects pigs against lethal infection with classical swine fever virus. *Virology* **2004**, *322*, 143–157.
256. Renson, P.; Le Dimna, M.; Gabriel, C.; Levai, R.; Blome, S.; Kulcsar, G.; Koenen, F.; Le Potier, M.F. Cytokine and immunoglobulin isotype profiles during CP7_E2alf vaccination against a challenge with the highly virulent Koslov strain of classical swine fever virus. *Res. Vet. Sci.* **2014**, *96*, 389–395.
257. Farsang, A.; Levai, R.; Barna, T.; Fabian, K.; Blome, S.; Belak, K.; Balint, A.; Koenen, F.; Kulcsar, G. Pre-registration efficacy study of a novel marker vaccine against classical swine fever on maternally derived antibody positive (MDA+) target animals. *Biologicals* **2017**, *45*, 85–92.

2.2. Persistent infection

Like other pestiviruses, e.g. Bovine viral diarrhea virus (BVDV), CSFV is able to cross the placental barrier and infect the fetuses throughout the time of pregnancy. While the virus can cross this barrier, maternally derived antibodies that could protect the offspring are whithheld and only transferred by colostrum uptake. This leads to infection of a fully susceptible host with a developing immune system. For the outcome of the prenatal infection, the stage of gestation and virulence of the infecting strain are crucial factors. Infection of the dam in the first trimesters of gestation may lead to resorption of the fetuses or abortion, whereas infection at the end of gestation could also lead to the birth of transiently infected piglets or stillbirth. The crucial time for the genesis of persistently infected piglets is in mid gestation between days 50 and 70 (Kaden et al. 2005; Liess 1987; Stewart, Carbrey, and Kresse 1973). Not all of the exposed piglets have to be persistently infected and also all other above mentioned outcomes are possible during this time. However, the most dangerous consequence is the birth of persistently infected (PI) offspring. These animals develop an innate central immunotolerance due to infection in an early stage of development of the immune system of the fetus. The adaptive immune system of these animals does not recognize the virus as pathogen and thus, no immune response is initiated. Furthermore, key mechanisms of the innate immune system are affected due to interference of the interferon synthesis (Peterhans, Jungi, and Schweizer 2003; Peterhans and Schweizer 2010).

Given the fact, that no immune response is mounted, CSFV can replicate to high titers and is shed throughout the lifespan of the animal. Yet, these animals are usually born rather healthy and may enter trade. In this case, the disease can be introduced into new susceptible herds and holdings, without being detected at first. However, the long-term fate of these PI animals seems to be fatal. It is acknowledged opinion that persistently infected animals will eventually show the so-called “late-onset” form of CSF. The latter is characterized by detoriating health and various unspecific clinical signs and secondary infections, which complicate the correct diagnosis of these animals (Frey et al. 1980; Meyer et al. 1981; Hermanns et al. 1981).

The importance of PI animals is best characterized for BVDV infection in cattle. Even when the prevalence of PI animals is low on the population level, their impact on disease spread and maintenance is crucial. For this reason, detection and removal of these animals is an important pilar in any BVDV control effort (Lindberg and Houe 2005; Schweizer and Peterhans 2014). The same seems to apply for perpetuation of CSFV within wild boar populations in endemic

regions. The presence of PI animals in combination with a high density of wild boar can play a crucial role for endemic situations (Kern et al. 1999). To prevent PI animals in endemic settings, safe and highly efficient vaccines, which protect dam and piglets equally, should be used,.
Recently it was shown, that infection with a moderately virulent CSFV strain shortly after birth (up to 48 hours) is also able to induce an infection course in piglets that resembles that of persistency. Despite intensive discussion whether this course is comparable to persistence induced by transplacental infection, this course is now known as “postnatal persistence”. The affected animals do not develop an immune response against the virus but constantly shed the virus (Cabezon et al. 2015; Munoz-Gonzalez et al. 2015b). The problem with these animals is, that vaccination is ineffective, not least because of interference. In this context, the persistently infected animals are already carrying high amounts of virus and therefore, no other CSFV is able to replicate in these animals (Munoz-Gonzalez et al. 2015a).

2.3. Emergency vaccination in the European Union

Within the European Union (EU) the measures for controlling CSF are defined in the Council Directive 2001/89/EC and the diagnostic manual accompanying it (Commission Decision 2002/106/EC) (European Commission 2001). This legal framework was established to eradicate CSF and to prevent the spread of the disease in the EU. According to these regulations, prophylactic vaccination is prohibited and in case of detection of CSF, a strict stamping-out campaign is carried out. The implication of these stamping out campaigns is, that numerous mostly uninfected pigs have to be culled in case of an outbreak in an area with a high density of pig production. To be able to prevent this kind of scenario, the possibility of an emergency vaccination has been laid down for both domestic pigs and wild boar. In case of an outbreak situation, countries will be allowed to present plans for emergency vaccination campaigns to the European Commission. Concerning the vaccine type no restrictions are laid down, and therefore, both conventional live attenuated vaccines and marker vaccines are possible for application (European Commission 2001). The problem with the use of a conventional live attenuated vaccine would be that severe trade restrictions are imposed on areas and countries with conventional vaccination against CSFV.
In detail, Council Directive 2001/89/EC prescribes: No living pigs leave the vaccination area, unless to be transported to a slaughterhouse designated by the competent authority and

situated within the vaccination area or close to that area for immediate slaughter or to a rendering plant or to a suitable place where they are immediately killed and their carcasses are processed under official supervision, all fresh pig meat produced from pigs vaccinated during the emergency vaccination is either processed or marked and treated, and semen, ova and embryos collected from the pigs to be vaccinated during the 30 days prior to vaccination are traced and destroyed under official supervision. All these provisions shall apply for a minimum of six months following completion of the vaccination operations in the area in question (European Commission, 2001). In addition, the OIE free status can only be recovered three months after the last case and slaughter of all vaccinated animals.

However, both EU and OIE foresee derogations that can be made if a marker vaccine and a reliable and validated DIVA concept is in place.

It has to be stressed that emergency vaccination was so far only implemented in wild boar and a very limited area of Romania. Among the reasons is the lack of tested exit scenarios and the unpredictable behavior of trade partners.

Among the vaccines that are available within the EU are two live attenuated vaccines that are based on the so-called C-strain of CSFV. The products are PESTIFFA® (Merial) and Pestiporc CSFV® (IDT Biologika). Due to the above mentioned trade restrictions, these highly safe and efficacious vaccines are difficult to use in outbreak scenarios. The focus would probably be on marker vaccines. Among them is the E2 subunit vaccine Porcillis Pesti® (Intervet international BV). It is a marker vaccine based on baculovirus-expressed immunedominant CSFV envelope glycoprotein E2. The vaccine is safe but shows drawbacks especially in terms of early protection and protection against vertical transmission (van Oirschot 2003b).

One more promising candidate is the recently licensed live marker vaccine CP7_E2alf (Suvaxyn® CSF Marker, Zoetis), which is the first ever licensed genetically modified chimeric vaccine in the veterinary field.

2.4. The marker vaccine CP7_E2alf

The live marker vaccine CP7_E2alf is a chimeric pestivirus, based on the cytopathogenic BVDV-1 strain CP7 which serves as a backbone in the vaccine virus (Corapi, Donis, and Dubovi 1988; Reimann et al. 2004; Reimann, Blome, and Beer 2016; Reimann et al. 2010). In this backbone, the E2 coding region was replaced by the E2 coding region of the CSFV strain Alfort/187 (Meyers et al. 1996) (see Figure 1).

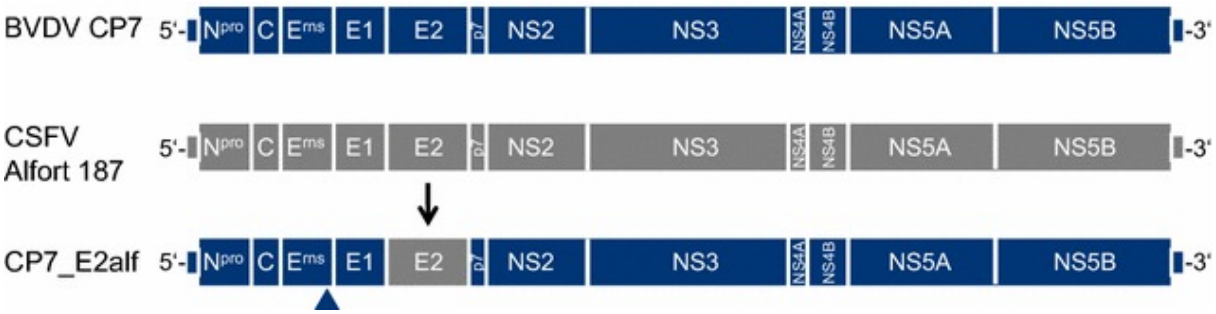


Figure 1: Schematic representation of "CP7_E2alf" and its parental viruses BVDV "CP7" (represented in blue) and CSFV "Alfort/187" (grey). The arrow indicates the position of the CSFV E2 (in grey) in the BVDV backbone (in blue). The arrowhead indicates the G479R mutation in BVDV-Erns, which is responsible for an efficient virus growth in porcine cells. Source: Blome et al. (2017 DOI: 10.3390/v9040086)

Differentiation of infected from vaccinated animals (DIVA) is achieved through detection of CSFV E^{rns} antibodies. In case of an infection with CSFV, the host produces antibodies against the E2 protein as well as the E^{rns} protein. A pig vaccinated with CP7_E2alf will only develop antibodies against the E2 protein of CSFV (Meyer et al. 2017). The DIVA principle therefore depends on specific and reliable detection systems of these two antibodies against CSFV (Schroeder et al. 2012; Pannhorst et al. 2015).

In the licensing process, the safety, stability and efficacy of the vaccine had to be demonstrated. (European Medicines Agency - Committee for Medicinal Products for Veterinary Use 2014; CORDIS 2013). One of the requirements was demonstration of genetic stability. The virus presented itself as highly stable *in vitro* and *in vivo*. Furthermore, there were no indications that the virus is more prone for mutation or genetic recombination than its parental viruses (Goller et al. 2015).

The efficacy of vaccination was proven in several studies, where protection against highly virulent strains was confirmed by challenges within one and two weeks after intramuscular vaccination (Leifer et al. 2009; Blome et al. 2014). With regard to the required investigation of the duration of immunity it was shown that a one shot intramuscular vaccination protected the animals against a challenge six month after vaccination (Gabriel et al. 2012). In domestic pigs and European wild boar, safety for intramuscular and oral vaccination was demonstrated (König, Lange, et al. 2007). No vaccine virus has been transmitted or shed by vaccinated animals (König et al. 2011). In organs, vaccine virus can be found in the tonsil of vaccinated

animals up to several days, but later on, only genome is detectable in the tonsil up to 42 days after intramuscular vaccination. In lymphatic organs, genome is detectable up to two weeks after vaccination. In the majority of studies with tested blood samples genome detection of the virus is possible for a short period (few days) or absent in most cases (König, Hoffmann, et al. 2007; Tignon et al. 2010). In addition to the genetic stability and the efficacy, it was also mandated to show the innocuousness in several species, susceptible for the parental viruses, especially since BVDV has a much broader host range than CSFV. In case of transmission of vaccine virus, the most likely transmission scenario would be an oro-nasal contact, especially with an open vaccine bait. Therefore the vaccine was tested with a single, high-dose oral inoculation in calves, goat kids, lambs and rabbits, including contact animals of the respective species to exclude possible transmission of vaccine virus. Vaccine virus was not found in any of the samples taken from vaccinated or control animals and furthermore none of the animals seroconverted (König et al. 2011).

