Classical and African swine fever: 
State-of-the-art diagnostics and control measures

Habilitationsschrift

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African swine fever (ASF) and classical swine fever (CSF) are severe multi-systemic viral diseases that are considered a major threat to profitable pig production worldwide. Both diseases can affect domestic pigs and wild boar. The comparable clinical picture is highly variable and can range from an almost inapparent infection to a hemorrhagic fever like illness with high mortality. Due to their impact on animal health and pig industry as well as their potential to cause trans-boundary outbreaks, both diseases are notifiable to the World Organization for Animal Health (OIE).

African swine fever is caused by a large DNA virus (ASFV) which is the only member of the genus *Asfivirus* and the *Asfarviridae* family. Vertebrate hosts are suids, but the transmission cycle can involve *Ornithodoros* soft ticks. Thus, ASFV is the only arthropod-borne DNA virus that is known so far. ASF is enzootic in most countries of Sub-Saharan Africa. In Europe it had been reported between the 1960ies and late 1990ies on the Iberian Peninsula and Sardinia, but sporadic outbreaks were also reported from France, the Netherlands, Malta, Italy (mainland), and Belgium. It was also present in South American and Caribbean countries, but has been eradicated. Since 2007, ASF has been reported in the Caucasus region, Russia, Ukraine, and Belarus. In January 2014, it entered the European Union and affects currently the Baltic member states and Poland.

Classical swine fever is caused by a small enveloped RNA virus (CSFV) which belongs to the genus *Pestivirus* within the *Flaviviridae* family. Natural hosts are again members of the family *Suidae*. After implementation of strict control measures, several countries succeeded in eradicating CSF. Nevertheless, in most parts of the world with significant pig production, CSFV is at least sporadically present. Within the EU, only Latvia is currently affected by CSF in wild boar. Last outbreaks in domestic pigs were reported from Lithuania.

A binding legal framework exists for the surveillance and control in most countries with industrialized pig production. Within the EU, general measures are laid down in Council Directives 2002/60/EC (ASF) and 2001/189/EC (CSF), and the corresponding details can be found in Commission Decisions 2003/422/EC (ASF) and 2002/106/EC (CSF). Integral part 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 strictly prohibited. However, provisions are
laid for an emergency vaccination scenario against CSF in both domestic pigs and European wild boar. To date, no vaccine exists against ASF.

The presence of both diseases at the Eastern borders of the EU present a constant threat for the introduction into Central Europe, including Germany. For this reason, rational and pragmatic approaches for disease prevention, diagnosis and control are needed.

Understanding disease dynamics, driving forces and possible intervention points is of utmost importance for the design of situation-adapted and optimized early warning and control strategies. The first topic of this thesis is therefore aiming at the basic knowledge, i.e. pathogenesis and host response upon ASF and CSF virus infection. It covers several applied studies that were carried out to characterize disease courses and host responses of domestic pigs and European wild boar upon infection with recent CSFV and ASFV isolates.

The second topic deals with the optimization of diagnostic approaches, especially in the wildlife host. The most recent outbreaks of CSF and ASF within the EU involved wild boar populations. Here, early warning and timely diagnosis is particularly difficult and appropriate sampling can be an important bottleneck. To provide a pragmatic solution that is suitable under field and laboratory conditions, diagnostic systems were evaluated that are based on a dry blood swab in combination with real-time (reverse transcription) polymerase chain reaction (RT-qPCR). Furthermore, diagnostic approaches were tested that allow genetic differentiation of infected from vaccinated wild boar upon oral vaccination with a conventional live attenuated vaccine.

The third topic covered in this thesis is vaccination against swine fevers. As mentioned above, emergency vaccination is among the control options that have to be considered in case of a CSF contingency, especially in areas with high pig density and for CSF control in the wildlife host. So far, this tool was only employed in Romania as part of an eradication program (Commission Decision 2006/802/EC), and in wild boar populations (e.g. in France, Slovakia, and Germany). Serious trade implications and technical considerations prevented the further use in domestic pigs. However, recent advances in vaccine development allow revisiting this option. In this framework, studies were undertaken to assess the chimeric marker vaccine candidate “CP7_E2alf” for oral and intramuscular vaccination. The presented studies were later on included into the data that were submitted to the European Medicines Agency (EMA) for central licensing of the vaccine. Only very recently, licensing was granted and “CP7_E2alf” is now available on the market (Suvaxyn® CSF Marker, Zoetis).

For ASF, the lack of vaccines can complicate control, and all eradication efforts have to rely on strict hygiene measures. Despite decades of so far unsuccessful research towards an
effective and safe ASF vaccine, the background data suggest that it could still be feasible. One promising fact is that it has been long-established that pigs which recover from infection with less virulent ASFV strains can be protected from challenge with related virulent viruses. To reconsider traditional approaches in combination with recent developments, a study was carried out using an inactivated ASFV preparation in combination with state-of-the-art adjuvants. This study is integrated into the vaccination topic.

The publications resulting from the above mentioned studies are presented under the three major topics “pathogenesis and host responses”, “optimization of diagnosis”, and “vaccination and control”. Each topic is accompanied by a brief section on the scientific background and a targeted gap analysis. With permission of the respective publishers, all original research contributions were included as they appear in the printed and/or online version of the publication. Where available, a review article precedes the manuscripts reporting on original research to set them into perspective and to provide additional background. Where permission to reproduce either the article in print-layout or the underlying Word format was not granted, these articles appear together with the abstract. In these cases, the original papers will be removed after the reviewing process (leaving the abstract). The requested detailed summary is presented following the discussion part of the thesis.
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3 CONTRIBUTIONS

3.1 Pathogenesis and host responses

3.1.1 Scientific background

Clinical signs of both ASFV and CSFV infections can vary considerably from peracute deaths to inapparent courses depending on virulence of the virus strain involved and different (partly unknown) host factors (Moennig et al., 2003; Sanchez-Vizcaino et al., 2015). Unspecific clinical signs predominate in both cases and differentiation among the swine fevers and from several other infectious diseases of swine is only possible based on laboratory diagnosis. Acute-lethal forms can be viral haemorrhagic fever like with severe thrombocytopenia, pulmonary oedema, petechial bleedings, and increased vascular leakage (Gomez-Villamandos et al., 2013). A further similarity concerns the primary target cells that are in both cases cells of the monocyte-macrophage lineage (the former reticuloendothelial system). For ASFV, a preference for mature macrophages has been shown that coincides with the expression of CD163 (Sanchez-Torres et al., 2003). In later stages of infection, both viruses replicate additionally in several other cell types (Gomez-Villamandos et al., 2013; Gomez-Villamandos et al., 2003a). It is widely acknowledged that the majority of lesions may be attributed rather to cytokine-mediated interactions triggered by infected and activated monocytes and macrophages than to direct virus actions (Gomez-Villamandos et al., 2003a; Lange et al., 2011). For ASFV, persistent infection is discussed for all animals that survive the acute phase (Sanchez-Vizcaino, 2006).

Infection with CSFV is followed by primary replication in the tonsils and subsequent spread to surrounding lymphoid tissues (Liess, 1987). The virus reaches 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 (Dunne, 1970; Ressang, 1973a, b). Apoptotic reactions as well as phagocytic and secretory activation can be observed in several macrophage populations (Carrasco et al., 2001; Choi et al., 2004; Gomez-Villamandos et al., 2000; Gomez-Villamandos et al., 2001; Gomez-Villamandos et al., 2003b; Knoetig et al., 1999; Narita et al., 2000; Nunez et al., 2005; Sanchez-Cordon et al., 2005). These activated macrophages seem to play a crucial role in pathogenesis while direct damage by the virus could be almost excluded for many lesions occurring in the course of CSFV infection. Especially in the acute-lethal course, CSF is
accompanied by severe lymphopenia and resulting immunosuppression as well as granulocytopenia (Pauly et al., 1998; Summerfield et al., 2000; Susa et al., 1992; Trautwein, 1988). Moreover, a marked thrombocytopenia starts very early after infection (Bautista et al., 2002; Heene et al., 1971; Weiss et al., 1973). 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 (Gomez-Villamandos et al., 2003a). Recently, massive activation and subsequent phagocytosis of platelets has been discussed as an etiological factor (Bautista et al., 2002) while DIC related correlates were not observed upon infection with a genotype 2.3 CSFV strain (Blome et al., 2013c). At least in vitro, endothelial cells are also activated and expression levels of pro-inflammatory and pro-coagulatory factors are increased (Bensaude et al., 2004). The pathogenic mechanism involved in haemorrhagic lesions may thus include damage of endothelial cells, causal involvement of thrombocytopenia (and DIC), erythrodiapedesis, and capillary vasodilatation and increased permeability (Gomez-Villamandos et al., 2000; Heene et al., 1971; Hoffmann et al., 1971; Trautwein, 1988; Weiss et al., 1973). However, several factors remain unclear and studies with different strains have given conflicting results.

