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# Biological and genomic characterization of recent African swine fever strains and field validation of surveillance tools

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# 1 Introduction

Ten years ago, African swine fever (ASF) was a so-called exotic animal disease in Europe, but nowadays the picture has changed drastically. The disease is currently one of the most important threats to the European pig farming sector and the wild boar population. After ASF reached Georgia in 2007, the disease was expected to either spread rapidly or die-out due to self-limitation, especially in the wild boar population. The current situation in Eastern Europe, where the disease can be regarded as endemic, shows, that none of these predictions of the disease spread held true. ASF was re-introduced into the Eastern part of the European Union (EU) in 2014 and spread since then slowly but constantly through the Baltic States and Poland. In 2017, the disease leapt over to the Czech Republic and single cases were reported from Romanian backyard farms. However, most cases occur within the wild boar population leading to new challenges regarding surveillance and early disease detection. The compliance of hunters and foresters is indispensable to get samples of fallen wild boar, the first hint for disease outbreak. So far, blood, organ samples or bone marrow are the common diagnostic samples. Nonetheless, the sampling procedure is inconvenient and constitutes the risk of contamination. To address this issue, a practical and easy-to-use sampling tool is required leading to the target of the first presented study. Previous experiments already showed that easy-to-use dry-swabs are suitable for this purpose. Hence, the conducted study strengthened this fact and extended the applicability of dry blood swabs to pathogen and antibody detection under field conditions together with point-of-care test systems. However, not only the surveillance of the disease offers new challenges. In order to explain the unexpected distribution pattern of ASF, a detailed knowledge about the disease dynamics and pathogenesis of relevant virus strains is fundamental. For this reason, the second study focused on peculiarities reported from North Eastern Estonia, where a relatively high antibody prevalence was found in hunted wild boar, raising the hypothesis of natural virus-attenuation. To approach this question, four animal trials including different pig breeds were carried out. All pigs were infected with a re-isolated African swine fever virus (ASFV) field-strain from North Eastern Estonia, representative strains were subjected to next-generation sequencing (NGS), and full-genomes have been investigated. A tailored PCR was subsequently designed to screen field samples for the variant virus strain that was discovered. Another issue of the endemic disease situation in Eastern Europe is the long-term fate of wild boar that recover from ASFV infection. The role of these survivors, acting as potential carriers that could help

the disease spread, is controversially discussed already since the first outbreaks in the EU in the 1960s. In order to evaluate, if and how long the recovered swine can transmit the disease, another study was conducted. Pigs were infected with a moderately virulent ASFV strain to obtain a representative number of surviving animals. The clinical course was assessed together with virus shedding and distribution. To proof if the recovered animals are able to transmit the disease, they were commingled to sentinels to investigate the potential transmission by pig-to-pig contact.

# 2 Literature review

## 2.1 Virus taxonomy, morphology and viral genome

*African swine fever virus* (ASFV) belongs to the genus *Asfivirus* and is the only member of the *Asfaviridae* family. Due to the sylvatic transmission pathway that includes soft ticks of the genus *Ornithodoros,* it is further classified as the only DNA arthropod-borne (ARBO) virus (Sanchez-Vizcaino et al. 2015). Molecular classification based on the partial sequencing of the gene encoding the p72 protein revealed 23 different circulating genotypes (Achenbach et al. 2017). The virion shows an icosahedral shape (Carrascosa et al. 1984) and with a size of 175-215 nm it is quite large compared to e.g. *Classical swine fever virus* with a size of 50 nm (Swiss Institute of Bioinformatics 2018). Four concentric layers constitute the virus particle: the central nucleoid, the core shell, the inner envelope and the icosahedral capsid (Salas et al. 2013). The precise origin of the inner envelope remains controversial but a complex wrapping mechanism involving intracellular membrane cisternae, similar to *Poxviruses*, seems to be the most likely (Rouiller et al. 1998). By budding through the plasma membrane, the extracellular virus acquires an additional external envelope (Breese et al. 1966) lacking glycoproteins which is atypical compared to the majority of viruses (del Val et al. 1986).