One additional concern in the registration process has been the interference of antibodies against BVDV with the vaccination. Because CP7_E2alf is based on a backbone of the BVDV strain CP7 this would play an important role in endemic areas with BVDV where contact between cattle and pigs is common and the pigs could be infected with BVDV and develop antibodies against BVDV. Full protection against an infection with CSFV was shown in pigs with pre-existing antibodies against BVDV-1 (Dräger et al. 2016). One aspect, which has to be taken into consideration, was the shown interference with the serological DIVA diagnostics, especially in regions where pigs are kept in close proximity with cattle.

In a supplemental study, full protection after oral vaccination was shown three weeks after vaccination, whereas clinical protection was already shown two weeks after vaccination; however, the possibility of virus transmission could not be excluded at that time (Blome et al. 2012). As early as two days after oral vaccination, partial protection was shown in a supplementary study with a moderately virulent CSFV strain (Renson et al. 2013).

All executed studies taken together (see table 1) fulfilled the requirements for CSF vaccines that are provided by the European Pharmacopoiea (Ph. Eur., monograph 07/2008:0065) and the OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (OIE Manual, chapter 2.8.3) and therefore “CP7_E2alf” was licensed as first live marker vaccine against CSF.

Table 1: Published studies on "CP7_E2alf" and their topics. Source: Blome et al. (2017, DOI: 10.3390/v9040086)

Topic	**Data covered by the article**	**Reference**
Vaccine design and construction	Laboratory protocols for chimera design	Reimann et al., 2015
	Construction of the chimera, sequence analysis, initial *in vitro* and *in vivo* tests	Reimann et al., 2004
Genetic stability	Stability over cell culture passages, search for recombinants in co-infection studies, stability *in vivo*	Goller et al., 2015
Safety	Assessment of shedding through feces, urine and semen, dissemination	Dräger et al., 2015
	Dissemination, onset of antibody responses, diagnostic tests	Tignon et al., 2010
	Innocuousness and safety in target and non-target species	König et al., 2011
	Detection and dissemination of vaccine virus	König et al., 2007
Efficacy	Efficacy in the presence of BVDV-1 antibodies, DIVA	Dräger et al., 2016
	Efficacy in MDA negative piglets, intramuscular and oral vaccination with challenge at 14 dpv with CSFV "Koslov"	Levai et al., 2015
	Efficacy in piglets with MDA, intramuscular (3 weeks/6 weeks) and oral vaccination (6 weeks), challenge 14 dpv with CSFV "Koslov"	Eble et al., 2014
	Efficacy against different genotypes of CSFV, intramuscular and oral vaccination (domestic pigs and wild boar), challenge 14 dpv/ 21 dpv	Blome et al., 2014
	Efficacy after intramuscular vaccination and DIVA (comparative trial with different chimeras), challenge 7 and 14 dpv with CSFV "Koslov"	Eble et al., 2013
	Efficacy in piglets with C-strain derived MDA (5 weeks/ 8 weeks), challenge with CSFV "Koslov" 14 dpv	Rangelova et al., 2012
	Duration of immunity study, intramuscular and oral vaccination, challenge six month post vaccination with CSFV "Koslov"	Gabriel et al., 2012
	Efficacy after oral vaccination (comparative trial with different chimeras), challenge 14 and 21 dpv	Blome et al., 2012
	Onset of immunity and vaccine dose, efficacy study, genetic stability, intramuscular and oral vaccination	Leifer et al., 2009
	Efficacy (and safety) of oral immunization of wild boar	König et al., 2007

Topic	Data covered by the article	Reference
DIVA diagnostics	Design and evaluation of an E^{rns} ELISA	Luo et al., 2015
	Evaluation of a discriminatory CSFV E^{rns} ELISA in an inter-laboratory trial	Pannhorst et al., 2015
	Differentiation of CSFV infection and "CP7_E2alf" vaccination using a multiplex microsphere immunoassay	Xia et al., 2015
	Design of two E^{rns} antibody ELISAs	Aebischer et al., 2013
	Inter-laboratory comparison test of possible discriminatory assays	Schroeder et al., 2012
	Development of a RT-PCR system for vaccine/field virus discrimination (genetic DIVA)	Liu et al., 2009
	Development of a RT-PCR system for vaccine/field virus discrimination (genetic DIVA)	Leifer et al., 2009
Field study	Oral vaccination of wild boar in faunistic hunting farms in Umbria, bait vaccination, comparative study in captive wild boar, vaccine stability	Feliziani et al., 2014
Supplemental studies	Cytokine and immunoglobulin isotype profiles	Renson et al., 2014
	Challenge two days after oral immunization, cytokine profiles	Renson et al., 2013

3 Objectives

Protection against transplacental transmission

In the framework of licensing, performance characteristics of CP7_E2alf as an emergency vaccine were the main focus. For this reason, harsh challenge models were implemented as a worst case scenario. Under these conditions, CP7_E2alf could not confer complete protection against vertical transmission in all studies and a warning, not to vaccinate breeding sows, was included in the product description. However, these challenge models do not mirror the current field situation where moderately virulent virus strains prevail and all production levels are vaccinated if the disease is endemic. Beyond that, the feared “carrier-sow syndrome” is not to be expected with highly virulent strains. To test the hypothesis that CP7_E2alf is able to confer protection against vertical transmission of a more recent, relevant and moderately virulent CSFV strain, an efficacy trial was performed according to the guidelines of the OIE Manual of Diagnostic Tests and Vaccines (OIE Manual, Chapter 2.8.3).

4 Publication

The reference section of the manuscript is presented in the style of the journal and is not included at the end of this document. The labeling of figures and tables corresponds to the published form of the manuscript.

4.1. Protection against transplacental transmission of moderately virulent classical swine fever virus using live marker vaccine "CP7_E2alf"

Julia Henke[1], Jolene Carlson[1,2], Laura Zani[1], Simone Leidenberger[1], Theresa Schwaiger[1,3], Kore Schlottau[1], Jens P. Teifke[3], Charlotte Schröder[3], Martin Beer[1] and Sandra Blome[1*]

[1] Institute of Diagnostic Virology, Friedrich-Loeffler-Institut, Suedufer 10, 17493 Greifswald - Insel Riems, Germany
[2] Institute of Immunology, Friedrich-Loeffler-Institut, Suedufer 10, 17493 Greifswald - Insel Riems, Germany
[3] Department of Experimental Animal Facilities and Biorisk Management, Friedrich-Loeffler-Institut, Suedufer 10, 17493 Greifswald - Insel Riems, Germany

Vaccine 2018
Volume 36, Issue 29
DOI: 10.1016/j.vaccine.2018.06.014

Publication

Vaccine 36 (2018) 4181–4187

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

Protection against transplacental transmission of moderately virulent classical swine fever virus using live marker vaccine "CP7_E2alf"

Julia Henke [a], Jolene Carlson [a,b], Laura Zani [a], Simone Leidenberger [a], Theresa Schwaiger [a,c], Kore Schlottau [a], Jens P. Teifke [c], Charlotte Schröder [c], Martin Beer [a], Sandra Blome [a,*]

[a] Institute of Diagnostic Virology, Friedrich-Loeffler-Institut, Suedufer 10, 17493 Greifswald - Insel Riems, Germany
[b] Institute of Immunology, Friedrich-Loeffler-Institut, Suedufer 10, 17493 Greifswald - Insel Riems, Germany
[c] Department of Experimental Animal Facilities and Biorisk Management, Friedrich-Loeffler-Institut, Suedufer 10, 17493 Greifswald - Insel Riems, Germany

ARTICLE INFO

Article history:
Received 22 September 2017
Received in revised form 4 May 2018
Accepted 5 June 2018
Available online 9 June 2018

Keywords:
Classical swine fever
Marker vaccine
Efficacy
Transplacental transmission
Differentiating infected from vaccinated animals

ABSTRACT

Classical swine fever (CSF) remains as one of the most important infectious diseases of swine. While prophylactic vaccination is usually prohibited in free countries with industrialized pig production, emergency vaccination is still foreseen. In this context, marker vaccines are preferred as they can reduce the impact on trade.

The live-attenuated Suvaxyn® CSF Marker vaccine by Zoetis (based on pestivirus chimera "CP7_E2alf"), was recently licensed by the European Medicines Agency. Its efficacy for the individual animal had been shown in prior studies, but questions remained regarding protection against transplacental transmission. To answer this question, a trial with eight pregnant sows and their offspring was performed as prescribed by the OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals. Six of the sows were intramuscularly vaccinated on day 44 of gestation, while the other two remained as unvaccinated controls. All sows were challenged with the moderately virulent CSFV strain "Roesrath" and euthanized shortly before the calculated farrowing date. Sows and piglets were grossly examined and necropsied. Organs (spleen, tonsil, lymph node, and kidney), EDTA-blood and serum were collected from all animals. All samples were tested for antibodies against CSFV glycoproteins E2 and E^{rns} as well as CSFV (virus, antigen and genome). It could be demonstrated that the vaccine complies with all requirements, i.e. no virus was found in the blood of vaccinated sows and their fetuses, and no antibodies were found in the serum of the fetuses from the vaccinated sows. All controls were valid.

Thus, it was demonstrated that a single dose vaccination in the sows efficiently protected the offspring against transplacental infection with a moderately virulent CSFV strain.

1. Introduction

Classical swine fever (CSF) is one of the most important diseases in swine, with a large impact on pig production worldwide [1]. Because of this, outbreaks are notifiable to the OIE [1]. To control the disease that can exhibit variable clinical courses, live-attenuated and E2 subunit vaccines exist and are commercially available. The former have been used in eradication programs throughout the world and are still being used in endemically affected countries [2,3].

In free countries, prophylactic vaccination is now usually prohibited but emergency vaccination is still foreseen [1]. In this context, marker vaccines are preferred as they can reduce the impact on trade [4,5].

Recently, pestivirus chimera "CP7_E2alf" (Suvaxyn® CSF Marker, Zoetis) was licensed by the European Medicines Agency as live marker vaccine against CSF. Towards filing of the vaccine dossier, experimental focus was placed on tests that would show suitability for emergency vaccination scenarios in countries with industrialized pig production, i.e. provision of early protection after single vaccination [6]. This meant early and harsh challenge in most efficacy tests with highly virulent CSFV strains. Challenge usually happened before antibodies were detectable. Under these circumstances, solid protection was shown for the individual animal (including sows), but transplacental transmission in pregnant animals could not be prevented in some cases. Due to this, a warning was included in the summary of product characteristics that states that sows should not be vaccinated, due to the risk of birth

* Corresponding author.
E-mail address: sandra.blome@fli.bund.de (S. Blome).

https://doi.org/10.1016/j.vaccine.2018.06.014
0264-410X/

of immunotolerant persistently infected offspring (see http://www.ema.europa.eu/docs/en_GB/document_library/EPAR_-_Product_Information/veterinary/002757/WC500185867.pdf, visited July 2017). This warning refers to the fact that in-utero infection with low and moderately virulent CSFV strains can result in what is referred to as the 'carrier sow' syndrome [7–9]. Piglets born to these sows can be persistently infected while appearing healthy at the time of farrowing [10,11]. These infections may go undetected for months but are accompanied by constant shedding of high amounts of virus. The latter serves as a cause for virus transmission.