ASFV enters the body via the tonsils or dorsal pharyngeal mucosa to the mandibular or retropharyngeal lymph nodes, from where the virus spreads through viraemia (Anderson et al., 1987). Haemadsorbing ASFV isolates are mainly found associated with erythrocytes (Quintero et al., 1986; Wardley and Wilkinson, 1977), but also with lymphocytes and neutrophils (Plowright et al., 1994). Also with ASF, the mechanisms involved in genesis of haemorrhagic lesions remains controversial. While some studies suggest that these lesions could be associated with viral replication in endothelial cells (Sierra et al., 1989), others dispute this hypothesis despite the fact that endothelial damage has been shown (Carrasco et al., 1997; Gomez-Villamandos et al., 1995). Release of cytokines by infected macrophages and DIC are also among the possible options (Anderson et al., 1987; Gomez-Villamandos et al., 2003a; Villeda et al., 1993). Thrombocytopenia is generally observed much later than with CSF, especially in the final phase of acute forms. It has been attributed to consumption of platelets due to coagulopathy (Villeda et al., 1993), to the direct effect of the virus on megakaryocytes (Gomez-Villamandos et al., 2003a), and to various immune-mediated processes involving immune complexes of ASF antigens and antibodies that cause aggregation of platelets (Edwards et al., 1985a, b). Nowadays it is generally accepted that the massive destruction of macrophages plays a major role in the impaired haemostasis due to the
release of active substances including cytokines, complement factors and arachidonic acid metabolites (Penrith, 2004). As with CSF, pigs infected with ASF generally suffer severe lymphopenia that could be attributed to apoptosis of lymphocytes (Oura et al., 1998b). Production of pro-inflammatory cytokines by infected macrophages is strongly implicated in induction of apoptosis in lymphocyte populations (Oura et al., 1998b; Salguero et al., 2002; Salguero et al., 2005). ASFV chronic disease that was mainly observed after infection of pigs with attenuated strains on the Iberian Peninsula may have an auto-immune component and lesions might result from the deposition of immune-complexes in tissues such as kidneys, lungs and skin with their subsequent binding to complement (Plowright et al., 1994).

3.1.2 Identified gaps

- Little is known about the host and virus factors that influence the clinical outcome
- Immune pathological aspects remain to be elucidated
- The genesis of haemorrhagic lesions is still unclear
- For ASF, neither chronic nor persistent infections are well understood, and their impact under field conditions needs to be elucidated

3.1.3 Contributions

In view of the above mentioned gaps, and to facilitate the understanding of disease dynamics, pathogenesis studies in the broader sense were carried out in European wild boar and domestic pigs using recent ASFV and CSFV isolates. These studies were accompanied by the design and validation tools for host response evaluation.

The following publications on the topic pathogenesis and host responses have been included into this habilitation thesis:


Pathogenesis of African swine fever in domestic pigs and European wild boar

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Pathogenese der Klassischen Schweinepest - Parallelen zu viralen hämorrhagischen Fiebern: Eine Übersicht

Pathogenesis of Classical swine fever– similarities to viral haemorrhagic fevers: A review

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Friedrich-Loeffler-Institut, Bundesforschungsinstitut für Tiergesundheit, Greifswald - Insel Riems\textsuperscript{b}

Berlinier und Münchener Tierärztliche Wochenschrift 2011

PMID: 21309164

Please read this part online.
Characterization of African swine fever virus Caucasus isolate in European wild boars

Claudia Gabriel\textsuperscript{a}, Sandra Blome\textsuperscript{a}, Alexander Malogolovkin\textsuperscript{b}, Stanislav Parilov\textsuperscript{b}, Denis Kolbasov\textsuperscript{b}, Jens P. Teifke\textsuperscript{a}, and Martin Beer\textsuperscript{a}

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Emerging Infectious Diseases 2011
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http://dx.doi.org/10.3201/eid1712.110430
http://wwwnc.cdc.gov/eid/article/17/12/11-0430_article

Please read this part online.
High virulence of African swine fever virus Caucasus isolate in European wild boars of all ages

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Emerging Infectious Diseases 2012
18(4):708

http://dx.doi.org/10.3201/eid1804.111813
Course and transmission characteristics of oral low dose infection of domestic pigs and European wild boar with a Caucasian African swine fever virus isolate

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Comparative analyses of host responses upon infection with moderately virulent Classical swine fever virus in domestic pigs and wild boar

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Comparative analyses of host responses upon infection with moderately virulent Classical swine fever virus in domestic pigs and wild boar

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Abstract

**Background:** Classical swine fever (CSF) is one of the most important viral diseases of pigs. Clinical signs may vary from almost inapparent infection to a hemorrhagic fever like illness. Among the host factors leading to different disease courses are age, breed, and immune status. The aim of this study was to compare host responses of different pig breeds upon infection with a recent moderately virulent CSF virus (CSFV) strain, and to assess their impact on the clinical outcome and the efficiency of immune responses. To this means, two domestic pig types (German Landrace and hybrids), were compared to European wild boar. Along with clinical and pathological assessments and routine virological and serological methods, kinetics of immune-cellular parameters were evaluated.

**Findings:** All animals were susceptible to infection and despite clinical differences, virus could be detected in all infected animals to similar amounts. All but one animal developed an acute disease course, two landrace animals recovered after a transient infection. One wild boar got chronically infected. Changes in the percentages of lymphocyte subsets in peripheral blood did not show a clear correlation with the clinical outcome. High and early titers of neutralizing antibodies were especially detected in wild boar and German Landrace pigs.

**Conclusions:** While differences among breeds did not have the expected impact on course and outcome of CSFV infection, preoad with facultative pathogens and even small differences in age seemed to be more relevant. Future studies will target the characterization of responses observed during different disease courses including cytokine reactions and further analyses of lymphocyte subsets.

**Keywords:** Classical swine fever virus, Host responses, Pathogenesis, Host factors

Findings

Clinical signs of classical swine fever (CSF) can range from an almost inapparent infection to a hemorrhagic fever like illness with high mortality. Factors influencing disease severity and outcome include the virulence of the CSF virus (CSFV) isolate as well as the age and immune status of the host [1-3]. However, neither beneficial nor detrimental host reaction patterns have been defined up to know, and the influence of breed-related factors remains unclear. Yet, indications exist that breed and race may have a relevant impact on the severity of the disease [1,4-6]. To target this issue, the presented study was undertaken to compare host responses of different pig breeds upon infection with a recent moderately virulent CSFV strain.

Six German landrace pigs (12 weeks of age), six hybrid pigs (8–10 weeks of age), and six European wild boar (12 weeks of age), were oronasally inoculated with $10^{5.5}$ tissue culture infectious doses 50% of the moderately virulent CSFV strain "Roesrath" (CSF1045). Three additional pigs of each breed acted as negative controls (housed separately). Clinical scores (CS) were assessed as previously described [7], and rectal body temperatures were recorded. While body temperatures of domestic pigs could be assessed daily, wild boar were measured upon blood collection only as they did not tolerate measurement without restraint. All animals were subjected to necropsy. For the execution of the experiment, all applicable animal welfare regulations, including EU Directive 2010/63/EC and institutional guidelines, were taken into consideration. The animal...
experiment was approved by the competent German authority (Landesamt für Landwirtschaft, Lebensmittelsicherheit und Fischerei Mecklenburg-Vorpommern) under reference number 7221.3-1.1-015/12.

Blood samples were collected in regular intervals from 0 to 28 days post inoculation (dpi). Peripheral blood mononuclear cells (PBMC) were subjected to multicolor immuno-staining for flow cytometry analysis of pig-cell surface markers using a BD FACSCanto™ flow cytometer (BD Biosciences). Virus isolation and neutralization tests (NT) were carried out as previously described [8]. All methodological details can be obtained from the author’s upon request.

Landrace pigs developed first clinical signs at 3 dpi. While four animals developed an acute-lethal course of the disease with severe clinical symptoms (see Figure 1 and Additional file 1: Table S1), two animals recovered. Clinical scores mirrored the disease outcome (see Figure 1) and mortality reached 66%. Post mortem examinations revealed CSF symptoms in all pigs with acute-lethal infection (see Additional file 1: Table S1). The surviving animals (LR#56 and LR#59) showed poor nutritional status and multifocal petechiae in the kidney.

Hybrid pigs showed first clinical signs from 3 dpi that worsened till the day of euthanasia (see Figure 1 and Additional file 1: Table S1). All animals succumbed to infection. In post-mortem examinations, all hybrid pigs showed typical CSF lesions and severe secondary infections.

In infected wild boar, first clinical signs were observed from 5 dpi, but raises in body temperature were only sporadically observed (see Additional file 2: Table S2). In total, 5 animals showed an acute-lethal disease course, while one animal survived till the end of the trial. Thus, mortality amounted to 83%. Post-mortem examinations revealed severe pathological lesions, both CSF specific and related to secondary infections (see Additional file 1: Table S1).

In the group of control pigs, unspecific symptoms were occasionally observed and led to euthanasia of one landrace pig at 7 dpi (dyspnea upon bleeding), and of one wild boar at 23 dpi (ruptured gall bladder, severe gastritis and enteritis).

Parameters indicative for the B-cell populations in peripheral blood are summarized in Figure 2: The percentage of cells with CD2+CD21+ phenotype (naïve B-cells) was down regulated in all infected groups. After an initial decline, an increase of CD2-CD21+ cells (phenotype of B-cells after activation) was observed in all infected groups (see Figure 2). Cells representing the phenotype of antibody producing plasma cells (CD2+CD21-) showed a percentage increase in all infected groups with highest changes in hybrid pigs from 7 dpi. With regard to T-cell populations (see Figure 3), all inoculated animals showed slightly elevated CD4+ T helper cells starting from 3 dpi compared to the controls (see Figure 3). The reaction was most pronounced in landrace pigs. Following the increase of helper cells, an increase of cells with a CD8+CD4- phenotype (cytotoxic T cells, CTL) was detectable. The highest percentage peak was observed in hybrid pigs. Furthermore, an increase in γδ-TCR-positive T cells was detectable in domestic pigs, especially in landrace pigs (see Figure 3).

