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(PD Dr. Martin G. Beer)

Classical and African Swine Fever in Domestic Pigs and European Wild Boar: Optimization of Control Strategies and Laboratory Diagnosis

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von Claudia Gabriel
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Berichterstatter:	Univ.-Prof. Dr. Sutter
Korreferenten:	Univ.-Prof. Dr. Dr. habil. Gareis
	Univ.-Prof. Dr. Ritzmann
	Univ.-Prof. Dr. Matiasek
	Univ.-Prof. Dr. Müller

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For my family

1 INTRODUCTION

Classical swine fever (CSF) and African swine fever (ASF) are highly contagious viral diseases. Due to their tremendous socioeconomic impact, both diseases are notifiable to the World Organisation for Animal Health (OIE). Classical swine fever is caused by a small enveloped RNA virus of the genus *Pestivirus* within the *Flaviviridae* family. Natural hosts are domestic pigs and European wild boar (Sus scrofa). African swine fever is caused by ASF virus (ASFV), a complex DNA virus of the genus *Asfivirus* within the *Asfarviridae* family. Besides the natural infection of different members of the Suidae family, ASFV is able to replicate in soft ticks of the genus Ornithodoros and is therefore classified as the only DNA arthropod borne virus. Both diseases can cause a wide range of unspecific symptoms and lesions, which can not be distinguished from a variety of other viral, bacterial and non-infectious diseases.

In the case of CSF outbreaks in domestic pig populations, a strict stamping out strategy without prophylactic vaccination is applied within the European Union (EU). Nevertheless, EU legislation also foresees the possibility of emergency vaccination campaigns using either conventional or marker vaccines. Conventional modified live vaccines have been successfully used to eradicate CSF from the EU and to control outbreaks in European wild boar populations. However, the use of these vaccines implicates severe trade restrictions for domestic pigs and their products. The use of available marker vaccines, namely E2 subunit vaccines, would allow a differentiation of infected from vaccinated animals (DIVA) based on serology and thus ease trade restrictions. Unfortunately, E2 subunit vaccines lack important properties like early onset of immunity and full protection against vertical transmission. For this reason, emergency vaccination with the marker vaccine was so far only implemented for industrialized pig holdings in Romania.

Different approaches have been followed to optimize the current control strategies and to develop new tools.

For optimization of current strategies, recently developed multiplex real-time reverse transcription polymerase chain reactions for the simultaneous detection and differentiation of field viruses from vaccine viruses have been designed and can be used for a “genetic DIVA” concept. The evaluation of this approach in the framework of oral emergency vaccination of wild boar is part of the presented work.

In terms of new tools, several research groups worked on the design of new marker vaccine candidates and accompanying discriminatory laboratory tests. Among various promising marker vaccine candidates that have been developed over the last decades, the chimeric pestivirus “CP7_E2alf” has proven to be an efficacious DIVA vaccine candidate for both, intramuscular vaccination of domestic pigs and oral vaccination of wild boar. To obtain market authorization, several mandatory immunogenicity, safety, and efficacy trials are required. Here, studies are reported that cover innocuousness in target and non-target species, duration of immunity and efficacy against challenge with different strains.

While Europe has a long history of CSF outbreaks, ASF is still considered exotic. However, the occurrence and persistence of ASF among domestic pigs and wild boar in the Russian Federation and the Trans-Caucasian Countries increases the risk of introduction into the EU. In contrast to CSF, control of ASF outbreaks can not draw on vaccination, as all attempts to develop an effective vaccine failed up to now. Therefore, knowledge about disease dynamics in different hosts including wild boar is of paramount importance for the design of disease control strategies and risk assessment. In the presented studies, the Caucasian ASFV Isolate was characterized in different age classes of wild boar.

2 LITERATURE REVIEW

2.1 Classical Swine Fever

2.1.1 Virus taxonomy, morphology and structure

Classical swine fever virus (CSFV), the causative agent of CSF, belongs to the genus *Pestivirus* within the *Flaviviridae* family (Thiel et al., 2005). The virus is antigenically closely related to the approved pestiviral species *Bovine viral diarrhea virus* (BVDV) *1* and *2*, *Border disease virus* (BDV), the atypical pestivirus isolated from a giraffe and a variety of unclassified pestiviruses (Harasawa et al., 2000; 2004; Stalder et al., 2005; Thabti et al., 2005; Vilcek et al., 2005; Kirkland et al., 2007; Stahl et al., 2007).

Classical swine fever virus is a small, enveloped, icosahedral virus with a diameter of 40-60 nm (Horzinek et al., 1967; Moennig and Plagemann, 1992) and has a single-stranded, positive-sense RNA genome of approximately 12.3 kb (Meyers et al., 1989; Moormann et al., 1996). The genome possesses one large open reading frame (ORF) flanked by two non-translated regions (NTRs) (Rümenapf et al., 1991a; Collett, 1992). The ORF codes for a polyprotein of about 3900 amino acids which is co- and posttranslationally processed by viral and cellular proteases into eleven viral proteins (Thiel et al., 1991; Rümenapf et al., 1993; Tautz et al., 1997; Xu et al., 1997; Bintintan and Meyers, 2010). Among these, four proteins constitute the structure, in particular the core (C) protein and the three envelope glycoproteins E^{rns}, E1, and E2. Furthermore, seven non-structural (NS) proteins are encoded, namely N^{pro}, p7, NS2-3, NS4A, NS4B, NS5A, and NS5B (Thiel et al., 1991; Elbers et al., 1996; Meyers and Thiel, 1996; Lattwein et al., 2012). The genome organization of *Pestiviruses* is depicted in Figure 1.

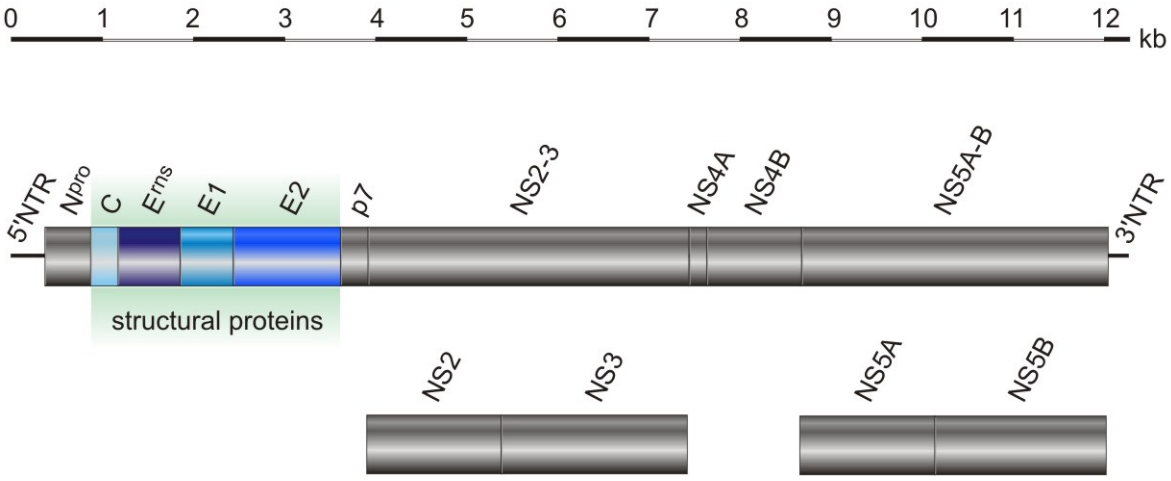

Fig. 1: Schematic representation of the genome organization of *Pestiviruses*. Genome regions encoding structural proteins are colored blue, whereas genome regions encoding non-structural proteins are shown in grey (modified from Meyers and Thiel, 1996). The width of rectangles is scaled according to the different lengths of genome fragments.

For immune response and thus vaccine development, the envelope glycoproteins E2 and E^{rns} are of major importance.

The envelope glycoprotein E2 is known to be the major immunogen of Pestiviruses including CSFV. It was also shown that it presents a virulence determinant (van Gennip et al., 2004; Risatti et al., 2005b; Risatti et al., 2006; Risatti et al., 2007), plays a major role in virus attachment and entry into target cells (Hulst and Moormann, 1997; Weiland et al., 1999), and is essential for virus progeny (van Gennip et al., 2002). In addition, it was identified as a target for cytotoxic T cells (Ceppi et al., 2005). The protein is present as disulfide-linked complex as homodimer or as stable heterodimer with the structural protein E1 (Weiland et al., 1990; Thiel et al., 1991; Rümenapf et al., 1991a). Cysteins in the C-terminal or N-terminal half of E2 were shown to lead to intermolecular or intramolecular disulfide bonds, respectively. Using a panel of monoclonal antibodies, Wensvoort (1989) identified four antigenic domains (A, B, C, and D) on the E2 protein, from which the highly conserved domain A contains a linear epitope of the amino acid sequence "TAVSPTTLR" (Lin et al., 2000; Qi et al., 2008; Qi et al., 2009). Advanced epitope mapping studies now indicate that the E2 protein consists of only three domains which contain at least 14 linear or discontinuous epitopes (Lerch, 2006).

The envelope glycoprotein E^{rns} is the second antigen inducing antibodies in the host (Weiland et al., 1992; König et al., 1995). This protein was characterized as a ribonuclease and is

presumed to be also involved in the determination of virulence and host adaptation (Schneider et al., 1993; Windisch et al., 1996; Hulst et al., 1998; Meyers et al., 1999; Hausmann et al., 2004). Recent studies showed that the deletion of the pestiviral E^{rns} protein leads to a lack of virus progeny (Widjojoatmodjo et al., 2000; Reimann et al., 2007), whereas mutated E^{rns}-coding sequences of several recombinant pestiviruses lead to clinical attenuation (Meyers et al., 1999; Meyer et al., 2002; Tews et al., 2009). The protein is stabilized by four intramolecular disulfide bridges, possesses seven to nine putative N-linked glycosylation sites, and is usually present in form of disulfide-bond homodimers (Rümenapf et al., 1993; Langedijk et al., 2002; Sainz et al., 2008). An unusual membrane anchor mediates the association to the surface of the virion (Fetzer et al., 2005; Tews and Meyers, 2007). Furthermore, E^{rns} is secreted in considerable amounts from infected cells (Rümenapf et al., 1993; Schneider et al., 1993).

The other structural and nonstructural proteins have various functions in RNA replication and production of virus progeny, for instance as protease (NS3), or as RNA dependent RNA polymerase (NS5B) (Meyers et al., 1989; Weiland et al., 1990; Greiser-Wilke et al., 1992; Rümenapf et al., 1993; Stark et al., 1993; Tamura et al., 1993; Warrener and Collett, 1995; Elbers et al., 1996; Tautz et al., 1997; Xu et al., 1997; Zhong et al., 1998; Steffens et al., 1999; Harada et al., 2000; Tautz et al., 2000; Qu et al., 2001; La Rocca et al., 2005; Ruggli et al., 2005; Tellinghuisen et al., 2006; Bauhofer et al., 2007; Murray et al., 2008; Ivanyi-Nagy et al., 2008; Fernandez-Sainz et al., 2009; Fiebach et al., 2011).

Based on phylogenetic analyses of nucleotide sequences of fragments of the 5'-NTR, and the E2 encoding genome regions, CSFV strains can be divided into three genogroups (1-3). Genogroups 1 and 2 are further subdivided into three subgroups (CSFV 1.1, 1.2, 1.3, 2.1, 2.2, and 2.3), whereas genogroup 3 consists of four subgroups (CSFV 3.1, 3.2, 3.3, and 3.4) (Paton et al., 2000; Greiser-Wilke et al., 2006). Clusters, especially of subgroup 2.3 can be defined based on the partial 5'-NRT sequence. These clusters are often linked to geographic distribution and used for molecular epidemiology (Fritzemeier et al., 2000; Leifer et al., 2010b).

2.1.2 Epidemiology, clinical signs, lesions and pathogenesis

Susceptible hosts for CSFV are different members of the *Suidae* family, particularly domestic pigs (*Sus scrofa domesticus*) and European wild boar (*Sus scrofa scrofa*) (Depner et al., 1995; Blacksell et al., 2006). In a recent study in South Africa, the susceptibility of Common Warthogs (*Phacochoerus africanus*) and Bushpigs (*Potamochoerus larvatus*) for CSFV was demonstrated (Everett et al., 2011).

Classical swine fever virus can be transmitted both horizontally and vertically. Horizontal transmission takes places through direct or indirect contact between infected and susceptible pigs. Important indirect routes include feeding of virus contaminated garbage and mechanical transmission via contact to humans or agricultural and veterinary equipment (van Oirschot, 1999). Upon contact, infection usually occurs through the oronasal route, or less frequently via conjunctiva, mucus membranes, skin abrasions, insemination, and the use of contaminated instruments (de Smit et al., 1999a; Floegel et al., 2000; Moennig and Greiser-Wilke, 2008; Pasick, 2008). Infected pigs show high-titer viremia and shed virus at least from the beginning of clinical disease until death or specific antibodies have developed. The main excretion routes are by saliva, lacrimal secretions, urine, feces, and semen (Ressang, 1973a; van Oirschot, 1999; Pasick, 2008). In contrast, chronically infected pigs shed the virus continuously or intermittently until death (van Oirschot, 1999). Vertical transmission from pregnant sows to fetuses is possible throughout all stages of gestation and can lead to persistently infected offspring.

Classical swine fever can cause a wide range of clinical syndromes after an incubation period of seven to ten days (Moennig, 2000). The clinical course can vary considerably, depending on different host factors and the virulence of the corresponding CSFV isolate. Among the host factors, the age of affected animals, the breed, the immune status as well as secondary infections determine the outcome of the disease (Kaden et al., 2000b; Moennig et al., 2003). Different courses of CSF can be distinguished: peracute, acute, chronic, and prenatal forms such as persistent infections.

After infection with highly virulent CSFV isolates peracute courses can occur. In this case, piglets die peracutely after a short period with high fever but without any CSFV specific symptoms (Dunne, 1970).

The acute course can either lead to death (acute lethal form) or recovery (acute transient form) of the animal. Acute lethal forms with severe clinical signs are mostly seen in weaner and

young fattening pigs after infection with moderately or highly virulent isolates. Initial signs are high fever, anorexia, lethargy, huddling, conjunctivitis, enlarged lymph nodes, respiratory symptoms and constipation followed by diarrhea. Neurological signs like a staggering gait, incoordination and convulsions are frequently seen. During the second or third week after infection, typical hemorrhages of the skin may appear on the ear, tail, abdomen and the inner side of the limbs (Moennig et al., 2003). As CSFV causes severe leucopenia and thrombocytopenia, secondary infections of the respiratory or gastrointestinal tract may mask or overlap typical signs of CSF, contributing in a further deterioration in health and misleading of farmers and veterinarians (Schmidt and Kaaden, 1968; Depner et al., 1999; Moennig et al., 2003). Affected animals usually die ten to 20 days post infection (Blome, 2006). The pathological findings include lesions in the lymphoreticular system and petechiae and ecchymoses in several organs (Kleiboeker, 2002; Moennig et al., 2003). Regularly, infarctions of the spleen are observed and considered highly characteristic, almost pathognomic for CSF (van Oirschot, 1999; Kleiboeker, 2002). Complicated by bacterial secondary infections, purulent to necrotic tonsillitis as well as catarrhal to fibrinous bronchopneumonia may be present (Kleiboeker, 2002). A nonpurulent meningoencephalitis is seen in almost every pig (Gruber et al., 1995; Gómez-Villamandos et al., 2006).

Apart from acute lethal courses, transient or subacute infections may appear associated with low virulent strains or with an increasing age of the infected animals. In these cases, atypical and less pronounced clinical signs and the production of antibodies occur and the animal recovers completely. The term “atypical course” arose due to the fact that clinical signs are not indicative for CSF (Depner, 2006).

The chronic form of CSF is seen when the host’s immune system fails to establish an effective immune response against the virus and is associated with 100% mortality (Moennig et al., 2003; Moennig and Greiser-Wilke, 2008). Initially, clinical signs are similar to the acute form, followed by non-specific signs like anorexia, lethargy, intermittent fever, chronic enteritis, and wasting (Moennig et al., 2003). Periods of acute clinical disease and general improvement in the clinical condition may alternate and last for several months until the pigs die (Depner et al., 1996; van Oirschot, 1999; Moennig et al., 2003). Pathological changes are less pronounced, especially hemorrhages and infarctions are almost always absent (Kleiboeker, 2002). Most striking lesions are atrophy of the thymus and depletion of lymphocytes in peripheral lymphoid organs (van Oirschot, 1999). Necrosis and ulceration on the ileum, the ileocecal valve and colon are common (Moennig et al., 2003).

The prenatal form of CSF arises from the fact that CSFV is able to cross the placenta of pregnant sows, whereas antibodies cannot be transferred from the pregnant sow to the fetuses (van Oirschot and Terpstra, 1977; Meyers and Thiel, 1996). While sows mostly develop mild or subclinical courses of the disease, the outcome of transplacental infection of fetuses depends on the time of gestation and virus virulence. Infection in the early stage of pregnancy can lead to abortions, stillbirths, mummification and malformations. Infection from about 50-70 days of pregnancy, however, may result in persistently infected piglets (Moennig et al., 2003). Being immunotolerant to CSFV, these piglets stay seronegative and shed virus continuously and therefore play an important role in spreading of the disease (Kleiboeker, 2002). The piglets may remain healthy for months, then develop the so-called "late-onset" course of CSF which is characterized by retarding of growth, wasting, conjunctivitis, dermatitis and diarrhea. All animals affected by this disease course eventually die. Occasionally congenital tremor occurs (van Oirschot and Terpstra, 1977; van Oirschot, 1999; Kleiboeker, 2002).

After natural oronasal infection, the epithelial cells of the tonsillar crypts are the primary site of virus replication (Ressang, 1973a; Ressang, 1973b; Liess, 1987). Subsequently, the virus spreads via lymphatic vessels to the regional lymph nodes, followed by secondary dissemination into the spleen, bone marrow, visceral lymph nodes and lymphoid structures of the small intestine through the vascular system (Ressang, 1973a). Whilst secondary replication of CSFV occurs in lymphoid tissue and in circulating leukocytes and mononuclear cells, the level of viremia rises rapidly and results in the invasion of parenchymatous organs (van Oirschot, 1999). The spread of virus throughout the pig is usually completed in five to six days (Ressang, 1973a). The typical clinical picture of acute lethal CSF in form of a hemorrhagic disease is caused by direct and indirect interactions of the virus with its main target cells, namely macrophages (Gómez-Villamandos et al., 2001), endothelial cells (Heene et al., 1971; Trautwein, 1988), dendritic cells (Carrasco et al., 2004), lymphoreticular cells and epithelial cells (Moennig, 2000).

According to Gómez-Villamandos (2003) macrophages may play a major role in the pathogenesis of CSF as viral infection, apoptosis, phagocytotic and secretory activation, and cell count changes appear in these cells after infection. Lymphopenia, thrombocytopenia and hemorrhages, however, were postulated not to be attributed to a direct effect of the virus on lymphocytes, endothelial cells and platelets (Gómez-Villamandos et al., 1998; Summerfield et al., 1998; Gómez-Villamandos et al., 2000; Sato et al., 2000; Sánchez-Cordón et al., 2002).

Instead, it is assumed that these processes may especially be due to mediators released by various activated macrophage populations, in particular tumor necrosis factor-alpha (TNF-α), Interleukin (IL) 1α, IL-1β, IL-6, and platelet-activating factor (PAF) (Gómez-Villamandos et al., 2003; Lange et al., 2011).

2.1.3 Immune response

After CSFV infection, cellular as well as humoral immune mechanism are involved in the development of immunity against the virus. As natural CSFV infections are accompanied by severe lymphopenia, the immune responses in CSF are delayed. During the acute course of disease, pigs develop only low levels or even no neutralizing antibodies that may be detectable from two weeks after infection (Liess et al., 1977; Artois et al., 2002). Transplacental infection of fetuses can lead to immunotolerance, thus no antibodies can be detected in persistently infected piglets (Liess, 1987). If animals recover, high antibody titers against the glycoproteins E^{rns} and E2, and the non-structural protein NS3 appear and may persist lifelong (Moennig and Greiser-Wilke, 2008).

Neutralizing antibodies are mainly raised against the envelope protein E2. As these are sufficient for protective immunity, marker vaccine concepts are based on this antigen (Weiland et al., 1990; van Zijl et al., 1991; Weiland et al., 1992; Hulst et al., 1993; van Rijn et al., 1996; Bouma et al., 1999; van Gennip et al., 2002).

To a lesser extent, the host's immune system produces antibodies against the envelope protein E^{rns} (Weiland et al., 1992; König et al., 1995). As most marker vaccines do not induce E^{rns} antibodies, marker assays based on the detection of E^{rns} antibodies allow a serological DIVA strategy.

Furthermore, antibodies are raised against the highly conserved NS3 protein. These antibodies are not specific for CSFV, but show cross-reactivity with other pestiviruses, and thus are not suitable for most DIVA approaches (Paton et al., 1991).

Generally, animals developing neutralizing antibodies are protected against subsequent CSF infection (Terpstra and Wensvoort, 1988). However, certain vaccination-challenge studies revealed protection in the absence of neutralizing antibodies (Aynaud and Launais, 1978; Rümenapf et al., 1991; Suradhat et al., 2001; Ganges et al., 2005), indicating that cellular immune mechanism are likewise involved in conferring protection. Indeed, the role of protective T-cell immunity could be confirmed, as specific epitopes for the stimulation of helper T-cells ($CD4^+$ T-cells) and cytotoxic T-lymphocytes ($CD8^+$ T-cells) have been identified on the viral proteins E2 and NS2-3 (Pauly et al., 1995; Armengol et al., 2002; Piriou et al., 2003; Ceppi et al., 2005; Ganges et al., 2005; Rau et al., 2006). Although several questions regarding the role of cellular and innate immunity remain open, it can be stated that a balance between humoral and cellular immunity seems to play a pivotal role for the development of optimized immune responses (Anonymous, 2009).

2.1.4 History, global distribution and economic impact

The origin of CSF, formerly called "hog cholera", still remains unclear. Chronicles go back as far as to the early 19th century. Accordingly, CSF was first observed in 1833 in Ohio, USA, or in 1822 in France (Edwards et al., 2000). For long time assumed to be a bacterium, the causative agent was found to be a filterable virus in 1903 (De Schweinitz and Dorset, 1904). During the late 19th century, the disease evolved to one of the most important diseases of swine with worldwide distribution. Nowadays, CSF has been successfully eradicated in Australia, Canada, the US and most EU Member States, whereas the disease is prevalent in Central and South America, the Caribbean, and many Asian countries (Moennig and Greiser-Wilke, 2008). Apart from the occurrence of CSF in Madagascar and South Africa, the situation in Africa remains uncertain (Sandvik et al., 2005; Penrith et al., 2011). Despite intensive efforts to eradicate the disease in Europe, CSF was continuously observed over the last decades in several European countries in either wild boar or domestic pig populations (Edwards et al., 2000; Artois et al., 2002; Pol et al., 2008; Moennig and Greiser-Wilke, 2008; Floegel-Niesmann et al., 2009; Blome et al., 2010; Leifer et al., 2010b), partially reaching endemicity in wild boar (Moennig, 2000). These epidemics and related control measures caused major socio-economic damage, as seen during the epidemic in The Netherlands in 1997/1998, where over 11 million pigs were slaughtered and the direct costs were estimated at about US $ 2 billion (Stegeman et al., 2000; Terpstra and de Smit, 2000). Since the 1980s, almost all isolates from Europe belong to genogroup 2 (Paton et al., 2000), and recent CSF outbreaks in the European wild boar population were mainly associated with CSFV isolates of subgroup 2.3 (Kaden et al., 2004; Pol et al., 2008; Leifer et al., 2010b).

Particular attention must be given to wild boar populations, as infected wild boars may act as a reservoir for CSFV. Usually infected via contaminated food, persistence of CSF in wild boar populations may occur over long periods and is believed to benefit from large population sizes, accompanied by high densities of most susceptible young animals (Moennig, 2000; Artois et al., 2002; Penrith et al., 2011). Introduction of CSF from wild boar into domestic pig populations may occur through direct or indirect contact, and was supposed to be the reason for 59% of primary outbreaks in domestic pig herds in Germany between 1993 to 1998 (Fritzemeier et al., 2000).

A disease distribution map based on available data of the first half-year of 2011 according to the World Organisation for Animal Health web site (http://www.oie.int) is shown in Figure 2. Nevertheless, according to Paton and Greiser-Wilke (2003) the true extent of the disease

might be underrated, not least because of insufficient surveillance resources, political and economic pressure not to notify the presence of the disease and masking effects of vaccination.