Excluding sows from vaccination can be feasible and advisable in emergency situations, but if the vaccine should be used in endemically affected countries to control the disease on a longer term, vaccination of sows is necessary to protect both sows and piglets [12]. As most virus strains circulating nowadays are moderately virulent [13], protection against these strains is probably much more relevant than early protection against highly virulent strains that are no longer circulating.

To test the hypothesis that " CP7_E2alf "is able to confer protection against vertical transmission of a relevant, moderately virulent CSFV strain, an efficacy test (protection against transplacental infection) was conducted according to the guidelines of the OIE Manual of Diagnostic Tests and Vaccines (OIE Manual, Chapter 2.8.3, paragraph 2.3.3. ii).

2. Material and methods

2.1. Experimental design

Following the guidelines of the OIE Manual, eight pregnant sows and their fetuses were used in this study. The pregnant sows were purchased from a commercial breeding farm with a high veterinary hygiene standard and brought to the high containment facilities at the Friedrich-Loeffler-Institut (FLI), Greifswald-Insel Riems in Germany. All sows were tested to confirm the absence of pestiviruses and antibodies against pestiviruses prior to the start of the trial. Upon arrival, the sows were randomly allocated either to the control group (two sows) or the vaccinated group (six sows). Sows were provided *ad libitum* access to water and were fed commercial feed for breeding sows. All applicable animal welfare regulations, including EU-directive 2010/63/EC and institutional guidelines, were followed. The animal experiment was approved by the competent authority (Landesamt für Landwirtschaft, Lebensmittelsicherheit und Fischerei Mecklenburg-Vorpommern, LALLF MV) under reference number 7221.3-1-077/16 (FLI 19/16).

On day 44 of gestation, the sows of the vaccine group were vaccinated intramuscularly (deep into the right neck using a 2 mL single-use syringe and a 20 G needle) with a single dose (1 mL, $10^{5.25}$ tissue culture infectious doses 50% ($TCID_{50}$)/mL) of Suvaxyn® CSF Marker, provided by Zoetis (batch T24070) while the control group remained unvaccinated. Twenty-one days after vaccination, both groups (vaccine and control group) were challenged oronasal with 5 mL of challenge material derived from an animal experiment at the FLI where whole blood was collected from swine infected with CSFV strain "Roesrath" (genotype 2.3, originating from Germany 2009, EU reference laboratory data base entry CSF1045). The blood had been defibrinated, and prepared as challenge material. The applied dose was $10^{5.25}$ $TCID_{50}$ per mL. Vaccine and challenge virus were back-titrated after administration to confirm the titer. Sows of both groups were sampled at seven and nine days post challenge. Whole blood (with EDTA) and serum were collected to detect viremia and seroconversion.

Following the OIE guidelines, the sows were humanely euthanized approximately one week prior to farrowing. All sows and their fetuses (reproductive performance see supplementary table 1) were examined grossly at necropsy. Serum and EDTA-treated whole blood, as well as samples of tonsil, lymph node, spleen and kidney were collected from all animals.

2.2. Additional treatments

All sows presented with lameness upon arrival and were treated with meloxicam, a nonsteroidal anti-inflammatory drug (Metacam, Boehringer Ingelheim, 20 mg/mL). During the study, some sows developed abscesses on the skin, especially on the ventrum and on the forelegs. These were cleaned daily and treated with cod liver oil zinc ointment. In the case of deep scratches and bite wounds, they were also treated with oxytetracycline spray (Engemycin Spray, MSD, 25 mg/ml).

2.3. Clinical monitoring

Rectal temperatures and clinical scores following the system proposed by Mittelholzer et al [14] were collected daily to determine the health status of the sows, especially after the challenge. Fever was defined as a rectal body temperature of >40 °C for two consecutive days. The observed parameters were liveliness, bearing, breathing, gait, skin, eyes, fecal consistency, and feed intake. Each was assigned a score from 0 (within normal limits) to 3 (severely abnormal).

2.4. Laboratory tests

2.4.1. Sample preparation and nucleic acid extraction

All laboratory tests were carried out in accordance with the EU Diagnostic Manual for CSF (Commission Decision 2002/106/EC) and the Technical Annex accompanying it. To obtain serum, native blood samples were centrifuged at 2031*g* for 20 min at room temperature. The resulting serum was aliquoted and stored at −80 °C. All tissue samples were cut in small pieces (3–4 mm) for homogenization with a metal bead in phosphate-buffered saline (PBS). Tissue pieces were homogenized with a TissueLyser (Qiagen). Viral RNA was extracted using the NucleoMag VET extraction kit (Macherey-Nagel) with the KingFisher extraction platform (Thermo Fisher Scientific). An internal control RNA (IC2) was added to all extractions [15]. Nucleic acids were subsequently tested in the accredited routine CSFV-specific RT-qPCR that is established at the Germany National Reference Laboratory for CSF as CSF-System one [16]. All RT-qPCRs were performed with a Bio-Rad CFX 96 Real-Time Detection System (Bio-Rad). Results were recorded as quantification cycle (Cq) values.

Peripheral-blood mononuclear cells (PBMCs) were prepared by adding 1 mL of dextran sulfate solution (5%) to 5 mL of EDTA-blood. After one hour at room temperature, the opaque supernatant was centrifuged at 2000 rpm for 10 min, washed twice with PBS and finally resuspended in 2 mL of PBS.

2.4.2. Virus isolation

Virus isolation was performed with 100 μL homogenized organ material or PBMCs by incubation on porcine kidney (PK15) cells in 24-well plates for 72 h. Subsequently, plates were heat fixed and stained with an indirect immuno-peroxidase staining, using an anti-CSFV-E2 monoclonal mouse antibody mix and a polyclonal goat anti-mouse secondary antibody conjugated with horseradish peroxidase (Thermo Fisher Scientific).

Titrations to confirm the administered vaccine and virus doses were performed according to standard procedures as endpoint dilutions on PK15 cells. These titers were also obtained by indirect immuno-peroxidase staining.

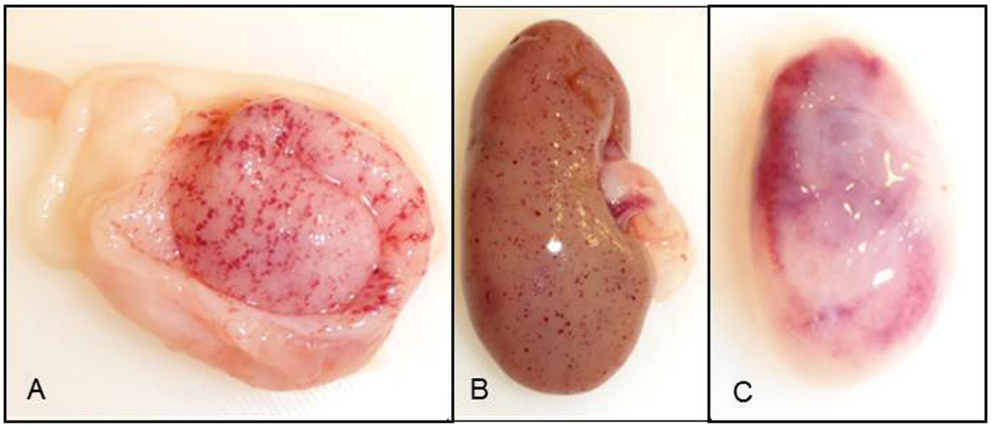


Fig. 1. Organs of one of the fetuses from naïve control sow 4913 with typical CSF signs. Bladder (A) and kidney (B) with petechial hemorrhages. Fetal lymph node (C), enlarged and hemorrhagic.

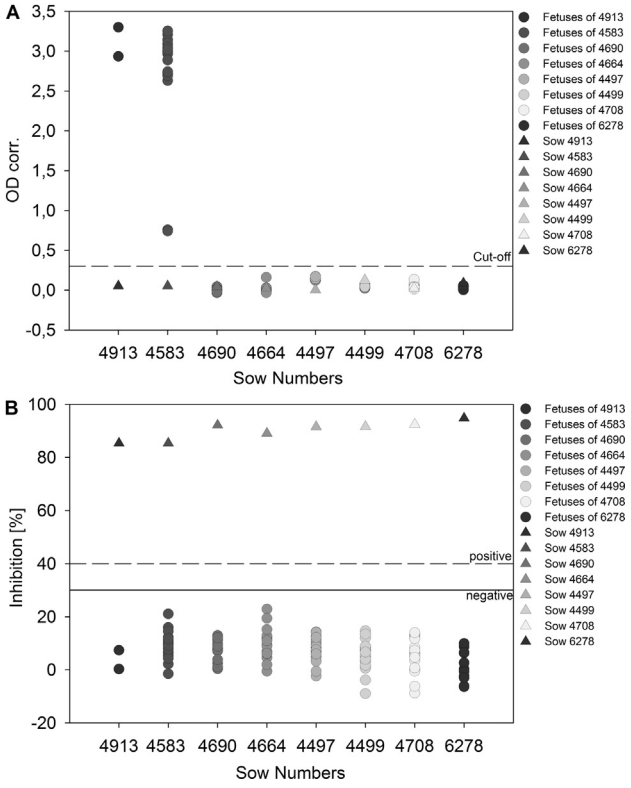


Fig. 2. Results of the IDEXX CSFV Antigen ELISA (A) and the IDEXX CSFV E2 antibody ELISA (B) in sows and their fetuses at the end of the trial. The values are presented as corrected optical densities and percentage of inhibition, respectively. Control sows are 4913 and 4583, all others belong to the vaccinated group.

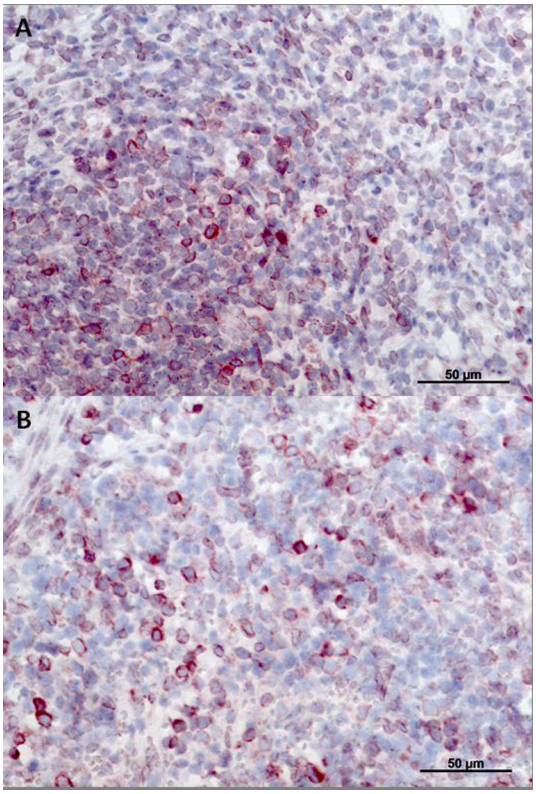


Fig. 3. Magnification 40X. Lymph node (A) and spleen (B) of a naïve control fetus 49 from sow 4913 infected with CSFV. Anti-CSFV staining is present within the mononuclear cells in the germinal centers of the lymph node and white pulp of the spleen.

2.4.3. Serological assays and antigen detection

Antigen detection was carried out on all sera using the Herd-Chek CSFV Ag/Serum ELISA (IDEXX Laboratories) following the manufacturer's protocol.