Virus isolation was positive for all samples from infected animals taken at 7 and 10 dpi. Thereafter, virus detection mirrored the clinical status and most tonsil samples taken at necropsy were virus isolation positive.

With regard to antibody detection, landrace pigs showed one weak-positive NT result at 10 dpi (see Figure 4). At

![Clinical Score](chart.png)

**Figure 1** Group mean values (mean value ± standard deviation) for clinical score points of European wild boar (WB), commercial fattening hybrids (HY) and German Landrace (LR) pigs. Each race/breed was divided into one group for infection with CSFV "Rosrath" (infected) and one group acting as negative control (control). During the cause of disease total numbers of pigs decreased due to euthanasia.
14 dpi, neutralization tests were positive for 2 out of 4 pigs. At 21 and 28 dpi, all remaining pigs were found positive with high homologue titers in surviving pigs (see Figure 4).

Hybrid pigs became positive in NTs from 14 dpi (two animals). From 21 dpi, all remaining pigs were found positive (see Figure 4).

In wild boar, first antibodies were detected at 10 dpi with 2 out of 6 animals in the NTs. From 14 dpi, all tested wild boar were positive in the NTs with the homologue virus (see Figure 4).

Classical swine fever may cause most variable clinical syndromes and it is generally acknowledged that disease courses are influenced by both virus and host factors. On the host’s side, age and immune status are main parameters that influence disease course and outcome [9]. However, breed factors were also often discussed to play an important role. Depner et al. [1] showed that German landrace pigs were more severely affected than crossbred animals. Influence of breed was also seen when susceptibility was assessed in indigenous Moo Laat and improved Large White/Landrace [4]. In contrast, no differences were seen by Bunzenthal [10].

In the presented study, two domestic pig breeds were compared to European wild boar in a CSFV infection experiment. All animals proved to be susceptible to CSFV, and all but one animal enrolled in this study developed an acute course of CSF. For all hybrids and all but one wild boar, infection led to acute-lethal disease. The remaining wild boar showed both moderate antibody titers and high viral loads by the end of the trial. Based on these findings, a chronic disease course can be assumed. In the group of landrace pigs, two animals recovered after an acute-transient disease course, the others showed again an acute-lethal disease course. The clinical picture of hybrids was apparently influenced by their preload of secondary pathogens of the respiratory tract that were not sufficiently controlled by metaphylactic antibiotic treatment. Necropsy gave rise to suspicions of *Actinobacillus pleuropneumoniae* and *Haemophilus parasuis* infections. In addition to these secondary infections, hybrids were slightly younger than wild boar and landrace pigs (about two to three weeks), and the body weight was markedly lower than that of the landrace pigs. Taken together, these facts might also have influenced the clinical picture in the hybrid pigs.

In terms of serological responses, wild boar showed earliest responses. However, by the end of the trial, titers of neutralizing antibodies were similar or even higher in landrace pigs. In hybrids, E2 antibodies were only detected late in some animals and to lower titers.
Figure 3 T-cell related responses upon infection. Percentage of T cell subpopulations of blood lymphocytes is given: cytotoxic T cells: CD8+CD4-; T-helper cells/memory T-helper cells: CD4+CD8-/CD4+CD8low. Bottom row shows percentage of γδTCR+ T cells of all T cells during infection. Filled symbols represent data from infected animals in comparison to uninfected controls (open symbols).

Figure 4 Mean values of antibody responses of infected groups of each race (WB: wild boar; HY: commercial fattening hybrid; LR: German Landrace). Results of the neutralization tests using CSFV strain "Roesrath" are shown in (a), in which antibody titers are represented as log_{10} ND50.
reflects the clinical picture but contrasts tendencies seen in the responses of lymphocyte phenotypes (with regard to percentages of cells with plasma cell phenotype).

Despite the fact that the majority of leukocytes will be active outside the blood compartment, changes in the percentages of different lymphocyte phenotypes were investigated in blood samples as the only matrix that allowed kinetics in individual animals. With regard to B-cell responses in peripheral blood, some breed-dependent patterns were observed that were however not statistically significant among the different groups. Upon infection, all animals showed a down regulation of CD2 + CD21+ cells (phenotype of naive B-cells), this could be either due to depletion or an indication of B-cell activation. As especially domestic pigs showed an increase of cells presenting the phenotype of primed and activated B-cells (CD2-CD21+) after 7 dpi, activation could be suggested. Interestingly, the increase of cells displaying the phenotype of antibody producing plasma cells (CD2 + CD21-) was highest in hybrid pigs. This is in contrast to both clinical course and serology. However, due to the lack of additional plasma cell markers at the time point of the experiment (CD79a) and the possible impact of lymphocyte depletion, these results have to be viewed with caution and need further investigation. All investigated breeds showed slightly elevated helper cells from 3 dpi. Following the increase of helper cells, an increase of CD8 + CD4- CTLs was detectable. Strongest CTL proliferation was seen again in hybrid pigs. Preceding CTL proliferation, probably virus-mediated decrease of CD8 + CD4- T cells was detectable in all animals. This is in line with previous studies that showed that CSFV is able to suppress porcine 

\[ \gamma \delta \] TCR positive T cells was detectable. While differences among breeds did not have the expected impact on course and outcome of CSFV infection, preoad with facultative pathogens and even small differences in age seemed to be more relevant. Future studies will target the characterization of responses observed during different disease courses including cytokine reactions and further analyses of lymphocyte subsets.

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
AP carried out the animal trial, participated in the conception and design of the presented study, investigated samples from the related animal trial using serological and virological methods, performed blood count analyses, and drafted the manuscript. UB analyzed cellular responses upon infection. MB conceived the study, and participated in its design and coordination and helped to critically revise the manuscript. JP was involved in the execution of the animal trial and the related laboratory analyses. SB supervised the whole study and was involved in both the conception and execution of the animal trial. Moreover, SB critically revised the manuscript. All authors read and approved the final manuscript.

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References

Additional files
Additional file 1: Table S1. Overview on clinical presentation and disease courses upon infection with CSFV strain “Roesrath”.

Additional file 2: Table S2. Rectal body temperatures upon infection with CSFV strain “Roesrath” (0–28 days post infection). Fever was defined as a body temperature >40°C for at least two consecutive days.

Temperatures >40°C but <40.5°C in red. WB = wild boar, HY = hybrid pigs, LR = landrace pigs, inf = infected, ctr = negative control, nd = not determined.


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3.2   Optimization of diagnosis

3.2.1   Scientific background

Rapid and reliable diagnosis is of utmost importance for the timely implementation of control measures against swine fevers. For ASF and CSF, laboratory methods as well as sampling and shipping guidelines can be found in the respective EU Diagnostic Manuals (Commission Decision 2003/422/EC for ASF, and 2002/106/EC for CSF) and the OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals. Nowadays, rapid qPCR protocols dominate the direct detection of ASFV. There are several well established assays that are used in reference laboratories throughout the EU and beyond (Fernandez-Pinero et al., 2013; King et al., 2003; Tignon et al., 2011). Recently, some of these assays have been commercialized, and more are under way. Moreover, ASFV detection has been included in multiplex assays with CSFV and other porcine pathogens (Haines et al., 2013; Wernike et al., 2013). Yet, also gel-based (multiplex) PCR systems are still in use (Aguero et al., 2004; Basto et al., 2006). Alternative assays including isothermal amplification methods such as loop-mediated isothermal amplification (LAMP) or PCR variations such as Linear-After-The-Exponential PCR (LATE-PCR) assays have been developed (James et al., 2010; Ronish et al., 2011). Despite the advantages of PCR, virus isolation is still a desirable tool as it allows generating a virus isolate for further testing. The most sensitive method to isolate ASFV is still the traditional cultivation on primary porcine macrophages (peripheral blood mononuclear cells, bone marrow cells or lung macrophages). Viral growth can be visualized through either haemadsorption or immune-staining (Carrascosa et al., 2011). The haemadsorption phenomenon is based on the capacity of pig erythrocytes to adhere to the surface of ASFV infected cells (Malmquist, 1960). The method can be very sensitive but is time consuming and may yield false-negative results for virulent, non-haemadsorbing ASFV strains (Oura et al., 2013). Different permanent cell lines have been tried for the cultivation of ASFV (Carrascosa et al., 2011). However, their performance with field isolates is often variable to poor. As a rapid antigen test on cryostat sections or smears, a fluorescent antibody test can be carried out (Heuschele and Hess, 1973). This test is mainly useful for acute infections due to the fact that antigen-antibody complexes in subacute or chronic courses will negatively influence the sensitivity (Sanchez-Vizcaino, 2006). The same holds true for the antigen ELISA. This test is only recommended on herd-basis and in combination with at least
an antibody detection technique. Several enzyme-linked immunosorbent assays (ELISA) are available for the detection of ASFV antibodies. Confirmatory serological assays are immunoblotting and indirect immune-peroxidase tests (Gallardo et al., 2013; Pastor et al., 1989).

For CSFV, primary detection will be performed using well established RT-qPCR systems (Hoffmann et al., 2005; Hoffmann et al., 2011; Le Dimna et al., 2008; Leifer et al., 2011; McGoldrick et al., 1998; Paton et al., 2000) of which quite a few have been commercialized. Also here, alternatives have been designed such as LAMP assays (Chen et al., 2009; Chen et al., 2010; Chowdry et al., 2014), primer-probe energy transfer real-time PCR (Liu et al., 2009b; Zhang et al., 2010) or recently insulated isothermal RT-PCR (Lung et al., 2015).