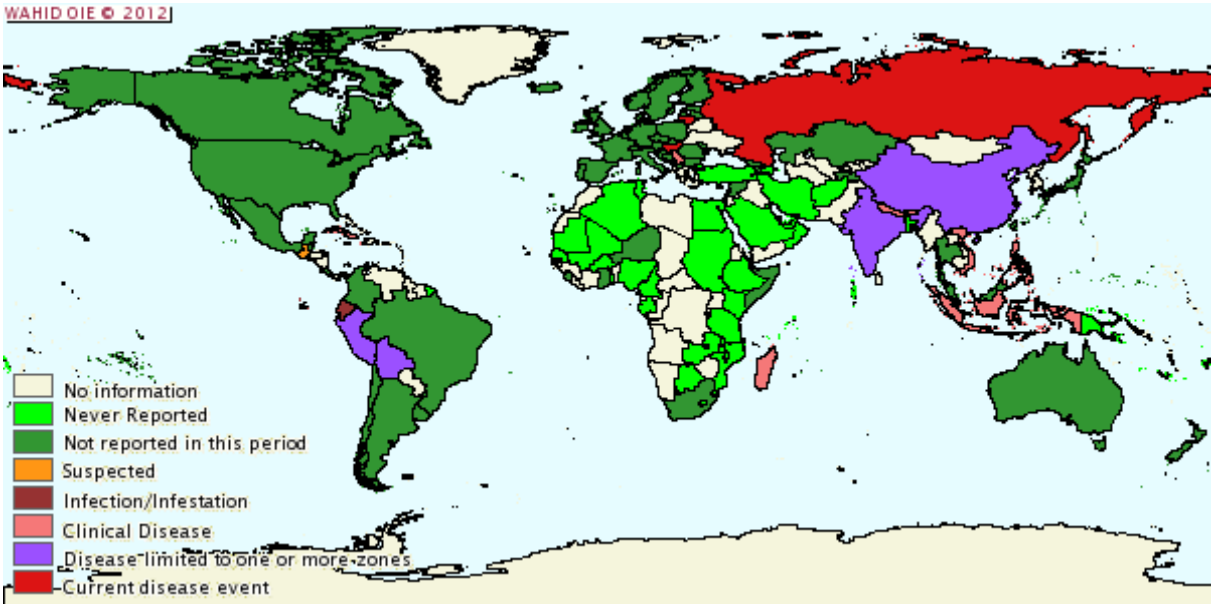

Fig. 2: Classical swine fever distribution in wild and domestic pigs, reporting period January to June 2011 (OIE, 2012a).

2.1.5 Control strategies and laboratory diagnosis

The majority of countries with significant pig production has statutory control measures for CSF in place, but the efficacy and success of these measures varies in accordance with economical factors including the status of veterinary and laboratory infrastructure (Edwards et al., 2000). Integral part of these control measures are most often potent vaccines, especially live attenuated variants. Although vaccination in itself does not bring about disease eradication, vaccines have proven to present powerful tools for animal disease control. Through the use of mandatory vaccination campaigns in combination with strict veterinary sanitary measures, eradication of CSF has been achieved in many industrialized pig populations worldwide (van Oirschot, 2003b; Greiser-Wilke and Moennig, 2004a; Dong and Chen, 2007). Nevertheless, complete eradication of the disease could not be achieved and sporadic outbreaks keep occurring. In addition, endemically infected feral pig populations in some EU member states complicate the situation (Anonymous, 2009).

Within the EU, control and eradication measures for CSF are laid down in Council Directive 2001/89/EC and Commission Decision 2002/106/EC (Anonymous, 2001; Anonymous, 2002a). In case of a CSF outbreak in domestic pigs, a strict stamping out strategy is applied since 1990 in order to prevent further spreading of the virus (Greiser-Wilke and Moennig, 2004a). This implies culling of infected herds and potential contact herds, establishment of protection and surveillance zones and movement restrictions. Prophylactic vaccination is prohibited, but emergency vaccination of domestic pigs and wild boar populations is among the legal options. So far, emergency vaccination has been mainly applied for the control of CSF outbreaks in wild boar populations. Several Member States made use of the possibility of oral immunization, among these were Germany, France, Slovakia, Romania, Luxembourg, and Bulgaria. Due to the fear of trade restrictions, emergency vaccination of domestic pigs was so far only implemented in Romania (Anonymous, 2006).

Control measures have to be accompanied by reliable diagnostic tools. Due to the high variability of clinical and pathological signs, laboratory tests are needed to confirm or rule out CSF in case of suspicions, and in the framework of monitoring and surveillance activities.

Both direct and indirect detection methods are available for CSF laboratory diagnosis. Detailed laboratory procedures including sampling are laid down in the EU Diagnostic Manual (Commission Decision 2002/106/EC) and the accompanying Technical Annex

(Anonymous, 2002a; Anonymous, 2003a) as well as the *Manual of Diagnostic Tests and Vaccines for Terrestrial Animals* (OIE, 2008b).

The "gold standard" for direct detection of the agent is virus isolation on susceptible porcine cell cultures (Blome et al., 2006; Grummer et al., 2006) in combination with direct or indirect immune staining, e.g. immunofluorescence test (IFT) or immunoperoxidase test (IPT). Although this method is laborious and time-consuming, it is an indispensable method for the confirmation of CSF outbreaks (Anonymous, 2002a; OIE, 2008b) and allows the establishment of strain collections and genotyping (Greiser-Wilke et al., 2007).

Detection of antigen on fixed cryosections of tissues by IFT or IPT can be used as a rapid laboratory test in the case of suspicion, but requires experienced personnel for correct interpretation (Turner et al., 1968; de Smit et al., 2000b). Due to the limited sensitivity of the method, negative results can not be used for ruling out a CSFV infection (Teifke et al., 2005; Kaden et al., 2007).

For surveillance on a herd basis, fully automatable antigen capture enzyme-linked immunosorbent assays (ELISAs) yield results within 4 hours. In consequence of rather low sensitivity and specifity (Kaden et al., 1999; Dewulf et al., 2004), this technique must not be used for individual animals, and is increasingly replaced by reverse transcription polymerase chain reaction (RT-PCR) (Anonymous, 2002a).

Nowadays, the detection of viral RNA by RT-PCR has become a most valuable tool for diagnostic and research. Several gel-based RT-PCR protocols (Liu et al., 1991; Roehe and Woodward, 1991; Katz et al., 1993; Harding et al., 1994; Vilcek et al., 1994; Díaz et al., 1998; Agüero et al., 2004; Liu et al., 2007) as well as real-time RT-PCR (rRT-PCR) assays (McGoldrick et al., 1998; McGoldrick et al., 1999; Barlič-Maganja and Grom, 2001; Risatti et al., 2003; Uttenthal et al., 2003; Hoffmann et al., 2005; Risatti et al., 2005a; Hoffmann et al., 2006; Liu et al., 2007; Leifer et al., 2011; Eberling et al., 2011) have been developed so far and are either based on the detection of pestiviral or CSFV genome. Being considered to be one of the most sensitive method, RT-PCR can be used on pooled samples and for preclinical diagnosis (Paton and Greiser-Wilke, 2003; Depner et al., 2006; Le Potier et al., 2006; Depner et al., 2007; Le Dimna et al., 2008). Recently, RT-PCR assays have been developed that distinguish between wild-type CSFV and several attenuated lapinized vaccine strains (Li et al., 2007c; Pan et al., 2008; Zhao et al., 2008; Leifer et al., 2009a; Leifer et al., 2010a; Zhang et al., 2011b). Advanced multiplex real-time RT-PCR assays allow simultaneous detection and differentiation of viruses belonging to different virus families and are used to detect

disease clusters or pathogens with similar clinical picture (Agüero et al., 2004; Cheng et al., 2008; Giammarioli et al., 2008; Jiang et al., 2010; Liu et al., 2011).

Recent approaches, like reverse transcription loop-mediated isothermal amplification (RT-LAMP) (Chen et al., 2009; Yin et al., 2010; Zhang et al., 2010a; Zhang et al., 2011a) and microarray technology (Deregt et al., 2006; LeBlanc et al., 2009; LeBlanc et al., 2010) yielded first promising results and may have significant potential for future diagnostic tools. The same applies for modern and alternative probes for real-time RT-PCR, for example primer-probe energy transfer (PriProET) technology (Liu et al., 2009b; Zhang et al., 2010b).

For serology, the neutralization test (NT) is the most sensitive method. Although it is time-consuming, work intensive, and requires a high biosafety standard, it allows the discrimination between highly cross-reactive antibodies arising after CSFV or other pestivirus infections (Anonymous, 2002a; Blome et al., 2006; OIE, 2008b). Thereby, the NT is an indispensable tool, since CSF outbreaks entail mandatory control measures, whereas BDV or BVDV infections in pigs do not impose legal regulations (Greiser-Wilke et al., 2007).

Enzyme-linked immunosorbent assays for the detection of antibodies against the envelope E2 protein (Wensvoort et al., 1988; Moser et al., 1996) are widely used for screening and monitoring purposes (Pejsak et al., 1993; de Smit et al., 1999b; Zupancić et al., 2002; Vengust et al., 2006), and especially to evaluate oral vaccination campaigns in wild boar (Kaden et al., 2002; Kaden et al., 2005). In addition, two commercially available ELISAs for the detection of E^{rns} specific antibodies have been developed as accompanying tests for E2 subunit marker vaccines (Moormann et al., 2000; Floegel-Niesmann, 2003). Although the evaluation of two available discriminatory E^{rns} ELISAs has revealed deficiencies in both sensitivity and specificity compared to conventional E2 antibody ELISAs (Floegel-Niesmann, 2001; Floegel-Niesmann, 2003), they remain useful tools for CSFV serology.

2.1.6 Vaccination

Due to the tremendous socio-economic impact, the development of potent vaccines posed and still poses a challenge for research groups worldwide. For CSFV vaccination campaigns, different vaccination scenarios are possible, requiring different characteristics of vaccines (van Oirschot, 2003b). While vaccination in endemically infected areas is meant to prevent economic losses and may be a first step towards eradication, emergency vaccination campaigns during epidemics are mainly intended to prevent spread of the disease (van Oirschot, 2003a). According to Dong and Chen (2007), a perfect vaccine that could be used in all scenarios should induce reliable protection against horizontal transmission within a short time, induce full protection against vertical transmission, protect against a broad range of viral variants, be innocuous and safe in vaccinated animals and other species, be easy to use, and acceptable in terms of consumer protection. In addition, the perfect vaccine should have marker properties, allowing differentiation of infected from vaccinated animals (DIVA), accompanied by a reliable test system for disease surveillance and confirmation. Finally, vaccine production should be easy and at low cost using a standardized protocol. So far, a vaccine meeting all these criteria does not exist.

2.1.6.1 Conventional vaccines

Conventional vaccines comprise live attenuated vaccines and inactivated vaccines. Whereas inactivated vaccines are barely in use, live attenuated vaccines present the “gold standard”, especially in terms of efficacy (van Oirschot, 2003b; Blome et al., 2006).

In the early 20th century, a first generation of vaccines against CSF were developed, initially consisting of virus and porcine serum, followed by a crystal-violet vaccine in 1936 (Pehl, 1954; Saulmon, 1973). Due to the low safety and efficacy of these primary vaccines, further investigations aimed at the development of live attenuated vaccines. Among commonly used strains are the Lapinized Philippines Coronel (LPC) strain, the Chinese vaccine strain (C-strain) or so-called “Chinese hog cholera lapinized virus” (HCLV), the low-temperature-adapted Japanese guinea-pig exaltation-negative (GPE^-) strain, the French cell culture adapted Thiverval strain, and Mexican PAV strains (Dong and Chen, 2007).

So far, the most frequently used vaccines derive from the C-strain. The origin of this strain is not exactly known (Xia et al., 2011). It is likely that a vast number of chinese strains are used for the production of commercial vaccines, and all of them are attenuated by extensive serial passages in rabbits before adaptation to cell culture (van Oirschot, 2003b; Greiser-Wilke and

Moennig, 2004a). For C-strain formulations used in Europe it was shown that reliable protection is provided as early as four days after a single vaccination (Terpstra et al., 1990; Dahle and Liess, 1995; Kaden and Lange, 2001). Furthermore, immunity has been proven to persist for at least six to eleven months, probably even lifelong (Terpstra et al., 1990; Ferrari, 1992; Kaden and Lange, 2001; van Oirschot, 2003b). Transplacental infection with field virus is prevented (Kaden et al., 2008). Besides this remarkable efficacy, these vaccine strains are highly safe in both, target and non-target species (Kaden et al., 2010). Side effects are neither seen in pregnant sows nor immunosuppressed pigs (European Commission, 2003; van Oirschot, 2003b). In addition, production of these vaccines is facile and cost-saving, the vaccines do not require adjuvants and are suitable for oral vaccination of wild boar populations. Recently, several RT-PCR assays have been developed allowing genetic differentiation of C-strain vaccine and field virus strains (Zhao et al., 2008; Leifer et al., 2009a; Zhang et al., 2011b). Serological differentiation between C-strain vaccinated and infected animals is not possible (Beer et al., 2007).

Besides efforts to attenuate CSFV by passage through rabbits, other attempts aimed at attenuation of CSFV strains ALD and Alfort by serial cell culture passages under low temperature (29-30°C), resulting in the attenuated GPE^- and Thiverval vaccine strains, respectively (Sasahara et al., 1969; Aynaud et al., 1971). Commercial vaccines derived from the above-mentioned strains show similar performance as C-strain vaccines, and likewise lack serological marker properties.

The considerations set out above show that almost perfect vaccines against CSF are available, but all of them showing one serious drawback: they do not allow a serological DIVA strategy. In the case of emergency vaccination scenarios using conventional live attenuated vaccines, incising trade restrictions for vaccinated animals and their products would be applied (Greiser-Wilke and Moennig, 2004a).

2.1.6.2 E2 subunit marker vaccines

Up to now, marker vaccines were successfully used in the control of Aujeszky's disease (Greiser-Wilke and Moennig, 2004b) or bovine herpesvirus 1 infection (van Oirschot et al., 1996). Generally, two types of marker concepts can be distinguished: positive and negative marker vaccines, whereas the latter is most commonly used. Here, the differentiation is based on the absence of one or more microbial proteins in the vaccine, in contrast to the presence in the wild-type pathogen. Consequently, while infected animals develop specific antibodies against that specific protein, no specific "marker" antibodies can be detected in solely vaccinated animals. Using a protein-specific antibody test, infected and vaccinated individuals can thus be distinguished.

For CSFV, two E2 subunit vaccines have been licensed in Europe that represent the first generation of marker vaccines. They contain recombinant CSFV E2 expressed in a baculovirus sytem (Hulst et al., 1993). The marker concept is based on the fact that field-virus infected animals react positive in an E^{rns}-specific antibody ELISA, while vaccinated animals only develop a CSFV E2-specific antibody response.

Over the last years, a large number of studies were conducted to characterize these subunit vaccines (Bouma et al., 1999; Ahrens et al., 2000; Dewulf et al., 2000; Lipowski et al., 2000; Moormann et al., 2000; de Smit et al., 2000a; Depner et al., 2001; Uttenthal et al., 2001; de Smit et al., 2001a; Klinkenberg et al., 2002; Terzić et al., 2003; van Aarle, 2003; Dortmans et al., 2008). During these studies, safety of the E2 subunit vaccines was in general confirmed, but their efficacy was found not to be comparable with C-strain vaccines or other live attenuated vaccines, since results of vaccination-challenge and transmission studies were rather variable (van Oirschot, 2003b). Disadvantages compared to live attenuated vaccines can be seen in the late onset of immunity and incomplete protection against vertical transmission, both depending on the challenge strain used (European Commission, 2003). In order to obtain a reliable protection against horizontal and vertical transmission of CSFV, these vaccines require parenteral double vaccination campaigns (de Smit et al., 2000a; Ziegler and Kaden, 2002). Moreover, they are ineligible for oral administration, and thus not suitable for oral vaccination particularly of endemically infected wild boar populations during emergency vaccination campaigns.

Nowadays, only one subunit vaccine is still available on the market, containing the E2 glycoprotein of CSFV strain "Alfort/Tübingen".

2.1.6.3 Novel marker vaccines

Novel marker vaccines against CSF should in principle combine the outstanding efficacy of live attenuated vaccines with a reliable serological DIVA strategy. In order to achieve this goal, research activities mainly concentrated on the following strategies: immunogenic CSFV peptides, DNA vaccines, viral vector vaccines, trans-complemented deleted CSFV genomes (replicons), and chimeric pestiviruses (Beer et al., 2007).

Immunogenic CSFV peptides

For these vaccines, subunits are used instead of inactivated whole virus particles. The E2 subunit vaccines mentioned above are therefore also assigned to this group. These vaccines are based on recombinant proteins or so-called immunogenic peptides. So far, all peptide vaccines against CSFV contain either one peptide (mono-peptide vaccines, mPV) or a mixture of different peptides (multi-peptide-vaccines, MPV) covering different parts of the antigenic domains of the CSFV glycoprotein E2, in particular of the domains BC or A (Dong et al., 2002; Dong et al., 2005; Dong et al., 2006; Dong and Chen, 2006a; Liu et al., 2006a; Dong and Chen, 2006b; Liu et al., 2006b). The serological marker principle is based on the detection of E^{rns} or NS3 specific antibodies, for instance while using different blocking ELISAs. For mono-peptide-vaccines, however, detection can even rely on E2 domains that are not present in the vaccine. Besides serological marker properties, the advantage of this vaccine strategy is that it poses no risk for pathogen replication. Nevertheless, peptide vaccines require parenteral administration, adjuvants and multiple vaccination schemes. Dong et al. (2005) showed that double vaccinations (each time 50 µg peptide per pig) with a multi-peptide-vaccine provided complete protection against lethal challenge infection.

Several vaccination-challenge studies have shown that in most cases synthetic peptides indeed elicite neutralizing antibodies, but fail to induce complete protection against clinical disease, viremia and virus shedding. One example is the approach to use dendrimeric peptide vaccine candidates which target different B-cell epitopes (Tarradas et al., 2011). None of the evaluated vaccine candidates were able to confer complete protection upon CSFV challenge infection.

In recent years, several attempts were made to develop further E2 subunit vaccines by the use of various expression systems. To these candidates belongs the “E2his” vaccine produced in the mammary gland of goats after adenoviral transduction, which contains the extracellular domain of the glycoprotein E2 (Toledo et al., 2008). One major advantage of this expression

system might be the preservation or implementation of the complex tertiary structure of the E2 glycoprotein. This vaccine proved to induce an early, reliable, and long-lasting protection against CSFV challenge after a single application (Barrera et al., 2010). Further candidates, for instance in *Picia pastoris* expressed E2 also showed potential, as yeast-expressed yE2 induced a reliable protection after double vaccination and allowed a DIVA diagnostic based on the detection of E^{rns} specific antibodies (Lin et al., 2009).

Besides E2 subunit vaccines, also other proteins were tested for their suitability. For recombinant NS3 protein, Voigt et al. (2007) demonstrated no protection against lethal challenge infection, though stimulation of a specific immune response could be shown.

Therefore, CSFV peptide vaccines are in summary still in the experimental stage.

DNA vaccines

All DNA vaccine prototypes described so far are based on plasmid constructs that express the CSFV glycoprotein E2 (Andrew et al., 2000; Yu et al., 2001; Ganges et al., 2005; Wienhold et al., 2005; Andrew et al., 2006). The marker principle is based on the detection of E^{rns} or NS3 specific antibodies. Partially, co-expression of genes for cytokines (such as IL-3, IL-12, and IL-18) or regulatory cell surface molecules (CD154) was implemented in order to enhance their immunogenic potential (Wienhold et al., 2005; Andrew et al., 2006). To protect pigs against challenge infection with highly virulent CSFV, however, high dosages and multiple vaccinations were required.

Viral vector vaccines

After intensive studies on development of viral vector vaccines, this strategy still encourages several research groups. Especially vaccinia virus and pseudorabies virus vectors were already described throughout the 1990s (Rümenapf et al., 1991; Rümenapf et al., 1991; König et al., 1995; Peeters et al., 1997). In most cases, the virus recombinants express CSFV E2. Thus, the DIVA principle is based on the detection of E^{rns} or NS3 specific antibodies. Protection against lethal CSF could be shown for vaccinia virus recombinants expressing CSFV glycoprotein E2 and/or E^{rns}, albeit high titers and intravenous administration were required (Rümenapf et al., 1991c; König et al., 1995). Concerns have been expressed regarding possible sporadic pathogenicity of the previously used vaccinia virus strain for non-vaccinated humans. Modern, highly attenuated vaccinia virus vectors could solve this problem (Dong and Chen, 2007).

Therefore, further viral vector system were tested or discussed to be feasible alternatives, including porcine adenoviral vectors (Hammond et al., 2000; Hammond et al., 2001b; Hammond et al., 2003; Hammond and Johnson, 2005), swinepox virus vectors (Hahn et al., 2001a), parapox virus vectors (Hahn et al., 2001b) as well as fowlpox and canarypox viral vectors (Dong and Chen, 2007). The two latter *Avipoxviruses* have the great advantage that infection of vertebrates leads to an abortive infection, since productive replication is only seen in avian species. During investigations on adenoviral vector vaccines, Hammond et al. (2001a) discovered that a prime-boost vaccination using naked plasmid DNA and subsequent administration of recombinant porcine adenovirus, both expressing the CSFV E2 gene, was able to induce protective immunity in weaner pigs. However, only 75% of pre-weaned piglets were protected from disease.

Summarizing, some of the vector vaccines mentioned above are able to completely protect vaccinated pigs from lethal challenge infection. Nevertheless, reliable data from vaccination trials are missing for several of the candidate vaccines, in particular regarding to immunity against the viral vector. Usage of certain vectors may be problematic due to possible interference with serological surveillance programs, including the application of pseudorabies virus vectors in countries free of Aujeszky's disease. Up to now, vector vaccines remain prototypes, and licensing of a candidate is not yet in sight.

Trans-complemented deletion mutants (replicons)

Another promising approach comprises the construction of *trans*-complemented deletion mutants. While replication-competent chimeric pestiviruses may in theory revert to virulent viruses, *trans*-complemented deletion mutants do not exhibit that risk (Beer et al., 2007). For CSFV vaccine development, *trans*-complemented CSFV E^{rns} or E2 deletion mutants were constructed (Widjojoatmodjo et al., 2000; van Gennip et al., 2002; Maurer et al., 2005; Frey et al., 2006). While RNA transfection of CSFV deletion mutants into porcine kidney cells was shown to lead to autonomous replication without the production of virus progeny (replicons), trans-complementation was achieved by RNA transfection into E^{rns} or E2 expressing recombinant cell lines. Complemented virions are replication-deficient during the second replication cycle and thus referred to as DISC (defective in second cycle). Immunization of pigs using trans-complemented DISC virions may induce protective immunity against lethal challenge infection. It was shown that the vaccination efficiency was dependent on the application route (van Gennip et al., 2002; Frey et al., 2006). In particular, intradermal injection of the replicon A187del^{Erns} elicited complete protection against lethal challenge, whereas oral application induced only partial protection (Frey et al., 2006). Similarly, van Gennip et al. (2002) observed full protection after intradermal inoculation of the E^{rns}-complemented virus strain Flc23, but the intramuscular and intranasal route only mediated partial and even no protection, respectively. As with subunit marker vaccines, vaccinated animals can be distinguished from wild-type virus infected pigs by the absence of specific antibodies against the deleted protein. *Trans*-complemented E2 deletion mutants were shown to be less potent (van Gennip et al., 2002; Maurer et al., 2005).

Another, rather exotic approach is the use of Semliki Forest Virus replicons that serve as vector for a CSFV E2 DNA vaccine (pSFV1CS-E2). A high dose (three-time application of 600 µg) could induce a reliable protection (Li et al., 2007a). Double vaccination with a lower dose (100 µg) protected against lethal CSFV, but could not prevent fever peaks and short periods of viremia (Li et al., 2007b).

Recent studies aimed at the improvement of replicon-based vaccines. Therefore, IFN-α/β-inducing replicons were developed and the effect of a co-expression of granulocyte macrophage colony-stimulating factor (GM-CSF) was examined (Suter et al., 2011). Based on these data, IFN-α/β-inducing replicons seem to have a positive effect on the B- and T-cell immune response and hence enhance the efficacy of vaccines.

Chimeric pestiviruses

Among the most promising marker vaccines candidates against CSFV are chimeric pestiviruses based on infectious cDNA clones of CSFV or BVDV (Moormann et al., 1996; Ruggli et al., 1996; Meyers et al., 1996a; Meyers et al., 1996b; Vassilev et al., 1997b). Primarily developed for basic molecular research purposes, these cDNA clones even permit the construction of deletion mutants and replicons.

Several chimeric pestiviruses have been described so far, and some of them have been extensively studied in the target species (Vassilev et al., 1997a; de Smit et al., 2001c; Reimann et al., 2004; Rasmussen et al., 2007). Among the best characterized chimeras, the strain CP7_E2alf is currently under investigation within the EU funded research project "Improve tools and strategies for the prevention and control of classical swine fever" (CSFV_goDIVA, KBBE-227003). Based on the cytopathogenic BVDV strain "CP7" expressing the E2 glycoprotein from CSFV "Alfort/187" instead of BVDV E2 (Reimann et al., 2004), this chimeric pestivirus was chosen after large comparative trials to be further characterized for licensing purposes (Blome et al., 2012a). In this context, the CP7_E2alf candidate marker vaccine proved to be comparable with the conventional C-strain vaccine in both domestic pigs and European wild boar (Koenig et al., 2007a; Koenig et al., 2007b; Leifer et al., 2009b; Tignon et al., 2010). Nevertheless, controversial discussions were raised, especially on a possible risks of a BVDV backbone. Reimann et al. (2004) describe an altered cell tropism of this chimera in favor of porcine kidney cells as compared to bovine cell lines. However, there is a lack of reliable data concerning host tropism and safety in non-target species. A discriminating serological test has to be a CSFV E^{rns} antibody specific assay. For a genetic DIVA approach, two rRT-PCR assays have been developed by Leifer et al. (2009a) and Liu et al. (2009a). Efforts to develop chimeric viruses with modified epitope patterns, e.g. the vaccine candidate CP7_E1E2alf_TLA (Reimann et al., 2010), did not result in the intended improvement of DIVA diagnostic properties. The construct CP7_E1E2alf_TLA is likewise based on infectious cDNA of the BVDV strain "CP7" and contains the E1 and E2 genome coding regions of CSFV "Alfort/187". The replacement of both proteins allows optimal heterodimerization of E1 and E2, thus promoting virus assembly and growth. In order to establish an additional E2 based DIVA diagnostic, the CSFV specific TAVSPTTLR epitope (Lin et al., 2000; Liu et al., 2006a) was substituted by the corresponding antigenic epitope of BVDV strain "CP7". As all in vitro tests revealed promising results, efficacy and marker properties of different vaccine titers were investigated in target species. While

protection against lethal challenge infection could be proven in principle, a reliable E2 DIVA diagnostic failed.

