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Design and validation of optimized tools for Classical and African swine fever surveillance and pathogenesis research

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For my family

1 INTRODUCTION

Classical and African swine fever belong to the most important contagious diseases of pigs worldwide and are notifiable to the World Organization for Animal Health (OIE). While outbreaks of classical swine fever (CSF) have a long and ongoing history in Europe, up to now African swine fever (ASF) was considered an exotic disease within EU Member States. However, very recently the disease has been introduced into the European wild boar and domestic pig population in Poland, Lithuania, Latvia, and Estland. Hence, both diseases pose a high risk to the whole European pig industry and wildlife.

Regardless of very similar clinical pictures that are not discriminable without laboratory diagnosis, the causative agents differ greatly. Classical swine fever is caused by a small enveloped positive single-stranded RNA virus belonging to the genus *Pestivirus* within the *Flaviviridae* family. The causative agent of ASF is a complex DNA virus of the genus *Asfivirus* within the *Asfarviridae* family representing the only DNA arthropod-borne (ARBO) virus.

Classical swine fever virus (CSFV) isolates of recent European outbreaks are characterized by their moderate virulence. The clinical picture can range from an almost inapparent infection to a lethal hemorrhagic fever like illness. High variability in disease course and outcome are a challenge for both disease surveillance and pathogenetic research. Although several studies aimed to analyze basic pathogenetic mechanisms, responsible factors have never been elucidated entirely. While on the host's side, age and immune status are acknowleged parameters, the virulence of the isolate seems to be decisive on the agent's side. Moreover, the influence of the genetic background of the host has been discussed. To define host responses linked to different disease courses and outcome, a first animal trial was conducted with a moderately virulent CSFV strain and different pig breeds including European wild boar. In a second trial, the impact of the age was revisited in combination with the assessment of tools for active swine fever surveillance.

Dysregulation of immune responses, especially cytokine reactions, seems to play a crucial role in CSF pathogenesis. Up to now, there has been a serious lack of appropriate and reliable tools for cytokine gene expression analyses, especially in pigs. To overcome this shortcoming, a harmonized TaqMan-based RT-qPCR protocol for the detection of seven swine fever relevant cytokines was developed and fully validated. This assay is now available for future studies and could be implemented also for other swine diseases including ASF.

Beyond the studies focusing on underlying mechanisms, diagnostic approaches for active and passive disease surveillance in wild boar have been targeted. Sample submission is usually the bottleneck of wildlife surveillance under field conditions, even in times of increased risk. Pragmatic approaches for sampling and transport could improve the compliance of hunters. In view of the fact that an introduction of ASF and CSF is usually accompanied by an increased mortality, animals found dead have to be sampled even if they are already in various stages of decay. To this means, a dry-/ semi-dry blood swab method was implemented enabling an easy handling under field and laboratory conditions. Moreover, a simple approach for sampling of live animals for active surveillance, i.e. a “rope-in-a-bait” method, was evaluated with respect to the frequently observed subclinical CSF forms in older animals.

2 LITERATURE REVIEW

2.1 Classical Swine Fever

2.1.1 Virus taxonomy, morphology, global distribution and economic impact

The causative agent of classical swine fever (CSF) is Classical swine fever virus (CSFV) which belongs to the genus *Pestivirus* within the *Flaviviridae* family (Lindenbach, 2013). It represents a small, enveloped, positive single-stranded RNA virus (Horzinek, 1967; Lindenbach, 2013; Meyers, 1989; Moennig, 1992).

The RNA genome consists of approximately 12.3 kb and includes one large open reading frame (ORF) flanked by two non-translated regions (NTRs) (Collett, 1992; Rümenapf et al., 1991a). Virus particles are composed of eleven viral proteins comprising four structural and seven non-structural (NS) proteins. In detail, the core (C) protein along with three envelope glycopreoteins (E1, E2, and Erns) constitutes the virion, and Npro, p7, NS2-3, NS4A, NS4B, NS5A, and NS5B are NS proteins (Elbers et al., 1996; Lattwein et al., 2012; Thiel et al., 1991).

CSFV strains can be assigned to three distinct genotypes with three to four subtypes (Paton et al., 2000; Postel et al., 2012). This classification is based on the nucleotide sequences of fragments of the 5′-non-translated region (5′-NTR), and of the region encoding the glycoprotein E2 (Greiser-Wilke et al., 2006; Paton et al., 2000). Due to the fact that the appearance of different subtypes is often linked to particular geographical distributions, genetic typing enables to trace disease spread and outbreak dynamics (Depner et al., 2006; Paton et al., 2000).

Classical swine fever virus is prevalent worldwide except for Australia, Canada, the United States and most of the EU Member States in which eradication programs have been successfully implemented. While the current situation in Africa remains unknown (apart from reported cases in Madagascar and South Africa) (Penrith et al., 2011; Sandvik et al., 2005), CSFV is prevalent in Central and South America, the Carribbean and many parts of Asia (Moennig, 2008). Although a decreasing number of outbreaks was reported during the last decade (Postel et al., 2013), CSFV keeps reoccurring also in several European countries in domestic pigs and wild boar (Blome et al., 2010; Edwards et al., 2000; Floegel-Niesmann et al., 2009; Leifer et al., 2010; Moennig, 2008; Pol et al., 2008). Especially the South-Eastern European countries are affected although clear reports are rare (Edwards et al., 2000; Vargas

Teran et al., 2004). However, recent outbreaks in Lithuania and Latvia demonstrate the permanent risk of reintroduction (Postel et al., 2013). Interestingly, current CSF restriction zones in Latvia overlap with ASF outbreaks (Animal Disease Notification System, ADNS, visited September 11th 2014).

Over the last two decades, most outbreaks among European domestic pigs and wild boar were caused by moderately virulent strains of genotype 2, especially subtype 2.3 (Bartak and Greiser-Wilke, 2000; Biagetti et al., 2001; Blome et al., 2010; Depner et al., 2006; Leifer et al., 2010). The most recent CSFV occurrence in Germany in 2009 was caused by the strain "Roesrath" of genotype 2.3. This isolate, found in North Rhine-Westphalia, Germany, originated from a wild boar piglet. Wild boar get usually infected through contaminated food and may act as important reservoir for CSFV. Large wild boar population sizes (as present in Germany and other European countries) may facilitate virus persistence over a long time (Moennig, 2000; Penrith et al., 2011). In several European countries, CSFV occurrence in wild boar even reaches an endemic state. Because of the permanent risk to spill over into the domestic pig population, CSFV infections in wild boar represent a constant threat to the whole domestic pig industry and wildlife. Moreover, expensive control-measures are required in cases of disease occurence resulting in tremendous socio-economic damage. To state an example, the fatality of financial losses had been revealed during the CSFV epidemic in The Netherlands in 1997/1998 (Stegeman et al., 2000). The related control measures included the slaughtering of more than 11 million pigs and incurred expenses were estimated at about US $ 2 billion (Stegeman et al., 2000; Terpstra and de Smit, 2000).

The officially confirmed global CSF situation from January 2013 to August 2014 is illustrated in the disease distribution map according to the OIE in Fig. 1 (available on the OIE website http://www.oie.int).

Figure 1: CSFV outbreak distribution in domestic pigs and wild boar; reporting period: January 2013 to August 2014.

2.1.2 Epidemiology, disease surveillance and control strategies

Members of the *Suidae* family, i.e. domestic pigs and European wild boar, but also Common Warthogs (*Phacochoerus africanus*) and bushpigs (*Potamocherus larvatus*) are susceptible hosts (Blacksell et al., 2006; Depner et al., 1995; Everett et al., 2011).

CSF can be transmitted directly by inhaling or ingesting the virus, indirectly through fomites contaminated with CSFV (e.g. vehicles, equipment, clothing) or by treatment with contaminated medicinal equipment (van Oirschot, 1999).

Apart from horizontal transmission, a vertical infection to fetuses via the placenta is possible in infected sows throughout all stages of gestation. If infection occurs during the first weeks of pregnancy, persistently infected piglets may be born and develop the late-onset form of CSF (Moennig, 2000; van Oirschot, 1999). Similarly to chronically infected pigs, these animals shed virus constantly over the whole course and by that can have a high epidemiological impact (van Oirschot, 2004). Another epidemiological risk arises from the fact that CSFV may persist in uncooked meat for long periods. This can be crucial in terms of illegal feeding of e.g. smuggled meat or swill (Wooldridge et al., 2006). Further risk factors for viral spread over long distances include movements of people between herds and transport of swine during the incubation time (for slaughtering, sale or breeding). Besides, local spreads through neighbourhood infections may also contribute to CSFV dissemination. These most likely occur when farm densities are high and distances between them are small (Boender et al., 2007).

Because of the potential to spread over long distances and cause outbreaks in areas where CSF was eradicated, e.g. CSF outbreaks in South Africa, The Netherlands, Great Britain, and France (Sandvik et al., 2005; Simon et al., 2013; Widjojoatmodjo et al., 1999), disease surveillance is not only required for endemic areas. Only timely detection and intervention can lower the impact on both, pig industry and wildlife (De la Torre et al., 2013) and prove that the disease is not hiding within the domestic pig or wild boar population. This also applies for ASF.

For a successful eradication, cost-effective control strategies adjusted to local conditions and pig production systems are necessary. Backyard holdings, like the ones in South-Eastern Europe, represent an epidemiological risk by allowing contact between both, domestic pigs and wild boar and therefore provide ideal preconditions for virus introduction and transmission (Blome et al., 2010). Due to implementation of modern pig production systems in Western European countries the risk of CSFV introduction has been significantly reduced.

However, during the last years CSFV was reported sporadically also in these countries which are otherwise free from CSFV (Germany, Italy, France). In more than two-thirds of cases disease outbreaks were associated with contacts between domestic pigs and wild boar (Fritzemeier et al., 2000; Laddomada, 2000). Therefore samplings from wild boar are of utmost importance for successfull disease surveillance.

Serological (Rossi et al., 2005) and virological monitoring systems were implemented using either blood or organ samples (Mintiens et al., 2005). A scientific opinion issued by the European Commission (Scientific Opinion XXIV/B3/R09/1999) recommends a multi-faceted approach to control and eradicate CSF in wild boar population comprising the reduction of population density, intensive diagnosis and good hygiene practice during hunting. In addition to the reduction of wild boar population, an oral vaccination strategy of wild boar using the modified live C-Strain vaccine was implemented (Kaden and Lange, 2001; Kaden et al., 2000). Inclusion of vaccination can limit costs and virus dissemination (van Oirschot, 2003). However, the major disadvantage of conventional modified live vaccines are massive trade restrictions (Terpstra and de Smit, 2000). Therefore, marker vaccines able to differentiate infected from vaccinated animals (DIVA) were developed comprising the commercial available E2 subunit marker vaccine (Beer et al., 2007; Moormann et al., 2000) and the recently developed marker vaccine candidate CP7_E2alf (Reimann et al., 2004). The latter was recently shown to be equally protective as the C-Strain vaccine (Blome et al., 2012; Blome et al., 2014b; Eble et al., 2012; Gabriel et al., 2012; König et al., 2011). However, for rapid eradication a strict stamping out policy is widely conducted including culling of infected pigs, in-contact herds, movement restrictions, non-hazardous removal of carcasses as well as the establishment of protection and surveillance zones (Garner et al., 2001; Greiser-Wilke and Moennig, 2004). These measures are laid down in the Council Directive 2001/89/EC and Commission Decision 2002/106/EC. Although a prophylactic vaccination is prohibited an emergency vaccination is a legal tool for limiting CSFV spread. However, in order to avoid trade restrictions emergency vaccination was conducted only in Romania so far (Anonymous, 2006).

2.1.3 Clinical courses, signs and lesions

The clinical picture of CSF is highly variable, ranging from an almost inapparent infection to a hemorrhagic fever like illness (Moennig, 2000). In general, peracute, acute, chronic and prenatal disease courses can be distinguished. The peracute form is characterized by a short period of high fever and a rapid death without the development of CSFV specific signs (Dunne, 1970).

Acute courses may either lead to death (acute-lethal) or to full recovery including production of neutralizing antibodies and complete virus clearance (acute-transient). Severe acute courses usually start with high fever, anorexia, huddling, conjunctivitis, respiratory and gastro-intestinal signs (constipation followed by diarrhea) as well as general depression. Secondary infections mostly affect the respiratory and gastrointestinal tract as a result of leukopenia and thrombocytopenia. Pigs showing an acute-lethal disease course may additionally develop hemorrhagic lesions including petechiae and ecchymoses as well as cyanosis and central nervous disorders (staggering gait, incoordination, convulsions) (Moennig et al., 2003). These signs mostly occur between 14 to 21 days after infection. Hemorrhages of the skin typically appear on ears, abdomen, anogenital region, tail and the inner side of the limbs (Moennig et al., 2003). In cases of acute-lethal courses pigs often die within 10 to 20 days after infection (Blome et al., 2006). In contrast, clinical signs during acute-transient forms are mild and often not indicative for CSF (atypical course of infection). These forms usually result in a complete convalescence (Depner, 2006).

The chronic course is accompanied by non-specific symptoms including recurrent fever, loss of body weight and growth retardation due to anorexia and chronic enteritis. However, during the initial phase after infection signs may resemble the acute forms (Moennig et al., 2003). As a result of general immune suppression, secondary infections are frequently observed. After several month of alternating acute clinical periods and general improvements all chronically infected animals succumb to infection (Moennig et al., 2003; van Oirschot, 1999).

Transplacentarly infections of the fetus result in a prenatal disease course. While pregnant sows only develop mild or subclinical symptoms the effect in fetuses is dependent on the time of gestation. CSFV-infections during the very early phase of pregnancy may result in abortions, stillbirth, mummification and malformations. In cases of transplacental infections between day 50 and 70, immunotolerant and persistently infected piglets are born. Although this infection may be inapparent for month, these animals shed virus continuously until finally developing the late-onset form and die. Characteristic clinical signs comprise growth

retardation, loss of body weight, conjunctivitis, dermatitis, diarrhea and occasionally congenital tremor (Kleiboeker, 2002).

The pathological signs of acute CSF forms include hemorrhagic symptoms (petechiae, ecchymoses) in numerous organs, lesions within the lymphoreticular system and infarcts of the spleen (Kleiboeker, 2002; Moennig et al., 2003; van Oirschot, 1999). Furthermore, an inflammation in different stages of severity affecting the gastro-intestinal tract, particularly the ileum, ileocaecal valve and colon (from catarrhal to necrotic and ulcerative lesions), may occur. Bacterial secondary infections commonly cause inflammations at the tonsil and the respiratory tract (Kleiboeker, 2002). In addition, a non-purulent meningoencephalitis may develop (Gruber et al., 1995).

In contrast to the acute form, pathological findings are less pronounced in cases of chronic CSF (Kleiboeker, 2002). Here, atrophy of the thymus and symptoms of the gastro-intestinal tract including necrosis and ulceration of the ileum, the ileocaecal valve and the colon are characteristic signs (Moennig et al., 2003; van Oirschot, 1999). Haematological changes upon CSFV infection comprise leukopenia, thrombocytopenia and in certain cases also anaemia (Thiel, 1996; Trautwein, 1988).

2.1.4 Pathogenesis and immune response

Upon oronasal CSFV infection, primary replication takes place in tonsils and other local lymphoreticular tissues. Subsequently, virus progeny reaches regional lymph nodes via lymphatic vessels and enter the blood circulation (Dunne, 1973; Liess, 1987; Ressang, 1973). Thereafter, virus is disseminated into the spleen, bone marrow, visceral lymph nodes, intestinal lymphatic mesh, and other parenchymatos organs.

Thereby, viral replication takes place within immune cells, particularly monocytes, macrophages and dendritic cells, which represent primary target cells (Carrasco et al., 2004; Gomez-Villamandos et al., 2001; Moennig, 2000). Furthermore, endothelial and epithelial cells can get initially infected (Ressang, 1973; Trautwein, 1988) until during later disease stages almost every cell type is susceptible towards CSFV (Knoetig et al., 1999).

After an incubation time of 3 to 10 days a clinical onset with highly varying stages of severity may occur (Moennig, 2000). Virus shedding already begins prior to occurence of clinical signs (de Smit et al., 1999; Floegel et al., 2000; Moennig, 2008; van Oirschot, 1999) and sustains until development of antibodies or death. CSFV is excreted in large amounts via saliva and also, to a lesser extent, in urine, faeces, semen as well as ocular and nasal secretions (Pasick, 2008; Ressang, 1973; van Oirschot, 1999).

CSFV infection may either lead to peracute death or result in complete convalescence. Several factors from both, the virus' and the host's side, has been discussed to influence the clinical picture and outcome (Depner et al., 1997; Depner et al., 1995; Kaden et al., 2004). On the agent's side, the virulence of the CSFV isolate seems to play the major role and, to a lesser extent, the dose and route of infection (Kaden et al., 2004). It was reported that moderately or low virulent strains of wild boar origin may lead to a more severe disease course or higher viral loads when introduced into domestic pigs (Kaden et al., 2000; Kaden et al., 1999). On the host's side, age and immune status are assumed to play the key role (Depner et al., 1997; Depner et al., 1995; Kaden et al., 2004; Moennig et al., 2003). In addition, genetically variances leading to distinct innate antiviral immune responses in different breeds or races were suggested to influence the disease severity (Blacksell et al., 2006; Depner et al., 1997). However, the entire influences of host factors are still far from being understood. Neither beneficial nor detrimental reaction pattern have been clearly defined to date.

Serious courses predominate in young pigs while the clinical pictures in adult swine are usually mild or even subclinical (Moennig et al., 2003). Thus, a peracute disease course occurs when highly virulent CSFV isolates infect young piglets (Dunne, 1970). Acute-lethal disease forms with severe clinical signs mainly develop in weaner pigs and young fattening pigs after infection with moderately to highly virulent strains (Moennig et al., 2003). Transient or subacute infections mostly appear in combination of low virulent isolates with an increasing age of the hosts (Depner, 2006) and chronic CSF forms only develop when effective immune responses have failed to establish (Moennig et al., 2003; Moennig, 2008).

In general, secondary or concomitant infections are characteristic for CSF. They occur in consequence of a severe immunosuppression promoted by the depletion of both, B- and T-lymphocytes (Summerfield et al., 2001). Immunosuppressive events were associated with the relatively late humoral and cellular immune response which is typical for CSF (Thiel, 1996; Trautwein, 1988).

In detail, a short-term leukocytosis occurs very early after infection followed by a leukopenia, particularly affecting lymphocytes (B- and T-cells) (Gomez-Villamandos et al., 2000; Lee et al., 1999; Markowska-Daniel et al., 1999; Pauly et al., 1998; Sato et al., 2000; Summerfield et al., 1998; Susa et al., 1992). Thereby, T-cell depletion developes rapidly after infection while B-lymphocytes are depleted in later disease stages (Susa et al., 1992). The kinetics of T-cell depletion are dependent on the cell subset and the virulence of the isolate. While αβ-T-lymphocytes are generally depleted irrespective of the virulence, γδ-T-cells are mainly reduced after highly virulent infections (Lee et al., 1999; Summerfield et al., 2000; Summerfield et al., 2001). In comparison to highly virulent strains, a delay of this kind of kinetic was reported upon infection with low virulent isolates (Markowska-Daniel et al., 1999; Summerfield et al., 1998). However, this difference may only be observed during early stages of disease. In advanced stages severe lymphocyte depletions occur irrespective of the virulence and therefore even develope in absence of severe clinical signs (Summerfield et al., 2001).

The lymphocyte depletion during CSF is caused by lymphocyte apoptosis, which is more correlated to virus-host interactions and the release of immunological mediators than to direct virus-associated cell damage (Summerfield et al., 2001). Several proinflammatory cytokines including Interleukin (IL)-1α, IL-1β, TNF-α, IL-6 and in addition Interferon (IFN)-α were suggested to induce apoptosis in lymphocytes or other leukocytes during CSF (Choi et al., 2004; Nunez et al., 2005; Sanchez-Cordon et al., 2002; Summerfield et al., 2006).

Apart from apoptosis, a redistribution of lymphocytes from peripheral blood to lymphoid tissues due to local inflammatory reactions may also be involved in decreasing peripheral lymphocyte counts (Knoetig et al., 1999).

Clinical signs occurring in acute-lethal forms may resemble viral haemorrhagic fevers which are also caused by members of the *Arenaviridae*, *Bunyaviridae*, *Togaviridae* and *Filoviridae* (Bray, 2005; Gomez-Villamandos et al., 2003; Lange et al., 2011; Mahanty and Bray, 2004). The underlying pathogenetic mechanism has not been clearly defined up to now. An immuno-pathogenesis including a dysregulated cytokine release, which is presumed to play a key role in pathogenesis of other viral haemorrhagic fevers was also suggested for CSF (Bray, 2005; Lange et al., 2011; Mahanty and Bray, 2004). A close correlation between viral infection, inflammation process and coagulation dysfunctions resulting in haemorrhagic lesions was reported (Lange et al., 2011; Moennig et al., 2003).
In detail, an overexpression of the proinflammatory cytokines IL-1α, IL-6, and IL-8, along with pro-coagulation factors including tissue factor (TF), vascular endothelial cell growth factor (VEGF), E-selectin and other factors may lead to coagulation dysfunctions by activating platelets and endothelial cells and in addition, increase vascular permeability and vasodilatation (Bensaude et al., 2004; Lange et al., 2011). Further suggested cytokines are IL-10, IL-12, and IFN-γ (Jamin et al., 2008). As IL-10 and IFN-γ were reported to play a role during filoviral haemorrhagic fevers (Bray and Geisbert, 2005; Mahanty and Bray, 2004), a similar role in the pathogenesis of CSF could be assumed.
In cases of complete convalescence, protective immunological responses were successful. Upon CSFV infection protection is mediated by both, humoral and cellular-mediated immune response. However, a stronger focus on the cellular level was shown, especially concerning mediation of early protection (Piriou et al., 2003; Sanchez-Cordon et al., 2005).

With regard to the humoral response, quantitative and qualitative changes in B-lymphocytes and immunoglobulins (IgM-positive and IgG-positive) occur upon CSFV-infection (Sanchez-Cordon et al., 2006). In lymphoid organs an early increase of B-cells including plasma cells was detected. In detail, IgM^+ cells were shown to rise from day seven post infection (dpi) and IgG^+ cells increase from 11 dpi onwards (Urbaneck, 1987) until virus-specific neutralizing antibodies occur between 10 to 21 dpi in the peripheral blood (Piriou et al., 2003).
The B-cell increase as well as their differentiation into immunoglobulin-producing plasma cells require stimulatory signals mediated through several cytokines, including IL-4, IFN-γ,

and IL-2, released by activated monocytes/macrophages and T-cells following CSFV infection (Clark, 1994; Nunez et al., 2005; Parker, 1993; Sanchez-Cordon et al., 2005; van Miert, 1995). This differentiation mechanism was suggested to be dependent on an increased IL-4 level secreted by T-cells, an eventual predominance of IL-4 over IL-2, and a late decrease of IFN-γ (Sanchez-Cordon et al., 2005; Sanchez-Cordon et al., 2006). The delayed occurrence of neutralizing antibodies may be explained by this relatively late change from cell-mediated to humoral immune response, which is characteristic for CSFV infection. Concordantly to the late development of antibodies, IgG^+ cell increase occurs in advanced disease stages until they outnumber the initially predominant IgM^+ cells (Depner, 1994; Laevens et al., 1999; Piriou et al., 2003). The protective value of neutralizing antibodies against CSFV infection was demonstrated *in vivo* and *in vitro* (Terpstra and Wensvoort, 1988).

CSFV-specific antibodies are primarily targeted against the envelope glycoprotein E2 which is known to be the major immunogen of pestiviruses and additionally targeted by cytotoxic T-cells (Ceppi et al., 2005; Risatti et al., 2005; Risatti et al., 2006; Risatti et al., 2007). The envelope glycoprotein E^{rns} represents an additional antibody target and is moreover important for host adaption (Konig et al., 1995; Weiland et al., 1992; Windisch et al., 1996).

Upon CSFV infection, the cell-mediated immune response is primarily focused on the early and middle stages of disease (Sanchez-Cordon et al., 2005). This response includes quantitative changes in T-lymphocyte populations comprising cytotoxic T-cells (CTL; $CD4^-CD8^+$), T-helper cells ($CD4^+CD8^-$), and mature T-cells ($CD3^+$) (Arnaud et al., 1996; Kearse et al., 1995; Roitt, 1998) along with qualitative changes in cytokine expressions by T-cells (IFN-γ, IL-2, IL-4) (Suradhat et al., 2001).

With regard to the kinetic of T-cell response, an initial activation of cytotoxic T-cells (CD4-CD8+ cells) and T-helper cells (CD4+CD8- cells) was shown shortly after infection (Narita et al., 1996; Piriou et al., 2003; Sanchez-Cordon et al., 2005). During the further disease course a focus on cytotoxic T-cell response was suggested (Lee et al., 1999; Narita et al., 2000; Pauly et al., 1998) which may contribute to the defense mechanisms against CSFV-infection (Doherty, 1992; Pauly et al., 1995; Summerfield et al., 1996). The defending ability of T-cells was demonstrated in cases of full protection towards CSFV despite neutralizing antibodies were absent (Rümenapf et al., 1991b; Suradhat et al., 2001).

In addition, an activation of memory T-cells ($CD4^+CD8^+$) was observed during disease course (Summerfield et al., 1996; Zuckermann and Husmann, 1996).

2.2 African Swine Fever

2.2.1 Virus taxonomy, global distribution, control strategies and economic impact

The causative agent of ASF is African swine fever virus (ASFV) of the genus *Asfivirus* within the *Asfarviridae* family. African swine fever virus represents a large, complex, double-stranded DNA virus (Takamatsu, 2011) and is the only known DNA virus which can be transmitted by arthropods (ARBO-virus = arthropod borne virus). Soft ticks of the genus *Ornithodoros* (Kleiboeker and Scoles, 2001) may serve as potential vector, in particular *Ornithodoros erraticus* which was identified in Spain and Portugal, and the *Ornithodoros moubata* complex in Africa. So far, 22 main ASFV genotypes have been identified; some of them with haemadsorbing abilities (Bastos et al., 2003; Gallardo et al., 2009).

As ASF had been considered as an exotic disease until recently by especially impairing pig productions considerably in Africa (Penrith and Vosloo, 2009), ASFV was lately introduced into the European wild boar and domestic pig population including outbreaks within EU Member States (OIE, 2014).

Before ASFV spread across Europe, epidemics and repeating outbreaks were mostly restricted to several African countries of the sub-Saharan region including Madagascar and the Iberian Peninsula. Apart from the long-lasting ASFV presence in Africa, the endemic situation in Sardinia, Italy, in domestic pig and wild boar population has retained since 1978. Outside of Sardinia, ASFV outbreaks in Europe had just occurred occasionally and were eradicated eventually (Costard et al., 2013; Giammarioli et al., 2011; Laddomada et al., 1994).

Then, ASFV was introduced in Georgia in 2007 (Chapman et al., 2011) from where the virus spread throughout the Trans-Caucasian Countries, the Islamic Republic of Iran (Rahimi et al., 2010) and the Russian Federation (Khomenko et al., 2013). In the latter, ASFV has become endemic since 2008 and still continues to spread (Khomenko et al., 2013). Subsequently, ASFV expanded its geographical distribution into other Eastern European countries (e.g. Armenia, Azerbaijan, Ukraine) (Khomenko et al., 2013) by affecting both, wild boar and the domestic pigs. Recent outbreaks were affirmed in Belarus, Latvia, Lithuanian and Poland (WAHID, status report as of July 29th 2014) where the disease has not been controlled until now. Current speads are illustrated in the ASFV distribution map in Fig. 2. ASFV isolates of genotype 2 were identified to be responsible for the current spread across Europe (Gallardo et al., 2009; Rowlands et al., 2008).

Due to the fact that neither a vaccine nor a treatment against ASFV is available, the disease represents a serious problem in many countries by causing tremendous economic losses

through extensive culling. To give an example, overall losses due to ASF were estimated at 30 billion RUB or 1 billion USD through culling of more than 600.000 pigs from 2007 to mid-2012 (Khomenko et al., 2013) in the Russian Federation where all sectors of pig industry and production have been affected until now (Gogin et al., 2013; Khomenko et al., 2013). Nevertheless, outbreaks have to be controlled by a stamping out policy including the implementation of movement restrictions of swine and their products (Khomenko et al., 2013). In addition, eradication programs are further complicated by the fact that control measures are difficult to implement in mostly affected areas where equipment of veterinary services and personnel training is insufficient. Moreover, the compliance of the population is often low because of the scarceness in food through culling measures which often concerns the poorest households and farmers (Khomenko et al., 2013).

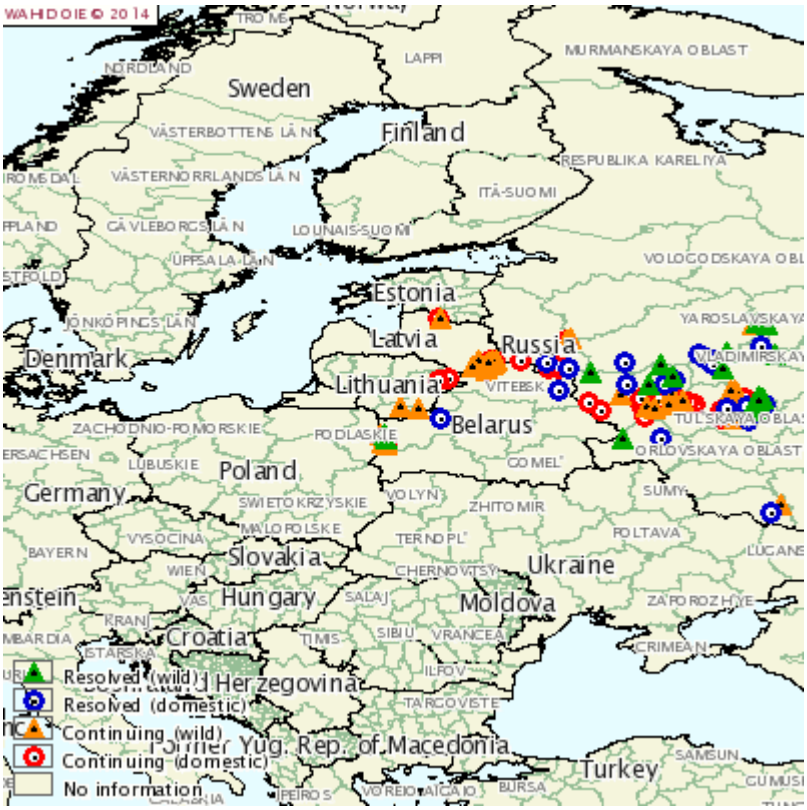

Figure 2: ASFV outbreak distribution in domestic pigs and wild boar; reporting period: January 2013 to August 2014.

2.2.2 Epidemiology and risk factors

ASFV can infect domestic pigs and different wild *Suidae*. In addition, soft-tick species can act as non-vertebral hosts and vectors under special conditions. Moreover, stable-flies (*Stomoxys*

spp.) as well as probably other blood-suckling insects may serve as mechanical vectors (Mellor, 1987). While the infection of domestic pigs is likely to cause a clinical disease, milder forms or asymptomatic carrier states may occur in feral pigs, especially in African wild suids (Costard et al., 2013). Susceptible wild suids include warthogs *(Phacochoerus africanus),* bushpigs (*Potachoerus larvatus*), red river hogs (*Potamochoerus porcus*) and Giant Forest hogs *(Hylochoerus meinertzhangi).* Amongst them, the widely distributed Warthogs are considered to be the original and most important vertebrate ASFV reservoir in Africa and therefore playing a major epidemiological role (Anderson et al., 1998; Jori and Bastos, 2009; Plowright et al., 1969; Thomson, 1985). Soft ticks (*O.moubata*) display another virus reservoir in Africa which may retain and transmit ASFV over long periods (Plowright et al., 1969). Apart from direct transmissions, transstadional, transovarial, and sexual transmissions are possible enabling a viral persistence for up to 15 month even in absence of susceptible hosts (Plowright et al., 1974; Plowright et al., 1970; Rennie et al., 2001). Like *O.moubata* in Africa, *O.erraticus* was considered as biological vector and reservoir in Spain.

In contrast to warthogs, which remain clinically inapparent, in Europe, wild boar and feral pigs were shown to be equally susceptible towards ASFV as domestic pigs (Jori and Bastos, 2009; McVicar et al., 1981). For virus maintenance in wild boar populations a direct contact to domestic pigs or other infectious sources seem to be necessary. Otherwise ASFV outbreaks in wild boar would be self-limiting (Laddomada et al., 1994; Mur et al., 2012a; Perez et al., 1998; Ruiz-Fons et al., 2008). However, disease occurrence in wild boar is always accompanied by the risk to spill over into the domestic pig population (Costard et al., 2013). Upon an introduction of the highly contagious ASFV to previously free areas the disease spreads rapidly by showing morbidities up to 100 % and high mortality rates (Blome et al., 2013). In contrast, in endemic regions in which chronic or subclinical forms were reported to occur more often, mortality rates may be lower (Allaway et al., 1995; Fasina et al., 2010; Owolodun et al., 2010; Thomson, 1985). In these areas, chronically infected or clinically recovered pigs may play a role in disease persistence and pose a risk for sporadic outbreaks or spreading into disease-free areas (Allaway et al., 1995; Boinas et al., 2004; Sanchez-Vizcaino et al., 2012).

The complexity of ASF epidemiology is underlined by the existence of several transmission cycles, namely the sylvatic cycle, the domestic cycle, the transmissions from sylvatic to domestic pigs or back as well as the tick-pig-cycle. In Europe, the domestic cycle plays the major epidemiological role. It is restricted to domestic pigs and includes the transmission either directly via contact between susceptible swine or indirectly via contaminated fomites

(clothing, equipment, vehicles) (Mur et al., 2012c; Plowright, 1994; Sanchez-Vizcaino et al., 2012) as well as the ingestion of contaminated meats and products (Farez and Morley, 1997). The sylvatic cycle occurs exclusively in Africa and involves warthogs and ticks of the *O.moubata* complex (Plowright, 1994; Plowright et al., 1969; Thomson, 1985). Briefly, it comprises the infection of suckling warthogs via ticks (*O.moubata*) which is necessary for virus maintenance as a vertical and horizontal transmission between warthogs is not possible (Thomson, 1985). Other tick-pig cycles were identified in Africa, the Iberian Peninsula and also in some areas of Spain by involving *O.erraticus* (Caiado et al., 1988; Haresnape and Mamu, 1986; Haresnape and Wilkinson, 1989; Haresnape et al., 1988; Perez-Sanchez, 1994).
A particular contribution to the potential risk of local and international spread is displayed by pig trades/movements as well as a lack of biosecurity practices in endemic areas (Costard et al., 2013). In these regions, backyard holdings are often predominating and disease control measures may be implemented only poorly. Due to the fact that ASFV remains viable and infectious not only in blood but also in porcine tissues for long periods the access of pigs to carcasses, frozen or insufficiently cooked or pured meat represents a further risk of infection (Farez and Morley, 1997). These risk factors may not only lead to persistence within one region but also to a spread across Europe (Costard et al., 2013; Mur et al., 2012b; Mur et al., 2012c; Sanchez-Vizcaino et al., 2012; Wieland et al., 2011).

2.2.3 Clinical courses, signs and lesions

Similarly to CSF, ASFV can cause a wide range of clinical syndromes in both, domestic pigs and wild boar, ranging from peracute to almost inapparent clinical forms. Concordantly, highly variable pathomorphological findings may be observed (Blome et al., 2013; Kleiboeker, 2002; Penrith and Vosloo, 2009; Takamatsu, 2011).

The incubation period varies from 2 to 7 (in rare cases up to 14) days (Mebus, 1988). The peracute course is characterized by a sudden death of pigs without obvious clinical signs (Kleiboeker, 2002). Clinical symptoms during acute disease forms typically comprise initial high fever, severe depression, anorexia, reddened skin at the acra (ears, tail, distal extremities, ventral areas of chest and abdomen), and conjunctivitis. Furthermore, respiratory findings, tachypnoe, tachycardia and cyanosis as well as gastro-intestinal signs like vomiting and diarrhea (watery to bloody) may be observed. Central nervous symptoms including incoordination, ataxia and convulsions may additionally appear particularly during final stages of the disease. In addition, fertility disruptions are possible leading to abortions in pregnant sows (Schlafer and Mebus, 1987). During acute-lethal forms, also petechiae and epistaxis may appear, leading to death mostly within 6 to 13 days after infection (Gomez-Villamandos et al., 2003). In wild boar, the main clinical signs are severe depression, anorexia, diarrhea and respiratory distress (Gabriel et al., 2011; Ruiz-Fons et al., 2008). Pathomorphological findings can include hemorrhages in several organs, swollen and haemorrhagic lymph nodes (particularly affecting gastrohepatic and renal lymph nodes), a congestive splenomegaly with necrotic foci, petechiae in kidneys, ecchymoses in serosae, alveolar haemorrhages and oedema of the lungs (Arias, 2002; Blome et al., 2013; Colgrove et al., 1969; Kleiboeker, 2002; Rodriguez et al., 1996).

In less acute disease courses, typical clinical signs comprise respiratory findings (dyspnea, coughing, sneezing) and gastro-intestinal symptoms (watery diarrhea to obstipation) (Blome et al., 2013). Less pronounced clinical signs occur during subacute courses characterized by recurrent fever, anorexia, wasting as well as milder gastro-intestinal (diarrhea), and respiratory clinical signs (agitation-associated cough and dyspnea). In addition, pregnant sows may abort. This disease course may either lead to death within 15 to 45 days or full convalescence after 30 to 45 days (Arias, 2002; Kleiboeker, 2002). These forms are accompanied by less severe pathomorphological findings including pneumonia, serofibrinous pleuritis and pericarditis (Arias, 2002; Kleiboeker, 2002).

Clinical signs of chronic disease courses are highly variable and unspecific like recurrent fever, wasting, growth retardation, respiratory signs, chronic skin ulcers, arthritis and abortions. Secondary infections may cause additional signs. It was reported that these forms can last 2 to 5 month by showing low mortality rates (less than 30%) (Arias, 2002; Kleiboeker, 2002). Necropsy of chronically infected pigs may either reveal hemorrhages, enlarged lymph nodes, interstitial pneumonia, fibrinous pericarditis and splenomegalie or a complete absence of lesions (Arias, 2002; Kleiboeker, 2002).

Changes of blood count parameters upon ASFV infection mainly include leukopenia and thrombocytopenia and are dependent on the isolates virulence and host factors. Highly virulent strains were reported to cause severe alterations in white blood cells resulting in a decrease of lymphocyte counts and a parallel increase of neutrophils while red blood counts remain unchanged (Detray and Scott, 1957; Greig and Plowright, 1970; Wardley and Wilkinson, 1977). However, reports gave different indications concerning significant changes in total white blood cell and platelet counts (Gomez-Villamandos et al., 1997). Moderately virulent strains were shown to have only minor effects on total white blood cell counts including slight increases in neutrophils and decreases in lymphocytes (Knudsen and Genovesi, 1987). In subacute forms, a transient lymphopenia and thrombocytopenia were observed. During chronic disease courses, marked differences in blood counts were observed, mainly concerning changes of immune cell proportions, in particular B-lymphocytes and macrophages (Blome et al., 2013; Ramiro-Ibanez et al., 1997).

2.2.4 Pathogenesis and immune response

Virus dissemination and target cells

Outside the sylvatic cycle, ASFV enters the host organism oronasally via the tonsils or the mucosa on the dorsal pharynx and reaches the mandibular or retropharyngeal lymph nodes. Subsequently, the virus spreads systemically throughout the body (Greig, 1972; Plowright, 1994). Approximately 8 hours post infection (hpi), primary viraemia appears and from 15 to 24 hpi secondary viraemia is detectable (Blome et al., 2013; Colgrove et al., 1969). When viral dissemination has completed after approximately 30 hpi, ASFV can be found in almost all tissues especially in spleen, lymph nodes and other organs owning high proportions of cells of the mononuclear phagocyte system (Blome et al., 2013; Heuschele, 1967).

Monocytes/macrophages are considered as main target cells for ASFV (Malmquist, 1960; Sanchez-Torres et al., 2003; Sierra et al., 1990). However, their susceptibility seems to be dependent on the maturation state by suggesting that mature macrophages are more susceptible than young monocytes. The expression of several macrophage specific markers (e.g. CD163, CD107a) as well as the presence of SLA II antigens may benefit permissiveness towards ASFV (Blome et al., 2013; Chamorro et al., 2005; Rodriguez et al., 1996; Sanchez-Torres et al., 2003). Further initial susceptible cells comprise dendritic cells (Gregg et al., 1995), granulocytes and neutrophils (in particular the immature state) (Carrasco et al., 1996; Gomez-Villamandos et al., 1997). Moreover, during advanced stages of disease, ASFV replicates in additional cell types including endothelial cells, hepatocytes and platelet precursor cells (megakaryocytes).

During viraemia, ASFV genotypes with haemadsorbing abilities may be found associated with erythrocytes (Quintero et al., 1986; Wardley and Wilkinson, 1977), but also with lymphocytes and neutrophils (Plowright, 1994).

Influencing factors on clinical course and outcome

As already stated for CSF, the highly variable clinical course and outcome is dependent on both, factors on the agent's and the host's side (Costard et al., 2013).

Concerning the virus, the clinical form is mainly influenced by the virulence but also by the dose and the route of exposure (Costard et al., 2013; Kleiboeker, 2002). On the host's side, several factors like previous presence of the agent in the corresponding pig population and genetical factors were discussed to play a role.

Acute to peracute forms mostly develop after introduction of highly virulent ASFV strains into a naïve pig population whereas chronic to subacute courses mainly occur in regions in which infections have become more frequent (Allaway et al., 1995; Costard et al., 2013; Fasina et al., 2010; Owolodun et al., 2010; Thomson, 1985). Several factors were discussed to be responsible for the milder to subclinical forms in Africa compared to the fatal outcomes caused by the same isolates in European pigs. One hypothesis includes an acquired immunity from previous exposure to lower viral doses or to related viruses of reduced virulence (Penrith et al., 2004). Furthermore, genetically reasons were suggested for reduced susceptibility of African local pig breeds. However, it was shown that this increased resistance is not heritable (Penrith et al., 2004). Apart from the fact that chronic forms are more likely to occur upon infection with low virulent strains, another reason is presumed to be responsible for ASFV occurrence in the Iberian Peninsula from 1960 to 1995. Here, the employment of live attenuated vaccines during the 1960s is a suspected source of infection (Sanchez-Vizcaino et al., 2012).

In general, acute and peracute forms were presumed to be correlated to highly virulent strains, subacute courses to moderately, and chronic courses to low virulent isolates. However, the above mentioned facts clarify that disease courses are influenced by further factors which are far from being understood to date.

The role of cytokines

The increasing phagocytic and secretory activity of several macrophage subpopulations upon ASFV-infection results in an unregulated release of active substances including cytokines, complement factors and arachidonic acid metabolites (Blome et al., 2013; Penrith, 2004).

The enhanced secretion of proinflammatory cytokines like IL-1, IL-6, and TNF-α (Murtaugh et al., 1996) is timely correlated to the onset of fever, vascular damage, and changes in lymphoid structures (Salguero et al., 2002). Furthermore, the expression levels of IL-1α and TNF-α correlate with the development of interstitial oedema and the formation of fibrin microthrombi in septal capillaries (Carrasco et al., 2002). Like IL-1 and TNF-α, also IL-6 was shown to enhance the acute-phase-reactions and inflammatory events. IL-6 is also capable of activating endothelial cells and inducing their apoptosis. The same procoagulatory effect was shown for TNF-α with the difference that this cytokine is additionally able to activate the anticoagulant status of the vascular endothelium (Blome et al., 2013). The presumably particular importance of TNF-α during pathogenesis of ASF is further underlined by it's ability to enhance vasodilatation and vascular permeability (Gomez del Moral, 1999). TNF-α

is mainly produced by mature macrophages primarily targeted by ASFV (Chamorro et al., 2005). IL-1, IL-6, and TNF-α are known to enhance acute phase reactions, which are characteristic for highly virulent ASFV infections. In addition, these cytokines may also stimulate the production of further acute-phase-indicators like C-reactive protein (CRP), serum-amyloid A (SAA), and haptoglobin (Gabay and Kushner, 1999).

Besides the involvement of proinflammatory cytokines in lymphopenia by mediating lymphocyte apoptosis, their role in development of haemorrhagic lesions is presumably of higher importance than direct viral cell damage (Blome et al., 2013). This is evidenced by the fact that first haemorrhagic lesions in lymphoid organs occur exactly when local monocytes/ macrophages get infected and destroyed (Salguero et al., 2005). Coinciding with that, neighboring endothelial cells get stimulated by released cytokines leading to a procoagulant state of the endothelium and final and activation of coagulation cascade (Blome et al., 2013). Direct infections of endothelial cells only occur in later disease stages when haemorrhages had been developed for several days (Gomez-Villamandos et al., 1997; Perez et al., 1994).

IFN-α, IL-12, and IL-2 may be of additional importance in ASFV pathogenesis as they are involved in development of cellular immunity. Increases of IFN-α and IL-12 were detected in several *in vitro* studies in dependence of the isolates virulence (Gil et al., 2008). In addition, an IL-2 decrease was suggested to contribute to the development of lesions because of its relevant influence on regulatory and effector T-cells (Canals et al., 1995). Therefore, it was suggested that the survival seems to be influenced by cytokine reaction pattern at least partly (Blome et al., 2013). Another positive relationship between survival and cytokine production was shown for IFN-γ. An increased level of ASFV-specific IFN-γ producing T-cells may promote convalescence (Takamatsu et al., 2013). Besides, it was suggested that controlling the IFN-γ expression by the virus may determine the virulence of the ASFV isolate (Afonso et al., 2004).

Apart from that, cytokines do not seem to play a major role in chronic ASF forms. Here, an auto-immune component was suggested in which lesions might result from deposition of immune-complexes in lungs, kidneys, skin and other tissues with subsequent binding to complement (Plowright, 1994).

Finally, cytokines may additionally be involved in the development of thrombocytopenia resulting in a haemorrhagic syndrome during acute and subacute ASF forms. This may be due to an impairment of platelets and coagulation changes (Blome et al., 2013). However, the genesis of thrombocytopenia remains unknown to date.