In contrast to ASFV, 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). Detection of antigen on fixed cryosections of tissues is possible using fluorescence antibody assays or immune-peroxidase test (de Smit et al., 2000; Turner et al., 1968). The available antigen ELISAs are recommended for the use on herd-base only. While the sensitivity of panpesti-specific assays is usually at least comparable with virus isolation, most CSF specific assays lack sensitivity (Blome et al., 2006). Serological screening is performed using different commercially available E2 antibody ELISAs. In addition, neutralization assays allow, to a certain extent, differentiation of pestivirus antibodies and are used for confirmation (Greiser-Wilke et al., 2007).

In the case of CSF, DIVA assays are needed for the use with marker vaccines. Commercially available tests target the detection of antibodies directed against glycoprotein E^{nt} (Blome et al., 2006; Floegel-Niesmann, 2001, 2003). Recently, additional diagnostic tests have been developed that could accompany vaccine strain “CP7_E2alf” as discriminatory assays. One is an ELISA format with screening and confirmation part (Aebischer et al., 2013), the other a microsphere immunoassay (Xia et al., 2015).

Due to the increased sensitivity of diagnostic tools, 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 that allow to differentiate between vaccine and field viruses (Huang et al., 2009; Leifer et al., 2009a; Li et al., 2007; Liu et al., 2009a; Widen et al., 2014; Zhao et al., 2008).

Sampling can be the bottleneck of swine fever diagnosis, especially in the case of wild boar, but also in remoter areas. Alternative sampling strategies and sample matrices have been tested for ASF and CSF especially for wildlife specimens and under rural conditions (Braae et
al., 2013; de Carvalho Ferreira et al., 2014; Michaud et al., 2007; Mouchantat et al., 2014; Prickett and Zimmerman, 2010). However, most of them are not in routine use.

### 3.2.2 Identified gaps

- Reliable discriminatory tests are needed to accompany DIVA vaccines
- Field validation of genetic DIVA concepts was needed
- Sample collection and submission is the main bottleneck, pragmatic approaches that facilitate compliance are needed

### 3.2.3 Contributions

The following publications have been included into this thesis:

Assessment of classical swine fever diagnostics and vaccine performance

Sandra Blome\textsuperscript{a}, Alexandra Meindl-Böhmer\textsuperscript{a}, Willie Loeffen\textsuperscript{b}, Barbara Thuer\textsuperscript{c}
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Revue scientifique et technique (OIE) 2006
PMID: 17361768

http://web.oie.int/boutique/extrait/13blome10271040.pdf
Alternative sampling strategies for passive classical and African swine fever surveillance in wild boar

Anja Petrov\textsuperscript{a}, Ulrich Schotte\textsuperscript{b}, Jana Pietschmann\textsuperscript{a}, Carolin Dräger\textsuperscript{a}, Martin Beer\textsuperscript{a}, Helena Anheyer-Behmenburg\textsuperscript{b}, Katja Goller\textsuperscript{a}, and Sandra Blome\textsuperscript{a}

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\textit{Veterinary Microbiology} 2014

http://dx.doi.org/10.1016/j.vetmic.2014.07.030

Please purchase this part online.
Alternative sampling strategies for passive classical and
African swine fever surveillance in wild boar –
Extension towards African swine fever virus antibody detection

Sandra Blome, Katja V. Goller, Anja Petrov, Carolin Dräger, Jana Pietschmann,
and Martin Beer

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Insel Riems, German

Veterinary Microbiology 2014
http://dx.doi.org/10.1016/j.vetmic.2014.09.018
Please purchase this part online.
Genetic differentiation of infected from vaccinated animals after implementation of an emergency vaccination strategy against classical swine fever in wild boar

Sandra Blome\textsuperscript{a}, Claudia Gabriel\textsuperscript{a}, Christoph Staubach\textsuperscript{b}, Immanuel Leifer\textsuperscript{c}, Günter Strebelow\textsuperscript{a}, and Martin Beer\textsuperscript{a} \\

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\textit{Veterinary Microbiology} 2011

http://dx.doi.org/10.1016/j.vetmic.2011.05.039

Please purchase this part online.
3.3 Vaccination and control

3.3.1 Scientific background

Highly efficacious and safe live attenuated CSF vaccines exist since decades (van Oirschot, 2003). The underlying virus strains (e.g. the C-strain of CSFV or the guinea-pig exaltation negative GPE-strain) were attenuated through serial passages in either animals (rabbits) or cell culture, and have been implemented in mandatory control programs that led to the eradication of CSF from several countries in combination with strict hygiene measures (Greiser-Wilke and Moennig, 2004). These vaccines are still in use in several Asian countries including China and were adapted to a bait format for oral immunization of wild boar (Kaden et al., 2002; Kaden et al., 2010; Luo et al., 2014). While these vaccines have usually outstanding virtues in terms of onset and duration of immunity, the main drawback is the lack of a serological marker concept (van Oirschot, 2003) that would allow differentiating field strain infected from vaccinated animals (DIVA concept). In general, there are no legal obligations to use a certain type of vaccine for an emergency vaccination scenario. Yet, due to the trade restrictions that are imposed on pigs vaccinated with conventional live attenuated vaccines, only marker vaccines are considered a feasible option for domestic pigs (Blome et al., 2013b). Up to very recently, only E2 subunit vaccines were available on the market. These vaccines are safe but show drawbacks especially in terms of early protection (van Oirschot, 2003). 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. The ideal vaccine would fulfill all of the following requirements (postulated by Terpstra and Kroese (1996); modified by Dong and Chen (2007) for marker vaccines): no short- or long-term side effects in vaccinated animals, genetic stability in both target and non-target species, stable and easy production under standard conditions, low costs of production, early onset of a robust and life-long immunity, efficacy against all virus variants, prevention of a carrier status, prevention of horizontal and vertical transmission, and availability of a highly sensitive and specific DIVA diagnostic test. It is obvious that meeting all demands is quite illusive. However, several promising attempts have been made. Among the concepts that have been tried are different vector vaccines, recombinant attenuated vaccines (live and inactivated), subunit vaccines based on different expression systems, and RNA/DNA vaccines (Beer et al.,
2007; Blome et al., 2013b). It became evident that attenuated deletion vaccines and chimeric constructs showed high potential. The European Medicines Agency (EMA) recently licensed one of the chimeric marker vaccine candidates, “CP7_E2alf”, after extensive testing in the framework of an EU-funded research project (Blome et al., 2012a; Blome et al., 2014b; Eble et al., 2012; Feliziani et al., 2014; Gabriel et al., 2012; Koenig et al., 2007a; Koenig et al., 2007b; König et al., 2011; Leifer et al., 2009b; Rangelova et al., 2012; Reimann et al., 2004; Renson et al., 2014; Renson et al., 2013). These recent developments allow revisiting emergency vaccination strategies for both domestic pigs and European wild boar.

In the set of control tools for ASF, effective and safe vaccines are still missing. Complexity of the large DNA virus, replication of the virus in cells of the monocyte/macrophage lineage, the lack of neutralizing antibodies, and immune modulation accomplished by the virus are factors that complicate rational design of vaccine candidates.

Up to now, all inactivated ASF vaccine preparations lacked efficacy (Blome et al., 2014a; Mebus, 1988; Stone and Hess, 1967), and while it has been reported that attenuated ASFV strains can induce protective immunity in general (Dixon et al., 2013), reproducibility was low, and all candidates showed either severe side effects or lack of efficacy. Other concepts like DNA vaccines showed only partial protection (Argilaguet et al., 2012; Lacasta et al., 2014).

Unfortunately, the mechanisms underlying protection are so far only poorly understood. Especially the role of humoral responses, i.e. antibody production, is discussed controversially (Escribano et al., 2013; Neilan et al., 2004). While it has been demonstrated that cytotoxic CD8+-T-cells are essential for protection (Dixon et al., 2013; Oura et al., 2005), the relevant viral antigens are still unknown, and only in silico analyses have been performed to date. Currently, additional deletion mutants are pursued, and alternative inactivated vaccines are discussed. Moreover, T-cell antigens expressed in different vector systems are tested.
3.3.2 Identified gaps

- Up to now, marker vaccines were missing that combine safety, early onset of immunity, and DIVA potential
- Candidate vaccines such as chimeric pestiviruses need further investigation towards licensing
- A safe and efficacious vaccine against ASFV does not exist, and traditional approaches were unsuccessful; alternative approaches are needed

3.3.3 Contributions

The following contributions to the topic vaccination and control have been included into this habilitation thesis:

Möglichkeiten und Grenzen der Impfstoffentwicklung in der Veterinärmedizin am Beispiel der Klassischen Schweinepest

*Possibilities and limitations in veterinary vaccine development using the example of classical swine fever*

Sandra Blome, Claudia Gabriel, Martin Beer

Friedrich-Loeffler-Institut, Institut für Virusdiagnostik, Greifswald - Insel Riems

Berliner und Münchener Tierärztliche Wochenschrift 2013

PMID: 24511823

Please purchase this part online.
Comparative evaluation of live marker vaccine candidates “CP7_E2alf” and “flc11” along with C-strain “Riems” after oral vaccination