Further approaches

Ideally, an optimal marker vaccine may arise from an efficacious and safe vaccine strain by mutations in solely one antigenic domain (Kortekaas et al., 2010). Unfortunately, it was shown that logical efforts do not necessarily succeed. For instance, a chimeric CSFV "Riems" construct developed by Wehrle et al. (2007) which expresses E2 genes with a replaced domain A of the corresponding region of BDV strain "Gifhorn" could not show convincing results. More promising approaches arised from targeted mutations in the TAVSPTTLR epitope of the E2 protein (Risatti et al., 2006; Holinka et al., 2009), though initially not succeeding for the C-strain (Kortekaas et al., 2010). A non-chimeric C-strain mutant (vFlc-ΔPTa1) with several targeted amino acid deletions in the TAVSPTTLR epitope established stability after adaptive mutations in cell culture and was shown to induce reliable protection against lethal challenge infection (Kortekaas et al., 2010; Kortekaas et al., 2011). Nevertheless, this strategy demands further investigations, particularly as several ELISAs proved to be unsuitable as accompanying DIVA test.

2.2 African Swine Fever

2.2.1 Virus taxonomy, morphology and structure

African swine fever virus (ASFV) is a large DNA virus classified as the only member in the genus *Asfivirus* within the *Asfarviridae* family (Dixon et al., 2005). Due to the ability to replicate in soft ticks of the genus *Ornithodoros*, and to be transmitted by these vectors, ASFV is the only arthropod-borne (ARBO) DNA virus.

The complex virus particle consists of a core, surrounded by two lipid bilayers, an icosahedral capsid and an external envelope (Tulman et al., 2009). The virion core, about 80 nm in diameter, is composed of an electron-dense nucleoid, enclosed by a multi-protein layer, also termed core shell or matrix. The core is surrounded by two lipid bilayers, also called the inner membran, which is in turn covered by a capsid. The capsid is formed by 1892 to 2172 hexagonal capsomeres (Breese and DeBoer, 1966; Carrascosa et al., 1984). The mature virions acquire an external membrane by virion budding through the cellular plasma membrane and have a final size of about 200 nm.

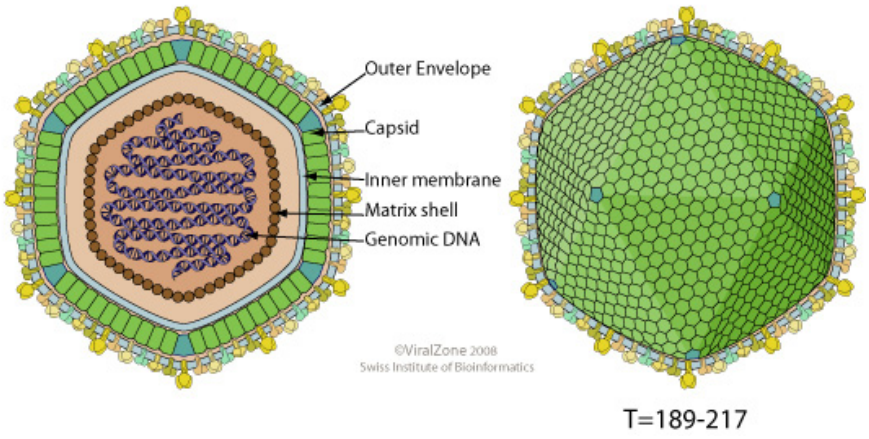

Fig. 3: Morphology of African swine fever virions[1].

The linear double-stranded DNA genome with 170 to 190 kbp of length contains between 160 and 175 ORFs located on both strands of the DNA (Tabares et al., 1980; Blasco et al., 1989; Yanez et al., 1995; Dixon et al., 2008), terminal hairpin loop structures (Gónzalez et al., 1986), terminal inverted repeats (Sogo et al., 1984), and encodes six multigene families (Dixon et al., 2008).

[1] Source: ViralZone, http://www.expasy.org/viralzone, Swiss Institute of Bioinformatics.

The genome codes for a large number of proteins, including essential enzymes and factors required for replication and transcription (Dixon et al., 2004). Furthermore, over 50 structural proteins have been identified, some of them being highly antigenic and valuable tools for serological diagnostics. These include (1) the VP72 protein, the main component of the virus capsid (López-Otín et al., 1990), (2) the protein p12, which is localized on the external membrane and plays a major role in virus attachment (Alcamí et al., 1992), (3) the most important integral membrane protein p54 in the external envelope of the virion (Rodríguez et al., 1996), and (4) the phosphoprotein p30 (Afonso et al., 1992).

2.2.2 Epidemiology, clinical signs, lesions and pathogenesis

The natural vertebrate hosts of ASFV are African wild pigs. Albeit, domestic pigs and European wild boar are likewise susceptible to an ASFV infection (Wilkinson, 1984). In addition, ASFV has the ability to replicate in soft ticks of the genus *Ornithodoros*, including *O. erraticus* on the Iberian Peninsula, and ticks of the *O. moubata complex*, which are prevalent in Africa (Sánchez-Vizcaíno, 2006). These ticks are considered ASFV reservoirs and competent vectors. In addition, stable flies (*Stomoxys* spp.) were identified as mechanical vectors, maintaining ASFV for at least 48 hours (Mellor et al., 1987). This is probably also true for other insects, especially other blood-sucking ectoparasites.

The outcome of ASF in a certain area is depending on the virulence of virus strains, host susceptibility, presence of tick vectors, and the possible interaction between suid hosts and vectors. Therefore, three different cycles or epidemiological scenarios can be distinguished in general: the “sylvatic cycle”, the “intermediate enzootic cycle”, and the “domestic cycle”. In addition, the “enzootic domestic cycle” and a “peculiar enzootic domestic cycle” have been described (EFSA, 2009).

The “sylvatic cycle” is based on the interaction between wild pigs and *Ornithodoros* ticks. In the African wildlife, warthogs (*Phacochoerus* spp.) play a major role as reservoirs for ASFV. Bush pigs (*Potamochoerus* spp.) and the Giant Forest hog (*Hylochoerus meinertzhangeni*) are also susceptible hosts for ASFV, but their role in the epidemiology of the disease remains unclear (Jori and Bastos, 2009). These wildlife hosts, however, do not exhibit clinical signs and show low or even absent viremia. Moreover, horizontal and vertical transmission between these animals has not been described (Thomson et al., 1980; Penrith et al., 2011). Key players in the sylvatic cycle are young viremic warthogs and *Ornithodoros* ticks. Infection occurs in the burrow, where young warthogs spend the first weeks of their life. Transient viremia in

warthogs, which lasts for two to three weeks, is sufficient to infect ticks in turn (Thomson et al., 1980). The ticks may become persistently infected (Basto et al., 2006a). Sporadic outbreaks among domestic pigs may occur due to transmission through ASFV infected ticks (EFSA, 2009). This sylvatic cycle model has been described in most South and East African countries, but can not be deployed to the whole African continent (EFSA, 2009).

The "intermediate enzootic cycle" involves domestic pigs and *Ornithodoros* ticks which act as vectors and reservoirs. It was shown that adult and nymph ticks can survive for at least 5 years and thus pose a persistent source of infection. While transsexual, transstadial and transovarial transmission of ASFV occurs in *O. moubata*, the latter route has not been observed in *O. erraticus* (Arias and Sánchez-Vizcaíno, 2002). These facts may explain that ASFV can be maintained in areas without pigs, and can be reintroduced into previously diseased areas (EFSA, 2009). For instance, long-term maintenance of the virus in Spanish ticks was presumed by Oleaga-Perez et al. (1990) and proven in pig-pens in enzootic regions in Malawi (Haresnape and Wilkinson, 1989) and abandoned pig-pens in Madagascar (Ravaomanana et al., 2010).

The "domestic cycle" is restricted to domestic pigs. The main transmission routes include direct contact between infected pigs, often due to uncontrolled movements, and indirect transmission through contaminated fomites or ingestion of infected pork products. This scenario leads to typical extensive outbreaks.

Adaptation of the virus to domestic pigs and their free-ranging descendants, however, may lead to an "enzootic domestic cycle" of ASF, as chronic or asymptomatic carriers allow the maintenance of the disease. This scenario may explain enzootic ASF in West and South African countries (Mebus and Dardiri, 1980; Haresnape et al., 1987; Penrith et al., 2004; EFSA, 2009). As such outbreaks are not self-limiting they pose a constant risk for disease spread (Penrith and Vosloo, 2009).

In some areas of Madagascar, an unusual enzootic cycle has been observed. As the role of vectors and virus adaptation remains unclear, this cycle was called "peculiar enzootic domestic cycle" (EFSA, 2009).

In Europe, three distinct scenarios have been observed. First of all a *"domestic-domestic scenario without ticks"* which was seen in Portugal and Spain. The involvement of *O. erraticus* in Portugal and Spain engendered a *"domestic-domestic scenario with ticks"*. Thirdly, a complex *"free-ranging with/without wild boar and/or vector scenario"* has been assigned to be the prevailing scenario in Sardinia and the Caucasus (EFSA, 2009).

As with CSF, clinical signs of ASF in domestic pigs can vary considerably, depending on the virulence of the ASFV strain, the infection route, and dose. After an incubation period of 3 to 15 days, peracute, acute, subacute or chronic courses of disease can occur (Kleiboeker, 2002).
The peracute course is seen after infection with highly virulent strains. Pigs suddenly die without any previous clinical signs and develop only few or even no lesions (Kleiboeker, 2002).
The acute course is seen after infection with highly virulent strains and is initially characterized by high fever, accompanied by tachycardia and tachypnoe. Reddening of the skin, especially on ears, tail, distal extremities and ventral areas of chest and abdomen are frequently observed. Additionally, vomiting, (hemorrhagic) diarrhea and conjunctivitis can be present. Anorexia, apathia, cyanosis and incoordination are seen in the final stage of disease. In domestic pigs, the mortality rate is up to 100%, and death occurs within 6 to 13 days. In pregnant sows, abortion may occur. Dying pigs usually have little or no detectable antibodies. In the case of survival, animals are virus carriers for periods of 6 months or more. Lesions associated with acute ASF include hemorrhages in several organs, swollen and hemorrhagic lymph nodes, congestive splenomegaly, and effusion in all body cavities (Kleiboeker, 2002; Arias and Sánchez-Vizcaíno, 2002; EFSA, 2009).
During the subacute course, induced by infection with moderately virulent viruses, clinical signs are less pronounced, and thus can be readily confused with other febrile swine diseases, particularly CSF. Pigs show irregular remittent fever, anorexia, a loss of condition and mild diarrhea. Upon agitation, coughing and dyspnea as well as death due to cardiac failure may be observed. Pregnant sows may abort. Transient lymphopenia and thrombocytopenia may occur. Animals may die within 15 to 45 days, or recover after 30 to 45 days of illness. The mortality rate varies between 30 to 70%, depending on the strain involved and the age of affected animals. Necropsy may reveal similar findings to those seen in acute ASF, but less severe. Despites regularly observed enlarged and hemorrhagic spleen and lymph nodes, frequent signs are pneumonia, serofibrinous pleuritis, and serofibrinous pericarditis (Kleiboeker, 2002; Arias and Sánchez-Vizcaíno, 2002; EFSA, 2009).
The chronic form is caused by infection with low virulent viruses and is characterized by a variety of clinical and rather unspecific signs. Animals usually show a loss of body weight, runting, and growth retardation. Chronic skin ulcers or necrosis may appear. Fever peaks, respiratory signs, arthritis, abortions and secondary infections may mislead veterinarians to diagnose ASF. Clinical disease may last 2 to 5 months, and the mortality rate is less than 30%. Lesions may be absent, or enlarged lymph nodes, interstitial pneumonia and fibrinous

pericarditis may be present. In addition, splenic enlargement, hemorrhages, or focal necrosis may occur (Kleiboeker, 2002; Arias and Sánchez-Vizcaíno, 2002; EFSA, 2009).
Wild boar and feral pigs are susceptible to both natural and experimental infection and show similar clinical signs and mortality rates (McVicar et al., 1981). Initial descriptions from ASFV infected wild boar in Spain showed peracute and acute courses, whereas later findings included subacute, chronic and even inapparent disease (Wilkinson, 1984; Bech-Nielsen et al., 1995; Pérez et al., 1998). Gross lesions in European wild boar and feral pigs are equal to those seen in domestic pigs (Ruiz-Fons et al., 2008). Although wild boar shed virus in similar quantities as domestic pigs, their role in epidemiology remains unclear (Laddomada et al., 1994; Beltran-Alcrudo et al., 2008; Mur et al., 2012).
In general, specific antibodies are detectable from approximately six days post infection. Despite the fact that antibodies are detectable for a long time (at least 10 month), they do not neutralize the virus (Arias and Sánchez-Vizcaíno, 2002).

ASFV enters the body via the tonsils or dorsal pharyngeal mucosa and gets to the mandibular or retropharyngeal lymph nodes, from where the virus spreads through viremia (EFSA, 2009). Main target cells for virus replication in the early stages of infection are cells of the monocyte/macrophage lineage. In addition, dendritic cells can be infected. In the later stages, a variety of cell types are involved in virus replication including endothelial cells, platelets and their precursors, neutrophils, and hepatocytes. Hemadsorbing ASFV isolates can be found associated with erythrocytes, but also with lymphocytes and neutrophils. Replication in macrophages and several virus encoded proteins help the virus to evade host defences. Through manipulation of macrophage functions, both the innate and acquired immune responses can be targeted (reviewed by Dixon et al., 2008).
Especially the mechanisms involved in genesis of hemorrhagic lesions that occur after infection with highly virulent ASFV strains remain controversial. Nowadays it is generally accepted that the massive destruction of macrophages plays a major role in the impaired hemostasis due to the release of active substances including cytokines, complement factors and arachidonic acid metabolites (Anderson et al., 1987). Some studies also suggest that hemorrhagic 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 (Gómez et al., 1995; Carrasco et al., 1997). Release of cytokines by infected macrophages and disseminated intravascular coagulation (DIC) are also among the possible options (Anderson et al., 1987; Villeda et al., 1993; Gómez-Villamandos et al., 2003).

Thrombocytopenia is generally observed much later than with CSF 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 (Gómez-Villamandos et al., 2003), and to various immune-mediated processes involving immune complexes of ASF antigens and antibodies that cause aggregation of platelets (Edwards et al., 1985a; Edwards et al., 1985b). As with CSF, pigs infected with ASF generally suffer severe lymphopenia that could be attributed to apoptosis of lymphocytes. Production of pro-inflammatory cytokines by infected macrophages is strongly implicated in induction of apoptosis in lymphocyte populations (Oura et al., 1998; Salguero et al., 2002; Salguero et al., 2004; Salguero et al., 2005). ASFV chronic disease 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).

2.2.3 History, global distribution and economic impact

African swine fever is endemic in most sub-Saharan countries where it often involves a sylvatic transmission cycle. In domestic pigs it was first described in Kenya in 1921 (Montgomery, 1921). Thereafter it was reported tin many countries of East and South Africa. Besides the endemic situation, epidemics and introductions into free areas occasionally occur. For example, ASF was introduced to Madagascar in 1998 and Mauritius in 2007 (Penrith, 2009).

The first cases of ASF outside Africa were reported in 1957 in Portugal. Although this first outbreak could be eradicated, a further outbreak occurred in 1960 which also affected Spain, and ASF remained endemic on the Iberian peninsula until the mid 1990s. Further outbreaks in Europe included Malta (1978), Italy (1967, 1980), France (1964, 1967, 1970), Belgium (1985), and The Netherlands (1986). The Caribbean and Brazil were affected in the 1980s. While the disease was successfully eradicated from most European countries, the Caribbean and Brazil, ASF remains endemic in Sardinia, and affects domestic as well as wild pigs (Costard et al., 2009). The worldwide distribution of ASF in the first half of 2011 is depicted in Figure 4.

In June 2007, a further transcontinental spread was reported, as ASF was confirmed in the Republic of Georgia. It has been suggested that the virus was introduced by galley waste that was available to free-ranging pigs around the Black Sea port of Poti (Penrith, 2009). Sequence analysis of this isolate revealed a close relationship to isolates of genotype II, which were previously described from Mozambique, Madagascar and Zambia (Rowlands et al., 2008). In the following weeks, the disease spread on the whole territory of Georgia and to Abkhazia and South Ossetia. Subsequently, ASF was confirmed in neighboring countries, including Armenia (August 2007) and Azerbaijan (November 2007). In November 2007, infection of a wild boar was reported in the Russian Republic of Chechnya near the border with Georgia. At the end of 2007, ASF was widely spread in the Caucasus (Gulenkin et al., 2011).

In 2008, Georgia, Armenia, and Azerbaijan declared that all outbreaks were resolved. In total, Georgia reported 58 outbreaks, and Armenia and Azerbaijan confirmed 13 and 2 outbreaks, respectively. However, another outbreak occurred in Armenian wild boar in 2010 (OIE, 2012d).

In 2008, the disease continued spreading, and was almost exclusively seen in the Southern territories of the Russian Federation, but with a clear tendency to move north- and eastwards. In total, 35 cases of ASF in domestic pigs and 19 cases in wild boar were reported in 2008 in

the Russian region (Gulenkin et al., 2011). The number of outbreaks expanded in 2010, as 77 outbreaks were reported, of which 18 were among wild boar (OIE, 2012d).
In addition to the general tendency to move north- and eastwards, spread over long distances was observed, e.g. when ASF was introduced into a private subsidiary holding in the Orenburgskaya oblast, close to the border with Kazakhstan (2008). Another outbreak occurred in the Leningradskaya oblast in 2009, which is located in a distance of 1500 km from the affected southern Caucasian region, close to the borders with Finland and Estonia. Two more outbreaks in the Saint Petersburg region occurred in 2010 and 2011. Further remote outbreaks were recorded in the Murmanskaya Oblast (March 2011), and recently in the Respublika Kareliya (January 2012), both close to the border with Finland (OIE, 2012d). Long distance spread was restricted to domestic pigs and probably caused by swill-feeding. Through implementation of strict quarantine measures and culling of affected pig holdings, the disease was eradicated in these cases.
In the period from the first outbreak in 2007 to March 2012, a total number of 209 outbreaks has been confirmed in the Russian Federation (OIE, 2012d). The corresponding outbreak map is depicted in Figure 5.

African swine fever has been shown to have a devastating impact on both, epidemic and endemic areas. Total economic losses, mainly composed of costs for eradication and surveillance, but also due to effects on pork production and trade restrictions, are most apparent in countries with industrialized pig production.
This was seen in Spain, where the last five years of the Spanish eradication program were estimated to have cost US $92 million (Arias and Sánchez-Vizcaíno, 2002). The outbreak in Cuba in 1980 led to total direct costs of US $9.4 million (Costard et al., 2009).
Besides the potential impact on the commercial pig sector, tremendous losses are also inflicted on poorer pig producers, especially in developing countries. This is seen in many African countries, where the restart of pig production is often hampered by insufficient financial resources of farmers (Costard et al., 2009).

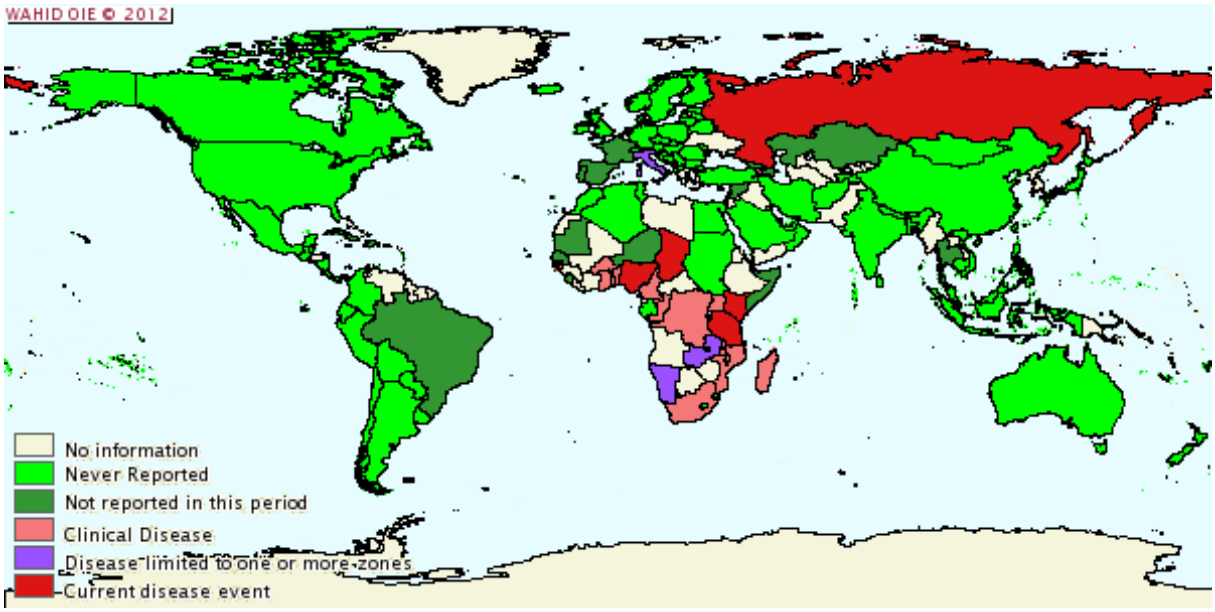

Fig. 4: African swine fever distribution in wild and domestic pigs, reporting period January to June 2011(OIE, 2012b).

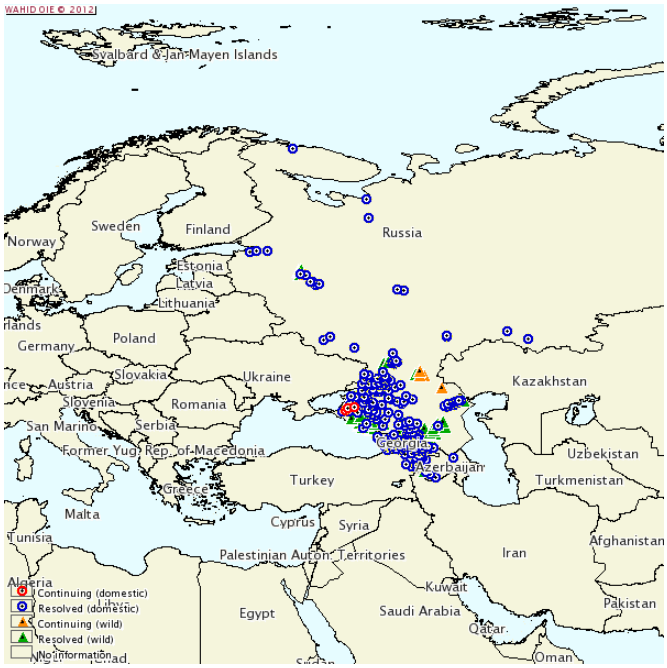

Fig. 5: African swine fever outbreak map in Trans-Caucasian Countries and the Russian Federation in wild and domestic pigs, reporting period January 2007 to March 2012 (OIE, 2012c).

2.2.4 Control strategies and laboratory diagnosis

As no vaccine exists, only strict sanitary measures can be applied in cases of disease outbreaks. Early detection in the field, rapid reporting and laboratory diagnosis can limit the extent of disease spread and reduce outbreak size and duration. All countries with an industrialized pig production enforce a stamping out strategy that includes culling of infected and contact herds, establishment of restriction zones, movement restrictions, epidemiological investigations, and surveillance. Although competent tick vectors do not exist in all regions, epidemiological investigations have to assess abundance of soft ticks (Costard et al., 2009). Within the EU, these control measures are laid down in Council Directive 2002/60/EC (Anonymous, 2002b) and Commission Decision 2003/422/EC (Anonymous, 2003b).

As ASF must be considered as differential diagnosis of any acute febrile hemorrhagic syndrome seen in pigs and drastic control measures are accompanied by serious economical consequences, laboratory examinations are essential for confirmation of the disease. Detailed laboratory procedures as well as a guide for sampling are elaborated in the EU Diagnostic Manual (Anonymous, 2003b), and the *Manual of Diagnostic Tests and Vaccines for Terrestrial Animals* (OIE, 2008a).

Direct detection of the agent is recommended when introduction of the disease is suspected in countries free from ASF.

Isolation of ASFV is possible on primary and permanent cell cultures. The traditional method for growing ASFV is based on peripheral blood mononuclear cell (PBMC) cultures. In these cultures, the presence of ASFV is detected after addition of homologous erythrocytes by hemadsorption, a phenomenon based on the capacity of pig erythrocytes to adhere to the surface of ASFV infected cells (Malmquist and Hay, 1960; OIE, 2008a). The hemadsorption test (HAT) represents a sensitive and specific method, but non-hemadsorbing isolates have been reported (Pini and Wagenaar, 1974; Gonzague et al., 2001). Apart from HAT, virus isolation can be performed either by inoculation of blood or tissue samples from suspected animals into primary leukocyte cultures derived from peripheral blood, bone marrow or lung washing, or in susceptible permanent cell cultures (Carrascosa et al., 2011).