The viral genome consists of linear double-stranded DNA with a length of 170 to 193 kilobase pairs (kbp) (Chapman et al. 2008). It contains between 151 and 167 open reading frames (ORF) resulting in more than 50 identified structural proteins and around hundred non-structural proteins. Those are not only required for replication and morphology but also involved in the evasion of host defense systems (Dixon et al. 2004).

The viral polypeptides can be divided in early and late classes according to their time of occurrence in the infected cells (Carvalho et al. 1986). One of the structural proteins synthesized in early times of infection (Afonso et al. 1992) is p30. Since it is the most abundantly expressed protein in the early infection phase, and has been found in the supernatant of infected cells, secretion was anticipated but refuted by Prados et al. (1993). However, it is one of the highly immunogenic viral proteins inducing a strong antibody response in pigs (Letchworth et al. 1984). Another protein of the early class is p22 which is one of the external viral structural proteins and was also found incorporated transiently into membranes of infected cells (del Val et al. 1986). With its N-terminal hydrophobic region it shows characteristics of a signal peptide (von Heijne 1985). P54 is one of the structural proteins assigned to the late class: it has been described as one of the proteins essential for

virus viability (Rodriguez et al. 1996) and plays an essential role in the viral morphogenesis by forming disulfide-linked homodimers in the replication factories (Rodriguez et al. 2004). Another late protein, is the before mentioned p72 major capsid protein (Lopez-Otin et al. 1990) which also plays an important immunogenic role. High loads have been located in the endoplasmic reticulum cisterna, which is incorporated in the viral morphogenesis by wrapping the viral structure (Garcia-Escudero et al. 1998). P72 was shown to be externally and internally located in the intracellular virus (Cobbold et al. 1996) protecting the virus membrane from trypsin and other proteases. As the encoding gene is highly conserved (Yu et al. 1996), the protein is suitable for virus classification and is basis for both direct and indirect diagnostic methods

After an early nuclear phase, the viral replication occurs in perinuclear cytoplasmic viral assembly sites involving enzymes that are immediately expressed after virus entry (Dixon et al. 2013). DNA replication intermediates consisting of predominantly head-to-head and tail-to-tail concatemers similar to vaccinia virus have been detected (Gonzalez et al. 1986, Rojo et al. 1999).

## 2.2 Clinical signs, lesions and pathogenesis

*Clinical picture and differential diagnoses*

ASF induces a broad range of severe but unspecific clinical signs in infected pigs. The disease course is similar to CSF and differential diagnosis based on clinical signs and lesions is not possible. Apart from CSF, Porcine Reproductive and Respiratory Syndrome (PRRS), Porcine Dermatitis and Nephropathy Syndrome, Aujeszky's disease, Swine Erysipelas, several bacterial infections, and coumarin poisoning range among the differential diagnoses (Kleiboeker 2002, OIE World Organisation for Animal Health 2013). Peracute forms, characterized by sudden death of the infected animals as well as chronic disease courses without any obvious sign of the disease can occur (Blome et al. 2013). However, the acute disease course is the common form in animals infected with higher virulent strains (Gabriel et al. 2011). After an incubation period of 2 to 7 days (in rare cases up to 14 days) (Mebus 1988) the infected animals start showing the eponymous high fever together with other unspecific clinical signs like lethargy, reddened skin (especially at the acra), anorexia and conjunctivitis. Respiratory problems such as coughing and tachypnoe accompanied by tachycardia and cyanosis have been observed. In addition, digestive findings such as vomiting and diarrhea (watery or bloody) can occur. In later stages of the disease, neurological signs (paddling, convulsions) and walking difficulties