Sandra Blome\textsuperscript{a}, Andrea Aebischer\textsuperscript{b}, Elke Lange\textsuperscript{a}, Martin Hofmann\textsuperscript{b}, Immanuel Leifer\textsuperscript{a,b}, Willie Loeffen\textsuperscript{c}, Frank Koenen\textsuperscript{d}, and Martin Beer\textsuperscript{a}

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Veterinary Microbiology 2012

http://dx.doi.org/10.1016/j.vetmic.2012.02.015

Please purchase this part online.
Efficacy of marker vaccine candidate CP7_E2alf against challenge with Classical swine fever virus isolates of different genotypes

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\textit{Veterinary Microbiology} 2014


Please purchase this part online.
Towards licensing of CP7_E2alf as marker vaccine against classical swine fever
– Duration of immunity

Claudia Gabriel\textsuperscript{a}, Sandra Blome\textsuperscript{a}, Alicia Urniza\textsuperscript{b}, Sandra Juanola\textsuperscript{b}, Frank Koenen\textsuperscript{c}, and Martin Beer\textsuperscript{a}

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Vaccine 2012

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Please purchase this part online.
Modern adjuvants do not enhance the efficacy of an inactivated African swine fever virus vaccine preparation

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Please purchase this part online.
4 DISCUSSION

4.1 Pathogenesis and host responses

This part of the thesis focused on studies that were undertaken to better understand the pathogenesis and course of ASF and CSF as well as the resulting host responses in the domestic and European wildlife host. Ultimate goal was to generate knowledge that would explain disease dynamics, and that could help in an applied way to design risk-based and rational control measures including optimize vaccine approaches. To this means, infection models were chosen that represent the current situation, i.e. recent European virus isolates.

4.1.1 African swine fever

When ASF was introduced into Georgia (2007) and subsequently into several Trans-Caucasian countries and the Russian Federation, an exotic disease became a tangible threat to the European Unions pig industry. From the beginning, both domestic pigs and European wild boar were affected, and this triggered an additional alarm in countries that had experienced long lasting outbreaks of CSF in their still growing wild boar population. As known from recent CSF outbreaks, an endemic situation in the wild boar population can significantly complicate disease control also in the livestock population and presents a constant threat for introduction into the domestic sector (Fritzemeier et al., 2000). In case of CSF, most of these cases have been resolved by now, but quite frequently with the help of oral immunization campaigns. The absence of the latter option fueled the concerns with regard to ASF introduction. In the beginning, only little was known about ASF disease courses, the threat of endemic situations, and the possible influence of different host factors such as age and immune status. Indications existed from one experimental infection and field experience (Spain and Sardinia) that the wild boar would probably show susceptibility and courses comparable to domestic pigs rather than African wild pigs (McVicar et al., 1981). Moreover, it seemed that wild boar populations were not able to maintain ASF by themselves (Costard et al., 2013; Laddomada et al., 1994).

To generate supplemental data on disease courses in European wild boar, three experiments were executed. The first one (Gabriel et al., 2011) was conducted in collaboration with Russian partners and involved two study parts. The first part involved wild boar piglets and domestic contacts to mirror the population part that was considered most susceptible (young animals). This was meant to provide basic data on general susceptibility, virus virulence in
wild boar, and the outcome of contact infection in domestic pigs. The oro-nasal inoculation was carried out using a representative ASFV strain from Armenia (2008). Based on routine genetic typing, this strain is identical to the ones reported from Georgia and other countries of the affected area (Gallardo et al., 2014) and thus represented the current threat. The second part was carried out using older wild boar (9 month) and intramuscular inoculation with a Chechen ASFV isolate. Irrespective of the inoculation route, all animals developed an acute-lethal disease course upon inoculation and there was no doubt that wild boar are as susceptible as domestic pigs. This high virulence was recently confirmed in other international studies (Mur et al., 2014). The clinical signs were severe but most unspecific, and hemorrhagic lesions were seen only in a minority of animals (more often during necropsy). None of the animals developed a detectable antibody response. The course of infection in the domestic contact animals showed that contagiosity was rather low in the absence of blood contact. One animal that was in very close contact to the infected pen-mates throughout the study showed clinical signs very late and only after another animal had to be euthanized within the pen leading to a few drops of blood on the floor. This behavior could be explained using the virological data. While blood showed strong positive reactions in PCR assays, oropharyngeal and fecal swabs were only weak positive. Comparison of genome loads suggests that blood contains more than 1000 times more virus than feces or saliva (Blome et al., 2013a). Extrapolated to the field situation, ASF could perfectly creep through holdings and populations after a single introduction as long as extensive blood contact does not occur. This behavior is probably expected for a virus that is optimized for vector transmission. However, it should be kept in mind for the assessment of epidemiological situations and also when training veterinarians and farmers on “highly contagious” diseases. It would be fatal to wait for a disease that spreads with foot-and-mouth disease speed and looks like a hemorrhagic fever in pigs.

Following this initial trial, age dependence of ASF courses was assessed in a limited study with three full-grown adult wild boar (between 4 and 10 years of age) and one piglet (Blome et al., 2012b). Inoculation was carried out orally with the above mentioned ASFV strain from Armenia. Again, all animals developed an acute-lethal disease course and died or were euthanized within 10 days post infection. Clinical signs were once more severe but unspecific. No indications were seen for an age dependence of clinical courses and no antibodies were detectable. Despite the limitations of the study due to group size, a strong age dependence as it is usually seen with the recent CSFV strains does not seem to play a role. In the end, this would be beneficial for disease control as it lowers the risk of chronic infections and long
term persistence. However, it also demonstrates that the surveillance with a strong focus on piglets that is suitable for CSF should be discussed for ASF.

Feeding the obtained data into a mathematical model, a true state of endemicity (defined as an infection persisting from generation to generation in the same population) can be doubted for Southern Russia (Lange et al., 2014), and these estimates confirm the assumption that wild boar are most often rather victims than culprits. However, this does not prevent the virus from staying in the wild boar population over long time periods. In fact, constant reintroduction seems to happen, especially through deposition of domestic pig carcasses but also direct contacts (Gogin et al., 2013; Khomenko et al., 2013). Thus, scientifically endemicity may not be present, but still, the disease prevails.

Most (re-)introduction scenarios will involve low-dose oral infection, either through contaminated swill or contact to infected carcasses or their parts. It has been reported that the inoculation dose could have an influence on course and outcome of ASFV infection (Sanchez-Vizcaino et al., 2015). Should low dose infection lead to long-term carriers or chronic infections, disease control would be much more complicated. Consequently, the third study addressed the course and transmission characteristics after oral low dose infection of domestic pigs and wild boar (Pietschmann et al., in press). To ensure comparability, the Armenian ASFV strain was used again. It was demonstrated that very low doses (less than 10 and 100 HAU, respectively) are sufficient to infect weak or runting animals by the oro-nasal route while this dose was not enough to induce infection in healthy animals. Interestingly, some of the weak animals did not present clinical signs indicative for ASF, especially almost no fever. In contrast, no changes were observed regarding the onset, course and outcome of infection as assessed by diagnostic tests. This behavior of runting animals was also seen with CSF (Blome and Lange, unpublished results), and could be an indication of a compromised immune response in general. Despite the fact that only a small proportion of animals got directly infected, all pen- and stable-mates got infected after amplification of ASFV by the weak animals, and developed acute-lethal disease courses that were comparable among all animals. Again, no antibodies were detected. Thus, no indications exist for prolonged or chronic individual courses upon low dose infection in either species. However, contact infection occurred in a rather scattered way and confirms again moderate contagiosivity that is strongly linked with blood contact. Prolonged courses on herd or population level are imaginable and could be important disease drivers in wild boar populations and under backyard settings.
4.1.2 Classical swine fever

Unlike the highly virulent ASFV strains that are currently circulating in Eastern Europe, most recent CSFV strains are moderately virulent and belong to genotype 2 (Bartak and Greiser-Wilke, 2000; Biagetti et al., 2001; Blome et al., 2010; Depner et al., 2006; Leifer et al., 2010b). These virus strains show an age dependent clinical course and can lead to a wide range of clinical syndromes. It can be discussed whether these strains present the optimum of well-adapted CSFV strains that are particularly prone to establish endemicity in the wildlife host (Lange et al., 2012). Despite intensive research efforts, host and virus factors leading to different disease outcomes and the perceived change in virulence are far from being understood, and it seems that several host factors play a role, including genetic background and immune status (Blacksell et al., 2006; Depner et al., 1997). Moreover, reports exist that moderately or low virulent strains of wild boar origin may show more severe disease courses or higher viral loads upon infection when introduced into the domestic pig population (Kaden et al., 1999; Kaden et al., 2000). Additional results (Blohm and Blome, unpublished) also indicated that wild boar may show a particularly strong cellular immune response.

In an attempt to define beneficial and detrimental host reaction pattern, a study was carried out that involved two different domestic pig breeds and European wild boar. To mirror the current field situation, a well characterized CSFV strain (CSF1045, “Roesrath”) was used that was isolated from a German wild boar piglet in 2009 (Leifer et al., 2010b). The outcome of the study showed that all animals were equally susceptible to CSFV infection and the majority of animals developed and acute-lethal course. However, two domestic pigs recovered completely, and one wild boar showed signs for chronic infection. Despite the fact that no clear correlation was seen between cellular immune responses and clinical outcome, one can discuss that survivors did show overall moderate responses. This needs further investigation but could confirm the assumption that CSF has an immune-pathological aspect. The surviving animals showed strong reactions in all antibody tests including the E\textsuperscript{ns} ELISA that could accompany marker vaccines as discriminatory test. A secondary finding in this regard was the high sensitivity of the assay in terms of early antibody detection. The E\textsuperscript{ns} ELISA is often discussed as being less sensitive (Floegel-Niesmann, 2001, 2003; Schroeder et al., 2012) but this may only be true for the detection of infection in vaccinated animals.