Immunofluorescence staining, also referred to as Fluorescent antibody test (FAT), allows fast detection of antigen on cryostat sections or impression smears, as well as the identification of non-hemadsorbing virus strains (Bool et al., 1969). Due to the fact that sensitivity significantly decreases due to the formation of antigen-antibody complexes in subacute or

chronic courses, the test is evidentiary only in the case of positive reactions (Sánchez-Vizcaíno, 2006).

Nowadays, PCR techniques are most valuable and widely used, as they are highly sensitive, specific, and fast. First described by Steiger et al. (1992), various assays have been developed, including gel-based protocols (Agüero et al., 2004; Basto et al., 2006b), advanced real-time PCRs (McGoldrick et al., 1998; King et al., 2003; Zsak et al., 2005; McKillen et al., 2007; McKillen et al., 2010; Tignon et al., 2011; Fernández-Pinero et al., 2012), and multiplexed assays for the simultaneous detection of different viral swine pathogens (Agüero et al., 2004; Giammarioli et al., 2008). A recently developed in-situ hybridization assay (Ballester et al., 2010) and a LAMP assay (James et al., 2010) show potential for use in ASF diagnostics.

Serological tests, however, are recommended in endemically infected areas or epidemics with low virulent strains. Enzyme-linked immunosorbent assays are commercially available and most commonly used for large-scale screening (Pastor et al., 1990; Sánchez-Vizcaíno, 2006). For ELISA-positive or inconclusive results, the OIE Manual (2012a) recommends confirmation by the use of alternative tests, such as the indirect fluorescent antibody test (Pan et al., 1974), immunoperoxidase staining or immunoblotting (Pastor et al., 1989).

2.2.5 Vaccination

All efforts to develop an effective vaccine against ASF failed so far (EFSA, 2009). Still, vaccination seems feasible as protection against virulent strains can be achieved through inoculation of pigs with closely related strains of low virulence (Leitão et al., 2001; Boinas et al., 2004). Recent efforts included development of different deletion mutants with one or more deleted genes, subunit vaccines based on recombinant proteins or DNA vaccines. So far, none of these vaccines conferred complete protection (Sánchez-Vizcaíno et al., 2012). Under the threat posed by the outbreaks in Russia, research has been intensified, e.g. under the 7th Framework Programme of the EU.

3 OBJECTIVES

Classical swine fever

Optimization of conventional vaccination

During CSF outbreaks and subsequent emergency vaccination campaigns among wild boar in Germany 2009, the use of highly sensitive real-time RT-PCR assays led to an increase in viral genome detections at regional level. In order to establish a rapid and reliable method to differentiate field virus from vaccine virus, a "genetic DIVA" approach based on a real-time multiplex RT-PCR was implemented and evaluated.

Assessment of marker vaccine candidate CP7_E2alf

To obtain market authorization for a CSFV marker vaccine candidate, several mandatory trials covering all aspects of immunogenicity and safety are required. In this context, marker vaccine candidate CP7_E2alf was evaluated.

- Safety data on CP7_E2alf in target species had been scarce, and innocuousness in non-target species had not been proven. Safety aspects in domestic pigs and wild boar were addressed in several vaccination and challenge experiments and summarized. Appropriate vaccination experiments in calves, goats, lambs, and rabbits were conducted.
- As the duration of immunity after oral and intramuscular vaccination with CP7_E2alf was unknown, this aspect was investigated in an animal experiment in domestic pigs.
- After vaccination with CP7_E2alf, protection against lethal challenge with highly virulent CSFV strains of genotype 1.1 was proven, whereas efficacy against recent CSFV strains of genotype 2.3 was not yet evaluated. Therefore, additional vaccination and challenge experiments in domestic pigs and wild boar were conducted using CSFV challenge strains of both genotypes.

African swine fever

Characterization of a recent Caucasian isolate in wild boar

After the introduction and spread of ASF in several Trans-Caucasian Countries and Russia since 2007, disease dynamics of circulating isolates in wild boar was unknown. Caucasian ASFV isolates were characterized in animal experiments in European wild boar of different age classes.

4 RESULTS

The publications are grouped according to their topic.

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

The poster "Efficacy of CP7_E2alf pilot vaccine batches after intramuscular and oral vaccination" is depicted as presented at the 8th ESVV Pestivirus Symposium. The respective manuscript is in preparation.

Genetic differentiation of infected from vaccinated animals after implementation of an emergency vaccination strategy against classical swine fever in wild boar

Sandra Blome[a], Claudia Gabriel[a], Christoph Staubach[b], Immanuel Leifer[c], Günter Strebelow[a], and Martin Beer[a]

[a] Institute of Diagnostic Virology, Friedrich-Loeffler-Institute, Südufer 10, 17493 Greifswald-Insel Riems, Germany

[b] Institute of Epidemiology, Friedrich-Loeffler-Institute, Seestr. 55, 16868 Wusterhausen, Germany

[c] Institute of Virology and Immunoprophylaxis, Sensemattstr. 293, 3147 Mittelhaeusern, Switzerland

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Genetic differentiation of infected from vaccinated animals after implementation of an emergency vaccination strategy against classical swine fever in wild boar

Abstract

Oral emergency vaccination against classical swine fever is a powerful tool to control disease outbreaks among European wild boar and thus to safeguard domestic pigs in affected regions. In the past, when virus detection was mainly done using virus isolation in cell culture or antigen enzyme-linked immunosorbent assays, modified live vaccine strains like C-strain "Riems", were barely detectable after oral vaccination campaigns. Nowadays, the use of highly sensitive molecular techniques has given rise to an increase in vaccine virus detections. This was also the case during the 2009 outbreak among German wild boar and the subsequent vaccination campaigns. To guarantee a rapid differentiation of truly infected from C-strain vaccinated animals, a combination of differentiating multiplex rRT-PCR assays with partial sequencing was implemented. Here, we report on the rational and use of this approach and the lessons learned during execution. It was shown that positive results in the recently developed vaccine strain (genotype) specific rRT-PCR assay can be taken as almost evidentiary whereas negative results should be confirmed by partial sequencing. Thus, combination of multiplex rRT-PCR assays as a first line differentiation with partial sequencing can be recommended for a genetic DIVA strategy in areas with oral vaccination against classical swine fever in wild boars.

1. Introduction

Classical swine fever (CSF) is among the most important diseases impairing pig production world wide. Clinical signs are mainly dependent on the age of the animal and the virulence of the virus strain. Infection can lead to a wide range of symptoms, from mild transient disease to a haemorrhagic syndrome with high lethality (Moennig et al., 2003). The disease is caused by a small enveloped RNA virus of the Genus Pestivirus within the *Flaviviridae* family (Fauquet and Fargette, 2005). Apart from domestic pigs, CSF virus (CSFV) affects European wild boar where the disease seems to run quite similar courses. Over the last decades, several European Union (EU) Member States including Germany, France, and Slovakia, were confronted with outbreaks among wild boar that had a clear tendency to establish endemicity (Anonym, 2009). Since it was shown that infected wild boar populations can be a major cause

for primary outbreaks in domestic pigs (Fritzemeier et al., 2000), strict control measures were implemented based on binding EU legislation (Anonym, 2001). Control measures often included oral vaccination of affected wild boar populations with a conventional modified live vaccine based on the C-strain of CSFV (von Rüden et al., 2008). Despite intensive efforts and obvious success of oral vaccination in terms of safeguarding domestic pigs, CSF kept reoccurring and/or persisting in some areas, especially where high population densities were present. One of the most recent outbreaks among wild boars occurred in Germany 2009, where CSF was detected in wild boar populations in the Federal States of Rhineland-Palatinate and North-Rhine Westphalia (Leifer et al., 2010a). In accordance with EU legislation, restriction zones (infected area and surveillance zone) were declared and oral vaccination of wild boar was started almost immediately. The vaccination scheme comprised three double vaccination campaigns per year. Thereafter, all animals shot or found dead in the restriction zones were subjected to diagnostic investigations. Methods for both virus and antibody detection were employed. While during outbreaks in the 1990ies, pathogen detection was mainly done through virus isolation in cell culture and to a lesser extent using antigen enzyme-linked immunosorbent assays (ELISA), nowadays molecular methods such as real-time reverse transcription polymerase chain reaction (rRT-PCR) were implemented in diagnostic routine. The use of these highly sensitive methods led to a phenomenon that was not observed to this extent during previous outbreaks: Following baiting campaigns, several samples were observed that gave positive results in CSFV specific rRT-PCR assays but were later on confirmed as vaccine virus detections through partial sequencing. In order to achieve a rapid decision whether field or vaccine virus was detected, a genetic DIVA strategy was implemented based on recently published multiplex rRT-PCR assays (Leifer et al., 2009, 2010b).

The presented short communication describes the components, use, and outcome of this approach in the framework of the above mentioned outbreak among German wild boar in 2009 and discusses the implications for the optimal use in the future.

2. Materials and methods

2.1. Data collection and analysis

Data were collected and retrospectively analysed at the German National Reference Laboratory (NRL) for CSF during and after the 2009 outbreak among German wild boar. All samples described were part of the routine diagnostic work and had given a positive result in a CSFV-specific rRT-PCR assay (Hoffmann et al., 2005) at the regional level but were later on confirmed as vaccine virus detection.

2.2. RNA extraction, RT-PCR and sequencing

Total RNA was extracted from blood and organ samples using the RNeasy mini kit (Qiagen), and from serum using the QIAamp Viral RNA mini kit (Qiagen) following the manufacturer's recommendations.

Extracted RNAs were simultaneously subjected to partial sequencing and a multiplex rRT-PCR assay for the detection and differentiation of CSFV and C-strain "Riems" (Leifer et al., 2009). The assay combined the well established CSFV-specific duplex rRT-PCR published by Hoffmann et al. (2005) with a vaccine strain specific rRT-PCR targeting 77 nucleotides of the E^{rns} encoding region. After detection of variant sequences in the primer-binding site of the forward primer of the C-strain "Riems" specific real-time RT-PCR, the amended assay published by Leifer et al. (2010b) was implemented instead of the above mentioned one. This amended assay detects not only C-strain "Riems" but all CSFV strains of genotype 1.1.

Two regions of the CSFV genome were used for phylogenetic analyses after partial sequencing: (1) a 150 nt fragment of the 5' NTR; and (2) a 190 nt fragment of the E2 gene (Paton et al., 2000). DNA fragments obtained by standard one-step RT-PCR using the SuperScript™ III One-Step RT-PCR System with Platinum® Taq (Invitrogen), were isolated from agarose gels with the QIAquick® Gel Extraction Kit (Qiagen). Sequencing was carried out using the BigDye® Terminator v1.1. Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA).

Nucleotide sequences were obtained with a 3130 Genetic Analyzer (Applied Biosystems). All reactions were carried out as published previously (Greiser-Wilke et al., 2006).

Nucleotide sequences were edited and analysed using BioEdit 7.0.9 (Hall, 1999) and Genetics Computer Group (GCG) software version 11.1 (Accelrys Inc., San Diego, USA).

Subsequently, phylogenetic trees were calculated with the Neighbor-joining method as implemented in MEGA 4.0 (Tamura et al., 2007).

3. Results

Following the 2009 outbreaks of CSF in German wild boar, 89 samples were sent to the NRL for differentiation and confirmation after positive CSFV-specific rRT-PCR results at regional level. Out of these 89 samples, 30 were later on confirmed as vaccine virus detection by partial sequencing, and three additional samples were positive only in the multiplex rRT-PCR assay indicating vaccine virus detection that could not be confirmed by sequencing due to negative conventional RT-PCR (details for these samples are presented in Table 1).

The original differentiating multiplex rRT-PCR assay detected 27 out of 33 samples (82%) correctly as C-strain positive. After amendment, an additional sample could be detected (28 out of 33, 85%). Four samples were only positive in the CSFV-specific assay and were later confirmed as C-strain “Riems” detection by partial sequencing. In one case, only the C-strain specific assay was positive, and an additional sample was only detectable in the conventional RT-PCR assay and subsequent sequencing.

Table 1

Diagnostic specimens with vaccine virus confirmation by partial sequencing that were sent to the National Reference Laboratory for CSF after positive results in a CSFV-specific rRT-PCR at regional level. Results of the differentiating multiplex rRT-PCR assay (Leifer et al., 2009) are presented as cycle threshold (ct) values. Discrepant results are presented with grey background. Values with asterisks were obtained with the amended multiplex assay (Leifer et al., 2010b).

		Multiplex rRT-PCR		Sequencing
NRL-No.	Material	*C-strain*	*CSFV*	*Genotype*
K22/09_1	Spleen	36	35	not available
K24/09_1	Blood	37	no ct	not available
K25/09_1	Blood	no ct	33	1.1 C-strain
K25/09_3	Blood	31	33	1.1 C-strain
K26/09_1	Spleen	33	32	1.1 C-strain
K26/09_2	Spleen	32	32	1.1 C-strain
K27/09_1	Spleen	37	38	not available
K27/09_2	Spleen	35	35	1.1 C-strain
K28/09	Tonsil	30	27	1.1 C-strain
K30/09	Blood	35	34	1.1 C-strain
K35/09_2	Blood	32	35	1.1 C-strain
K35/09_3	Spleen	31	34	1.1 C-strain
K39/09	Spleen	no ct	37	1.1 C-strain
K43/09_1	Lymph node	37	32	1.1 C-strain
K43/09_3	Tonsil	25	29	1.1 C-strain
K49/09	Spleen	no ct	no ct	1.1 C-strain
K60/09	Blood	no ct	37	1.1 C-strain
K66/09_1	Spleen	36	38	1.1 C-strain
K66/09_2	Tonsil	21	23	1.1 C-strain
K71/09_1+2	Blood	31	30	1.1 C-strain
K71/09_3+4	Blood	29	28	1.1 C-strain
K72/09	Blood	39	39	1.1 C-strain
K76/09	Blood	no ct/33*	39	1.1 C-strain
K77/09	Blood	39/32*	36	1.1 C-strain
K82/09	Spleen	no ct	35	1.1 C-strain
K07/10	Blood	37	39	1.1 C-strain
K08/10	Blood	30	34	1.1 C-strain
K14/10	Spleen	37	36	1.1 C-strain
K15/10	Blood	32	36	1.1 C-strain
K18/10	Blood	39	37	1.1 C-strain
K19/10	Blood	35	35	1.1 C-strain
K25/10_1	Spleen	36	38	1.1 C-strain
K27/10	Blood	36	40	1.1 C-strain

4. Discussion

Oral vaccination of CSF-infected wild boar populations has been used in the EU to control the disease and thus to markedly reduce the risk of introduction into domestic pigs (von Rüden et al., 2008). In the past, the vaccine strain C-strain "Riems" was barely detected after oral vaccination campaigns. However, nowadays the use of highly sensitive molecular techniques has given rise to an increase in vaccine virus detections. This was also the case during the 2009 outbreaks among German wild boars and the subsequent vaccination campaigns. For a period of at least 14 days after baiting, vaccine strain detections occurred. Taking into account the huge number of tested wild boar, these findings present a minority, but nevertheless, discrimination from field virus detection is needed to circumvent unnecessary prolongation of control measures in this region. To this means, a genetic DIVA (differentiation of infected from vaccinated animals) strategy was implemented at the German NRL to rapidly rule out or confirm field virus detection after positive rRT-PCR results at regional level. The strategy combined both recently developed multiplex rRT-PCR assays for simultaneous detection of CSFV and the vaccine strain in special (Leifer et al., 2009, 2010b) and partial sequencing (Greiser-Wilke et al., 2006). As published recently (Leifer et al., 2010b), the multiplex rRT-PCR assay had to be amended after detection of primer binding site escape variants in the vaccine formulation. This amended assay is less specific for C-strain "Riems" virus as it also detects other CSFV isolates of genotype 1.1. Fortunately, this reduced strain specificity is of lesser importance for Central Europe as only animals infected with field strains of genotype 2 were identified during the last few decades. The loss of full specificity has also one unintended advantage. In areas with the same favourable epidemiological situation, the assay could now also be used with other vaccine strains of genotype 1.1.

Retrospective analysis of 33 cases where vaccine virus detection could be confirmed in subsequent tests showed that 85% of vaccine strain positive samples could be detected based on the amended multiplex rRT-PCR assay. Three samples with low genome load were only positive in the rRT-PCR assay and could not be confirmed by sequencing. As these results were repeatable under different conditions, no indications exist that these are false positive results. Five samples were only detectable in the conventional RT-PCR followed by sequencing. This outcome is surprising as rRT-PCR is generally regarded as more sensitive. Nevertheless, also these results were repeatable. There is no confirmatory assay that could reliably rule out that these unconfirmed detections could have been contaminations. At least the epidemiological background strengthened the outcome.

Thus, a positive result in the vaccine strain (genotype) specific assay could be used for direct confirmation of vaccine strain detection. There is only a very limited risk that the animal could be positive for both vaccine and field strains. This case could not be detected using the multiplex rRT-PCR alone. As the respective sample would come from an infected area with strict measures anyway, and the animal in question would be removed from the population already, this risk might be acceptable. Negative results in the vaccine strain specific assay combined with a positive result in the CSFV-specific assay will be subjected to partial sequencing anyway as field virus is suspected in these cases. Therefore, it is recommended to use the rRT-PCR assay as a first line confirmation combined with partial sequencing for samples with negative results in the vaccine assay and positive result in the CSFV-specific assay.

Beside the diagnostic approach, one could discuss adaptation of the hunting rest after baiting. As mentioned above, vaccine virus detections normally occurred in the first 14 days after baiting. In contrast, only five days hunting rest are implemented to guarantee undisturbed uptake of baits. After this period, baiting areas are checked and empty blisters as well as unused baits are collected. With three double vaccination campaigns a year, this can already mean an impact on hunting conventions, especially in autumn. As reliable discrimination of vaccine virus from field strains is possible, no adaptations towards a longer hunting rest were implemented in Germany.

5. Conclusion

Despite the experience of the past that C-strain “Riems” was barely detectable after oral vaccination campaigns, the use of highly sensitive molecular techniques has nowadays given rise to an increase in vaccine virus detections. To guarantee a rapid differentiation of truly infected from C-strain vaccinated animals, a combination of differentiating multiplex rRT-PCR assays with partial sequencing is recommended. Positive results in the vaccine strain genotype specific rRT-PCR assay can be taken as almost evidentiary whereas negative results should be confirmed by partial sequencing. The genetic DIVA concept that was proven here for wild boar, could be furthermore transferred to C-strain vaccination programs in domestic pigs, e.g. during an emergency vaccination campaign.

Conflict of interest

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Innocuousness and safety of classical swine fever marker vaccine candidate CP7_E2alf in non-target and target species

Patricia König, Sandra Blome, Claudia Gabriel, Ilona Reimann, and Martin Beer

Institute of Diagnostic Virology, Friedrich-Loeffler-Institut, Südufer 10, 17493 Greifswald-Insel Riems, Germany

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Innocuousness and safety of classical swine fever marker vaccine candidate CP7_E2alf in non-target and target species

Abstract

Chimeric pestivirus CP7_E2alf is a promising live marker vaccine candidate against classical swine fever. Prior to a possible application in the field, several safety aspects have to be addressed. Due to the fact that CP7_E2alf is based on a bovine viral diarrhea virus backbone, its behavior in ruminants is of particular interest. In the framework of this study, its innocuousness in non-target species was addressed by inoculation of calves, young goats, lambs, and rabbits. To this means, high titres of CP7_E2alf were applied orally to three animals of each species. Additional animals were left as unvaccinated contact controls. During the study, all animals remained clinically healthy, and neither fever nor leukopenia were observed. Virus could not be isolated from purified white blood cells or from nasal or faecal excretions. Moreover, none of the animals (inoculated or contact control) seroconverted. In the target species, innocuousness, shedding and transmission of vaccine virus was addressed in different animal trials that were carried out primarily for the purpose of efficacy, potency or duration of immunity studies. In all experiments, CP7_E2alf proved to be completely safe for the vaccinees and unvaccinated contact controls. Furthermore, no shedding or transmission was detected in any of the experiments. Even after parental vaccination, vaccine virus genome was barely detectable in blood or organ samples of vaccinated animals. Thus, CP7_E2alf can be regarded as completely safe for both target and non-target species.

1. Introduction

Due to its relevance for animal health and pig industry, classical swine fever (CSF) is among the most important infectious diseases of pigs and notifiable to the World Organization for Animal Health (Office International des Epizooties, OIE) [1,2]. The causative agent, Classical swine fever virus (CSFV), is an enveloped RNA virus of the genus *Pestivirus* within the *Flaviviridae* family [3]. It is antigenically closely related to the other members of the genus *Pestivirus*, namely Bovine viral diarrhea virus and border disease virus [2].

While mandatory vaccination against CSF is carried out in several countries world wide, the European Union (EU) follows a strict stamping out strategy without prophylactic vaccination,

but foresees the possibility to use emergency vaccination of both domestic pigs and wild boar [4]. To make use of this provision with an acceptable strategy, efficacious and safe vaccines are needed that allow differentiation of infected from vaccinated animals (DIVA) [5]. Unfortunately, the available subunit vaccines show drawbacks in terms of application (double, parenteral), onset of immunity and protection from vertical transmission [6,7]. Several research groups seek to develop an optimized vaccine using different approaches. A promising candidate of the new generation of marker vaccines is the chimeric pestivirus CP7_E2alf [8]. This recombinant virus proved to be a safe and efficacious DIVA vaccine candidate for oral and intramuscular vaccination of domestic pigs and wild boar in several studies [5,8–10].

As the viral backbone of CP7_E2alf is a BVDV strain, which could have a much broader host range than CSFV itself, the virulence of CP7_E2alf in ruminant non-target species is most important to assess prior to any field trials. In this context, Dong and Chen [11] requested a full evaluation on the safety not only in pigs, but also in cattle and sheep. Here, we report on studies that were carried out in order to prove innocuousness and aspects of safety in target and non-target species.

2. Materials and methods

2.1. Vaccine virus, cell culture, and virus propagation

The chimeric CP7_E2alf virus used for vaccination was described by Reimann et al. in 2004 [8]. CP7_E2alf is based on bovine viral diarrhea virus (BVDV) strain “CP7” expressing the E2 glycoprotein of CSFV strain “Alfort 187”. Differentiation of CSFV field strain infection from CP7_E2alf vaccination would be possible by a commercial available CSFV specific E^{rns} antibody ELISA.

CP7_E2alf virus for both, inoculation and neutralization assays, as well as CSFV “Alfort 187” were propagated on permanent porcine kidney cells (PK15 cells, obtained from the Collection of Cell Lines in Veterinary Medicine, FLI, Insel Riems, Germany). Viral titres were obtained through end point titrations as desribed previously [9].

Cells and viruses were grown in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% BVDVfree foetal bovine serum at 37 °C in a humidified atmosphere containing 5% CO_2.

2.2. Innocuousness in non-target species

Innocuousness in non-target species was addressed by a single, high-dose oral inoculation of calves, young goats, lambs, and rabbits. All animals were males at an age of 3–5 months. Upon inoculation, clinical signs, virus excretion, viraemia, and antibody status were closely monitored. The experiments were carried out in the high containment unit of the Friedrich–Loeffler-Institut (FLI), Isle of Riems, Germany, taking into account all applicable animal welfare regulations and standards.

In the ruminant species, three animals received 2 ml cell culture supernatant containing 2.66×10^7 tissue culture infectious doses 50% ($TCID_{50}$) CP7_E2alf each. Three rabbits received 1.5 ml cell culture supernatant containing 1.99×10^7 $TCID_{50}$ CP7_E2alf. All inocula were back-titrated after application. In order to assess the possible transmission of CP7_E2alf, co-housed contact control animals were added. To this means, one calf, two young goats, two lambs, and two rabbits were placed in direct contact to the vaccinees and sampled likewise.

Rectal body temperature and clinical signs were recorded daily for all animals over a period of 14 days post inoculation. Nasal swabs were collected daily from the ruminant species for a time period of 14 days post inoculation (dpi). Over the same period, rectal swabs were taken from the rabbits. Swabs were processed for virus isolation in cell culture as described previously [9]. Every second day from 0 to 14 dpi, EDTA blood samples were collected from all inoculated animals. Contact controls were sampled from 6 to 20 dpi. EDTA blood was used to obtain a full differential blood count using a CELL-DYN® 3700 hematology analyzer (Abbott). For virus isolation, leukocytes were prepared from EDTA blood samples after alkaline erythrocyte lysis as described previously [9]. Isolation of vaccine virus was performed by inoculation of $2–4 \times 10^6$ leukocytes or 100 µl nasal swab fluid on PK15 cells in quadruplicates on 48-well cell culture plates. After 6 days of blind passage, supernatants were transferred to a fresh subculture of PK15 cells. Indirect immunofluorescence staining was performed with the pestivirus NS3-specific monoclonal antibody BVD/C16 [12] and an Alexa Fluor®488 conjugated F(ab')2 fragment of goat anti-mouse IgG (Molecular Probes®, Invitrogen). Standard immunofluorescence analysis was carried out using an Olympus IX51 fluorescence microscope.

Serum samples for serology were collected in weekly intervals and tested in neutralization assays against CP7_E2alf and different commercially available antibody enzyme-linked immunosorbent assays (ELISA). In detail, the following antibody ELISAs were used according to the manufacturer's instructions: A BVDV NS3-blocking ELISA (*Ceditest*

BVDV, former Cedi-Diagnostics), a BVDV E2 ELISA (*Barvac BVDV*, Svanova), and two CSFV E2 blocking ELISAs (*Chekit CSF-Sero*, former Bommeli; *Ceditest CSFV-Ab* (2.0), former Cedi-Diagnostics).