It was suggested that an impairment of thrombocytopoiesis through infection and destruction of megakaryocytes is responsible for platelet decrease (Rodriguez et al., 1996). Despite the fact that bone marrow lesions may be observed during ASFV-infection other studies prefer the hypothesis of peripheral consumption of platelets as main reason for thrombocytopenia (Gomez-Villamandos et al., 1997). Indications for microthrombi correlated to disseminated intravascular coagulation exist (Rodriguez et al., 1996), which could result in peripheral platelet consumption. Possibly involved cytokines are the platelet activating factor (PAF) which is probably secreted by activated macrophages and the tissue factor/coagulation factor III (TF) produced by endothelial cells. While PAF may contribute to activation and degranulation of platelets during ASFV-infections (Gomez-Villamandos et al., 1996), TF was demonstrated to activate the coagulation system and by that, trigger the generation of microthrombi and peripheral platelet consumption (Vallee et al., 2001).
In conclusion, probably both, an impairment of thrombocytopoiesis and a peripheral platelet consumption may lead to the frequently observed thrombocytopenia (Blome et al., 2013).

Humoral immune response

Regarding the humoral immune response, the existence of an antibody-mediated protection is controversially discussed. After a complete absence of neutralizing antibodies during ASF had been suggested (Dimmock, 1993) several studies indicated that developing antibodies may at least lead to a delayed disease onset and in addition, reduce viraemia titers (Onisk et al., 1994). These factors were considered to determine the survival after infection (Escribano et al., 2013). In addition, several authors demonstrated neutralizing capacities of immune sera and monoclonal antibodies against several ASFV-isolates (Borca et al., 1994; Gomez-Puertas et al., 1996; Zsak et al., 1993).
However, a failure of protection after immunization and challenge of pigs with the inactivated ASFV strain “Armenia08” (Blome et al., 2014a) was reported recently resulting in an acute-lethal disease course in all animals. This is in line with observations from previous studies, posing that inactivated ASFV does not induce the production of neutralizing antibodies (Hess, 1981; Vinuela, 1985). Recent studies even indicate an accelerated disease course when antibodies are present (Blome et al., 2014a). In addition, indications exist that antibodies against the A104R/histone may probably promote an effective immune response while others against the K196R/thymidine kinase were frequently found in clinically diseased pigs (Reis et al., 2007).

Cellular immune response

Apart from the fact that the role of ASFV-specific antibodies and its potential for viral neutralization is doubtful so far, a common agreement concerning the major role of cellular-mediated immune responses towards ASFV seem to exist.

A resistance was demonstrated for pigs which survived infection with less-virulent isolates or developed a chronic course. Despite the fact that this was only observed for homologous or closely related isolates, this observation evidenced the development of a protective immune response against ASFV (Alonso et al., 1997; Canals et al., 1992; King et al., 2011; Leitao et al., 2001; Malmquist, 1963; Martins et al., 1993; Oura et al., 2005; Revilla et al., 1992). However, the underlying mechanism has not been identified up to now. It was suggested that moderately or non-virulent ASFV-strains may induce the proliferation of ASFV-specific memory T-cells (Alonso et al., 1997; Canals et al., 1992; Casal et al., 1987; Revilla et al., 1992; Scholl et al., 1989).

The adaptive cellular immune response, in particular the cytotoxic T-cell subset seems to play a major role in mediating antiviral protection. This was demonstrated by showing that a depletion of $CD8^+$ lymphocytes resulted in a loss of protection against related virulent viruses *in vivo* (Oura et al., 2005). The depletion of $CD8^+$ lymphocytes led to a severe clinical disease course and high viraemia upon homologue challenge while control animals were protected completely. In detail, this study was conducted by employing the non-virulent OURT88/3 ASFV isolate for immunization. Approximately after 5 weeks, one group of pigs was treated with an anti-CD8α monoclonal antibody (mAb) intravenously for 5 to 6 days. At day 2 of mAb inoculation, a challenge was performed with the virulent OURT88/1. In contrast to the isotype control mAb treated animals, all pigs with an effective $CD8^+$ depletion succumbed to infection (Oura et al., 2005; Takamatsu et al., 2013). This finding is in line with other studies in which a close relationship between the presence of ASFV-specific $CD8^+$ T-cells and the protection against challenge virus upon immunization was observed (Argilaguet et al., 2012). In particular, double positive cytotoxic T-cells with the $CD4^+CD^{8high+}$ phenotype seem to be mainly involved as they were found in higher proportions in peripheral blood mononuclear cells (PBMC) from ASF immune pigs when compared to clinically diseased pigs.

Furthermore, an involvement of the innate immune response was suggested after showing an increased activity of natural killer (NK) cells upon asymptomatic, non-virulent infections (Leitao et al., 2001). In contrast, a lower NK activity was detected in pigs showing a severe clinical picture. The observed induction of antiviral protection upon challenge infection may

be a result of their antigen-specific memory (Paust et al., 2010; Paust and von Andrian, 2011). This was observed for non-pathogenic ASFV isolates so far (Takamatsu et al., 2013).

As mentioned above, IFN-γ seems to play an important role in mediating antiviral protection. This is supported by the observed positive relationship between the amount of IFN-γ producing T-cells and the degree of protection towards ASFV-challenge (Esparza et al., 1988; King et al., 2011; Revilla et al., 1992). Main IFN-γ producers during ASFV-infection were characterized as $CD4^+CD8^+$ T-cells comprising mainly CTLs ($CD8^{high}$), but also to a lesser extent T memory cells ($CD4^+CD8^{low}$) (Takamatsu et al., 2013).

In general, immune responses towards ASFV are impaired by the infection of professional antigen-presenting cells (APCs), coding for several immune evasion genes, and inducing an extensive apoptosis of lymphocytes resulting in a severe lymphopenia (Oura et al., 1998; Ramiro-Ibanez et al., 1996; Ramiro-Ibanez et al., 1997; Salguero et al., 2004).

3 OBJECTIVES

Classical swine fever

Assessment of host responses towards moderately virulent CSFV

Moderately-virulent CSFV isolates of genotype 2.3 have predominated in Europe over the last two decades. These isolates are characterized by a highly variable, mostly age-dependent clinical picture. Besides, immune status and genetic background were reported to play a role in disease severity and outcome. So far, little is known about the reaction pattern leading to these differences and their impact on disease dynamics. For this reason, a recent CSFV isolate was employed in infection studies with weaner pigs of different breeds, and for a trial with subadult wild boar. Analyses focused on virological and immunological tests.

Swine fever pathogenesis

Development and validation of multiplex RT-qPCRs for seven porcine cytokines

Dysregulation of immune responses is suspected to play an important role in the pathogenesis of severe infections including classical and African swine fever. Especially proinflammatory cytokines were reported as key factors. To assess normalized gene expression profiles of seven cytokines that were previously reported in this context, a highly specific and sensitive Taqman-based RT-qPCR was established.

Swine fever surveillance

Implementation of disease surveillance and early warning tools

Despite of the emerging spread of ASFV across Europe and the continuing reoccurence of CSFV, current disease surveillance is insufficient regarding the massive lack of sample submissions from both fallen and living animals. With the purpose to provide pragmatic approaches for passive and active swine fever surveillance adequate sampling tools were validated and implemented. For optimization of passive swine fever surveillance dry/semi-dry blood swabs were proven to be suitable. Secondly, ‘‘rope-in-a-bait’’sampling swabs were evaluated for CSFV in order to improve early warning strategies.

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.

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

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

Abstract

Background

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

Findings

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

Conclusions

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

Keywords

Classical swine fever virus, Host responses, Pathogenesis, Host factors

Findings

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

Six German landrace pigs (12 weeks of age), six hybrid pigs (8–10 weeks of age), and six European wild boar (12 weeks of age), were oronasally inoculated with $10^{5.5}$ tissue culture infectious doses 50% of the moderately virulent CSFV strain "Roesrath" (CSF1045). Three additional pigs of each breed acted as negative controls (housed separately). Clinical scores (CS) were assessed as previously described [7], and rectal body temperatures were recorded. While body temperatures of domestic pigs could be assessed daily, wild boar were measured upon blood collection only as they did not tolerate measurement without restraint. All animals were subjected to necropsy.

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

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

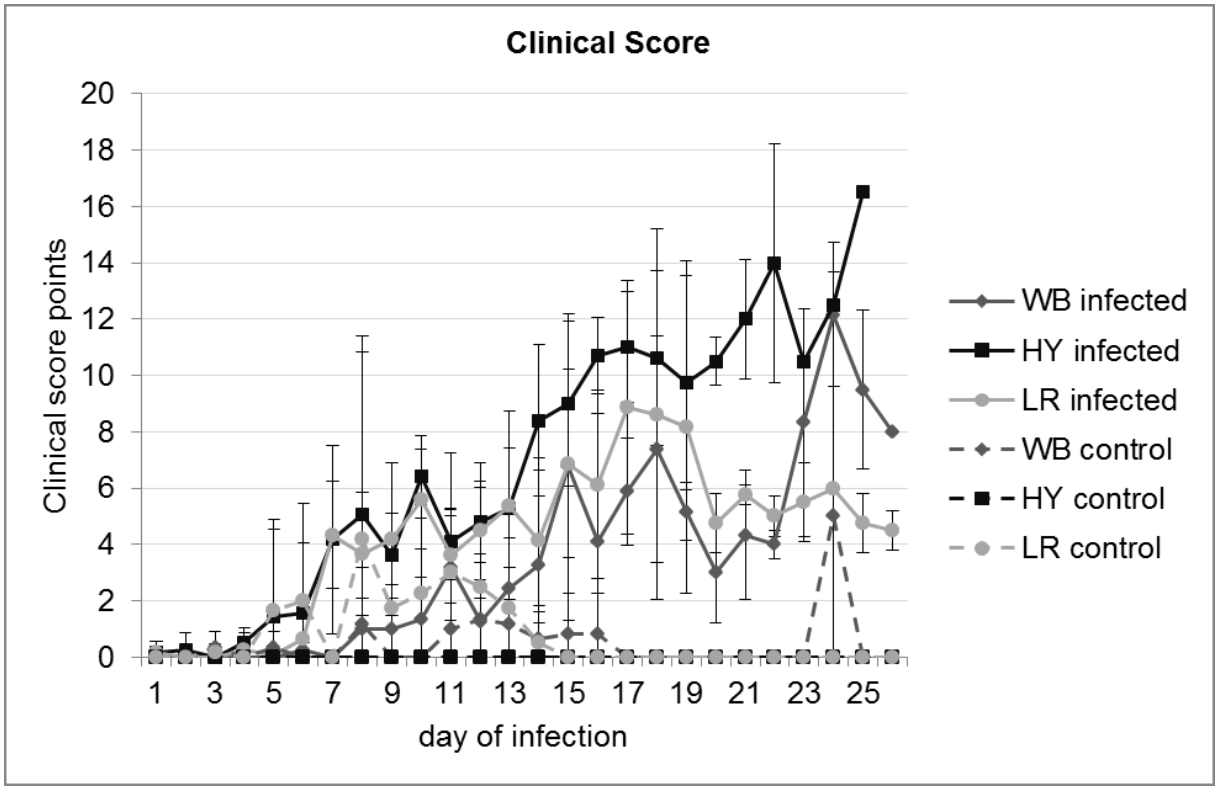

Figure 1 Group mean values (mean value ± standard deviation) for clinical score points of European wild boar (WB), commercial fattening hybrids (HY) and German Landrace (LR) pigs. Each race/breed was divided into one group for infection with CSFV "Roesrath" (infected) and one group acting as negative control (control). During the cause of disease total numbers of pigs decreased due to euthanasia.

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

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

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

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

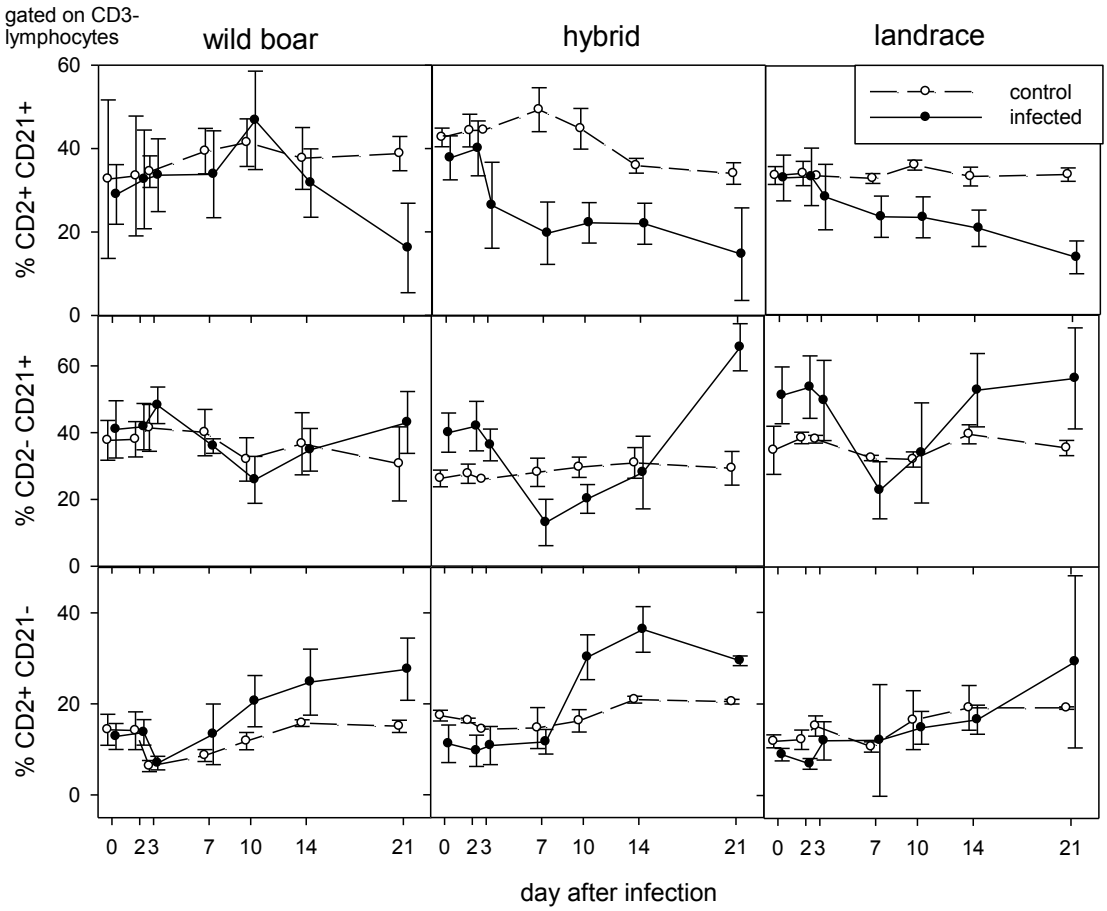

Figure 2 B-cell related responses upon infection. Blood lymphocytes were immune-stained to determine the frequency of different B cell subpopulations by FACS analysis: conventional B cells: CD3-CD2 + CD21+; activated B cells upon antigen contact: CD3-CD2-CD21+ and antibody forming and/or memory B cells: CD3-CD2 + CD21-. Filled symbols represent data from infected animals in comparison to uninfected controls (open symbols).

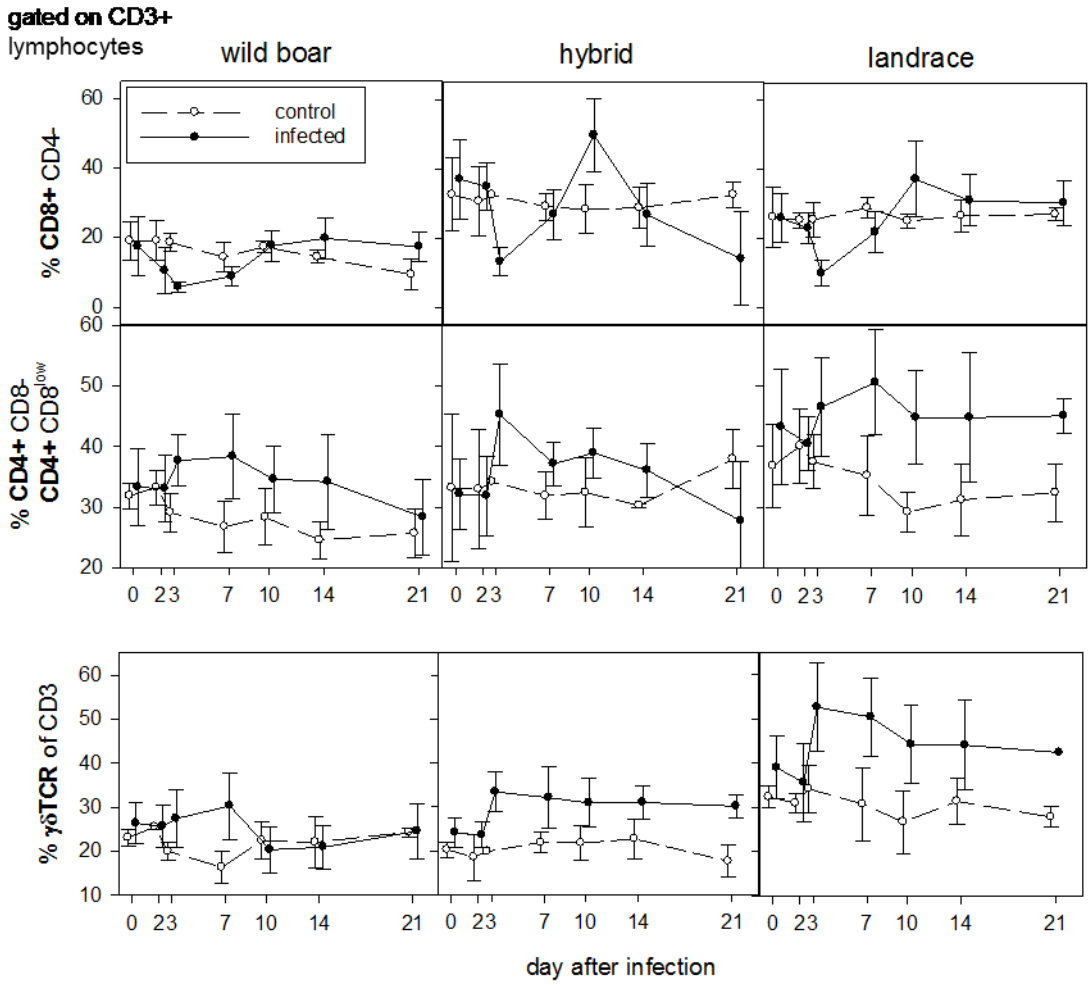

Figure 3 T-cell related responses upon infection. Percentage of T cell subpopulations of blood lymphocytes is given: cytotoxic T cells: CD8 + CD4-; T-helper cells/memory T-helper cells: CD4 + CD8-/CD4 + CD8low. Bottom row shows percentage of γδTCR + T cells of all T cells during infection. Filled symbols represent data from infected animals in comparison to uninfected controls (open symbols).

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

With regard to antibody detection, landrace pigs showed one weak-positive NT result at 10 dpi (see Figure 4). At 14 dpi, neutralization test were positive for 2 out of 4 pigs. At 21 and 28 dpi, all remaining pigs were found positive with high homologue titers in surviving pigs (see Figure 4).

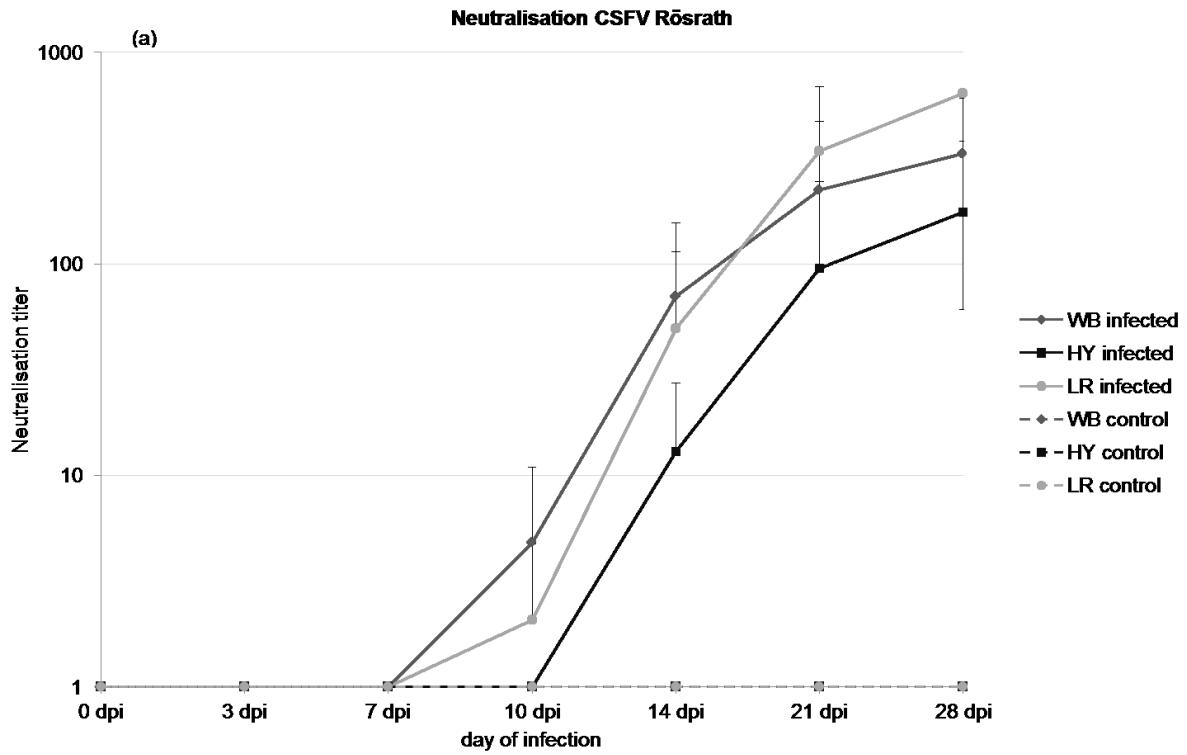

Figure 4 Mean values of antibody responses of infected groups of each race (WB: wild boar; HY: commercial fattening hybrid; LR: German Landrace). Results of the neutralization test using CSFV strain "Roesrath" are shown in **(a)**, in which antibody titers are represented as $\log_{10}$ ND_{50}.

Hybrid pigs became positive in NTs from 14 dpi (two animals). From 21 dpi, all remaining pigs were found positive (see Figure 4).
In wild boar, first antibodies were detected at 10 dpi with 2 out of 6 animals in the NTs. From 14 dpi, all tested wild boar were positive in the NTs with the homologue virus (see Figure 4).

Classical swine fever may cause most variable clinical syndromes and it is generally acknowledged that disease courses are influenced by both virus and host factors. On the host's side, age and immune status are main parameters that influence disease course and outcome [9]. However, breed factors were also often discussed to play an important role.

Depner et al. [1] showed that German landrace pigs were more severely affected than crossbred animals. Influence of breed was also seen when susceptibility was assessed in indigenous Moo Laat and improved Large White/Landrace [4]. In contrast, no differences were seen by Bunzenthal [10].

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

In terms of serological responses, wild boar showed earliest responses. However, by the end of the trial, titers of neutralizing antibodies were similar or even higher in landrace pigs. In hybrids, E2 antibodies were only detected late in some animals and to lower titers. This reflects the clinical picture but contrasts tendencies seen in the responses of lymphocyte phenotypes (with regard to percentages of cells with plasma cell phenotype).

Despite the fact that the majority of leukocytes will be active outside the blood compartment, changes in the percentages of different lymphocyte phenotypes were investigated in blood samples as the only matrix that allowed kinetics in individual animals. With regard to B-cell responses in peripheral blood, some breed-depended patterns were observed that were however not statistically significant among the different groups. Upon infection, all animals showed a down regulation of CD2 + CD21+ cells (phenotype of naïve B-cells), this could be either due to depletion or an indication of B-cell activation. As especially domestic pigs showed an increase of cells presenting the phenotype of primed and activated B-cells (CD2-

CD21+) after 7 dpi, activation could be suggested. Interestingly, the increase of cells displaying the phenotype of antibody producing plasma cells (CD2 + CD21-) was highest in hybrid pigs. This is in contrast to both clinical course and serology. However, due to the lack of additional plasma cell markers at the time point of the experiment (CD79a) and the possible impact of lymphocyte depletion, these results have to be viewed with caution and need further investigation. All investigated breeds showed slightly elevated helper cells from 3 dpi. Following the increase of helper cells, an increase of CD8 + CD4- CTLs was detectable. Strongest CTL proliferation was seen again in hybrid pigs. Preceding CTL proliferation, probably virus-mediated decrease of CD8 + CD4- T cells was detectable in all animals. This is in line with previous studies that showed that CSFV is able to suppress porcine T cells [11] and to induce killing of T cells [12]. In domestic pigs an increase of γδ TCR positive T cells was detectable, more pronounced in landrace pigs. The γδ T cells are discussed as antigen presenting cells in swine [13]. Clearly, changes in lymphocyte subsets need further investigation, especially with regard to harmful pattern and involvement of the immune system in the pathogenesis of CSF as was suggested by several authors [14-16].

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

Additional file 1: Table S1 Overview on clinical presentation and disease courses upon infection with CSFV strain “Roesrath”. Onset refers to the occurrence of clinical signs excluding fever. Duration is given as the number of consecutive days (int. = intermittent, d = day). Unspecific symptoms (Unspec.) include depression and changes in general bearing, reduced liveliness, lack of appetite, conjunctivitis, gastro-intestinal and respiratory signs. Neurological signs (Neurol.) include ataxia, hind leg paresis, and uncontrolled shivering. Haemorrhagic signs (Haem.) refer to skin haemorrhages and cyanosis. Pathological lesions are presented for the lymphatic organs in particular (To. = tonsil, Lnn. = lymphnodes, Sp. = spleen) . Moreover, haemorrhagic lesions (Haem.) and secondary infections (Sec.Inf.) are accounted for. Additional abbreviations: WB = wild boar; HY = hybrid pigs; LR = landrace pigs; INF = inoculated animal; NC = uninfected control animal; DOE = day of euthanasia; AL = acute-lethal disease course; AT = acute-transient disease course, CH = chronic illness.

Animal	Breed	Status	Fever	Onset	Duration	CS max	Clinical Symptoms			Outcome	DOE	Pathology					Seroconversion	
			[T°C ≥40]				*Unspec.*	*Neuro.*	*Haem.*			*To.*	*Lnn.*	*Sp.*	*Haem.*	*Sec. Inf.*	*E2*	*Erns*
#WB08	WB	INF	10 dpi	5 dpi	11 d	9.0	x			AL	15 dpi	x	x				x	x
#WB09	WB	INF	12 dpi	8 dpi	16 d	15.0	x	x		AL	23 dpi	x	x		x	x	x	x
#WB10	WB	INF	int.	5 dpi	13 d	13.0	x	x		AL	17 dpi	x	x		x	x	x	x
#WB13	WB	INF	10 dpi	10 dpi	15 d	11.5	x	x		AL	24 dpi	x	x		x	x	x	x
#WB15	WB	INF	int.	5 dpi	24 d	11.5	x	x		CH	28 dpi	x	x			x	x	x
#WB16	WB	INF		7 dpi	9 d	9.5	x			AL	15 dpi	x	x			x	x	x
#WB07	WB	NC									28 dpi							
#WB12	WB	NC		4 dpi	1 d	1.0	x				28 dpi							
#WB14	WB	NC		2 dpi	int.	15.0	x	x			23 dpi							
#DP49	HY	INF	3-24 dpi	3 dpi	22 d	16.5	x	x	x	AL	24 dpi	x	x	x	x	x	x	x
#DP50	HY	INF	3-18 dpi	3 dpi	16 d	14.5	x	x	x	AL	18 dpi		x		x	x	x	x
#DP51	HY	INF	4-17 dpi	5 dpi	13 d	14.0	x	x		AL	17 dpi		x			x	x	x
#DP52	HY	INF	3-7 dpi	3 dpi	5 d	16.5	x			AL	7 dpi					x		
#DP53	HY	INF	5-21 dpi	5 dpi	17 d	17.0	x	x	x	AL	21 dpi		x		x	x	x	x
#DP54	HY	INF	3-19 dpi	6 dpi	19 d	11.0	x	x	x	AL	20 dpi	x	x		x	x	x	x
#DP43	HY	NC	20 dpi								28 dpi							
#DP44	HY	NC	int.								28 dpi							
#DP45	HY	NC	int.								28 dpi							
#DP55	LR	INF	3 - 11 dpi	6 dpi	6 d	8.0	x	x		AL	11 dpi		x		x			x
#DP56	LR	INF	int.	4 dpi	25 d	7.0	x	x		AT	28 dpi				x		x	x
#DP57	LR	INF	int.	5 dpi	14 d	15.0	x	x	x	AL	18 dpi	x	x		x	x	x	x
#DP58	LR	INF	3-17 dpi	5 dpi	13 d	15.5	x	x	x	AL	17 dpi	x	x		x	x	x	x
#DP59	LR	INF	4-20 dpi	6 dpi	23 d	6.0	x	x		AT	28 dpi		x		x	x	x	x
#DP60	LR	INF	4-7 dpi	3 dpi	5 d	7.5	x	x		AL	7 dpi		x		x			
#DP46	LR	NC	4 dpi	4 dpi	int.	12.5	x	x			7 dpi							
#DP47	LR	NC	int.	8 dpi	6 d	6.0	x	x			28 dpi							
#DP48	LR	NC	int.								28 dpi							

Additional file 2: Table S2 Rectal body temperatures upon infection with CSFV strain "Roesrath" (0–28 days post infection). Fever was defined as a body temperature >40°C for at least two consecutive days. Temperatures >40°C but <40.5 are marked iin yellow, temperatures >40.5°C in red. WB = wild boar, HY = hybrid pigs, LR = landrace pigs, inf = infected, ctr = negative control, nd = not determined.

		0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28
WB (inf)	WB#08	nd	39,1	37,9	39,6	nd	nd	nd	38,4	nd	nd	40,8	nd	39,2	nd	nd	nd													
	WB#09	nd	39,1	39,2	38,8	nd	39,6	nd	39,9	nd	nd	39,3	nd	41,1	nd	nd	nd	nd	nd	nd	nd	nd	39,3	nd	nd					
	WB#10	nd	38,9	39,5	39,9	nd	40,2	nd	39,9	nd	nd	39,6	nd	40,9	nd	nd	nd	nd	40,3											
	WB#13	nd	39,3	39,2	39,3	nd	nd	nd	39,4	nd	nd	40,1	39,8	39,6	nd	nd	nd	nd	nd	nd	nd	nd	39,2	nd	nd	nd				
	WB#15	nd	38,8	40,1	39,1	nd	39,6	nd	39,8	nd	nd	39,8	nd	39,9	nd	nd	nd	nd	nd	nd	nd	nd	40,1	nd	nd	nd	nd	nd	nd	40,1
	WB#16	nd	38,5	39,1	39,3	nd	nd	nd	37,6	nd	nd	39,9	nd	39,0	nd	nd	nd													
HY (inf)	HY#49	39,4	39,0	38,7	40,0	40,8	41,0	41,6	41,4	41,1	40,9	40,6	40,4	40,9	41,2	40,7	41,9	41,4	40,7	41,2	41,2	41,2	41,0	41,0	41,1	41,0				
	HY#50	39,4	39,3	39,3	40,3	40,5	41,1	40,9	40,2	42,0	41,2	41,1	41,2	41,4	40,9	41,1	41,3	41,3	41,2	41,2										
	HY#51	38,2	38,4	39,0	39,8	40,5	40,7	41,2	40,8	41,4	42,5	40,7	40,5	41,6	41,3	41,2	40,8	41,3	41,3											
	HY#52	39,4	39,7	39,3	40,4	40,6	41,3	41,6	41,3																					
	HY#53	38,8	38,7	38,4	39,0	39,7	40,5	41,3	41,4	40,8	41,5	40,6	41,8	40,1	40,6	41,4	41,3	41,5	41,6	41,9	41,8	41,1	41,0							
	HY#54	39,8	39,1	39,3	40,3	40,3	40,5	41,9	40,8	40,4	41,1	41,0	40,7	40,5	41,3	40,9	41,2	41,3	40,2	41,0	40,6									
	LR#55	39,4	39,1	39,7	41,0	41,4	40,5	41,2	41,4	41,8	41,9	41,2	40,8																	
LR (inf)	LR#56	39,7	38,7	38,5	40,4	41,3	40,7	40,8	41,4	41,2	41,5	40,2	40,6	40,2	40,3	39,2	40,7	39,9	39,8	39,4	39,3	39,4	39,7	39,9	39,2	39,5	39,3	39,4	39,3	39,1
	LR#57	39,7	40,0	39,4	40,0	40,5	41,4	41,9	41,5	41,7	41,2	40,7	41,9	41,7	41,8	41,4	41,7	41,6	41,6	41,7										
	LR#58	39,3	39,3	39,4	40,7	40,9	41,3	41,8	40,5	41,5	40,8	41,0	41,6	41,5	40,9	41,6	41,8	42,0	41,5											
	LR#59	39,0	38,7	39,6	39,3	40,0	40,3	40,5	41,5	40,8	41,2	40,7	40,5	40,2	40,8	40,4	40,3	41,0	40,8	40,3	40,7	41,3	39,6	39,7	39,7	39,9	39,7	39,6	39,6	39,1
	LR#60	39,7	39,4	39,0	39,7	40,8	41,3	41,4	41,5																					
WB (ctr)	WB#07	nd	38,4	38,2	39,1	nd	nd	nd	38,8	nd	nd	39,3	nd	nd	nd	39,6	nd	nd	nd	nd	nd	nd	39,1	39,1	nd	nd	nd	nd	nd	nd
	WB#12	nd	39,1	38,8	38,8	nd	nd	nd	38,9	nd	nd	39,0	nd	nd	nd	39,3	nd	nd	nd	nd	nd	nd	38,3	39,1	nd	nd	nd	nd	nd	nd
	WB#14	nd	39,1	38,5	38,4	nd	nd	nd	38,8	nd	nd	39,8	nd	nd	nd	39,4	nd	nd	nd	nd	nd	nd	38,8	nd	nd					
HY (ctr)	HY#43	38,7	38,8	38,7	38,7	39,4	39,4	39,3	39,0	39,3	39,2	39,4	39,6	39,6	39,7	39,7	39,7	39,3	39,5	39,8	39,8	40,3	39,6	39,7	39,8	39,6	39,6	39,6	39,6	39,5
	HY#44	39,1	39,0	39,1	39,1	39,8	39,4	39,6	39,2	39,4	39,3	39,5	39,5	39,1	39,9	39,8	39,3	39,8	40,3	39,8	40,8	40,1	39,3	40,0	40,1	40,1	39,7	39,7	39,8	39,4
	HY#45	39,2	38,7	39,2	38,9	39,8	39,7	39,4	39,1	39,5	39,3	39,6	39,8	39,7	40,1	39,8	39,7	39,7	39,7	40,2	40,0	39,9	39,7	39,8	39,9	39,4	39,8	39,8	39,9	39,3
LR (ctr)	LR#46	39,3	39,1	39,0	38,5	40,0	39,6	39,0	38,3																					
	LR#47	39,0	39,1	38,8	39,2	39,5	39,8	39,7	39,5	40,1	40,1	40,1	40,2	39,5	39,4	39,5	39,8	40,8	40,6	39,9	40,1	40,5	40,5	39,9	40,1	40,1	39,2	38,8	39,2	38,8
	LR#48	39,3	38,4	39,3	39,5	39,8	40,6	39,3	39,6	39,1	39,1	39,3	39,1	39,2	39,4	39,5	39,0	39,3	38,9	39,3	39,5	39,7	39,6	39,4	40,3	40,1	39,2	39,8	39,6	39,5

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

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

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4.2 Novel rope-based sampling of classical swine fever shedding in a group of wild boar showing low contagiosity upon experimental infection with a classical swine fever field strain of genotype 2.3

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Novel rope-based sampling of classical swine fever shedding in a group of wild boar showing low contagiosity upon experimental infection with a classical swine fever field strain of genotype 2.3

Abstract

Several classical swine fever (CSF) epidemics in wild boar and domestic pigs in Europe during the last decades have been caused by CSF virus (CSFV) strains of genotype 2.3. This genotype is known to be virulent leading to high morbidity and mortality. We experimentally infected two eight months old wild boar with $10^{5,5}$ $TCID_{50}$ of CSFV genotype 2.3 and kept the animals together with five noninoculated wild boar of the same age. Our original purpose was to evaluate a non-invasive sampling method based on saliva collection using ”rope-in-a-bait” sampling baits. While expecting high morbidity, high level of virus shedding and some mortality, we actually observed a subclinical course of infection with an unexpected low contagiosity. The two inoculated animals infected only three contact animals while two contact animals remained uninfected. These findings substantially add to our epidemiological understanding of CSFV circulation in wild boar populations. CSFV infected animals older than six months and in good condition may not shed sufficient virus to transmit infection to all seronegative in-contact animals. The contagiosity in relation to the animal’s age is discussed. This supports the hypothesis of silent perpetuation of CSFV in wild boar populations for several months if the wild boar density is sufficiently high. The feasibility of the “rope-in-a-bait” sampling method could be proven during the short viraemic phase of infected animals during the second week of infection.

Key words

Classical swine fever (CSF), wild boar, contagiosity, subclinical course, non-invasive sampling “rope-in-a-bait”

Novel rope-based sampling of classical swine fever shedding in a group of wild boar showing low contagiosity upon experimental infection with a classical swine fever field strain of genotype 2.3

1. Introduction

Classical swine fever (CSF) is caused by an RNA virus of the genus Pestivirus of the *Flaviviridae* family. The disease is often fatal, affecting pigs and wild boar (*Sus scrofa*) alike, and causes major economic losses especially in countries with an intensive pig production system (Horst et al., 1999). The role of wild boar in CSF is primarily of epidemiological interest since they are regarded as a reservoir for CSF virus (CSFV) and a possible source of infection for domestic pigs (Artois et al., 2002; Staubach et al., 2013). Therefore, the presence of CSFV in wild boar populations represents a high risk for domestic pigs. Under these conditions adequate surveillance which enables an early detection of CSF in the wild boar population is crucial. However, collecting a statistically significant number of samples from wild boar for early detection of infection, e.g. for demonstration of the presence of virus, viral RNA or antigen, is logistically difficult and up to now linked exclusively to hunting or trapping activities (Alexandrov et al., 2013). Furthermore, sampling methods for antibody detection provide a retrospective analysis of the disease situation, but are not useful for early warning.

Our primary aim in this study was to explore the efficiency of CSF viral RNA detection in saliva samples collected by a non-invasive method using specific sampling baits consisting of ropes imbedded in a bait matrix ("rope-in-a-bait"). For validating the method we conducted an experimental infection of wild boar to probe animals which shed CSFV and which can be sampled regularly. In particular, we wanted to assess the sensitivity of the novel "rope-in-a-bait" sampling technique by comparison with blood tests and oronasal swabs.

2. Materials and methods

Seven wild boar were used in the experiment. The animals were eight months old, weighing around 45 kg each. They were all in good condition and kept in the same stable. Two randomly selected wild boar (A1 and A2) were inoculated oronasally with 4 ml of cell culture medium containing $10^{5.5}$ $TCID_{50}$ (tissue culture infectious dose 50%) of CSFV isolate 2.3 „Rösrath". The CSFV strain "Rösrath" (CSF1045, GenBank accession number GU233734) was originally isolated from a wild boar piglet in Germany in 2009 and has been previously described by Leifer et al. (2010) as a strain of moderate virulence causing varying clinical

pictures of different severities ranging from unspecific symptoms to haemorrhagic lesions. The challenge virus was obtained from the German National Reference Laboratory for CSF and passaged twice on PK15 cells prior to use. The inoculum was from the same virus stock used in earlier experiments showing a higher virulence.

Clinical signs were recorded daily. Blood samples (Monovette® EDTA KE/9 ml resp. Serum/9 ml Sarstedt, Numbrecht, Germany) for virological and serological investigations as well as oronasal swabs (Copan Rayon Regular Tip cat. no. 155C, Hain Lifescience GmbH, Nehren, Germany) were taken twice a week during the first 4 weeks, and once a week afterwards. The last samples were taken 81 days post infection. For collecting oronasal swabs and blood samples the animals had to be sedated using Tiletamin and Zolezepam (0,5-1 ml Zoletil® 100 per animal). While the animals were under sedation the body temperature was also measured. Increased body temperatures between 40 °C and 41 °C were regarded as an effect caused by the handling and agitation of the animals while body temperatures above 41 °C where regarded as fever.

For non-invasive sampling we used rope-in-a-bait sampling baits which were manufactured in analogy to the CSF oral vaccine baits (Faust et al., 2007) by embedding a raw cotton rope with a length of 10 cm and a diameter of 0.8 cm (Kanirope GmbH, Dortmund, Germany) in a cereal-based bait matrix. At least seven sampling baits were distributed every morning on the floor of the pen. The chewed cotton ropes were collected either the same day or next morning.

RNA was extracted from all samples using the MagAttract Virus Mini M48 Kit for automated extraction (Qiagen GmbH, Hilden, Germany) according to the manufacturer's recommendations. For CSF viral RNA detection real time reverse transcription PCR (RTqPCR) according to the protocol of Hoffmann et al. (2005) was used. Samples with C_q values below 40 were considered as positive.

For antibody detection a commercially available CSF antibody ELISA (IDEXX CSFV Ab, Idexx Laboratories, Inc., Westbrook Maine, USA) was used according to the protocol of the producer. Additionally, selected serum samples were tested in the virus neutralisation assay (NT) according to the OIE manual of diagnostic tests and vaccines for terrestrial animals (OIE, 2008).

After completion of the trial necropsy was performed.

3. Results

Liveliness and appetite of all animals remained normal during the whole observation period and no clinical signs of CSFV infection were seen (for example hemorrhages in the skin, central nervous symptoms). The only sign which could be attributed to CSF was fever in some of the animals during the second week of infection (Fig 1). However, fever did not affect their lively roaming and eating behaviour.

The virological and serological results are shown in Table 1. The first RTqPCR positive results were detected on day 3 post inoculation (3 dpi) in the blood sample of one inoculated animal (A1) and on 6 dpi of the second inoculated wild boar (A2). Three contact animals (C3, C4, C5) reacted positive one week later (10 dpi), while two contact animals (C6 and C7) remained negative during the entire observation period. Seroconversion started in the inoculated animals 2 weeks post infection (10 dpi), followed by the three positive RTqPCR contact animals (13 and 17 dpi). CSF specific antibodies could not be detected in serum samples from the two wild boar with negative RTqPCR results. These two animals were repeatedly tested negative until 81 dpi.

In the "rope-in-a-bait" samples, positive RTqPCR reactions were measured on 12 dpi (C_q 36) and 13 dpi (C_q 35) while only one oronasal swab from 10 dpi gave a positive RTqPCR signal (C_q 37).

At necropsy no gross pathological signs of a CSFV infection were seen. Viral RNA was detected in tonsils (Cq 33-34), salivary gland (Cq 37), mandibular lymphnotes (Cq 33-36) but not in spleen.

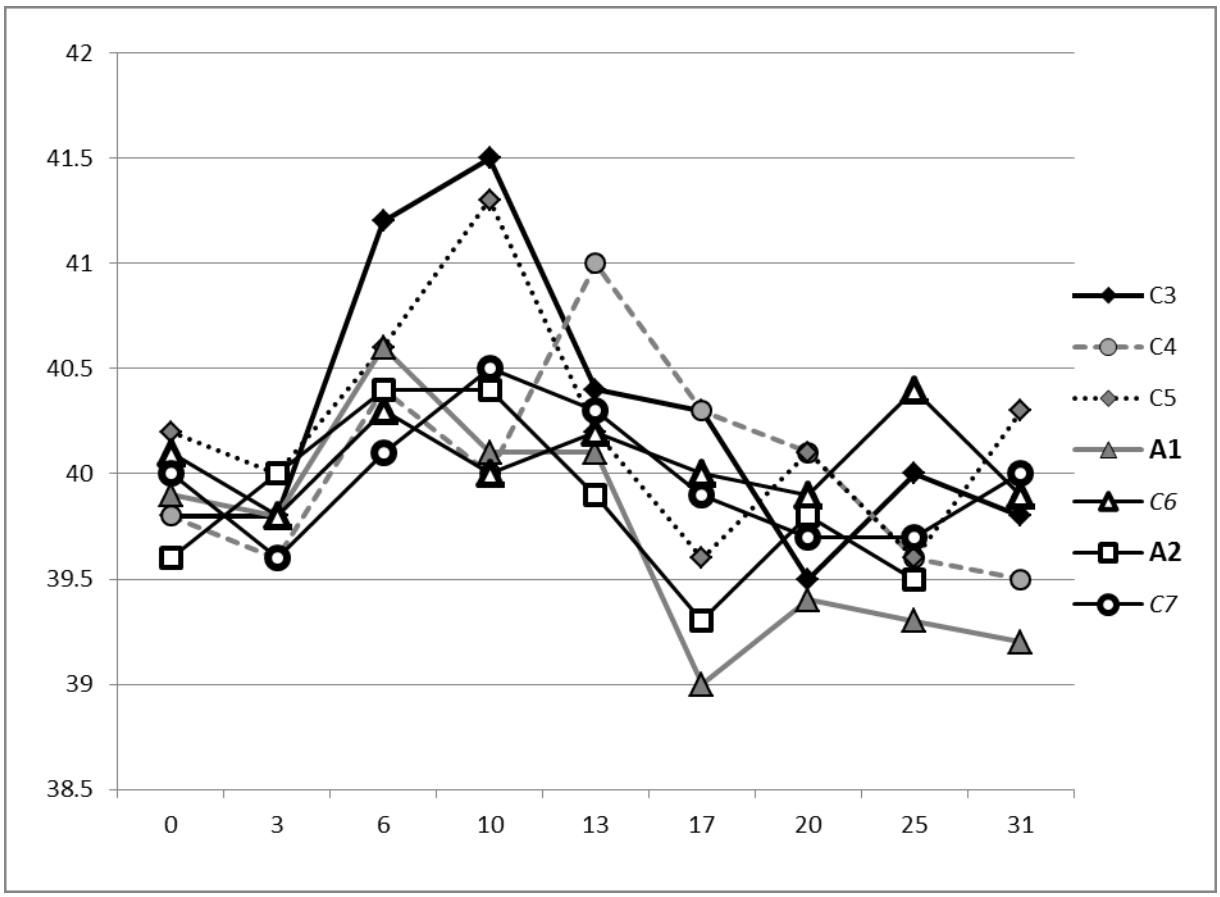

Fig. 1. Rectal temperature (°C) of wild boar infected with CSFV.
A1 and A2 (bold face) were inoculated with $10^{5,5}$ $TCID_{50}$ Rösrath genotype 2.3 CSFV.
C3, C4 and C5 were infected by contact, C6 and C7 remained uninfected (italic).