In addition, detection of CSFV E2 specific antibodies was performed using the IDEXX CSFV Ab ELISA (IDEXX Laboratories). CSFV E^{rns} specific antibodies were detected by the PrioCHECK CSFV E^{rns} ELISA (Thermo Fisher Scientific) and the Pigtype CSFV E^{rns} ELISA (Qiagen).

Neutralization peroxidase-linked antibody assays (NPLA) were also performed to show freedom of antibodies against pestiviruses using Border disease strain "Moredun" and Bovine viral diarrhea strain "CP7", respectively, on SFT-R (sheep fetal thymus) and KOP (bovine esophagus) cells. Neutralizing antibody titers against CSFV "Roesrath" and "Alfort/187" were also completed on PK15 cells. Titers were calculated as 50% neutralization dose (ND_{50}) using indirect immune peroxidase staining after an incubation of 72 h.

2.4.4. Flow cytometry analyses

To assess the induction of a cellular immune and memory response of peripheral blood mononuclear cells (PBMCs) EDTA blood from day seven post challenge was analyzed by flow cytometry as previously described [17]. Briefly, leukocytes were isolated by density gradient centrifugation using pancoll animal (Pan

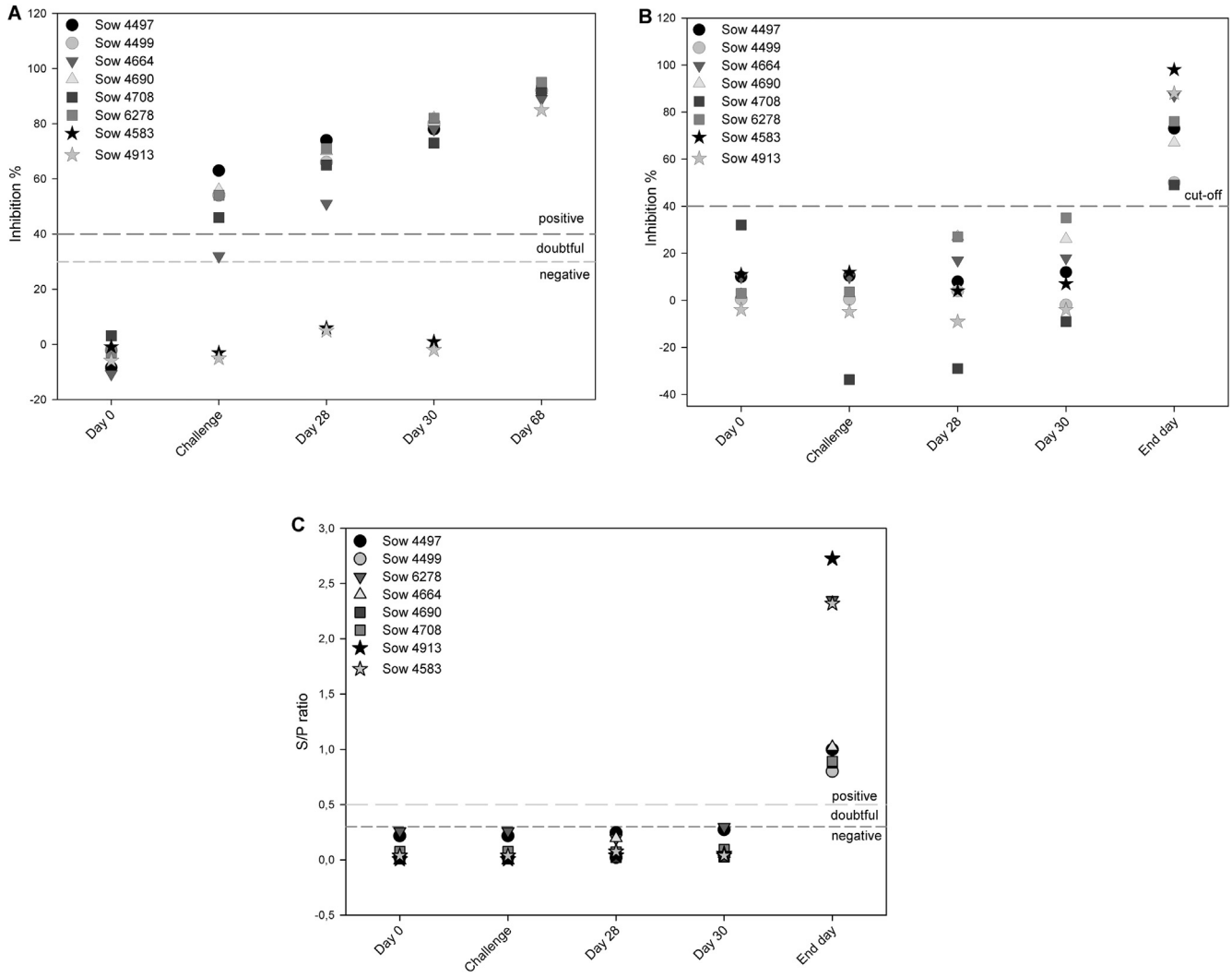


Fig. 4. Results of the IDEXX CSFV E2 antibody ELISA (A), the PrioCHECK CSFV Erns antibody ELISA (B) and the pigtype Erns antibody ELISA (C). The values are presented in percentage of inhibition and S/P ratio (sample-to-positive-control-ratio). Stars represent control sows (Sow 4583 and Sow 4913), vaccinated sows are represented by circles, squares and triangles.

Biotech). Immune cell subsets were identified using the following antibodies: anti-pig CD4 (mouse IgG2b, clone 74-12-4, in-house) with secondary anti-mouse IgG2b PerCP (dianova), anti-pig CD8α (FITC, clone 11/295/33, SouthernBiotech) and anti-pig CD8β (mouse IgG2a, clone PG164A, in-house) with secondary anti-mouse IgG2a AlexaFlour647 (dianova). After permeabilization of cells with the Intracellular Fixation & Permeabilization Buffer Set (eBioscience™) proliferating cells were visualized with Brilliant Violet™ 420-conjugated anti-human Ki67 antibody (clone B56, BD Biosciences). Perforin was stained using PE-conjugated anti-human perforin antibody (clone δG9, BD Biosciences). All analyses were run on BD Canto II flow cytometer, FACS DIVA (BD Biosciences) and FlowJo software (Tree Star Inc.).

2.4.5. Immunohistochemistry

For the detection of viral antigen by immunohistochemistry, tissues were collected, fixed in 10% neutral buffered formalin and paraffin embedded. Tissue sections were heated to 110 °C for 10 min in 10 mM citric buffer (pH 6.0) in a deckloaking chamber. Anti-CSFV Monoclonal Antibody BIO 275 (BioX diagnostics) (1:200) and a secondary anti-mouse biotinylated antibody (1:200) were applied. For a positive control, an RT-qPCR positive tonsil from a domestic pig infected with CSFV "Alfort/ 187" was used. A tonsil from a slaughter pig from the abattoir was used as a negative control.

3. Results and discussion

3.1. Clinical and pathological observations

Throughout the vaccine trial, all sows remained healthy and showed no fever or other signs of CSF. Two sows developed multiple abscesses, which were not related to CSFV or the vaccine. The sows of the control group had normal temperatures throughout the trial and showed only mild depression on day 13 after the challenge.

The gross observations at necropsy showed no CSF-related lesions in the vaccinated sows or their fetuses. Some of the fetuses of the control sows had "classical" signs of CSF (Fig. 1), including petechiae in the kidneys and the bladder as well as enlarged and marbled lymph nodes [18]. Furthermore, skin petechiae could be seen in some of these fetuses (Fig. 1).

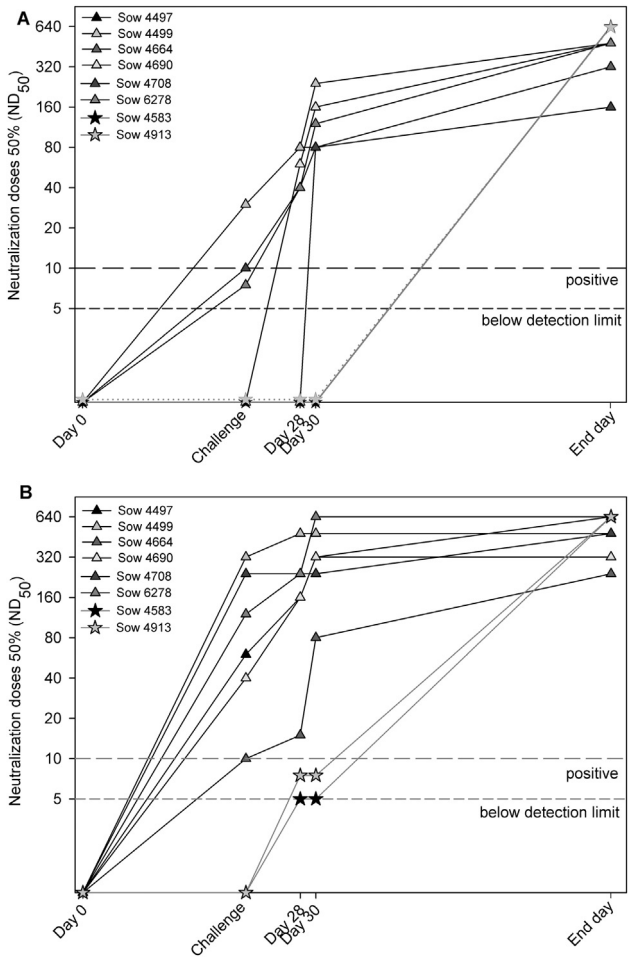


Fig. 5. Antibody responses in neutralization peroxidase linked antibody assays (NPLA), using the CSFV "Rösrath" (A) and CSFV "Alfort/187" (B) as test viruses. Titers are presented as group mean neutralization doses 50% (ND50).

3.2. Virus detection

Requirements: The test is valid if virus is found in at least 50% of the fetuses from the control sows (excluding mummified fetuses). No virus should be found in the blood of vaccinated sows and in fetuses from vaccinated sows.

All vaccinated sows were negative for CSFV in all performed tests throughout the trial. In contrast, both control sows were tested positive for CSFV by RT-qPCR in blood (Cq values 23.5–31.0), by antigen ELISA in serum (Fig. 2), and by virus isolation from PBMCs on days seven and nine after challenge. At the end of the trial, sera of the control sows were negative in the antigen ELISA again. However, the organ pools of the control sows collected at the end day were positive in the RT-qPCR but negative by virus isolation. It has to be noted that the PCR results of the control sows showed a decrease of Cq-values from day seven to day nine after challenge infection, indicating that the virus was able to considerably replicate in the unvaccinated sows despite the lack of obvious clinical signs. The latter underlines the possible impact of moderately virulent CSFV strains when it comes to breeding animals. Under field conditions, the infection would probably have gone unnoticed till persistently infected piglets would have spread the virus.

The samples of the fetuses of the vaccinated sows tested negative for CSFV in RT-qPCR, antigen ELISA (Fig. 2) and virus isolation. This was in clear contrast to the samples of the fetuses from the control sows, as nearly all organ samples were positive in qPCR (semi-quantitative results are presented in Supplemental Fig. 1) and virus isolation. Only one mummy and two stillborn piglets of sow 4583 were negative by virus isolation. Beyond that, all fetuses from the control sows tested positive with the serum antigen ELISA (Fig. 2).