such as staggering are described. Animals suffering the acute course commonly die within the first two weeks after onset of clinical signs. Chronically infected animals often show a prolonged disease course over several months with joint swelling and wasting along with secondary infections (European Food Safety Authority (EFSA) 2009). Abortions of pregnant sows especially within the first days of high fever are reported from experimentally infected animals (Schlafer et al. 1987). The same is described for natural infections in outbreak situations where abortion rates up to 90% are described (Mc Daniel 1978). Stable loads of viral antigen have been found in tissues of necrotic uterus and ovaries whereas the occurrence of antigen in fetal tissues seems to be unreliable and dependent on the employed strain. Thus, vertical transmission of the disease remains possible but unlikely (Schlafer and Mebus 1987, Antiabong et al. 2006). The course of the disease in animals infected with highly virulent strains is not age dependent under experimental conditions (Blome et al. 2012), although field observations in Estonia report higher antibody prevalence in wild boar piglets, indicating that milder disease courses could occur in this age class (Nurmoja et al. 2017).

*Virus dissemination and pathogenesis*

Regarding the dynamics of viremia and virus shedding, studies show that stable loads of antigen are detectable in blood starting with the onset of clinical signs. Throughout the evaluated disease courses, viral shedding via saliva, nasal fluids and faeces is much lower compared to the amount of virus in blood and limited to the acute phase of the disease (Gabriel et al. 2011, Guinat et al. 2014, Nurmoja et al. 2017).

Apart from the arthropod-driven infections, where the virus primarily infects Langerhans cells in the skin (Bernard et al. 2016), the virus enters the body via the oronasal route. Primary replication takes place in the lymphatic tissues of the oropharynx, i.e. tonsils and regional lymph nodes. Main target cells are monocytes and, to a lesser extent, lymphocytes (Munoz-Moreno et al. 2015). In the late stages of infection, several cell types are infected. Monocyte derived macrophages show viral replication and the following secretory activation has a pro-inflammatory, pro-coagulant profile leading to hemorrhages (through activation and dysregulation of endothelial cells) and lymphoid depletion (by apoptosis of lymphocytes) (Gomez-Villamandos et al. 2003, Gomez-Villamandos et al. 2013). In the blood fraction, 90% of the virus is associated with erythrocytes (Wardley et al. 1977) which is not surprising for an arthropod-borne virus. The viral particles attached to the red cell surface can ultimately lead

to hemolysis in infected animals (Karalyan et al. 2016) causing hemolytic anemia contributing to the typical picture of a hemorrhagic fever in acute lethal disease courses.

*Pathological findings*

The mentioned hemorrhages characterize the picture of pathological lesions after acute-lethal disease courses. Necropsy of these animals reveal generalized or single enlarged, hemorrhagic lymph nodes of marbled appearance or ebony colour (Gabriel et al. 2011, Nurmoja et al. 2017). The spleen of acutely affected animals can be highly enlarged with rounded edges and a friable consistency. Petechiae occur in the renal pelvis and cortex, as well as the mucosa of the urinary bladder, epicardium, endocardium, pleura and the gastric mucosa (Mebus et al. 1979, Hervás et al. 1996, Sanchez-Vizcaino et al. 2015). In chronically infected animals, necrotic lesions of skin or tonsil, arthritis and different kinds of secondary bacterial infections, like pleural adhesions and fibrinous pericarditis are common (Sanchez-Botija 1982).

## 2.3 Global distribution, epidemiology and risk factors

*History and global spread*

ASF was described for the first time by Montgomery in 1921 (Mebus 1988) when he described the outbreak in Kenya between 1909 and 1915. He already mentioned contact to wild suids as source of infection. Later it was hypothesized, that the virus was already present in its natural wildlife host, such as African wild suids, for a long time (Penrith et al. 2004). The first outbreak on the European continent took place in 1957 in Portugal where contaminated waste from international airports was fed to domestic pigs. After a second introduction of the disease in 1960 the disease remained endemic in Portugal until the mid of 1990ies (Costard et al. 2009). During this time, the disease spread to several European countries such as Belgium, the Netherlands, France, and Italy. In all these places the disease was successfully eradicated apart from Sardinia where ASF is endemic until today (Gallardo et al. 2015). After this first wave of minor outbreaks it remained silent on the European continent until 2007. In this year, the first cases of African swine fever were reported from Georgia. It is most likely, that free-ranging pigs got infected by contact to dumped port waste near the Black sea harbour of Poti (FAO/OIE mission 2007). Starting from this initial introduction, the disease spread through the neighbouring countries, such as the Russian Federation, Armenia and Azerbaijan, following