4. Discussion

The experimental trial presented in this paper was aimed to validate a non-invasive sampling method for the detection of CSF viral RNA in saliva of wild boar. The unexpected subclinical course in the five infected animals and the absence of infection in two sentinel animals did not allow a thorough evaluation of the non-invasive sampling method. Virus excretion through saliva could only be demonstrated with two sampling baits and one oronasal swab during the second week of infection. The low virus dose in the saliva is reflected by the high C_q values (>30) measured. Presumably, insufficient amounts of virus had been excreted during the viraemic phase which lasted less than one week. Since measurements of temperature and blood sampling could not be performed every day, the temperature curve and the viral RNA

detection in oronasal swabs may not reflect accurately the duration of virus shedding and clinical course.

CSFV isolates belonging to the 2.3 genotype have caused several CSF epidemics in wild boar and domestic pigs in Europe during the last decades (von Rüden et al., 2008; Leifer et al., 2010; Postel et al., 2013; Staubach et al., 2013). These strains are classified as moderate virulent causing age-dependent disease outcomes with severe clinical signs and high mortality in young animals, and milder, often transient courses in older animals. Hence, we expected in our experimental trial distinct clinical signs of CSF as well as a lethal outcome for some of the animals. Further, as shown in previous experiments, we expected an extended virus excretion (Depner et al., 1995; Depner et al., 1996). Based on our results, we hypothesized that the reason for the low contagiosity might be (i) either a change in virus virulence, (ii) a partial protection by fortuitous presence of antibodies against bovine viral diarrhea (BVD) virus, or (iii) a natural robustness associated with physiological host factors. For excluding an eventual virus mutation we sequenced PCR products of different positive blood samples from inoculated (6 dpi) and contact animals (10/13 dpi) (Schaarschmidt et al., 2000) and could prove that the subclinical infection was indeed caused by CSFV 2.3 „Rösrath“ (data not shown). Furthermore, we also excluded an unnoticed prior BVDV infection by retesting the sera of 0 dpi in a BVD virus neutralization antibody test in which all sera proved to be negative (data not shown). Hence, the role of host factors influencing the course of the disease remained the only plausible hypothesis.

It is known that host factors may significantly influence the outcome of a CSFV infection (Depner at al., 1997). Younger pigs are not yet fully immunocompetent (Suradhat et al., 2007). This might be an explanation why the severity of infection (mortality) is higher in piglets than in adults. Field data from the CSF epidemics in wild boar in Germany have shown that CSFV is mainly detected in animals younger than one year. Around 80% of virus positive samples originated from this age group (Kern et al., 1999; von Rüden at al., 2008). Unfortunately, the exact age of young wild boar (e.g. younger or older than 6 months) had not been reported. In our experiment the wild boar were eight months old sub-adults. They were in excellent condition which might have positively influenced the mild course of infection.

Other possible determinants affecting the outcome of CSFV infections may be factors associated to virus dose (Dahle and Liess, 1995). However, this can be excluded here since the inoculated pigs received a very high virus dose ($>10^{5,5}$ $TCID_{50}$). From experimental

studies it is known that inoculation doses of 300 TCID are sufficient to infect an animal (Depner et al., 1995).

The results of this infection experiment might help to explain the virus silent perpetuation over several months in a wild boar population. The age (older than six months) together with a good condition of the animal might influence the outcome of CSF. Under field conditions this means that during certain periods of time when fewer young piglets (< six months) are present in the population (e.g. towards the end of the year, before the farrowing period starts) subclinical courses may sustain the epidemic if the wild boar density is sufficiently high, e.g. more than 2 animals per square kilometer (EFSA 2009).

The results described in this communication underline the need to examine more closely the age dependency of CSF in wild boar, particularly in animals between six and 12 months of age.

In conclusion, the most striking finding in our experiment was that two contact animals did not become infected, although they had permanent direct contact with infected animals throughout the viraemic phase. The contagiosity of CSF seems to be dependent from age and the condition of the animal. Limited virus shedding may not lead to fast spread but rather subtle transmission that may go unnoticed for long time periods. Attributing a particular virulence feature to a field strain, e.g. high or low virulent, can be misleading and not predict the outcome of the infection which results from the complex interaction between pathogen and host. The feasibility of the “rope-in-a-bait” sampling method could be proven although the animals had a very short viraemic phase and virus excretion via saliva was poor.

Furthermore, the results of this experimental trial lead to the recommendation to determine the exact age primarily of hunted young boar in the context of epidemiological investigations during a CSF epidemic.

Table 1

Comparison of rectal temperature, C_q-values and results of antibody detection by ELISA. On the days not shown all RTqPCR reactions were negative. Between 17 dpi and 81 dpi all serum samples were ELISA positive. A1 and A2 were inoculated with $10^{5,5}$ $TCID_{50}$ Rösrath genotype 2.3 CSFV and C3, C4, C5, C6 and C7 were contact animals.

	-1 dpi	3 dpi	6 dpi	10 dpi	12/13 dpi	17 dpi	81 dpi
A1							
temperature[1]	39.9	39.8	40.6	40.1	40.1	39.0	*nd[2]
EDTA blood	no c_q	**37**	**32**	**35**	no c_q	no c_q	*nd
oronasal swab	no c_q	no c_q	no c_q	no c_q	no c_q	no c_q	*nd
ELISA[3]	-[4]	-	-	-	+[3]	++[3]	*nd
A2							
temperature	39.6	40.0	40.4	40.4	39.9	39.3	*nd
EDTA blood	no c_q	no c_q	**32**	**34**	**37**	no c_q	*nd
oronasal swab	no c_q	no c_q	no c_q	no c_q	no c_q	no c_q	*nd
ELISA	-	-	-	(+)[3]	+	++	*nd
C3							
Temperature	39.8	39.8	**41.2**	**41.5**	40.4	40.3	39.4
EDTA blood	no c_q	no c_q	no c_q	**35**	no c_q	no c_q	nd
oronasal swab	no c_q	no c_q	no c_q	**37**	no c_q	no c_q	nd
ELISA	-	-	-	-	+	++	++[3]
C4							
Temperature	39.8	39.6	40.4	40.0	**41.0**	40.3	40.2
EDTA blood	no c_q	no c_q	no c_q	**31**	**34**	**35**	nd
oronasal swab	no c_q	no c_q	no c_q	no c_q	no c_q	no c_q	nd
ELISA	-	-	-	-	-	+	++
C5							
Temperature	40.2	40.0	40.6	**41.3**	40.2	39.6	39.2
EDTA blood	no c_q	no c_q	no c_q	**36**	**33**	**37**	nd
oronasal swab	no c_q	no c_q	no c_q	no c_q	no c_q	no c_q	nd
ELISA	-	-	-	-	-	+	++
C6							
Temperature	40.1	39.8	40.3	40.0	40.2	40.0	39.2
EDTA blood	no c_q	no c_q	no c_q	no c_q	no c_q	no c_q	nd
oronasal swab	no c_q	no c_q	no c_q	no c_q	no c_q	no c_q	nd
ELISA	-	-	-	-	-	-	-
C7							
Temperature	40.0	39.6	40.1	40.5	40.3	39.9	**41.5**
EDTA blood	no c_q	no c_q	no c_q	no c_q	no c_q	no c_q	nd
oronasal swab	no c_q	no c_q	no c_q	no c_q	no c_q	no c_q	nd
ELISA	-	-	-	-	-	-	-
"Rope in a bait" (accumulative sample)							
rope sample	no c_q	no c_q	no c_q	no c_q	**36/35**	no c_q	nd

[1] Rectal (°C), fever (>41.0) showing in bold

[2] Not done

[3] % inhibition: positive (+) 30-40, + >40-70, ++ >70; negative –< 30

[*] Euthanised at 46 dpi.

Conflict of interest

The authors affirm that no financial or personal relationship existed that could have inappropriately influenced the content of this manuscript or the opinions expressed.

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4.3 Alternative sampling strategies for passive classical and African swine fever surveillance in wild boar

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Alternative sampling strategies for passive classical and African swine fever surveillance in wild boar

Abstract

In view of the fact that African swine fever (ASF) was recently introduced into the wild boar population of the European Union and that classical swine fever (CSF) keeps reoccurring, targeted surveillance is of utmost importance for early detection. Introduction of both diseases is usually accompanied by an increased occurrence of animals found dead. Thus, fallen wild boar are the main target for passive surveillance. However, encouraging reporting by hunters and sampling of these animals is difficult. Partly, these problems could be solved by providing a pragmatic sampling approach. For this reason, we assessed the applicability of three different dry/semi-dry blood swabs, namely a cotton swab, a flocked swab, and a forensic livestock swab, for molecular swine fever diagnosis. After nucleic acid extraction using manual and automated systems, routine quantitative real-time polymerase chain reactions (qPCR) were carried out. Results obtained from swabs or their fragments were compared to results generated from EDTA blood.

It was shown that reliable detection of both pathogens was possible by qPCR. Shifts in genome copy numbers were observed, but they did not change the qualitative results. In general, all swabs were suitable, but the forensic swab showed slight advantages, especially in terms of cutting and further storage. Robustness of the method was confirmed by the fact that different extraction methods and protocols as well as storage at room temperature did not have an influence on the final outcome. Taken together, swab samples could be recommended as a pragmatic approach to sample fallen wild boar.

Keywords

Passive surveillance, wild boar, swine fever, alternative sampling, swabs

1. Introduction

Both African and classical swine fever are among the most important and devastating viral diseases of domestic pigs and wild boar (Edwards et al., 2000; Sanchez-Vizcaino et al., 2013) and are notifiable to the World Organization for Animal Health (OIE). Recently, African swine fever (ASF) was introduced into the wild boar population of the European Union (EU), but also classical swine fever (CSF) keeps reoccurring (WAHID interface, visited May 10th 2014). As was observed with classical swine fever (CSF), disease occurrence in the wild boar population is often accompanied by spill over into the domestic pig population (Fritzemeier et al., 2000), with severe socio-economic consequences. Similar pattern were recently seen with African swine fever (ASF) that was also transmitted from wild boar to domestic pigs and back (Gogin et al., 2013). Only timely detection and intervention can lower the impact on both pig industry and wildlife and therefore, appropriate surveillance and warning systems are needed for countries at risk (De la Torre et al., 2013).

As introduction of both diseases into a naïve wild boar population is usually accompanied by high morbidity and mortality (Artois et al., 2002; Costard et al., 2013), and thus an increased occurrence of animals found dead, passive surveillance is crucial. However, the number of sample submissions from fallen wild boar is usually very low, even in times of increased risk. This could be partly due to the fact that sampling and/or transport of wild boar carcasses in various stages of decay is difficult and in some cases even nauseating. Thus, encouraging hunters to report and sample fallen wild boar could be facilitated by provision of an easy to handle and pragmatic sampling and transport approach.

In the presented pilot study, dry blood swabs were investigated. To this means, different swabs were immersed in EDTA blood samples from experimentally infected wild boar and domestic pigs and subsequently subjected to molecular swine fever diagnosis using different nucleic acid extraction methods and specific quantitative real-time polymerase chain reaction (qPCR) techniques. Preparatory methods were chosen to allow detection of both diseases at the same time. Additional samples were tested to assess field applicability and transferability to other sample matrices including organ swabs.

2. Materials and methods

2.1. Swabs

Three different swabs, namely a routine cotton swab (COPAN), a flocked swab (FLOQSwabs, COPAN), and a forensic livestock swab (Genotube, Prionics) were used.

2.2 Processing and testing of samples

As a first proof of concept experiment, the above mentioned cotton swabs were immersed in EDTA blood samples from experimentally infected animals (n=7 for ASF, collected at 4 dpi; n=11 for CSF, collected at 4, 5, 7, 10 dpi). Samples were chosen to represent animals in the clinical phase of infection and had been stored at -70°C until further use. The resulting blood swabs were stored three days (ASF) or over night (CSF) at room temperature to mimic sample transport without cooling. For nucleic acid extraction, swabs were dipped into the AVL buffer of the QIAamp Viral RNA Mini Kit (Qiagen) and used to stir it. After removal of the swab, all subsequent extraction steps were carried out according to the manufacturers instructions. A slight modification concerned the addition of an internal control DNA/RNA (5 µl per reaction with 2 x 105 copies per µl). Subsequently, qPCR or reverse transcription qPCR (RT-qPCR) was performed according to the protocols published by King et al.(2003) for ASF, and Hoffmann et al. (2005) for CSF. The PCR reactions were carried out using a Bio-Rad CFX Cycler (Bio-Rad Laboratories) and its accompanying software. Results were presented in a semi-quantitative way as quantification cycle (cq) values.

In a second pilot experiment, three different swabs (cotton, flocked and forensic) were used along with one manual and one automated nucleic acid extraction system. This time, swab fragments were subjected to nucleic acid extraction to ensure a retesting option.

For ASF, 10 samples from wild boar experimentally infected with ASF virus “Armenia08” (including samples from 6, 8 and 9 dpi) were used to soak the above mentioned swabs in parallel. After storage over night at room temperature, small fragments (about pinhead sized) were cut from the swabs with sterile scissors and used to extract nucleic acids through either the manual QIAamp Viral RNA Mini Kit (Qiagen) or the automated EZ1 Virus Mini Kit v2.0 (Qiagen) with slight modifications (modification details are available from the authors upon request). For CSF, 5 samples from animals infected with CSFV strains recently isolated in Israel (“CSF01047”, 2009, genotype 2.1) and Germany (“CSF1045”, 2009, genotype 2.3) were subjected to the protocol described above. Downstream handling was done as in the pilot trial, but here, a dilution series of a synthetic standard with known copy numbers was used to quantify genome copies in the respective samples. Results were compared among the

different swabs and extraction methods, and with results from EDTA blood samples (pre-existing data from the respective animal trials or parallel extractions).

Based on the results of the two pilot experiments, additional Genotube samples were tested for both ASF (n=30) and CSF (n=19). The parental blood samples were tested in parallel. The samples were chosen from the clinical phase of infection from both domestic pigs and wild boar. The CSFV panel included in addition a dilution series of a strong positive blood sample (1:2 to 1:1024) to assess the dose response. Here, pinhead-sized swab fragments were used for nucleic acid extraction using the QIAamp Viral RNA Mini Kit (Qiagen) after 24 h storage at room temperature. All samples were subsequently tested in the above mentioned PCR systems, and results were presented in a semi-quantitative way as quantification cycle (cq) values. In addition, flexibility and robustness were assessed through the inclusion of Genotube organ swabs. These swabs were taken from different blood and organ samples of experimentally ASFV and CSFV infected animals directly upon thawing of the material and after 7 days at 37°C in an incubator. Downstream handling was done as described above.

Finally, Genotube samples were generated from 96 negative wild boar blood samples of varying quality (almost optimal to dark haemolytic with impurities) to assess field applicability. In this case, nucleic acids were extracted using the QIAsymphony DSP Virus/Pathogen Mini Kit (Qiagen) on the respective instrument.

3. Results

In the first pilot trial, comparison of RT-PCR results showed no qualitative differences among EDTA and swab samples irrespective of the initial genome load. However, while quantitative ASF results were again comparable in terms of cq-values, marked shifts were observed between CSF blood and swab samples (see supplementary table 1). The latter showed up to 8 cq values difference.

Supplementary Table 1: Comparison of blood and blood swab samples in real-time PCR (cq-values) after nucleic acid extraction using the QIAamp Viral RNA Mini Kit (Qiagen). dpi = days post infection, gt = genotype

		QIAamp Viral RNA Mini Kit (Qiagen)	
		EDTA Blood	cotton swab, immersed in AVL-buffer
#76 - 4 dpi	CSF1045 gt 2.3	33.38	38.77
#76 - 7 dpi	CSF1045 gt 2.3	23.45	28.81
#75 - 4 dpi	CSF1045 gt 2.3	35.41	37.34
#75 - 7 dpi	CSF1045 gt 2.3	26.93	31.30
#100 - 7 dpi	CSF1045 gt 2.3	19.99	23.26
#100 - 10 dpi	CSF1045 gt 2.3	18.18	26.41
#167 - 7 dpi	CSF1045 gt 2.4	22.45	26.24
#90 - 4 dpi	CSFV "Koslov" gt 1.1	15.38	19.93
#90 - 5 dpi	CSFV "Koslov" gt 1.1	12.10	17.19
#91 - 4 dpi	CSFV "Koslov" gt 1.1	18.56	20.97
#91 - 5 dpi	CSFV "Koslov" gt 1.1	16.68	18.19
#HS1 - 4 dpi	ASPV Armenia08	19.84	19.50
#HS2 - 4 dpi	ASPV Armenia08	18.91	19.22
#HS 3 - 4 dpi	ASPV Armenia08	18.16	19.44
#HS 4 - 4 dpi	ASPV Armenia08	18.46	19.42
#HS 5 - 4 dpi	ASPV Armenia08	20.50	20.41
#HS 9 - 4 dpi	ASPV Armenia08	20.15	19.61
#HS 11 - 4 dpi	ASPV Armenia08	22.20	25.09

Comparing the different swabs of the second pilot experiment treated with the manual extraction method, PCR results were roughly similar with differences mainly within one log step for all swabs. In general, slightly higher genome loads were found in flocked and forensic swabs (see figure 1 and supplementary table 2). In case of ASF, comparison with parallel extractions from EDTA blood using the same methodology, did not reveal any qualitative differences. Nevertheless, quantitative divergence was obvious but stayed within one log step or slightly above (see figure 1 and supplementary table 2).

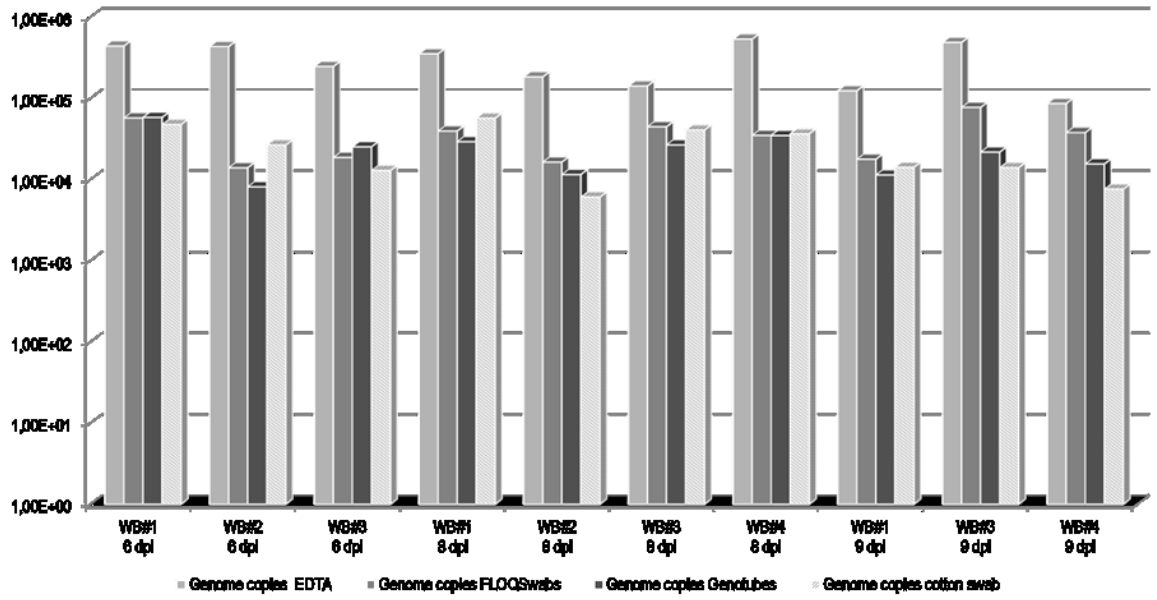

Fig. 1.: Comparison of blood and different blood swab samples after manual nucleic acid extraction using the QIAamp Viral RNA Mini Kit (Qiagen) and ASFV specific qPCR. Results are presented as genome copy numbers per µl based on a synthetic standard. Samples were taken from experimentally infected wild boar between days 6 and 9 post infection (dpi) with ASFV "Armenia08".

For CSF, qualitative results from EDTA blood were also confirmed using swab samples. Quantitative differences were observed in the same manner (see supplementary table 3). Comparison of different extraction methods using the same swabs showed no qualitative changes. Slightly higher genome loads were detected upon nucleic acid extraction using the EZ1 automated system for both ASF and CSF (see figure 2 and supplementary table 4 for ASF, and supplementary table 3 for CSF). One Genotube sample showed negative results upon EZ1 extraction in both target and internal control PCR.

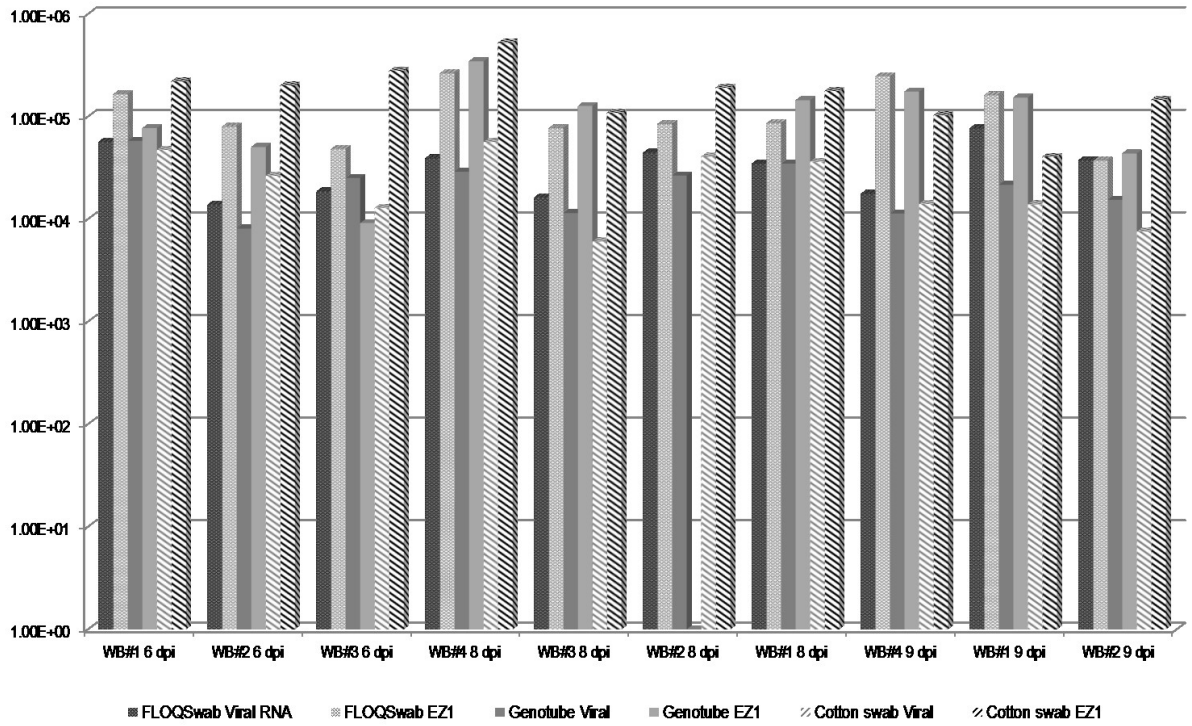

Fig. 2.: Comparison of different blood swab samples after manual and automated nucleic acid extraction and ASFV specific qPCR. The manual extraction was carried out using the QIAamp Viral RNA Mini Kit (Qiagen), while automated extraction was performed employing the EZ1 Virus Mini Kit v2.0 (Qiagen). Results are presented as genome copy numbers per µl based on a synthetic standard. Samples were taken from experimentally infected wild boar between days 6 and 9 post infection (dpi) with ASFV "Armenia08".

Testing of the additional Genotube samples showed matching qualitative results (see figures 3 and 4), and quantification cycle values differed in the range seen in the pilot trials. Testing of the CSF dilution series showed the expected dose response in both EDTA blood and Genotube samples (see figure 4). Using blood and organ samples from experimentally infected animals, Genotubes were shown to be suitable for organ swabbing, even if the samples were stored for 7 days at 37°C (see supplementary table 4). No qualitative differences occurred. However, blood samples showed the lowest variance. Comparative studies with FTA cards showed similar results on ASF samples stored for one week at 37°C (see supplementary table 4). All field samples gave negative results in the ASFV and CSFV specific PCRs while detection of internal controls was possible.

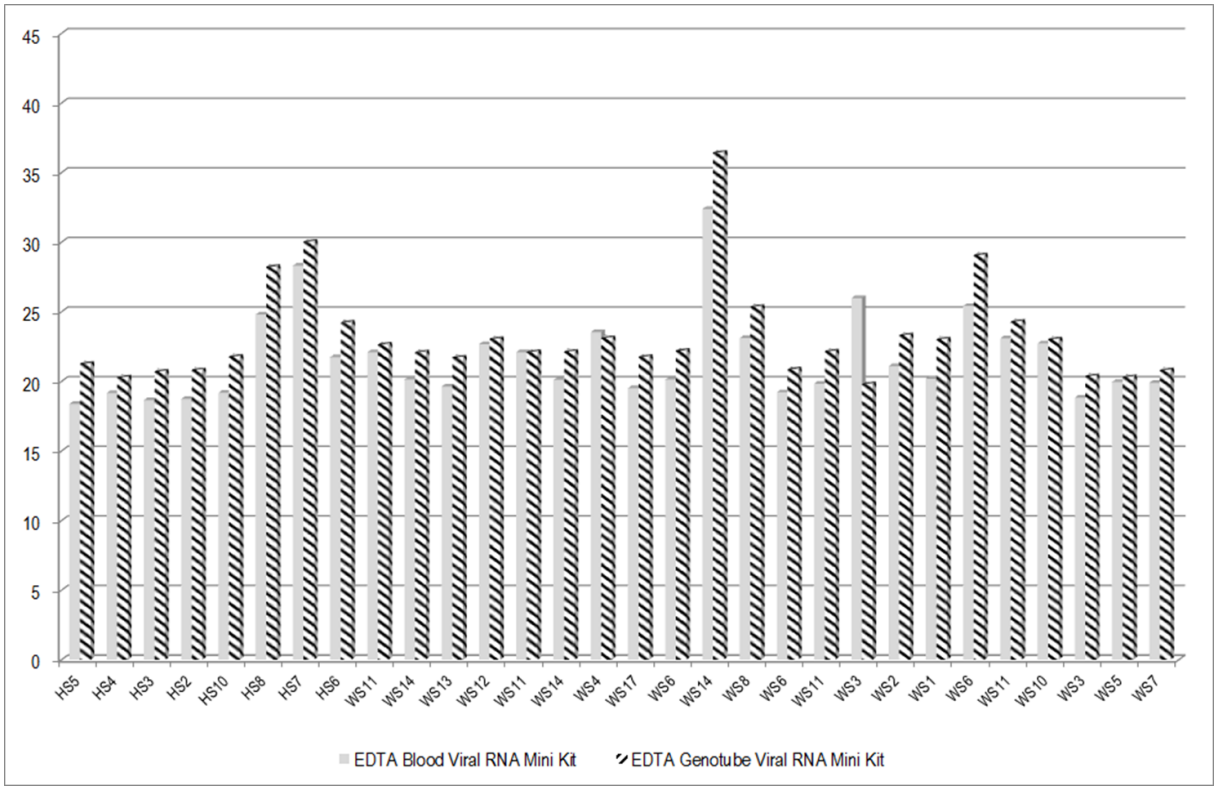

Fig. 3.: Testing of ASFV forensic swab (Genotube) samples in comparison with the parental EDTA blood sample after nucleic acid extraction using the QIAamp Viral RNA Mini Kit (Qiagen). Results are depicted as quantification cycle values.

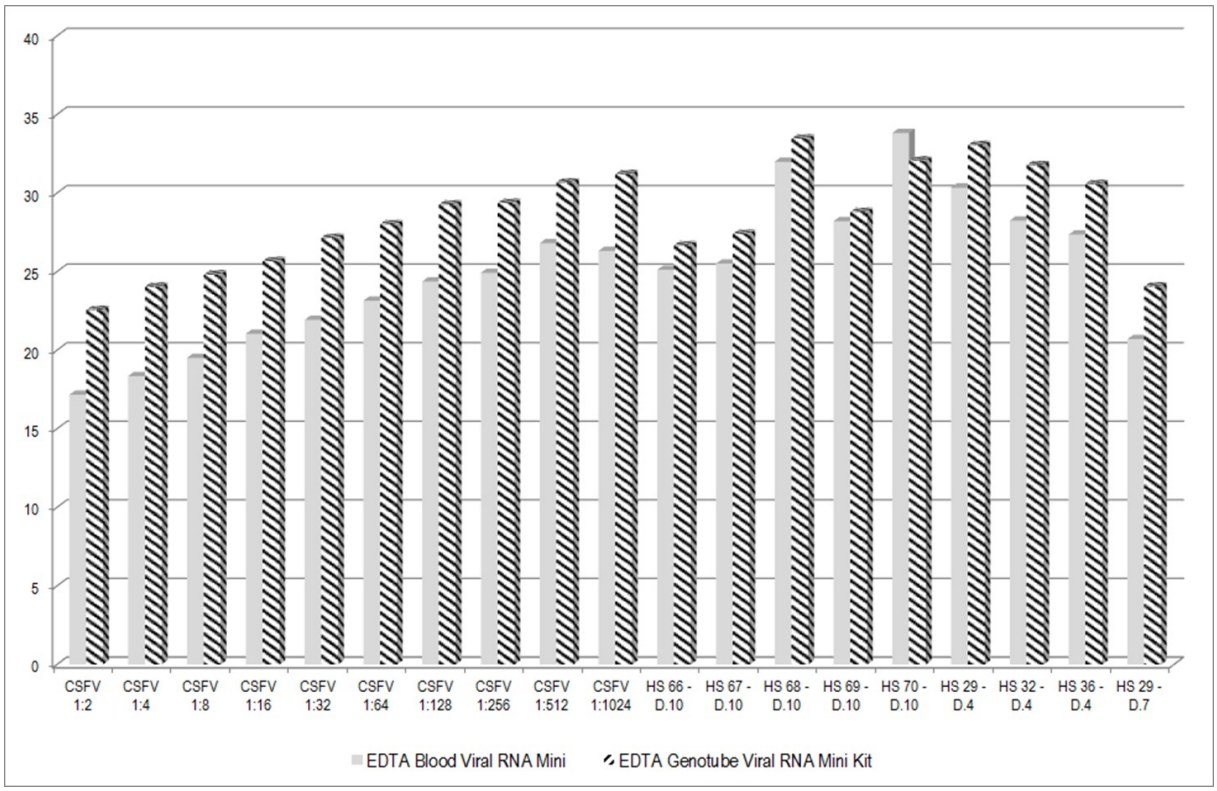

Fig. 4.: Testing of CSFV forensic swab (Genotube) samples in comparison with the parental EDTA blood sample after nucleic acid extraction using the QIAamp Viral RNA Mini Kit (Qiagen). Results are depicted as quantification cycle values.

4. Discussion

The recent introduction of ASF into the wild boar population of the EU has stressed the necessity of targeted surveillance and early warning actions (De la Torre et al., 2013). Based on the assumption that an increase in wild boar mortality could be observed upon introduction of either ASF or CSF, fallen wild boar are the primary target for passive surveillance. In most countries, hunters will observe and sample fallen wild boar and thus, early warning relies on their compliance. Lowering the effort involved in sampling and transport could increase willingness to cooperate.

Among the options are different swab samples that could be send via ordinary mail or courier to the competent laboratory. In general, rectal (faecal) or oropharyngeal swabs are easiest to obtain. Yet, these samples were shown to contain much lower genome loads than organs or blood, especially in the case of ASF (Blome et al., 2013). In contrast, dry blood samples, e.g. on filter papers or FTA cards (Braae et al., 2013; Michaud et al., 2007), have been shown to contain high viral loads and work for both RNA and DNA viruses. While filter papers would still need an additional device to drop blood onto the device or to somehow soak it, swab sampling would minimize the necessary contact to the carcass (that can remain in the ecosystem while the region is free from swine fever) and allow shipment in the swab tube.

In the presented study, different swabs were tested for their suitability along with three different nucleic acid extraction methods to assess robustness of the approach. The most promising swab was tested in more detail.

It was shown that both ASFV and CSFV could be detected in nucleic acids derived from swab samples irrespective of the nucleic extraction method. In general, both testing of swabs by just immersing them in lysis buffer or by cutting small fragments was possible. To allow a retesting of samples in case of doubtful results or technical problems, fragment generation and testing is recommended. In this regard, the forensic Genotube swab had the advantage that cutting a suitable fragment was easiest and least contamination prone. Moreover, this device has been shown to be suitable for long term storage of ASF samples (K. Goller, unpublished data), and retesting of CSF samples after 35 days and two months confirmed stability also for

the viral RNA (data not shown). In terms of genome load, the flocked swab was slightly superior (figure 1 and supplementary table 2), but fragment generation was most difficult.
As expected, shifts in genome load were seen when comparing results from EDTA blood and the corresponding swabs. These differences were in the range of roughly one log step (about 3 cq-values) for ASFV and slightly higher for CSFV. When judging on these differences and their impact on the fitness for purpose, it has to be kept in mind that the relevant fallen animals died from the disease in question and will therefore contain very high copy numbers of the respective pathogen. For this reason, the loss of sensitivity is probably acceptable, even if field samples would mean swabs of reddish fluid from any accessible part of the body rather than blood like in the presented study. In this regard, testing of field samples showed that even suboptimal samples did not negatively influence the diagnostic specificity (100 % based on 96 field blood samples from shot wild boar). Flexibility of the approach was further proven through inclusion of different organ samples. Although blood samples were again shown to be most robust, all organ samples from acutely infected animals were found positive in qPCR, even after seven days at 37°C. Here, results were comparable with FTA cards. A general drawback of swab (or FTA card) sampling alone is the loss of syndromic surveillance that could be carried out on the whole carcass. However, this drawback might be tolerable if the number of samples for targeted swine fever surveillance can be considerably increased. Suitability for other diseases should be tested as it can be assumed that other pathogens would be also detectable.

5. Conclusions

Blood swabs are suitable for a reliable ASF and CSF virus detection. Different swabs and extraction protocols could be robustly used. Forensic swabs showed advantages in terms of fragment preparation and further storage. Swabbing of organs is possible where no blood is available.
Taken together, swab samples could be recommended as a pragmatic approach to sample especially fallen wild boar for passive swine fever diagnosis.

Supplementary Table 2: Comparison of blood and different blood swab samples for ASF virus detection. Results of qPCR are presented as cq values and genome copy numbers per µl. The table includes results obtained from EDTA blood after nucleid acid extraction using the QIAamp DNA Mini Kit. These results were obtained in the framework of the animal trial. WB = wild boar; dpi = days post infection

			QIAamp DNA Mini		QIAamp Viral RNA Mini							
			EDTA blood 200 µl		EDTA blood parallel 75 µl		FLOQSwab, fragment		Genotube, fragment		cotton swab, fragment	
Animal	Type	dpi	cq	copies/µl	cq	copies/µl	cq	copies/µl	cq	copies/µl	cq	copies/µl
Bache 3	WB, adult	6 dpi	14.87	3.12E+06	17.60	4.38E+05	20.66	5.69E+04	20.64	5.77E+04	20.94	4.73E+04
Paula	WB, adult	6 dpi	15.75	1.75E+06	17.63	4.31E+05	22.78	1.39E+04	23.59	8.07E+03	21.81	2.64E+04
Fridolin	WB, piglet	6 dpi	15.87	1.62E+06	18.47	2.45E+05	22.34	1.86E+04	21.89	2.51E+04	22.89	1.28E+04
Keiler	WB, adult	8 dpi	15.02	2.83E+06	17.93	3.51E+05	21.22	3.92E+04	21.67	2.90E+04	20.67	5.64E+04
Fridolin	WB, piglet	8 dpi	16.71	9.35E+05	18.92	1.82E+05	22.54	1.63E+04	23.06	1.14E+04	24.02	6.03E+03
Paula	WB, adult	8 dpi	15.71	1.67E+06	19.30	1.41E+05	21.04	4.43E+04	21.80	2.65E+04	21.17	4.05E+04
Bache 3	WB, adult	8 dpi	15.83	1.80E+06	17.31	5.32E+05	21.40	3.48E+04	21.41	3.46E+04	21.34	3.62E+04
Keiler	WB, adult	9 dpi	19.50	1.51E+05	19.51	1.23E+05	22.41	1.77E+04	23.08	1.13E+04	22.76	1.41E+04
Bache 3	WB, adult	9 dpi	15.92	1.57E+06	17.45	4.85E+05	20.21	7.67E+04	22.12	2.16E+04	22.77	1.39E+04
Paula	WB, adult	9 dpi	21.84	3.28E+04	20.04	8.59E+04	21.28	3.76E+04	22.62	1.54E+04	23.68	7.57E+03

Supplementary Table 3: Comparison of blood and different blood swab samples for detection of CSFV genome. Results include different extraction protocols. RT-qPCR results are depicted as cq values and/or genome copy numbers per µl. n.d. = not done

		QIAamp Viral RNA	QIAamp Viral RNA		EZ1 AL-Puffer		QIAamp Viral RNA		EZ1 AL-Puffer		QIAamp Viral RNA		EZ1 AL-Puffer	
Animal	Inoculum	EDTA blood	FLOQSwab, fragment		FLOQSwab, fragment		Genotube, fragment		Genotube, fragment		cotton swab, fragment		cotton swab, fragment	
365	CSFV "CSF1047", gt 2.1	26.43	27.77	1.02E+04	28.26	7.54E+03	26.91	1.74E+04	31.32	1.12E+03	29.54	3.40E+03	30.27	2.16E+03
366	CSFV "CSF1047", gt 2.1	23.09	25.37	4.54E+04	26.11	2.87E+04	24.27	9.00E+04	29.24	4.10E+03	27.48	1.23E+04	28.20	7.81E+03
367	CSFV "CSF1047", gt 2.1	26.89	29.54	3.39E+03	30.17	2.30E+03	32.24	6.32E+02	33.76	2.47E+02	31.90	7.80E+02	32.23	6.39E+02
368	CSFV "CSF1047", gt 2.1	25.07	27.51	1.20E+04	28.21	7.76E+03	26.31	2.54E+04	30.00	2.56E+03	27.88	9.56E+03	28.55	6.27E+03
76	CSFV "CSF1045", gt 2.3	23.45	n.d.	n.d.	27.55	1.17E+04	28.22	7.69E+03	30.44	1.94E+03	27.64	1.11E+04	30.27	2.16E+03

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Conflict of interest statement

The authors declare that they have no competing interests.

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Letter to the Editor

4.4 Alternative sampling strategies for passive classical and African swine fever surveillance in wild boar – Extension towards African swine fever virus antibody detection

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Alternative sampling strategies for passive classical and African swine fever surveillance in wild boar – Extension towards African swine fever virus antibody detection

To the Editor:

We recently reported on the use of blood swab samples for passive classical and African swine fever (CSF and ASF) surveillance in wild boar (Petrov et al., in press). Upon availability of the article online, we were asked by national and international colleagues whether this approach would be suitable also for antibody detection.

While antibody detection might not be the primary focus of diagnostic investigations in fallen animals, we think that an approach that would allow both pathogen and antibody detection in one easy-to-collect sample matrix, combined with simple shipment, and long-term storage, would be optimal under field conditions.

For this reason, we tried to rapidly answer this question on a limited set of sero-positive and sero-negative blood samples from animal experiments. Given the current epidemiological situation of ASF in the European Union wild boar population (cases in several Eastern Member States with a tendency to spread, see OIE WAHID), we feel that the audience of Veterinary Microbiology would benefit from a brief addendum to the above mentioned article. For this reason, we report on the outcome of our initial studies here, while further validation is still in progress.

Study design and outcome

A total of 42 porcine EDTA blood samples was employed to test applicability of swab fragments for antibody detection. The expected status of the blood sample was related to the corresponding serum sample of the same animal and sampling day. The result of the p72 antibody ELISA (Ingezim PPA Compac, Ingenasa, Madrid, Spain) was used as a reference. Genotube swabs (Prionics, Zurich, Switzerland), were dipped into the respective blood sample and left to dry for at least 12 h at room temperature. Thereafter, diamond-shaped fragments (app. 5 mm lateral length) were cut with sterile scissors and transferred to the ELISA system. To test samples close to the “worst-case-scenario”, 30 samples were included that had been stored for more than 21 months at 4°C. This set comprised 12 samples from sero-negative animals, and 18 samples from sero-positive animals. The latter had been immunized twice with an inactivated preparation of genotype II ASFV Armenia08 (Blome et

al., 2014). Samples were included from days 28 to 41 post immunization. The second set of samples comprised animals that had been inoculated with ASFV OURT88/3 (genotype I, non-hemadsorbing). These samples (n = 10) had been taken 29 days post inoculation and were stored approximatelyone month at 4°C. Also here, negative animals (n=2) were included. To compare the performance with dried blood on filter papers as foreseen in the ELISA protocol (see below), we tested 14 samples also on this matrix (the second set of samples and two long-term storage samples, see table 1).

The commercially available ID Screen® African Swine Fever Indirect antibody ELISA (ID.vet, Grabels, France) allows a protocol for dried blood on filter papers. We used this protocol to test the swab fragments. The original protocol foresees the use of two filter paper punches with a diameter of 6 mm. We replaced them with two of the above mentioned Genotube fragments and performed all subsequent steps according to the manufacturer's instructions.

Based on the above mentioned set of samples, we could clearly demonstrate that antibody detection is possible also from Genotube swabs (see table 1). Fourty out of 42 samples were in complete agreement with the serological status, and an additional sample that had a doubtful status was detected positive. Only one doubtful sample gave a negative result. Comparison of dried blood on filter paper and on Genotube swabs gave similar results (see table 1), also in terms of raw data values (data not shown). No false positive reactions occurred, even with samples stored for several months (see table 1).

Table 1: EDTA blood sample details and results. The status of the sample was defined by a p72 antibody ELISA (Ingezim PPA Compac, Ingenasa) of the corresponding serum sample. The storage time is depicted in month (M). DPI = days post inoculation; neg = negative according to the test criteria; dbt = doubtful according to the test criteria; pos = positive according to the test criteria; nd = not done; inact. = inactivated

Genotube	Animal ID	DPI	Storage	Virus	Status	Result swab	Result filter
1	HS1	0	21M	/	neg	neg	nd
2	HS2	0	21M	/	neg	neg	nd
3	HS3	0	21M	/	neg	neg	nd
4	HS4	0	21M	/	neg	neg	nd
5	HS5	0	21M	/	neg	neg	nd
6	HS6	0	21M	/	neg	neg	nd
7	HS7	0	21M	/	neg	neg	nd
8	HS8	0	21M	/	neg	neg	nd
9	HS9	0	21M	/	neg	neg	nd
10	HS11	0	21M	/	neg	neg	nd
11	HS12	0	21M	/	neg	neg	nd
12	HS13	0	21M	/	neg	neg	nd
13	HS3	28	21M	Armenia08 inact.	neg	neg	nd
14	HS4	28	21M	Armenia08 inact.	neg	neg	nd
15	HS6	28	21M	Armenia08 inact.	neg	neg	nd
16	HS7	28	21M	Armenia08 inact.	**pos**	**pos**	nd
17	HS8	28	21M	Armenia08 inact.	**pos**	**pos**	nd
18	HS9	28	21M	Armenia08 inact.	**pos**	**pos**	nd
19	HS11	28	21M	Armenia08 inact.	**pos**	**pos**	nd
20	HS8	35	21M	Armenia08 inact.	**pos**	**pos**	nd
21	HS12	28	21M	Armenia08 inact.	**pos**	**pos**	nd
22	HS13	28	21M	Armenia08 inact.	**pos**	**pos**	nd
23	HS4	41	21M	Armenia08 inact.	*dbt*	neg	nd
24	HS6	41	21M	Armenia08 inact.	*dbt*	**pos**	nd
25	HS7	41	21M	Armenia08 inact.	**pos**	**pos**	nd
26	HS8	41	21M	Armenia08 inact.	**pos**	**pos**	nd
27	HS9	41	21M	Armenia08 inact.	**pos**	**pos**	nd
28	HS11	41	21M	Armenia08 inact.	**pos**	**pos**	nd
29	HS12	41	21M	Armenia08 inact.	**pos**	**pos**	**pos**
30	HS13	41	21M	Armenia08 inact.	**pos**	**pos**	**pos**
31	HS1	29	1M	OURT88/3	**pos**	**pos**	**pos**
32	HS2	29	1M	OURT88/3	**pos**	**pos**	**pos**
33	HS3	29	1M	OURT88/3	**pos**	**pos**	**pos**
34	HS4	29	1M	OURT88/3	**pos**	**pos**	**pos**
35	HS5	29	1M	OURT88/3	**pos**	**pos**	**pos**
36	HS6	29	1M	OURT88/3	**pos**	**pos**	**pos**
37	HS7	29	1M	OURT88/3	**pos**	**pos**	**pos**
38	HS8	29	1M	OURT88/3	**pos**	**pos**	**pos**
39	HS9	29	1M	OURT88/3	**pos**	**pos**	**pos**
40	HS10	29	1M	OURT88/3	**pos**	**pos**	**pos**
41	HS1	0	1M	/	neg	neg	neg
42	HS2	0	1M	/	neg	neg	neg

Despite the fact, that further validation is clearly needed and ongoing, these initial results are most promising and could prompt the inclusion of antibody detection from swabs in the field. Due to the very high virulence of the ASFV strains currently circulating in Eastern Europe (Gabriel et al., 2012; Blome et al., 2013), antibody detection is still a rather rare finding. However, to obtain a full picture of the epidemiological situation, and to fulfill all legal requirements (e.g. Commission Decision 2003/422/EC), the search for antibodies is mandatory. Another important issue would be to isolate the causative virus strains for further characterization. In this respect, preliminary studies showed that ASFV isolation from Genotube swabs was possible in blood monocyte derived macrophage cultures while CSFV could not be isolated (data not shown). Probably, the latter could be obtained from RNA transfection.