Thus, all of the requirements regarding virus detection were fulfilled, since all of the fetuses of the control group were positive for viral genome in PCR and viral antigen in ELISA. Furthermore, no virus (neither viral antigen nor genome) was detected in blood or organs of the vaccinated sows throughout the whole trial and no virus (again neither replicating virus nor viral genome) was detected in the organ pools of their fetuses.

3.3. Supplementary antigen detection in fetal tissues

In fetuses with gross lesions and positive RT-qPCR results, immunohistochemistry was performed. Positive staining for CSFV antigen was apparent in the mononuclear phagocytic cells and lymphocytes of the lymph node, spleen, tonsil, and kidney (see Fig. 3). Although no RT-qPCR was completed with bone marrow, liver, and lung, CSFV antigen could also be detected by immunohistochemistry in some fetuses. Faint staining specific for CSFV antigen was also apparent in the extramedullary hematopoietic cells scattered throughout the liver.

3.4. Antibody detection

Requirement: Antibodies against CSFV should not be found in the serum of the fetuses from the vaccinated sows.

For samples taken from the sows, antibody ELISAs (IDEXX CSFV Ab, PrioCHECK CSFV E^{rns} and Pigtype CSFV E^{rns}) were performed on the day of vaccination (0 dpv), the day of challenge (21 dpv), day seven and nine after challenge (28 and 30 dpv), and at the end of the experiment on 65–68 dpv. All sows tested negative in all E2 and E^{rns} antibody ELISAs and also NPLA (see below) prior to vaccination. The control sows remained negative also in the blood samples from 21, 28 and 30 dpv, but were positive for anti-E2 and E^{rns} antibodies at the end of the experiment (Fig. 4).

In contrast, all vaccinated sows tested positive (five animals) or doubtful (one animal) for E2 antibodies by ELISA at the day of challenge. From 28 dpv onward, all vaccinated sows were positive in the E2 antibody ELISA. In the E^{rns} ELISAs, on the other hand, the vaccinated sows tested negative in all samples except on the last day at 65–68 dpv. Sera collected from the fetuses were tested negative in all antibody ELISAs.

The results of the ELISAs, particularly the discriminatory E^{rns} assays, confirm the marker concept of the vaccine. Antibodies against E^{rns} were only seen after the challenge (Fig. 4) [19].

Sera were also subjected to neutralization assays. On the day of vaccination, all sows tested negative for neutralizing antibodies against all tested pestiviruses. The control sows remained negative for neutralizing antibodies against CSFV strains "Roesrath" and "Alfort/187" on 28 and 30 dpv, while all of the vaccinated sows were positive beginning on 21 dpv and remained positive until the end of the experiment. On 21 dpv, two of the vaccinated sows tested negative for neutralizing antibodies against CSFV strain "Alfort/187", but tested positive on day 7 and 9 after challenge as well as on the last day of the experiment (Fig. 5).

In the NPLA, the control sows tested positive for neutralizing antibodies against CSFV strains "Alfort/187" and "Roesrath" on the last day, but were negative in all earlier samples. All fetuses tested negative for neutralizing antibodies against both CSFV strains in NPLA.

Therefore, the requirement regarding the detection of antibodies was fulfilled. The absence of antibodies in the fetuses of the control sows indicates either acute-lethal or persistent infection.

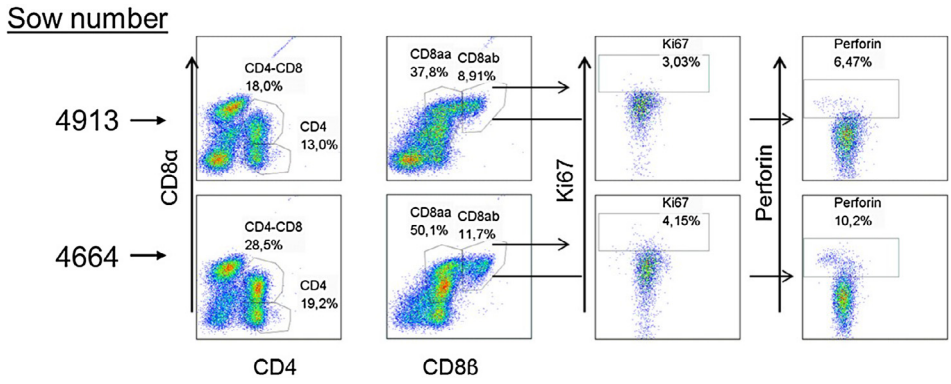


Fig. 6. Representative dot plots from sow 4913 (naïve sow, upper part of the figure) and sow 4664 (vaccinated sow, lower part of the figure) in flow cytometric analysis of PBMCs. Comparison of CD4/CD8 double positive T-cells, cytotoxic T-cells (CD8αβ), proliferation and perforin production.

The latter would confirm occurrence of the feared carrier-sow-syndrome.

3.5. Flow cytometric analyses of cellular immune response

A flow cytometric analysis of PBMCs from all sows was performed seven days after challenge. Representative dot plots from sows 4913 (control) and 4664 (immunized) are shown in Fig. 6. Immunized sows showed an increased frequency of CD4/CD8 double positive T-cells, which are known to be mature antigen-experienced T-cells. In line with this, the overall frequency of CD8 cells was higher in immunized sows compared to control sows. The cytotoxic T-cells (CD8αβ) were increased in immunized animals and showed higher capacity to proliferate (Ki67-positive cells) as well as to produce perforin, which in turn mediates cytotoxicity in infected cells. To determine the capacity of antigen-experienced T-cells from immunized animals further re-stimulation-studies are needed.

4. Conclusions

It was demonstrated that pregnant sows and their fetuses were fully protected with a single dose of the DIVA vaccine "CP7_E2alf". Vertical transmission of a relevant, moderately virulent CSFV was completely prevented. Also in terms of virus detection in control animals, all requirements of the OIE manual of standards for diagnostic tests and vaccines were fulfilled. In addition, reliable and accurate serological differentiation between infected and vaccinated animals was demonstrated. Thus, this study adds to former safety and efficacy studies of Suvaxyn® CSF Marker (Zoetis).

In the context of emergency vaccination, the previous experience that protection might be incomplete upon early challenge with highly virulent strains should still be taken into consideration. The decision to vaccinate sows has to depend upon the risk assessment implemented by the authorities during potential outbreak situations.

Funding and conflicts of interest

The research received financial support by Zoetis. Moreover, the vaccine was provided by the manufacturer. No other conflicts of interest exist.

Authors' contributions

Sandra Blome (SB) and Martin Beer (MB) designed the study, and Julia Henke (JH), Jolene Carlson (JC), Charlotte Schröder (CS), Laura Zani (LZ) and SB conducted the animal trial. Laboratory work was carried out by JH, JC, LZ, Kore Schlottau (KS), Simone Leidenberger (SL), and Theresa Schwaiger (TS). Necropsy and pathological studies were conducted by JC, Jens P. Teifke (JPT), JH, and SB. The manuscript was prepared by JH and critically revised by SB and MB.

Acknowledgements

The authors would like to thank Ulrike Kleinert and Ulrike Wilhelm for their excellent laboratory work. We also thank Gabriele Czerwinski for her exceptional work with the tissue preparation and immunohistochemistry. Additionally, the authors would like to acknowledge all animal caretakers and technical staff involved in this study.

Appendix A. Supplementary material

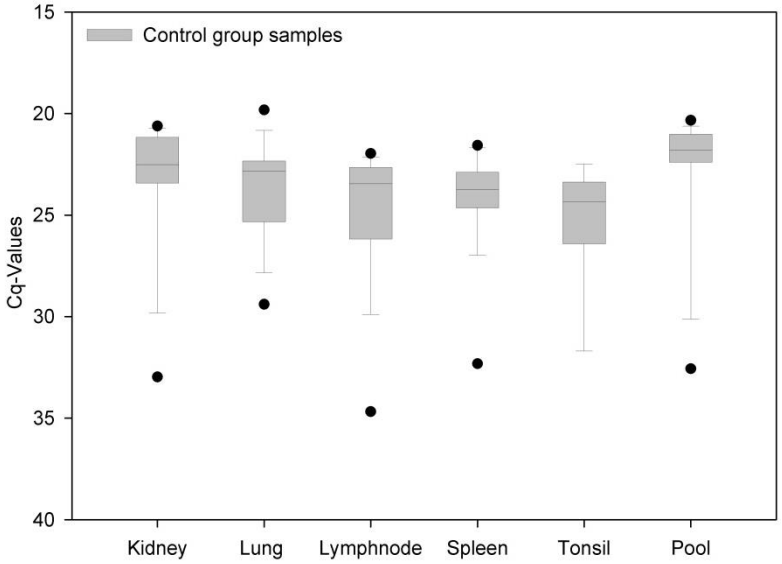
Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.vaccine.2018.06.014.

References

[1] Edwards S, Fukusho A, Lefevre PC, Lipowski A, Pejsak Z, Roehe P, et al. Classical swine fever: the global situation. Vet Microbiol 2000;73:103–19.
[2] van Oirschot JT. Vaccinology of classical swine fever: from lab to field. Vet Microbiol 2003;96:367–84.
[3] Greiser-Wilke I, Moennig V. Vaccination against classical swine fever virus: limitations and new strategies. Anim Health Res Rev 2004;5:223–6.
[4] van Oirschot JT. Emergency vaccination against classical swine fever. Dev Biol (Basel) 2003;114:259–67.
[5] Dong XN, Chen YH. Marker vaccine strategies and candidate CSFV marker vaccines. Vaccine 2007;25:205–30.
[6] Blome S, Wernike K, Reimann I, König P, Moss C, Beer M. A decade of research into classical swine fever marker vaccine CP7_E2alf (Suvaxyn® CSF Marker): a review of vaccine properties. Vet Res 2017;48(1):51.
[7] Dahle J, Liess B. A review on classical swine fever infections in pigs: epizootiology, clinical disease and pathology. Comp Immunol Microbiol Infect Dis. 1992;15:203–11.
[8] Plateau E, Vannier P, Tillon JP. Atypical hog cholera infection: viral isolation and clinical study of in utero transmission. Am J Vet Res 1980;41:2012–5.
[9] Terpstra C. Epizootiology of swine fever. Vet Q 1987;9(Suppl. 1):50S–60S.
[10] Depner KR, Müller A, Gruber A, Rodriguez A, Bickhardt K, Liess B. Classical swine fever in wild boar (*Sus scrofa*)–experimental infections and viral persistence. Dtsch Tierarztl Wochenschr 1995;102:381–4.
[11] Moennig V, Floegel-Niesmann G, Greiser-Wilke I. Clinical signs and epidemiology of classical swine fever: a review of new knowledge. Vet J 2003;165:11–20.
[12] Munoz-Gonzalez S, Sordo Y, Perez-Simo M, Suarez M, Canturri A, Rodriguez MP, et al. Efficacy of E2 glycoprotein fused to porcine CD154 as a novel chimeric subunit vaccine to prevent classical swine fever virus vertical transmission in pregnant sows. Vet Microbiol 2017;205:110–6.
[13] Beer M, Goller KV, Staubach C, Blome S. Genetic variability and distribution of Classical swine fever virus. Anim Health Res Rev 2015;16:33–9.