While differences among the breeds did not have the expected influence, even small changes in microbial preload (facultative pathogens) and age seemed to impact on the outcome of infection. It is an important fact that chronically infected animals are found even under our
limited experimental conditions. These animals could be crucial for long-term perpetuation of the disease and may explain how virus can persist even in areas with high surveillance coverage (Leifer et al., 2010b). The same might be true for animals with persistent infection that can occur upon prenatal CSFV infection (Liess, 1984). As long as population immunity is high, either through natural infection or vaccination, these animals do not necessarily play a role. However, after some time, the young generation will be susceptible again (after disappearance of maternally derived antibodies) and a new epidemic wave could start if chronically/persistently infected animals are still shedding virus. Despite the fact that both chronically and persistently infected animals will eventually die from CSF, they are usually quite healthy for several weeks or months in which they constantly shed the virus. It is not known how long such animal could survive under natural conditions. However, their epidemiological impact is high.

The outcome of the presented study may also confirm that even moderately virulent CSFV strains will lead to considerable mortality among young animals. Thus, an introduction of CSF into a free area will also lead to the increased occurrence of fallen animals in the wild boar population. These animals are thus a key factor for early swine fever detection in general.

4.2 Optimization of Diagnosis

This part of the thesis dealt with the optimization and validation of diagnostic tools for CSF and ASF diagnosis making use of the recent advances in technology. It covers two studies that aimed at assessment and validation of swab samples for ASF and CSF diagnosis in wild boar, and a field validation study that employed a genetic DIVA concept for wild boar vaccinated with “C-strain Riems”.

4.2.1 Alternative sampling strategies

Introduction of qPCR technology into routine diagnosis has led to a tremendous increase in sensitivity but also robustness of swine fever diagnosis. When talking about optimization of sampling and testing strategies, the aim is regularly to detect a pathogen even earlier, faster, in lower amounts, and at lower costs. Here, we tried to use the advantages of qPCR to make thinks easier to handle while still assuring fitness for purpose, i.e. to give improvements back to the field.
Currently, both ASF and CSF are present in the wild boar population at the Eastern border of the EU and especially ASF threatens to spread. Taking into account the above mentioned experimental studies and field data, introduction into free areas would most likely lead to considerable mortality and thus to an increase in wild boar found dead. These animals are consequently a major target for an early warning system of both ASF and CSF. This system has to rely on the cooperation of hunters, game wardens, foresters, and probably also those responsible for public easement (if animals killed in road accidents are sampled). Naturally, fallen wild boar are often not easy to find, and sampling of half-rotten carcass is challenging. For this reason, we tried to find an option that would increase the compliance of the parties thereto. African swine fever virus is found to tremendous titers in blood and bloody fluids of diseased animals (Blome et al., 2013a), especially if they died from the disease, and is detectable in this matrix over several months irrespective of the storage conditions (unpublished results by my working group). Taking into account the possible decomposition of carcasses, we targeted blood as the most likely source of high viral loads. As a sampling device, we tried different dry swabs with the rational that it lowers the direct contact with the carcass and that the swab brings its primary receptacle for shipment already with it. Overall, the forensic Genotube swab showed best results. The design of the inner receptacle leads to rapid drying of the swab and preserves the specimen. It could be shown that swab samples allowed reliable detection of CSFV and ASFV and that the sample was stable over several weeks at room temperature (Petrov et al., 2014b). By swabbing different matrices we could also show that any kind of bloody swab is sufficient for swine fever detection by PCR. While PCR is suitable for primary diagnosis, isolation of the virus strain for further characterization is desirable especially in primary outbreaks. Using swab material, ASFV could be isolated in several cases. For CSFV, this was not successful. However, transfection of CSFV RNA could help to overcome this drawback. The latter method is already in use at the EU Reference Laboratory for CSF in Hannover (communication at the Annual Meeting of Swine Fever Laboratories). It was also shown that the dry blood swab is suitable for the detection of ASFV antibodies using a commercial ELISA protocol that was designed for filter papers (Blome et al., 2014c).

The swab has many advantages, but also some possible drawbacks. The most important could be the loss of syndromic surveillance. However, this could be partly overcome using the swab for additional pathogens. This approach would also be feasible for FTA cards or filter papers that have been used to detect swine fevers in Africa (Braae et al., 2013; Michaud et al., 2007).
The latter approaches may have the disadvantage that additional tools (e.g. single-use pipets) are needed to obtain rather clean samples.

Another inherent drawback is the fact that the swab still needs to be taken from an animal that has been found or shot. This problem could be overcome using non-invasive strategies such as rope-in-a-bait approaches (Mouchantat et al., 2014) or other sample matrices such as faeces (de Carvalho Ferreira et al., 2014). For the former, it has to be kept in mind that sick, virus positive animals may not show interest in baits, and that due to this fact, rope-in-a-bait devices may be more appropriate for antibody detection in analogy to oral-fluid sampling (Mur et al., 2013; Prickett and Zimmerman, 2010). In the later study that evaluated the potential of faeces and tissue samples as a basis for non-invasive sampling strategies for ASFV in wild boar, it was demonstrated that even in the acute phase (0-21 days after infection), virus can be detected in faeces only in 50-80% of the time. Still, there could be potential in combining swabs, ropes and fecal samples to obtain a broader view.

4.2.2 Genetic differentiation of infected from vaccinated animals

Oral emergency vaccination of wild boar is a powerful tool to safeguard domestic pigs in areas with CSF in the wildlife host (Kaden et al., 2003; von Rüden et al., 2008). In the framework of the vaccination campaign, all wild boar shot or found dead have to be inspected by an official veterinarian and are examined for CSFV in accordance with the EU Diagnostic Manual (Commission Decision 2002/106/EC). In the past, vaccine virus detections have been very scarce, but nowadays, the implementation of highly sensitive PCR methods as led to much higher numbers. As the continuation of measures and the recovery of the free status of a country or region depend on the last detection of field virus, a rapid decision is necessary whether field or vaccine virus was detected. To avoid the necessity of sequencing approaches, a genetic DIVA concept has been designed that was based on a discriminatory multiplex RT-qPCR assay (Leifer et al., 2009a; Leifer et al., 2010a). Implementation of this approach under field conditions showed a good accordance with sequencing results and a positive result can be regarded as evidentiary under the current field conditions. It guarantees swift decision within roughly a day. This method is not only suitable for wild boar vaccination but could also be implemented in emergency vaccination campaigns in domestic pigs. A similar PCR system is available for CP7_E2alf (Leifer et al., 2009a) and could help especially in the early phase of vaccination campaigns.
4.3 Vaccination

This part of the thesis focused on the assessment of the chimeric marker vaccine candidate “CP7_E2alf”, and on a small study that was conducted to re-assess inactivated ASFV vaccines.

4.3.1 Pre-licensing assessment of chimeric pestivirus “CP7_E2alf”

During the last decades, a vast number of CSF marker vaccine candidates have been developed, and research activities mainly focused on new innovative ideas and their realization (Beer et al., 2007; Blome et al., 2013b; Dong and Chen, 2007). Among the most promising approaches were chimeric pestiviruses of which “CP7_E2alf” (Reimann et al., 2004) was assessed in the framework of this thesis.

The CP7_E2alf experiments were carried out within the EU-funded research project 'Improve tools and strategies for the prevention and control of classical swine fever' (CSFV_GODIVA), and were part of the dossier that was later on submitted to EMA. The studies that have been included in this thesis are only a fraction of the work that has been achieved within the project, in several cases with the help of my working group and our collaborators within the Friedrich-Loeffler-Institut (Eble et al., 2013; Eble et al., 2014; Feliziani et al., 2014; Koenig et al., 2007a; Koenig et al., 2007b; Konig et al., 2011; Leifer et al., 2009a; Levai et al., 2015; Rangelova et al., 2012; Renson et al., 2014; Renson et al., 2013; Xia et al., 2015).

Here, three studies are included that targeted the initial comparative testing of two possible vaccine candidates (Blome et al., 2012a), and two efficacy studies that were embedded in the pre-licensing evaluations of “CP7_E2alf” (Blome et al., 2014b; Gabriel et al., 2012).

The comparative testing comprised marker vaccine candidates “CP7_E2alf” and “flc11”. As a gold-standard comparator, the live attenuated vaccine “C-strain Riems” was included. The study design foresaw a single oral vaccination with the respective vaccine and highly virulent challenge after 14 and 21 days, respectively. A analogous experiment was carried out with single intramuscular vaccination and challenge after 7 and 14 days at the facilities of another project partner, the Central Veterinary Institute (CVI) in Lelystad, the Netherlands (Eble et al., 2012). Both experiments were later on assessed using a scoring system that had been developed within the project consortium and included not only the above mentioned studies but also the available background data on several aspects such as safety, DIVA potential, and stability. Challenge times were chosen to include the requirements for CSF vaccines that are provided by the European Pharmacopoeia (Ph. Eur., monograph 07/2008:0065) and the OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (OIE Manual, chapter
2.8.3). In these guidelines that refer to intramuscular vaccination, challenge after 14 days is prescribed. As it is known that the onset of immunity upon oral vaccination might be slightly delayed when compared with intramuscular schemes (Kaden and Lange, 2001), 21 days were included. For the intramuscular part, an emergency vaccine should confer protection prior to 14 days. Therefore, challenge after 7 days was incorporated. In addition to the vaccination-challenge trial, multiple vaccinations with both candidates were carried out to assess the DIVA potential.