Easy sampling and testing by using swabs for both pathogen and antibodies could facilitate this task and present a pragmatic approach also for other scenarios, e.g. for wild-life monitoring in Africa.

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4.5 Development and validation of a harmonized TaqMan-based triplex real-time RT-PCR protocol for the quantitative detection of normalized gene expression profiles of seven porcine cytokines

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Development and validation of a harmonized TaqMan-based triplex real-time RT-PCR protocol for the quantitative detection of normalized gene expression profiles of seven porcine cytokines

Abstract

Dysregulation of cytokine responses plays a major role in the pathogenesis of severe and life-threatening infectious diseases like septicemia or viral hemorrhagic fevers. In pigs, diseases like African and classical swine fever are known to show exaggerated cytokine releases. To study these responses and their impact on disease severity and outcome in detail, reliable, highly specific and sensitive methods are needed. For cytokine research on the molecular level, real-time RT-PCRs have been proven to be suitable. Yet, the currently available and most commonly used SYBR Green I assays or heterogeneous gel-based RT-PCRs for swine show a significant lack of specificity and sensitivity. The latter is however absolutely essential for an accurate quantification of rare cytokine transcripts as well as for detection of small changes in gene expressions. For this reason, a harmonized TaqMan-based triplex real-time RT-PCR protocol for the quantitative detection of normalized gene expression profiles of seven porcine cytokines was designed and validated within the presented study. Cytokines were chosen to represent different immunological pathways and targets known to be involved in the pathogenesis of the above mentioned porcine diseases, namely interleukin (IL)-1β, IL-2, IL-4, IL-6, IL-8, tumor necrosis factor (TNF)-α and interferon (IFN)-α. Beta-Actin and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) served as reference genes for normalization. For absolute quantification a synthetic standard plasmid was constructed comprising all target cytokines and reference genes within a single molecule allowing the generation of positive control RNA. The standard as well as positive RNAs from samples, and additionally more than 400 clinical samples, which were collected from animal trials, were included in the validation process to assess analytical sensitivity and applicability under routine conditions.

The resulting assay allows the reliable assessment of gene expression profiles and provides a broad applicability to any kind of immunological research in swine.

1. Introduction

Cytokines are important mediators that orchestrate cellular functions including inflammatory responses and innate immune reactions. However, excessive activation of the innate immune system in response to pathogens can lead to pathological inflammatory consequences [1], and dysregulation of cytokine responses plays a major role in the pathogenesis of severe and life-threatening infectious diseases including viral haemorrhagic fevers [2]. Another example is the "cytokine storm" that is held responsible for the exceptionally high morbidity and mortality in human highly pathogenic influenza virus infections [3]. Hence, cytokine profiles, especially when targeting a set of cytokines expressed within a certain microenvironment [4, 5], can provide important insights into the development of infectious diseases, which are characterised by an immune pathogenesis such as classical swine fever (CSF), a severe porcine infection that can be accompanied by haemorrhagic lesions. For CSF, a cytokine dysregulation is suspected to be decisive for clinical severity [6]. Similar responses are known for African swine fever (ASF) [7], a disease that recently gained importance through its introduction into several Eastern European countries [8, 9].

Cytokines can be targeted at various levels, from assessment of cellular expression profiles using mRNA detection by RT-PCR, to measurement of intracellular proteins by fluorescence-activated cell sorter staining and quantification of secreted cytokine proteins by the use of bioassays, enzyme-linked immunosorbent assays, radioactive immunosorbent assays, and microarrays [10]. For pathogenesis studies, a combination of expression and protein detection methodologies is usually advisable. However, assessment of expression profiles in pigs is so far severely hampered by the lack of fully validated and reliable diagnostic tools. While several PCR systems for porcine cytokine detection were developed during the last years, most of them either comprised non-standardised heterologous conventional RT-PCR systems [11-13] or were performed using intercalating fluorescent dyes such as SYBR-Green I [14-18]. However, these techniques have clear disadvantages compared to TaqMan based PCRs particularly with regard to the lack of sensitivity and specificity which are however essential for the accurate quantification of rare cytokine transcripts and the detection of small changes in gene expression.

With the pig as target species, the presented study reports on the design and validation of a harmonized approach for specific detection of cytokine gene expression profiles in swine.

Cytokines were chosen to represent different reaction pattern of the immune system (Th1 and Th2 responses), and mediators that are known to be involved in the pathogenesis of important porcine infections such as CSF. To this means, a harmonized multiplexed one-step TaqMan 5' nuclease [19] protocol for specific detection and quantification of seven cytokines, namely interleukin (IL)-1β, IL-2, IL-4, IL-6, IL-8, tumor necrosis factor (TNF)-α and interferon (IFN)-α, was designed and validated. Two reference genes were included to allow reliable normalization.

2. Materials and methods

2.1 Selection of primers and probes

Primers and probes for seven porcine cytokines (IL-1β, IL-2, IL-4, IL-6, IL-8, IFN-α, TNF-α) and two reference genes (β-Actin, GAPDH) were either selected from previous studies [20-25] or designed using "Primer-BLAST" (NCBI GenBank). Sequences and corresponding references are shown in table 1. For corresponding alignments Mega 5- and BioEdit software (IBIS Biosciences Carlsbad, USA) were utilized. For all cytokines, probes were labeled with 6-Carboxyfluorecein (FAM), the β-Actin probe with hexachloro-6-carboxyfluorescein (HEX) and the GAPDH probe with Texas Red (TR). The synthesis of oligonucleotides was carried out by biomers.net (Ulm, Germany).

2.2 In vitro generation of positive control RNA

2.2.1 Generation of peripheral blood mononuclear cells (PBMCs)

Approximately 50 ml of porcine EDTA blood were overlayed with the equal amount of lymphocyte separation medium LSM 1077 (PAA Laboratories GmBH, Pasching, Austria) and a density gradient centrifugation at 580 *g* for 40 min at 20°C without brake was performed. The leucocyte phase was collected and washed with 0.8 mM EDTA solubilized in phosphate buffered saline (PBS^-) for removal of separation medium. Thereafter, the remaining erythrocytes were removed through lysis with buffered ammonium chloride solution (containing 153mM NH_4Cl, 10mM KHCO3, 1 mM EDTA to 1 l (pH 7.4)). To this means, the threefold volume of lysis buffer was added and incubated at 4°C for 15 min. The resulting PBMC suspension was washed with PBS^- and cultured in DMEM medium containing 10% fetal bovine serum, 20 mM HEPES including penicillin-streptomycin ("Anti-Anti (100X)"), Antibiotic-Antimycotic from GIBCO by Life Technologies, Carlsbad, California, USA) at

approximately 10^7 cells/ml. After 16 h of incubation at 37°C in a 5% CO_2 atmosphere, non-adherent cells where removed through washing with sterile pre-warmed PBS^- , and the cleaned PBMC cell suspension was incubated for one to three days for maturation under the same culture conditions until exposure to different cytokine-stimulators.

2.2.2 In-vitro stimulation of cytokines

Different mitogens and antigens were used for stimulation of the desired cytokines as previously described [26-29]. Details are depicted in table 2. The stimulating agents were obtained from Sigma (Sigma-Aldrich, St. Louis, Missouri, USA). After a maturation time for PBMCs of one to three days, stimulators were utilized at the following concentrations: Lipopolysaccharide (LPS) 20 µg/ml, all other stimulating agents (Peptidoglycan, PGN; Concanavalin A, ConA; phytohaemagglutinin; PHA; pokeweed mitogen, PWM) 5 µg/ml. Along with the stimulating agents, cells were incubated for approximately 18 h at 37°C with 5% CO_2 in 6-well plates until the expected cytokine expression optimum was reached. For RNA extraction, cells were harvested and subjected to RNA extraction using the methods described below.

Table 2: In-vitro cytokine stimulation

The stimulating agents are presented along with the corresponding target cytokines as well as background information about their functionality.

LPS=*Salmonella typhimurium* lipopolysaccharid; PGN= *Staphylococcus aureus* peptidoglycan; ConA= Concanavalin A; PWM=Pokeweed mitogen; PHA= Phytohemagglutinin

Cytokine	Stimulating agents	Manner of stimulation
IL-1β	LPS	– LPS: a component of the outer gram positive bacteria membrane, antigenic effect on PBMCs PGN: a stabilizing macro molecule in the cell wall of gram positive bacteria; antigenic effect on PBMCs – ConA: a lectin from the jack bean, mitogenic effect (especially on T-cells) – PHA: a herbal lectin, mitogenic effect (especially on T-cells) – PWM: a lectin of the American pokeweed, activating effect on B- and T-cells
IL-4	PWM + ConA + PHA	
IL-2	PWM + ConA + PHA	
IL-6	PWM + ConA + PHA	
IL-8	LPS	
IFN-α	PGN + LPS + ConA	
TNF-α	PGN + LPS + ConA	

2.3 RNA isolation

RNA extraction of different sample matrices was performed using Trizol Reagent (Life Technologies) in combination with the automated MagAttract Virus Mini M48 Kit (QIAGEN GmbH, Hilden, Germany) on the King Fisher 96 Flex instrument (Thermo Scientific) as previously described [30].

2.4 Analyses of expression stability of reference genes

Confirmatory analyses of stable expressions of β-Actin and GAPDH comprised the following tests. Firstly, *in vivo* generated PBMCs (see section 2.2.1) were exposed to different stimulating agents (see section 2.2.2 and table 2) while several wells were left untreated by incubating them only with cell culture media each time. PBMC RNA was extracted in different time intervals after stimulation (after 1, 12, 18, 24, 36, 48 and 60 hours) and RT-qPCRs targeting β-Actin and GAPDH were performed comparing the quantification cycle (Cq)-values of stimulated and untreated PBMC RNA. Secondly, RNA from EDTA blood derived from pigs infected with the highly virulent CSFV-strain "Koslov" were used in RT-qPCR. Cq-values of β-Actin and GAPDH were detected prior to infection and compared to measurements at different time intervals after infection.

2.5 Construction of synthetic standard RNA

A synthetic gene comprising all target cytokines and reference genes (see figure 1) was constructed and synthesized by GeneArt Gene Synthesis (Life Technologies). The Kanamycin-resistant gene was transformed in corresponding resistant bacteria after permeabilization. The bacteria plasmid was purified with QIAfilter Plasmid Maxi Kit (Qiagen, Venlo, Netherlands) and the nucleic acid concentration was determined with a NanoDrop 2000c Spectrophotometer (PEQLAB Biotechnologie GmbH, Polling, Austria). To verify the transformation process the plasmid was sequenced using the Big Dye Terminator v1.1 Cycle sequencing Kit (Applied Biosystems). Nucleotide sequences were read with an automatic sequencer (3130 Genetic Analyzer, Applied Biosystems) and analyzed using the Genetics Computer Group software version 11.1 (Accelrys Inc., San Diego, USA). Thereafter, the DNA-plasmid was used for synthesis of heterologous RNA. It was cleaved at the attached NOD1-restriction site with NOD1 enzyme (New England Biolabs, Ipswitch, Massachusetts, USA) and linearized DNA strands were filtrated and eluted by using

QIAquick Nucleotide Removal Kit (Qiagen). The obtained DNA was in vitro transcribed using T7 RNA Polymerase (Promega Corporation, Madison, USA). Subsequently, the DNA matrix was removed through DNase I digestion (RQ1 RNase-Free DNase, Promega). The gained RNA was visualized by 1% agarose gel electrophoresis. RNeasy Mini Kit (Qiagen) was utilized in combination with Trizol Reagent (Life Technologies) and DNA digestion (with RNase-Free DNase Set (Qiagen)) for a final RNA cleanup. Finally, RNA concentration was determined using the NanoDrop spectrophotometer (Peqlab) and the concentration was set to $2x10^9$ copies/µl. A 10-fold standard dilution series was generated in RNA-safe buffer (50 ng/µl of carrier polyA-RNA, 0.05% Tween-20, 0.05% sodium azided in RNase-free water). Dilutions from $2x10^1$ to $2x10^6$ copies/µl were employed for subsequent RT-qPCRs.

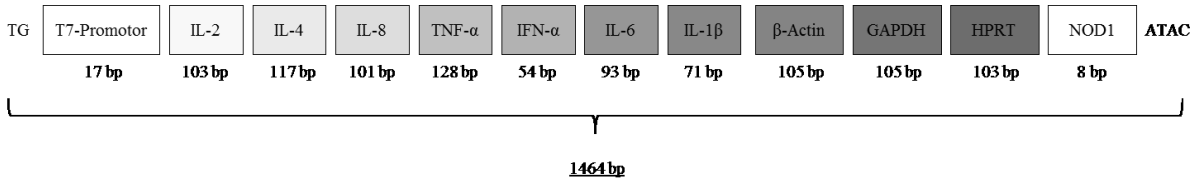

Figure 1: Composition of the synthetic standard gene comprising all target cytokines (IL-2, IL-4, IL-8, TNF-α, IFN-α, IL-6, IL-1β) and internal reference genes (β-Actin, GAPDH, HPRT (Hypoxanthin-Guanin-Phosphoribosyltransferase), starting with the T7-promotor sequence and concluding with the NOD1 restriction site as initial point for linearization and transformation to RNA. Each target cytokine was included with a nucleotide overhang of approximately 50 base pairs (Takamatsu et al.) prior to forward primer sequence. In total, the synthetic standard gene comprises 1464 bp.

2.6 RT-qPCR

Prior to implementation in RT-qPCR systems, 10-fold dilution series of each *in-vitro* generated positive control RNA and the synthetic standard RNA were amplified in conventional RT-PCR, visualized in 3% agarose gel electrophoresis and verified by sequencing. Sequence data were obtained from the NCBI GenBank and corresponding alignments were carried out with Mega 5- and BioEdit software (IBIS Biosciences Carlsbad, USA).

In a first step, single-tube assays were designed for each cytokine or reference gene as basis for the subsequent development of the triplex RT-qPCR protocol using the AgPath-ID One-

Step RT-PCR reagents (Ambion-Applied Biosystems, Thermo Fisher Scientific by Life technologies) for simultaneous detection of one target cytokine and two reference genes.

Subsequently, tests for the reduction of the total mastermix reaction volume from 25 µl to 12.5 µl were carried out. Thereby, the mastermix for a single reaction comprised 0.25 µl RNase-free water, 6.25 µl 2X RT- PCR Buffer, 0.5 µl of 25X RT- PCR Enzyme Mix, 1 µl FAM-labelled cytokine-primer-probe mix, 1 µl of each reference gen-primer-probe mix and finally 2.5 µl of template RNA.

All RT-qPCRs were performed with a Bio-Rad CFX 96 Real-Time Detection Systems (Bio-Rad, Hercules, CA, USA). Protocols for all cytokine RT-qPCRs were adjusted to the same thermal profile: reverse transcription at 45°C for 10 min, followed by PCR activation for 10 min at 95°C and 45 cycles including denaturation phase at 95°C for 15 sec, annealing at 57°C for 20 sec and elongation for 30 sec at 72°C. Data were collected during the annealing phase.

Oligonucleotide concentrations were optimized through checkerboard titrations. Furthermore, confirmatory tests for the absence of residual DNA in isolated RNA samples were performed by deployment of the one- and two-step RT-qPCR chemistry from Promega. For that purpose, RNA samples were tested using both systems under the same conditions while the two-step assay was carried out without adding the enzyme for reverse transcription.

Moreover, reproducibility of all assays was tested using the standard RNA in triplicates and deviations of Cq-value were determined.

In addition, 402 samples gathered from pigs infected or vaccinated with CSFV (leucocyte samples) and infected with ASFV (EDTA blood samples) during several animal trials carried out at the Institute of Diagnostic Virology, Friedrich-Loeffler-Institut, Insel Riems, Germany (sample repository) were tested in all seven triplex RT-qPCRs (see table 3). In the experimental studies involving live animals, all applicable animal welfare regulations, including EU Directive 2010/63/EC and institutional guidelines, were taken into consideration. The animal experiments were approved by the competent German authority (Landesamt für Landwirtschaft, Lebensmittelsicherheit und Fischerei Mecklenburg-Vorpommern) under reference numbers 7221.3-1.1-015/12 and 7221.3-1.1-018/12.

Table 3: Samples from different animal trials (n=402) used for assay validation. Samples were chosen to represent different pig species (wild boar, domestic pigs) and inoculation status (CSFV infection/ vaccination, ASFV infection, corresponding control animals). Moreover, PBMC and EDTA blood samples were included.

Sample status	Sample matrix	Domestic pigs	Wild boar
Control	EDTA	3	3
	PBMC	49	24
CSFV infected	PBMC	109	45
CSFV vaccinated	PBMC	97	55
ASFV infected	EDTA	17	/

3. Results

3.1 Confirmation of identity and stable expression of reference genes

Identity of all cytokines and reference genes could be confirmed by alignments with sequences available through NCBI GenBank. No indications for possible cross-reactions were observed. Absence of residual DNA in extracted RNA samples was proven by tests for no-reverse transcription (RT) as described in section 2.6.

RT-qPCRs conducted for the assessment of β-Actin- and GAPDH as reference targets (see section 2.4) revealed no coherent changes in expression levels after different stimulation or infection and thus, suitability for gene expression normalization was confirmed.

3.2 Sensitivity

For first assessment of analytical sensitivity, a 10-fold dilution series of the positive control RNA derived from the synthetic plasmid was employed for testing each cytokine and reference gene. The resulting standard curves from all single tube assays are shown in supplementary figure S1, starting from $2x10^7$ copies/µl as highest standard to $2x10^1$ copies/µl as lowest. Corresponding Cq-values and efficiencies for all assays are listed in supplementary table S1. The last employed standard dilution of to $2x10^1$ copies/µl could be detected by each assay except for β-Actin which showed a detection limit of $2x10^2$ copies/µl. Cq-values of standard RNA dilutions ranged between 15 for the highest standard and 35 for the lowest (see supplementary table S1).

Sensitivity was further analyzed by testing *in vitro*-generated positive RNA in 10-fold dilutions from 10^{-1} to 10^{-7}. The measured Cq-values as well as limits of detections are shown

in supplementary table S1 ranging from a dilution of 10^{-3} (for IL-6 and IFN-α) to more than 10^{-7} for IL-8.

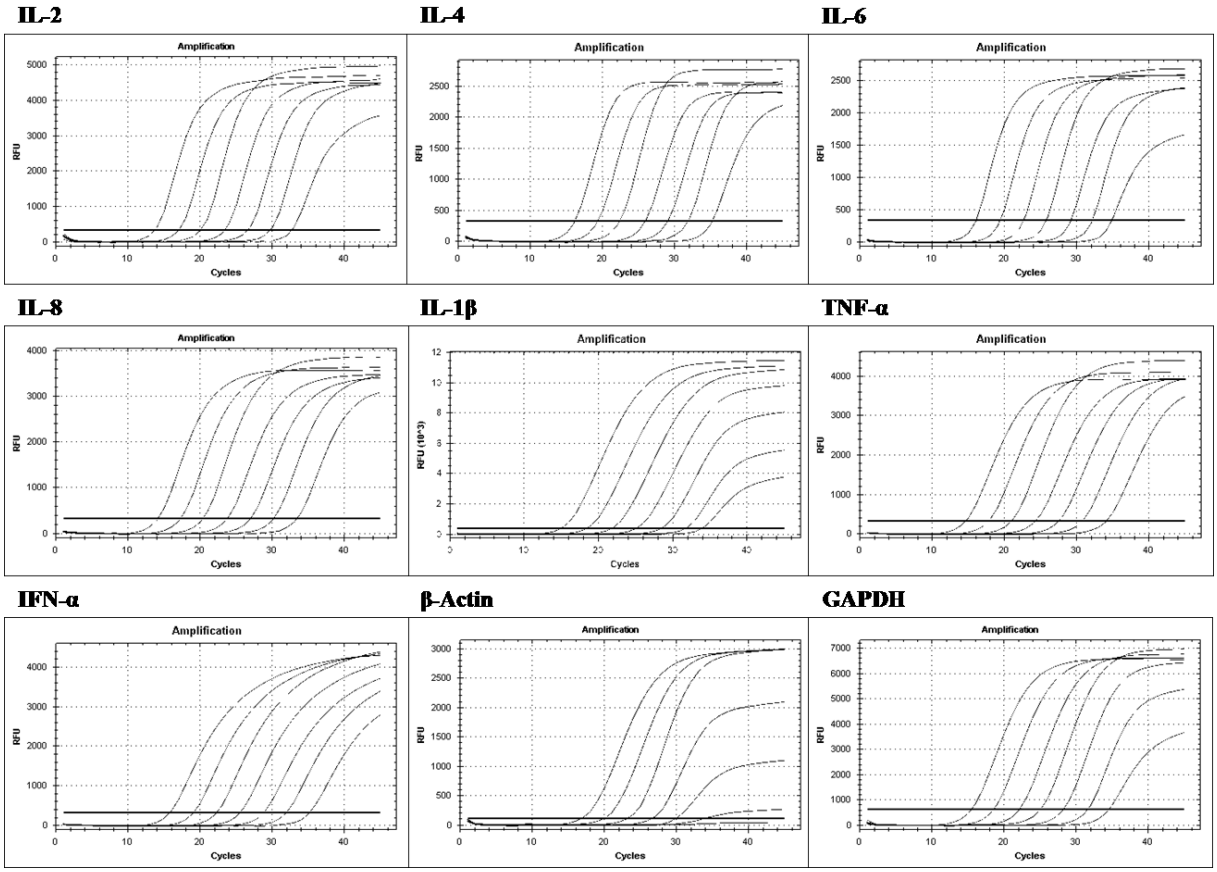

Figure S1: Amplifications of all single assays of 10-fold diluted standard RNA ranging from $2x10^1$ to $2x10^7$ copies/µl. The cytokines IL-2, IL-4, IL-6, IL-8, IL-1β, TNF-α, and IFN-α were measured in FAM; β-Actin in HEX and GAPDH in TR. The vertical axis demonstrates fluorescence levels (RFUs), the horizontal axis shows the number of cycles.

Finally, the applicability of all assays for routine pig samples (EDTA blood and white blood cells) could be demonstrated by testing a total number of 402 samples from pigs infected with different CSFV strains, ASFV "*Armenia08"*or from CSFV vaccinated pigs. Exemplary results for normalized TNF-α and IL-8 expression ($\Delta\Delta C_q$) after infection of two different pig breeds with the highly virulent CSF virus strain "Koslov" are depicted in figures 2 and 3, respectively. Additionally, detailed information comprising the results of all seven triplex assays including Cq values and corresponding normalized gene expressions are provided in supplementary table S5. To link gene expression with protein detection, exemplary results are depicted in supplementary figure 2.

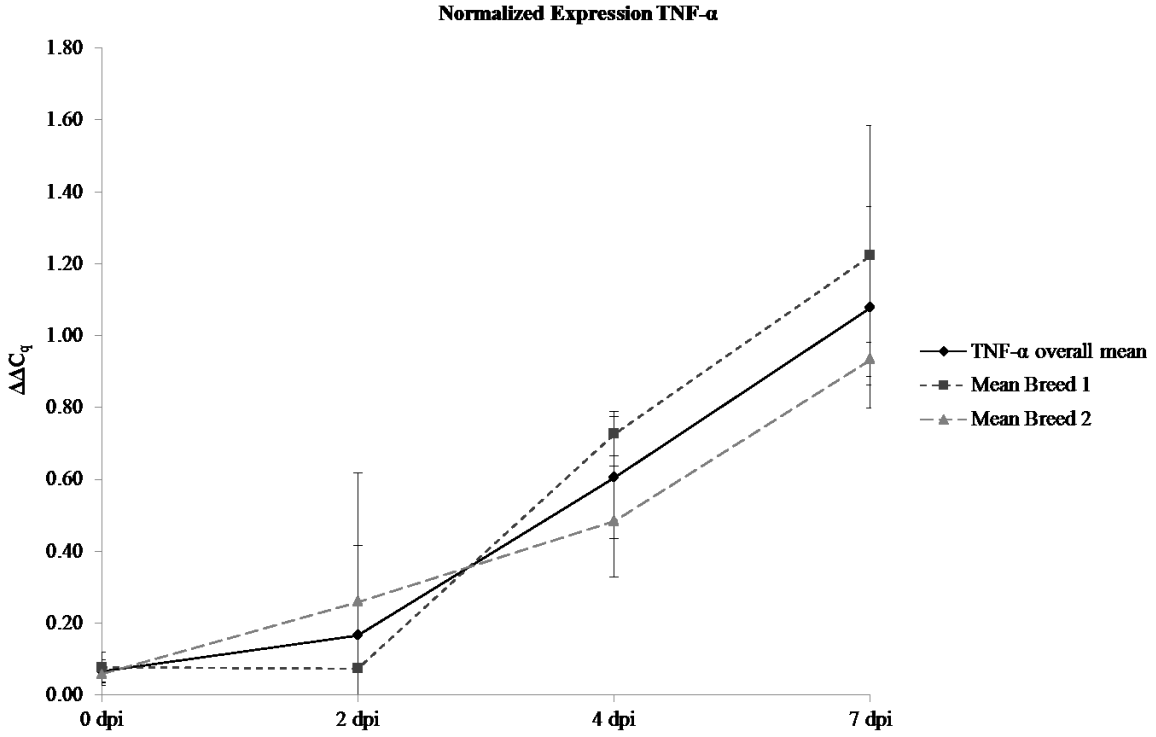

Figure 2: Detection of normalized TNF-α gene expression (ΔΔCq) in leukocyte samples from CSFV infected pigs. Samples were obtained from two different pig breeds at days 0, 2, 4, and 7 post infection (dpi). Results are given as mean values: in total from all animals (TNF-α overall mean) and separately for each breed (Mean Breed 1, Mean Breed 2). Bars indicate standard deviations.

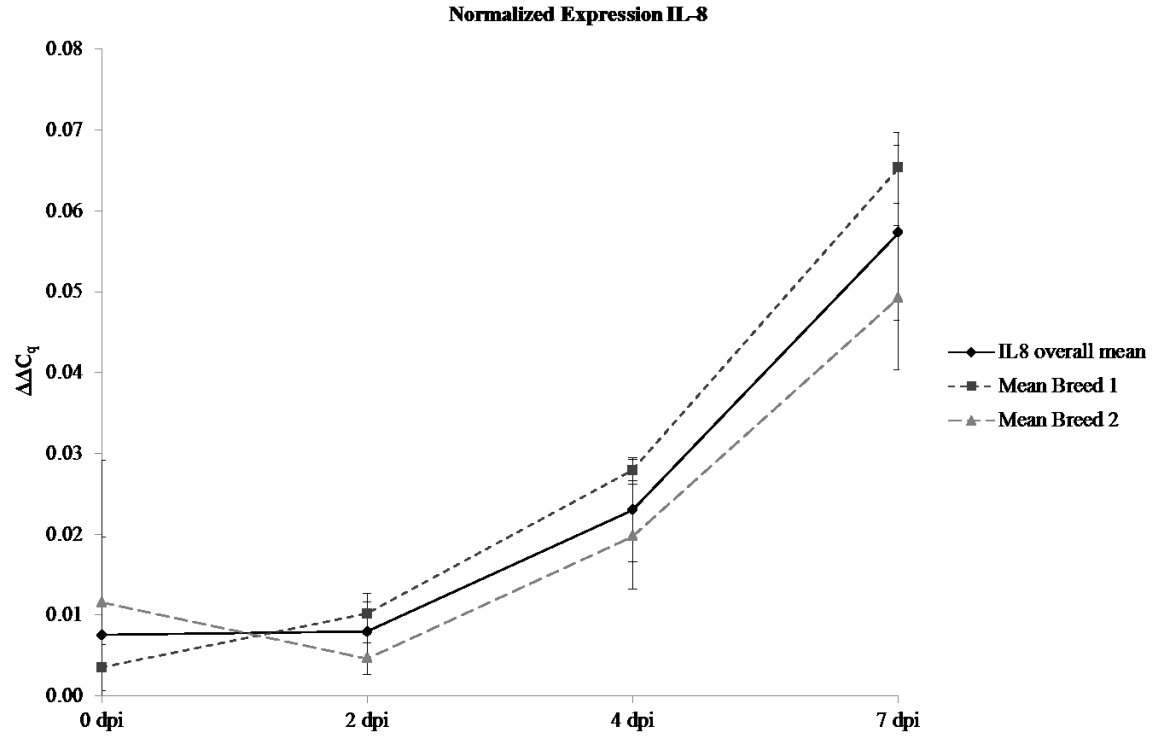

Figure 3: Detection of normalized IL-8 gene expression ($\Delta\Delta C_q$) in leukocyte samples from CSFV infected pigs. Samples were obtained from two different pig breeds at days 0, 2, 4, and 7 post infection (dpi). Results are given as mean values: in total from all animals (IL-8 overall mean) and separately for each breed (Mean Breed 1, Mean Breed 2). Bars indicate standard deviations.

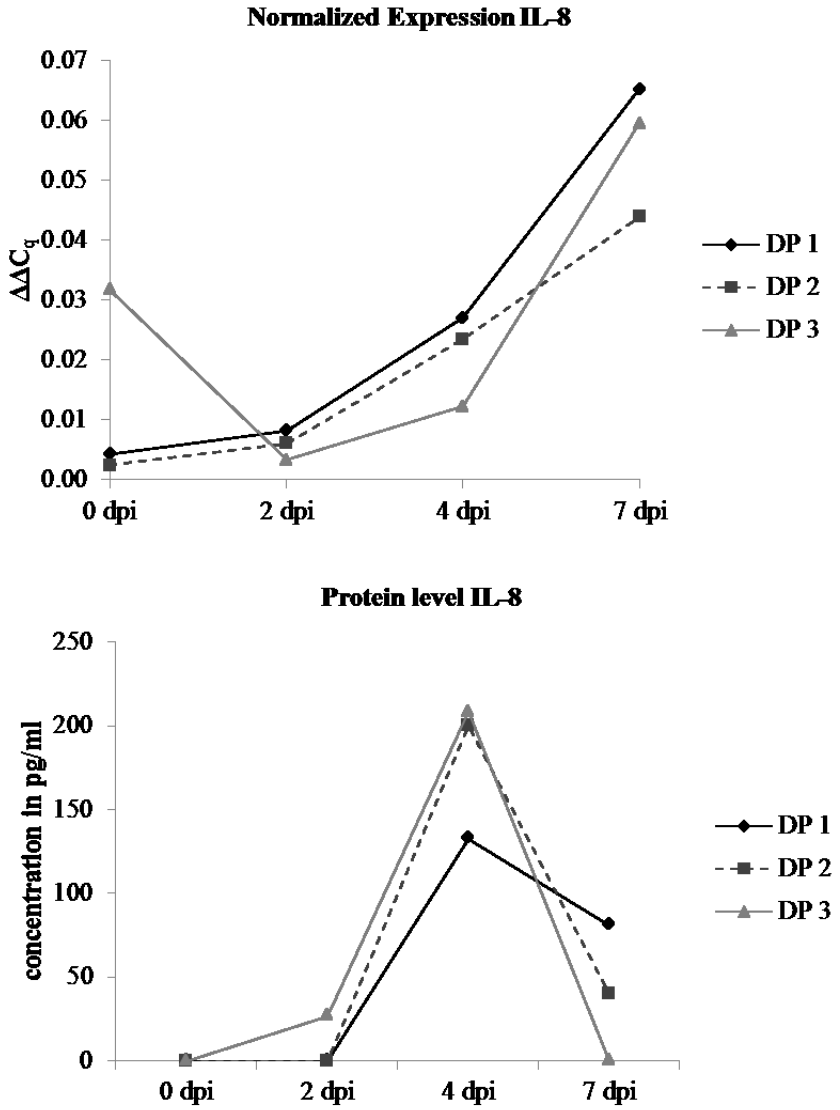

Figure S2a: Exemplary comparison of gene expression and cytokine protein detection using ELISA systems. Comparison of IL-8 gene expression (Normalized Expression, ΔΔCq) analyzed by triplex real-time RT-qPCR with protein detection using a commercial available ELISA system for IL-8 (CytoSet^{TM} (Novex, Life technologies), given as concentration in pg/ml. Optical densities were determined using the TECAN infinite F200 Pro- ELISA-reader (Tecan Austria GmBH, Austria). For calculation of results, the Magellan 7.0 software (Tecan) was employed. Three different domestic pigs (all adult) were included into comparison.

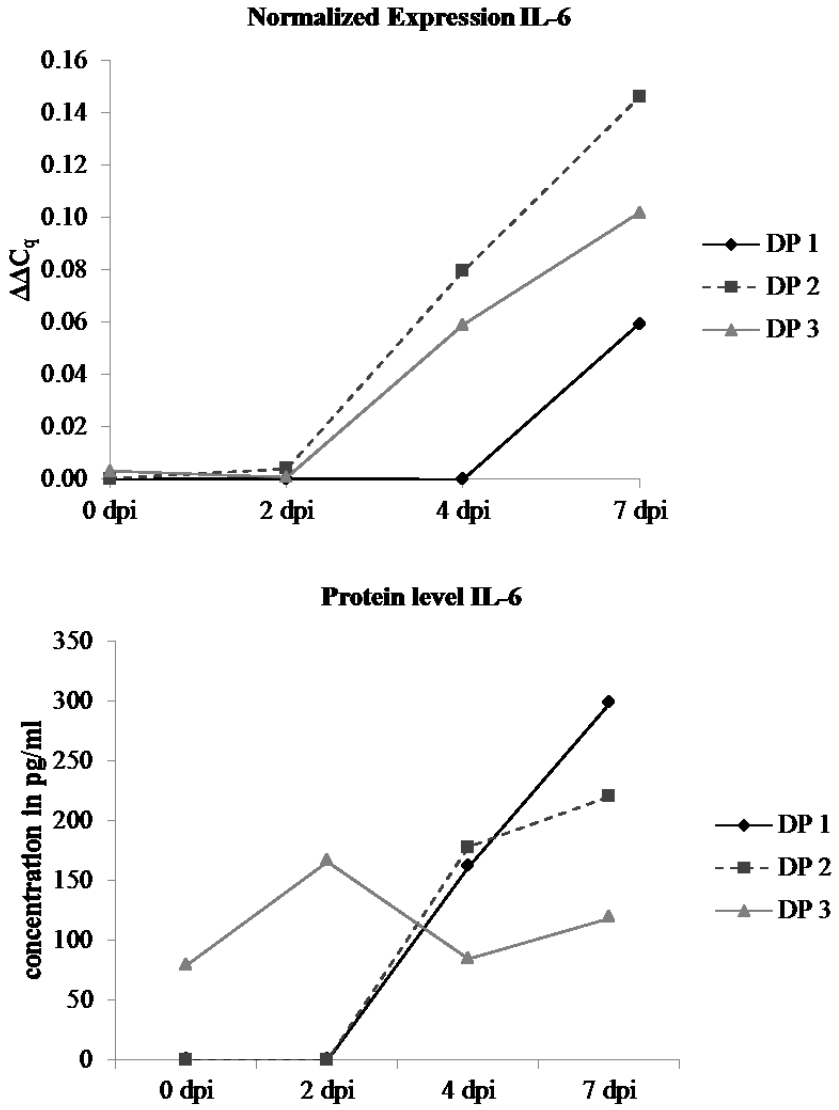

Figure S2b: Exemplary comparison of gene expression and cytokine protein detection using ELISA systems. Comparison of IL-6 gene expression (Normalized Expression, ΔΔCq) analyzed by triplex real-time RT-qPCR with protein detection using a commercial available ELISA system for IL-6 (CytoSet^{TM} (Novex, Life technologies), given as concentration in pg/ml. Optical densities were determined using the TECAN infinite F200 Pro- ELISA-reader (Tecan Austria GmBH, Austria). For calculation of results, the Magellan 7.0 software (Tecan) was employed. Three different domestic pigs (all adult) were included into comparison.

3.3 Implementation of seven cytokine triplex RT-qPCR assays

In order to detect one cytokine and two reference genes simultaneously a triplex protocol was developed (as described in section 2.6). Checkerboard titrations of all primers and probes revealed the following optimal and harmonized concentrations: for all cytokines the

harmonized protocols uses 10 pmol primers, 2.5 pmol probe, and for reference genes 2.5 pmol primers, 1.25 pmol probe.

A comparison between single and triplex assays was performed for each cytokine by using the standard RNA and *in vitro*-generated positive RNA in 10-fold dilutions. The comparative results are presented in supplementary table S2.

The analyses revealed variations of Cq-values between single and multiplex assays of less than 2 Cq-values in all cases apart from two exceptions, in which higher deviations were found within the last dilution steps of positive RNA (IFN-α, IL-1β). Each single and triplex assay was able to detect the lowest deployed standard (see supplementary table S2). Furthermore, losses of end fluorescence levels (End RFUs) did not obviously influence final results (see figure 4).

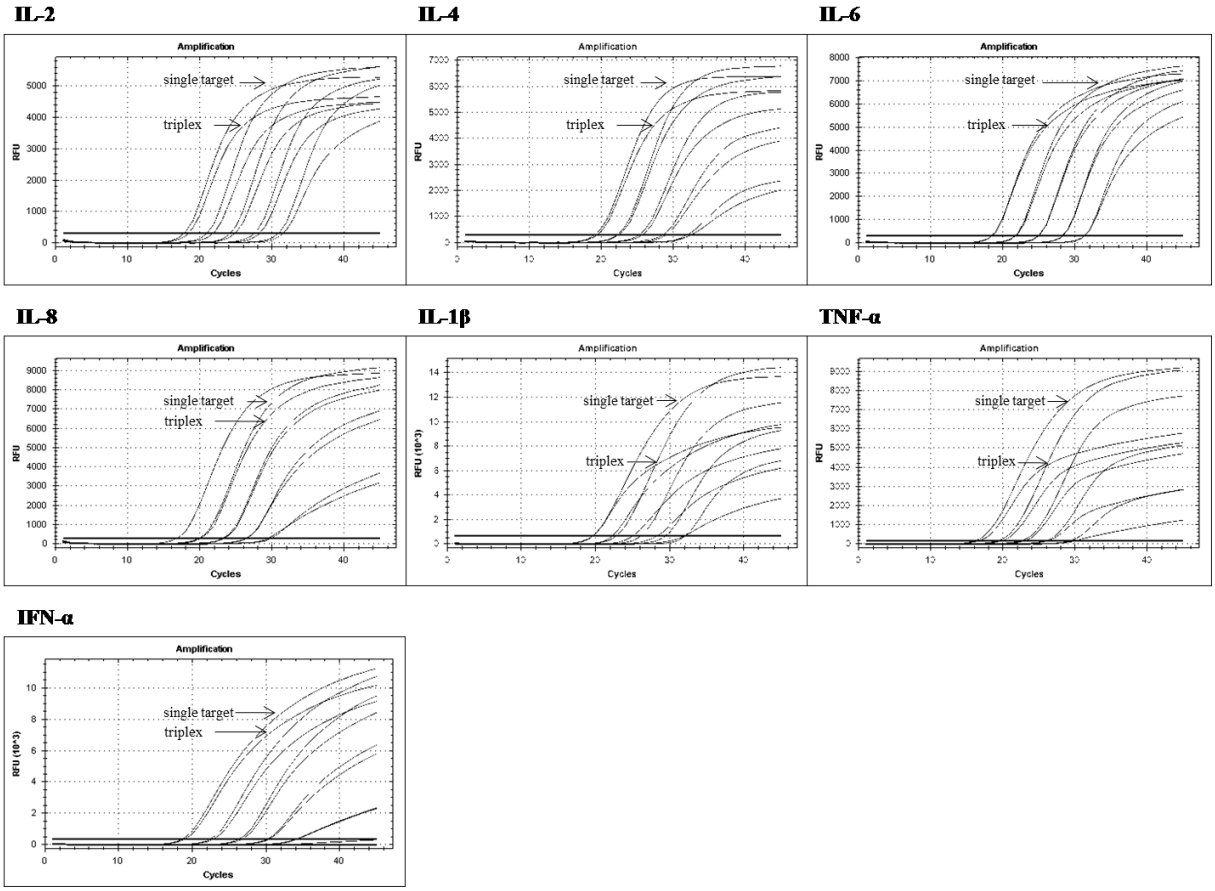

Figure 4: Comparative amplifications of 10-fold diluted standard RNA ranging from $2x10^2$ to $2x10^7$ copies/µl of single target and multiplex assays of all target cytokines (FAM). The vertical axis demonstrate fluorescence levels (relative fluorescence units, RFU), the horizontal axis shows the number of cycles. Comparative illustrations of end fluorescence levels of standard curves are shown illustrating higher end fluorescences in single target

compared triplex assays in which target cytokines were detected simultaneously with β-Actin (HEX) and GAPDH (TR).

All validation experiments of the triplex RT-qPCR protocol were performed with a total mastermix reaction volume of 12.5 µl including 2.5 µl RNA-template. Comparative analyses of full (25 µl) and halved (12.5 µl) approaches revealed no notable differences of Cq-values, detected genome copies and fluorescence levels (RFUs) as shown in supplementary table S3. Cq-losses higher than 3 were only observed in some of the lowermost dilution steps within the standard- and positive RNA 10-fold dilution series. For example, the lowest standard of $2x10^1$ copies/µl revealed Cq-losses in assays targeting IL-2, IL-8 and IL-1β (FAM) or by targeting β-Actin (HEX) in the IFN-α triplex assay and GAPDH (TR) in the IL-2-,TNF-α and IFN-α triplex assays respectively. Apart from that, no notable differences of Cq-values or absolute quantities were measured. End fluorescence levels revealed differences between approximately 500 to maximum deviations of 3000 (see supplementary table S3).
Finally, reproducibility was tested as described in section 2.6 and could be confirmed by showing no notable differences in Cq-values between the standard RNA triplicates in all assays (see supplementary table S4). Cytokine Cq-deviations were below 1 in the majority of cases which corresponds to a less than with a less than 3-fold deviation. In general, variation was mainly observed in higher dilutions (lowest target concentrations, see supplementary table S4).

4. Discussion

Cytokines are powerful mediators of the immune system and have a key role in the selection of immunological pathways and link innate and adaptive immune responses. To date, many basic immune pathological mechanisms e.g. for haemorrhagic diseases like CSF and ASF have not been clearly defined showing the need for reliable detection tools in order to characterize beneficial or detrimental reaction patterns. The selection of target cytokines for this study pursued the objective of covering a preferably wide range of immunological events in swine. While IL-2 can be regarded as indicator for the Th1 pathway, IL-4 is indicative for the Th2 response respectively [31]. The endogenous pyrogen TNF-α is of great importance as it can provoke shock symptoms upon systemically release. Yet, it also has beneficial abilities through a local restrictive effect after infection [31]. Especially in the context of CSF

pathogenesis, it has been proven one of the most crucial cytokines [32]. In this context, inclusion of the TNF-α induced proinflammatory cytokines IL-1β and IL-6 seems reasonable for their potential of acting either pyrogenic or activating monocytes and natural killer cells [31]. In contrast, IL-8 which can be produced e.g. by T-helper cells can be indicative for the Th2 pathway possessing the abilities of attracting neutrophil granulocytes, lymphocytes and of contributing to angiogenesis [31]. An IL-8 dysregulation is suspected to be involved in CSF development [20]. Finally, as an effective mediator of antiviral resistance, IFN-α is particularly involved in important mechanisms of innate immunity [33] and was therefore included in the established assay. Taken together, the selected cytokines represent valuable immunological markers by giving information about complex immunological responses.

Among the most suitable techniques for molecular cytokine research is the highly sensitive one-step RT-qPCR system [34] which allows quantitative analyses as well as multiplexing. As already stated by Huggett et al., the enhanced specificity of TaqMan-based real-time assays is greatly advantageous for immunological research since many cytokines appear in such low abundances that detection of their mRNA by real-time RT-PCR represents the only method which is sensitive enough for reliably measuring their expression *in vivo* [24, 35]. So far, gel-based PCR systems have been applied widely for cytokine detection [11, 13] despite their disadvantage of being not truly quantitative and often leading to an underestimation of total mRNA levels because of common depletion of reagents during the reaction [36]. Consequently, PCR products are not proportional to the amount of initial target when visualised on a gel [37]. The most widely used SYBR-Green I assays [38-42] have the disadvantages of potentially generating primer-dimers, the indiscriminately binding to all double-stranded DNA which might lead to a formation of secondary structures, and to a possibly limiting primer-concentration as well as to an overestimation of target-DNA [43]. To overcome these problems and to add specificity, a fluorogenic probe based approach waschosen in the presented study. The probe detection system in TaqMan PCRs make those assays clearly advantageous in comparison to SYBR Green and conventional PCR methods as they provide a high level of target specificity [19]. Specificity is particularly difficult to prove in immunological assays as truly negative biological samples are difficult to obtain (e.g. stress reactions or previous pathogen contact). However, “negative” control samples were involved in the establish procedure either originating from *in vitro* generated PMBCs or from pigs of untreated control groups. To prove the “diagnostic” performance of the established assay more than 400 samples were collected during several animal trials including CSFV

vaccination and infection as well as ASFV infection and were tested in all seven triplex assays. However, due to the above mentioned reasons, comparative evaluations of true "positive" and "negative" pigs concerning specific cytokine gene expressions was problematic. During the development procedure, the "Assay validation pathway" [44] was implemented as far as for this purpose possible by detecting analytical performance characteristics. The assessment of repeatability revealed a high level of agreement between triplicates of synthetic standard RNA by showing only minor deviations while increased variations were exclusively found in dilution steps with lowest concentrations as shown in supplementary table 4. Furthermore, limits of detections were assessed for determining the analytical sensitivity for each, single-target test (see supplementary table 1), comparative analyses between single target and multiplex assays (see supplementary table 2) as well as the comparison between a full mastermix reaction volume of 25 µl and a halved volume of 12.5 µl (see supplementary table 3). Indeed detection limits partly showed decreases in triplex assays compared to single target PCRs and also in the halve approach compared to the full, but these were measured in negligible amounts or exclusively within the least dilutions of the standard RNA or biological control RNA. Thereby, it could be proven that RT-qPCR chemistry as well as the sample volume could be successfully halved making the assay much more cost-effective and that simultaneous detection of one target cytokine and two reference genes is possible which allows an accurate determination of gene expression profiles by normalization. Another advantage of inclusion of two reference genes is to control varying amounts of input RNA used in the reverse transcription step [34]. This is particularly useful regarding the high variability of biological sample material. Different stimulation and infection experiments were successfully conducted for further confirmation of stable β-Actin and GAPDH expressions [45] despite this was already shown by preliminary studies [46].