[14] Mittelholzer C, Moser C, Tratschin J-D, Hofmann MA. Analysis of classical swine fever virus replication kinetics allows differentiation of highly virulent from avirulent strains. Vet Microbiol 2000;74:293–308.
[15] Hoffmann B, Depner K, Schirrmeier H, Beer M. A universal heterologous internal control system for duplex real-time RT-PCR assays used in a detection system for pestiviruses. J Virol Methods 2006;136:200–9.
[16] Hoffmann B, Beer M, Schelp C, Schirrmeier H, Depner K. Validation of a real-time RT-PCR assay for sensitive and specific detection of classical swine fever. J Virol Methods 2005;130:36–44.
[17] Wang Z, Burwinkel M, Chai W, Lange E, Blohm U, Breithaupt A, et al. Dietary *Enterococcus faecium* NCIMB 10415 and zinc oxide stimulate immune reactions to trivalent influenza vaccination in pigs but do not affect virological response upon challenge infection. PLoS One 2014;9:e87007.
[18] Blome S, Staubach C, Henke J, Carlson J, Beer M. Classical swine fever-an updated review. Viruses 2017;9.
[19] Meyer D, Fritsche S, Luo Y, Engemann C, Blome S, Beyerbach M, et al. The double-antigen ELISA concept for early detection of Erns -specific classical swine fever virus antibodies and application as an accompanying test for differentiation of infected from marker vaccinated animals. Transbound Emerg Dis 2017.

Supplemental Figures



Supplemental Figure 1: Results of the qPCR of the samples from the fetuses of the control sows. The values are presented as Cq-values in form of a box-plot diagram.

Supplemental table 1: Fetal counts

Status	Sow No.	Piglets	Mummies	Stillborn	Live
Naive	4913	2	0	0	2
Naive	4583	22	2	4	16
Vaccinated	6278	13	0	0	13
Vaccinated	4664	15	0	0	15
Vaccinated	4497	24	1	0	23
Vaccinated	4499	19	0	0	19
Vaccinated	4708	23	3	0	20
Vaccinated	4690	15	0	0	15

5 Discussion and outlook

5.1. Discussion

Given the fact that CSF is still one of the most important diseases of pigs worldwide, vaccination is constantly under debate for both endemic and emergency settings. In endemically affected countries, vaccination is used to lower the disease burden. Vaccination is then embedded into a mandatory control program (Greiser-Wilke and Moennig 2007; Postel et al. 2018; van Oirschot 2003a). Under these circumstances, vaccines are usually applied to breeding stock and different age classes of weaners or young fattening animals. Emergency vaccination in response to massive outbreaks is a tool to prevent further spread of the disease and to protect free areas from introduction. Both vaccination to kill and vaccination to live are discussed among stakeholders. Yet, emergency vaccination of breeding animals is viewed with caution as these animals will remain in the population and may cause diagnostic problems and disturb trade. Moreover, old reports of carrier-sow-syndromes in herds that were emergency vaccinated in the incubation period exist and created mistrust. In any case, before vaccination of breeding animals there has to be a risk assessment based on solid data, especially for new marker vaccines such as CP7_E2alf. While studies in reproductive boar were carried out to assess the shedding of vaccine virus in semen (Dräger et al., 2016), there were still open questions regarding protection against vertical transmission and thus suitability for sow vaccination. The latter question was key part of the presented study.

As mentioned above, the “carrier-sow-syndrome”, resulting from vertical transmission from subclinical sows to their fetuses, is the most feared phenomenon in vaccinated sows without full protection. It has to be kept in mind that viral persistence happens through immunotolerance. The placenta type of pigs prevents the transfer of maternal antibodies and other maternal immune components (Bruno Machado Bertasoli 2015; Sinkora and Butler 2009) and thus, prevention of the initial event, i.e. transplacental transmission and therefore infection of the fetus, is the main target.

Previous studies with the live attenuated C-strain vaccine already investigated the protection against transplacental transmission of CSFV. One of these studies investigated the protective effect of an oral vaccination of pregnant sows approximately five weeks after insemination. Two experiments were carried out, one with a highly virulent and the other with a moderately virulent CSFV-strain for challenge in mid-gestation. All fetuses of the vaccinated sows, were

virologically and serologically negative, whereas the fetuses of the control sows tested positive for CSFV (Kaden et al. 2008). So it was shown, that vaccination with the C-strain vaccine protects against transplacental transmission of CSFV.

In a study conducted with the subunit marker vaccine Porcilis® Pesti full protection against transplacental transmission of CSFV could not be shown. The sows were vaccinated two times before insemination and developed neutralizing antibodies five weeks after the first vaccination. Although the vaccinated sows were protected against a challenge with a low virulent CSFV strain, in one out of ten litters fetuses were viraemic and organ samples of some of these fetuses were positive for CSFV (Ahrens et al. 2000). In two studies similar results were shown with the two vaccines Bayovac CSF Marker and Porcilis® Pesti. It was shown, that neither a one shot vaccination (day 46 of gestation) , nor a two shot vaccination (carried out 25 and 46 days of gestation) were able to completely prevent transplacental transmission of CSFV (Depner et al. 2001). Another study also compared a one and two shot vaccination with a CSFV E2-subunit vaccine. In this case, vaccination was carried out four weeks before insemination and for the twice vaccinated group again two weeks after insemination. The sows were challenged with a moderately virulent CSFV-strain six weeks after insemination. The fetuses of the twice vaccinated sows were protected against an infection with CSFV, whereas in the group of the once vaccinated sows in one out of nine litters viraemic fetuses were found (de Smit et al. 2000). Taken together all these studies, performed with first generation marker vaccines, showed difficulties in full protection against transplacental transmission of CSFV, especially in a scenario with one shot vaccination during pregnancy.

For CP7_E2alf, unpublished results are included in the vaccine dossier that show lack of protection in some cases when a highly virulent CSFV strain was used for early challenge (CORDIS 2013). For this reason, a warning was included in the summary of product characteristics: “Sows should not be vaccinated, due to the risk of birth of immunotolerant persistently infected offspring(European Medicines Agency - Committee for Medicinal Products for Veterinary Use 2015).” In our opinion, this warning is an overestimation, as infection of the dam with a highly virulent strain would lead to severe clinics in the sow and therefore the animal would be recognized as infected with CSFV and removed from the farm. Furthermore, a highly virulent strain would normally lead to the death of the fetuses. In all these cases, there would be no persistent infection but abortion and stillbirth. In literature, only low and moderately virulent strains are able to induce tolerance (Dahle and Liess 1992;

Depner et al. 1995; Kern et al. 1999; Moennig, Floegel-Niesmann, and Greiser-Wilke 2003; Rossi et al. 2005). Based on this background, it was important to show that vaccination of sows could protect the offspring against transplacental infection with one of the recent moderately virulent CSFV field strains from Europe which would be likely to generate PI animals.

The efficacy test presented in this thesis was conducted according to the guidelines for CSF vaccines of the OIE Manual of Diagnostic Tests and Vaccines (OIE Manual, Chapter 2.8.3). The OIE efficacy testing is based on an emergency scenario with an one shot vaccination during pregnancy in a narrow timeframe before mid-gestation when the genesis of PI animals is most likely. Since most of the sows nowadays, especially in large breeding farms, are hormonally synchronized, it is most likely that the exact date of insemination is known. So the possibility to determine an exact vaccination timepoint which does not contain the risk for undetected genesis of PI animals is given in most of the breeding facilities. Using the vaccine in a farm with no knowledge of the stage of gestation would not be advisable. The worst case scenario would be, that despite the succesful vaccination of the sow, PI animals emerge, since the vaccination would have been too late for the development of an appropriate immune response of the sow to prevent the transmission of virus through the placental barrier.

In our study eight pregnant sows and their fetuses were used. On day 44 of gestation, six sows were vaccinated intramuscularly with a single dose of CP7_E2alf (Suvaxyn® CSF Marker, Zoetis), while two sows remained unvaccinated. Twenty-one days after vaccination, all sows were challenged with the moderately virulent CSFV strain “Rösrath”.

These results of the study showed full protection of the vaccinated sows against the challenge infection. On the day of the challenge, all vaccinated sows tested positive or at least doubtful (one sow) in the performed E2-ELISA and four out of six sows were positive for neutralizing antibodies in the performed neutralization peroxidase-linked antibody assays (NPLA). One week later, day 28 after vaccination and one week after the challenge, all vaccinated sows tested positive for neutralizing antibodies in NPLA and for anti-E2 antibodies in the antibody ELISA. These results are the foundation for the protection of the fetuses against transplacental transmission of CSFV “Rösrath”. Neutralization through antibodies of the sow and therefore prevention of transmission of CSFV across the placental barrier most likely protects the fetuses from the infection with CSFV. The immune system of the fetuses would not be able to mount an immune response against the virus as shown for the fetuses of the control sows.

All samples of the fetuses of the vaccinated sows tested negative for CSFV in RT-qPCR, antigen ELISA and virus isolation. The samples of the fetuses of unvaccinated control sows tested positive in all those tests, so viral genome and infectious virus was detected in blood and organs of these animals. Furthermore, in some of the fetuses of the control group "classical" signs of CSF could be observed. Some had petechiae in the skin, the kidneys and the bladder, compared to the animals of the vaccinated group where no signs of infection were found. Given the virus detection in many organs of the control piglets, it can be assumed that several animals would have been persistently infected.

An important finding was also, that the unvaccinated sows showed almost no clinical signs but shedding and vertical transmission. Thus, the used challenge virus would create a severe, high impact problem in the field. Worldwide, the strains of CSFV seem to be developing from high virulent to moderately virulent strains (Edwards et al. 2000; Lange et al. 2012). Moderately virulent CSFV strains entail different problems than highly virulent strains: an infection with those strains can go unnoticed in older animals as it was demonstrated in different studies including the presented (Lohse, Nielsen, and Uttenthal 2012; Tarradas et al. 2014).

These moderately virulent strains are also associated with the recently described phenomon of postnatal persistence, a course of the disease where piglets get persistently infected when challenged with a moderately virulent CSFV strain in the first 48 hours after birth (Cabezon et al. 2015; Munoz-Gonzalez et al. 2015b). The affected animals do not seroconvert but constantly shed virus. Given the high viral load in their bodies, CSF vaccination fails completely. This is mainly due to superinfection exclusion or interference (Munoz-Gonzalez et al. 2016). This scenario was already shown for vaccination with the C-strain vaccine. The persistently infected animals were vaccinated six weeks after challenge with a moderately virulent CSFV strain but did not develop a specific immune response and no neutralizing antibodies were detected (Munoz-Gonzalez et al. 2015a). The problem of unsuccessful vaccination is mostly of importance in endemic countries, where mandatory vaccination is carried out. For emergencies it does not seem to play a crucial role but should still be considered because excessive monitoring would be implemented in affected areas. Nonetheless, for the application of the vaccine in either situation, it has to be tested whether solid protection of the sows could prevent postnatal persistence through maternally derived antibodies. First results of a corresponding study show that this effect can be achieved by the live marker vaccine (Henke et al., manuscript in preparation). The results of this study would influence the

decision for utilization of the live marker vaccine CP7_E2alf in an emergency vaccination program. With the study presented in this publication it was demonstrated, that it might not be necessary to exclude sows and breeding farms from vaccination programs neither in emergency situations nor endemically affected countries. These data were generated following the official OIE guidelines and provide a very solid experimental basis. With a solid and a thoroughly thought through vaccination program it could be beneficial to also include breeding farms in areas with high density of pig production.