It was demonstrated that all vaccines were safe and yielded full protection three weeks post oral vaccination. With challenge two weeks after single oral vaccination, only “CP7_E2alf” was able to confer full protection against lethal challenge under the circumstances of the experiment. Taking into account that all other experimental parts yielded comparable results for all vaccines, and that “CP7_E2alf” yielded good results with the accompanying DIVA assay, the strain was chosen as final candidate within CSFV_GODIVA.

Among the general characteristics that an optimal CSF vaccine should have, is efficacy against all strains and types of field viruses. Due to the requirements for efficacy studies that the challenge of pigs has to be done by a suitable route with a dose of a virulent strain of CSFV that kills at least 50% of the non-vaccinated control piglets in less than 21 days (OIE, 2008), only a few challenge strains are suitable and can fulfill these prerequisites. Among these strains is the genotype 1 strain “Koslov” that has been used for almost all challenge experiments within the project and also beyond. Strictly speaking, this challenge is quite homologues for all available vaccines as they are derived from genotype 1 strains (including C-strain, Thiverval, and also the subunit vaccines). As already mentioned, most recent CSFV strains belong to genotype 2 and show a moderate, age-dependent virulence. Even the acute-lethal disease courses may not lead to death within 21 days. This means that an efficacy study using a challenge with those strains carries the risk that the outcome is invalid with regard to the OIE/Ph. Eur. requirements. These circumstances and the fact that serotypes do not play an acknowledged role for CSFV (Luo et al., 2014) have so far prevented such trials. To nevertheless provide cross-protection data, two recent field strains of sub-genotypes 2.1 and 2.3 were employed for efficacy studies along with the highly virulent strain “Koslov” (Blome et al., 2014). The field strain representing sub-genotype 2.1 (CSF1047) was isolated in Israel 2009 (David et al., 2011) and was used to challenge five domestic weaners 14 days post intramuscular vaccination. As representative for the most prevalent sub-genotype 2.3, the above mentioned CSFV strain “Roesrath” (CSFV1045, Germany 2009) was employed. It was used to challenge a group of domestic pigs 14 days post intramuscular vaccination and a
group of wild boar 21 days upon oral vaccination. The study demonstrated that solid protection was achieved against all employed genotypes (1.1, 2.1, and 2.3) and thus, broad applicability under relevant field conditions can be assumed.

As another part of the licensing procedure, duration of immunity data had to be generated for “CP7_E2alf”. As mentioned in the OIE Manual and the PhrEur, duration of immunity after vaccination against CSF shall not be less than 6 months. This has to be demonstrated in at least ten vaccinated pigs. A corresponding study (Gabriel et al., 2012) was undertaken for both intramuscular and oral vaccination with “CP7_E2alf”. Within the study, it was demonstrated that the duration of immunity after single intramuscular vaccination was at least 6 months and thus, the vaccine complied with the requirements. In the trial part with oral vaccination, one animal did not respond to vaccination and succumbed to challenge infection. Apart from this non-responder, all animals were also completely protected upon challenge six month after vaccination.

4.3.2 Efficacy of inactivated African swine fever virus vaccines

While we discuss how to obtain the ideal vaccine against CSF, holding several good options in our hands, we are still searching for any type of effective vaccine for ASF. The traditional means of vaccine production, including inactivated preparations, failed to confer solid protection. The latter had been tried in the 1960ies and 70ies (Bommeli et al., 1981; Stone and Hess, 1967) and were then abandoned. However, looking in detail, all trials had animals that survived challenge infection, and almost all animals developed antibodies. This fact let us re-assess this approach with two state-of-the-art adjuvants where we hoped to stimulate also cellular immune responses that are known to be crucial in ASF protection (Oura et al., 2005).

To go for the option that looked most feasible, we decided to use a strictly homologues system with an ASFV “Armenia” BEI-inactivated preparation, and challenge with the same “active” virus. To cut a long story short, no protection was conferred despite the fact that antibodies were produced by all animals. From temperature data, even a harmful effect cannot be excluded. The latter could be explained by an antibody-mediated enhancement of infection. This would prove that the induced antibodies were not able to neutralize the virus, and that opsonisation led to high rates of infected macrophages. The lack of virus neutralization could be confirmed in several in vitro attempts with pre-challenge sera and different protocols for a neutralization assay.

In the end, this study outcome is not only negative for this approach; it also indicates that other approaches that rely on antibody production might fail.
4.4 Conclusions and Perspectives

For the pathogenesis and host responses part, the main lessons learned include the following:

- Wild boar are comparable to domestic pigs in terms of ASFV susceptibility and course/outcome of infection.
- The ASFV strains circulating in the Trans-Caucasus, the Russian Federation and other Eastern European countries show high virulence and lead to acute-lethal disease courses in the majority of cases. No age dependence is observed under experimental conditions.
- No indications exist from experimental studies that low doses of ASFV would carry a higher risk of chronic infections or the induction of a carrier state.
- Contagiosity of ASFV is only moderate without blood contact.
- For CSFV, breed related factors could not be defined within the limits of our trials.
- Most young animals infected with recent CSFV strains will succumb to infection after an acute-lethal disease course. However, some chronic cases are likely as they even appeared under experimental conditions. These animals can have tremendous impact on the epidemiology of CSF.

The generated data on ASF can now feed into the discussion with regard to appropriate control measures. In fact, this happened already when the data complemented the background data that were used to draft the EFSA Scientific Opinions on African swine fever in 2010 and 2014. The above mentioned experimental data have also an impact on the choice of diagnostic systems and were taken into consideration for the below mentioned establishment of alternative sampling options for early warning systems in wild boar. Especially the complete lack of antibody detection in all our trials puts the focus on virus detection methods for surveillance and early warning systems in free areas.

In the meantime, ASF has further spread. In this way, our assumptions and decisions are put to an unwanted test. So far, mostly wild boar are affected, but the disease was also found in a couple of industrialized pig farms and in backyard holdings. The slow spread among wild boar seems to confirm the moderate to low contagiosity that was indicated above, and the continuous reports of fallen animals of all age classes underline the occurrence of acute-lethal disease courses. However, according to personal communications (Z. Pejsak, Poland), the
death toll apparently does not lead to a reduction in wild boar numbers. Low antibody prevalences in the affected areas could indicate that at least some animals survive infection. The role of these survivors in the epidemiology of ASF remains unclear. The animals are discussed to be persistently infected. In this case, they could play a crucial role in the perpetuation of ASFV infection and favor an endemic state. Here, additional studies under field and laboratory conditions are clearly needed. An approach towards the understanding of critical host responses could be detailed research into the reservoir host, the warthog and its African relatives. In contrast to the disease courses in domestic pigs and European wild boar, the warthog does not present clinical signs but seems to be persistently infected (Jori and Bastos, 2009). The same may hold true for bushpigs (Anderson et al., 1998; Oura et al., 1998a). Interestingly, neither horizontal nor vertical transmission seem to occur in the warthog and maintenance of the virus within warthog populations is dependent on the soft tick *Ornithodoros moubata* which inhabits warthog burrows (Jori et al., 2013; Plowright et al., 1969). Factors and reaction pattern leading to these differences are not well defined and present an important knowledge gap in ASF research, also towards vaccine and control design. To address this question, a warthog challenge model is planned in the framework of a new research project. The project parts that will be covered by my working group shall include the *in vitro* comparison of macrophages and dendritic cells from warthogs, domestic pigs and European wild boar, implementation of an infection model and appropriate readouts for pathogenesis studies in warthog and subsequent studies. These studies will make use of our recently developed research tools such as cytokine assays (Petrov et al., 2014a).

One of the most important findings with regard to CSF was the occurrence of chronic infections even under experimental conditions. To supplement the studies on host responses with the impact of the virus (through genetic changes or quasispecies composition), sequence analyses were now carried out using next-generation sequencing. The preliminary results do not show an impact of the virus on different disease courses (M. Jenckel, manuscript in preparation). Further studies are needed to understand the host factors that are important for the induction of chronic infection. Cytokine reactions will be one of the future targets and the above mentioned assays will be extended towards additional cellular factors (e.g. toll-like receptors).
For the diagnostic parts, the following conclusions can be drawn:

- Blood swabs are suitable for the reliable detection of ASFV and CSFV and can be used as an alternative sampling strategy for fallen wild boar. These swabs are also suitable for the detection of antibodies against ASFV.
- Genetic DIVA approaches can facilitate the rapid differentiation of field and vaccine strains of CSFV in case of emergency vaccination.

Based on the promising results with blood swabs, we included this sampling method into the official method collection for swine fevers as an alternative for surveillance in free areas. This „Official Collection of Methods for the Sampling and Investigation of Materials of Animal Origin for Notifiable Animal Diseases (Method Collection)“ that is published and updated by the FLI implements Article 27 (paragraph 4) of the German Animal Health Law and lists the test principles recommended for application in Germany for the laboratory diagnosis of notifiable animal diseases. Follow-up under field conditions will complete the studies conducted in the framework of this thesis. For now, an instructive document has been designed and is available for download on the webpage of the FLI.