First implementation of the assays in different animal trials (see examples in supplementary table 5) showed that the choice of sample matrices and sample handling (especially leukocyte preparation and freeze/thawing) had a strong impact on the detection of cytokine gene expression. While *in vitro* stimulations proved that the respective cytokine mRNAs were reliably detected (see results for positive RNAs), diagnostic samples often resulted in negative results (if normalized gene expression was assessed). In this context, further validation and optimization for sample transport (direct cooling), preparation (avoiding freeze/thawing) and extraction (direct sample suspension in Trizol or equivalents) is clearly needed.

5. Conclusions

A one-step TaqMan-based triplex RT-qPCR protocol was established and validated for the accurate and reliable detection and quantification of seven porcine cytokines (IL-1β, IL-2, IL-4, IL-6, IL-8, TNF-α, IFN-α) representing immunological markers by covering a broad range of host responses. These real-time assays were successfully harmonized by using a unique RT-qPCR protocol along with the same chemistry, temperature profile and synthetic standard resulting in a simple, cost-efficient, specific and highly sensitive assessment of normalized gene expression profiles. This novel and versatile tool will aid not only studies of swine fever pathogenesis but also swine immunology in general.

Table 1: Sequences of primers and probes. FAM 5' modification was used for IL-1β, IL-2, IL-4, IL-6, IL-8, IFN-α, TNF-α; HEX for β-Actin; Texas Red for GAPDH. BHQ (Black-Hole-Quencher)-1was used for 3' modifications of IL-1β, IL-2, IL-4, IL-6, IFN-α, β-Actin and GAPDH; BHQ-2 was used for of TNF-α and IL-8. Corresponding references are given in the right column. Sequences marked with "this study" were created by the use of "Primer-BLAST" available on NCBI GenBank. F= Forward Primer; R= Reverse Primer; P= Probe; bp=base pairs.

Gen-name	Forward- (F) and Reverse- (R) primer	Probe (P)	Product length	Reference
IL-1β	F-GTGCTGGCTGGCCCACA R- GAACACCACTTCTCTCTTCA	CTCTCCACCTCCTCAAAGGG	71 bp	F/P: this study; R: Lange, 2010
IL-4	F-GTCTGCTTACTGGCATGTACCA R-GCTCCATGCACGAGTTCTTTCT	CCACGGACACAAGTGCGACATCACCTTAC	117 bp	F/R: Duvigneau et al., 2005 P: this study
IL-2	F-TGCTGATCTCTCCAGGATGC R-CCTCCAGAGCTTTGAGTTCTTCTACTA	AAGCAGGCTACAGAATTGAAACACCTT	103 bp	F: this study; R: Yang et al., 2012 [47]; P: Duvigneau et.al. 2005 [21]
IL-6	F-CTGGCAGAAAACAACCTGAACC R-TGATTCTCATCAAGCAGGTCTCC	TGGCAGAAAAAGACGGATGC	93 bp	F/R: Duvigneau et al., 2005 [21]; P: this study
IL-8	F-AAGCTTGTCAATGGAAAAGAG R-CTGTTGTTGTTGCTTCTCAG	TCTGCCTGGACCCCAAGGAAAAGT	101 bp	F/R/P: Lange, 2010 [20]
IFN-α	F-TGGTGCATGAGATGCTCCA R-GCCGAGCCCTCTGTGCT	CAGACCTTCCAGCTCT	54 bp	F/R/P: Bautista et al., 2004 [48]
TNF-α	F-AACCTCAGATAAGCCCGTCG R-ACCACCAGCTGGTTGTCTTT	CCAATGCCCTCCTGGCCAACG	128 bp	F/R/P: Lange, 2010 [20]
β-Actin	F-AGCGCAAGTACTCCGTGTG R-CGGACTCATCGTACTCCTGCTT	TCGCTGTCCACCTTCCAGCAGATGT	105 bp	F modified /R/P: Toussaint et al., 2007 [49]
GAPDH	F-ACATGGCCTCCAAGGAGTAAGA R-GATCGAGTTGGGGCTGTGACT	CCACCAACCCCAGCAAGAGCACGC	105 bp	F/R/P: Demissie et al.,2004 [21]

Table S1: RT-qPCR results of all single target assays. Results were obtained including the 10-fold dilution series of the synthetic standard RNA and *in vitro* generated positive RNA in all assays. The standard RNA dilution is given as copies/µl, ranging from $2x10^1$ to $2x10^7$. *In vitro* generated positive RNA obtained through specific stimulation of PBMCs is declared as "positive RNA" and given as 10-fold dilution steps ranging from 10^{-1} to 10^{-7}. The "Limit of detection" describes the last dilution step with a positive RT-qPCR result.

Target gene	**IL-2**	**IL-4**	**IL-6**	**IL-8**	**IL-1β**	**TNF-α**	**IFN-α**	**β-Actin**	**GAPDH**
Efficiency in %	106.1	106.4	107.1	103.9	90.1	103.3	103.9	97.9	103.8
Standard RNA copies					*Cq-values*				
$2x10^1$	32.94	35.47	35.09	33.74	35.91	34.39	35.46	N/A	35.65
$2x10^2$	30.00	32.15	32.32	30.44	33.46	31.07	32.24	33.46	31.88
$2x10^3$	26.84	29.31	29.36	27.27	29.87	27.80	29.13	29.69	28.94
$2x10^4$	23.59	26.20	26.01	24.07	25.98	24.57	25.82	26.36	25.41
$2x10^5$	20.23	22.65	22.85	20.65	22.32	21.25	22.56	23.00	22.09
$2x10^6$	17.24	19.60	19.58	17.53	18.45	18.10	19.35	19.70	18.97
$2x10^7$	13.93	16.39	16.23	14.39	14.98	14.94	16.09	16.51	16.36
Limit of detection positive RNA	10^{-5}	10^{-4}	10^{-3}	$> 10^{-7}$	10^{-6}	10^{-4}	10^{-3}		
Positive RNA dilution				*Cq-values*					
10^{-1}	22.85	30.27	29.07	21.13	23.29	28.81	31.75		
10^{-2}	26.26	33.19	32.21	24.42	27.1	32.27	35.27		
10^{-3}	29.61	36.19	34.83	27.83	30.15	36.17	39.05		
10^{-4}	32.95	40.14	N/A	31.1	32.84	39.92	N/A		
10^{-5}	36.2	N/A	N/A	34.25	35.07	N/A	N/A		
10^{-6}	N/A	N/A	N/A	37.09	39.09	N/A	N/A		
10^{-7}	N/A	N/A	N/A	38.15	N/A	N/A	N/A		

Table S2: Comparison of single target and triplex RT-qPCR results. Cq-values and total amounts (given as copies/µl) of target cytokines (IL-2, IL-4, IL-6, IL-8, IL-1β, TNF-α and IFN-α) from the 10-fold diluted standard RNA series ranging from $2x10^2$ to $2x10^6$ copies/µl and of positive RNA dilutions of 10^{-1} to 10^{-5} are comparatively illustrated between single target and triplex assays (FAM). Corresponding deviations between Cq-values of single and multiplex assay as well as PCR-Efficiencies (E) and Baseline Thresholds (Thresholds) are additionally provided. PC RNA= *in vitro* generated positive RNA; Std=Standard; E= Efficiency of RT-qPCR; NTC= H_2O used as negative control; N/A=no Cq-value detectable

Dilutions of pos. RNA and Std.	IL-2					IL-4				
	Single Target Cq-values	Multiplex Cq-values	*Deviations*	Single Target Copies/µl	Multiplex Copies/µl	Single Target Cq-values	Multiplex Cq-values	*Deviations*	Single Target Copies/µl	Multiplex Copies/µl
PC RNA 10^{-1}	22.71	23.14	*-0.43*	5.81E+04	6.94E+04	29.07	29.29	*-0.02*	1.42E+03	1.68E+03
PC RNA 10^{-2}	26.21	26.41	*-0.20*	4.93E+03	6.97E+03	32.62	33.39	*-0.77*	1.12E+02	9.50E+01
PC RNA 10^{-3}	29.44	29.78	*-0.34*	5.12E+02	6.46E+02	36.59	42.62	*-6.03*	6.53E+00	1.48E-01
PC RNA 10^{-4}	32.64	33.05	*-0.41*	5.36E+01	6.48E+01	N/A	N/A	/	N/A	N/A
PC RNA 10^{-5}	35.99	37.8	*-1.81*	5.09E+00	2.29E+00	N/A	N/A	/	N/A	N/A
Std $2x10^2$	30.83	31.45	*-0.62*	2.00E+02	2.00E+02	31.22	32.45	*-1.23*	2.00E+02	2.00E+02
Std $2x10^3$	27.47	28.21	*-0.74*	2.00E+03	2.00E+03	27.85	29.01	*-1.16*	2.00E+03	2.00E+03
Std $2x10^4$	24.15	24.8	*-0.65*	2.00E+04	2.00E+04	24.63	25.65	*-1.02*	2.00E+04	2.00E+04
Std $2x10^5$	20.93	21.77	*-0.84*	2.00E+05	2.00E+05	21.56	22.32	*-0.76*	2.00E+05	2.00E+05
Std $2x10^6$	17.73	18.32	*-0.59*	2.00E+06	2.00E+06	18.47	19.36	*-0.89*	2.00E+06	2.00E+06
E in %	102.1 %	102.2 %				104.5 %	101.5 %			
NTC	N/A	N/A				N/A	N/A			
Threshold:	300	300				300	300			
	IL-6					**IL-8**				
	Single Target Cq-values	Multiplex Cq-values	*Deviations*	Single Target Copies/µl	Multiplex Copies/µl	Single Target Cq-values	Multiplex Cq-values	*Deviations*	Single Target Copies/µl	Multiplex Copies/µl
PC RNA 10^{-1}	27.33	26.74	*0.59*	3.53E+03	5.35E+03	19.38	19.56	*-0.18*	3.13E+05	3.14E+05
PC RNA 10^{-2}	30.4	30.07	*0.33*	3.94E+02	4.94E+02	22.86	22.65	*0.21*	2.53E+04	3.40E+04
PC RNA 10^{-3}	33.36	33.04	*0.32*	4.80E+01	5.96E+01	26.32	26.26	*0.06*	2.07E+03	2.53E+03
PC RNA 10^{-4}	36.21	35.46	*0.75*	6.29E+00	1.06E+01	33.52	32.53	*0.99*	1.13E+01	2.78E+01
PC RNA 10^{-5}	N/A	N/A	/	N/A	N/A	34.66	33.98	*0.68*	4.97E+00	9.79E+00
Std $2x10^2$	31.27	31.26	*0.01*	2.00E+02	2.00E+02	29.52	29.88	*-0.36*	2.00E+02	2.00E+02
Std $2x10^3$	28.23	28.22	*0.01*	2.00E+03	2.00E+03	26.42	26.49	*-0.07*	2.00E+03	2.00E+03
Std $2x10^4$	24.92	24.88	*0.04*	2.00E+04	2.00E+04	23.21	23.37	*-0.16*	2.00E+04	2.00E+04
Std $2x10^5$	21.64	21.7	*-0.06*	2.00E+05	2.00E+05	19.9	20.14	*-0.24*	2.00E+05	2.00E+05

Std 2x10^6	18.41	18.38	*0.03*	2.00E+06	2.00E+06	16.86	17.06	*-0.20*	2.00E+06	2.00E+06
E in %	104.0 %	104.1 %				106.1 %	105.4 %			
NTC	N/A	N/A				N/A	N/A			
Threshold:	300	300				300	300			
Dilutions of pos. RNA and Std.	**IL-1β**					**TNF-α**				
	Single Target Cq-values	Multiplex Cq-values	*Deviations*	Single Target Cq-values	Multiplex Cq-values	Single Target Cq-values	Multiplex Cq-values	*Deviations*	Single Target Copies/µl	Multiplex Copies/µl
PC RNA 10^{-1}	25.54	26.45	*-0.91*	1.12E+04	1.94E+04	27.72	26.96	*0.76*	1.23E+03	1.26E+03
PC RNA 10^{-2}	28.79	29.85	*-1.06*	1.08E+03	1.70E+03	30.88	31.19	*-0.31*	1.35E+02	6.72E+01
PC RNA 10^{-3}	31.78	34.27	*-2.49*	1.24E+02	7.19E+01	35.55	36.81	*-1.26*	6.18E+00	1.38E+00
PC RNA 10^{-4}	N/A	N/A	*/*	N/A	N/A	N/A	N/A	*/*	2.30E-02	N/A
PC RNA 10^{-5}	40.34	N/A	*-4.66*	2.58E-01	N/A	N/A	N/A	*/*	N/A	N/A
Std 2x10^2	30.91	33.32	*-2.41*	2.00E+02	2.00E+02	30.38	29.87	*0.51*	2.00E+02	2.00E+02
Std 2x10^3	28.13	29.34	*-1.10*	2.00E+03	2.00E+03	26.97	26.06	*0.91*	2.00E+03	2.00E+03
Std 2x10^4	24.9	25.94	*-1.04*	2.00E+04	2.00E+04	23.55	22.8	*0.75*	2.00E+04	2.00E+04
Std 2x10^5	21.47	23.03	*-1.56*	2.00E+05	2.00E+05	20.3	19.62	*0.68*	2.00E+05	2.00E+05
Std 2x10^6	18.28	20.4	*-2.12*	2.00E+06	2.00E+06	17.16	16.46	*0.70*	2.00E+06	2.00E+06
E in %	105.7 %	104.6 %				100.4 %	99.8 %			
NTC	N/A	N/A				N/A	N/A			
Threshold:	250	450				350	150			
	IFN-α									
	Single Target Cq-values	Multiplex Cq-values	*Deviations*	Single Target Copies/µl	Multiplex Copies/µl					
PC RNA 10^{-1}	26.66	25.05	*1.61*	1.11E+03	3.50E+03					
PC RNA 10^{-2}	30.02	30.16	*-0.14*	1.16E+02	1.06E+02					
PC RNA 10^{-3}	32.46	N/A	*-12.54*	2.26E+01	N/A					
PC RNA 10^{-4}	N/A	N/A	*/*	N/A	N/A					
PC RNA 10^{-5}	43.87	N/A	*-1.13*	1.05E-02	N/A					
Std 2x10^2	28.97	28.98	*-0.01*	2.00E+02	2.00E+02					
Std 2x10^3	26.08	25.78	*0.30*	2.00E+03	2.00E+03					
Std 2x10^4	22.35	23.15	*-0.80*	2.00E+04	2.00E+04					
Std 2x10^5	19.09	19.13	*-0.04*	2.00E+05	2.00E+05					
Std 2x10^6	15.34	15.46	*-0.12*	2.00E+06	2.00E+06					
E in %	95.9 %	98.0 %								
NTC	N/A	N/A								
Threshold:	150	150								

Table S3: Comparison of RT-qPCRs between a total mastermix reaction volume of 25 µl and the halved volume of 12.5 µl.

10-fold dilution series of synthetic standard RNA ("std. dilutions") ranging from $2x10^{1}$ to $2x10^{6}$ copies/µl and *in vitro* generated positive RNA ("pos. RNA") including the dilutions steps 10^{-1} to 10^{-5} were used for assessment of the applicability of a halved mastermix reaction volume of 12.5 µl instead of 25 µl. Cq-values, total amounts (in copies/µl) as well as end fluorescence levels (End RFUs) for each triplex assay are comparatively provided for each channel (target cytokines IL-2, IL-4, IL-6, IL-8, IL-1β, TNF-α , IFN-α in FAM; β-Actin in Hex; GAPDH in Texas Red). Fat letters indicate deviations of more than three Cq-values.

Std=Standard; PC RNA=*in vitro* generated positive RNA; N/A=no Cq-value detectable; V-MM=total mastermix reaction volume including RNA-template

IL-2 triplex assay

Pos. RNA and std. dilutions	IL-2 *FAM*						β-Actin *HEX*						GAPDH *Texas Red*					
	Cq-values		Copies/µl		End RFUs		Cq-values		Copies/µl		End RFUs		Cq-values		Copies/µl		End RFUs	
V-MM in µl	25	12.5	25	12.5	25	12.5	25	12.5	25	12.5	25	12.5	25	12.5	25	12.5	25	12.5
PC RNA 10^{-1}	21.30	21.31	5.49E+04	7.23E+04	7075	5966	21.16	21.56	2.86E+05	3.21E+05	5014	3397	20.64	21.04	6.24E+05	7.50E+05	10413	6370
PC RNA 10^{-2}	24.72	25.01	4.56E+03	5.06E+03	5856	4286	24.37	25.07	3.13E+04	2.88E+04	4196	3171	23.78	24.4	7.19E+04	7.26E+04	9158	6208
PC RNA 10^{-3}	28.03	28.11	4.14E+02	5.42E+02	3523	2259	27.57	27.83	3.47E+03	4.33E+03	2685	1911	27.1	27.57	7.30E+03	7.97E+03	6710	4019
PC RNA 10^{-4}	31.46	31.87	3.42E+01	3.62E+01	844	572	30.54	31.53	4.49E+02	3.41E+02	1029	691	30.14	30.82	9.03E+02	8.31E+02	3432	2131
PC RNA 10^{-5}	N/A	N/A	N/A	N/A	33.2	21.1	**35.9**	**N/A**	1.13E+01	N/A	196	70.4	36.98	38.33	8.14E+00	4.45E+00	415	302
Std $2x10^{1}$	**32.49**	**40.68**	2.00E+01	2.00E+01	551	229	37.54	34.34	2.00E+01	2.00E+01	143	167	**36.09**	**N/A**	2.00E+01	2.00E+01	482	23.5
Std $2x10^{2}$	28.81	29.48	2.00E+02	2.00E+02	2652	1465	31.65	32.28	2.00E+02	2.00E+02	628	455	31.83	32.96	2.00E+02	2.00E+02	1835	909
Std $2x10^{3}$	26.06	26.27	2.00E+03	2.00E+03	5305	3528	28.51	29.03	2.00E+03	2.00E+03	1863	1268	29.08	29.44	2.00E+03	2.00E+03	4075	2463
Std $2x10^{4}$	22.88	23.20	2.00E+04	2.00E+04	6891	4694	25.05	25.53	2.00E+04	2.00E+04	3554	2432	25.5	26.17	2.00E+04	2.00E+04	7014	4273
Std $2x10^{5}$	19.39	19.87	2.00E+05	2.00E+05	7071	5022	21.51	22.21	2.00E+05	2.00E+05	4387	3220	22.24	23.05	2.00E+05	2.00E+05	8443	5391
Std $2x10^{6}$	16.3	16.69	2.00E+06	2.00E+06	7294	5104	18.41	18.93	2.00E+06	2.00E+06	4819	3570	19.14	19.62	2.00E+06	2.00E+06	9243	6001

IL-4 triplex assay

Pos. RNA and std. dilutions	IL-4 *FAM*						β-Actin *HEX*						GAPDH *Texas Red*					
	Cq-values		Copies/µl		End RFUs		Cq-values		Copies/µl		End RFUs		Cq-values		Copies/µl		End RFUs	
V-MM in µl	25	12.5	25	12.5	25	12.5	25	12.5	25	12.5	25	12.5	25	12.5	25	12.5	25	12.5
PC RNA 10^{-1}	27.66	28.20	2.40E+03	2.24E+03	9498	6133	21.76	23.00	3.19E+05	2.55E+05	4306	2399	20.41	21.06	5.89E+05	5.65E+05	9186	5964
PC RNA 10^{-2}	31.09	31.57	2.08E+02	2.08E+02	5714	3765	25.22	26.17	2.86E+04	3.32E+04	3532	2025	23.85	24.23	5.86E+04	6.10E+04	8205	5604
PC RNA 10^{-3}	36.28	36.95	5.10E+00	5.10E+00	1382	1211	28.36	29.36	3.21E+03	4.28E+03	2403	1413	26.99	27.38	7.15E+03	6.68E+03	6491	4891
PC RNA 10^{-4}	44.79	N/A	1.19E-02	N/A	309	27.9	31.26	32.45	4.24E+02	5.86E+02	1101	621	31.00	30.59	4.88E+02	6.94E+02	3092	2746
PC RNA 10^{-5}	N/A	N/A	N/A	N/A	26.6	23	**36.37**	**N/A**	1.20E+01	N/A	267	124	33.42	32.54	9.62E+01	1.77E+02	1503	1641
Std $2x10^{1}$	34.82	34.85	2.00E+01	2.00E+01	2507	2083	44.87	N/A	2.00E+01	2.00E+01	142	140	39.66	38.13	2.00E+01	2.00E+01	310	375
Std $2x10^{2}$	31.08	31.73	2.00E+02	2.00E+02	6475	4015	32.29	34.58	2.00E+02	2.00E+02	659	317	32.59	32.35	2.00E+02	2.00E+02	1693	1362
Std $2x10^{3}$	27.98	28.21	2.00E+03	2.00E+03	10606	7445	29.13	30.08	2.00E+03	2.00E+03	1845	1056	28.68	29.08	2.00E+03	2.00E+03	4635	3361
Std $2x10^{4}$	24.65	25.12	2.00E+04	2.00E+04	13346	8699	25.6	26.81	2.00E+04	2.00E+04	3531	1945	25.18	25.86	2.00E+04	2.00E+04	7807	5079
Std $2x10^{5}$	21.58	21.83	2.00E+05	2.00E+05	13488	9773	22.63	23.24	2.00E+05	2.00E+05	4264	2791	22.16	22.52	2.00E+05	2.00E+05	9061	6704
Std $2x10^{6}$	18.14	18.62	2.00E+06	2.00E+06	13527	9775	19.02	20.09	2.00E+06	2.00E+06	4686	3046	18.67	19.26	2.00E+06	2.00E+06	9543	7334

IL-6 triplex assay

Pos. RNA and std. dilutions	IL-6 *FAM*						β-Actin *HEX*						GAPDH *Texas Red*					
	Cq-values		Copies/µl		End RFUs		Cq-values		Copies/µl		End RFUs		Cq-values		Copies/µl		End RFUs	
V-MM in µl	25	12.5	25	12.5	25	12.5	25	12.5	25	12.5	25	12.5	25	12.5	25	12.5	25	12.5
PC RNA 10^{-1}	25.96	26.32	5.41E+03	5.69E+03	7286	6193	21.83	22.27	2.62E+05	2.79E+05	3645	3020	20.89	21.25	5.27E+05	7.01E+05	7334	5988
PC RNA 10^{-2}	29.26	29.74	4.67E+02	4.66E+02	5613	4929	25.20	25.98	2.81E+04	2.29E+04	3124	2603	24.22	24.78	5.38E+04	6.00E+04	6897	5614
PC RNA 10^{-3}	32.88	33.51	3.16E+01	2.95E+01	2507	2218	28.38	28.96	3.42E+03	3.06E+03	2145	1859	27.36	27.94	6.25E+03	6.68E+03	5597	4620
PC RNA 10^{-4}	42.02	41.59	3.57E-02	7.93E-02	445	466	31.42	32.19	4.59E+02	3.46E+02	985	812	30.64	31.09	6.62E+02	7.43E+02	3345	2781
PC RNA 10^{-5}	N/A	N/A	N/A	N/A	10.5	7.35	36.02	38.2	2.17E+01	6.01E+00	278	221	34.59	34.69	4.41E+01	6.07E+01	1068	1047
Std 2x10^{1}	33.37	35.16	2.00E+01	2.00E+01	2366	1545	N/A	N/A	2.00E+01	2.00E+01	111	102	**36.66**	**N/A**	2.00E+01	2.00E+01	609	200
Std 2x10^{2}	30.26	30.62	2.00E+02	2.00E+02	5476	4920	32.84	32.98	2.00E+02	2.00E+02	602	569	32.41	32.86	2.00E+02	2.00E+02	1820	1497
Std 2x10^{3}	27.58	28.04	2.00E+03	2.00E+03	7324	6791	29.02	29.71	2.00E+03	2.00E+03	1601	1340	29.09	29.92	2.00E+03	2.00E+03	3668	2832
Std 2x10^{4}	24.34	24.69	2.00E+04	2.00E+04	7714	8032	25.65	26.09	2.00E+04	2.00E+04	2390	2390	25.52	26.20	2.00E+04	2.00E+04	4981	4670
Std 2x10^{5}	21.16	21.47	2.00E+05	2.00E+05	9291	8358	22.22	22.70	2.00E+05	2.00E+05	3313	2918	22.3	23.10	2.00E+05	2.00E+05	6635	5459
Std 2x10^{6}	17.78	18.18	2.00E+06	2.00E+06	9202	8472	18.85	19.42	2.00E+06	2.00E+06	3546	3180	19.01	19.74	2.00E+06	2.00E+06	6966	5926

IL-8 triplex assay

Pos. RNA and std. dilutions	IL-8 *FAM*						β-Actin *HEX*						GAPDH *Texas Red*					
	Cq-values		Copies/µl		End RFUs		Cq-values		Copies/µl		End RFUs		Cq-values		Copies/µl		End RFUs	
V-MM in µl	25	12.5	25	12.5	25	12.5	25	12.5	25	12.5	25	12.5	25	12.5	25	12.5	25	12.5
PC RNA 10^{-1}	19.24	19.80	2.67E+05	2.68E+05	10266	8673	26.38	26.19	2.54E+04	2.12E+04	2400	2250	24.67	24.99	5.73E+04	5.56E+04	5079	4673
PC RNA 10^{-2}	22.61	23.10	2.42E+04	2.68E+04	10114	8570	30.68	29.67	1.70E+03	1.87E+03	1326	1361	28.45	28.42	4.47E+03	5.24E+03	3866	3470
PC RNA 10^{-3}	26.16	26.49	1.95E+03	2.51E+03	9416	8001	34.90	33.54	1.19E+02	1.27E+02	458	522	32.07	32.16	3.91E+02	3.99E+02	2190	1759
PC RNA 10^{-4}	30.31	30.80	1.02E+02	1.25E+02	6355	4637	N/A	N/A	N/A	N/A	10.1	5.26	43.21	N/A	2.13E-01	N/A	248	4.58
PC RNA 10^{-5}	32.32	33.08	2.44E+01	2.54E+01	3397	2226	N/A	N/A	N/A	N/A	3.65	62.6	39.37	41.11	2.85E+00	8.44E-01	382	302
Std 2x10^{1}	**32.25**	**36.55**	2.00E+01	2.00E+01	3568	1016	41.61	43.00	2.00E+01	2.00E+01	159	152	**38.18**	**N/A**	2.00E+01	2.00E+01	460	208
Std 2x10^{2}	29.66	30.07	2.00E+02	2.00E+02	6818	5307	34.41	32.74	2.00E+02	2.00E+02	472	653	33.06	33.17	2.00E+02	2.00E+02	1677	1364
Std 2x10^{3}	26.27	26.92	2.00E+03	2.00E+03	9762	8296	30.03	29.82	2.00E+03	2.00E+03	1571	1465	29.63	29.90	2.00E+03	2.00E+03	3591	3081
Std 2x10^{4}	22.96	23.54	2.00E+04	2.00E+04	10497	9354	26.63	26.31	2.00E+04	2.00E+04	2520	2571	26.23	26.41	2.00E+04	2.00E+04	5122	4870
Std 2x10^{5}	19.57	20.09	2.00E+05	2.00E+05	11554	11194	23.13	22.77	2.00E+05	2.00E+05	3248	3616	22.86	23.02	2.00E+05	2.00E+05	6253	6762
Std 2x10^{6}	16.29	16.99	2.00E+06	2.00E+06	11002	10190	19.58	19.72	2.00E+06	2.00E+06	3303	3571	19.37	19.88	2.00E+06	2.00E+06	6352	6599

IL-1β triplex assay

Pos. RNA and std. dilutions	IL-1β *FAM*						β-Actin *HEX*						GAPDH *Texas Red*					
	Cq-values		Copies/µl		End RFUs		Cq-values		Copies/µl		End RFUs		Cq-values		Copies/µl		End RFUs	
V-MM in µl	25	12.5	25	12.5	25	12.5	25	12.5	25	12.5	25	12.5	25	12.5	25	12.5	25	12.5
PC RNA 10^{-1}	24.07	24.83	3.84E+05	2.97E+05	1896	1577	27.07	27.72	1.68E+04	1.16E+04	4159	2990	26.71	27.76	3.33E+04	1.51E+04	7785	5294
PC RNA 10^{-2}	27.40	27.54	5.66E+04	6.94E+04	1133	959	30.51	30.65	1.54E+03	1.37E+03	2502	1889	30.12	30.63	3.08E+03	1.80E+03	5471	3859
PC RNA 10^{-3}	30.97	31.55	7.31E+03	8.10E+03	406	326	33.64	33.37	1.75E+02	1.88E+02	897	807	33.32	35.62	3.30E+02	4.50E+01	2481	1027
PC RNA 10^{-4}	N/A	N/A	N/A	N/A	12.9	12.2	N/A	N/A	N/A	2.21E+02	86.8	823	N/A	N/A	N/A	N/A	-43.4	4.29
PC RNA 10^{-5}	N/A	N/A	N/A	N/A	10.5	7.89	N/A	N/A	N/A	1.40E+02	3.55	643	N/A	N/A	N/A	N/A	-61.5	5.23
Std 2x10^{1}	N/A	N/A	2.00E+01	2.00E+01	11.9	11.6	**39.50**	**35.83**	2.00E+01	2.00E+01	208	386	**N/A**	**37.98**	2.00E+01	2.00E+01	5.42	709
Std 2x10^{2}	N/A	N/A	2.00E+02	2.00E+02	30.5	26.7	33.33	33.17	2.00E+02	2.00E+02	1005	925	34.08	33.52	2.00E+02	2.00E+02	2276	2229
Std 2x10^{3}	33.49	34.78	2.00E+03	2.00E+03	257	185	30.18	30.17	2.00E+03	2.00E+03	2838	2176	30.62	30.54	2.00E+03	2.00E+03	5306	4428
Std 2x10^{4}	28.87	29.14	2.00E+04	2.00E+04	925	670	27.01	27.10	2.00E+04	2.00E+04	4562	3354	27.52	27.49	2.00E+04	2.00E+04	7687	6117
Std 2x10^{5}	25.11	25.15	2.00E+05	2.00E+05	1693	1444	23.46	23.85	2.00E+05	2.00E+05	5503	4557	24.16	24.23	2.00E+05	2.00E+05	8765	7970
Std 2x10^{6}	21.38	21.78	2.00E+06	2.00E+06	2138	1637	20.12	20.52	2.00E+06	2.00E+06	5816	4394	20.84	21.10	2.00E+06	2.00E+06	9275	7427

TNF-α triplex assay

Pos. RNA and std. dilutions	TNF-α *FAM*						β-Actin *HEX*						GAPDH *Texas Red*					
	Cq-values		Copies/µl		End RFUs		Cq-values		Copies/µl		End RFUs		Cq-values		Copies/µl		End RFUs	
V-MM in µl	25	12.5	25	12.5	25	12.5	25	12.5	25	12.5	25	12.5	25	12.5	25	12.5	25	12.5
PC RNA 10^{-1}	27.16	27.33	1.29E+03	1.13E+03	4057	3296	26.38	27.05	1.04E+04	1.05E+04	4248	2486	26.13	26.03	2.77E+04	2.70E+04	8996	6823
PC RNA 10^{-2}	30.57	30.71	1.21E+02	1.05E+02	1856	1692	29.75	29.80	8.80E+02	1.41E+03	2447	1482	29.47	29.24	2.53E+03	2.82E+03	6342	5056
PC RNA 10^{-3}	**42.03**	**38.82**	4.19E-02	3.49E-01	325	532	31.07	32.88	3.31E+02	1.48E+02	993	548	33.27	32.80	1.66E+02	2.32E+02	2883	2562
PC RNA 10^{-4}	N/A	N/A	N/A	N/A	-6.66	3.38	**30.29**	**34.57**	1.56E+01	4.30E+01	621	309	**39.57**	**N/A**	1.82E+00	N/A	680	5
PC RNA 10^{-5}	N/A	N/A	N/A	N/A	4.14	4.58	34.24	34.51	3.22E+01	4.49E+01	483	293	N/A	N/A	N/A	N/A	2.26	1.86
Std 2x10^{1}	36.74	34.36	2.00E+01	2.00E+01	614	961	33.99	32.97	2.00E+01	2.00E+01	619	439	**38.93**	**43.27**	2.00E+01	2.00E+01	734	388
Std 2x10^{2}	30.00	29.96	2.00E+02	2.00E+02	2372	2113	31.53	32.25	2.00E+02	2.00E+02	1319	602	33.01	33.21	2.00E+02	2.00E+02	2905	2153
Std 2x10^{3}	26.35	26.35	2.00E+03	2.00E+03	5488	4615	28.78	29.55	2.00E+03	2.00E+03	2920	1666	29.79	29.53	2.00E+03	2.00E+03	5799	4812
Std 2x10^{4}	23.15	23.18	2.00E+04	2.00E+04	8864	6514	25.72	26.28	2.00E+04	2.00E+04	4693	2698	26.59	26.35	2.00E+04	2.00E+04	8735	6459
Std 2x10^{5}	19.92	20.02	2.00E+05	2.00E+05	9890	7939	22.37	23.01	2.00E+05	2.00E+05	5370	3421	23.38	23.19	2.00E+05	2.00E+05	9375	7516
Std 2x10^{6}	16.64	16.76	2.00E+06	2.00E+06	10544	7974	19.08	19.78	2.00E+06	2.00E+06	5705	3403	20.14	20.01	2.00E+06	2.00E+06	9841	7365

IFN-α triplex assay

Pos. RNA and std. dilutions	IFN-α *FAM*						β-Actin *HEX*						GAPDH *Texas Red*					
	Cq-values		Copies/µl		End RFUs		Cq-values		Copies/µl		End RFUs		Cq-values		Copies/µl		End RFUs	
V-MM in µl	25	12.5	25	12.5	25	12.5	25	12.5	25	12.5	25	12.5	25	12.5	25	12.5	25	12.5
PC RNA 10^{-1}	28.70	29.12	3.86E+02	3.28E+02	5112	3778	26.40	26.77	1.62E+04	9.03E+03	3224	2521	25.22	26.05	3.31E+04	2.84E+04	10948	5972
PC RNA 10^{-2}	33.33	35.69	1.26E+01	2.87E+00	866	518	29.65	29.43	2.63E+03	1.44E+03	1287	1325	28.64	29.24	2.71E+03	2.94E+03	6050	3819
PC RNA 10^{-3}	**40.28**	**N/A**	7.47E-02	N/A	263	92.8	33.80	34.89	2.55E+02	3.36E+01	335	249	31.28	32.79	3.94E+02	2.35E+02	3407	1591
PC RNA 10^{-4}	N/A	N/A	N/A	N/A	10.6	6.42	N/A	N/A	N/A	N/A	9.86	39	37.03	38.02	5.91E+00	5.74E+00	573	412
PC RNA 10^{-5}	N/A	N/A	N/A	N/A	120	3.06	N/A	N/A	N/A	N/A	1.01	0.8	42.08	42.51	1.47E-01	2.38E-01	222	209
Std 2x10^{1}	32.95	33.36	2.00E+01	2.00E+01	1101	944	**41.20**	**N/A**	2.00E+01	2.00E+01	44.9	34.9	**34.91**	**N/A**	2.00E+01	2.00E+01	1035	4.79
Std 2x10^{2}	29.29	29.42	2.00E+02	2.00E+02	5006	4057	32.08	32.37	2.00E+02	2.00E+02	587	528	32.55	34.92	2.00E+02	2.00E+02	2275	822
Std 2x10^{3}	26.38	26.49	2.00E+03	2.00E+03	9063	7534	28.52	29.00	2.00E+03	2.00E+03	1795	1504	29.28	29.89	2.00E+03	2.00E+03	4972	3037
Std 2x10^{4}	23.46	23.46	2.00E+04	2.00E+04	11364	9274	25.50	25.41	2.00E+04	2.00E+04	3145	2683	26.06	26.44	2.00E+04	2.00E+04	8219	4945
Std 2x10^{5}	20.27	20.24	2.00E+05	2.00E+05	12809	9514	22.17	22.28	2.00E+05	2.00E+05	4142	3165	22.64	23.16	2.00E+05	2.00E+05	10561	5610
Std 2x10^{6}	17.11	17.13	2.00E+06	2.00E+06	13043	10420	18.99	19.02	2.00E+06	2.00E+06	4499	3688	19.44	20.18	2.00E+06	2.00E+06	11520	6293

Table S4: Assessment of reproducibility by the use of a synthetic standard RNA dilution series as triplicates. Mean Cq-values of the 10-fold standard RNA dilution series ranging from $2x10^1$ to $2x10^6$ copies/µl are given for each channel (FAM, HEX, TR) for all cytokine triplex real-time RT-qPCR assays (IL-2, IL-4, IL-6, IL-8, IL-1β, TNF-α , IFN-α, β-Actin, GAPDH) along with corresponding Cq-deviations between the triplicates.

Std=Standard; „/“=no Cq-value detectable

IL-2 triplex assay							**IL-1β triplex assay**						
Standard dilution series	**IL-2 *FAM***		**β-Actin *HEX***		**GAPDH *TR***		***Standard dilution series***	**IL-1β *FAM***		**β-Actin *HEX***		**GAPDH *TR***	
	Cq mean	*Cq std. deviation*	Cq mean	*Cq std. deviation*	Cq mean	*Cq std. deviation*		Cq mean	*Cq std. deviation*	Cq mean	*Cq std. deviation*	Cq mean	*Cq std. deviation*
Std $2x10^1$	34.20	*0.09*	37.00	*0.53*	35.99	*0.94*	*Std $2x10^1$*	34.62	*0.12*	36.58	*0.26*	34.53	*0.26*
Std $2x10^2$	31.29	*0.05*	34.67	*0.20*	33.55	*0.31*	*Std $2x10^2$*	32.28	*0.12*	36.24	*0.55*	32.34	*0.16*
Std $2x10^3$	28.01	*0.07*	30.94	*0.16*	30.03	*0.11*	*Std $2x10^3$*	29.22	*0.06*	32.01	*0.12*	29.04	*0.05*
Std $2x10^4$	24.69	*0.06*	27.59	*0.24*	26.78	*0.12*	*Std $2x10^4$*	26.08	*0.04*	28.46	*0.06*	25.80	*0.06*
Std $2x10^5$	21.45	*0.12*	24.31	*0.40*	23.64	*0.39*	*Std $2x10^5$*	22.76	*0.08*	24.87	*0.17*	22.43	*0.06*
Std $2x10^6$	18.14	*0.01*	20.65	*0.14*	20.15	*0.03*	*Std $2x10^6$*	19.41	*0.05*	21.48	*0.08*	19.17	*0.06*
IL-4 triplex assay							**TNF-α triplex assay**						
	IL-2 *FAM*		**β-Actin *HEX***		**GAPDH *TR***			**IL-1β *FAM***		**β-Actin *HEX***		**GAPDH *TR***	
	Cq mean	*Cq std. deviation*	Cq mean	*Cq std. deviation*	Cq mean	*Cq std. deviation*		Cq mean	*Cq std. deviation*	Cq mean	*Cq std. deviation*	Cq mean	*Cq std. deviation*
Std $2x10^1$	36.36	*0.50*	35.71	*0.50*	35.30	*1.41*	*Std $2x10^1$*	34.28	*1.23*	35.96	*0.70*	37.14	*0.05*
Std $2x10^2$	32.18	*0.41*	34.96	*1.66*	31.88	*0.55*	*Std $2x10^2$*	29.47	*0.08*	33.65	*0.26*	32.25	*0.42*
Std $2x10^3$	28.84	*0.08*	30.45	*0.21*	28.06	*0.05*	*Std $2x10^3$*	26.24	*0.03*	30.78	*0.06*	29.17	*0.09*
Std $2x10^4$	25.65	*0.16*	27.04	*0.32*	24.92	*0.17*	*Std $2x10^4$*	23.04	*0.07*	27.44	*0.11*	25.78	*0.25*
Std $2x10^5$	22.44	*0.04*	23.77	*0.15*	21.61	*0.06*	*Std $2x10^5$*	19.68	*0.02*	24.14	*0.01*	22.49	*0.04*
Std $2x10^6$	19.24	*0.15*	20.35	*0.24*	18.43	*0.10*	*Std $2x10^6$*	16.44	*0.05*	20.88	*0.07*	19.27	*0.11*

IL-6 triplex assay

	IL-2 *FAM*		**β-Actin *HEX***		**GAPDH *TR***	
	Cq mean	*Cq std. deviation*	Cq mean	*Cq std. deviation*	Cq mean	*Cq std. deviation*
Std 2x10^1	34.12	*0.80*	/	/	/	/
Std 2x10^2	30.50	*0.10*	32.62	*0.29*	34.21	*0.49*
Std 2x10^3	28.02	*0.01*	29.05	*0.19*	29.94	*0.16*
Std 2x10^4	24.95	*0.05*	25.72	*0.17*	26.53	*0.08*
Std 2x10^5	21.59	*0.11*	22.49	*0.13*	23.29	*0.13*
Std 2x10^6	18.33	*0.02*	19.21	*0.07*	20.13	*0.04*

IL-8 triplex assay

Standard dilution series	**IL-2 *FAM***		**β-Actin *HEX***		**GAPDH *TR***	
	Cq mean	*Cq std. deviation*	Cq mean	*Cq std. deviation*	Cq mean	*Cq std. deviation*
Std 2x10^1	34.31	*0.76*	/	/	/	/
Std 2x10^2	29.19	*0.15*	33.10	*0.41*	/	/
Std 2x10^3	26.06	*0.10*	29.51	*0.13*	30.89	*0.31*
Std 2x10^4	22.79	*0.14*	25.90	*0.37*	27.55	*0.14*
Std 2x10^5	19.50	*0.10*	22.74	*0.03*	24.31	*0.06*
Std 2x10^6	16.40	*0.02*	19.62	*0.09*	21.15	*0.02*

IFN-α triplex assay

	IL-1β *FAM*		**β-Actin *HEX***		**GAPDH *TR***	
	Cq mean	*Cq std. deviation*	Cq mean	*Cq std. deviation*	Cq mean	*Cq std. deviation*
Std 2x10^1	34.55	*0.77*	36.57	*1.02*	35.86	*1.00*
Std 2x10^2	31.69	*0.13*	35.14	*0.19*	32.73	*0.35*
Std 2x10^3	28.30	*0.17*	30.65	*0.44*	29.35	*0.19*
Std 2x10^4	25.23	*0.09*	27.21	*0.06*	26.07	*0.07*
Std 2x10^5	22.02	*0.05*	23.95	*0.20*	22.85	*0.01*
Std 2x10^6	18.56	*0.03*	20.33	*0.17*	19.33	*0.08*

Table S5: Testing of experimental samples.