the main transportation routes (Beltrán-Alcrudo et al. 2008, Khomenko et al. 2013). The isolates circulating in the Trans-Caucasian region showed high virulence accompanied by high lethality rates in case reports and under experimental conditions (Gabriel et al. 2011). Therefore, two potential scenarios of disease spread were initially assumed: Either the disease would spread rapidly or die out due to self-limitation. Both models were proven wrong and ASF spread slowly but constantly forward. In 2012 and 2013, Ukraine (Dietze et al. 2012) and Belarus (WAHID 2013) reported their first cases, respectively. In January 2014, ASF was detected for the first time in the Eastern part of the European Union. Four wild boar were found dead in Lithuania and Poland not far from the Belarussian border (Gallardo et al. 2014). Today, the disease is endemic in the Baltic States and further outbreaks have been reported from the Czech Republic and Romania. The outbreak in wild boar of the Czech Republic in June 2017 was 400-500 kilometres away from the nearest known outbreak in Poland or Ukraine and locally limited to the area of Zlín (ISZAM 2017). Consequently, a lot of effort has taken place in trying to stop further disease spread. Among the measures were fencing of a designated high-risk area (repellents and electric fences), initial hunting ban, removal of carcasses and limited access to the infected zone. These measures were meant to keep the potentially infected wild boar in the restricted area. In the surrounding regions, intensified and concerted hunting actions were carried out to reduce the susceptible wild boar population. Both hunting and search for carcasses was facilitated through the payment of incentives. However, in December 2017 the disease was confirmed in five dead wild boar south of the high risk area (WAHID 2017) leading to a controversial discussion about the most effective disease control also regarding the outbreak situation in Moldova (WAHID 2017).

*Transmission routes and risks*

Hosts of the disease are all members of the *Suidae* family including domestic pigs (*Sus scrofa domesticus*) and wild boar (*Sus scrofa scrofa*). African wild suids like bushpigs (*Potamochoerus larvatus*), red river hogs (*Potamochoerus porcus*), Giant Forest hogs (*Hylochoerus meinertzhangi)* and warthogs (*Phacochoerus africanus*) are susceptible as well, but show only mild or asymptomatic clinical courses (Thomson et al. 1980, Anderson et al. 1998, Costard et al. 2009, Jori et al. 2009). Warthogs (*Phacochoerus africanus*) are regarded as the original and most important vertebrate host in Africa and play a major epidemiological role in the sylvatic cycle together with soft ticks of the genus *Ornithodoros*, the only non-vertebrate host of the

disease (Burrage 2013). ASF can be transmitted via direct or indirect pig contact or the before mentioned tick-vector. This second transmission route can be neglected in Central and most parts of Eastern Europe due to the lack of soft ticks in the natural arthropod fauna (Pietschmann et al. 2016, European Centre for Disease Prevention and Control 2018). Epidemiological studies revealed human factors as the main risk for disease spread over larger distances (Costard et al. 2009). Transport of untreated pork meat and the inadequate disposal of virus positive material such as swill-feeding led to several new outbreaks particularly in backyard farms. The tenacity of the virus in raw and cooled pork products is very high (Mebus et al. 1993). Studies have shown, that the virus can be inactivated safely by temperatures over 70°C (Plowright et al. 1967), but is still infectious in 399 days old Parma ham. In this context, the role of wild boar carcasses succumbed to the disease has been discussed (Probst et al. 2017). Wild boar could get infected by contact to those infectious bodies and contribute to the disease spread.