However, in the context of emergency vaccination, the previous experience that protection might be incomplete upon early challenge with highly virulent strains should still be taken into consideration and has to be seen by implementing adapted diagnostic procedures. The decision to vaccinate sows therefore has to depend upon the risk assessment implemented by the authorities during potential outbreak situations, and this thesis provides the necessary data to allow implementation of a live marker vaccine under suitable conditions.

5.2. Outlook

We could demonstrate that the licensed live marker vaccine CP7_E2alf (Suvaxyn® CSF Marker) is a powerful tool for CSF control and could be used for breeding farms if indicated. Vaccination of breeding sows could then also help to overcome additional problems such as the recently described phenomenon of postnatal persistence in endemically affected countries, a course of the disease where piglets get persistently infected when challenged with a moderately virulent CSFV strain in the first 48 hours after birth (Cabezon et al. 2015; Munoz-Gonzalez et al. 2015b). The affected animals do not seroconvert but constantly shed virus. Given the high viral load in their bodies, CSF vaccination fails completely. This is mainly due to superinfection exclusion or interference (Munoz-Gonzalez et al. 2016). In this context, it has to be tested whether solid protection of the sows could prevent postnatal persistence through maternally derived antibodies. First results of a corresponding study show that this effect can be achieved by the live marker vaccine (Henke et al., manuscript in preparation).

Another aspect which also needs further evaluation is the performance and possible optimization of marker tests. These test systems have to be further evaluated with different samples of vaccinated animals at different time points after vaccination and different numbers of administered vaccinations, to show the reliability of these systems especially in emergency scenarios (Schroeder et al. 2012).

So far, the vaccine is only licensed for active immunization by intramuscular injection of pigs from seven weeks of age onwards (European Medicines Agency - Committee for Medicinal Products for Veterinary Use 2015). For the future it is important that the vaccine will be also licensed for oral vaccination and the utilization in wild boar, since oral vaccination of wild boar populations in threatened areas is stated as option by the European communities in an emergency outbreak situation to prevent transmission of CSF from wild boar to domestic pigs and vice versa (Council Directive 2001/89/EC). A first field trial, carried out in Italy, gave promising results using the bait formulation which is generally used for C-strain vaccination, either as single or double vaccination (Feliziani et al. 2014). The application of a marker vaccine in the field would clearly improve the monitoring of CSF outbreaks, which is nearly impossible with conventional live attenuated vaccines without the DIVA principle (Rossi et al. 2015).

In summary, efficacious vaccines exist against CSFV. Some of them allow DIVA concepts and can prevent trade restrictions. However, the vaccines have to be embedded into a control program with clear exit scenarios and risk assessment for different parts of the pig value chain.

If these prerequisted are met, vacccination, especially with marker vaccines such as CP7_E2alf, is a most powerful tool to control CSF.

6 Summary

Classical swine fever is one of the most important diseases in swine and despite implemented eradication programs still present in many countries worldwide. Recently, the live marker vaccine “CP7_E2alf” (Suvaxyn® CSF Marker, Zoetis), has been licensed by the European Medicines Agency. However, data are still missing regarding the use on breeding farms, expecially in breeding sow herds. A major concern was the protection against vertical transmission of CSFV. Since transmission of virus in mid-gestation can lead to persistently infected offspring, it was important to show that the vaccine is able to protect the fetuses against an infection with CSFV. Along these lines, a study was conducted according to the guidelines of the OIE Manual of Diagnostic Tests and Vaccines. A relevant, moderately virulent CSFV strain was used for challenge purposes.

It was demonstrated that the vaccine protected the fetuses completely against an infection with CSFV and fulfilled all requirements of the guidelines. No virus was found in the blood of vaccinated sows and their fetuses, and also no antibodies were found in the serum of the fetuses from the vaccinated sows. Furthermore, all of the fetuses of the control group tested positive for viral genome in qPCR and tested positive in virus isolation.

This study provided solid data, that it might not be necessary to exclude sows and breeding farms from (emergency) vaccination programs.

7 Zusammenfassung

Die Klassische Schweinepest (KSP) gehört bis heute zu den wichtigsten Infektionskrankheiten im Bereich der Schweineproduktion und ist trotz massiver Bekämpfungsmaßnahmen weltweit immer noch in vielen Ländern verbreitet.

Vor kurzem wurde der Lebendmarkerimpfstoff “CP7_E2alf” (Suvaxyn® CSF Marker, Zoetis) nach eingehender Prüfung durch die Europäische Arzneimittel-Argentur (European Medicines Agency, EMA) zugelassen. Trotzdem bestehen noch einige Wissenlücken, insbesondere in Bezug auf den Einsatz in Zuchtsauenbetrieben. Ein besonderes Augenmerk lag hierbei auf dem Schutz vor transplazentarer Übertragung des Virus auf die Föten, da es bei einer solchen Übertragung im mittleren Drittel der Trächtigkeit zur Geburt von persistierent infizierten Ferkeln kommen kann, welche eine Immuntoleranz gegenüber dem Virus zeigen und es durch Ausscheidung unkontrolliert weiterverbreiten können. In der vorliegenden Studie wurde geprüft, ob eine Impfung der Muttersau den Fetus vor einer Infektion mit dem Virus der Klassichen Schweinepest (KSPV) schützt. Der Versuchsaufbau entsprach den Vorgaben des Diagnosehandbuchs der Weltorganisation für Tiergesundheit. Als Material für die Belastungsinfektion der Tiere wurde ein aktueller moderat virulenter KSPV-Stamm verwendet.

Es konnte gezeigt werden, dass eine einmalige Impfung der Sau die Föten vor einer Infektion mit KSPV schützt. Es konnten kein Virus und keine Antikörper im Blut und den Organen der Föten nachgewiesen werden, darüber hinaus wurden alle Tiere der Kontrollgruppe positiv in der Virusisolierung und im Genormnachweis getestet. Die gewonnenen Ergebnisse erfüllen somit alle Anforderungen des Handbuchs.

Die in dieser Studie gewonnen Daten zeigen umfassend, dass es nicht unbedingt nötig ist, Sauen und Zuchtbetriebe von (Not)-Impfprogrammen auszuschließen.

8 References

Ahrens, U., V. Kaden, C. Drexler, and N. Visser. 2000. 'Efficacy of the classical swine fever (CSF) marker vaccine Porcilis Pesti in pregnant sows', *Vet Microbiol*, 77: 83-97.

Blome, S., A. Aebischer, E. Lange, M. Hofmann, I. Leifer, W. Loeffen, F. Koenen, and M. Beer. 2012. 'Comparative evaluation of live marker vaccine candidates "CP7_E2alf" and "flc11" along with C-strain "Riems" after oral vaccination', *Vet Microbiol*, 158: 42-59.

Blome, S., C. Gabriel, S. Schmeiser, D. Meyer, A. Meindl-Bohmer, F. Koenen, and M. Beer. 2014. 'Efficacy of marker vaccine candidate CP7_E2alf against challenge with classical swine fever virus isolates of different genotypes', *Vet Microbiol*, 169: 8-17.

Blome, S., C. Staubach, J. Henke, J. Carlson, and M. Beer. 2017. 'Classical Swine Fever-An Updated Review', *Viruses*, 9.

Bruno Machado Bertasoli, Amilton Cesar dos Santos, Rayan Silva de Paula, Alan S. Barbosa, Gerluza Aparecida Borges da Silva and Erika Cristina Jorge 2015. 'Swine placenta and placentation', *Brazilian Journal of Biological Sciences*, 2: 199-207.

Cabezon, O., A. Colom-Cadena, S. Munoz-Gonzalez, M. Perez-Simo, J. A. Bohorquez, R. Rosell, I. Marco, M. Domingo, S. Lavin, and L. Ganges. 2015. 'Post-Natal Persistent Infection With Classical Swine Fever Virus in Wild Boar: A Strategy for Viral Maintenance?', *Transbound Emerg Dis*.

Corapi, W. V., R. O. Donis, and E. J. Dubovi. 1988. 'Monoclonal antibody analyses of cytopathic and noncytopathic viruses from fatal bovine viral diarrhea virus infections', *J Virol*, 62: 2823-7.

CORDIS. 2013. "Final Report Summary - CSFV_GODIVA." In.

Dahle, J., and B. Liess. 1992. 'A review on classical swine fever infections in pigs: epizootiology, clinical disease and pathology', *Comp Immunol Microbiol Infect Dis*, 15: 203-11.

de Smit, A. J., A. Bouma, E. P. de Kluijver, C. Terpstra, and R. J. Moormann. 2000. 'Prevention of transplacental transmission of moderate-virulent classical swine fever virus after single or double vaccination with an E2 subunit vaccine', *Vet Q*, 22: 150-3.

Depner, K. R., A. Bouma, F. Koenen, D. Klinkenberg, E. Lange, H. de Smit, and H. Vanderhallen. 2001. 'Classical swine fever (CSF) marker vaccine. Trial II. Challenge study in pregnant sows', *Vet Microbiol*, 83: 107-20.

Depner, K. R., A. Muller, A. Gruber, A. Rodriguez, K. Bickhardt, and B. Liess. 1995. 'Classical swine fever in wild boar (Sus scrofa)--experimental infections and viral persistence', *Dtsch Tierarztl Wochenschr*, 102: 381-4.

Dräger, C., C. Schröder, P. König, B. Tegtmeyer, M. Beer, and S. Blome. 2016. 'Efficacy of Suvaxyn CSF Marker (CP7_E2alf) in the presence of pre-existing antibodies against Bovine viral diarrhea virus type 1', *Vaccine*, 34: 4666-71.

European Commission. 2001. 'COUNCIL DIRECTIVE 2001/89/EC'.

European Medicines Agency - Committee for Medicinal Products for Veterinary Use. 2014. 'CVMP assessment report for Suvaxyn CSF Marker

(EMEA/V/C/002757/0000)'. http://www.ema.europa.eu/docs/en_GB/document_library/EPAR_-_Public_assessment_report/veterinary/002757/WC500185869.pdf.

———. 2015. 'EPAR - Product Information (Suvaxyn CSF Marker)', Accessed 25.09.2018. http://www.ema.europa.eu/docs/en_GB/document_library/EPAR_-_Product_Information/veterinary/002757/WC500185867.pdf.

Feliziani, F., S. Blome, S. Petrini, M. Giammarioli, C. Iscaro, G. Severi, L. Convito, J. Pietschmann, M. Beer, and G. M. De Mia. 2014. 'First assessment of classical swine fever marker vaccine candidate CP7_E2alf for oral immunization of wild boar under field conditions', *Vaccine*, 32: 2050-5.

Frey, H. R., B. Liess, H. B. Richter-Reichhelm, K. von Benten, and G. Trautwein. 1980. 'Experimental transplacental transmission of hog cholera virus in pigs. I. Virological and serological studies', *Zentralbl Veterinarmed B*, 27: 154-64.

Gabriel, C., S. Blome, A. Urniza, S. Juanola, F. Koenen, and M. Beer. 2012. 'Towards licensing of CP7_E2alf as marker vaccine against classical swine fever-Duration of immunity', *Vaccine*, 30: 2928-36.

Goller, K. V., C. Drager, D. Hoper, M. Beer, and S. Blome. 2015. 'Classical swine fever virus marker vaccine strain CP7_E2alf: genetic stability in vitro and in vivo', *Arch Virol*, 160: 3121-5.

Greiser-Wilke, Irene, and Volker Moennig. 2007. 'Vaccination against classical swine fever virus: limitations and new strategies', *Animal Health Research Reviews*, 5: 223-26.