All seven triplex RT-qPCRs were performed with different sample matrices (leukocytes, EDTA-blood) in order to prove suitability for field samples. The following sample amounts were included in all assays:

45 wb and 85 dp infected with CSFV strain "Roesrath" along with corresponding negative controls (neg.ctr.): 24 wb, 49 dp;

24 samples from CSFV strain "Koslov" infected pigs; 17 samples from ASFV strain "Armenia" infected pigs with additional neg. ctr. (3 wb; 3 dp);

83 samples from pigs vaccinated against CSFV with "C-strain" vaccine (31 wb; 52 dp);

69 samples from pigs vaccinated against CSFV with the marker vaccine candidate "CP7_E2alf" (24 wb; 45 dp);

Relevant data from PCR assays are listed below:

Cq-values for target cytokines (IL-6, IL-8, TNF-α, IFN-α, IL-1β, IL-2, IL-4; FAM labled), for reference gene 1 (β-Actin; Ref 1) using HEX, for reference gene 2 (GAPDH; Ref 2) Texas Red (TR) labeled;

along with corresponding gene expression values of each target cytokine as ΔΔCq. Animal identities (Animal ID) are given as ear tag numbers (ET).

wb = wild boar; dp = domestic pig; DPI = days post inoculation; Vacc. = Vaccine

Animal ID	Breed	Age	Inoculum	DPI	IL-6	Ref 1	Ref 2	$\Delta\Delta C_q$	IL-8	Ref 1	Ref 2	ΔΔCq	TNF-α	Ref 1	Ref 2	ΔΔCq	IFN-α	Ref 1	Ref 2	ΔΔCq	IL-1β	Ref 1	Ref 2	ΔΔCq	IL-2	Ref 1	Ref 2	ΔΔCq	IL-4	Ref 1	Ref 2	ΔΔCq
ET 08	wb	weaner	CSFV "*Roesrath*"	0	36.95	28.20	29.13	0.0009	37.75	30.21	30.51	0.0003	33.30	29.84	30.46	0.0472	31.37	30.64	30.21	0.3347	32.34	30.66	31.21	0.1031	38.94	30.47	30.33	0.0017	N/A	28.41	30.92	N/A
				3	26.94	17.81	20.08	0.0061	24.49	22.38	21.47	0.0206	26.61	23.14	21.81	0.0285	26.91	23.44	21.73	0.0358	26.65	23.64	23.12	0.0362	N/A	23.54	21.81	N/A	N/A	23.02	22.51	N/A
				7	27.11	21.08	21.47	0.0108	20.10	21.91	21.17	1.7518	22.79	22.73	21.59	0.4470	23.34	21.43	22.78	0.4208	25.89	21.89	22.18	0.0122	31.57	21.46	21.76	0.0001	35.03	22.77	20.48	0.0000
				10	30.55	22.11	23.37	0.0026	25.29	22.59	22.76	0.1025	24.38	24.40	23.80	0.5529	23.03	22.33	24.20	1.1592	31.34	23.25	23.89	0.0006	32.31	22.95	23.59	0.0003	34.73	24.02	22.18	0.0001
				15	28.77	21.46	20.53	0.0004	20.02	21.58	20.80	0.3488	25.31	22.09	21.21	0.0410	28.49	22.70	21.08	0.0075	24.10	22.44	22.13	0.1084	N/A	22.77	21.17	N/A	N/A	21.85	22.08	N/A
ET 09	wb	weaner	CSFV "*Roesrath*"	0	40.42	29.41	30.18	0.0002	N/A	33.28	34.51	N/A	38.49	31.74	32.41	0.0044	32.71	33.41	31.74	0.5704	33.28	32.80	32.95	0.1946	38.75	32.48	32.20	0.0076	N/A	31.54	33.93	N/A
				2	34.39	27.94	28.73	0.0042	30.23	27.97	27.66	0.1306	29.69	30.05	28.24	0.4236	N/A	29.89	29.26	N/A	31.68	28.20	29.00	0.0089	N/A	28.46	27.20	N/A	N/A	28.94	28.68	N/A
				3	32.05	24.16	25.34	0.0036	23.14	23.98	24.71	1.4027	29.20	25.71	25.31	0.0512	36.07	24.22	26.54	0.0006	29.61	25.10	26.10	0.0085	30.80	25.06	25.47	0.0031	34.61	25.75	24.05	0.0003
				7	35.75	23.98	24.78	0.0002	25.16	24.42	24.37	0.3579	26.86	26.13	25.10	0.2759	27.18	24.36	26.20	0.2653	31.57	25.10	25.54	0.0016	N/A	24.55	25.26	N/A	31.21	25.72	23.75	0.0029
				10	31.30	22.70	22.95	0.0017	25.28	22.64	22.57	0.0986	24.67	24.08	23.35	0.3469	23.27	22.36	23.91	0.8997	32.44	23.24	23.54	0.0002	N/A	22.53	23.41	N/A	N/A	23.74	21.87	N/A
				14	35.45	25.64	25.89	0.0007	27.04	25.92	25.24	0.2163	29.80	27.62	26.26	0.0882	N/A	26.40	27.27	N/A	33.21	26.97	26.92	0.0014	22.82	25.93	26.02	0.3926	N/A	27.05	24.30	N/A
				21	41.37	27.18	27.00	0.0000	27.24	26.51	26.13	0.3888	30.62	28.55	26.32	0.0660	N/A	28.12	27.56	N/A	37.19	27.19	27.55	0.0001	N/A	27.00	25.12	N/A	N/A	27.96	27.30	N/A
				23	31.51	24.13	23.88	0.0017	26.27	23.59	22.96	0.0916	26.82	25.65	23.28	0.1251	N/A	24.32	24.05	N/A	29.91	24.08	24.26	0.0017	38.38	23.63	22.45	0.0000	N/A	24.78	24.17	N/A
ET 10	wb	weaner	CSFV "*Roesrath*"	0	42.55	29.18	29.16	0.0000	37.99	32.31	32.57	0.0009	34.30	30.20	30.78	0.0294	31.39	31.79	30.47	0.5342	32.30	31.51	31.88	0.1771	N/A	32.07	30.81	N/A	N/A	31.51	31.87	N/A
				1	34.06	26.25	27.82	0.0022	30.81	25.67	27.10	0.0317	29.87	27.80	27.40	0.1278	29.81	26.26	28.64	0.1763	31.01	26.10	28.05	0.0053	N/A	25.78	26.51	N/A	30.13	26.83	28.03	0.0100
				2	38.71	30.51	30.75	0.0009	33.64	30.04	29.83	0.0511	33.60	33.35	30.73	0.1955	N/A	33.03	32.28	N/A	33.27	30.20	30.96	0.0102	N/A	30.56	29.10	N/A	N/A	31.27	31.05	N/A
				3	28.00	21.48	20.91	0.0055	20.81	21.52	20.46	0.7373	24.57	23.26	21.17	0.1357	23.59	22.05	22.20	0.3580	25.83	22.24	21.66	0.0120	30.78	21.76	21.26	0.0002	35.50	23.16	19.86	0.0000
				7	32.76	23.45	24.32	0.0012	25.25	23.89	23.96	0.2444	26.02	25.34	24.63	0.3217	24.91	23.63	25.35	0.7408	32.84	24.47	25.00	0.0004	N/A	24.52	24.82	N/A	34.07	25.02	23.25	0.0002
				10	39.18	29.66	29.78	0.0007	30.66	30.73	29.23	0.3396	33.76	31.88	30.12	0.0884	N/A	32.75	33.11	N/A	41.70	31.17	30.81	0.0000	N/A	30.07	30.05	N/A	40.16	31.34	28.41	0.0001
				14	33.90	25.81	26.13	0.0023	28.33	26.47	26.01	0.1378	30.03	27.66	26.48	0.0822	N/A	26.82	27.83	N/A	34.23	27.08	27.34	0.0008	32.91	26.76	26.39	0.0015	31.82	27.22	25.05	0.0048
				17	30.32	22.82	23.49	0.0023	24.14	22.59	22.29	0.2323	25.67	24.69	23.12	0.1928	23.79	22.83	23.15	0.5269	29.06	23.10	23.42	0.0017	N/A	22.65	22.38	N/A	N/A	23.57	23.82	N/A
ET 13	wb	weaner	CSFV "*Roesrath*"	0	35.91	27.04	27.49	0.0007	31.12	28.06	28.74	0.0106	31.18	27.85	28.45	0.0542	30.24	28.73	28.30	0.2012	31.05	28.72	29.56	0.0775	42.85	28.94	28.46	0.0000	N/A	27.84	29.23	N/A
				1	37.23	31.16	31.92	0.0047	39.88	31.36	30.51	0.0012	38.82	34.94	31.13	0.0095	N/A	36.00	35.69	N/A	36.34	31.44	32.39	0.0024	N/A	31.29	29.82	N/A	N/A	32.74	32.17	N/A
				2	33.98	27.95	28.45	0.0050	30.23	28.11	27.57	0.1333	29.47	30.34	28.21	0.5409	N/A	29.77	29.38	N/A	31.13	28.24	28.91	0.0134	N/A	28.27	27.08	N/A	N/A	28.65	28.52	N/A
				3	N/A	27.53	27.57	N/A	26.80	28.24	28.44	0.2477	39.61	28.11	28.36	0.0002	34.35	29.07	28.20	0.0126	33.00	29.10	29.80	0.0234	40.61	29.22	28.72	0.0002	N/A	28.52	29.62	N/A
				7	25.56	19.06	20.21	0.0105	18.57	19.02	19.70	1.1648	20.84	21.00	20.48	0.6624	19.66	19.48	21.11	1.5411	24.64	20.08	20.60	0.0097	31.75	19.32	20.55	0.0000	34.93	20.73	19.22	0.0000
				10	30.14	21.93	23.22	0.0031	25.48	22.45	22.61	0.0815	24.64	24.16	23.34	0.3633	22.79	22.30	24.03	1.2794	31.74	23.43	24.01	0.0005	N/A	22.99	23.52	N/A	N/A	23.95	21.96	N/A
				14	34.02	24.38	24.35	0.0007	26.22	25.11	24.02	0.1932	28.49	26.25	24.67	0.0801	44.21	24.87	25.76	0.0000	31.96	25.77	25.67	0.0016	N/A	24.73	24.64	N/A	27.86	26.02	23.36	0.0320
				21	39.40	29.45	29.45	0.0002	27.93	28.82	28.65	1.2890	29.53	31.34	29.13	1.0060	N/A	32.20	31.22	N/A	33.06	29.39	29.90	0.0063	N/A	28.96	27.43	N/A	N/A	29.97	29.44	N/A
				24	29.64	20.93	21.44	0.0010	24.02	21.01	20.38	0.0742	23.02	22.78	20.96	0.3078	22.10	20.98	21.34	0.4791	27.99	21.23	22.00	0.0013	N/A	20.36	20.00	N/A	27.03	21.43	21.53	0.0018
ET 15	wb	weaner	CSFV "*Roesrath*"	0	32.22	26.27	26.29	0.0051	27.63	26.23	26.52	0.0375	29.18	27.01	27.22	0.1105	28.52	27.36	26.97	0.2653	28.64	27.49	28.07	0.1739	37.52	27.43	27.07	0.0005	N/A	27.01	28.15	N/A
				1	39.26	30.36	30.84	0.0006	N/A	29.82	29.46	N/A	34.72	32.68	30.21	0.0580	N/A	34.13	33.98	N/A	35.17	30.15	30.97	0.0024	N/A	30.76	29.20	N/A	N/A	30.79	31.10	N/A
				2	33.28	27.02	27.95	0.0052	29.97	27.21	26.73	0.0869	29.09	28.72	27.34	0.2986	33.36	27.97	28.50	0.0257	30.97	27.06	28.08	0.0076	N/A	27.49	26.64	N/A	26.32	27.29	28.26	0.2571
				3	33.15	25.36	25.97	0.0031	24.96	25.46	25.61	0.8839	30.62	27.38	26.28	0.0464	N/A	25.99	27.54	N/A	31.47	26.42	26.74	0.0041	N/A	25.68	26.26	N/A	N/A	27.14	24.97	N/A
				10	37.66	26.76	26.83	0.0003	27.68	27.09	26.29	0.2924	29.42	28.68	27.24	0.2279	N/A	28.11	28.75	N/A	35.89	27.80	27.73	0.0003	N/A	27.39	27.17	N/A	28.93	28.39	25.67	0.0728
				7	28.42	20.93	22.62	0.0060	24.05	21.78	22.24	0.1549	23.47	23.41	23.00	0.5648	22.05	21.60	23.46	1.3782	28.55	22.62	23.20	0.0030	N/A	22.31	23.11	N/A	27.01	23.22	21.63	0.0126
				14	37.04	25.42	25.91	0.0001	27.53	25.50	26.45	0.0315	31.13	25.38	26.16	0.0111	33.16	27.02	26.17	0.0072	32.15	26.43	27.71	0.0086	N/A	26.40	26.47	N/A	N/A	25.32	27.39	N/A
				21	29.33	23.02	23.52	0.0051	26.07	22.15	22.51	0.0542	24.15	24.35	23.15	0.5098	22.64	22.44	23.38	1.1066	29.88	22.22	23.30	0.0007	N/A	22.91	21.93	N/A	N/A	23.33	23.82	N/A
				28	29.50	22.73	22.29	0.0027	26.28	21.41	21.22	0.0228	24.27	23.85	21.74	0.2402	24.58	22.79	22.36	0.2277	29.22	21.87	22.41	0.0007	N/A	22.22	20.90	N/A	N/A	23.03	22.52	N/A
ET 16	wb	weaner	CSFV "*Roesrath*"	0	37.48	25.51	26.51	0.0001	31.10	26.23	27.16	0.0036	31.10	26.57	27.26	0.0248	29.34	27.27	27.28	0.1617	30.29	27.25	28.33	0.0538	N/A	27.16	27.26	N/A	N/A	25.87	28.09	N/A
				3	34.54	27.54	27.19	0.0020	27.59	28.83	28.06	0.1449	37.17	27.85	27.78	0.0006	34.31	28.96	27.76	0.0108	32.30	29.21	29.26	0.0332	N/A	29.22	28.23	N/A	N/A	28.62	29.65	N/A
				7	33.38	23.98	24.61	0.0010	26.04	24.19	24.24	0.1722	25.73	25.68	25.05	0.5085	24.74	24.09	25.69	1.0996	35.27	25.29	25.71	0.0001	35.30	23.98	24.89	0.0001	24.22	25.37	23.50	0.3837
				10	37.39	24.33	24.97	0.0001	27.25	24.87	24.40	0.0987	27.41	26.51	25.28	0.2276	26.37	24.54	25.92	0.4490	33.65	25.39	25.50	0.0004	N/A	24.46	25.16	N/A	N/A	25.88	23.67	N/A
				14	34.79	24.04	24.04	0.0003	25.84	24.28	23.41	0.1540	27.68	25.62	24.15	0.0960	32.62	24.65	25.17	0.0047	32.39	25.55	25.35	0.0009	N/A	24.17	24.09	N/A	17.33	25.39	22.57	1.5980
				15	32.52	23.45	24.07	0.0013	27.39	23.75	23.57	0.0465	25.99	25.36	24.29	0.2960	24.34	23.48	25.11	0.9596	33.65	24.64	24.75	0.0002	37.05	23.79	24.27	0.0000	31.18	25.09	22.97	0.0018
ET 07	wb	weaner	neg.ctr."*Roesrath*"	0	40.05	30.51	30.23	0.0003	35.65	33.42	31.71	0.0053	36.00	31.24	31.72	0.0172	30.99	31.94	30.67	0.7928	32.64	32.07	32.63	0.2169	N/A	32.99	31.31	N/A	N/A	32.69	32.72	N/A
				1	N/A	32.79	32.86	N/A	N/A	31.96	31.42	N/A	38.98	37.14	32.37	0.0277	N/A	38.76	38.06	N/A	42.19	32.05	33.18	0.0000	N/A	31.33	30.44	N/A	N/A	32.41	33.48	N/A
				2	N/A	30.85	29.76	N/A	N/A	30.59	29.50	N/A	N/A	29.97	29.13	N/A	N/A	30.58	29.35	N/A	29.71	21.74	22.83	0.0005	N/A	31.24	30.20	N/A	N/A	30.68	29.76	N/A
				3	34.09	25.93	25.92	0.0019	26.46	26.03	25.78	0.4006	30.19	27.57	26.25	0.0661	35.72	26.17	27.64	0.0022	30.34	26.57	26.67	0.0099	N/A	26.31	26.23	N/A	32.07	27.35	24.81	0.0038
				7	24.41	18.33	19.08	0.0125	18.02	18.52	18.71	1.0336	21.05	20.17	19.38	0.2967	19.83	18.92	20.18	0.8183	22.92	19.29	19.64	0.0196	30.90	18.64	19.27	0.0000	35.40	20.02	18.14	0.0000
				10	35.80	24.18	24.69	0.0002	25.80	24.03	24.14	0.1855	26.41	26.10	25.28	0.3963	25.65	24.42	26.06	0.7436	33.17	25.15	25.64	0.0005	N/A	24.51	25.10	N/A	N/A	25.53	23.40	N/A
				14	32.92	23.42	24.62	0.0012	23.68	23.64	24.11	0.6992	26.15	25.61	24.89	0.3516	26.54	24.41	25.90	0.3766	29.58	24.72	25.43	0.0061	31.50	24.27	25.04	0.0012	40.55	25.14	23.50	0.0000
				28	30.93	22.15	23.29	0.0011	25.52	22.04	22.20	0.0694	23.61	24.05	22.84	0.6102	22.61	21.95	22.89	0.8074	N/A	44.64	41.44	N/A	N/A	22.39	21.47	N/A	N/A	23.17	23.48	N/A
ET 12	wb	weaner	neg.ctr."*Roesrath*"	0	38.86	29.63	28.65	0.0004	37.98	31.96	31.04	0.0005	34.16	29.74	30.30	0.0174	32.31	30.11	30.69	0.3519	32.89	31.82	31.73	0.1218	39.05	31.68	30.80	0.0029	24.22	30.72	31.84	1.2600
				1	33.50	26.49	27.87	0.0036	31.65	25.92	27.06	0.0188	31.02	27.78	27.41	0.0561	29.93	26.71	28.79	0.1992	32.69	26.63	28.28	0.0019	N/A	26.26	26.50	N/A	N/A	27.05	28.21	N/A
				2	38.91	29.10	30.57	0.0005	33.82	28.63	29.75	0.0266	32.80	30.66	29.65	0.0934	37.81	30.82	32.09	0.0109	33.23	28.59	30.62	0.0054	N/A	28.50	29.12	N/A	32.59	29.47	30.59	0.0088
				3	28.27	19.49	20.56	0.0021	19.35	19.48	20.20	0.9359	23.09	21.43	20.86	0.1830	22.64	20.20	21.96	0.3344	25.33	20.84	21.47	0.0101	33.12	20.35	21.01	0.0000	29.27	21.21	19.58	0.0006

				7	26.65	19.19	20.55	0.0057	22.08	19.53	20.17	0.1417	21.19	21.29	20.97	0.6766	19.52	19.49	21.27	1.7976	27.39	20.25	20.81	0.0014	N/A	19.76	21.03	N/A	17.91	21.02	19.50	1.8960
				10	29.95	21.58	22.68	0.0026	23.44	21.95	22.37	0.2625	23.29	23.46	23.06	0.6654	22.57	22.07	23.49	1.1456	28.11	22.67	23.26	0.0043	N/A	22.24	23.11	N/A	25.00	23.36	21.61	0.0572
				14	30.14	21.43	22.54	0.0021	21.66	21.94	22.06	0.8064	23.20	23.56	22.82	0.6761	22.73	21.90	23.24	0.8866	28.16	22.76	23.05	0.0040	N/A	22.19	22.80	N/A	33.72	23.21	21.29	0.0001
				21	27.26	19.14	20.57	0.0022	19.89	18.81	19.35	0.4550	22.36	20.85	20.02	0.1832	20.84	19.95	20.66	0.6372	24.40	18.98	20.41	0.0054	31.52	18.86	18.95	0.0000	37.12	19.97	20.78	0.0000
				28	31.60	23.17	24.16	0.0013	25.80	23.16	23.15	0.1168	24.75	25.06	23.84	0.5449	23.65	23.26	23.92	0.8747	30.12	23.06	23.90	0.0009	N/A	22.76	22.33	N/A	N/A	24.00	24.24	N/A
ET 14	wb	weaner	neg.ctr."*Roesrath*"	0	39.42	28.87	29.29	0.0002	32.28	29.45	29.40	0.0087	29.87	26.61	29.43	0.0558	30.26	27.61	29.64	0.4978	32.36	30.57	30.48	0.0762	N/A	30.61	29.81	N/A	N/A	29.59	30.81	N/A
				3	N/A	27.85	30.35	N/A	N/A	32.39	40.00	N/A	34.09	30.43	27.04	0.0311	29.58	32.22	26.80	0.1303	30.20	27.60	28.16	0.0602	N/A	27.60	26.98	N/A	33.08	26.22	27.72	0.0015
				7	25.86	18.57	19.71	0.0061	18.39	19.00	19.19	1.1068	21.10	20.40	20.00	0.3835	19.38	19.15	20.74	1.4713	24.52	19.57	20.19	0.0077	27.57	19.03	20.05	0.0007	N/A	20.22	18.79	N/A
				10	29.60	21.33	22.57	0.0030	23.16	21.46	22.18	0.2537	23.42	23.41	23.05	0.5960	22.30	21.65	23.34	1.1329	28.14	22.28	22.90	0.0033	32.40	21.96	22.93	0.0001	35.09	23.12	21.45	0.0000
				14	29.28	21.47	22.42	0.0037	23.64	21.91	21.87	0.1904	23.44	23.52	22.77	0.5528	22.45	22.09	23.34	1.1900	28.37	22.52	23.02	0.0031	N/A	22.15	22.83	N/A	27.62	23.11	21.33	0.0070
				21	26.12	19.57	21.11	0.0071	20.43	19.87	20.07	0.5777	22.54	21.65	20.54	0.2544	21.46	20.50	21.18	0.5993	26.03	20.18	21.29	0.0031	24.27	20.32	19.42	0.0152	N/A	21.00	21.28	N/A
				23	29.51	21.29	22.34	0.0017	25.49	21.16	21.52	0.0410	22.28	23.18	22.01	0.8637	21.90	21.22	22.04	0.7632	30.87	21.45	22.37	0.0002	N/A	20.89	20.75	N/A	N/A	22.16	22.56	N/A
ET 49	dp	weaner	CSFV "*Roesrath*"	0	37.80	26.21	26.56	0.0001	30.11	26.35	26.63	0.0065	29.92	27.00	27.36	0.0681	28.26	27.54	26.92	0.3297	29.28	27.39	28.02	0.1041	41.19	27.72	27.32	0.0001	N/A	26.28	28.03	N/A
				1	N/A	31.14	32.45	N/A	N/A	30.07	30.77	N/A	44.12	33.15	32.07	0.0002	N/A	35.24	37.03	N/A	39.60	30.42	32.12	0.0001	N/A	30.21	30.54	N/A	N/A	31.55	32.50	N/A
				2	36.96	31.54	32.10	0.0069	N/A	31.18	30.40	N/A	36.30	35.59	31.17	0.0720	N/A	36.02	36.56	N/A	37.61	30.60	32.92	0.0008	N/A	30.62	29.53	N/A	39.28	31.80	32.31	0.0002
				3	31.53	21.71	21.36	0.0003	21.89	22.41	22.19	0.1834	26.34	22.19	22.25	0.0294	29.03	23.11	22.03	0.0082	28.06	23.47	23.28	0.0131	38.41	23.11	22.19	0.0000	34.10	22.32	23.09	0.0030
				7	35.10	23.95	25.27	0.0004	26.41	24.29	24.79	0.1657	26.27	25.83	25.65	0.4512	26.17	24.32	26.40	0.5618	34.78	25.03	25.95	0.0002	27.13	24.30	25.57	0.0379	21.94	25.36	24.10	1.8500
				10	30.51	22.26	23.51	0.0030	25.57	22.62	23.19	0.0988	25.09	24.45	24.16	0.3883	23.42	23.05	25.04	1.5266	32.53	23.38	24.11	0.0003	N/A	22.46	23.79	N/A	23.72	23.88	22.28	0.2193
				14	N/A	31.01	31.51	N/A	27.18	25.53	26.05	0.2265	30.58	28.36	27.13	0.0884	N/A	26.99	28.62	N/A	32.53	27.98	28.15	0.0051	N/A	26.49	26.72	N/A	25.09	27.63	25.51	0.8732
				21	33.37	27.13	26.78	0.0033	27.94	26.98	25.55	0.2263	30.00	28.94	26.66	0.1323	N/A	27.99	27.09	N/A	33.46	27.32	26.83	0.0008	N/A	26.80	25.04	N/A	N/A	27.53	26.69	N/A
				24	30.30	22.65	23.24	0.0020	25.57	22.22	22.12	0.0693	24.27	24.45	22.82	0.4336	N/A	26.59	23.38	N/A	35.64	26.16	26.91	0.0001	N/A	22.55	21.76	N/A	N/A	23.58	23.44	N/A
ET 50	dp	weaner	CSFV "*Roesrath*"	0	35.53	26.13	26.81	0.0005	32.75	26.54	27.27	0.0012	30.26	27.20	27.93	0.0700	28.84	28.02	27.63	0.3326	30.21	28.05	28.77	0.0860	N/A	28.01	27.69	N/A	33.56	26.83	28.57	0.0024
				1	38.11	27.21	28.91	0.0002	35.73	26.59	27.89	0.0017	33.07	29.11	28.48	0.0301	32.12	28.41	29.83	0.1121	32.70	27.18	29.17	0.0031	N/A	27.45	27.53	N/A	N/A	28.00	29.04	N/A
				2	N/A	30.46	30.58	N/A	35.99	29.85	30.03	0.0095	31.68	31.90	30.07	0.3689	N/A	32.62	32.25	N/A	33.23	29.92	30.70	0.0088	N/A	29.48	29.02	N/A	N/A	30.56	30.83	N/A
				3	N/A	27.53	28.74	N/A	26.48	28.62	29.44	0.4967	37.13	29.09	29.54	0.0018	34.42	29.57	29.27	0.0204	33.50	29.25	30.45	0.0216	N/A	29.49	29.57	N/A	N/A	27.92	30.34	N/A
				7	39.05	26.09	27.28	0.0001	26.83	26.64	27.22	0.6209	29.40	28.30	27.76	0.2430	38.40	27.12	29.35	0.0009	39.27	27.55	28.40	0.0000	N/A	26.67	27.96	N/A	33.61	28.08	26.52	0.0029
				10	30.07	21.75	23.24	0.0031	24.84	22.32	22.58	0.1206	23.63	23.53	23.30	0.5848	22.60	22.20	23.80	1.3060	29.78	22.74	23.60	0.0014	N/A	22.37	23.35	N/A	29.44	23.37	21.92	0.0025
				14	36.68	26.48	28.02	0.0008	24.63	24.94	25.56	0.9218	27.50	26.78	26.17	0.3168	N/A	25.85	27.72	N/A	30.26	26.19	27.10	0.0107	33.77	25.56	26.23	0.0005	24.64	26.67	24.60	0.6421
				18	30.62	23.37	24.45	0.0031	26.58	23.19	23.35	0.0728	24.65	25.26	24.03	0.6674	24.44	23.33	24.24	0.5812	30.98	23.43	24.51	0.0006	28.23	23.26	22.79	0.0073	N/A	24.32	24.66	N/A
ET 51	dp	weaner	CSFV "*Roesrath*"	0	N/A	29.75	30.15	N/A	N/A	30.38	30.65	N/A	36.45	30.04	30.47	0.0055	31.16	31.07	30.75	0.5398	33.80	31.25	31.79	0.0537	44.64	31.09	30.41	0.0000	N/A	30.06	31.74	N/A
				2	34.75	27.40	28.43	0.0024	35.23	30.17	31.13	0.0272	29.19	29.04	28.08	0.4031	28.64	28.05	29.28	0.9123	31.11	27.00	28.70	0.0083	N/A	27.49	27.24	N/A	N/A	28.15	28.62	N/A
				3	28.47	18.92	20.48	0.0014	20.14	19.50	20.26	0.5566	22.23	21.34	21.10	0.3485	22.37	20.22	22.10	0.4268	25.48	20.75	21.88	0.0101	22.61	20.07	21.13	0.0577	35.38	21.02	19.66	0.0000
				10	32.28	23.22	24.11	0.0015	26.05	23.06	23.61	0.0947	24.91	25.00	24.59	0.6104	23.83	22.95	24.85	1.0416	32.53	24.10	24.58	0.0004	N/A	23.47	24.36	N/A	N/A	24.62	22.98	N/A
				14	30.34	21.43	22.75	0.0019	23.63	21.71	22.15	0.1969	23.15	23.32	22.95	0.6761	22.35	21.59	23.38	1.0843	29.50	22.70	23.42	0.0016	N/A	21.39	22.97	N/A	33.84	23.13	21.47	0.0001
				17	30.86	21.26	23.20	0.0009	25.75	21.63	21.83	0.0445	23.24	23.47	22.55	0.5836	21.99	21.61	22.36	0.9133	29.86	21.46	22.44	0.0004	N/A	21.78	21.38	N/A	N/A	22.78	23.38	N/A
ET 52	dp	weaner	CSFV "*Roesrath*"	0	N/A	26.49	28.13	N/A	42.63	26.46	27.93	0.0000	33.21	27.35	28.60	0.0115	29.10	28.19	28.32	0.3700	32.28	27.83	29.26	0.0215	N/A	28.38	28.82	N/A	N/A	26.28	29.40	N/A
				1	39.19	29.49	30.25	0.0004	34.14	29.48	29.18	0.0234	33.50	31.14	29.68	0.0680	N/A	30.92	31.10	N/A	34.15	29.00	29.90	0.0024	N/A	29.20	28.58	N/A	N/A	30.00	30.31	N/A
				2	31.83	24.85	26.18	0.0039	31.64	27.20	27.72	0.0376	26.89	26.24	25.46	0.3160	26.13	25.11	26.04	0.6168	29.94	24.62	26.26	0.0039	N/A	24.53	24.63	N/A	19.04	25.21	26.30	1.4201
				3	N/A	28.28	29.34	N/A	28.24	29.33	30.68	0.2573	N/A	28.60	30.03	N/A	N/A	29.75	29.41	N/A	36.90	29.78	31.05	0.0028	N/A	29.82	30.10	N/A	N/A	28.61	31.18	N/A
				7	28.94	20.05	21.18	0.0019	21.69	20.35	20.58	0.2810	21.65	22.15	21.43	0.7659	21.34	20.30	21.97	0.8604	27.82	21.09	21.56	0.0017	13.74	20.68	21.52	0.0670	22.28	21.85	20.15	0.1508
				10	31.48	23.19	23.90	0.0024	25.16	22.90	23.40	0.1551	24.66	24.77	24.21	0.5912	23.56	23.05	24.67	1.2146	31.86	23.71	24.34	0.0005	24.14	23.34	24.16	0.1565	N/A	24.34	22.76	N/A
				14	35.53	25.03	26.26	0.0006	25.21	25.32	26.03	0.8195	27.81	27.06	26.53	0.3163	40.28	25.79	27.76	0.0001	32.17	26.21	27.20	0.0026	24.72	25.76	26.48	0.5061	N/A	26.55	25.11	N/A
ET 53	dp	weaner	CSFV "*Roesrath*"	0	39.17	27.19	28.40	0.0001	35.30	27.60	28.46	0.0004	33.72	27.68	29.02	0.0103	29.74	28.83	28.91	0.3606	31.57	29.08	30.14	0.0735	N/A	29.10	29.35	N/A	N/A	28.50	30.31	N/A
				1	N/A	28.79	30.84	N/A	38.08	29.04	29.51	0.0014	36.09	30.45	30.30	0.0106	38.99	29.94	32.59	0.0042	33.62	29.02	30.72	0.0048	N/A	28.69	29.06	N/A	N/A	29.46	30.76	N/A
				2	37.60	28.85	29.53	0.0007	31.48	28.51	28.86	0.0994	30.17	31.00	29.18	0.5768	41.76	30.28	30.58	0.0004	32.14	28.94	29.49	0.0095	N/A	28.36	28.06	N/A	N/A	29.50	30.04	N/A
				21	32.20	25.23	25.31	0.0025	27.39	25.04	24.20	0.1053	27.32	27.01	24.82	0.2400	31.61	25.52	25.40	0.0128	32.06	25.31	25.64	0.0008	N/A	24.69	23.57	N/A	N/A	25.70	25.46	N/A
ET 54	dp	weaner	CSFV "*Roesrath*"	0	39.67	26.13	27.31	0.0000	31.43	26.41	27.58	0.0034	30.35	26.57	27.75	0.0498	29.74	27.91	27.81	0.1809	30.30	27.59	28.76	0.0693	39.45	27.57	27.72	0.0002	40.07	26.07	28.52	0.0000
				2	37.15	28.14	29.13	0.0007	31.51	27.71	28.07	0.0554	29.03	29.52	28.50	0.6163	37.73	29.56	29.85	0.0034	31.87	28.18	29.34	0.0086	N/A	27.91	27.46	N/A	N/A	29.87	29.36	N/A
				3	N/A	27.12	28.11	N/A	26.98	27.95	27.93	1.1005	30.89	29.59	28.74	0.1874	N/A	28.38	30.64	N/A	34.72	28.75	29.21	0.0018	35.06	27.95	28.74	0.0010	36.23	29.06	27.19	0.0007
				7	29.69	21.58	23.06	0.0036	25.03	22.01	22.46	0.0910	23.46	23.62	23.30	0.6802	22.18	21.78	23.52	1.3625	31.22	22.47	23.30	0.0004	35.00	22.58	23.26	0.0000	N/A	23.43	21.91	N/A
				10	29.44	21.35	22.45	0.0032	25.14	21.71	22.14	0.0689	23.47	23.54	23.07	0.6049	21.99	21.25	23.18	1.1569	31.81	22.27	22.79	0.0002	37.24	21.75	22.82	0.0000	N/A	22.97	21.33	N/A
				20	26.49	21.32	22.30	0.0145	22.08	21.19	21.28	0.4312	25.49	23.40	21.82	0.0890	25.37	22.42	22.61	0.1265	28.16	21.78	22.56	0.0016	N/A	21.87	20.68	N/A	N/A	22.50	22.62	N/A
ET 55	dp	weaner	CSFV "*Roesrath*"	0	33.30	24.91	26.48	0.0029	28.97	25.78	26.36	0.0786	27.23	27.05	26.83	0.5248	23.96	25.34	28.18	6.8720	29.61	26.23	27.57	0.0209	N/A	25.64	26.74	N/A	30.00	26.75	25.42	0.0175
				1	31.46	24.05	25.47	0.0031	29.26	23.80	24.51	0.0201	27.58	25.65	25.07	0.1376	23.95	23.76	25.18	1.3112	28.60	23.31	25.15	0.0048	29.24	23.43	24.02	0.0057	37.27	24.35	25.51	0.0000
				2	31.82	25.92	27.02	0.0074	29.76	25.50	25.84	0.0406	27.50	27.32	26.52	0.4290	28.74	26.36	27.28	0.2379	29.84	25.10	27.18	0.0068	N/A	25.78	25.59	N/A	N/A	28.35	28.66	N/A
				3	35.81	24.83	25.74	0.0004	26.48	25.01	25.28	0.2381	26.93	26.78	26.04	0.4504	26.12	24.99	26.79	0.8416	32.24	25.73	26.31	0.0016	34.91	25.25	25.91	0.0002	25.26	26.14	24.60	0.3417
				7	28.44	21.33	22.25	0.0060	24.05	21.71	21.92	0.1358	22.97	23.16	22.60	0.6402	21.41	21.11	22.99	1.5427	29.63	22.08	22.63	0.0009	24.37	21.26	22.54	0.0377	24.44	22.71	21.21	0.0602
				10	35.49	24.62	25.47	0.0004	27.85	25.04	25.13	0.0880	26.62	26.54	25.99	0.5067	25.87	24.75	26.52	0.8399	36.14	25.61	26.10	0.0001	N/A	24.85	25.92	N/A	27.79	26.09	24.47	0.0504

				11	38.60	26.85	24.96	0.0000	28.00	27.37	25.58	0.0291	30.86	27.03	25.40	0.0180	30.13	28.30	25.34	0.0686	31.34	28.11	26.83	0.0196	N/A	28.15	25.58	N/A	N/A	26.58	26.41	N/A
ET 56	dp	weaner	CSFV "*Roesrath*"	0	38.27	28.43	29.13	0.0004	39.68	29.69	29.57	0.0000	32.13	28.93	29.40	0.0551	29.92	29.89	29.31	0.5250	31.20	30.05	30.27	0.1381	N/A	30.16	29.30	N/A	N/A	29.41	30.40	N/A
				1	N/A	29.47	31.90	N/A	N/A	28.93	30.93	N/A	42.69	32.12	31.54	0.0003	N/A	32.22	34.38	N/A	37.49	29.69	32.09	0.0005	N/A	30.13	30.60	N/A	N/A	30.59	31.33	N/A
				2	37.19	31.34	31.99	0.0053	38.57	30.91	30.99	0.0031	32.33	34.16	30.93	0.6777	N/A	38.47	34.23	N/A	33.87	31.08	31.75	0.0114	N/A	31.04	29.79	N/A	N/A	30.95	31.26	N/A
				3	31.66	24.56	24.57	0.0042	24.60	24.87	24.18	0.5764	26.06	26.21	24.86	0.4547	N/A	25.34	26.62	N/A	28.92	25.49	25.63	0.0139	N/A	24.84	24.68	N/A	32.06	25.97	23.51	0.0016
				7	28.36	20.77	22.05	0.0049	25.08	21.02	21.55	0.0463	23.14	22.94	22.41	0.4975	21.13	21.07	22.67	1.6576	30.62	21.78	22.28	0.0003	34.51	21.23	22.35	0.0000	N/A	22.55	21.02	N/A
				10	28.99	21.44	22.49	0.0046	26.02	22.06	22.02	0.0401	23.32	23.30	22.73	0.5504	21.92	21.34	23.12	1.2319	30.39	22.37	22.87	0.0006	N/A	21.63	22.76	N/A	25.05	23.09	21.27	0.0446
				14	34.87	26.37	26.51	0.0016	27.33	26.67	26.19	0.3124	28.78	28.16	26.85	0.2611	N/A	27.68	28.29	N/A	31.55	27.87	27.64	0.0087	N/A	26.92	26.76	N/A	30.72	28.17	25.33	0.0163
				21	32.98	22.11	23.75	0.0003	25.15	21.35	21.96	0.0647	23.64	23.65	22.74	0.5021	21.32	21.74	22.79	1.7691	29.98	21.54	23.01	0.0004	N/A	21.62	21.51	N/A	N/A	22.82	23.34	N/A
				28	33.30	22.52	24.06	0.0003	26.36	22.68	23.05	0.0642	24.25	24.48	23.52	0.5688	23.35	22.45	23.68	0.7528	31.18	22.40	23.82	0.0003	16.94	22.58	22.44	0.7508	N/A	23.63	24.12	N/A
ET 57	dp	weaner	CSFV "*Roesrath*"	0	38.07	29.13	29.26	0.0006	N/A	30.12	29.86	N/A	32.88	29.43	30.07	0.0485	29.77	30.48	29.59	0.7798	31.75	30.64	31.13	0.1525	N/A	30.85	30.17	N/A	N/A	29.06	30.57	N/A
				1	N/A	32.52	33.36	N/A	N/A	33.47	31.82	N/A	N/A	34.67	33.90	N/A	N/A	38.14	36.68	N/A	42.06	32.83	33.45	0.0001	N/A	18.26	30.95	N/A	26.29	33.97	33.73	0.9218
				2	39.39	28.82	30.14	0.0003	35.02	29.13	28.83	0.0097	30.95	30.55	29.16	0.2820	41.38	30.70	30.89	0.0006	32.95	28.59	29.92	0.0053	N/A	28.84	28.15	N/A	N/A	29.49	30.51	N/A
				3	29.50	17.46	19.64	0.0002	26.79	29.32	28.93	0.4115	35.58	27.87	28.43	0.0024	35.00	29.34	28.60	0.0101	32.25	29.10	29.93	0.0419	40.21	29.00	28.69	0.0002	N/A	28.35	29.50	N/A
				7	30.87	23.16	23.96	0.0037	24.79	23.30	23.61	0.2449	24.85	24.96	24.28	0.5664	23.60	23.03	24.89	1.2660	31.63	24.13	24.58	0.0008	N/A	23.52	24.27	N/A	16.51	24.60	22.66	1.0240
				10	28.91	21.24	22.36	0.0044	24.27	21.81	22.11	0.1289	22.67	23.18	22.69	0.8187	21.72	21.27	23.07	1.3454	29.06	22.48	22.96	0.0018	N/A	21.84	22.69	N/A	N/A	22.94	21.31	N/A
				14	33.92	25.81	26.03	0.0022	25.18	25.67	25.42	0.7649	27.46	27.13	26.15	0.3642	N/A	26.67	27.82	N/A	30.77	26.64	27.17	0.0087	N/A	25.98	26.06	N/A	N/A	27.10	24.78	N/A
				18	30.50	22.91	24.02	0.0025	24.99	22.59	22.86	0.1540	24.24	24.67	23.45	0.5950	23.67	22.79	23.66	0.6740	30.82	23.08	23.94	0.0005	N/A	22.67	22.21	N/A	N/A	23.91	24.11	N/A
ET 58	dp	weaner	CSFV "*Roesrath*"	0	37.24	25.68	26.71	0.0001	32.65	26.95	27.37	0.0015	30.49	26.63	27.40	0.0408	28.85	27.12	27.16	0.2066	30.09	27.09	28.29	0.0581	N/A	27.29	27.33	N/A	35.29	25.97	28.15	0.0003
				1	36.09	27.01	29.67	0.0013	43.08	27.22	28.33	0.0000	31.79	29.44	29.17	0.1067	32.23	28.33	30.68	0.1361	32.61	27.31	29.46	0.0038	N/A	27.59	27.94	N/A	N/A	28.45	29.49	N/A
				2	N/A	30.42	32.32	N/A	N/A	30.51	31.05	N/A	32.62	33.06	31.88	0.5314	N/A	30.69	34.20	N/A	34.36	31.15	32.05	0.0089	N/A	30.60	30.18	N/A	N/A	29.38	29.18	N/A
				3	29.06	16.76	19.47	0.0002	28.43	30.22	29.80	0.2173	35.81	29.54	29.40	0.0050	36.53	30.47	29.26	0.0064	32.60	30.29	30.64	0.0620	N/A	30.32	29.88	N/A	N/A	29.71	30.62	N/A
				7	29.03	21.66	23.00	0.0058	24.41	22.03	22.44	0.1403	24.01	23.76	23.28	0.4826	22.10	21.62	23.60	1.4089	30.33	22.93	23.43	0.0009	N/A	22.06	23.30	N/A	29.32	23.19	21.98	0.0026
				10	27.39	20.20	21.27	0.0061	23.67	20.17	20.82	0.0725	21.92	21.88	21.70	0.6372	19.96	19.93	21.96	1.9613	29.23	21.09	21.92	0.0007	19.67	20.14	21.84	0.6781	29.65	21.37	20.08	0.0006
				14	N/A	30.55	31.05	N/A	29.68	30.60	30.49	0.9806	35.72	32.46	32.83	0.0698	N/A	32.78	38.63	N/A	N/A	31.58	31.95	N/A	N/A	31.15	31.54	N/A	N/A	32.58	29.94	N/A
				17	31.77	24.43	25.45	0.0027	25.76	24.07	24.35	0.2534	26.79	26.30	25.13	0.3075	33.18	24.87	25.38	0.0034	32.02	24.74	25.73	0.0007	N/A	24.55	24.07	N/A	N/A	25.33	25.64	N/A
ET 59	dp	weaner	CSFV "*Roesrath*"	0	34.10	27.74	27.60	0.0034	31.58	27.97	27.64	0.0050	29.98	27.71	28.08	0.1065	29.32	28.82	28.04	0.3570	30.14	28.82	29.14	0.1325	N/A	29.13	28.18	N/A	N/A	28.01	29.16	N/A
				1	35.06	26.10	28.10	0.0011	33.03	26.02	26.95	0.0070	30.25	27.85	27.55	0.1047	26.91	26.40	28.43	1.2865	31.79	25.79	27.96	0.0025	30.20	25.99	26.24	0.0140	N/A	27.05	28.23	N/A
				2	33.57	27.97	28.12	0.0061	31.25	28.02	27.07	0.0524	28.60	29.41	27.64	0.5928	37.71	29.26	28.63	0.0021	30.98	27.94	28.49	0.0117	N/A	27.89	26.75	N/A	N/A	28.45	29.25	N/A
				3	N/A	29.09	30.25	N/A	30.80	30.24	30.07	0.3446	37.19	32.12	30.95	0.0119	N/A	34.30	34.74	N/A	36.06	31.25	31.43	0.0034	N/A	30.10	30.88	N/A	27.81	32.27	29.32	0.0004
				7	35.14	23.91	25.72	0.0004	27.93	24.28	25.24	0.0669	28.44	26.49	26.28	0.1565	27.04	25.24	27.19	0.5570	32.42	25.05	26.37	0.0011	N/A	24.85	26.13	N/A	N/A	25.97	24.70	N/A
				10	30.97	22.84	24.01	0.0031	25.58	22.88	23.30	0.1108	24.27	24.33	24.13	0.6485	23.15	22.88	24.50	1.4430	31.36	23.78	24.33	0.0008	N/A	23.41	24.20	N/A	35.76	24.27	22.67	0.0000
				14	35.05	26.12	26.70	0.0014	27.92	25.95	26.28	0.1684	29.96	27.65	27.04	0.1043	N/A	26.66	28.39	N/A	32.51	27.18	28.17	0.0040	N/A	26.24	26.96	N/A	24.99	27.42	25.50	0.8744
				21	29.52	22.53	23.32	0.0035	25.25	22.23	22.34	0.0942	23.66	24.52	23.12	0.7570	23.59	22.60	23.13	0.5568	28.07	22.31	23.42	0.0028	N/A	22.62	21.79	N/A	N/A	23.56	23.63	N/A
				28	33.26	22.88	24.03	0.0004	26.22	22.28	23.02	0.0611	24.50	24.43	23.58	0.4752	23.81	22.63	24.00	0.6529	30.22	23.04	23.99	0.0008	N/A	21.53	22.21	N/A	N/A	23.34	24.14	N/A
ET 60	dp	weaner	CSFV "*Roesrath*"	0	33.93	25.42	26.18	0.0011	28.37	25.95	26.22	0.0179	28.90	26.27	26.89	0.0940	27.40	27.22	26.66	0.4924	28.91	27.15	27.64	0.1096	N/A	27.27	26.80	N/A	N/A	26.00	27.79	N/A
				2	30.45	24.00	24.86	0.0050	27.75	24.06	24.01	0.0542	25.14	25.13	24.39	0.5124	27.60	24.22	24.85	0.1090	28.21	23.61	25.14	0.0071	N/A	23.75	23.51	N/A	N/A	26.87	27.29	N/A
				3	31.07	21.85	23.01	0.0009	26.17	27.31	27.63	0.2247	33.06	27.55	28.01	0.0112	33.05	28.12	27.66	0.0188	32.52	28.57	28.99	0.0209	43.75	28.33	28.02	0.0000	N/A	27.25	28.82	N/A
				7	30.98	23.85	24.24	0.0047	23.57	23.11	23.84	0.5813	25.71	25.09	24.58	0.3617	24.60	23.43	25.13	0.7919	29.31	24.34	24.99	0.0056	N/A	23.80	24.55	N/A	34.68	24.93	23.22	0.0001
ET 43	dp	weaner	neg.ctr."*Roesrath*"	0	36.49	26.24	27.72	0.0004	31.59	27.00	27.82	0.0040	30.77	27.02	28.12	0.0491	28.99	27.74	27.97	0.3043	30.75	28.14	29.11	0.0681	N/A	28.14	28.11	N/A	N/A	26.95	29.31	N/A
				1	40.47	28.18	30.13	0.0001	40.41	27.53	29.14	0.0001	35.66	29.73	29.39	0.0081	35.42	29.51	30.93	0.0243	33.90	27.62	29.82	0.0018	N/A	27.49	28.07	N/A	N/A	28.22	30.24	N/A
				2	N/A	30.18	29.50	N/A	34.71	28.25	28.76	0.0087	31.06	30.02	29.18	0.2207	29.44	28.92	30.60	1.1155	33.03	27.85	29.50	0.0033	N/A	28.24	27.97	N/A	N/A	29.05	30.13	N/A
				3	37.30	26.19	27.13	0.0003	26.03	26.36	26.93	0.8922	29.41	28.15	27.51	0.2114	40.56	26.60	29.08	0.0001	32.01	27.26	28.05	0.0057	N/A	26.77	27.45	N/A	19.35	27.53	26.08	1.5602
				7	29.04	20.02	20.10	0.0012	19.28	20.15	19.58	1.0007	22.65	21.83	20.37	0.2416	21.45	20.44	21.20	0.6371	26.67	21.00	20.56	0.0028	33.53	20.32	20.32	0.0000	34.07	21.78	19.13	0.0000
				10	34.28	24.56	25.66	0.0010	26.20	24.65	25.25	0.2520	27.78	26.30	26.14	0.2197	26.03	24.09	26.71	0.6383	31.68	25.51	26.33	0.0022	32.43	24.98	25.96	0.0011	N/A	26.05	24.64	N/A
				14	30.63	22.71	24.33	0.0042	22.15	23.62	24.17	2.0596	24.74	24.85	24.58	0.6490	25.83	23.33	25.34	0.3517	27.43	23.92	24.96	0.0201	N/A	23.43	24.61	N/A	N/A	24.69	23.29	N/A
				21	30.43	21.74	23.03	0.0013	24.19	21.58	22.06	0.1440	22.98	23.53	22.48	0.6992	22.25	21.43	22.55	0.7697	28.89	21.30	22.45	0.0008	32.53	21.78	21.19	0.0001	N/A	22.41	23.16	N/A
				28	39.81	26.40	26.95	0.0000	26.73	25.66	25.84	0.3739	27.99	27.62	26.28	0.3070	N/A	26.61	27.18	N/A	31.45	25.64	27.08	0.0023	N/A	26.20	25.31	N/A	N/A	26.65	27.12	N/A
ET 44	dp	weaner	neg.ctr."*Roesrath*"	0	N/A	34.80	35.66	N/A	42.20	30.35	30.55	0.0000	32.80	29.55	30.07	0.0532	29.96	30.47	29.89	0.7538	31.80	30.78	30.98	0.1458	N/A	31.22	31.09	N/A	N/A	28.91	30.95	N/A
				1	N/A	31.73	32.80	N/A	N/A	30.65	31.21	N/A	38.24	33.64	31.53	0.0106	N/A	35.27	36.31	N/A	40.94	30.06	32.34	0.0000	N/A	31.10	30.77	N/A	N/A	32.04	33.92	N/A
				2	N/A	28.02	30.07	N/A	28.61	26.12	27.29	0.1916	29.57	27.74	27.49	0.1606	22.80	26.07	27.97	0.8700	33.04	26.05	27.68	0.0010	N/A	26.38	26.49	N/A	N/A	27.05	28.08	N/A
				3	N/A	27.94	28.32	N/A	28.26	29.22	30.01	0.1947	N/A	29.00	29.72	N/A	36.40	30.64	30.15	0.0100	34.93	30.45	31.17	0.0146	41.90	30.08	29.96	0.0002	43.56	29.55	31.39	0.00
				7	28.40	20.20	20.46	0.0023	19.42	20.09	20.19	1.0901	23.00	22.13	21.08	0.2645	21.85	20.91	21.91	0.7294	26.76	21.14	21.53	0.0039	25.12	20.40	20.90	0.0092	33.24	21.50	19.66	0.0000
				10	31.68	22.22	23.63	0.0013	23.22	22.65	23.48	0.5611	24.79	24.34	24.16	0.4608	23.45	22.36	24.32	0.9165	29.58	23.38	24.15	0.0025	35.10	23.03	23.82	0.0000	N/A	24.05	22.56	N/A
				14	31.09	22.19	23.87	0.0022	21.21	22.09	23.06	1.6280	24.57	24.12	24.12	0.4916	23.78	22.90	24.54	0.9493	28.49	23.24	24.19	0.0054	N/A	22.87	24.05	N/A	27.59	24.24	22.73	0.0172
				21	31.25	22.70	23.86	0.0013	23.44	22.04	22.96	0.3973	24.23	24.31	23.45	0.5307	23.84	22.48	23.53	0.5142	28.19	22.23	23.72	0.0028	N/A	22.30	22.23	N/A	N/A	23.31	24.08	N/A
				28	39.45	28.18	29.12	0.0001	26.17	27.32	28.08	2.1867	30.55	29.47	28.32	0.1934	N/A	30.67	29.32	N/A	33.64	27.45	29.17	0.0016	N/A	27.95	27.39	N/A	N/A	28.29	29.01	N/A
ET 45	dp	weaner	neg.ctr."*Roesrath*"	0	N/A	28.05	28.50	N/A	N/A	28.99	29.38	N/A	33.51	29.10	29.69	0.0243	30.26	29.79	29.38	0.4101	32.45	30.12	30.43	0.0606	39.17	30.87	30.49	0.0018	N/A	29.27	30.94	N/A