It is an interesting coincidence that parts of the current ASF restriction zones in Latvia overlap with CSF restriction zones. Both diseases are still present and are more or less simultaneously reported (OIE WAHID interface, visited March 3rd 2015). This underlines the necessity of differential diagnosis as it is e.g. implemented in the implemented diagnostic approaches.

The successful implementation of a genetic DIVA concept paves that way also for the use in domestic pig populations. While the approach was here implemented for “C-strain Riems”, a similar approach is also available for marker vaccine “CP7_E2alf” (Leifer et al., 2009a). It could supplement the tools that are available for a possible emergency vaccination scenario.
Concluding from the vaccination and control part of the thesis, the following could be demonstrated:

- In a comparative trial, chimeric pestivirus “CP7_E2alf” was shown to be at least as protective as C-strain “Riems” vaccine after oral vaccination.
- “CP7_E2alf” is able to confer solid protection against challenge with different CSFV genotypes, including relevant field strains of genotype 2.
- Duration of immunity is at least 6 months after single intramuscular or oral vaccination. Non-responders may occur upon oral vaccination.
- State-of-the-art adjuvants do not enhance the efficacy of inactivated ASFV vaccine preparations.
- Antibodies against ASFV (as measured by routine serological tests) did not have a positive impact on disease outcome. An antibody enhancement phenomenon cannot be excluded.

For CP7_E2alf, some questions remain to be answered. While studies in reproductive boar to assess the shedding of vaccine virus in semen and further studies with regard to genetic stability have been carried out very recently (Dräger et al., submitted; Goller et al., manuscript in preparation), there are still open questions with regard to efficacy of “CP7_E2alf” (and other live vaccines) in animals with antibodies against ruminant pestiviruses (Bovine viral diarrhea virus and Border disease virus). Studies addressing this issue are planned for the near future.

However, including the above mentioned findings on “CP7_E2alf” efficacy, a dossier was submitted to EMA, and very recently, “CP7_E2alf” has been officially licensed for intramuscular vaccination (Suvaxyn CSF Marker) of domestic pigs.

Thus, for the first time, an emergency vaccination scenario is feasible that would allow deviations from the trade restrictions for vaccinated animals.
African swine fever (ASF) and classical swine fever (CSF) are among the most important viral diseases of domestic and wild pigs. Outbreaks of either disease are accompanied by tremendous socio-economic consequences that threaten the livelihood of small farmers and the profitable production in industrialized settings. Thus there is an urgent need for state-of-the-art control measures including diagnostic tests for timely diagnosis and disease surveillance.

This habilitation thesis covers different aspects that are needed for rational strategy design, i.e. generation of background data, design and evaluation of control tools in terms of vaccines, and the optimization of diagnostic systems.

The first point, generation of background data, was addressed in experimental studies with domestic pigs and European wild boar that targeted the pathogenesis of swine fevers in the broader sense. In detail, the presented original contributions include three studies that were carried out to characterize a representative Caucasian ASFV strain. It was demonstrated that European wild boar are fully susceptible to ASFV upon oral and intramuscular inoculation, and that the chosen ASFV strain is highly virulent. Under the conditions of the experiments, all animals developed an acute-lethal disease course and succumbed to infection within roughly 10 days. No antibodies were detected by routine screening and confirmation techniques. Neither an age nor dose dependence was observed. Contagiosity of the virus was moderate without direct blood contact. To address the impact of breed and race on the clinical course and outcome of CSF, different domestic pig breeds and European wild boar were inoculated with a recent, moderately virulent CSFV strain from Germany. Under the circumstances of this study, no breed dependence was observable with regard to virological results. However, small differences in age and preload with facultative pathogens seemed to play a role. Almost all animals showed an acute-lethal disease course. Still, two animals recovered, and one developed a chronic form of CSF.

Based on these studies, it can be expected that an introduction of ASF or CSF into a free area or population would lead to observable mortality. Thus, fallen animals would be the best target for an early warning system. For the wildlife host, this system has to rely on the cooperation of hunters, game wardens, and foresters. Naturally, fallen wild boar are often not easy to find, and sampling is challenging and therefore a bottleneck. To increase the
compliance of the people involved and thus the number of samples, alternative sampling methods were investigated in combination with routine polymerase chain reaction (PCR) technology. It could be shown that dry blood swabs are suitable for molecular swine fever diagnosis. The samples on the swabs were stable for several weeks and allowed reliable detection of ASF and CSF. Moreover, the swab samples were proven suitable for ASF antibody detection.

A second diagnostic challenge was the rapid genetic differentiation of C-strain vaccinated from CSF field virus infected wild boar in areas with oral immunization. Here, a recently developed differentiating multiplex reverse transcription PCR was employed under field conditions. It could be proven that this genetic DIVA (differentiation of infected from vaccinated animals) approach is suitable under field conditions. This method helps to rule out or confirm new cases within a day and thus increases efficacy of control measures.

The last part covered vaccination as control tool. Recent advances in CSF vaccine development allowed revisiting emergency vaccination strategies for both domestic pigs and European wild boar. The concepts that were followed to design a new generation of marker vaccines, their advantages and disadvantages, and the conclusions thereof are outlined in a review article. The presented original contributions cover different parts of the assessment of marker vaccine strain “CP7_E2alf” on its way to licensing.

The first contribution refers to the comparative testing of two chimeric marker vaccine candidates, namely “flc11” and “CP7_E2alf”, along with the gold standard “C-strain” in a trial with oral immunization of domestic pigs. This trial was part of the preliminary testing that led to the choice of “CP7_E2alf” as final candidate within the EU project CSFV_goDIVA (project reference 227003; funded under FP7-KBBE). In this experiment, both chimeric viruses proved safety and efficacy upon challenge with a highly virulent CSFV strain 21 days after vaccination. With a challenge 14 days after vaccination, “CP7_E2alf” was able to confer full clinical protection while 50% of “flc11” and 17% of “C-strain” vaccinated pigs succumbed to challenge infection. The discriminatory marker test worked well with vaccinated animals even after multiple vaccination but showed weaknesses in detecting infection in vaccinated animals.

Further, a study is presented that was carried out to proof efficacy against relevant CSFV genotypes after oral immunization of wild boar and intramuscular vaccination of domestic pigs. The study could confirm that “CP7_E2alf” pilot vaccine batches confer solid protection not only against highly virulent CSFV strains of a homologues genotype but also against recently circulating strains of genotypes 2.1 (Israel) and 2.3 (Germany).
Finally, a duration of immunity study is reported that was carried out in accordance with the requirements of the OIE manual of diagnostic tests and vaccines for terrestrial animals. The test was carried out for oral and intramuscular vaccination of domestic pigs. It was shown that all animals that seroconverted upon vaccination were protected from a highly virulent challenge after six month. One orally vaccinated animal did not respond to vaccination and suffered from acute-lethal CSF upon challenge. Thus, intramuscular vaccination fulfilled the OIE requirements, and with oral vaccination, only the non-responder was not protected.

The last contribution reports on a study that was undertaken to assess the impact of modern adjuvants on the efficacy of inactivated ASFV preparations. While all immunized animals showed specific antibody responses upon double vaccination, none of the animals was protected against strictly homologous challenge. All animals developed an acute lethal ASF. The outcome of the study clearly shows that modern adjuvants do not enhance the efficacy of ASFV vaccine preparations.

Taken together, the presented studies feed into the strategy design for ASF and CSF and will hopefully facilitate control.
6 LIST OF PUBLICATIONS

6.1 Topic 1: Pathogenesis and host responses

  
  Own contribution: Compilation of background data and writing of the manuscript draft.

  
  Own contribution: Compilation of background data (in part), provision of figures, and revision of the manuscript.

  
  Own contribution: Conception, supervision and execution of the animal trial, part of the laboratory investigations, evaluation of data, and revision of the manuscript.

  
  Own contribution: Conception, supervision and execution of the animal trial, part of the laboratory investigations, data analyses, and drafting of the manuscript.

  
  Own contribution: Conception, supervision and execution (in parts) of the animal trial, part of the laboratory investigations, data analyses, and revision of the manuscript.

*Own contribution: Conception, supervision and execution of the animal trial (in part), evaluation of data, revision of the manuscript.*

6.2 **Topic 2: Vaccination**


*Own contribution: Compilation of background data and writing of the manuscript draft.*


*Own contribution: Execution of the animal trials (in part), collection and evaluation of data, and drafting of the manuscript.*


*Own contribution: Conception and execution of the animal trial at the FLI (in part), laboratory investigations, collection and evaluation of data, and drafting of the manuscript.*


*Own contribution: Drafting of the study design, execution of the animal trial (in part), collection and evaluation of data, and revision of the manuscript.*


*Own contribution: Conception and, execution of the animal trial (in part), laboratory investigations, collection and evaluation of data, and drafting of the manuscript.*
6.3 Topic 3: Optimization of Diagnosis

  
  *Own contribution: Collection of background data, literature search, drafting of the manuscript.*

  
  *Own contribution: Drafting of the study design, organization of laboratory work, execution of laboratory investigations (in part), and revision of the manuscript.*

  
  *Own contribution: Drafting of the study design, organization of laboratory work, execution of laboratory investigations (in part), and drafting of the manuscript.*

  
  *Own contribution: Study design, organization of laboratory work, execution of laboratory investigations (in part), and drafting of the manuscript.*
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