Neutralization assays were carried out according to the OIE manual of diagnostic tests and vaccines for terrestrial animals with slight modifications regarding incubation times. In brief, replicates of two-fold dilutions of heat-inactivated (30 min, 56 °C) serum samples (50 µl) were incubated with 100 $TCID_{50}$/50 µl CP7 _E2alf in microtitration plates (2 h, 37 °C). After addition of 5×10^4 PK15 cells and incubation over 6 days, cultures were subjected to immunofluorescence as described above. Titres were expressed as log 2 of the reciprocal of dilutions that caused 50% neutralization (ND_{50}).

After completion of the study, all animals were slaughtered and subjected to post-mortem examinations. During necropsy, tonsil samples were collected.

2.3. Innocuousness and safety aspects in target species

In the target species, namely domestic pigs and wild boar, innocuousness, shedding and transmission of vaccine virus was addressed in different animal trials that were carried out primarily for the purpose of efficacy, potency or duration of immunity studies (4 trials with oral vaccination, two trials with intramuscular vaccination and one combined trial). In the framework of these studies, a total of 48 domestic pigs and 8 wild boar were orally vaccinated with CP7_E2alf, 35 domestic pigs were intramuscularly vaccinated, and 23 domestic pigs and 3 wild boar acted as co-housed unvaccinated contact controls. Upon immunization, vaccine recipients and in-contacts were closely monitored for adverse effects (clinical signs and body temperature). Clinical signs were recorded according to the clinical score system proposed by Mittelholzer et al. in 2000 [13]. Nasal swabs and EDTA blood samples were collected and investigated in virus isolation and real-time reverse transcription polymerase chain reaction (RT-PCR) as described elsewhere [5]. Serological response was investigated using commercially available E2 and E^{rns} antibody ELISAs (*HerdChek* CSFV Ab, IDEXX; *PrioCheck® CSFV E^{rns}*, Prionics) according to the manufacturer's instructions. Moreover, neutralization assays were performed using test virus CSFV "Alfort 187" as described above.

3. Results

3.1. Back titration of the administered virus suspensions

To prove infectivity of the inoculum, and to obtain the true titre, all virus suspensions were back titrated. For the suspension used in cattle, sheep, and goats, a mean virus titre of $10^{7.37}$ $TCID_{50}$ per inoculation dose was determined. The inoculum used in rabbits had a virus titre of $10^{7.06}$ $TCID_{50}$.

3.2. Innocuousness in non-target species

Following oral application of CP7_E2alf, all animals remained clinically healthy and showed no side effects. Neither elevation of body temperatures nor leukopenia (see Fig. 1 for leukocyte counts in calves) was observed throughout the observation period. By cultivation of purified white blood cells on susceptible cells, no CP7_E2alf viraemia was detected. Virus could not be re-isolated from nasal or faecal excretions. None of the animals seroconverted for CP7_E2alf by the end of the trial at 49 days post inoculation, and no ELISA reactivity for BVDV- or CSFV E2-specific antibodies was observed (data not shown). In addition, none of the unvaccinated contact animals seroconverted as demonstrated by neutralization assays and ELISA testing.

3.3. Innocuousness and safety aspects in target species

In none of the animal trials, clinical signs upon vaccination were observed in vaccine recipients. Moreover, neither shedding nor transmission of CP7_E2alf to contact controls was observed. All contact controls stayed negative in virological (virus isolation and real-time RT-PCR from leukocyte preparations and swab samples) and serological tests (neutralization assays and antibody ELISA) prior to a possible challenge infection (where carried out in the framework of the trial).

In rare cases (five animals), vaccine virus RNA could be detected at one sampling point (7 days post oral vaccination) from leukocyte preparations in a CP7_E2alf specific real-time RT-PCR assay [14] with very high cycle threshold values (>38), but never from swab samples.

Vaccine recipients developed neutralizing antibody titres between 14 and 21 days post vaccination, but stayed negative in the accompanying DIVA ELISA.

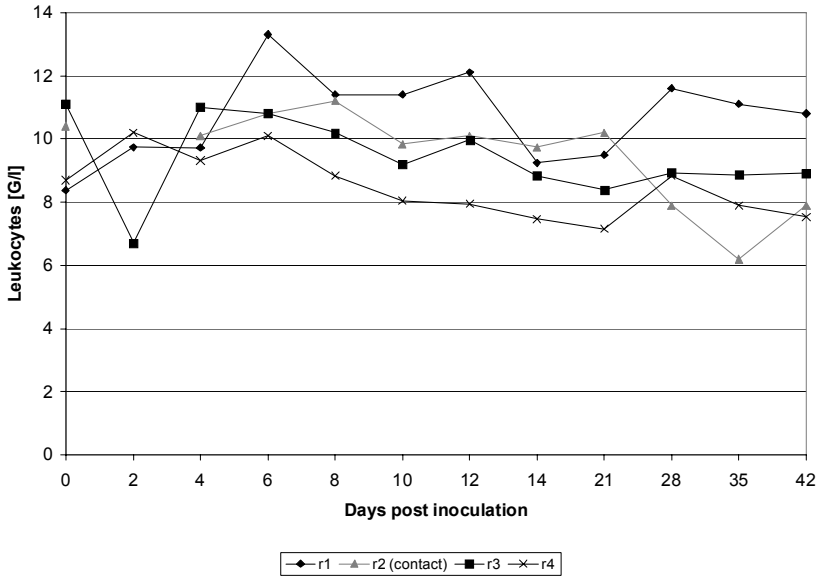

Fig. 1. Leukocyte counts [G/l] for calves orally inoculated with CP7_E2alf (r1, r3, r4) and the contact control animal (r2). Values were obtained using the CELL-DYN® 3700 hematology analyzer (Abbott). Leukocyte counts below 5.5 G/l were regarded as leukopenia.

4. Discussion

Among the most promising marker vaccine candidates against CSF are chimeric pestiviruses [6]. Several approaches in this direction have been published and substantiate the above mentioned claim [8,12,15–17]. One of these candidates is CP7_E2alf [8]. It has already proven its potential in terms of immunogenicity both after oral and intramuscular vaccination [5,9,10] and is currently investigated in an EU funded research project (CSFV_goDIVA). Due to the fact that this candidate vaccine is based on a cytopathogenic BVDV strain, safety aspects are important to answer, especially for ruminant species [11].

In a vaccination scenario with CP7_E2alf, vaccine virus could theoretically be shed by vaccinated animals (especially free-ranging wild boar), which could lead to possible contact with ruminant species. Cattle as the natural hosts are highly susceptible to oral BVDV infection. In general, clinical signs upon BVDV infection include respiratory symptoms like coughing, fever, diarrhea or mucosal lesions. The most sensitive clinical parameter is leucopenia even in animals with subclinical infection. For the non-cytopathic variant of the parental CP7 it was shown that it leads to only mild leucopenia and a short transient viraemia (P. König, unpublished data). Thus, it can be assumed that the cytopathic CP7 is even more attenuated due to restricted distribution in the host. Interspecies infection of sheep, goats and pigs normally results in seroconversion, accompanied by minor or lacking clinical symptoms.

In the above mentioned setting, the most likely transmission scenario would be an oro-nasal contact with the vaccine virus by contact to an open vaccine bait. To mimic this situation as a 'worst case scenario', our study included a direct high dose oral inoculation of cattle, sheep, and goats. Young animals were used to provide the highest possible susceptibility. As BVDV-specific antibodies were also detected in free-ranging rabbits [18], this species was included, despite the fact that BVDV antigen was never detected in this species.

It was shown that CP7_E2alf did not lead to viraemia or seroconversion in the investigated non-target species. Upon high-dose inoculation, no impairment of the health status or the differential blood cell counts was observed. Moreover, all contact controls remained negative in all virological and serological tests carried out. Infectivity of the inoculum could only be proven by back-titration of the administered virus suspension.

In several independent laboratory trials with pigs, CP7_E2alf was never shed or transmitted by vaccinated animals. Detection of vaccine virus RNA in leukocyte preparations shows that CP7_E2alf is capable of a limited replication in the target species.

Taking together the results obtained so far, both the likelihood of vaccine virus shedding and transmission as well as any effect in ruminants upon possible contact seems negligible. Finally, it has to be mentioned that the cytopathogenicity of the used BVDV backbone strain CP7 has to be judged as an additional safety factor since cytopathic BVDV strains are not able to induce a persistent infection and therefore cannot remain within a population.

5. Conclusions

In a pilot animal experiment, the BVDV/CSFV chimeric pestivirus CP7_E2alf was shown to be completely avirulent after oral application in calves, small ruminants, and rabbits. Neither viraemia nor virus excretion could be detected. No evidence for virus replication was found and no antibody response was observed at all.

Innocuousness in the target species was proven in several animal trials both after oral and intramuscular vaccination. CP7_E2alf never led to clinical signs in the vaccinated animals and was never transmitted to unvaccinated contact controls as proven by the lack of seroconversion and antigen detection.

In conclusion, interspecies transmission of vaccine virus from immunized pigs to ruminants or rabbits seems unlikely to occur under field conditions.

These data substantiate that CP7_E2alf exhibits an altered cell tropism in vitro as well as an altered host tropism in vivo and is completely safe for both target and non-target species.

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Towards licensing of CP7_E2alf as marker vaccine against classical swine fever–Duration of immunity

Claudia Gabriel[a], Sandra Blome[a], Alicia Urniza[b], Sandra Juanola[b], Frank Koenen[c], and Martin Beer[a]

[a] Institute of Diagnostic Virology, Friedrich-Loeffler-Institut, Südufer 10, 17493 Greifswald-Insel Riems, Germany

[b] Pfizer Olot S.L.U., Vall de Bianya, Spain

[c] CODA-CERVA, Groeselenberg 99, 1180 Ukkel, Belgium

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Towards licensing of CP7_E2alf as marker vaccine against classical swine fever– Duration of immunity

Abstract

Classical swine fever (CSF) marker vaccine candidate CP7_E2alf was tested in a "duration of immunity" trial according to the World Organisation for Animal Heath (OIE) guidelines. To this means, 15 weaner pigs were either orally or intramuscularly vaccinated with a single dose of CP7_E2alf vaccine produced under Good Laboratory Practice (GLP) conditions. Ten additional pigs were included as controls. Six months later, all animals were oronasally challenged with highly virulent CSF virus (CSFV) strain "Koslov". Upon vaccination, all but one orally and all intramuscularly vaccinated pigs developed rising and later on stable CSFV glycoprotein E2-specific antibodies. In contrast, no CSFV E^{rns}-specific "marker" antibodies were detectable prior to challenge infection. None of the co-housed control animals seroconverted. Upon challenge infection, all seropositive animals were protected from lethal challenge, whereas all control animals and the non-responder developed severe signs of CSF. One control animal recovered, the others had to be euthanised due to animal welfare reasons between days 4 and 7 post challenge infection. All protected animals showed quickly rising neutralizing antibodies reaching high titres by the end of the trial. At the end of the trial, the marker ELISA was positive for most challenged animals that survived the CSFV infection (27 out of 30). Using reverse transcription polymerase chain reaction, low level genome detection was seen in all vaccinated animals between days 4 and 10 post challenge infection, but no virus could be isolated from any samples of these animals. The OIE guidelines require seroconversion in at least 8 out of 10 vaccinated animals. This requirement was fulfilled. Moreover, only control animals should die. With this requirement, only the intramuscular vaccination fully complied as one orally vaccinated pig did not respond. Concluding, CP7_E2alf induced stable antibodies that led to protection from lethal challenge with highly virulent CSFV strain "Koslov" six months after vaccination, with the exception of one non-responder after oral vaccination.

1. Introduction

Classical swine fever (CSF) belongs to the most important viral diseases of domestic pigs that threaten industrialized pig production worldwide [1]. The notifiable disease can induce a wide range of clinical syndromes in both domestic pigs and wild boar ranging from almost inapparent infections to a haemorrhagic fever like illness [2]. It is caused by a small enveloped RNA virus of the genus Pestivirus within the *Flaviviridae* family [3].

To control CSF, several countries worldwide are implementing mandatory vaccination campaigns in combination with strict sanitary measures. Within the European Union (EU), this strategy was successfully followed until 1990 when prophylactic vaccination was banned and replaced by a stamping out policy to eradicate the disease [4,5]. Despite the ban of prophylactic vaccination, emergency vaccination could be used in case of an outbreak following an EU-approved emergency vaccination plan. Due to the immense economic consequences of CSF outbreaks, this provision is constantly under discussion among EU Member States. However, several obstacles, related to the available vaccines and the marketability of products from vaccinated animals, have led to the fact that the option of emergency vaccination, that is per se not limited to a certain vaccine type, was almost never implemented in domestic pigs.

As for the vaccines, two types are available on the European market: (a) modified live vaccines that have proven both efficacy and safety, and (b) an E2 subunit vaccine that allows differentiation of infected from vaccinated animals (DIVA) but lacks some properties that live vaccines exhibit, e.g. fast and robust protection after single application, complete block of transplacental infection or the possibility of oral immunization [6]. In terms of international trade, only DIVA vaccines are a feasible option as products from animals that were vaccinated with conventional live attenuated vaccines suffer severe trade restrictions.

In order to make the option of emergency vaccination acceptable for trade partners and consumers, new generations of potent vaccines are required that allow for a reliable DIVA approach [7]. As wild boar populations require oral vaccination, only live vaccines are suitable for all possible applications [5].

In order to overcome the above-mentioned impediments, several research groups worked on the development of an optimized vaccine using different approaches [7,8]. A promising candidate of this new generation of marker vaccines is the chimeric Pestivirus “CP7_E2alf”, which is based on the cytopathogenic bovine viral diarrhoea virus (BVDV) strain “CP7” expressing the E2 glycoprotein of CSFV strain “Alfort/187” [9]. This recombinant virus

proved to be a safe and efficacious DIVA vaccine candidate for oral and intramuscular vaccination of domestic pigs and wild boar [10–14], and was chosen after large comparative trials [24] as candidate for further development within the EU funded research project "Improve tools and strategies for the prevention and control of classical swine fever" (CSFV_goDIVA, KBBE-227003).

For CSF vaccines, detailed requirements are laid down in the European Pharmacopoeia and the World Organisation for Animal Health (OIE) manual of diagnostic tests and vaccines for terrestrial animals. Therefore, several studies on immunogenicity, safety, and efficacy are needed with vaccine batches produced under Good Laboratory Practice (GLP) conditions. Among the required studies is the assessment of duration of immunity. Here we report on the outcome of this trial after oral and intramuscular vaccination of domestic pigs with GLP-produced CP7_E2alf vaccine batches.

2. Materials and methods

2.1. Experimental settings

According to the OIE manual of diagnostic tests and vaccines for terrestrial animals, a test for duration of immunity must comprise at least 10 pigs inoculated with one dose of the respective vaccine, and two other animals kept as unvaccinated controls. Six months later, serum samples are tested for antibodies against CSF virus (CSFV). Subsequently, all pigs must be challenged with at least 10^5 PID_{50} of a virulent strain of CSFV, and observed for three weeks. The vaccine passes the test if at least eight pigs seroconvert and only controls die upon challenge infection.

To ensure data quality for in detail analyses, an approach was chosen with 15 orally vaccinated, 15 intramuscularly vaccinated, and 10 unvaccinated contact animals. To this means, 40 crossbred weaner pigs of approximately eight weeks of age (10–17 kg) were bought from a commercial pig farm and brought to the animal facilities of the Friedrich-Loeffler-Institut (FLI). Upon arrival, animals were allocated into three treatment groups using a generalized randomized block design blocking on body weight and pen location. Animals were kept litterless under appropriate high-containment conditions. All pigs were handled taking into account animal welfare regulations and standards according to EU Directive 2010/63/EU and institutional guidelines. They were fed a commercial feed for pigs of their age class combined with hay cobs, and had access to water ad libitum. After an

acclimatization phase of six days, sera were collected in order to evaluate the serological status of animals (freedom from pestivirus antibodies). Afterwards, 30 animals were vaccinated according to their assigned treatment group and all piglets were carefully observed for 60 min for any systemic reactions associated with vaccine administration. Four days after vaccination all treatment groups were co-mingled according to the randomisation plan. Following vaccination, sera were collected for serology in monthly intervals. Six months after vaccination, all animals were challenged with CSFV strain "Koslov", and serum and EDTA blood samples were collected at days 0, 4, 7, 10, 14, and 22 post challenge infection (dpc).

Rectal body temperature was measured on a daily basis. Fever was defined as a body temperature ≥40.0 °C for at least two consecutive days. Elevated body temperatures ≥40.0 °C for just one day were reported as such.

Clinical signs were recorded daily from 3 days prior to vaccination to 7 days post vaccination, and from 3 days before up to 21 days after challenge infection according to the protocol published by Mittelholzer et al. [15] with slight modifications regarding the evaluation of feed intake (assessment of interest in food upon supply).

Euthanasia of moribund animals and all remaining animals at the end of the trial was conducted by electro-stunning and exsanguination, followed by post-mortem examinations.

2.2. Vaccine and challenge viruses

Two different CP7_E2alf pilot vaccine batches for oral and intramuscular application, respectively, were produced under GLP conditions by Pfizer Olot S.L.U. (Spain). Vaccines were diluted in sterile diluent solution provided by Pfizer Olot S.L.U. (Spain) prior to administration based on pre-existing potency data (unpublished results). In detail, the liquid CP7_E2alf vaccine batch 081010 with stabilizer L2 intended for oral vaccination was thawed, mixed by pulse-vortexing and diluted 1:3 in sterile diluent solution to a calculated titre of $1 \times 10^{5.5}$ tissue culture infectious doses 50% ($TCID_{50}$) per ml. For oral immunization, 1.6 ml (the content of a standard blister for oral vaccination of wild boar) of the solution was administered to each animal orally using a 2 ml syringe without needle. For intramuscular vaccination, the lyophilized and L2 stabilized CP7_E2alf vaccine batch 191010 was reconstituted and diluted tenfold with sterile diluent solution to a calculated titre of $1 \times 10^{4.0}$ $TCID_{50}$/ml. Each animal of the intramuscularly vaccinated group received 1 ml of the latter by intramuscular injection in the right neck (deep into the muscles behind the ear using a 2 ml syringe and a 20 G needle). Due to bleeding at the injection site that was probably

accompanied by flow out of vaccine solution, one animal (#12) received an additional vaccine dose.

Challenge infection was carried out using CSFV strain "Koslov" which was obtained from the German National Reference Laboratory for CSF (FLI, Insel Riems, Germany). This strain belongs to genotype 1.1 and is highly virulent for all age classes of animals. Due to its severe and reproducible clinical course (accompanied by almost 100% mortality within 7–10 days), this strain has been used in several challenge trials with different vaccines including CP7_E2alf [11,12]. Severe unspecific and central nervous signs predominate upon "Koslov" infection. The "Koslov" virus used in this study originated from an animal experiment at the FLI where whole blood from a viraemic animal was collected. This material was subsequently defibrinated, aliquoted, and titrated at least three times. For challenge infection, the blood was diluted in PBS to a theoretical titre of $1 \times 10^{5.5}$ $TCID_{50}$/ml, and 2 ml were applied to each animal by oronasal inoculation using a 2 ml syringe without needle.

Vaccine and challenge virus dilutions were back-titrated at FLI in at least three independent virus titrations in order to obtain the true titres administered.

2.3. Cells and viruses

Cells and viruses were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% foetal bovine serum free of pestiviruses and anti-pestivirus antibodies at 37 °C in a humidified atmosphere containing 5% CO_2. Porcine kidney cell line 15 (PK15/5; CCLV-RIE0005), Madin–Darby bovine kidney cells (MDBK; CCLV-RIE261), and sheep fetal thymus-R cells (SFT-R; CCLV-RIE0043) were obtained from the Collection of Cell Lines in Veterinary Medicine (CCLV, FLI Insel Riems, Germany).

CSFV strain "Alfort/187", BVDV strain "NADL" and Border disease virus (BDV) strain "Moredun" were obtained from the German National Reference Laboratory for CSF (FLI Insel Riems, Germany) for use in neutralization tests.

2.4. Laboratory investigations

2.4.1. Processing of samples

EDTA blood as well as serum samples, which were obtained from native blood by centrifugation at 2031 × g at 20 °C for 20 min, were aliquoted and stored at −70 °C. Tissue samples of tonsil, spleen, and salivary gland were collected at necropsy and stored at −70 °C until further use. For real-time reverse transcription polymerase chain reaction (RT-qPCR) and virus isolation, tissue samples were homogenized in 1 ml DMEM using a TissueLyser II (QIAGEN GmbH, Hilden, Germany).

2.4.2. Virus detection

In order to assess the administered titres of vaccine and challenge viruses, virus titrations were performed by end point dilution on PK15 cells. The titres expressed as $TCID_{50}$/ml were obtained by indirect immuno-peroxidase staining of heat-fixated cells which was performed 72 h post inoculation using a mouse-anti-CSFV-E2 monoclonal antibody mix and a polyclonal goat anti-mouse horseradish peroxidase conjugated secondary antibody (Thermo Fisher Scientific, Waltham, USA).

Extraction of viral RNA from sera and EDTA blood samples was performed using the QIAamp® Viral RNA Mini Kit (QIAGEN GmbH, Hilden, Germany) according to manufacturer's recommendations. Homogenized tissue samples were processed using the automated MagAttract® Virus Mini M48 Kit in combination with the BioSprint 96 workstation (QIAGEN GmbH, Hilden, Germany). In both extraction methods, an internal control RNA (IC2) was added as described previously [16].

Viral genome was detected by RT-qPCR using the real-time PCR-cycler MX3005P™ (Stratagene, La Jolla, USA). To determine the absence of pestiviral infections in the animals prior to vaccination, all sera were tested in a pestivirus specific RT-qPCR as published previously [16]. For EDTA blood, tonsil, and spleen samples, a CSFV specific RT-qPCR protocol was used [17]. In addition, RT-qPCR of salivary gland samples was performed using the commercial Virotype® CSFV real-time RT-PCR test kit (Labor Diagnostik GmbH Leipzig, Leipzig, Germany) which is based on the protocol published by Leifer et al. [18]. Results were recorded as quantification cycle (cq) values. Amplified viral RNA was quantified using an in vitro transcribed RNA dilution series (encompassing parts of the 5´-non-translated region and the NS5A genome regions of CSFV) with defined copy numbers.

Virus isolation was carried out on all RT-qPCR positive blood samples and on all tissue samples according to the standard protocols laid down in the Technical Annex of European Commission Decision 2002/106/EC (EU Diagnostic Manual for CSF). Results were assessed after one virus passage using indirect immuno-peroxidase staining as described above.
Moreover, antigen detection was carried out on all sera collected after challenge infection using the HerdChek® CSFV Ag/Serum ELISA (IDEXX Laboratories, Hoofddorp, The Netherlands) following the standard protocol provided by the manufacturer.

2.4.3. Antibody detection

All sera were tested in neutralization peroxidase-linked antibody assays (NPLA) according to the EU Diagnostic Manual and the Technical Annex accompanying it. Neutralizing antibody titres against CSFV "Alfort/187" were determined on PK15 cells, those directed against BVDV "NADL" on MDBK cells, and against BDV "Moredun" on SFT-R cells. Indirect immuno-peroxidase staining was performed as mentioned above. Titres were calculated as neutralization doses 50% (ND_{50}). Titres exceeding 10240 ND_{50} were recorded as 10241 ND_{50}.
Furthermore, sera were tested for the presence of CSFV E2-specific antibodies with the HerdChek® CSFV Ab ELISA (IDEXX Laboratories, Hoofddorp, The Netherlands) and for the detection of CSFV E^{rns}-specific antibodies using the PrioCHECK® E^{rns} ELISA (Prionics Lelystad BV, Lelystad, The Netherlands) according to the manufacturer's instructions.

2.5. Data and statistical analysis

All data were recorded using Microsoft Excel 2010 (Microsoft Deutschland GmbH, Unterschleißheim, Germany). Box plots were created using SigmaPlot for Windows version 11.0 (Systat Software Inc., Chicago, USA).

3. Results

3.1. Back titration of vaccine and challenge viruses

In order to assess the titres administered, back titrations of vaccine and challenge viruses were conducted. The diluted CP7_E2alf pilot vaccine batch 081010 for oral use showed a mean titre of $10^{5.54}$ $TCID_{50}$/ml, whereas the pilot vaccine batch 191010 preparation for intramuscular use had a mean titre of $10^{4.49}$ $TCID_{50}$/ml. Back titration of "Koslov" challenge virus resulted in a mean virus titre of $10^{5.24}$ $TCID_{50}$/ml.

3.2. Observations before challenge infection

3.2.1. Clinical observations

Upon vaccination, neither local nor systemic adverse effects could be observed in vaccinated animals. Despite a sporadic occurrence of fever in three orally vaccinated animals and one unvaccinated animal which was not accompanied by any clinical signs, body temperatures of vaccinated and unvaccinated animals stayed within the physiological range.

3.2.2. Virus detection

Prior to vaccination, all animals were tested negative in the pestivirus specific RT-qPCR of serum samples, and EDTA blood samples collected before challenge infection were negative in the CSFV specific RT-qPCR. Furthermore, all sera were negative in virus isolation and antigen ELISA prior to challenge infection.

3.2.3. Antibody detection

Prior to vaccination, all animals were tested negative for neutralizing antibodies against CSFV, BVDV and BDV.