				1	39.36	30.36	31.44	0.0007	N/A	29.98	30.67	N/A	37.02	32.83	30.76	0.0146	N/A	33.25	34.22	N/A	36.63	29.83	31.32	0.0008	N/A	29.97	30.01	N/A	N/A	30.55	31.98	N/A
				2	35.04	26.13	28.04	0.0011	30.63	26.62	26.83	0.0459	28.77	28.23	27.44	0.3288	27.87	26.73	28.15	0.6702	31.08	26.37	28.12	0.0056	N/A	26.65	26.33	N/A	N/A	27.02	27.81	N/A
				3	N/A	27.48	28.58	N/A	27.13	27.72	28.31	1.0410	34.18	29.93	29.31	0.0262	N/A	29.55	33.68	N/A	33.21	28.48	29.83	0.0065	N/A	28.36	29.29	N/A	27.69	29.24	27.52	0.4531
				7	27.47	19.45	20.64	0.0036	18.30	19.88	20.19	2.2156	22.36	21.54	21.10	0.3426	20.34	19.94	21.63	1.3490	26.49	20.98	21.27	0.0041	32.72	20.37	21.05	0.0000	33.52	21.42	19.64	0.0000
				10	29.37	20.96	21.90	0.0025	23.98	20.90	21.59	0.0971	22.20	22.39	22.09	0.7114	21.22	20.82	22.40	1.2933	29.99	21.53	22.16	0.0005	N/A	21.11	22.13	N/A	35.29	22.25	20.77	0.0000
				14	N/A	30.18	31.82	N/A	28.49	30.66	31.27	2.9580	37.30	32.79	32.07	0.0201	N/A	34.07	35.90	N/A	N/A	31.94	32.62	N/A	35.26	31.38	31.94	0.0085	N/A	31.88	29.76	N/A
				21	30.16	22.39	23.71	0.0024	20.73	20.03	20.65	0.6039	23.08	22.01	21.08	0.2368	22.21	21.13	21.70	0.5340	25.80	20.32	21.75	0.0046	34.22	20.64	20.31	0.0000	34.71	21.37	21.94	0.0000
				28	41.48	28.30	28.84	0.0000	28.68	28.00	27.77	0.4172	30.93	30.07	28.39	0.1856	N/A	29.82	30.00	N/A	37.69	28.27	29.22	0.0001	N/A	28.48	27.34	N/A	N/A	28.55	28.98	N/A
ET 46	dp	weaner	neg.ctr."*Roesrath*"	0	N/A	30.22	30.22	N/A	N/A	31.44	30.92	N/A	35.84	30.48	31.01	0.0117	30.83	31.58	30.95	0.8599	34.02	31.57	31.71	0.0492	40.76	31.29	31.10	0.0008	N/A	30.82	32.45	N/A
				1	N/A	30.50	31.77	N/A	N/A	30.32	30.84	N/A	42.08	32.69	31.46	0.0005	N/A	33.64	34.64	N/A	40.68	30.15	31.68	0.0000	N/A	29.85	29.86	N/A	N/A	30.82	31.53	N/A
				2	N/A	30.33	31.11	N/A	N/A	30.13	30.14	N/A	32.45	32.37	30.36	0.2770	N/A	33.57	33.68	N/A	35.42	30.54	31.52	0.0027	N/A	30.58	29.69	N/A	N/A	31.27	31.62	N/A
				3	29.48	21.60	21.58	0.0015	22.92	22.32	22.12	0.0807	24.98	22.44	22.29	0.0850	26.66	22.97	22.13	0.0419	26.08	22.77	23.24	0.0429	N/A	23.10	22.24	N/A	21.48	22.12	22.93	0.46
				7	31.28	22.79	23.98	0.0024	22.62	23.01	23.44	0.9445	25.56	24.89	24.56	0.3721	23.42	23.11	25.00	1.5393	28.51	24.04	24.56	0.0080	N/A	23.48	24.38	N/A	19.01	24.53	23.03	0.9677
ET 47	dp	weaner	neg.ctr."*Roesrath*"	0	42.05	28.76	28.71	0.0000	N/A	29.94	29.37	N/A	31.42	29.47	29.41	0.1098	29.75	29.98	29.46	0.6376	31.30	30.56	30.70	0.1757	N/A	30.37	29.38	N/A	N/A	29.13	30.70	N/A
				1	N/A	29.19	30.59	N/A	N/A	29.04	29.27	N/A	32.94	31.32	30.10	0.1246	42.69	31.22	32.25	0.0005	34.42	28.88	30.26	0.0021	N/A	29.09	28.80	N/A	N/A	29.76	30.52	N/A
				2	N/A	32.60	32.62	N/A	N/A	32.13	31.48	N/A	34.62	42.53	32.27	3.7581	N/A	36.08	35.46	N/A	37.04	32.19	33.41	0.0026	37.06	31.68	31.15	0.0035	N/A	37.29	32.63	N/A
				3	27.23	19.35	20.86	0.0045	22.03	19.77	20.69	0.1897	22.07	21.54	21.36	0.4568	21.67	20.28	22.19	0.7292	25.71	21.10	22.11	0.0103	34.64	20.28	21.25	0.0000	31.96	21.40	20.01	0.0001
				7	28.10	20.27	21.51	0.0041	22.72	20.69	21.10	0.1841	22.14	22.22	21.82	0.6377	21.08	20.42	22.25	1.1804	27.51	21.34	21.80	0.0026	31.17	21.02	21.99	0.0002	19.06	22.03	20.53	1.9176
				10	28.27	20.94	22.06	0.0056	24.05	21.30	21.71	0.1103	22.32	22.61	22.21	0.7337	21.22	21.03	22.64	1.5167	29.56	21.76	22.39	0.0008	N/A	21.28	22.39	N/A	N/A	22.40	20.99	N/A
				14	36.05	27.28	28.11	0.0016	27.30	27.62	27.52	0.6900	30.94	29.53	28.30	0.1512	N/A	29.44	30.19	N/A	31.24	28.69	29.06	0.0237	N/A	28.03	28.14	N/A	17.51	28.89	26.70	2.0632
				21	30.35	22.00	23.66	0.0018	27.10	22.09	22.30	0.0237	24.56	24.22	23.35	0.3909	22.63	22.13	23.17	0.9267	30.54	22.92	24.03	0.0006	N/A	22.27	22.03	N/A	N/A	23.19	23.80	N/A
				28	34.75	25.66	26.99	0.0008	27.10	25.02	25.80	0.2266	28.50	27.33	26.45	0.2054	N/A	26.40	27.50	N/A	32.70	25.53	27.24	0.0009	32.54	26.00	25.44	0.0020	N/A	26.43	27.24	N/A
ET 48	dp	weaner	neg.ctr."*Roesrath*"	0	34.37	27.89	27.98	0.0033	33.14	28.16	28.12	0.0020	29.91	27.40	28.19	0.1046	28.51	28.49	28.17	0.5876	30.39	28.58	29.38	0.1113	43.49	28.61	28.09	0.0000	N/A	27.59	29.18	N/A
				1	N/A	28.88	30.22	N/A	N/A	28.11	29.04	N/A	34.23	30.26	29.84	0.0315	38.42	30.08	31.85	0.0051	34.53	28.43	30.04	0.0016	N/A	28.90	28.49	N/A	N/A	29.21	30.53	N/A
				2	35.89	29.15	30.85	0.0044	43.60	28.89	29.51	0.0000	33.36	31.17	30.33	0.0951	39.71	30.73	33.14	0.0041	33.60	29.41	30.84	0.0058	N/A	28.94	28.98	N/A	N/A	29.50	30.86	N/A
				3	30.79	22.82	23.91	0.0034	26.04	22.86	23.41	0.0830	25.18	24.65	24.27	0.4057	23.17	22.46	24.65	1.2913	30.41	23.57	24.26	0.0015	N/A	23.33	24.25	N/A	34.65	24.56	22.96	0.0001
				7	N/A	26.29	27.08	N/A	26.33	27.39	28.17	0.2469	36.84	27.07	28.06	0.0007	29.89	28.06	28.13	0.1922	31.72	28.21	29.28	0.0368	N/A	28.20	28.46	N/A	N/A	26.87	28.86	N/A
				14	38.46	26.65	27.38	0.0002	25.77	27.02	27.21	1.4653	30.58	28.63	27.81	0.1220	36.61	27.62	29.54	0.0037	31.64	27.85	28.38	0.0104	N/A	27.11	27.74	N/A	21.01	28.17	26.14	1.7600
				21	30.51	21.53	23.43	0.0013	25.10	21.79	22.28	0.0877	23.93	24.09	23.18	0.5526	22.35	22.17	23.03	1.0938	29.61	22.07	23.13	0.0007	N/A	21.90	21.68	N/A	N/A	23.38	23.80	N/A
				28	36.12	26.83	28.00	0.0007	26.88	26.07	26.65	0.5131	29.20	28.12	27.22	0.2149	N/A	26.51	28.30	N/A	32.45	26.13	27.80	0.0016	N/A	26.56	26.34	N/A	N/A	27.13	28.07	N/A
ET 09	wb	weaner	neg.ctr."*Armenia*"	0	28.62	17.87	19.84	0.0004	23.38	18.38	20.22	0.0090	24.64	18.44	20.76	0.0167	19.13	18.17	19.70	0.6650	28.92	20.27	22.03	0.0016	29.23	19.11	20.44	0.0010	14.60	17.16	21.26	1.5973
ET 12	wb	weaner	neg.ctr."*Armenia*"	0	30.41	17.65	19.97	0.0001	23.48	18.08	20.10	0.0073	25.29	18.38	20.61	0.0098	19.80	18.37	19.70	0.4480	27.19	19.01	20.89	0.0025	28.34	19.05	20.23	0.0017	32.83	18.11	21.12	0.0000
ET 15	wb	weaner	neg.ctr."*Armenia*"	0	29.55	17.09	19.49	0.0002	23.40	17.52	19.74	0.0058	24.18	17.47	20.08	0.0133	19.13	17.56	19.45	0.4963	27.67	18.22	20.62	0.0012	28.25	18.16	19.92	0.0012	22.12	17.22	21.09	0.0285
ET 22	dp	weaner	ASV"*Armenia*"	28	27.71	17.81	19.53	0.0007	23.55	17.61	20.09	0.0060	23.97	18.73	20.43	0.0264	18.46	18.25	19.25	0.9378	27.99	19.32	20.52	0.0014	30.44	19.10	20.15	0.0004	33.09	18.38	21.09	0.0000
				34	34.07	19.03	20.78	0.0000	24.48	20.06	21.41	0.0101	26.22	20.03	21.59	0.0124	21.86	20.14	21.01	0.3042	30.57	20.95	22.36	0.0007	38.10	20.50	21.29	0.0000	34.52	19.55	22.12	0.0000
ET 23	dp	weaner	ASV"*Armenia*"	28	29.00	18.19	20.05	0.0004	23.55	18.55	20.18	0.0082	23.45	18.60	20.45	0.0368	20.71	20.09	21.15	0.6982	30.26	20.62	22.06	0.0007	30.60	19.15	20.21	0.0004	35.17	18.14	20.97	0.0000
				31	30.95	19.20	20.93	0.0002	23.68	20.17	21.86	0.0220	25.14	20.09	21.68	0.0281	20.38	19.94	20.90	0.7666	29.70	20.82	22.21	0.0012	33.90	20.81	21.72	0.0001	23.52	19.74	22.44	0.0369
				34	33.10	19.90	21.27	0.0001	24.33	20.44	21.54	0.0132	26.74	20.38	21.85	0.0105	21.63	20.97	21.29	0.5209	30.27	21.17	22.43	0.0009	36.74	21.12	21.55	0.0000	36.04	19.94	22.22	0.0000
ET 24	dp	weaner	ASV"*Armenia*"	28	27.91	18.84	19.96	0.0011	24.08	19.33	20.62	0.0082	23.15	19.57	20.82	0.0719	18.84	19.30	19.80	1.2318	28.60	20.09	20.84	0.0013	30.62	20.09	20.26	0.0005	33.01	18.89	21.11	0.0000
				31	31.66	18.26	19.81	0.0001	24.01	19.05	20.46	0.0075	23.34	18.15	19.91	0.0283	19.15	18.75	19.60	0.7719	29.37	19.69	21.19	0.0007	32.68	19.40	20.33	0.0001	30.58	18.55	20.97	0.0001
				34	33.99	18.39	20.07	0.0000	24.76	19.38	20.63	0.0051	24.08	19.00	20.62	0.0285	20.30	19.42	20.24	0.5426	30.33	20.07	21.51	0.0005	39.80	19.94	20.77	0.0000	37.54	19.38	21.68	0.0000
ET 25	dp	weaner	ASV"*Armenia*"	28	32.26	19.73	21.73	0.0001	29.69	20.17	22.36	0.0003	26.12	20.56	22.46	0.0215	21.21	20.51	21.57	0.6547	31.33	21.16	22.80	0.0005	31.63	21.01	22.05	0.0006	N/A	20.06	22.72	N/A
				31	27.62	17.97	19.64	0.0009	23.45	18.64	20.13	0.0090	23.59	18.49	20.27	0.0303	18.55	18.33	19.30	0.9143	28.92	19.63	20.88	0.0009	30.52	19.10	20.03	0.0004	33.62	18.49	21.08	0.0000
				34	29.82	17.52	19.64	0.0002	23.47	18.38	20.29	0.0086	23.55	17.87	20.15	0.0242	19.38	18.46	19.66	0.6093	29.86	19.51	21.13	0.0005	34.55	19.05	20.36	0.0000	34.60	18.66	21.46	0.0000
				36	32.01	17.95	20.07	0.0000	22.29	18.29	20.22	0.0197	24.07	18.14	20.12	0.0182	19.69	18.65	20.21	0.6318	30.35	19.75	21.69	0.0004	37.74	19.13	20.50	0.0000	36.44	18.41	21.68	0.0000
ET 26	dp	weaner	ASV"*Armenia*"	28	26.51	17.40	18.16	0.0010	23.18	18.32	18.69	0.0060	21.73	17.96	18.80	0.0568	17.50	17.96	18.25	1.1709	27.97	19.05	19.57	0.0009	34.47	19.10	19.14	0.0000	34.60	18.59	20.18	0.0000
ET 27	dp	weaner	ASV"*Armenia*"	28	32.07	19.36	21.05	0.0001	25.56	19.75	21.37	0.0040	24.47	19.98	21.36	0.0390	21.23	20.59	21.76	0.7092	31.52	21.51	23.10	0.0005	31.01	20.53	21.39	0.0007	34.86	19.24	21.95	0.0000
				31	28.32	18.38	20.00	0.0007	23.35	19.21	20.27	0.0121	24.57	19.25	20.76	0.0230	19.37	19.25	19.86	0.8576	28.98	20.14	21.09	0.0011	32.19	19.96	20.44	0.0002	35.92	19.28	21.49	0.0000
				34	30.16	18.62	20.04	0.0002	24.52	19.46	20.51	0.0059	25.48	19.46	20.64	0.0125	20.75	19.67	20.28	0.4369	29.46	20.01	21.36	0.0008	35.11	19.82	20.44	0.0000	33.45	18.89	21.14	0.0000
				36	31.05	18.61	20.09	0.0001	23.84	19.21	20.72	0.0098	23.79	18.56	20.40	0.0280	20.07	19.03	20.27	0.5611	29.67	20.10	21.42	0.0007	37.42	19.63	20.59	0.0000	17.44	18.73	21.62	1.9902
ET 28	dp	weaner	ASV"*Armenia*"	0	30.41	18.89	20.71	0.0002	25.46	19.57	21.45	0.0043	25.91	19.70	21.55	0.0136	20.20	19.66	20.69	0.7364	30.55	20.50	22.23	0.0006	29.97	20.46	21.44	0.0014	33.84	19.79	22.61	0.0000
ET 29	dp	weaner	ASV"*Armenia*"	0	38.55	18.92	21.14	0.0000	25.26	18.69	21.14	0.0034	26.70	18.77	21.42	0.0054	21.41	19.24	20.93	0.2986	30.47	19.86	22.22	0.0005	29.61	19.36	21.26	0.0011	32.18	18.61	22.18	0.0000
ET 30	dp	weaner	ASV"*Armenia*"	0	32.26	18.74	21.05	0.0001	24.31	19.28	21.15	0.0082	25.09	19.00	21.13	0.0167	20.15	18.84	20.33	0.5113	30.41	20.09	21.87	0.0005	28.28	19.51	20.77	0.0026	32.15	18.66	21.80	0.0000
ET 13	dp	adult	CSFV "*Koslov*"	0	33.50	23.58	25.41	0.0030	31.26	23.77	25.70	0.0042	29.44	25.15	26.39	0.0486	27.59	24.98	26.01	0.0893	32.74	25.51	26.79	0.0017	34.62	25.77	27.11	0.0131	N/A	25.98	25.98	N/A
				2	35.14	24.19	23.34	0.0007	28.63	22.84	23.31	0.0082	27.50	24.28	24.24	0.0651	28.20	23.95	23.76	0.0198	29.41	23.83	24.21	0.0047	N/A	25.25	25.40	N/A	N/A	25.22	24.04	N/A
				4	26.41	23.24	21.31	0.0590	25.92	23.09	21.51	0.0270	22.40	24.17	22.43	0.7702	20.42	23.31	21.28	1.5452	30.69	23.94	22.06	0.0010	N/A	24.32	22.75	N/A	N/A	25.09	21.59	N/A
				7	25.20	24.03	19.91	0.1017	23.93	23.56	19.97	0.0653	20.87	24.46	20.80	1.2821	19.07	24.06	20.03	3.2779	29.15	25.20	20.45	0.0025	N/A	25.35	21.00	N/A	N/A	25.44	19.94	N/A
				8	24.54	23.31	19.66	0.1143	23.49	22.47	19.36	0.0541	20.33	23.87	20.36	1.3289	18.31	23.55	19.32	3.6944	28.50	23.28	19.91	0.0018	N/A	24.74	20.85	N/A	N/A	25.17	19.89	N/A

ET 14	dp	adult	CSFV "*Koslov*"	0	37.51	23.87	24.91	0.0002	35.10	24.06	25.02	0.0004	29.05	25.54	25.71	0.0557	27.00	24.64	25.41	0.0985	33.12	25.66	26.11	0.0011	34.69	25.64	26.59	0.0101	N/A	26.45	25.54	N/A
				2	32.22	24.01	23.44	0.0041	29.43	23.94	24.40	0.0092	27.81	24.92	24.53	0.0698	28.13	24.11	24.00	0.0236	29.85	24.90	24.82	0.0058	33.84	25.25	25.69	0.0112	N/A	25.64	24.22	N/A
				4	26.81	24.44	21.95	0.0796	26.10	23.41	21.84	0.0289	23.08	25.01	22.62	0.6829	20.70	23.73	21.63	1.6271	31.02	24.18	22.31	0.0009	N/A	24.78	23.15	N/A	N/A	25.74	22.35	N/A
				7	24.57	24.08	19.79	0.1462	24.29	24.11	20.02	0.0610	20.95	25.40	20.76	1.5515	18.87	25.13	19.92	5.0820	29.42	25.96	20.54	0.0027	N/A	25.22	20.77	N/A	N/A	26.21	20.09	N/A
ET 15	dp	adult	CSFV "*Koslov*"	0	N/A	24.80	24.00	N/A	30.15	24.27	24.13	0.0060	27.49	25.32	24.94	0.1063	25.74	25.15	24.50	0.2044	31.32	25.97	25.11	0.0032	34.21	26.09	25.92	0.0123	N/A	26.46	24.72	N/A
				2	39.25	25.76	25.01	0.0002	30.57	25.91	26.24	0.0131	29.25	27.23	26.10	0.0870	29.62	26.77	25.81	0.0361	31.94	26.93	26.38	0.0042	34.50	27.50	26.94	0.0221	N/A	27.53	25.53	N/A
				7	26.41	23.82	20.77	0.0594	24.22	23.54	20.81	0.0698	21.91	24.28	21.60	0.8358	20.10	24.42	20.87	2.3765	30.09	24.91	21.23	0.0015	N/A	25.07	22.04	N/A	N/A	25.53	20.84	N/A
ET 16	dp	adult	CSFV "*Koslov*"	0	34.43	27.75	26.25	0.0074	33.85	26.55	26.71	0.0024	31.95	28.72	27.63	0.0409	28.72	28.04	27.30	0.1671	33.90	28.45	27.84	0.0028	N/A	28.86	28.66	N/A	N/A	29.23	27.77	N/A
				2	33.73	24.08	23.71	0.0018	30.19	24.14	24.36	0.0060	28.69	25.14	24.99	0.0502	26.64	24.56	24.43	0.0891	29.75	25.21	25.06	0.0076	34.41	25.66	25.72	0.0090	N/A	26.09	24.52	N/A
				4	30.28	26.35	24.56	0.0371	28.59	25.40	24.56	0.0235	25.56	27.16	25.20	0.5928	23.46	26.21	24.37	1.3323	33.14	26.69	25.12	0.0011	N/A	27.89	25.99	N/A	N/A	28.33	25.09	N/A
				7	26.71	24.35	21.20	0.0656	25.12	23.47	21.18	0.0439	22.26	24.73	22.07	0.8777	20.47	25.23	21.22	2.6887	30.56	25.25	21.62	0.0014	N/A	25.79	22.34	N/A	N/A	26.23	21.50	N/A
ET 17	dp	adult	CSFV "*Koslov*"	0	36.93	29.16	27.76	0.0039	37.90	28.20	27.70	0.0004	33.24	30.22	28.81	0.0400	32.84	29.38	28.36	0.0210	38.08	30.14	28.76	0.0003	N/A	30.55	29.70	N/A	N/A	30.81	28.28	N/A
				4	27.67	24.01	22.30	0.0462	26.48	23.24	22.05	0.0234	23.31	24.90	23.13	0.6735	21.39	23.90	22.23	1.3023	30.73	25.04	22.50	0.0015	N/A	25.50	23.55	N/A	N/A	25.42	22.40	N/A
				7	27.22	24.65	21.52	0.0578	25.46	24.15	21.31	0.0443	22.61	25.60	22.31	0.9631	20.81	25.29	21.41	2.3078	30.75	25.08	22.02	0.0013	N/A	25.98	22.65	N/A	N/A	26.17	21.73	N/A
ET 18	dp	adult	CSFV "*Koslov*"	0	36.99	25.81	23.64	0.0004	27.64	25.39	23.63	0.0318	28.37	27.03	24.78	0.0943	28.09	26.28	24.33	0.0542	29.05	26.66	24.69	0.0174	37.14	27.64	25.80	0.0031	N/A	27.86	24.41	N/A
				2	41.47	23.85	23.17	0.0000	30.86	23.94	23.83	0.0032	28.22	25.15	24.26	0.0535	27.29	24.62	23.99	0.0499	30.29	25.10	24.60	0.0043	N/A	25.17	25.31	N/A	37.27	25.91	24.06	0.0000
				4	29.95	23.04	23.89	0.0140	28.17	23.09	23.49	0.0122	24.71	24.44	24.39	0.3744	22.81	23.58	23.50	0.6792	32.08	24.37	24.10	0.0008	30.76	25.09	25.17	0.0582	N/A	25.16	23.84	N/A
				7	28.25	25.89	22.76	0.0648	26.03	25.10	22.68	0.0595	23.73	26.57	23.68	0.9607	21.94	25.98	22.71	2.0225	31.75	26.46	23.32	0.0015	N/A	27.28	24.07	N/A	N/A	28.38	23.07	N/A
				8	28.65	25.88	23.28	0.0591	26.68	25.30	23.05	0.0470	23.67	26.98	23.74	1.1285	22.01	26.38	22.88	2.3215	31.86	26.64	23.87	0.0018	26.72	27.14	24.38	0.1256	22.77	27.53	23.30	0.0004
ET 44	wb	weaner	Vacc. "*C-Strain*"	0	23.52	24.42	19.24	0.2599	24.03	23.65	19.25	0.0506	20.33	25.82	20.17	2.1083	17.88	26.32	19.19	11.4453	27.93	25.69	19.88	0.0058	32.00	26.50	20.23	0.0077	N/A	26.56	19.40	N/A
				0.5	25.12	25.06	20.41	0.1676	24.42	24.70	20.28	0.0708	21.22	26.25	21.07	1.8192	19.13	26.57	20.16	7.2950	28.92	26.17	20.69	0.0043	N/A	26.73	21.42	N/A	N/A	27.32	20.47	N/A
				2	22.47	24.35	18.15	0.3483	24.21	24.09	17.57	0.0308	19.27	26.09	18.93	2.9653	16.75	25.92	18.19	15.7690	26.71	24.68	18.71	0.0068	N/A	26.86	19.22	N/A	22.52	27.97	18.41	0.0002
				3	27.89	25.63	20.65	0.0786	26.55	24.76	23.90	0.5601	22.44	26.04	21.03	0.7750	20.77	24.22	22.10	4.8192	27.42	26.15	22.56	0.0244	37.89	26.02	21.30	0.0001	N/A	26.46	21.83	N/A
				7	31.08	25.94	22.79	0.0246	21.96	24.06	21.84	3.9322	24.58	26.25	23.23	0.3902	23.49	25.11	24.13	1.9854	31.33	26.67	24.71	0.0036	34.99	26.10	23.48	0.0016	N/A	26.30	23.36	N/A
				14	27.09	26.31	20.07	0.1299	23.27	24.36	20.99	1.3993	21.26	26.32	20.40	1.5605	19.97	25.43	21.18	9.3636	29.24	27.27	21.68	0.0069	44.19	26.09	20.33	0.0000	N/A	27.57	20.62	N/A
				21	28.77	28.28	21.23	0.1237	25.27	26.66	22.30	1.2936	22.01	28.50	21.44	2.6308	21.90	28.48	22.56	11.1812	30.52	28.61	23.02	0.0066	27.65	27.89	21.38	0.1058	23.85	29.22	22.34	0.9038
				28	33.27	28.82	25.21	0.0336	25.99	27.05	25.99	3.4152	27.35	29.67	26.13	0.4469	25.50	28.32	26.77	3.7266	32.59	29.95	27.12	0.0095	34.24	29.76	25.81	0.0139	22.16	31.13	26.28	0.9987
ET 45	wb	weaner	Vacc. "*C-Strain*"	0	27.46	22.23	21.16	0.0220	23.16	21.26	21.03	0.0796	23.15	22.61	21.97	0.2830	21.82	22.06	21.38	0.4041	26.59	22.38	22.03	0.0106	33.28	22.95	22.86	0.0031	33.50	24.25	22.02	0.0000
				0.5	23.42	23.83	19.00	0.2163	23.05	23.46	18.45	0.0695	19.50	24.28	19.53	1.8985	17.03	24.06	18.29	7.4364	26.08	24.57	18.98	0.0112	N/A	25.48	20.04	N/A	N/A	25.24	18.88	N/A
				3	34.24	27.66	24.33	0.0101	25.03	24.03	22.72	0.7673	34.18	27.94	24.74	0.3757	25.62	26.53	26.03	1.4119	32.87	28.12	26.33	0.0033	N/A	28.36	25.16	N/A	N/A	28.77	25.34	N/A
				7	29.77	24.72	21.65	0.0263	27.11	26.23	25.47	1.0840	22.36	25.35	22.04	0.5418	22.28	23.88	23.00	2.0325	30.72	25.81	23.57	0.0029	36.61	25.06	22.08	0.0003	N/A	25.13	22.17	N/A
				14	27.22	26.23	18.61	0.0749	24.90	24.17	19.61	0.2911	37.30	26.25	18.96	2.5836	18.47	27.52	19.68	12.7585	28.36	26.26	20.34	0.0059	37.48	25.73	19.00	0.0001	N/A	26.21	19.39	N/A
				21	26.95	26.72	19.77	0.1465	23.92	25.54	20.79	1.2540	23.08	27.46	20.48	2.2500	19.70	27.15	20.83	18.1704	29.72	28.77	21.60	0.0075	36.80	28.05	20.38	0.0003	35.27	27.57	20.45	0.0009
				28	31.47	28.84	23.56	0.0597	25.05	27.14	24.62	3.9051	30.07	29.32	24.30	0.8583	23.58	27.93	24.98	6.6792	32.10	30.22	25.55	0.0085	N/A	29.63	24.15	N/A	22.22	31.40	24.29	1.0023
ET 46	wb	weaner	Vacc. "*C-Strain*"	0	27.42	26.66	22.22	0.1144	24.37	25.44	22.05	0.1492	23.06	27.42	22.91	1.4271	20.94	26.76	21.83	3.8575	30.66	27.22	22.43	0.0031	N/A	28.46	23.52	N/A	N/A	28.71	22.32	N/A
				0.5	25.12	25.69	20.33	0.1974	24.85	25.14	20.20	0.0593	21.37	27.06	21.24	2.1787	19.18	27.47	20.23	9.6023	28.72	26.98	20.93	0.0070	N/A	27.51	21.52	N/A	N/A	28.74	20.40	N/A
				2	N/A	26.86	24.19	N/A	26.60	25.77	24.22	0.8337	32.45	26.84	24.28	0.0538	27.18	25.47	26.06	0.3277	34.48	27.09	26.65	0.0008	40.05	27.57	25.44	0.0002	N/A	27.63	25.62	N/A
				3	25.42	26.03	20.83	0.2117	24.39	25.59	20.79	0.1051	21.79	27.15	21.56	1.9017	19.23	27.98	20.65	12.5157	28.59	27.36	21.15	0.0093	N/A	28.21	22.05	N/A	22.78	29.38	21.07	0.0004
				7	31.22	26.85	23.16	0.0335	25.21	25.22	25.40	2.5642	28.56	27.45	23.41	0.6020	23.59	25.85	24.31	2.5503	32.03	28.15	25.08	0.0040	37.94	27.36	23.79	0.0004	N/A	28.14	23.95	N/A
				14	26.04	25.61	18.52	0.1229	23.08	24.13	19.66	0.9198	21.05	25.62	19.18	2.2854	18.30	25.46	19.65	17.8865	28.24	26.56	20.41	0.0072	35.11	24.70	18.84	0.0002	N/A	25.04	19.50	N/A
				21	28.06	26.10	20.14	0.0701	24.85	24.99	21.09	0.6515	25.48	26.81	20.76	1.7086	20.32	25.70	21.22	8.1214	29.91	27.39	21.97	0.0049	N/A	27.31	20.64	N/A	N/A	28.14	21.02	N/A
				28	N/A	33.22	30.21	N/A	29.09	32.57	31.25	7.2345	38.50	33.82	30.79	N/A	32.68	33.37	33.25	1.3454	39.63	33.67	33.22	0.0016	N/A	33.19	31.07	N/A	22.90	36.37	31.86	1.0294
ET 51	wb	weaner	Vacc. "*C-Strain*"	0	23.18	23.48	18.21	0.1776	22.83	23.87	18.33	0.0848	19.24	24.44	18.94	1.9408	17.07	25.58	18.16	11.2767	27.82	26.15	18.91	0.0052	N/A	25.34	19.26	N/A	N/A	26.85	18.19	N/A
				0.5	24.73	24.78	19.74	0.1608	23.76	24.15	19.49	0.0728	20.76	25.74	20.50	1.7684	18.33	26.50	19.52	9.9517	27.94	25.56	20.10	0.0059	N/A	26.18	20.98	N/A	N/A	28.07	19.90	N/A
				1	24.04	23.65	19.18	0.1479	23.36	24.34	18.74	0.0779	19.83	25.29	19.70	2.1508	17.32	26.19	18.67	13.7166	27.15	25.18	19.11	0.0066	25.31	26.58	20.30	0.4666	23.58	25.72	19.21	0.0000
				2	23.78	25.32	19.16	0.2823	N/A	24.54	18.79	N/A	20.23	27.31	20.09	3.2668	17.83	26.35	19.21	12.0766	27.49	26.51	19.93	0.0104	43.28	26.49	19.86	0.0000	21.33	29.04	19.31	0.0009
				3	30.75	25.99	22.08	0.0245	26.28	24.82	23.06	0.5035	23.74	26.56	22.59	0.6242	23.05	25.40	23.39	2.2968	31.42	26.75	23.90	0.0026	27.61	27.25	22.61	0.1352	N/A	26.64	22.63	N/A
				14	27.45	26.42	19.11	0.0811	26.25	24.41	20.15	0.1611	20.75	26.10	19.65	1.6046	19.12	26.90	20.36	21.2182	29.26	26.80	21.09	0.0048	36.59	25.96	19.59	0.0002	N/A	26.77	20.05	N/A
				21	25.98	25.16	18.55	0.1122	25.80	22.87	19.38	0.1010	19.87	25.13	19.01	1.7633	18.06	24.87	19.73	17.6710	27.70	25.70	20.02	0.0072	37.88	25.59	18.89	0.0001	N/A	26.48	19.36	N/A
				28	N/A	29.54	27.05	N/A	27.26	28.54	28.32	5.5553	31.50	30.24	27.56	0.0486	29.36	29.28	29.26	0.8218	34.87	31.29	29.82	0.0072	37.39	30.40	27.80	0.0047	22.64	31.50	28.47	0.8679
ET 47	wb	weaner	Vacc. "*CP7_E2alf*"	0	28.29	26.66	23.01	0.0855	24.51	25.75	22.69	0.1786	23.76	27.83	23.55	1.2690	22.02	27.18	22.63	2.7461	31.29	27.06	23.25	0.0025	N/A	28.54	24.15	N/A	N/A	29.41	23.12	N/A
				0.5	23.78	23.09	19.14	0.1457	23.77	23.69	19.13	0.0580	19.88	25.92	19.73	2.4827	17.85	25.51	18.94	8.3716	27.05	25.72	19.52	0.0097	24.25	25.83	20.28	0.7097	N/A	27.61	19.16	N/A
				2	23.61	24.55	18.75	0.2194	23.35	24.49	18.41	0.0743	19.50	26.03	19.39	2.9098	17.15	27.14	18.37	18.7624	26.69	25.91	19.00	0.0112	N/A	26.88	19.58	N/A	N/A	25.82	19.22	N/A
				3	38.44	28.11	24.90	0.0011	24.52	24.97	21.40	0.8884	26.49	27.53	25.00	0.2827	27.53	26.78	26.19	0.4260	33.19	27.86	26.50	0.0026	N/A	28.32	25.42	N/A	36.22	28.70	25.56	0.0030
				7	28.39	26.23	20.46	0.0658	26.64	26.06	26.03	1.6842	22.25	26.31	21.23	1.0250	21.06	25.12	21.68	4.6281	30.57	26.25	22.42	0.0025	N/A	25.72	21.11	N/A	35.06	26.24	21.30	0.0010
				14	27.56	25.78	20.19	0.0867	23.18	24.72	21.09	1.7217	22.02	26.74	21.10	1.3300	20.59	25.93	21.63	8.3698	30.03	26.91	22.00	0.0039	35.52	27.20	20.75	0.0007	N/A	26.67	20.80	N/A
				21	26.21	27.44	18.21	0.1762	27.70	25.13	19.08	0.0554	19.72	27.61	18.80	4.0263	18.04	34.15	19.25	17.7440	28.55	28.10	20.00	0.0081	35.89	26.89	18.52	0.0002	21.87	29.41	18.42	0.8637
				28	35.80	29.79	26.72	0.0159	26.06	27.86	27.48	7.1240	28.75	29.80	27.00	0.2358	27.22	29.36	28.10	2.5501	33.99	30.52	29.11	0.0083	N/A	29.66	27.05	N/A	22.88	31.27	27.77	0.6743