## 2.4 Control strategies and diagnostics

*Legal framework of disease control*

The recent outbreak situation leaves Germany and the other neighbouring countries that are still disease-free but at high risk, severely alarmed. Several national strategies to prevent the introduction of the notifiable disease (TierGesG 2013) have been implemented: Since illegal import of contaminated pork products is under the main risk factors, the government is trying to sensitize the public to the issue with warning signs on traffic routes and airports (Bundesministerium für Ernährung und Landwirtschaft 2017). Furthermore, hunting on wild boar has been intensified to reduce the susceptible population. Since December 2017 cash rewards for hunted wild boar are paid e.g. in Mecklenburg-Western Pomerania, a Federal State with high wild boar density (Ministerium für Landwirtschaft und Umwelt Mecklenburg Vorpommern 2017). The EU regulates ASF control measures in Council Directive 2002/60/EC (RAT DER EUROPÄISCHEN UNION 2002) and Commission Decision 2003/422/EC (DIE KOMMISSION DER EUROPÄISCHEN GEMEINSCHAFTEN 2003). This legal framework is transposed in the German Schweinepest-Verordnung (Verordnung zum Schutz gegen die Schweinepest und die Afrikanische Schweinepest, (SchwPestV 1988)). The SchwPestV is currently under revision to accommodate necessary changes to the overall strategy. In order, to detect outbreaks of ASF as early as possible, passive surveillance is one of the main goals.

An introduction of the disease into naïve populations would lead to a high number of fallen wild boar. Therefore, hunters are told to report these carcasses to the local veterinary service and sample them for diagnostic workup. The same applies for hunted wild boar, showing the typical clinical or pathological signs (SchwPestMonV 2016). To facilitate reporting, an App for the use on smartphones has been established (Deutscher Jagdverband 2017). The optimal diagnostic sample is blood or organ material such as spleen or lung. If the carcass is already decomposed or skeletonized larger bones like the humerus or the femur are good alternatives (Deutscher Jagdverband und Friedrich-Loeffler-Institut 2017). However, these samples are difficult to take and in order to simplify the sampling process and to avoid contamination, swab sampling has been tested and showed good performance for pathogen and antibody detection. Without direct contact to the infectious material, hunters can soak the swab in body liquids or brush it on remaining organ tissues (Blome et al. 2014, Petrov et al. 2014, Carlson et al. 2017). If the applied tests reveal a positive result and the outbreak has been officially confirmed, control measures have to be implemented. In case of outbreaks in pig holdings the legal situation is quite clear stating culling of the infected herds and a total stand-still of the farms in the affected areas similar to other notifiable diseases. Treatment efforts of infected animals or vaccination are prohibited (SchwPestV 1988). Regarding the particular situation of ASF cases in wild boar, the legal specifications are less detailed and specified. Thus, the implementation of measures depends on the competent authorities. An expert group should be established including veterinarians, hunters, wildlife biologists and epidemiologists to assist the competent authorities. Local hunters are obliged to facilitate all means of disease control, e.g. carry out concerted hunting actions in certain areas while hunting could be banned in others, depending on the epidemiological situation. All feral pigs, shot or found dead in a certain area have to be inspected by an official veterinarian and tested for the disease. Furthermore, the measures to eradicate the disease should be checked for effectiveness by objective investigations (SchwPestV 1988, RAT DER EUROPÄISCHEN UNION 2002, DIE KOMMISSION DER EUROPÄISCHEN GEMEINSCHAFTEN 2003).

*Old and new diagnostic tools*

The national reference laboratory (NRL) for ASF provides several validated diagnostic tests in the Amtliche Methodensammlung (Friedrich-Loeffler-Institut 2016) according to EU Diagnostic Manual (Commission Decision 2003/422/EC). For genome detection, the *real-time* polymerase chain reaction (qPCR) according to King et al. (2003) is the routine test with slight