In neutralization assays of sera collected from orally vaccinated animals, antibodies against CSFV "Alfort" were first detected in 14 out of 15 animals 1 month post vaccination with titres ranging between 5 and 160 ND_{50} (see Fig. 1). Titres of these animals rose in the course of the experiment and were between 60 and 1920 ND_{50} prior to challenge infection 6 months post vaccination. One orally vaccinated animal (#4) showed no measurable antibodies in neutralization assays against CSFV "Alfort" until challenge infection (non-responder). Upon intramuscular vaccination, first measurable neutralizing antibodies against CSFV "Alfort"

were detected 1 month post vaccination with titres ranging from 10 to 120 ND_{50}. As in orally vaccinated animals, antibody titres rose in the course of time and final neutralizing antibody titres against CSFV "Alfort" ranged between 40 and 2560 ND_{50} prior to challenge infection.

CSFV E2-specific antibodies could be detected by the commercial antibody ELISA in all intramuscularly vaccinated animals and all but one orally vaccinated animals prior to challenge infection. In detail, 12 out of 15 orally vaccinated animals became positive in the CSFV E2-specific antibody ELISA at 1 month after vaccination, whereas one animal showed a doubtful result and two animals stayed negative by this time. From 2 months post vaccination, all but one (#4) orally vaccinated animals were positive showing increasing percentage of inhibition values which were stable and comparable until at least 6 months post vaccination. All intramuscularly vaccinated animals scored positive in the CSFV E2-specific ELISA at 1 month post vaccination showing increasing or stable percentage of inhibition values till challenge infection (Fig. 1).

None of the sera collected prior to challenge infection reacted positive in the CSFV E^{rns}-specific marker antibody ELISA.

All unvaccinated animals stayed negative in all serological examinations prior to challenge infection (Fig. 1).

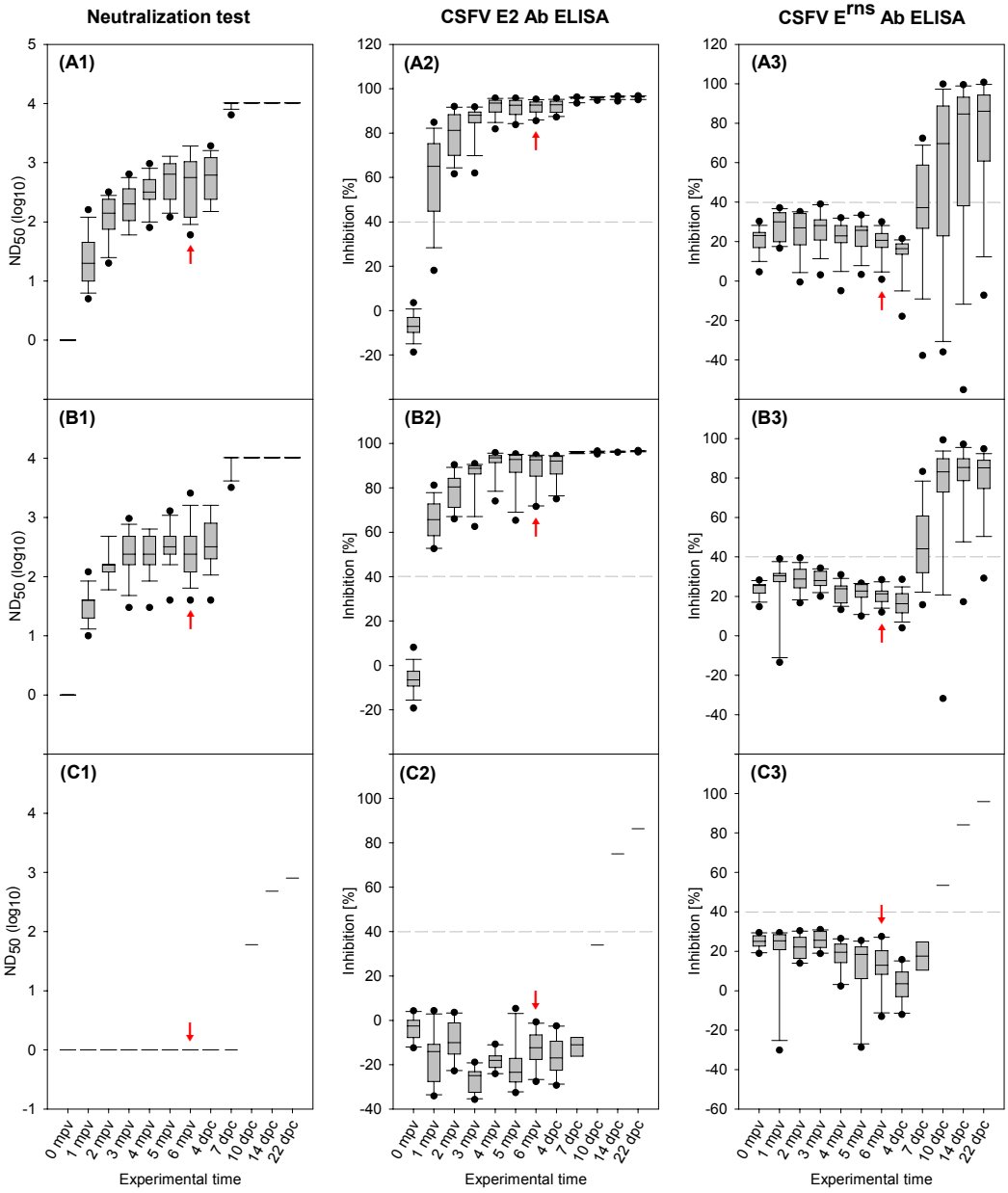

Fig. 1. Antibody response of orally vaccinated (A), intramuscularly vaccinated (B) and unvaccinated (C) domestic pigs upon vaccination with CP7_E2alf and after challenge infection using highly virulent CSFV strain "Koslov" six months later. Results of the neutralization tests using CSFV strain "Alfort/187" (A1–C1) are shown as group reactivity. Box plots are based on antibody titres represented as $\log_{10}$ ND_{50}. Results of the CSFV E2-specific antibody ELISA (A2–C2) and CSFV E^{rns}-specific antibody ELISA (A3–C3) are shown as group reactivity in percentage of inhibition and given as box plots. The dashed line represents the ELISA cut-off; red arrows mark the challenge infection time point.

mpv: months post vaccination; dpc: days post challenge infection. As complete non-responder, animal #4 was excluded. The boundaries of the boxes indicate the 25th and 75th percentiles, the line within the box marks the median and errors bars above and below the boxes indicate the 90th and 10th percentiles. Outlying points are graphed as dots.

3.3. Observations after challenge infection

3.3.1. Clinical observations

Upon challenge infection, four orally vaccinated animals showed fever up to 40.9 °C at 4 and 5 dpc. Additionally, three animals of this group had an elevated body temperature for one day (4, 6, 16, and 19 dpc). Animal #4, which did not seroconvert after oral vaccination, showed fever and typical signs of CSF starting 3 dpc and was euthanised in a moribund state with severe neurological signs including convulsions (maximum CS 9) at 7 dpc. Apart from this animal, none of the orally vaccinated animals showed clinical signs indicative for CSF after challenge infection.

In the intramuscularly vaccinated group, four animals had fever between 40.1 °C and 40.5 °C for two consecutive days (4 and 5 dpc for three animals, and 15 and 16 dpc for one animal), and two animals had an elevated body temperature at 4 dpc. None of these six animals showed other clinical signs. Another animal of this group showed fever up to 41.2 °C on three consecutive days (4–6 dpc), accompanied by slight depression at 4 and 5 dpc (CS 1–2). This animal showed an additional day of elevated body temperature after one day with a physiological body temperature. Furthermore, one animal showed recurrent fever up to 41.0 °C which was accompanied by a reduced liveliness and feed intake at 5 dpc (CS 2) and a persistent lameness caused by a lesion of one dew claw (CS 1).

Nine out of 10 unvaccinated animals had fever starting between 3 and 4 dpc and showed severe CSF specific signs including severe depression (lethargy, anorexia, shivering, reluctance to stand) and neurological signs (staggering gait, torticollis) with a maximum clinical scores ranging from 2 to 8. These animals were euthanised due to animal welfare reasons at 4 dpc (n = 4), 6 dpc (n = 4), and 7 dpc (n = 1). The remaining control animal showed fever from 3 to 16 dpc and clinical signs like reduced liveliness, moderate catarrhal conjunctivitis, and a slight staggering gait for a period of seven days (CS 1–3). The animal recovered until the end of the experiment showing a pronounced loss of body weight.

3.3.2. Virus detection

RT-qPCR of EDTA blood samples from orally vaccinated animals (excluding animal #4) showed weak positive results for 10 animals from 4 dpc (cq values >34, corresponding to less than 2.6×10^2 genome copies per µl) to last weak positive results at 10 dpc. Only the diseased animal #4 showed much higher and increasing genome loads in EDTA blood samples from 4

dpc till the day of euthanasia (cq values 28–16, corresponding to app. 7.5×10^3 to 5×10^7 genome copies per µl). An overview is given in Table 1.

Similarly, low copy numbers of viral RNA were detected in 12 out of 15 EDTA blood samples taken from intramuscularly vaccinated animals at 4 dpc. Quantification cycle values (values >31, corresponding to less than 1.4×10^3 genome copies per µl) were mostly decreasing over time (negative for all animals 14 dpc).

EDTA blood samples collected from unvaccinated control animals after challenge infection were positive in the CSFV specific RT-qPCR showing increasing genome loads (lowest cq values <17, corresponding to $>3.5 \times 10^7$ genome copies per µl) until euthanasia of the animals.

In the CSFV specific RT-qPCR, tonsil samples of all vaccinated animals (excluding animal #4) were positive with cq values between 28 and 36, corresponding to 7.7×10^3 and 2.5×10^1 genome copies per µl, respectively. The non-responder, animal #4, showed a cq value of 26 (3.7×10^4 genome copies per µl). In the orally vaccinated group, viral RNA could be detected in more than a third of spleen samples ($n = 6$) and in a large number of salivary gland samples ($n = 11$) (cq values mostly >30; $<7.8 \times 10^2$ genome copies per µl, except for animal #4). Eleven spleen and 13 salivary gland samples of intramuscularly vaccinated animals were positive in the CSFV specific RT-qPCR. Much higher CSFV genome loads were detected in tissue samples of the nine control animals which were euthanised due to severe suffering from CSF (1.4×10^3 to 5.1×10^5 genome copies per µl) while the surviving control animal #29 showed results comparable to the vaccinated animals.

Apart from animal #4, positive results in RT-qPCR of EDTA blood and tissue samples of vaccinated animals were not accompanied by any positive results in virus isolation. In contrast, sera from unvaccinated controls were always positive in virus isolation, except for serum samples taken from the surviving animal #29 starting 10 dpc. In addition, virus isolation was positive for all tissue samples of the unvaccinated controls except for the organ samples of animal #29. Detailed results of RT-qPCR and virus isolation on tissue samples are given in Table 2.

Beside animal #4, all vaccinated animals stayed clearly negative in antigen ELISA after challenge infection. For the unvaccinated group, all animals yielded positive results in antigen ELISA at least at the day of euthanasia, except for animal #32 (data not shown).

Table 1

RT-qPCR results of blood samples and results of virus isolation (VI) from serum samples after challenge infection 6 months post vaccination. −, not detected in RT-qPCR/negative in virus isolation; +, positive in virus isolation; ✞, animal was already euthanised by this time point; nd, not done. Positive RT-qPCR results are presented as copies per µl.

Pig #	Day post challenge infection											
	0		4		7[a]		10		14		22	
	RT-qPCR	VI	RT-qPCR	VI	RT-qPCR	VI	RT-qPCR	VI	RT-qPCR	VI	RT-qPCR	VI
Oral vaccination												
1	–	–	4.7×10^1	–	–	nd	–	nd	–	nd	–	nd
4	–	–	7.5×10^3	+	5.0×10^7	+	✞	✞	✞	✞	✞	✞
5	–	–	3.2×10^1	–	2.5×10^2	–	1.1×10^1	–	–	nd	–	nd
6	–	–	4.8×10^1	–	8.3×10^1	–	1.8×10^1	–	–	nd	–	nd
10	–	–	–	nd	–	nd	–	nd	–	nd	–	nd
11	–	–	4.7×10^1	–	8.5×10^1	–	1.0×10^1	–	–	nd	–	nd
14	–	–	2.6×10^0	–	–	nd	–	nd	–	nd	–	nd
15	–	–	1.4×10^0	–	4.0×10^0	–	–	nd	–	nd	–	nd
16	–	–	4.5×10^1	–	–	nd	–	nd	–	nd	–[b]	nd
18	–	–	5.5×10^1	–	1.4×10^1	–	4.3×10^0	–	–	nd	–	nd
23	–	–	–	nd	–	nd	–	nd	–	nd	–[b]	nd
34	–	–	–	nd	5.3×10^0	–	–	nd	–	nd	–	nd
35	–	–	2.2×10^1	–	–	nd	–	nd	–	nd	–	nd
37	–	–	2.5×10^2	–	5.1×10^1	–	4.8×10^0	–	–	nd	–	nd
40	–	–	-	nd	–	nd	–	nd	–	nd	–	nd
Intramuscular vaccination												
2	–	–	2.1×10^2	–	4.7×10^1	–	4.9×10^0	–	–	nd	–	nd
3	–	–	2.0×10^1	–	-	nd	–	nd	–	nd	–	nd
7	–	–	3.6×10^1	–	5.4×10^1	–	2.6×10^1	–	–	nd	–	nd
12	–	–	1.9×10^2	–	-	nd	1.2×10^1	–	–	nd	–	nd
13	–	–	4.8×10^2	–	1.3×10^3	–	8.3×10^1	–	–	nd	–[b]	nd
17	–	–	–	nd	1.4×10^1	–	–	nd	–	nd	–	nd
19	–	–	–	nd	–	nd	–	nd	–	nd	–	nd
24	–	–	1.1×10^1	–	–	nd	–	nd	–	nd	–[b]	nd
27	–	–	2.5×10^0	–	–	nd	–	nd	–	nd	–	nd
28	–	–	1.6×10^1	–	–	nd	–	nd	–	nd	–	nd
30	–	–	2.7×10^1	–	4.5×10^0	–	–	nd	–	nd	–[b]	nd
31	–	–	1.8×10^1	–	4.5×10^1	–	2.1×10^0	–	–	nd	–	nd
33	–	–	–	nd	6.1×10^0	–	–	nd	–	nd	–	nd
38	–	–	7.4×10^1	–	1.4×10^1	–	–	nd	–	nd	–	nd
39	–	–	1.0×10^3	–	1.1×10^3	–	2.9×10^1	–	–	nd	–	nd
Unvaccinated												
8	–	–	5.8×10^3	+	1.3×10^7	+	✞	✞	✞	✞	✞	✞
9	–	–	1.2×10^5	+	✞	✞	✞	✞	✞	✞	✞	✞
20	–	–	3.5×10^4	+	3.6×10^7	+	✞	✞	✞	✞	✞	✞
21	–	–	2.9×10^4	+	9.5×10^6	+	✞	✞	✞	✞	✞	✞
22	–	–	2.8×10^3	+	2.1×10^4	+	✞	✞	✞	✞	✞	✞
25	–	–	6.1×10^4	+	✞	✞	✞	✞	✞	✞	✞	✞
26	–	–	5.7×10^3	+	1.5×10^7	+	✞	✞	✞	✞	✞	✞
29	–	–	7.8×10^3	+	4.0×10^6	+	2.4×10^6	–	3.3×10^5	–	3.2×10^3	–
32	–	–	1.9×10^4	+	✞	✞	✞	✞	✞	✞	✞	✞
36	–	–	1.2×10^5	+	✞	✞	✞	✞	✞	✞	✞	✞

[a] Six days post challenge infection for samples from animals 8, 20, 21, and 22.

[b] Serum samples instead of blood samples.

Table 2

Detection of virus and viral RNA in tissue samples after oral and intramuscular vaccination with CP7_E2alf, and challenge infection 6 months post vaccination. VI, virus isolation; −, not detected in RT-qPCR/negative in virus isolation; +, positive in virus isolation; dpc, day post challenge infection. Positive RT-qPCR results are presented as copies per µl.

Pig #	Day of euthanasia (dpc)	Tonsil		Spleen		Salivary gland	
		RT-qPCR	VI	RT-qPCR	VI	RT-qPCR	VI
Oral vaccination							
1	22	1.5×10^{3}	−	−	−	7.5×10^{-1}	−
4	7	3.7×10^{4}	+	1.9×10^{6}	+	2.2×10^{5}	+
5	22	1.6×10^{2}	−	4.3×10^{0}	−	4.5×10^{0}	−
6	25	1.3×10^{2}	−	−	−	−	−
10	25	9.4×10^{2}	−	−	−	−	−
11	22	3.1×10^{2}	−	7.7×10^{2}	−	8.1×10^{0}	−
14	22	9.4×10^{1}	−	1.8×10^{1}	−	-	−
15	25	3.2×10^{2}	−	−	−	5.4×10^{1}	−
16	22	9.6×10^{2}	−	−	−	2.2×10^{1}	−
18	25	3.9×10^{2}	−	−	−	−	−
23	22	6.0×10^{2}	−	9.6×10^{0}	−	5.9×10^{0}	−
34	25	7.7×10^{3}	−	−	−	9.1×10^{1}	−
35	25	4.8×10^{2}	−	−	−	1.2×10^{1}	−
37	25	4.6×10^{3}	−	1.5×10^{1}	−	3.4×10^{1}	−
40	22	3.2×10^{2}	−	−	−	4.4×10^{1}	−
Intramuscular vaccination							
2	22	6.3×10^{2}	−	1.4×10^{2}	−	2.5×10^{0}	−
3	22	1.7×10^{2}	−	1.2×10^{2}	−	−	−
7	25	1.5×10^{3}	−	1.7×10^{1}	−	2.4×10^{2}	−
12	22	2.1×10^{3}	−	6.4×10^{2}	−	1.9×10^{1}	−
13	22	6.7×10^{3}	−	7.0×10^{1}	−	1.6×10^{2}	−
17	25	2.5×10^{1}	−	7.9×10^{0}	−	2.7×10^{0}	−
19	25	1.3×10^{3}	−	1.9×10^{1}	−	1.1×10^{0}	−
24	22	7.4×10^{3}	−	−	−	2.2×10^{1}	−
27	25	2.5×10^{3}	−	3.5×10^{1}	−	6.7×10^{1}	−
28	22	4.6×10^{2}	−	−	−	4.1×10^{1}	−
30	22	1.7×10^{2}	−	3.0×10^{0}	−	1.9×10^{2}	−
31	22	5.7×10^{2}	−	−	−	−	−
33	22	1.4×10^{2}	−	−	−	3.7×10^{0}	−
38	25	5.1×10^{2}	−	7.0×10^{0}	−	5.1×10^{1}	−
39	25	1.9×10^{3}	−	2.5×10^{2}	−	2.2×10^{1}	−
Unvaccinated							
8	6	1.1×10^{5}	+	3.8×10^{5}	+	3.3×10^{4}	+
9	4	1.5×10^{4}	+	1.1×10^{5}	+	2.9×10^{3}	+
20	6	1.6×10^{4}	+	1.1×10^{5}	+	8.8×10^{4}	+
21	6	1.1×10^{5}	+	6.2×10^{4}	+	3.6×10^{4}	+
22	6	1.6×10^{4}	+	4.2×10^{4}	+	4.9×10^{4}	+
25	4	4.5×10^{5}	+	1.1×10^{5}	+	1.6×10^{3}	+
26	7	5.6×10^{4}	+	5.1×10^{5}	+	1.7×10^{4}	+
29	25	8.3×10^{1}	−	8.5×10^{0}	−	5.2×10^{2}	−
32	4	7.6×10^{4}	+	9.9×10^{3}	+	1.4×10^{3}	+
36	4	1.2×10^{4}	+	6.1×10^{3}	+	9.4×10^{3}	+

3.3.3. Antibody detection

Upon challenge infection, neutralizing antibody titres against CSFV "Alfort" rose quickly in 14 out of 15 orally and all intramuscularly vaccinated animals, resulting in high antibody titres exceeding 10240 ND_{50} at 10 dpc. Furthermore, these animals remained strong positive in the CSFV E2-specific antibody ELISA after challenge (inhibition values >93% starting from 7 dpc), while the remaining animal #4 that suffered from CSF infection did not develop CSFV specific antibodies until euthanasia (7 dpc) (Fig. 1).

Marker E^{rns} antibodies could be detected as early as 7 dpc in five animals of the orally vaccinated group. Three days later, 10 orally vaccinated animals were positive in the CSFV E^{rns}-specific antibody ELISA, and 12 out of 14 orally vaccinated animals were clearly positive for E^{rns} antibodies at 22 dpc (Fig. 1).

As in the orally vaccinated group, E^{rns}-specific antibodies were first detectable at 7 dpc in the intramuscularly vaccinated group, but in contrast, more animals (n = 9) were tested positive in the CSFV E^{rns}-specific antibody ELISA. At the end of the trial (22 dpc), 14 out of 15 intramuscularly vaccinated animals were positive for E^{rns} antibodies (Fig. 1).

Nine out of 10 unvaccinated control animals stayed negative in all serological tests until euthanasia, whereas the unvaccinated animal #29 which recovered until the end of the experiment showed first doubtful results in the CSFV E2-specific ELISA and first positive results in the CSFV E^{rns}-specific antibody ELISA at 10 dpc. Neutralizing antibody titres against CSFV "Alfort" of this animal rose from 60 ND_{50} at 10 dpc to 800 ND_{50} at 22 dpc.

3.3.4. Post mortem examinations

In all but one unvaccinated control animal including the recovering animal #29, typical lesions for CSF were detected during post mortem examinations, for instance necrotizing and purulent tonsillitis, enlarged and haemorrhagic lymph nodes, spleen infarctions, and ascites. However, one unvaccinated animal that was euthanised in a moribund state at 4 dpc just showed an unspecific serous pericardial effusion.

Moreover, animal #4 that did not respond serologically to oral vaccination showed haemorrhages in the renal cortex and gall bladder wall, beside all CSF specific lesions mentioned above.

No CSF specific pathological signs were observed during necropsy in any of the remaining vaccinated pigs.

Apart from follicular hyperplasia seen in the majority of vaccinated as well as unvaccinated animals, typical signs for a previous infection with *Haemophilus parasuis* (Glässer's disease)

like adhesive pleurisy and pericarditis as well as fibrinous perihepatitis were observed frequently during post mortem examinations.

4. Discussion

Classical swine fever is among the most important diseases of domestic pigs worldwide and can cause tremendous economic losses. Thus, it is understandable that the development of potent vaccines was and is of paramount importance. Different vaccination scenarios exist and may require different vaccine characteristics [5]. While vaccination in endemically infected areas is used to prevent economic losses or as a first step towards eradication, emergency vaccination in case of outbreaks is intended to limit spread of the disease (freeze the outbreak situation). Especially the latter requires DIVA vaccines inducing an early onset of immunity. A special emergency vaccination scenario is the oral immunization of wild boar. Here, the measure aims at preventing introduction into the domestic pig population.

The perfect vaccine that would be suitable for all scenarios must therefore provide fast and solid protection against horizontal and vertical transmission, must be safe in target and non-target animals, and must be acceptable for consumers. Moreover, it should be easily applicable, also orally, and allow a DIVA principle. The DIVA vaccine has to be accompanied by a reliable diagnostic test system [7]. So far, such an optimal vaccine does not exist.

Over the last decades, intensive research activities have led to several most promising vaccine candidates, but so far, none of them came close to market authorization. In order to establish modern concepts for animal disease control, and CSF control in particular, licensing of promising candidates is necessary, even more than the continued search for the optimal vaccine. The here reported duration of immunity after single shot vaccination in accordance with the OIE manual of diagnostic tests and vaccines for terrestrial animals is one of the experiments of the CSFV_goDIVA project providing the necessary data required for licensing.

In the experimental setup, 15 animals were either orally or intramuscularly vaccinated with CP7_E2alf. In order to ensure and standardize oral vaccine uptake, the content of one blister was administered using a syringe. This deviates from the intended use in baits but guarantees both comparability of data among vaccinated animals in this study, and also with similar studies using other live attenuated vaccines, for example the C-strain “Riems” vaccine in the same manner [19,20]. The latter is the routine bait vaccine currently in use for CSF infected wild boar populations within the EU. For the C-strain “Riems” vaccine it was shown that the

onset of immunity was influenced by the mode of oral vaccination, but no effect was seen on the duration of immunity [21]. Bait vaccination always has a high degree of uncertainty regarding uptake in general and the ingested vaccine dose in special. Thus, it can only be viewed on population base.

Due to bleeding at the injection site, one animal (#12) received two vaccine doses (max. $2 \times 10^{4.49}$ $TCID_{50}$ in total) to account for the possible leakage. As this animal did not react differently in laboratory tests upon vaccination or challenge, this fact was later on disregarded for data analysis. Six months later, these animals were challenged together with 10 unvaccinated controls using highly virulent CSFV strain "Koslov".

Upon oral or intramuscular vaccination with CP7_E2alf, neither local nor systemic adverse effects were observed. Sporadic fever occurred without link to any other clinical signs. As several animals showed signs of an overcome infection with *H. parasuis* in necropsy, these reactions upon vaccination might be explained by this factor.

As all control animals stayed clearly negative in all serological tests (E2 and E^{rns} antibodies) prior to challenge infection, there is no evidence of vaccine virus transmission to contact animals. It has to be kept in mind that animals were only co-mingled from four days after vaccination. For this reason, it is impossible to judge on the early phase post vaccination. The issue of vaccine virus transmission was also addressed by König et al. [14]. In this context, it could be shown that vaccine virus transmission does not occur, even in the early phase post vaccination.