ET 50	wb	weaner	Vacc. "*CP7_E2alf*"	0	24.40	26.51	19.11	0.2702	23.12	24.67	18.89	0.1025	19.61	26.22	19.56	3.0314	17.18	27.21	18.25	18.1047	26.89	25.20	18.97	0.0077	N/A	27.01	20.12	N/A	22.83	27.97	19.03	0.0001
				0.5	24.05	25.09	19.39	0.2394	24.61	25.10	19.45	0.0545	20.46	26.84	20.34	2.7093	18.13	27.30	19.42	14.3073	27.82	27.24	20.13	0.0112	25.32	26.47	20.58	0.4954	22.77	29.04	19.67	0.0002
				2	24.48	26.12	19.60	0.2666	24.53	24.92	19.53	0.0561	20.27	26.50	20.19	2.6491	17.93	27.43	19.17	15.7433	26.77	26.64	19.85	0.0177	22.24	27.03	20.77	0.0138	22.80	27.93	19.54	0.0002
				3	30.30	26.43	21.73	0.0330	25.03	24.62	21.44	0.5854	23.77	27.59	22.52	0.8303	22.68	26.05	23.21	3.5110	30.30	27.74	23.48	0.0069	37.47	26.88	22.52	0.0003	N/A	27.64	22.65	N/A
				7	28.56	26.52	20.37	0.0633	25.69	25.41	22.93	0.8398	22.11	26.66	21.13	1.2272	21.03	26.34	21.71	7.3205	30.21	27.92	22.37	0.0053	35.56	26.62	20.92	0.0006	N/A	27.48	21.35	N/A
				14	27.14	27.08	19.45	0.1324	24.46	25.13	20.39	0.6809	21.16	26.42	20.11	1.5630	19.56	26.07	20.81	13.6570	28.84	27.70	21.26	0.0090	32.55	26.69	20.05	0.0027	N/A	27.75	20.20	N/A
				21	28.01	26.54	20.41	0.0894	25.05	25.28	21.44	0.7125	22.06	27.07	21.11	1.4367	21.08	25.64	21.91	5.9464	29.46	27.73	22.28	0.0083	36.30	27.12	21.04	0.0004	N/A	27.96	20.95	N/A
				28	29.73	28.04	22.62	0.0994	24.39	27.41	23.78	4.8006	23.99	29.42	23.16	1.5814	21.68	24.23	23.42	4.0976	31.33	29.81	24.66	0.0096	N/A	29.54	23.09	N/A	22.77	31.03	23.51	0.9634
ET 52	wb	weaner	Vacc. "*CP7_E2alf*"	0	23.02	24.11	18.30	0.2422	21.79	23.38	18.13	0.1334	19.42	25.13	19.16	2.2414	17.16	25.90	18.25	12.1276	27.82	26.07	18.93	0.0052	N/A	25.16	19.32	N/A	N/A	27.17	18.39	N/A
				0.5	25.17	24.91	19.63	0.1233	23.59	24.71	19.46	0.0924	20.66	25.87	20.38	1.8668	18.24	26.97	19.40	11.8327	28.34	25.66	19.90	0.0043	N/A	26.74	20.91	N/A	N/A	27.50	19.80	N/A
				1	23.81	24.69	19.70	0.2702	23.19	24.18	19.61	0.1080	20.59	25.83	20.62	2.0729	18.26	26.13	20.06	11.1781	27.56	25.72	20.46	0.0092	N/A	26.03	20.56	N/A	22.48	27.83	20.03	0.0002
				2	24.52	24.27	19.56	0.1486	N/A	23.57	19.80	N/A	20.38	25.62	20.28	2.0059	18.15	27.00	19.31	12.3355	27.26	26.11	20.02	0.0111	N/A	26.39	20.55	N/A	22.05	28.40	19.45	0.0004
				3	29.32	28.12	22.10	0.1106	24.67	26.28	23.11	2.2327	23.93	28.54	22.81	1.1064	22.02	27.56	23.29	9.7052	32.27	29.55	24.13	0.0037	32.81	28.54	22.44	0.0081	N/A	28.66	22.69	N/A
				14	26.40	25.80	18.76	0.1127	26.32	24.09	19.88	0.1276	20.29	25.79	19.29	1.7847	19.05	25.79	20.11	13.8748	29.15	26.61	20.67	0.0042	N/A	26.04	19.14	N/A	N/A	26.61	19.55	N/A
				21	25.93	25.24	19.08	0.1382	24.32	23.67	20.14	0.4295	20.57	25.38	19.72	1.4832	18.80	26.08	20.38	16.1332	28.75	26.19	20.96	0.0054	37.42	25.74	19.56	0.0001	N/A	26.66	19.72	N/A
				28	N/A	30.60	27.85	N/A	27.18	28.56	29.08	7.6693	33.86	30.94	28.30	0.0152	30.14	30.39	29.68	0.8093	35.11	32.07	30.77	0.0108	27.93	31.35	28.66	0.1985	22.73	32.64	29.63	0.5792
ET 41	dp	weaner	Vacc. "*CP7_E2alf*"	0	23.95	23.36	19.43	0.1548	22.83	23.16	19.47	0.0998	20.58	24.57	20.38	1.3900	18.08	24.15	19.31	5.2386	26.36	24.85	20.31	0.0157	N/A	24.44	20.26	N/A	N/A	28.60	19.37	N/A
				0.5	25.93	22.85	20.17	0.0499	22.38	22.03	19.98	0.1149	21.18	23.31	20.95	0.8215	19.62	23.23	20.05	1.7286	25.10	24.22	20.87	0.0380	36.66	23.86	21.44	0.0003	N/A	25.43	20.26	N/A
				1	24.52	23.71	18.84	0.1015	23.20	23.24	18.50	0.0609	19.78	24.33	19.43	1.5850	17.15	24.75	18.33	8.7080	26.04	24.46	19.01	0.0113	N/A	24.41	19.93	N/A	N/A	24.82	19.13	N/A
				2	23.55	23.77	18.95	0.1934	22.75	22.75	19.11	0.0846	20.06	25.22	19.81	1.9030	17.71	24.57	18.86	6.6134	25.51	24.62	19.40	0.0197	25.84	25.12	20.06	0.2048	22.41	25.05	19.03	0.0002
				3	32.27	25.46	22.37	0.0092	25.19	25.20	24.28	1.7329	24.01	26.45	22.89	0.5513	23.52	25.14	23.71	1.6921	30.84	26.14	24.42	0.0040	34.06	26.36	23.06	0.0026	N/A	25.65	23.27	N/A
				7	33.08	26.94	23.29	0.0118	25.46	24.13	23.42	0.7739	25.19	27.32	24.00	0.4630	24.44	26.21	24.72	1.8269	31.66	27.80	25.36	0.0051	36.31	27.60	23.91	0.0012	N/A	27.42	23.77	N/A
				14	27.42	25.63	20.00	0.0852	22.89	23.73	21.00	1.4729	21.47	25.27	20.52	0.9997	19.84	24.47	21.15	7.2642	28.51	25.80	21.81	0.0077	35.10	25.84	20.40	0.0005	N/A	25.86	20.38	N/A
				21	34.80	28.18	25.54	0.0123	29.81	26.08	26.62	0.2830	27.39	27.84	26.15	0.2443	26.36	27.15	27.14	1.5499	34.23	29.02	27.47	0.0025	35.50	28.22	26.40	0.0052	N/A	29.00	26.18	N/A
				28	35.19	29.40	26.14	0.0170	23.36	26.72	26.90	7.8256	28.09	29.95	26.82	0.3689	26.60	28.23	27.95	2.5370	31.98	30.64	28.66	0.0313	32.87	29.60	26.74	0.0407	N/A	30.27	27.20	N/A
ET 42	dp	weaner	Vacc. "*C-Strain*"	0	27.31	25.27	21.49	0.0654	23.15	24.39	21.61	0.2116	23.68	26.49	22.55	0.6927	20.96	25.58	21.70	2.5142	27.04	25.89	22.29	0.0260	N/A	26.15	22.71	N/A	N/A	27.30	21.85	N/A
				0.5	29.04	24.61	20.67	0.0146	24.21	23.27	20.59	0.0613	21.92	24.96	21.37	0.9273	20.19	23.90	20.66	1.7688	25.95	24.37	20.91	0.0218	N/A	25.95	22.27	N/A	N/A	27.18	20.83	N/A
				2	26.27	25.17	20.49	0.0882	25.22	24.52	20.63	0.0460	21.76	26.10	21.38	1.3894	19.91	25.93	20.55	3.9513	27.50	25.74	20.91	0.0112	N/A	26.22	21.81	N/A	22.42	25.61	20.89	0.0004
				3	32.33	26.22	23.20	0.0144	27.02	25.70	24.52	0.6965	25.00	27.06	23.91	0.4734	23.90	25.43	24.42	1.8403	32.88	27.09	25.34	0.0017	35.16	27.05	24.05	0.0022	35.85	27.49	24.53	0.0021
				7	32.94	27.82	23.49	0.0178	25.73	25.25	24.31	1.2687	25.46	27.55	24.20	0.4421	24.65	26.59	25.02	2.0054	31.47	27.61	25.38	0.0056	34.26	27.50	24.05	0.0043	N/A	28.20	24.15	N/A
				14	27.73	26.11	19.87	0.0786	24.95	25.10	20.94	0.6002	21.48	26.52	20.47	1.4605	20.05	25.47	21.07	8.6030	28.77	26.85	21.56	0.0081	36.81	27.25	20.29	0.0003	N/A	27.29	20.53	N/A
				21	29.61	26.10	21.10	0.0372	26.36	25.05	22.06	0.3627	22.17	26.64	21.43	1.2925	22.03	24.68	22.30	2.4905	30.55	27.70	22.79	0.0045	N/A	27.09	21.34	N/A	22.83	27.43	21.65	0.8749
				28	34.26	29.30	25.64	0.0245	26.16	28.15	26.47	5.1248	27.33	29.50	26.29	0.4545	25.92	28.90	27.11	3.8105	33.44	29.81	27.68	0.0060	38.25	29.98	25.96	0.0014	22.90	31.01	27.14	0.9756
ET 43	dp	weaner	Vacc. "*C-Strain*"	0	25.72	23.16	20.42	0.0668	21.94	23.06	20.61	0.2354	21.58	24.19	21.26	0.8966	19.53	24.04	20.56	2.8208	26.60	24.21	21.04	0.0137	N/A	25.14	21.65	N/A	35.59	28.85	20.63	0.0000
				0.5	25.70	24.18	19.30	0.0651	23.07	23.10	19.15	0.0769	20.20	24.30	20.00	1.4428	18.32	24.57	19.12	4.7553	25.01	24.52	19.64	0.0295	30.78	24.73	20.50	0.0106	N/A	25.17	19.51	N/A
				2	25.47	23.53	20.28	0.0830	22.52	22.41	20.06	0.1184	21.16	23.70	21.03	0.9463	19.35	23.58	20.24	2.4734	25.41	23.65	20.53	0.0225	N/A	23.99	21.38	N/A	22.86	25.01	20.41	0.0001
				3	35.93	26.01	24.03	0.0020	26.79	24.96	24.91	0.7301	26.65	26.04	24.39	0.1281	27.12	24.75	25.63	0.2291	30.78	26.34	26.50	0.0091	N/A	26.21	25.19	N/A	N/A	26.23	25.15	N/A
				7	33.83	27.18	23.71	0.0092	24.92	24.28	25.19	2.1213	25.39	26.71	24.28	0.3667	25.09	25.67	25.13	1.1136	32.05	27.69	25.72	0.0042	N/A	27.38	24.36	N/A	N/A	27.36	24.49	N/A
				14	27.35	25.33	19.82	0.0767	22.60	24.07	20.75	1.7947	21.45	25.61	20.40	1.0859	19.85	23.01	21.07	4.2242	28.48	25.72	21.51	0.0069	N/A	25.28	20.28	N/A	34.87	26.37	20.61	0.0009
				21	26.64	27.39	17.82	0.1190	24.38	25.33	18.85	0.4406	19.25	27.35	18.32	4.3719	17.90	32.60	19.05	12.7000	28.35	27.75	19.57	0.0072	27.90	27.66	18.17	0.0310	N/A	27.21	18.75	N/A
				28	28.02	27.42	19.66	0.0925	22.63	26.11	20.72	3.2919	21.40	28.24	20.51	2.7136	19.99	29.04	20.73	17.3316	28.35	28.34	21.60	0.0178	N/A	28.11	20.34	N/A	22.88	28.98	20.68	0.8901
ET 53	dp	weaner	Vacc. "*C-Strain*"	0	24.61	23.29	19.50	0.1038	22.50	23.62	19.64	0.1445	20.75	24.64	20.38	1.2801	18.80	24.91	19.47	4.2864	27.28	24.41	20.11	0.0066	38.18	25.27	20.85	0.0002	N/A	25.68	19.56	N/A
				0.5	25.64	23.39	19.15	0.0514	23.04	23.14	19.02	0.0763	20.08	24.07	19.74	1.3486	17.66	23.74	18.71	5.0040	24.92	24.29	19.33	0.0264	N/A	24.79	20.17	N/A	N/A	25.91	19.20	N/A
				1	24.10	24.75	19.38	0.2089	24.10	24.01	19.45	0.0563	20.55	25.94	20.28	1.9796	18.29	26.03	19.40	8.4830	26.33	26.46	19.99	0.0239	26.90	25.74	20.44	0.3987	35.56	25.14	19.36	0.0000
				2	26.35	25.31	20.52	0.0885	N/A	23.81	18.78	N/A	21.97	25.85	21.56	1.2090	19.53	25.39	20.39	4.0932	26.35	24.84	21.10	0.0204	N/A	25.94	21.75	N/A	22.27	28.55	20.53	0.0004
				3	38.95	26.73	25.32	0.0006	27.09	25.32	25.49	0.8332	27.89	26.45	25.53	0.0904	28.26	25.43	26.87	0.2011	31.09	27.13	27.48	0.0132	36.34	27.41	26.10	0.0023	N/A	27.11	26.19	N/A
				7	35.85	26.49	24.32	0.0027	25.87	24.41	26.01	1.6315	26.56	26.59	25.09	0.2046	26.21	25.25	25.94	0.5779	30.83	26.94	26.44	0.0104	37.62	26.37	25.04	0.0006	N/A	27.38	25.10	N/A
				14	36.94	25.44	20.49	0.0003	24.18	24.03	21.77	0.9402	22.61	25.54	21.12	0.6048	20.88	23.74	21.80	3.4049	28.75	25.91	22.46	0.0084	N/A	25.21	21.16	N/A	N/A	26.31	21.23	N/A
				21	26.71	25.80	18.95	0.0996	26.23	25.04	19.99	0.1885	20.06	26.59	19.35	2.7552	19.22	27.13	20.10	19.5731	29.55	26.71	20.70	0.0033	44.46	25.97	19.23	0.0000	35.26	27.14	19.84	0.0007
				28	32.66	29.29	24.63	0.0467	26.42	28.40	25.76	3.6501	26.56	30.40	25.65	0.8293	24.80	29.00	26.13	6.1493	33.89	30.95	26.88	0.0047	44.49	30.74	25.47	0.0000	21.72	32.48	26.32	0.7924
ET 54	dp	weaner	Vacc. "*C-Strain*"	0	24.79	25.35	18.98	0.1460	21.31	23.98	18.96	0.2426	20.04	25.51	19.73	2.0313	17.64	25.52	18.63	8.7617	26.47	24.93	19.36	0.0109	39.23	26.74	19.97	0.0001	36.38	28.38	18.84	0.0000
				0.5	25.02	23.57	19.08	0.0772	23.48	24.39	20.24	0.0708	19.99	25.49	19.83	2.1530	17.89	24.96	18.72	6.3303	25.52	24.98	19.37	0.0217	N/A	26.22	20.20	N/A	N/A	27.06	19.15	N/A
				1	25.79	25.34	20.24	0.1153	24.01	24.92	21.48	0.0832	21.55	26.36	21.16	1.5801	19.64	26.44	20.39	5.3049	26.24	26.43	20.86	0.0338	36.81	26.54	21.44	0.0006	35.63	25.69	20.37	0.0000
				2	27.16	24.75	21.76	0.0666	24.62	22.46	20.79	0.0943	22.58	26.30	22.43	1.2267	20.43	26.14	21.49	4.0225	26.73	26.42	22.13	0.0361	37.04	26.61	22.93	0.0009	22.28	28.57	22.01	0.0006
				3	39.37	27.88	25.45	0.0007	25.66	25.22	25.20	1.7912	27.31	28.03	25.91	0.2532	27.63	27.03	27.07	0.5900	31.07	28.16	27.66	0.0197	35.56	28.44	26.13	0.0049	N/A	29.11	26.16	N/A
				7	35.19	27.32	24.15	0.0049	25.08	25.97	26.31	4.8583	26.62	27.11	24.86	0.2145	26.04	25.59	25.72	0.6771	30.98	27.27	26.22	0.0096	42.38	26.72	24.73	0.0000	N/A	27.81	24.83	N/A
				14	35.13	26.42	24.45	0.0042	26.43	24.44	25.32	0.9038	25.64	25.91	24.85	0.2875	25.50	24.91	26.05	0.8895	29.48	25.90	26.54	0.0206	35.42	26.28	25.03	0.0021	N/A	25.98	24.95	N/A
				21	28.33	28.63	19.24	0.0971	26.18	26.02	20.43	0.3087	20.66	28.02	19.84	3.3602	19.48	30.48	20.41	19.4897	30.30	28.96	21.12	0.0045	37.09	27.78	19.59	0.0002	22.72	28.82	20.23	0.8205

				28	37.40	30.20	26.94	0.0074	26.82	29.11	28.11	8.1161	29.63	30.54	27.74	0.2081	27.39	28.91	28.44	2.1793	36.21	30.89	29.06	0.0018	27.10	30.58	27.52	0.4532	21.68	33.17	28.63	0.9962
ET 55	dp	weaner	Vacc. "*C-Strain*"	0	26.68	22.91	20.89	0.0398	23.08	22.00	20.13	0.1059	22.25	23.89	21.72	0.6347	20.33	23.49	20.81	1.4827	27.44	23.55	21.27	0.0066	N/A	24.26	22.20	N/A	N/A	28.67	21.23	N/A
				0.5	25.71	22.63	20.28	0.0551	23.96	23.80	20.05	0.0451	20.53	23.17	20.88	1.1401	19.23	22.95	19.97	2.0073	25.88	22.37	20.75	0.0116	37.80	24.09	21.66	0.0002	22.40	25.25	20.41	0.0001
				1	25.69	24.78	20.39	0.1089	24.30	22.77	21.38	0.0565	21.09	25.57	21.04	1.6244	19.16	25.27	20.12	4.6323	26.99	25.26	20.59	0.0125	N/A	25.27	21.28	N/A	22.71	23.81	20.42	0.0002
				2	27.24	24.33	21.48	0.0516	23.82	24.13	20.38	0.0866	22.53	24.07	22.16	0.6421	20.88	23.99	21.37	1.4328	26.81	23.55	21.83	0.0124	24.19	25.29	22.81	1.4875	22.35	27.67	21.75	0.0003
				3	37.72	25.41	24.44	0.0007	26.07	23.85	24.83	0.7932	28.89	25.30	24.41	0.0216	28.88	25.00	26.41	0.0952	31.49	25.97	27.07	0.0060	43.71	26.12	25.45	0.0000	N/A	26.04	25.69	N/A
				7	N/A	25.50	23.90	N/A	26.07	23.65	25.21	0.8542	27.23	24.87	23.90	0.0502	26.10	24.15	25.47	0.3631	30.44	25.61	26.17	0.0083	35.73	25.47	24.67	0.0013	34.17	25.60	24.79	0.0037
				14	27.71	25.81	20.18	0.0800	24.10	23.65	21.03	0.6751	21.23	25.32	20.47	1.1815	20.27	25.03	21.40	7.1388	28.63	25.61	22.07	0.0072	31.79	25.21	20.42	0.0032	N/A	24.75	20.75	N/A
				21	27.21	25.36	19.47	0.0753	25.01	24.14	20.59	0.3783	21.22	26.05	20.57	1.5505	20.16	24.70	20.94	5.8441	30.23	26.53	21.52	0.0025	35.90	26.38	20.13	0.0003	22.79	27.63	20.41	0.6516
				28	32.87	28.41	25.03	0.0356	25.78	27.65	26.11	4.9122	26.91	28.94	25.76	0.4265	25.22	27.63	26.42	3.1617	33.43	29.38	27.09	0.0043	N/A	29.09	25.62	N/A	35.80	30.95	26.34	0.0075
ET 56	dp	weaner	Vacc. "*CP7_E2alf*"	0	28.78	24.59	20.59	0.0166	22.30	22.78	20.20	0.2318	22.01	25.45	21.30	0.9852	19.41	24.87	20.33	3.6856	26.82	25.17	20.96	0.0155	34.78	26.12	21.76	0.0022	N/A	26.49	20.70	N/A
				0.5	24.77	22.85	20.03	0.0970	23.19	24.42	20.03	0.0898	21.16	23.58	21.20	0.9565	19.30	23.82	20.30	2.8138	25.77	24.30	20.78	0.0233	16.87	23.89	21.32	0.8256	22.98	26.54	20.09	0.0001
				1	25.33	24.56	20.12	0.1171	24.14	24.35	20.31	0.0726	21.20	26.00	20.90	1.6381	19.42	25.90	20.15	4.7838	26.57	26.13	20.51	0.0215	N/A	27.14	21.23	N/A	39.97	25.70	20.32	0.0000
				2	25.56	25.01	20.44	0.1281	24.17	23.78	19.98	0.0760	21.20	26.23	21.11	1.8500	19.45	25.84	20.34	4.9165	27.01	25.51	20.76	0.0141	N/A	26.10	21.59	N/A	N/A	28.11	20.58	N/A
				3	37.42	25.94	25.49	0.0013	25.74	25.01	24.40	1.2040	28.22	26.06	25.43	0.0614	29.37	25.61	27.45	0.1206	31.97	27.24	27.95	0.0085	33.79	26.52	26.24	0.0088	N/A	27.38	26.80	N/A
				7	32.78	26.91	23.52	0.0150	25.91	24.95	26.29	2.0654	25.11	26.63	23.96	0.3893	24.32	25.38	24.83	1.5504	31.49	27.35	25.31	0.0049	34.22	27.13	24.15	0.0041	34.98	26.94	23.76	0.0024
				14	28.70	26.12	19.61	0.0410	23.96	25.35	21.03	1.2518	21.07	26.28	20.15	1.6085	19.58	26.29	20.95	15.3127	28.79	26.76	21.39	0.0073	28.31	25.88	20.10	0.0277	22.45	26.10	20.47	0.5774
				21	28.05	26.66	20.21	0.0855	25.35	24.57	21.30	0.4497	21.58	27.01	20.84	1.7942	20.43	26.48	21.32	10.2411	30.25	27.32	21.98	0.0037	35.61	27.21	20.74	0.0006	22.92	28.93	21.02	0.9468
				28	N/A	27.84	21.50	N/A	27.33	29.61	30.55	8.2845	32.76	31.19	29.41	0.0512	31.35	30.09	30.86	0.4747	41.99	31.72	31.56	0.0001	40.15	30.94	29.81	0.0020	22.44	33.16	30.47	0.5687
ET 57	dp	weaner	Vacc. "*CP7_E2alf*"	0	26.03	24.03	20.16	0.0661	22.71	23.77	19.51	0.1457	21.15	25.41	20.88	1.4301	18.99	24.76	19.94	4.1669	26.03	24.55	20.42	0.0186	N/A	25.42	21.35	N/A	N/A	26.55	20.36	N/A
				0.5	24.72	24.25	19.38	0.1242	23.08	24.26	20.32	0.1009	20.37	25.40	20.34	1.9417	18.80	25.02	19.47	4.4171	25.04	25.51	20.06	0.0457	27.65	25.03	20.59	0.0792	N/A	26.02	19.58	N/A
				1	26.29	24.93	20.63	0.0849	24.03	23.59	20.45	0.0814	21.25	25.78	21.21	1.6523	19.53	25.45	20.42	4.2321	26.24	25.76	20.94	0.0281	N/A	26.18	21.80	N/A	22.99	26.85	20.77	0.0002
				2	25.81	24.29	20.57	0.0927	24.10	23.11	20.47	0.0682	21.34	25.21	21.31	1.3840	19.47	25.14	20.36	3.9047	26.91	24.35	20.78	0.0105	N/A	26.05	21.97	N/A	22.48	27.62	20.88	0.0002
				7	27.08	25.02	19.15	0.0668	24.14	23.29	20.15	0.4309	20.12	25.27	19.33	1.7198	19.33	24.93	20.05	8.3013	29.04	25.73	20.93	0.0038	34.14	24.60	19.38	0.0005	N/A	25.15	20.00	N/A
				14	29.20	25.59	20.92	0.0387	23.31	24.37	22.05	1.9921	22.67	25.95	21.93	0.8685	20.40	24.01	22.10	5.8575	28.82	26.58	22.72	0.0108	35.81	26.05	21.63	0.0005	29.73	26.82	21.97	0.0222
				28	N/A	27.86	19.89	N/A	26.13	24.45	21.16	0.2509	21.78	26.24	21.07	1.3186	21.01	25.09	21.44	4.3762	30.25	27.06	22.02	0.0035	37.79	26.53	20.67	0.0001	22.78	28.29	21.06	0.9051
				21	28.91	25.73	20.19	0.0382	25.12	26.73	24.63	3.3188	25.27	28.85	24.31	0.7954	23.36	27.63	24.82	6.6678	32.07	29.20	25.56	0.0064	24.24	29.03	24.16	0.6795	22.41	30.38	24.47	0.8956
ET 58	dp	weaner	Vacc. "*CP7_E2alf*"	0	25.90	22.86	20.61	0.0582	23.95	24.52	19.83	0.0667	21.30	24.19	21.36	1.0889	19.33	24.28	20.21	3.1092	27.45	23.91	20.99	0.0067	N/A	25.02	21.88	N/A	23.05	27.01	20.62	0.0001
				0.5	24.99	24.41	19.64	0.1190	25.05	25.92	22.91	0.0401	20.57	25.50	20.59	1.8985	18.78	26.02	19.72	6.7031	27.50	25.52	20.08	0.0080	N/A	25.85	20.84	N/A	N/A	25.24	19.90	N/A
				1	28.43	26.35	23.08	0.0731	26.43	24.05	20.17	0.0616	23.90	27.31	23.72	1.0677	22.38	27.24	23.05	2.5074	31.08	27.35	23.18	0.0031	N/A	27.35	24.27	N/A	22.84	27.85	23.16	0.0005
				2	25.15	23.36	20.38	0.0994	23.87	25.71	21.46	0.0813	20.95	24.18	21.06	1.2233	18.80	23.97	20.05	3.8638	26.92	23.67	20.41	0.0074	38.24	25.03	21.62	0.0002	22.68	28.83	20.47	0.0001
				3	35.49	25.85	24.79	0.0032	24.95	24.52	19.42	0.2938	26.47	26.00	24.99	0.1748	26.48	25.11	25.96	0.4592	32.86	25.82	26.87	0.0020	N/A	25.91	25.23	N/A	N/A	27.21	25.95	N/A
				7	26.49	26.18	18.54	0.1122	27.64	24.86	25.77	0.5655	19.40	26.26	18.67	3.1220	18.44	27.56	19.42	30.6636	29.16	27.04	20.29	0.0042	35.42	25.67	18.49	0.0002	N/A	27.29	19.39	N/A
				14	28.19	24.75	20.05	0.0416	23.43	23.14	20.95	0.8561	21.30	25.18	20.44	1.0665	20.05	23.57	21.19	4.6402	29.84	25.33	22.04	0.0028	34.23	24.74	20.35	0.0006	35.45	26.57	20.82	0.0007
				21	27.48	26.02	19.02	0.0685	27.77	24.93	20.08	0.0710	20.56	26.93	19.73	2.4504	19.80	27.55	20.28	15.9397	29.97	27.47	20.94	0.0033	N/A	26.83	19.62	N/A	22.70	28.84	20.03	0.7858
				3	35.49	25.85	24.79	0.0032	24.95	24.52	19.42	0.2938	26.47	26.00	24.99	0.1748	26.48	25.11	25.96	0.4592	32.86	25.82	26.87	0.0020	N/A	25.91	25.23	N/A	N/A	27.21	25.95	N/A
ET 59	dp	weaner	Vacc. "*CP7_E2alf*"	0	26.52	25.11	21.29	0.0949	25.11	23.92	18.44	0.0851	22.59	26.72	22.26	1.2900	21.10	27.06	21.55	3.4569	28.76	27.66	22.00	0.0121	32.42	26.37	22.47	0.0122	N/A	24.39	21.17	N/A
				0.5	23.80	24.41	18.87	0.1957	23.99	23.99	19.82	0.0438	20.03	25.32	19.77	1.9694	17.72	26.69	18.87	12.9838	26.42	24.88	19.24	0.0106	N/A	26.14	20.05	N/A	N/A	28.23	19.10	N/A
				3	33.58	25.91	23.48	0.0068	24.86	25.26	18.98	0.3353	25.73	26.07	24.27	0.2348	25.67	24.59	25.11	0.5037	31.23	26.00	25.57	0.0043	N/A	26.04	24.27	N/A	N/A	27.04	24.30	N/A
				7	26.75	26.66	18.32	0.1038	26.41	24.13	24.53	0.6284	20.23	27.33	19.18	2.9335	18.51	30.56	19.64	16.2498	29.08	27.47	20.45	0.0053	38.21	27.76	19.17	0.0001	22.78	28.00	19.44	0.5341
				14	38.91	26.92	20.30	0.0001	24.58	25.34	21.40	0.9604	22.37	26.56	21.10	0.9811	20.36	26.49	21.78	12.6841	29.26	27.34	22.24	0.0084	35.91	26.71	20.75	0.0005	N/A	28.08	21.03	N/A
				21	29.45	28.06	17.45	0.0242	26.59	26.41	18.58	0.1398	19.57	28.24	18.25	4.5280	17.85	36.63	18.99	9.3400	28.74	28.73	19.44	0.0071	35.36	27.93	17.96	0.0004	N/A	30.08	18.59	N/A
				28	33.18	28.86	25.19	0.0356	26.56	27.89	26.33	3.5001	26.67	29.19	25.66	0.5275	25.19	27.70	26.51	3.4156	33.90	29.34	27.30	0.0032	23.37	29.30	25.61	0.2167	22.69	31.56	26.71	0.5867
ET 60	dp	weaner	Vacc. "*CP7_E2alf*"	0	26.39	23.51	19.96	0.0431	21.93	23.04	19.02	0.2361	21.02	25.27	20.63	1.3822	18.95	24.03	19.97	3.4437	25.07	25.20	20.31	0.0440	32.31	25.06	20.96	0.0054	N/A	28.02	19.96	N/A
				0.5	24.73	23.01	19.21	0.0816	23.50	24.02	19.29	0.0561	20.39	23.68	19.91	1.0630	17.87	24.18	18.81	5.1676	25.19	23.18	19.35	0.0154	N/A	24.56	20.26	N/A	N/A	26.19	19.31	N/A
				1	24.76	24.33	19.94	0.1469	23.85	24.02	19.29	0.0628	20.02	24.53	20.27	1.8566	17.86	25.20	19.21	8.2244	25.33	24.16	19.88	0.0228	N/A	25.59	20.90	N/A	23.00	25.93	19.86	0.0001
				3	38.02	26.23	24.53	0.0007	22.36	22.76	20.44	1.2440	27.55	26.49	24.87	0.0928	28.28	25.82	26.39	0.1919	29.83	25.92	26.58	0.0164	38.34	26.21	25.23	0.0004	N/A	26.66	25.25	N/A
				7	28.80	25.08	19.57	0.0276	24.27	23.97	25.45	3.1971	21.46	25.09	20.21	0.8611	19.97	23.54	20.94	4.4626	28.07	25.06	21.33	0.0071	34.78	24.84	20.02	0.0004	N/A	25.74	20.37	N/A
				14	35.73	25.65	20.88	0.0008	23.28	24.30	21.82	1.8353	23.16	25.64	21.35	0.4602	21.41	23.38	22.09	2.2919	29.56	25.57	22.64	0.0045	35.36	25.40	21.29	0.0005	35.16	27.05	21.35	0.0011
				21	27.99	27.20	19.20	0.0764	27.42	25.32	20.25	0.1062	20.64	27.47	19.70	2.7292	20.12	29.09	20.47	18.3327	30.05	28.24	21.09	0.0042	36.56	26.49	19.45	0.0002	22.33	27.61	20.16	0.7693
				28	N/A	29.54	28.07	N/A	27.94	28.35	29.01	4.3277	32.97	30.09	28.26	0.0213	30.98	28.95	30.03	0.3070	37.14	30.38	30.59	0.0014	39.24	30.28	28.86	0.0021	22.63	32.42	30.21	0.9834
				2	42.31	25.67	24.19	0.0000	24.67	24.47	25.25	2.7072	N/A	29.87	29.52	N/A	28.37	25.21	26.49	0.1511	30.04	26.12	26.68	0.0155	N/A	25.94	25.02	N/A	N/A	26.20	25.25	N/A
ET 285	dp	weaner	Vacc. "*CP7_E2alf*"	0	24.21	23.39	19.00	0.1172	23.17	23.64	19.05	0.0808	20.16	24.85	19.79	1.6164	18.01	25.03	18.90	6.3616	26.23	24.83	19.50	0.0130	N/A	25.73	20.33	N/A	N/A	25.64	19.04	N/A
				0.5	25.52	23.98	20.39	0.0957	24.50	23.50	20.20	0.0486	21.23	24.90	21.09	1.2669	19.37	24.79	20.16	3.5046	27.19	24.89	20.65	0.0098	N/A	25.02	21.53	N/A	N/A	26.40	20.48	N/A
				1	23.62	23.15	19.47	0.1802	23.73	22.88	19.01	0.0464	20.69	24.72	20.58	1.4378	18.12	24.56	19.67	6.5260	27.00	25.20	20.31	0.0111	20.43	24.54	20.29	0.4982	22.47	27.31	19.59	0.0002

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5 DISCUSSION

5.1 Host related influences on classical swine fever disease course and outcome

Classical swine fever virus isolates of genotype 2.3 were responsible for most of the recent CSF-outbreaks among European domestic pigs and wild boar (Bartak and Greiser-Wilke, 2000; Biagetti et al., 2001; Blome et al., 2010; Depner et al., 2006; Leifer et al., 2010). These strains are moderately virulent and lead to highly variable clinical pictures that present a challenge for both outbreak detection and research on pathogenesis. To date, only little is known about the underlying mechanisms and principles with regard to virus characteristics and specific host reaction pattern that lead to the observed differences. It was shown that age and immune status of the animal have a major impact on disease severity (Moennig et al., 2003). In addition, studies with animals of different breeds gave rise to the assumption that the genetic background may also influence the clinical course of CSF infection with moderately virulent strains (Blacksell et al., 2006; Depner et al., 1997). In some studies, domestic pigs were shown to present more severe disease courses upon infection with CSFV strains of wild boar origin (Kaden et al., 2000; Kaden et al., 1999).

In the two here presented studies with animals of different breeds and age classes, efforts were directed towards the characterization of beneficial and detrimental reaction pattern on the individual animal level, and on gaining basic knowledge for the estimation of disease dynamics in animal groups.

In order to mirror the current field situation, the well characterized CSFV strain “Roesrath” (CSF1045) was employed to inoculate the animals. This virus was originally isolated from a German wild boar piglet in 2009 (Leifer et al., 2010) and was used as challenge virus in a previously published animal trial (Blome et al., 2014). In addition to the available sequencing information concerning CSFV subgroup 2.3 (Leifer et al., 2010), the genetic identity of the employed isolate was further confirmed by sequencing of different experimental samples (Mouchantat et al., 2014a). Apart from the isolate’s representative characteristics, it allows direct comparison with previously published trials employing similar strains of the same subgenotype, namely “CSF0634” (Bunzenthal, 2003), and “Visbek/Han95” (Depner et al., 1997).

In the first approach, weaner pigs with a different genetic background, in detail two domestic breeds and European wild boar, were employed (Petrov et al., 2014a). The domestic pig breeds, i.e. crossbred fattening pigs (hybrids) and purebred German landrace, were chosen due to their practical relevance in pig industry. While hybrids are commercially used for fattening, German landrace pigs are used as parental line for breeding purposes. Their wild relatives, European wild boar, were included in order to target basic differences in host immune responses and due to their key role in epidemiology and disease surveillance.

According to previous studies, hybrids were suspected to show superiority in terms of immunological functions (Buschmann, 1985) and landrace pigs were assumed to be most susceptible (Depner et al., 1997). Furthermore, wild boar were expected to show a strong cellular response. The latter assumption was based on previous, so far unpublished observations by the author group.

In contrast to our expectations, no significant genetically determined influence was observed (Petrov et al., 2014a). Finally, all animals were susceptible to infection and despite clinical differences, virus could be detected in all infected animals to similar amounts. All but one animal developed an acute disease course. One wild boar showed laboratory results that indicated a chronic disease course. Despite the fact that hybrids showed strong cellular immune responses, all of these animals succumbed to infection. In contrast, two German landrace pigs recovered after having shown only moderate cellular reactions. Thus, indications exist that dysregulation of immune responses might play a role in CSF pathogenesis and that moderate responses are beneficial. In the experimental work with wild boar, difficulties in clinical scoring were revealed. In the end, scores were not indicative for the disease outcome in this species. This complication might be due to the fact that the clinical evaluation system according to Mittelholzer et al. (2000) was hardly applicable to wild boar as their behavior differed greatly from that of domestic pig breeds and led probably to concealment of clinical signs. In order to reflect the health status of the animals in a proper way, more parameters such as viraemia, blood cell counts, and post mortem findings should be included in the future.

Concluding, minor differences in age and body weight as well as preload with facultative pathogenes outweight the impact of genetic background on disease outcome. However, substantial variations were observed among individuals ranging from acute-lethal to chronic forms or complete convalescence. Additional studies are currently targeting the definition of individual host responses, e.g. on cytokine level, and further characterization of the virus.

The second trial was conducted with the same isolate and infectious dose with a group of subadult wild boar. In contrast to the previous trial, all infected animals developed a subclinical form and seroconverted (Mouchantat et al., 2014a). This outcome confirmed previous observations and shows that on population level all disease courses will be present. It also shows that both virus and antibody detection methods are needed in surveillance.

5.2 Detection of porcine cytokine gene expression profiles by RT-qPCR

To further target individual host responses that might have led to the observed differences in CSF disease course and outcome, suitable parameters had to be defined. As the above mentioned studies indicated that dysregulation of immune responses plays a key role in CSF pathogenesis, cytokines were chosen as an additional target as they are known to link and orchestrate different parts of the immune system. Generally, cytokines are among the parameters that are suspected to influence disease course and outcome (Lange et al., 2011). In this context, especially TNF-α, IL-1β, IL-1α, and IL-6 were discussed (Jamin et al., 2008). The proinflammatory cytokines TNF-α and IL-1 seem to be involved in the induction of apoptosis in different leukocyte populations (Choi et al., 2004) that is well described for swine fever infection (Sanchez-Cordon et al., 2005). In analogy to filoviral infections, IL-6 could be suspected to be involved in changes in endothelial permeability (Lange et al., 2011).
Nevertheless, up to now, there has been a serious lack of appropriate and reliable tools for cytokine gene expression analyses, especially in pigs. Despite the fact that enormous progress has been made in the field of PCR-technologies, SYBR-Green based assays (Charerntantanakul et al., 2013; Ferrari et al., 2013) and conventional gel-based PCRs (Choi et al., 2006; Techau et al., 2007) are still commonly implemented for immunological purposes. However, these techniques possess significant deficits in terms of sensitivity and specificity.
In order to close this methodological gap, a harmonized TaqMan-based triplex RT-qPCR protocol was developed and validated for the quantitative detection of normalized gene expression profiles of seven porcine cytokines (Petrov et al., 2014c, in press). Cytokines were selected according to their proposed role in pathogenesis and as key cytokines for immunological pathways, namely IL-1β, -2, -4, -6, -8, TNF-α, and IFN-α (Lange et al., 2011). To further complement the system, two additional cytokines, i.e. IL-10 and IFN-γ, have been added recently (so far unpublished data).
The resulting assays were shown to be highly sensitive and specific and are harmonizied in terms of PCR chemistry, cycling conditions, and handling. In addition, they are economic due

to the validation for a small-volume (12.5 µl) approach and easy to conduct with common real-time PCR equipment. The novel assays are now available for *in vivo* and *in vitro* studies and have been included in ongoing pathogenesis studies.

However, a challenge is posed by potential instabilities of cytokine mRNA. Cytokines are tightly regulated factors *in vivo* for their crucial role in coordinating immune responses (Mino and Takeuchi, 2013; Reznik et al., 2014). Regulation strategies include modifications of cytokine mRNA stabilities, translations and degradations. The necessity of physiological regulation mechanisms was already shown in other contexts (Costa-Pereira, 2014; Kother et al., 2014; Masuda et al., 2013; Payne et al., 2014; van Vliet et al., 2013). Although mRNA degradations are essential for avoiding uncontrolled cytokine expressions and releases (Damgaard and Lykke-Andersen, 2013), they might interfere accurate gene expression analyses. This especially applies to IL-2 and IL-4 which are essential for the Th1/Th2 balance (Jungi, 1996; Murphy, 2009; Zhou et al., 1994). Their detection is further complicated by rare mRNA amounts due to a limited number of highly specialized producer cells and short expression times (Murphy, 2009). In contrast, assays for other cytokines, e.g. IL-8 (Jungi, 1996), with manifold functions and producer cells appear to be more robust. In addition, expressions of cytokines are markedly influenced by storage times and conditions (Duvigneau et al., 2003). While some expression levels can be artificially increased upon inadequate storage, other mRNAs may not be detectable anymore. This finding was confirmed during validation studies with more than 400 samples from experimentally infected animals (Petrov et al., 2014c, in press). Sample handling and processing, especially freeze-thawing, seems to be a crucial for possible mRNA degradation. In this respect, there is still a need for further optimization. Future studies will target the evaluation of proper handling methods for leucocyte isolations, ideal storage conditions and times as well as effects of freeze-thawing cycles on mRNA stability.

Cytokine mRNA encodes for highly regulated proteins (Reznik et al., 2014) which will also be targeted in future studies. Available test systems include commercially available ELISA systems (ready for use kits and antibody pairs), radioactive immunosorbent assays, Luminex based systems, bioassays as well as micro arrays. Bioassays (Baarsch et al., 1991; Pauli et al., 1994) and ELISAs (Nuntaprasert et al., 2004; Nuntaprasert et al., 2005; Splichal et al., 2003) were mostly implemented in the past and are still well suited for single parameter analysis nowadays (Dong et al., 2013; G et al., 2014; Wyns et al., 2013). During the last years, technical advances were made, e.g. by developing several Luminex-based assays allowing a multiparameter analysis with even small sample volumes (Bjerre et al., 2009; Bongoni et al.,

2013; Graham et al., 2010; Wyns et al., 2013). However, numerous difficulties must be resolved before usage. One problem is the lack of validation for porcine serum and other biological samples. So far, several validation experiments, including spike-recovery protocols and validation on experimental samples, were conducted in order to overcome technical and biological limitations (data unpublished).

A combination of the new tools with optimized flow cytometry analyses and routine serological tests will cover a broad spectrum of host responses in future animal trials. Besides the great potential to reveal new insights in disease development of CSF, the applicability is also expandable to other diseases of swine with immune pathogenetic background, e.g. ASF.

5.3 Optimization of swine fever surveillance and early warning tools

In January 2014, an exotic disease was introduced into the wild boar population of the EU that had been spreading in Eastern Europe since 2007, and that has a clinical picture that is indistinguishable from CSF: African swine fever. Up to now, four EU Member States, namely Lithuania, Latvia, Poland, and Estland (OIE WAHID, visited September 18th 2014), are affected, and the disease already spread to domestic pigs. Taking previous transmission pattern into account, the risk of introduction into additional free areas is high (Costard et al., 2013; Edwards et al., 2000; Vargas Teran et al., 2004).

A crucial factor for the control of animal diseases such as CSF and ASF is early detection. Depending on disease characteristics, active and/or passive surveillance can be crucial. Both systems are relying on compliance of hunters and sufficient sample submissions. Especially the latter is oftern lacking, even in times of increased risk.

In order to establish pragmatic detection workflows for the swine fevers that could increase compliance and sample submissions, two non-invasive methods were validated in the framework of our studies using either dry blood swabs (Petrov et al., 2014b) for passive ASF and CSF surveillance, or a “rope-in-a-bait” sampling method for active (Mouchantat et al., 2014a) CSF surveillance. Also the highly variable disease forms observed during both experimental trials with a recent CSFV isolate (Mouchantat et al., 2014a; Petrov et al., 2014a) demonstrate the necessity of including both, live and fallen animals, in disease surveillance.

The design of the workflows profited from the significant technological and methodological advances in diagnostic techniques that were achieved over the last few years. Nowadays, neither sample handling nor test sensitivity can be regarded as diagnostic bottle necks.

Accurate laboratory methods for serological and virological analyses are laid down in the EU Diagnostic Manual and the *Manual of Diagnostic Tests and Vaccines for Terrestrial Animals* for CSF and ASF (OIE, 2008a; OIE, 2008b). Furthermore, diagnostic procedures were tested and validated previously (Donahue et al., 2012; Gallardo et al., 2009; Hoffmann et al., 2011; Schroeder et al., 2012). Especially the application of highly sensitive real-time PCR protocols allowed pragmatic approaches (Haines et al., 2013; Hoffmann et al., 2009; Leifer et al., 2011; Tignon et al., 2011).

With regard to the dry blood swabs, it was shown that qualitative CSFV and ASFV genome detection in blood, feces and organ samples is possible even after storage under suboptimal climatic conditions (storage experiment at 37°C for 7 days prior to swab sampling). These results suggest the potential applicability to samples from carcasses even in various stages of decay. The fact that qualitative results were irrespective of the swab type and extraction method gives additional evidence concerning the robustness of this method. This system will be further validated under field conditions.

Apart from detection of viral genome, also serological analyses are required by law (Commission Decision 2003/422/EC). In cases of ASFV, it could be recently shown that forensic livestock swabs (their fragments) represent also an appropriate matrix for antibody detection by ELISA (Blome et al., 2014c, in press).

However, the major weakness is the loss of syndromic surveillance in the field. To overcome parts of this problem, applicability to swab samples for other pathogens should be explored.

In our second approach, detection of CSFV genome in saliva from rope baits was evaluated as pragmatic tool for early warning (Mouchantat et al., 2014a). So far, an adequate sample collection from living animals in the field involves special hunting or trapping activities (Alexandrov et al., 2013). On the contrary, the distribution and collection of rope baits do not require any additional equipment, is easy to handle and to transport for laboratory analysis. It could be shown that the system is suitable in principle. Moreover, its applicability was proven for foot-and-mouth diseased pigs recently (Mouchantat et al., 2014b).

Taken together, pragmatic approaches are now available for field application that could markedly lower the threshold of sample submission and increase compliance of hunters. In general, transfer is also possible to backyard settings or areas with semi-wild pigs improving the control measures in both CSF and ASF outbreak scenarios.

6 SUMMARY

Classical swine fever is among the most devastating diseases of pigs worldwide. Outbreaks are accompanied by tremendous socio-economic consequences. During the last two decades, CSFV isolates of genotype 2.3 have predominated in Europe. Their most characteristic feature is the moderate virulence leading to highly variable clinical pictures and outcomes. Several influencing factors were identified such as age and immune status on the host's side as well as virulence of the isolate on the agent's side. Furthermore, an impact of genetic background and individual factors were proposed. However, little is known about host responses leading to different disease courses and other basic pathogenetic mechanisms. With aim of characterising host responses, two experimental infection trials were performed with pigs of different breeds and ages. The strong age-dependence could be confirmed by observing subclinical courses in the older group and predominantly severe forms in younger animals irrespective of the genetic background. The breed-related impact was only minor compared to the influence of age and body weight as well as preload with facultative pathogens. However, significant differences were revealed among individuals that merit further investigation.

To identify underlaying immune-pathogenetic mechanisms, seven porcine cytokines were targeted for their suggested key role in swine fever pathogenesis. To this means, a TaqMan-based RT-qPCR for the assessment of normalized gene expression profiles of IL-1β, -2, -4, -6, -8, TNF-α, and IFN-α was developed and validated. This highly specific and sensitive assay is harmonized in terms of procedure, PCR chemistry, and cycling conditions enabling a rapid and economic mRNA detection. This assay will be used to target beneficial and detrimental host reaction pattern not only for swine fever but also for other porcine diseases with an immune-pathogenetic background.

In terms of disease surveillance, the lack of easy and swift sample submissions for laboratory diagnosis, even in times of increased risk, presents the major bottleneck. Therefore, pragmatic approaches for easy conductable sampling strategies were implemented. With regard to passive swine fever surveillance, a robust dry-/ semidry blood swab technique for CSFV and ASFV detection was validated and established. Furthermore, "rope-in-a-bait" sampling swabs were tested in terms of CSF in order to facilitate early warning strategies for optimization of active surveillance. An implementation for other diseases is conceivable providing a worthy contribution to the entire control of infectious animal diseases.

7 ZUSAMMENFASSUNG

Die Klassische Schweinepest (KSP) zählt weltweit zu den verheerendsten Schweinekrankheiten und verursacht im Ausbruchsfall immense sozio-ökonomische Verluste. Verantwortlich für die europäischen Ausbrüche der vergangenen zwei Jahrzehnte waren vorrangig Virusisolate vom Genotyp 2.3. Diese zeichnen sich insbesondere durch ihre moderate Virulenz aus, welche eine hohe Variabilität an klinischen Symptomen und Krankheitsausgängen zulässt. Einige beeinflussende Faktoren wurden sowohl auf Wirtsseite als auch auf Seiten des Erregers identifiziert. Hierzu zählen Alter und Immunstatus des Tieres sowie die Virulenz des jeweiligen Schweinepestisolates. Darüber hinaus wurden genetisch-bedingte Einflüsse und individuelle Faktoren in Betracht gezogen. Jedoch sind die grundlegenden pathogenetischen Mechanismen bislang nur unzureichend geklärt. Um die zugrunde liegenden Wirtsreaktionen näher zu charakterisieren, wurden zwei Infektionsversuche durchgeführt. Die infizierten Schweine unterschieden sich hinsichtlich Alter und Rasse. Die vorberichtlich starke Altersabhängigkeit konnte während der Versuche bestätigt werden, da die infizierten Tiere der älteren Gruppe ausschließlich subklinische Verläufe entwickelten, während schwere Verlaufsformen in allen jüngeren Tieren unabhängig von deren Rasse dominierten. Ein genetisch-bedingter Einfluss konnte nur geringfügig beobachtet werden, während Unterschiede im Alter und Gewicht der Tiere sowie eine Vorbelastung mit fakultativen Pathogenen eine größere Rolle gespielt haben. Jedoch zeigten sich starke individuelle Unterschiede im Krankheitsverlauf, die einer weiteren Betrachtung bedürfen.

Aufgrund der vermuteten Schlüsselrolle von Zytokinen in der Immunpathogenese der Schweinepest wurden sieben von ihnen als Messparameter ausgewählt. Für die Analyse normalisierter Genexpressionsprofile von Interleukin (IL)-1β, -2, -4, -6, -8, Tumor-Nekrose-Faktor-alpha (TNF-α), und Interferon-alpha (IFN-α) wurde ein TaqMan-basiertes real-time RT-PCR System entwickelt und validiert. Diese hoch spezifischen und sensitiven Assays wurden hinsichtlich der Durchführung, der PCR-Chemie, und des Temperaturprofils für die verschiedenen Zytokine vereinheitlicht und ermöglichen eine schnelle ökonomische Detektion der mRNA. Diese Technik gestattet nicht nur die Definition von Reaktionsmustern im Verlaufe der Schweinepest, sondern ist zudem auf jede andere Erkrankung des Schweines, der eine Immunpathogenese zu Grunde liegt, anwendbar.

Neben der KSP stellt insbesondere die aktuell bedrohliche Ausbreitung der Afrikanischen Schweinepest (ASP) innerhalb der Europäischen Union ein erhebliches Risiko dar. Trotz dieser alarmierenden Tatsache besteht derzeit ein bedenklicher Mangel an zur Diagnostik eingesandten Proben. Aus diesem Grund wurden pragmatische nicht-invasive Ansätze entwickelt, welche die Probennahme im Feld und dessen Transport erleichtern sollen. Für die passive Schweinepestüberwachung wurde eine robuste Methode auf der Basis von

getrockneten Blut-Tupfern etabliert und validiert. Diese ermöglicht sowohl die Detektion von Klassischer als auch von Afrikanischer Schweinepest. Des Weiteren wurde eine „Seil-im-Köder“ Technik für die Klassische Schweinepest implementiert, welche als vereinfachte Frühwarnstrategie die aktive Seuchenüberwachung optimieren soll. Eine Anwendung beider neu etablierten Beprobungsstrategien ist darüber hinaus für andere Seuchengeschehen denkbar, was einen bedeutenden Beitrag für die gesamte Tierseuchenbekämpfung darstellt.

8 ABBREVIATIONS

ASF	African swine fever
ASFV	African swine fever virus
BHQ	Black Hole Quencher
CSF	Classical swine fever
CSFV	Classical swine fever virus
DIVA	Differentiation of infected from vaccinated animals
DNA	Deoxyribonucleic acid
EFSA	European Food Safety Authority
ELISA	Enzyme-linked immunosorbent assay
EU	European Union
FAM	6-Carboxyfluorecein
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
HEX	Hexachloro-6-carboxyfluorescein
IFN	Interferon
IL	Interleukin
mAb	monoclonal antibody
NK	Natural killer cells
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
RNA	Ribonucleic acid
RT-qPCR	Reverse transcription polymerase chain reaction
spp.	*Species pluralis*, several species
TF	Tissue factor
TNF-α	Tumor necrosis factor-alpha
TR	Texas Red

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