Hermanns, W., G. Trautwein, H. Meyer, and B. Liess. 1981. 'Experimental transplacental transmission of hog cholera virus in pigs. V. Immunopathological findings in newborn pigs', *Zentralbl Veterinarmed B*, 28: 669-83.

Kaden, V., E. Lange, H. Steyer, B. Lange, R. Klopfleisch, J. P. Teifke, and W. Bruer. 2008. 'Classical swine fever virus strain "C" protects the offspring by oral immunisation of pregnant sows', *Vet Microbiol*, 130: 20-7.

Kaden, V., H. Steyer, J. Schnabel, and W. Bruer. 2005. 'Classical swine fever (CSF) in wild boar: the role of the transplacental infection in the perpetuation of CSF', *J Vet Med B Infect Dis Vet Public Health*, 52: 161-4.

Kern, B., K. R. Depner, W. Letz, M. Rott, S. Thalheim, B. Nitschke, R. Plagemann, and B. Liess. 1999. 'Incidence of classical swine fever (CSF) in wild boar in a densely populated area indicating CSF virus persistence as a mechanism for virus perpetuation', *Zentralbl Veterinarmed B*, 46: 63-7.

König, P., S. Blome, C. Gabriel, I. Reimann, and M. Beer. 2011. 'Innocuousness and safety of classical swine fever marker vaccine candidate CP7_E2alf in non-target and target species', *Vaccine*, 30: 5-8.

König, P., B. Hoffmann, K. R. Depner, I. Reimann, J. P. Teifke, and M. Beer. 2007. 'Detection of classical swine fever vaccine virus in blood and tissue samples of pigs vaccinated either with a conventional C-strain vaccine or a modified live marker vaccine', *Vet Microbiol*, 120: 343-51.

König, P., E. Lange, I. Reimann, and M. Beer. 2007. 'CP7_E2alf: a safe and efficient marker vaccine strain for oral immunisation of wild boar against Classical swine fever virus (CSFV)', *Vaccine*, 25: 3391-9.

Leifer, I., E. Lange, I. Reimann, S. Blome, S. Juanola, J. P. Duran, and M. Beer. 2009. 'Modified live marker vaccine candidate CP7_E2alf provides early onset of protection against lethal challenge infection with classical swine fever virus after both intramuscular and oral immunization', *Vaccine*, 27: 6522-9.

Liess, B. 1987. 'Pathogenesis and epidemiology of hog cholera', *Ann Rech Vet*, 18: 139-45.

Lindberg, A., and H. Houe. 2005. 'Characteristics in the epidemiology of bovine viral diarrhea virus (BVDV) of relevance to control', *Prev Vet Med*, 72: 55-73; discussion 215-9.

Lohse, L., J. Nielsen, and A. Uttenthal. 2012. 'Early pathogenesis of classical swine fever virus (CSFV) strains in Danish pigs', *Vet Microbiol*, 159: 327-36.

Meyer, D., S. Fritsche, Y. Luo, C. Engemann, S. Blome, M. Beyerbach, C. Y. Chang, H. J. Qiu, P. Becher, and A. Postel. 2017. 'The double-antigen ELISA concept for early detection of Erns -specific classical swine fever virus antibodies and application as an accompanying test for differentiation of infected from marker vaccinated animals', *Transbound Emerg Dis*.

Meyer, H., B. Liess, H. R. Frey, W. Hermanns, and G. Trautwein. 1981. 'Experimental transplacental transmission of hog cholera virus in pigs. IV. Virological and serological studies in newborn piglets', *Zentralbl Veterinarmed B*, 28: 659-68.

Meyers, G., N. Tautz, P. Becher, H. J. Thiel, and B. M. Kummerer. 1996. 'Recovery of cytopathogenic and noncytopathogenic bovine viral diarrhea viruses from cDNA constructs', *J Virol*, 70: 8606-13.

Moennig, V., G. Floegel-Niesmann, and I. Greiser-Wilke. 2003. 'Clinical Signs and Epidemiology of Classical Swine Fever: A Review of New Knowledge', *The Veterinary Journal*, 165: 11-20.

Munoz-Gonzalez, S., M. Perez-Simo, A. Colom-Cadena, O. Cabezon, J. A. Bohorquez, R. Rosell, L. J. Perez, I. Marco, S. Lavin, M. Domingo, and L. Ganges. 2016. 'Classical Swine Fever Virus vs. Classical Swine Fever Virus: The Superinfection Exclusion Phenomenon in Experimentally Infected Wild Boar', *PLoS One*, 11: e0149469.

Munoz-Gonzalez, S., M. Perez-Simo, M. Munoz, J. A. Bohorquez, R. Rosell, A. Summerfield, M. Domingo, N. Ruggli, and L. Ganges. 2015a. 'Efficacy of a live attenuated vaccine in classical swine fever virus postnatally persistently infected pigs', *Vet Res*, 46: 78.

Munoz-Gonzalez, S., N. Ruggli, R. Rosell, L. J. Perez, M. T. Frias-Leuporeau, L. Fraile, M. Montoya, L. Cordoba, M. Domingo, F. Ehrensperger, A. Summerfield, and L. Ganges. 2015b. 'Postnatal persistent infection with classical Swine Fever virus and its immunological implications', *PLoS One*, 10: e0125692.

Pannhorst, K., A. Frohlich, C. Staubach, D. Meyer, S. Blome, and P. Becher. 2015. 'Evaluation of an Erns-based enzyme-linked immunosorbent assay to distinguish Classical swine fever virus-infected pigs from pigs vaccinated with CP7_E2alf', *J Vet Diagn Invest*, 27: 449-60.

Peterhans, E., T. W. Jungi, and M. Schweizer. 2003. 'BVDV and innate immunity', *Biologicals*, 31: 107-12.

Peterhans, E., and M. Schweizer. 2010. 'Pestiviruses: how to outmaneuver your hosts', *Vet Microbiol*, 142: 18-25.

Postel, A., S. Austermann-Busch, A. Petrov, V. Moennig, and P. Becher. 2018. 'Epidemiology, diagnosis and control of classical swine fever: Recent developments and future challenges', *Transbound Emerg Dis*, 65 Suppl 1: 248-61.

Reimann, I., S. Blome, and M. Beer. 2016. 'Chimeric Pestivirus Experimental Vaccines', *Methods Mol Biol*, 1349: 239-46.

Reimann, I., K. Depner, S. Trapp, and M. Beer. 2004. 'An avirulent chimeric Pestivirus with altered cell tropism protects pigs against lethal infection with classical swine fever virus', *Virology*, 322: 143-57.

Reimann, I., K. Depner, K. Utke, I. Leifer, E. Lange, and M. Beer. 2010. 'Characterization of a new chimeric marker vaccine candidate with a mutated antigenic E2-epitope', *Vet Microbiol*, 142: 45-50.

Renson, P., M. Le Dimna, A. Keranflech, R. Cariolet, F. Koenen, and M. F. Le Potier. 2013. 'CP7_E2alf oral vaccination confers partial protection against early classical swine fever virus challenge and interferes with pathogeny-related cytokine responses', *Vet Res*, 44: 9.

Rossi, S., E. Fromont, D. Pontier, C. Cruciere, J. Hars, J. Barrat, X. Pacholek, and M. Artois. 2005. 'Incidence and persistence of classical swine fever in free-ranging wild boar (Sus scrofa)', *Epidemiol Infect*, 133: 559-68.

Rossi, S., C. Staubach, S. Blome, V. Guberti, H. H. Thulke, A. Vos, F. Koenen, and M. F. Le Potier. 2015. 'Controlling of CSFV in European wild boar using oral vaccination: a review', *Front Microbiol*, 6: 1141.

Schroeder, S., T. von Rosen, S. Blome, W. Loeffen, A. Haegeman, F. Koenen, and A. Uttenthal. 2012. 'Evaluation of classical swine fever virus antibody detection assays with an emphasis on the differentiation of infected from vaccinated animals', *Rev Sci Tech*, 31: 997-1010.

Schweizer, M., and E. Peterhans. 2014. 'Pestiviruses', *Annu Rev Anim Biosci*, 2: 141-63.

Sinkora, M., and J. E. Butler. 2009. 'The ontogeny of the porcine immune system', *Dev Comp Immunol*, 33: 273-83.

Stewart, W. C., E. A. Carbrey, and J. I. Kresse. 1973. 'Transplacental hog cholera infection in susceptible sows', *Am J Vet Res*, 34: 637-40.

Tarradas, J., M. E. de la Torre, R. Rosell, L. J. Perez, J. Pujols, M. Munoz, I. Munoz, S. Munoz, X. Abad, M. Domingo, L. Fraile, and L. Ganges. 2014. 'The impact of CSFV on the immune response to control infection', *Virus Res*, 185: 82-91.

Tignon, M., G. Kulcsar, A. Haegeman, T. Barna, K. Fabian, R. Levai, Y. Van der Stede, A. Farsang, R. Vrancken, K. Belak, and F. Koenen. 2010. 'Classical swine fever: comparison of oronasal immunisation with CP7E2alf marker and C-strain vaccines in domestic pigs', *Vet Microbiol*, 142: 59-68.

van Oirschot, J. T. 2003a. 'Emergency vaccination against classical swine fever', *Dev Biol (Basel)*, 114: 259-67.

———. 2003b. 'Vaccinology of classical swine fever: from lab to field', *Veterinary Microbiology*, 96: 367-84.

9 Abbreviations

BVDV	Bovine viral diarrhea virus
CSF	Classical swine fever
CSFV	Classical swine fever virus
DIVA	Differentiating infected from vaccinated animals
EC	European commission
ELISA	Enzyme-linked Immunosorbent Assay
EMA	European medicine agency
EU	European Union
FLI	Friedrich-Loeffler-Institut
KSP	Klassische Schweinepest
KSPV	Virus der Klassichen Schweinepest
NPLA	Neutralization peroxidase-linked antibody assays
OIE	World Organization for Animal Health
PI	Persistent infected
qPCR	Quantitative polymerase chain reaction

10 Acknowledgements

First, I would like to thank Prof. Dr. Sutter and the reviewers for their assessment of this work.

A very special gratitude goes to my mentor Prof. Dr. Martin Beer for the opportunity to work at his institute. Thank you for your ongoing support and valuable advice at any time.

My deepest appreciation goes to PD Dr. Sandra Blome, without you this work would never have been realized. Thank you for your throughout guidance, your patient encouragement in all situations and of course for the work-related discussions as well as the pleasant conversations after work.

A very heartfelt thanks goes to my colleagues in the lab. Simone Leidenberger, Laura Zani, Jolene Carlson, Theresa Schwaiger, Ulrike Kleinert, Ulrike Wilhelm, Markus Kreutzer and Aileen Stoll, thank you for your tireless support, for the nice talks and for being more than just colleagues. I will miss working with you all!

Furthermore, I want to express my gratitude to Dr. Charlotte Schröder and Bärbel Hammerschmidt for their support and help during animal trials.
As there are many more people without whom the animal trials wouldn't have been possible, I also want to express my gratitude to Frank Klipp, Steffen Brenz, Harald Manthei, Christian Loth and Matthias Jahn. Thank you for your help and patience in the stables and of course for all the coffees after long days of sampling.

And last but not least, I want to say thank you to the most important persons in my life: my parents, my sister and her family, my Grandmother and my partner Holger. Thank you for believing in me and for the consistent support through my life.