From one month after vaccination onwards, monthly serological tests were carried out to assess seroconversion of vaccinated animals. CSFV neutralizing antibodies were detected by neutralization assays in all intramuscularly and all but one orally vaccinated animals. These results were in general comparable with results from studies with shorter duration [12]. Orally vaccinated animal #4 remained negative throughout the vaccination phase of the experiment in all serological tests. This animal has to be regarded as a true non-responder. Explanations why this animal did not respond are rather speculative. Inactivation of vaccine virus by saliva enzymes and insufficient delivery are among these explanations. In general, oral immunization is much more error prone than intramuscular vaccination and non-responders have been observed in a few C-strain vaccination trials as well (Lange, personal communication). All other vaccinated animals showed rising and later on stable antibody responses both in the highly sensitive neutralization test and in the routine CSFV E2-specific antibody ELISA. By the day of challenge infection, 6 months post vaccination, antibody titres of both, intramuscularly and orally vaccinated animals (apart from animal #4) were moderate

to high. These results are comparable with those of C-strain "Riems" vaccination [21]. During the course of the vaccination part of the study, all animals (vaccinated and controls) remained negative for E^{rns} antibodies.

Thus, the requirement for at least eight out of 10 (80%) animals to seroconvert upon vaccination was met and the marker concept proved to work. In contrast to control animals that developed CSF specific lesion and were tested positive in all pathogen detection methods, all CP7_E2alf vaccinated animals that had seroconverted (all but animal #4) survived without noteworthy clinical signs, and virus isolations from all materials originating from these animals remained negative. Likewise, antigen ELISA results were negative throughout the challenge part of the trial. During necropsy, none of these animals showed signs indicative for CSF infection.

Nevertheless, a short fever peak was observed in several vaccinated animals (mainly at 4 dpc). These temperature peaks were in general not accompanied by other signs. Despite clinical protection and negative virus and antigen detections, viral genome was detectable in low quantities over a period of up to 7 days (4–10 dpc) in the majority of EDTA blood samples from vaccinated animals. Moderate genome loads were found in some organ samples, especially tonsils. This is in accordance with previous studies where limited replication of challenge virus was also observed [12]. Especially the high antibody titres in vaccinated animals seem to prevent shedding and isolation of live virus. Virus neutralization through antibodies also explains why tissue samples with moderate genome loads that were comparable or even higher than those of virus positive control animals were not detectable in virus isolation. This phenomenon is not only seen with CP7_E2alf vaccination but also with other live vaccines, even with the "gold standard" C-strain [24]. Inability to isolate virus despite positive RT-PCR results in the presence of antibodies is also known for experimental infections [22]. This limited replication may also explain the quickly rising neutralizing antibody titres that indicate a strong boost of immunity. In general, limited replication is also necessary for a clear response in the CSFV E^{rns}-specific marker antibody ELISA that is necessary for reliable investigations in the framework of an emergency vaccination scenario. In this study, a total of 27 out of 30 animals that survived challenge infection were positive in the E^{rns} ELISA by the end of the trial at 22 dpc (26 vaccinated, one unvaccinated but recovered). In addition, two non-reacting animals showed rising inhibition percentage values (32 and 29%, respectively). Based on these data, the inclusion of a doubtful range could probably increase the sensitivity of the marker test system under field conditions. The animals that stayed negative showed only low genome loads in RT-qPCR and thus almost no

challenge virus replication, but there was no obvious difference to other vaccinated animals that did react. So far, there is no indication that real-time RT-PCR positive animals with high antibody titres pose a risk in terms of transmission related to trade or use of products from such animals. The discrepancy between RT-PCR and virus isolation in general has been addressed in the context of CSFV after observation of quite similar phenomena in the field and after E2-subunit vaccination [23]. One reason for the discrepancy is the amplification of non-infectious viral RNA fragments by RT-PCR. Nevertheless, this point should be followed up in subsequent studies with the vaccine candidate.

According to the OIE manual of diagnostic tests and vaccines for terrestrial animals, only unvaccinated control animals should die upon challenge infection. Taken together with the requirement of 80% seroconversion in vaccinated animals (see above), this request seems somewhat contradictory. Probably, this requirement tried to take into account that it was shown for live attenuated vaccines that animals without measurable antibodies were protected upon early challenge. However, for duration of immunity, it can be assumed that an animal that did not respond after six months will not be protected. This was seen with the non-responder of this trial (animal #4). As the vaccine guidelines are mainly intended for intramuscularly applicable vaccines, discussion is needed regarding oral vaccines.

In conclusion, CP7_E2alf fulfills the requirements for duration of immunity for the part of intramuscular vaccination. Excluding the non-responder, duration of immunity was also shown for oral vaccination with CP7_E2alf.

5. Conclusions

Within the framework of this study it was confirmed that CP7_E2alf presents a safe and efficacious marker vaccine against CSF. Antibody titres elicited by one shot intramuscular or oral vaccination were stable for at least six months and provided protection against lethal challenge with a highly virulent CSFV strain. Despite short fever reactions upon challenge accompanied by detection of small quantities of viral genome, virus could never be isolated from vaccinated animals (which responded with detectable antibodies). High antibody titres seem to lead to full virus neutralization.

Not unexpectedly, oral vaccination proved to be more error prone than intramuscular vaccination. Especially for assessment of oral vaccination, OIE requirements are challenging and should be discussed in the future.

Based on OIE guidelines, intramuscular vaccination with CP7_E2alf passes the test for duration of immunity. Oral vaccination showed similar performance if the non-responder is excluded.

Towards licensing of CP7_E2alf, further studies are needed and will be conducted, especially on vaccine safety.

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Efficacy of CP7_E2alf pilot vaccine batches after intramuscular and oral vaccination

Claudia Gabriel[1], Sandra Blome[1], Sandra Juanola[2], Alicia Urniza[2], Frank Koenen[3], and Martin Beer[1]

[1] Institute of Diagnostic Virology, Friedrich-Loeffler-Institut (FLI), Greifswald – Insel Riems, Germany

[2] Pfizer Olot, S.L.U., Vall de Bianya (Girona), Spain

[3] Veterinary and Agrochemical Research Center (CODA-CERVA), Uccel, Belgium

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Claudia Gabriel[1], Sandra Blome[1], Sandra Juanola[2], Alicia Urniza[2], Frank Koenen[3], and Martin Beer[1]

[1]Institute of Diagnostic Virology, Friedrich-Loeffler-Institut (FLI), Greifswald – Insel Riems, Germany
[2]Pfizer Olot, S.L.U., Vall de Bianya (Girona), Spain
[3]Veterinary and Agrochemical Research Center (CODA-CERVA), Uccel, Belgium

Introduction

Classical swine fever (CSF) is one of the most important disease affecting pigs causing tremendous socio-economic losses. Within the European Union, a strict stamping out strategy without prophylactic vaccination is implemented. Nevertheless, Community legislation also foresees the possible use of emergency vaccination.
While vaccination using highly efficacious conventional vaccines does not allow differentiation between infected and vaccinated animals (DIVA), and vaccinated animals and their products are subjected to trade restrictions, currently available E2 subunit marker vaccines lack some important properties like early onset of immunity and full protection against vertical transmission. For this reason, several research efforts dealt with the design and characterization of new marker vaccines. Among these, one promising candidate is the chimeric pestivirus CP7_E2alf. The latter is currently assessed in accordance with requirements of the European Pharmacopoeia and the OIE Manual of Diagnostic Tests and Vaccines within the EU funded project "Improve tools and strategies for the prevention and control of classical swine fever" (CSFV_goDIVA). In this context, two animal trials were conducted to assess the efficacy of CP7_E2alf pilot vaccine batches for intramuscular (domestic pigs) and oral (wild boar) application after challenge infection using highly virulent CSFV strain "Koslov" (genotype 1.1) and moderately virulent CSFV strain "Rösrath" (genotype 2.3).

Study Design

In order to assess the efficacy of CP7_E2alf pilot vaccine batches the following study design was applied:

Intramuscular vaccination of domestic weaner pigs		**Oral vaccination of wild boar piglets**	
CP7_E2alf / Unvaccinated controls		CP7_E2alf / Unvaccinated controls	
Oronasal challenge infection of all pigs using either:			
Highly virulent CSFV strain **„Koslov"**	14 dpv	Highly virulent CSFV strain **„Koslov"**	21 dpv
Moderately virulent CSFV strain **„Rösrath"**	15 dpv	Moderately virulent CSFV strain **„Rösrath"**	21 dpv

Clinical signs and body temperatures were recorded daily. In addition, blood samples were collected regularly. At the end of the trials all animals were subjected to postmortem examinations.
Sera were tested serologically by the HerdChek® CSFV Ab ELISA (IDEXX) and neutralization tests using CSFV strain "Alfort 187" as test virus.
Furthermore, blood samples were tested in a CSFV specific real-time RT-PCR according to Hoffmann et al. (2005) [1]. Samples which were tested positiv by qRT-PCR were subjected to virus isolation.

Tab.1: Back titration of vaccines and challenge virus [$TCID_{50}$].

	Challenge infection using	
	Koslov	Rösrath
CP7_E2alf vaccines for i.m. vaccination	$10^{4.4}$	$10^{3.7}$
CP7_E2alf vaccines for oral vaccination	$10^{5.9}$	$10^{5.2}$
Koslov challenge virus	$10^{7.1}$	
Rösrath challenge virus	$10^{6.6}$	

Results

Part A: Challenge infection with CSFV strain "Koslov"
At the day of challenge using CSFV "Koslov", all orally vaccinated and all but one intramuscularly vaccinated animals showed measurable E2 antibodies (Fig. 1a). Titres rose quickly upon challenge and reached high levels by the end of the trial (Fig. 2a). Beside a temperature peak accompanied by slight depression four days post challenge (dpc) in domestic pigs, no symptoms were observed in vaccinated animals. The unvaccinated animals died at 7 dpc (wild boar) and 10 dpc (domestic pigs) showing CSF specific symptoms and lesions. The CSFV specific qRT-PCR of blood samples of vaccinated wild boar revealed positive results with cq-values between 33 to 37 at five days post challenge, whereas blood samples of unvaccinated pigs showed much higher genome loads with cq values between 12 to 18 (data not shown). Nevertheless, no challenge virus could be isolated from samples of vaccinated animals throughout the trial.

Part B: Challenge infection with CSFV strain "Rösrath"
In the trial covering challenge infection using CSFV strain "Rösrath", four out of five animals either orally or intramuscularly vaccinated, showed measurable E2 antibodies (Fig. 1b). As in part A, titres rose quickly upon challenge and reached high levels by the end of the trial (Fig. 2b). The unvaccinated wild boar died 7 and 12 dpc. In this experimental part, one control domestic pig died 16 dpc whereas the second domestic pig survived until the end of the trial (28 dpc). The CSFV specific qRT-PCR of blood samples of vaccinated animals sporadically revealed positive results with cq-values between 37 to 40, whereas blood samples of unvaccinated pigs showed higher genome loads (data not shown). Likewise, no challenge virus could be isolated from samples of vaccinated animals throughout the trial.

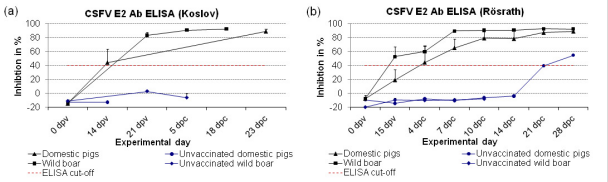

Fig. 1: Results of the CSFV E2 ELISA shown as average inhibition in %. Bars indicate standard deviation. (a) Challenge infection with highly virulent CSFV strain "Koslov". (b) Challenge infection with moderately virulent CSFV strain "Rösrath".

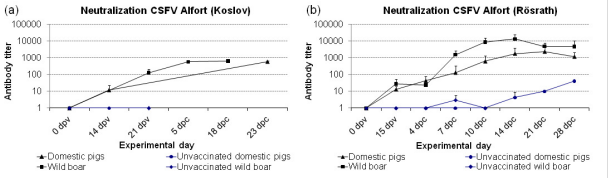

Fig. 2: Results of the neutralization tests against Alfort 187 shown as average ND_{50} titres. Bars indicate standard deviation. (a) Challenge infection with highly virulent CSFV strain "Koslov". (b) Challenge infection with moderately virulent CSFV strain "Rösrath".

Conclusion

The CP7_E2alf pilot vaccine batches proved to be safe after vaccination and were able to protect susceptible pigs from lethal challenge infection using the highly virulent CSFV strain "Koslov" as well as the moderately virulent CSFV strain "Rösrath". The latter represents recent field strains occurring in Europe.

Acknowledgements

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Correspondence address: claudia.gabriel@fli.bund.de

Characterization of African swine fever virus Caucasus isolate in European wild boars

Claudia Gabriel[a], Sandra Blome[a], Alexander Malogolovkin[b], Stanislav Parilov[b], Denis Kolbasov[b], Jens P. Teifke[a], and Martin Beer[a]

[a] Friedrich-Loeffler-Institut, Greifswald–Insel Riems, Germany

[b] National Research Institute for Veterinary Virology and Microbiology, Pokrov, Russia

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Characterization of African swine fever virus Caucasus isolate in European wild boars

Abstract

Since 2007, African swine fever has spread from the Caucasus region. To learn more about the dynamics of the disease in wild boars (*Sus scrofa*), we conducted experiments by using European wild boars. We found high virulence of Caucasus isolates limited potential for establishment of endemicity.

Introduction

African swine fever (ASF) is one of the most serious diseases affecting pigs (*1*). The causative agent, *African swine fever virus* (ASFV), is a complex DNA virus of the genus *Asfivirus* within the *Asfarviridae* family. Because of its ability to replicate in *Ornithodorus* ticks, ASFV can be classified as arthropod-borne virus (*2*). In domestic pigs, ASFV can cause a wide range of clinical signs, including hemorrhagic syndromes with high lethality. Little is known about ASF in European wild boars, although indications exist that the animals are highly susceptible (*3*).

In 2007, ASF affecting domestic pigs and wild boars was reported in the Caucasus region. The virus strain involved was related to isolates of genotype II, which are circulating in Mozambique, Madagascar, and Zambia (*4*). Especially in Russia, ASF recurs and shows a clear tendency to move northward (*5*). This unresolved situation increases the risk of introducing the virus into virus-free areas, and the involvement of wild boars raises special concerns. As seen with classical swine fever, the growing population of wild boars is problematic for animal disease control, particularly if the infection reaches endemicity (*6*). Therefore, knowledge about disease dynamics is vital for risk assessment and strategy design, particularly because no vaccine against ASF is available.

Therefore, animal experiments were carried out at the Friedrich-Loeffler-Institut (Greifswald–Insel Riems, Germany), and the National Research Institute for Veterinary Virology and Microbiology (NRIVVaMR, Pokrov, Russia). The aim was to define clinical signs, disease dynamics, and postmortem lesions in wild boars after intramuscular and oral infection with ASFV Caucasus isolates.

The Study

The study comprised 2 experimental parts: 1) oral infection conducted at the Friedrich-Loeffler-Institut and 2) intramuscular infection at NRIVVaMR. For oral infection, we used a 2008 isolate from Armenia. The experiment was conducted by using 6 wild boar piglets 9 weeks of age. Three domestic pigs were used as contact controls and were handled in the same manner as the wild boar piglets. The animals were kept under high-containment conditions. After acclimatization, the wild boars were infected orally with 2 mL of a spleen suspension containing 10^6 median tissue culture infectious dose ASFV/mL. Two days after infection, 3 domestic weaner pigs were added to the pen with the wild boar piglets. Starting from the day of infection, rectal temperature and clinical signs were recorded. Oral and fecal swabs were collected from the wild boars at 0, 1, 2, 3, 5, 6, and 7 days postinfection (dpi). In addition, blood samples were taken at 0, 2, 5, 6, and 7 dpi. Blood from the domestic pigs was sampled at 0, 6, 9, and 13 dpi. Necropsy was performed on all animals.

For real-time quantitative PCR (qPCR), viral DNA was extracted by using manual and automated extraction methods according to manufacturer instructions. Subsequently, qPCR was performed according to the protocol published by King et al. (*7*) with slight modifications by using an Mx3005P PCR Cycler (Stratagene, La Jolla, CA, USA).

For intramuscular infection, 4 wild boars 9 months of age were brought to the containment stables of the NRIVVaMR. One animal was inoculated intramuscularly with 1,000 hemadsorbing units 50% of a 2009 virus isolate from the Chechen Republic, which is identical to the isolate used in the oral trial in all genome fragments routinely sequenced. The remaining animals were housed together with the infected animal as contact controls.

Clinical signs of infection were recorded every day. Samples of visceral organs, skin, and hair were taken during necropsy and subjected to qPCR. Isolation of viral DNA was performed by using an in-house kit based on the modified method published by Boom et al. (*8*). The qPCR for ASFV detection was carried out according to the protocol published by King et al. (*7*) with a Rotorgene 6000 instrument (Corbett Research, Sydney, Queensland, Australia).

After oral infection, an acute fatal course of the disease developed in all wild boar piglets, and they died within 7 days. Apart from severe depression, slight diarrhea, and reduced feed intake, only high fever was observed starting 3–4 dpi. During postmortem examinations, enlarged and hemorrhagic lymph nodes (Figure 1) and hemorrhagic gastritis (Figure 2) were observed. Acute fatal ASF developed in 2 of the domestic pigs 11–12 dpi of the wild boars. These animals died 1 week later showing severe but unspecific symptoms. One domestic pig

became infected later. It only showed fever at 20 dpi and was euthanized on day 25. Infection of this animal was clearly linked to contact with blood from a moribund pen mate.

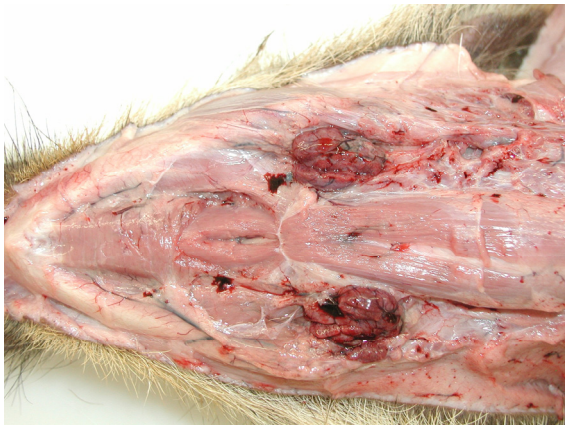

Figure 1. Ventral view of the head showing pathologic signs in a wild boar piglet after oral inoculation with 10^6 median tissue culture infectious dose of an African swine fever virus isolate from Armenia (experiment at the Friedrich-Loeffler-Institut). Note edematously enlarged and hemorrhagic mandibular lymph nodes. The animal died on day 7 postinfection.

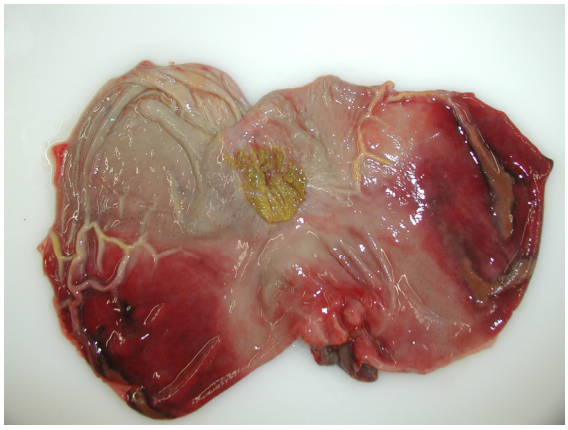

Figure 2. View of the mucosal surface of the dissected stomach showing representative gross lesions after oral inoculation of a wild boar with 10^6 median tissue culture infectious dose of an African swine fever virus isolate from Armenia (experiment at the Friedrich-Loeffler-Institut). The image illustrates acute gastritis; note diffuse mucosal hemorrhages affecting a large part of the mucosa. The animal died on day 7 postinfection.

During the clinical phase of the disease, qPCR was positive for all blood samples with first positive results 2 dpi. Oropharyngeal and fecal swabs were positive mainly on days 6 and 7. An overview of the qPCR results is presented in Table 1.

Table 1. Real-time PCR results of blood and swab samples after oral infection in study of African swine fever virus in European wild boars*

	Days post infection of wild boar										
Animal, sample source	0	1	2	3	5	6	7	9	13	17	20
Wild boar 1											
Blood	No C_t	ND	No C_t	ND	23						
Oropharyngeal swab	No C_t	No C_t	No C_t	No C_t	No C_t						
Fecal swab	No C_t	No C_t	No C_t	No C_t	No C_t						
Wild boar 2											
Blood	No C_t	ND	No C_t	ND	22	20	24				
Oropharyngeal swab	No C_t	No C_t	No C_t	No C_t	37	37	37				
Fecal swab	No C_t	No C_t	No C_t	No C_t	No C_t	38	No C_t				
Wild boar 3											
Blood	No C_t	ND	No C_t	ND	28	22	23				
Oropharyngeal swab	No C_t	No C_t	No C_t	No C_t	No C_t	38	34				
Fecal swab	No C_t	No C_t	No C_t	No C_t	37	34	33				
Wild boar 4											
Blood	No C_t	ND	No C_t	ND	25	26	26				
Oropharyngeal swab	No C_t	No C_t	No C_t	37	No C_t	34	37				
Fecal swab	No C_t	No C_t	No C_t	No C_t	30	29	33				
Wild boar 5											
Blood	No C_t	ND	39	ND	25	23					
Oropharyngeal swab	No C_t	No C_t	No C_t	No C_t	39	35					
Fecal swab	No C_t	No C_t	No C_t	No C_t	No C_t	29					
Wild boar 6											
Blood	No C_t	ND	No C_t	ND	23	24					
Oropharyngeal swab	No C_t	No C_t	No C_t	37	No C_t	34					
Fecal swab	No C_t	No C_t	No C_t	No C_t	35	32					
Domestic pig 1, blood	No C_t	ND	ND	ND	ND	39	ND	No C_t	21	20	
Domestic pig 2, blood	No C_t	ND	ND	ND	ND	No C_t	ND	No C_t	23	ND	
Domestic pig 3, blood	No C_t	ND	ND	ND	ND	No C_t	ND	No C_t	No C_t	ND	29†

*C_t, cycle threshold; ND, not done because of missing samples. Numbers indicate C_t values.
†Serum sample instead of whole blood sample was used.

On the third day after intramuscular inoculation, the infected wild boar showed depression, inappetence, and increased respiratory frequency. It died at 5 dpi showing hemorrhagic nasal discharge. The 3 contact animals showed similar symptoms at 8 dpi of the intramuscularly infected wild boar and died 2 days later. Postmortem examinations showed hemorrhages in multiple edematously enlarged lymph nodes, most prominent pulmonary hyperemia and alveolar edema, hyperplasia of the mesenteric lymph nodes, and acute gastritis with hemorrhages. Skin lesions were not present.

ASF genome was detected in the samples of visceral organs and lymph nodes of all animals. In samples of skin and kidneys, viral DNA was detected only in the infected animal. Results of qPCR are presented in the Table 2.

Table 2. Real-time PCR results of organ samples taken after intramuscular infection in a study of African swine fever virus in European wild boars*

Wild boar	Lung	Heart	Spleen	Lymph nodes
1	19	19	18	19
2	20	20	19	20
3	21	21	20	20
4	20	21	19	20

*Cycle threshold values indicated.

Conclusions

Knowledge about disease dynamics in domestic pigs and wild boars is a prerequisite for risk assessment and prevention strategy design. Unfortunately, wild boar data are scarce. To contribute to this information, animal trials were conducted for an experimental characterization of recent Caucasian ASFV isolates in wild boars.

We concluded that the Caucasian isolates are highly virulent in wild boars. Both oral and intramuscular infection resulted in 100% lethality.

PCR results showed that the ASFV genome is easily detected in blood and organ samples of diseased animals. Swab samples were positive in the clinical phase of infection but showed much lower genome loads. Shedding of ASFV through nasal discharge or feces, and thus overall contagiousness, seems to be limited.

Transmission to domestic pigs was delayed in comparison to transmission to wild boars. The most likely reason for this difference seems to be contact with blood. Although this factor could be observed most certainly for the contact wild boars, domestic pigs had only limited contact with blood.

On the basis of these data, it seems unlikely the Caucasian isolates have the potential to become endemic in European wild boar populations without a distinct change in virulence. So far no indications exist that the virulence of ASFV is changing in affected regions in Russia.

A risk factor for disease control could be the involvement of tick vectors. Until now, no indications exist that ticks are involved in ASFV outbreaks in the Caucasus region and Russia. Moreover, it has to be kept in mind that the wild boar's way of life does not facilitate

contact with soft ticks. Nevertheless, this possibility was not examined during this study and needs further investigation.

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Ms Gabriel is a veterinarian at the Friedrich-Loeffler-Institut, Institute of Diagnostic Virology. Her research focuses on pathogenesis, molecular epidemiology, and control of classical and African swine fever.

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High virulence of African swine fever virus Caucasus isolate in European wild boars of all ages

Sandra Blome[a], Claudia Gabriel[a], Klaas Dietze[b], Angele Breithaupt[a], and Martin Beer[a]

[a] Friedrich-Loeffler-Institut, Greifswald–Insel Riems, Germany

[b] Food and Agriculture Organization of the United Nations, Rome, Italy

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To the Editor:

African swine fever (ASF) is a serious disease that is currently affecting domestic pigs and wild boars in the Russian Federation. The disease is caused by *African swine fever virus* (ASFV; family *Asfarviridae*), and its continuing spread imposes a growing risk for introduction to disease-free areas with a high density of pigs and/or wild boars. We recently reported on the experimental characterization of ASFV Caucasus isolates in European wild boar piglets and juveniles (*1*), age classes that were deemed to be the most susceptible to ASFV. The extreme virulence of the virus strain led to an almost peracute disease and 100% mortality. On the basis of these data, a scenario of endemicity driven by chronically diseased animals or ASFV carriers seems unlikely. Nevertheless, ASF continues to occur in wild boars. The clinical course of some infectious diseases is age dependent; thus, we supplemented our previous study (*1*) with a limited study among adult wild boars to help clarify their role in the epidemiology of ASFV. To achieve this goal, we orally inoculated 1 boar (10 years of age), 2 sows (4 and 5 years, respectively), and 1 boar piglet with a 3×10^6 50% tissue culture infectious dose of the ASFV Caucasus isolate.

Severe, unspecific clinical signs (fever, depression, anorexia, dyspnea, ataxia) developed in all animals. Infection was confirmed by PCR of blood samples and fecal and oral swab samples obtained 6 days after inoculation. All animals died or were euthanized in a moribund state 8–9 days after inoculation, confirming that ASFV causes severe, acute disease and is fatal for 100% of infected adult European wild boars. No antibodies were detected in serum samples throughout the experiment.

The available data show no indication of chronic ASF disease or ASFV carrier states among adult wild boars, conditions that could potentially contribute to long-term persistence of disease in an affected region. In terms of risk assessment, the most likely routes for the introduction of ASFV into wild boar populations are spillover from domestic pigs, exposure to ASFV-contaminated carcasses under climate conditions favoring the persistence of infectious virus, contact with fomites, and consumption of ASFV-contaminated animal feed.

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5 DISCUSSION

5.1 Classical Swine Fever control

5.1.1 Optimization of conventional vaccination

Vaccination is an important tool to control the spread and intensity of CSF infection among wild boar (Anonymous, 2010). Over the last decades several EU Member States, including Germany, successfully implemented oral bait vaccination using the conventional modified live C-strain vaccine (Kaden et al., 2000a; Kaden et al., 2003; von Rüden et al., 2008; Rossi et al., 2010). As C-strain vaccinated animals cannot be differentiated serologically from infected animals, virological monitoring during and after vaccination programs is required. In infected areas, all wild boar shot or found dead have to be inspected by an official veterinarian and examined for CSF in accordance with the EU Diagnostic Manual (Anonymous, 2002a; Anonymous, 2010). Up to now, virus isolation and antigen ELISA have been routinely implemented for these investigations in combination with antibody ELISA to monitor seroprevalence. In the framework of emergency vaccination campaigns among European wild boar in two German Federal States in 2009, routine pathogen detection at regional level had shifted to modern, highly sensitive PCR applications. Using a CSFV specific rRT-PCR several vaccine virus detections occurred. This can be explained by the fact that vaccination using any live vaccine is accompanied by limited replication of the vaccine strain in the host, that is usually not detectable by virus isolation or antigen ELISA. In contrast, highly sensitive molecular techniques are able to detect viral nucleic acid for a limited period of time in blood and organ samples from replication sites (Koenig et al., 2007a). To ensure that restrictions and measures implemented after a CSF outbreak among wild boar with subsequent emergency vaccination are not unnecessarily prolonged, rapid differentiation of virus detections is required. Therefore, a genetic DIVA strategy was implemented at the German National Reference Laboratory for CSF based on a recently published multiplex rRT-PCR allowing differentiation of field virus infected from C-strain vaccinated animals (Leifer et al., 2009a).

This approach presents a first proof of principle for the implementation of a “genetic DIVA” strategy in the field, a concept first described by Beer et al. (2007). In fact, more than one third of samples coming from the affected region could be confirmed as vaccine virus detections (Blome et al., 2011).

So far, a genetic DIVA concept was only used for scenarios with wild boar immunization, but in general, it could be employed also in the framework of (emergency) vaccination campaigns in domestic pigs. Here, the approach could be part of a strategy to release animals from the restriction zones for slaughter after testing their freedom from CSFV by rRT-PCR. These strategies are currently under discussion among EU Member States and trade partners.

However, combining a highly efficacious modified live vaccine with a genetic DIVA concept seems to be the limit of optimization for conventional vaccines. As the use of these vaccines does not permit serological DIVA, the effect of vaccination can hardly be evaluated and the detection and differentiation of field virus induced antibodies is impossible. Thus, new generations of live marker vaccines are of paramount importance for long-term disease control. In this context, the chimeric pestivirus "CP7_E2alf" proved to be a most promising candidate for vaccination of domestic pigs and wild boar (Reimann et al., 2004; Koenig et al., 2007b), and was chosen for further evaluation towards licensing.

5.1.2 Assessment of marker vaccine candidate 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 (Dong and Chen, 2007; Beer et al., 2007). The ultimate goal, however, must be licensing of these candidates, making them an available tool for control. Therefore, an approach started in the framework of the EU funded FP7 project "Improve tools and strategies for the prevention and control of classical swine fever" (CSFV_goDIVA, KBBE-227003). After extensive comparative trials with two promising candidates in comparison with the "gold standard" C-strain (Blome et al., 2012a), chimeric pestivirus CP7_E2alf was chosen as candidate for authorization. Trials required by the European Pharmacopoeia (monograph 07/2008:0065) and the OIE Manual (OIE, 2008b) were distributed among the project partners. Our studies comprised innocuousness for target and non-target species and different aspects of efficacy including duration of immunity (König et al., 2011; Gabriel et al., 2012).

Innocuousness in ruminant species, especially cattle, is a prerequisite to embed a chimeric vaccine based on a BVDV backbone into a CSF control strategy especially in a country with mandatory BVDV control. In several EU Member States including Germany, vaccine virus transmission would have a high impact. Studies on the cell tropism of the engineered chimeric virus already showed an almost complete shift towards porcine cell lines (Reimann et al., 2004). In the presented study we demonstrated that oral inoculation with high titers of CP7_E2alf did not lead to virus detection or even seroconversion in cattle, goats, sheep, and rabbits (König et al., 2011). The latter were included as seroconversion had been reported in free-ranging German rabbits (Frölich and Streich, 1998). Apart from innocuousness in non-target species, no adverse effects were seen in target animals after both oral and intramuscular vaccination (domestic pigs and wild boar). Moreover, no indications exist that vaccine virus shedding occurred (König et al., 2011). As the vaccine is intended also for oral immunization of wild boar, baits will be distributed in the field. It is known from C-strain vaccination that despite covered distribution bait uptake by non-target species, especially badgers, mustelids, foxes, and raven, is quite frequently observed (Sophie Rossi, ONCSF, France, unpublished data). Thus, contact with vaccine virus cannot be excluded for other wildlife species. In general, it can be assumed that the reaction of wild ruminant species should be comparable to domestic ruminants and for this reason, the risk of replication and perpetuation of vaccine virus seems negligible. As neither BVDV nor CSFV replicates in species other than cloven-

hoofed animals, carnivores, birds, and other wild animals should not play a role. Nevertheless, additional studies could complement our data.

Besides studies on innocuousness and safety, several animal trials were carried out to assess the efficacy of the CP7_E2alf vaccine candidate. As both European Pharmacopoeia and OIE guidelines request challenge infection with a highly virulent CSFV strain, the well characterized strain "Koslov" was used as a standard. Like the "Alfort" strain, donor of the E2 of CP7_E2alf, "Koslov" belongs to genotype 1.1. The challenge model proved to be suitable in several trials in different laboratories. Moreover, it was valid for all age classes of domestic pigs as well as European wild boar. This approach has the disadvantage that genotype 1.1 does not present the current field situation in Europe, where moderate virulent strains of genotype 2 prevail. For this reason, a collaborative study was undertaken with the EU Reference Laboratory for CSF in Hannover using the recent 2.3 "Rösrath" strain from Germany (Leifer et al., 2010b) for challenge infection in intramuscularly vaccinated domestic pigs and orally vaccinated wild boar (poster presentation at the 8th ESVV Pestivirus Symposium in Hannover, 2011), and a 2.1 isolate from Israel (CSF1047) in intramuscularly vaccinated domestic pigs (unpublished data, manuscript in preparation). Efficacy was confirmed for all tested genotypes. Thus, not only protection against highly virulent challenge but also field suitability was proven.

Supplementing these studies, we conducted a study on the duration of immunity according to the OIE Manual, which requires a duration of immunity of at least 6 months (Gabriel et al., 2012). In detail, eight out of ten pigs vaccinated once with one vaccine dose should seroconvert, and only the two unvaccinated controls should die upon highly virulent challenge. These requirements were fulfilled for intramuscular vaccination. Due to the fact that one out of fifteen animals did not respond to oral vaccine application, the criterion that only unvaccinated animals should die was not met by oral vaccination. In fact, a non-responder has to be regarded as unvaccinated. Given the fact that all animals that seroconverted showed stable antibody titers and full protection against challenge with a CSFV strain of exceptionally high virulence, our data suggest that CP7_E2alf is comparable to C-strain vaccine (Kaden and Lange, 2001) or E2 subunit marker vaccine (de Smit et al., 2001b) in terms of duration of immunity.

If the criteria for a perfect marker vaccine as postulated by Dong and Chen (2007) would be used to assess CP7_E2alf, almost all demands are met.

First, it is postulated that a perfect marker vaccine should be safe and innocuous in target and non-target animals. Important data have already been compiled (König et al., 2011), and

further safety studies in pregnant animals are currently under execution (Berta Alberca, Pfizer Animal Health, Spain, personal communication). So far, no negative effects on gestation were reported. In order to generate data for regulatory submission at the European Medicines Agency (EMA), safety regarding local and systemic reactions upon vaccination is currently assessed under field-like conditions in a trial according to Good Clinical Practice (GCP) guidelines using a pilot vaccine batch produced under Good Laboratory Practice (GLP) conditions.

Furthermore, a marker vaccine that could be used in all possible scenarios must induce reliable protection against horizontal transmission within a short time. This criterion is met by vaccine candidate CP7_E2alf as was seen in previous studies (Leifer et al., 2009b; Blome et al., 2012a) and the studies presented here (Gabriel et al., 2012). Full protection also includes the block of transmission to susceptible animals. Previously, the "gold standard" C-strain was reported to induce sterile immunity (Dahle and Liess, 1995; van Oirschot, 2003b). However, this hypothesis needs to be revised. As seen in the framework of routine diagnostics of wild boar samples from areas with C-strain emergency vaccination, the implementation of highly sensitive molecular techniques enables detection of limited virus replication, which previously could not be observed by the use of virus isolation or antigen ELISA. Likewise to the detection of C-strain vaccine virus in the scenario mentioned above, rRT-PCR assays were able to detect a limited challenge virus replication even in C-strain vaccinated animals (Blome et al., 2012a). The same is true for other live modified vaccines including CP7_E2alf. In vaccinated and challenged animals, detection of viral nucleic acid was not accompanied by detection of infectious virus or shedding and for this reason does not present a risk (Gabriel et al., 2012). The strong antibody response suggests that challenge virus is rapidly neutralized.

Another demand is protection against a broad range of viral variants. As discussed above, CP7_E2alf confers protection against recent field strains of genotypes 2.1 and 2.3 as well as a highly virulent strain of genotype 1.1. Moreover, an important parameter is induction of protection against vertical transmission. For CP7_E2alf, preliminary studies suggest that vaccination completely prevents vertical challenge virus transmission (Gábor Kulcsár, CAO-DVMP, Hungary, personal communication). Throughout the studies, safety and easiness of application could be shown.

Discussions are necessary in terms of consumer protection. CP7_E2alf was recently downgraded to S1 status (no risk for the environment, animals and humans) by the German central committee for biological safety (ZKBS) based on all available data. Nevertheless,

acceptance of a genetically engineered organism is still controversially discussed and the authorization process will require additional data.

A crucial criterion is reliable differentiation of infected from vaccinated animals (DIVA), accompanied by a reliable test system for disease surveillance and confirmation. The test system used in this framework is a commercially available competitive E^{rns} ELISA (de Smit, 2000) which was initially developed as discriminatory test for E2 subunit vaccines (PrioCHECK® E^{rns} ELISA, formerly Ceditest Marker®). During evaluation of this test system, reduced sensitivity and specifity compared to conventional E2-based antibody ELISAs was reported (Floegel-Niesmann, 2001). So far, the test system was optimized and evaluated in the framework of the presented duration of immunity study. Here, non of the sera collected before challenge infection scored false positive, and 90% of the surviving animals became positive on day 22 post challenge, albeit a wide range of inhibition values was observed (Gabriel et al., 2012). However, it must be stated that the CSFV E^{rns} glycoprotein is less immunogenic than the major immunogen E2 glycoprotein. Based on our rRT-PCR data, it can be suggested that the negative results may be due to a very low E^{rns} antibody response linked to almost no challenge virus replication (Gabriel et al., 2012). Therefore, negative results may be interpreted incorrectly as “false negative”. Concluding, the current discriminatory marker ELISA is suitable for a diagnosis on a herd basis. Attempts for further enhancement are ongoing (Gerard van de Wetering, Prionics Leylystad, personal communication), and additional test systems are under development.

Based on the available data, CP7_E2alf presents a vaccine candidate with outstanding efficacy and safety that is comparable to the “gold standard” C-strain. Nevertheless, for successful implementation, the CP7_E2alf vaccine needs to be embedded in a suitable control strategy.

5.2 African Swine Fever

5.2.1 Characterization of a recent Caucasian isolate in wild boar

Since 2007, ASF, a so far rather exotic disease with a CSF-like clinical picture, poses a threat to the EU and other free areas. After the introduction into Georgia and other Trans-Caucasian Countries, the disease is constantly spreading on the territory of the Russian Federation and affects both domestic pigs and wild boar. In northwestern direction, it already reached the regions of Murmansk, Saint Petersburg, and Karelia. Moreover, it also moved southward, reaching Iran in 2008, where it concerned the wild boar population (Rahimi et al., 2010). Especially the involvement of wild boar raises concern. As was seen with CSF, infected wild boar populations can seriously hamper disease control and act as source of infection for domestic pigs (Fritzemeier, 1998; Anonymous, 2010). Taking into account the high wild boar density in several EU Member States, especially Germany (the official hunting bag 2010/11 was 585.244 animals (Anonymous, 2012)), it is understandable that an ASF introduction would mean a worst case scenario for disease control. Without the option of vaccination, eradication could be almost unachievable. Although the involvement of tick vectors is unlikely for many regions, it could add to the complexity of the problem where it occurs.

Course and outcome of the infection would depend amongst other factors on the virulence of the ASFV strain involved and the clinical course seen in affected animals. In the past, European wild boar were involved in ASF outbreaks on the Iberian Peninsula and Sardinia, and a wide range of clinical courses of disease was observed. However, no data were available on disease dynamics in European wild boar caused by the recent Caucasian ASFV isolates.

Regarding the clinical course, we found an acute disease linked with 100% mortality (Gabriel et al., 2011). An age dependence, as it is often seen for CSF and several other infectious diseases of wild boar, could not be observed (Blome et al., 2012b). A field report from Iran confirmed these first findings (Rahimi et al., 2010). Based on these data, it seems unlikely that chronic infections or carrier states appear which could contribute to long-term persistence in affected populations. However, it is an acknowledged fact that monitoring and understanding a disease in an open ecosystem is rather a complex exercise. This is due to the high number of parameters that influence the system e.g. the population structure and dynamics, the population size, and their unknown immunological status (Anonymous, 2010).

Despite our findings and assumptions, outbreaks among wild boar keep occurring in the Russian Federation. This contrasts past experiences with CSF outbreaks in Europe in the 1960s where highly virulent CSFV strains in a wild boar population with lower density showed a self-limiting dynamic (Hone et al., 1992). However, in recent decades also CSF epidemics often turned into endemics. Here, CSFV isolates were of moderate virulence, and confronted an increasing density and population size of wild boar. Interaction of viral and host factors led to persistence in the European wild boar population (Lange et al., 2012). Similar findings are reported from ASF outbreaks on the Iberian Peninsula and Sardinia, where epidemics also turned to endemic situations (Pérez et al., 1998; Jori and Bastos, 2009; Giammarioli et al., 2011; Mur et al., 2012). However, the factors contributing to the change in behavior remain unclear. Factors could be the evolution of strains with altered sequence or virulence characteristics, but so far, the Caucasian ASFV proved to be stable both in terms of virulence and genome sequence (Alexander Malogolovkin, NRIVVaMR, Russia, personal communication). Other factors could be human interventions such as swill feeding, disposal of dead pigs in wild boar habitats and semi-wild pig rearing. In-detail evaluation of the current situation and its key players is needed for reliable risk assessment.

5.3 Conclusions and outlook

Implementation of a genetic DIVA strategy accompanying oral immunization campaigns of wild boar in Germany proved that the concept is both practicable and reliable. The concept can be easily transferred to emergency vaccination scenarios in domestic pigs. A similar approach could be developed for other live attenuated vaccines including conventional vaccine strains and new generation marker vaccines such as CP7_E2alf. For the latter, molecular techniques are already described and were used under laboratory conditions (Leifer et al., 2009a).

In several laboratory trials, CP7_E2alf was comparable to the "gold standard" C-strain. It combines its outstanding efficacy and safety with a reliable serological marker system that ensures feasibility for emergency vaccination campaigns not only in wild boar but also in domestic pigs. Council Directive 2001/89/EC includes the provision that products from marker vaccinated animals could be exempted from trade restriction. Thus, emergency vaccination becomes a true option in case of CSF outbreaks in countries with industrialized pig production. To obtain market authorization, further studies under laboratory and field conditions are still needed. Nevertheless, it seems feasible that CP7_E2alf will soon be commercially available.

African swine fever has become a serious threat to European pig farming and the wild boar population. Introduction through illegal import of pork products, transport vehicles that did not undergo proper disinfection, and contact to infected swill can occur at any time. Especially the lack of a vaccine would implicate disastrous consequences especially for affected wild boar populations. Although our studies confirmed very high virulence of the Caucasian ASFV isolate for European wild boar, the outcome of such an introduction depends on a vast number of interacting factors. In conclusion, research towards a vaccine against ASF, preferably also applicable for oral vaccination, is of paramount importance.

6 SUMMARY

Classical and African swine fever are highly contagious, notifiable viral diseases affecting different members of the *Suidae* family, both showing tremendous impact on animal health and pig production.

Optimization of CSF control strategies comprised two different approaches. In a first step, the current strategy of oral immunization of wild boar using a conventional C-strain vaccine was supplemented with the implementation of genetic DIVA using a recently developed multiplex rRT-PCR assay. This approach facilitates a rapid and reliable differentiation of field virus infected from C-strain vaccinated wild boar, and thus presents a promising tool also for emergency vaccination scenarios in domestic pigs. However, the use of conventional modified live vaccines like the C-strain does not allow serological differentiation of infected from vaccinated animals which is a prerequisite for modern disease control.

Therefore, the second part comprised the evaluation of the new generation marker vaccine CP7_E2alf to generate data for the authorization process. In the framework of the presented studies, innocuousness in calves, goats, lambs, and rabbits as well as safety in domestic pigs and wild boar was proven. Moreover, protection against lethal CSF challenge infection after oral and intramuscular vaccination of domestic pigs was investigated, and confirmed for a duration of at least 6 months. Furthermore, CP7_E2alf proved outstanding efficacy against challenge infection of domestic pigs and wild boar with a highly virulent CSFV strain of genotype 1.1 and an isolate of genotype 2.3 representing strains currently prevalent in Europe.

Candidate CP7_E2alf showed all virtues of a safe and efficacious marker vaccine against CSF that would be suitable for intramuscular vaccination of domestic pigs and oral vaccination of wild boar. For this reason, licensing of CP7_E2alf will be beneficial for future CSF control.

African swine fever, one of the most important differential diagnoses of CSF, is currently affecting domestic pigs and wild boar on the territory of the Russian Federation. So far, the outbreak situation could not be resolved and the disease constantly spreads towards the EU. Disease dynamics, especially regarding wild boar, are almost unknown. For this reason, circulating ASFV isolates were characterized for the first time in animal experiments in European wild boar of different age classes. As an exceptionally high virulence was observed in all age classes of animals, endemic situations driven by chronically infected animals or carriers seem unlikely.

7 ZUSAMMENFASSUNG

Sowohl die Klassische als auch die Afrikanische Schweinepest sind hoch ansteckende, anzeigepflichtige virale Erkrankungen der *Suidae*. Ausbrüche dieser Erkrankungen sind mit immensen Auswirkungen auf die Tiergesundheit und Schweineproduktion verbunden.

Die Optimierung der Kontrollstrategien gegen die KSP umfasste zwei verschiedene Herangehensweisen. In einem ersten Schritt wurde die gegenwärtige Strategie der oralen Immunisierung von Wildschweinen mit einer C-Stamm basierten Vakzine durch die Nutzung einer unlängst entwickelten multiplex rRT-PCR ergänzt. Dieser Ansatz ermöglicht eine schnelle und verlässliche Differenzierung zwischen Feldvirus-infizierten und C-Stamm geimpften Wildschweinen und stellt damit auch ein vielversprechendes Instrument für die Notimpfung von Hausschweinebeständen dar. Nichtsdestotrotz erlaubt der Einsatz konventioneller Lebendvakzinen wie des C-Stammes keine serologische Differenzierung infizierter und geimpfter Tiere, was eine Grundvoraussetzung moderner Impfkonzepte darstellt.

Aus diesem Grund wurde im zweiten Teil der Markerimpfstoff CP7_E2alf evaluiert, um Daten für den Lizensierungsprozess zu erheben. Im Rahmen der präsentierten Studien wurde sowohl die Unbedenklichkeit für Kälber, Ziegen, Schaflämmer und Kaninchen als auch die Sicherheit für Haus- und Wildschweine gezeigt. Es konnte zudem bestätigt werden, dass nach oraler und intramuskulärer Impfung von Hauschweinen ein mindestens 6-monatiger Schutz gegenüber einer letalen Belastungsinfektion besteht. Die hervorragende Schutzwirkung konnte sowohl für eine Belastungsinfektion mit einem hochvirulenten KSPV-Stamm vom Genotyp 1.1 als auch für eine Belastungsinfektion mit einem Isolat des Genotyps 2.3 gezeigt werden. Virusstämme dieses Genotyps sind zur Zeit in Europa vorherrschend und repräsentieren somit die Feldsituation. Der Impfstoffkandidat CP7_E2alf zeigte alle Vorzüge eines sicheren und effizienten KSP-Impfstoffes für die intramuskuläre Impfung von Haus- und die orale Impfung von Wildschweinen. Aus diesem Grunde wird die Lizensierung von CP7_E2alf für die zukünftige Kontrolle der KSP vorteilhaft sein.

Die ASP gehört zu den wichtigsten Differentialdiagnosen der KSP. Gegenwärtig sind von dieser Erkrankung sowohl Haus- als auch Wildschweine auf dem Gebiet der Russischen Föderation betroffen. Bisher ist es nicht gelungen, die Ausbrüche zu kontrollieren, und die Seuche breitet sich konstant aus und droht die EU zu erreichen. Die Dynamik der Erkrankung ist vor allem in der Schwarzwildpopulation weitgehend unbekannt. Daher wurden erstmalig die aktuellen ASPV-Isolate in Tierversuchen mit Europäischen Wildschweinen unterschiedlicher Altersklassen charakterisiert. Die außerordentlich hohe, vom Alter der Tiere unabhängige Virulenz, die im Rahmen dieser Untersuchungen gezeigt wurde, macht eine durch chronisch erkrankte bzw. persistierend infizierte Tiere entstehende Endemiesituation unwahrscheinlich.

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9 ABBREVIATIONS

ASF	African swine fever
ASFV	African swine fever virus
BDV	Border disease virus
BVBV	Bovine viral diarrhea virus
cDNA	Complementary deoxyribonucleic acid
C-strain	Chinese vaccine strain
CSF	Classical swine fever
CSFV	Classical swine fever virus
DIC	Disseminated intravascular coagulation
DISC	Defective in second cycle
DIVA	Differentiation of infected from vaccinated animals
DNA	Deoxyribonucleic acid
EFSA	European Food Safety Authority
ELISA	Enzyme-linked immunosorbent assay
EMA	European Medicines Agency
EU	European Union
FAT	Fluorescent antibody test
GCP	Good Clinical Practice
GLP	Good Laboratory Practice
GM-CSF	Granulocyte macrophage colony-stimulating factor
GPE^-	Japanese guinea-pig exaltation-negative strain
HAT	Hemadsorption test
HCLV	Chinese hog cholera lapinized virus
IFT	Immunofluorescence test
IL	Interleukin
IPT	Immunoperoxidase test
LAMP	Loop-mediated isothermal amplification
LPC	Lapinized Philippines Coronel strain
mpV	Mono-peptide vaccine
mPV	Multi-peptide vaccine
NS	Non-structural protein

NT	Neutralization test
NTR	Non-translated region
OIE	*Office International des Épizooties*, World Organisation for Animal Health
ORF	Open reading frame
PAF	Platelet-activating factor
PBMC	Peripheral blood mononuclear cell
PriProET	Primer-probe energy transfer
RNA	Ribonucleic acid
rRT-PCR	Real-time reverse transcription polymerase chain reaction
RT-LAMP	Reverse transcription loop-mediated isothermal amplification
RT-PCR	Reverse transcription polymerase chain reaction
spp.	*Species pluralis*, several species
TNF-α	Tumor necrosis factor-alpha
USA	United States of America
VP	Virion protein
ZKBS	Zentrale Kommission für die biologische Sicherheit, German central committee for biological safety

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