modifications. This assay is listed in the Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (World Organization for Animal Health (OIE) 2017). For confirmation of doubtful results, the protocol according Tignon et al. (2011) is routinely performed at the NRL. Since ASFV is the only swine related virus showing hemadsorption, the standard method for direct detection of replicating ASFV is the hemadsorption assay (Malmquist et al. 1960, Sierra et al. 1991). It is based on the culture of the virus on macrophage cultures obtained from anticoagulated blood, lung lavage or bone marrow. Homologue erythrocytes are added to the culture after 24 hours of incubation and if replicating virus is present, hemadsorption can be observed as rosette formation around infected macrophages (European Union Reference Laboratory for ASF 2013). The hemadsorption reaction is linked to two different ASFV proteins (encoded in ORF EP402R and EP153R) responsible for the adhesion and stabilization of the erythrocyte on the cell surface (Galindo et al. 2000). Most ASFV strains show this phenomenon. Exceptions are e.g. found in some serogroups within genotype I (Leitao et al. 2001, Boinas et al. 2004) For antigen detection of non-hemadsorbing ASFV strains, immunofluorescence staining of permanent cell or macrophage cultures is suitable. Immunofluorescence or –peroxidase staining can also be used on cryosections.

Due to the presence of natural attenuated strains, leading to seroconversion in the recovering swine, antibody detection can be as important as reliable pathogen detection. It also helps to estimate the possible time point of disease introduction. In Germany, two commercial enzyme linked immunosorbent assays (ELISA) are approved for routine diagnosis of ASFV-specific antibodies. The INGEZIM PPA COMPAC K3 (INGENASA, Spain) is a blocking ELISA detecting antibodies against the viral protein (VP) 72 (Pastor et al. 1990). The second commercial kit named ID Screen® ASF (ID Vet, France) is an indirect system detecting antibodies against the viral proteins P32, P62 and P72. Doubtful results have to be confirmed by the indirect immunoperoxidase test (IPT) or immunoblotting (Cubillos et al. 2013).

# 3 Objectives

***Simplified sampling for improved African swine fever surveillance in the field***

Since passive surveillance in wild boar is currently the most effective strategy for early detection of ASFV, simple sampling-tools are required to increase the compliance of hunters and foresters to take samples from fallen wild boar. Dry blood swabs have already shown to be a suitable approach in earlier publications so the main goal of the present study was to demonstrate the suitability of the swab sampling method for both routine ASFV genome and antibody detection under field conditions.

***Biological and genomic characterization of recent African swine fever virus field strains from Estonia***

ASF was re-introduced into the EU in 2014, affecting domestic pigs and wild boar in the Baltic States and Poland. As high virulence has been observed in Caucasian ASFV strains, it was anticipated that the virus would either spread rapidly or die out due to self-limitation. In fact, the disease became endemic, spreading slowly but constantly in the wild boar population. As virus attenuation is among the explanations for this pattern, selected field-isolates were biologically characterized. For this purpose, four animal trials were carried out, including different pig breeds. Since the survival rates and clinical courses were rather variable, representative samples from each trial were subjected to next-generation sequencing and whole-genomes were investigated to see if there is a genetic basis for this variance. Subsequently, screening for variant viruses was carried out with tailored PCR systems.

***Evaluation of the potential carrier status of African swine fever virus infected pigs***

ASF survivors were suggested to play a crucial role in disease maintenance as so-called “silent carriers” but long-term studies with a representatively high amount of animals were lacking. Therefore, this study targeted the assessment of a potential ASFV carrier state of 30 pigs in total which were allowed to recover from infection with ASFV “Netherlands’86” prior to contact exposure to six healthy sentinel pigs for more than two months. Mainly viremia, virus shedding and seroconversion were analyzed to evaluate the epidemiological role of recovered animals.

# 4 Results

The publications for this thesis 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 Simplifying sampling for African swine fever surveillance: Assessment of antibody and pathogen detection from blood swabs

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**Summary**

African swine fever (ASF) is a notifiable disease with serious socio-economic consequences that has been present in wild boar in the Baltic States and Poland since 2014. An introduction of ASF is usually accompanied by increased mortality, making fallen wild boar and hunted animals with signs of disease the main target for early warning and passive surveillance. It is difficult, however, to encourage hunters and foresters to report and take samples from these cases. A pragmatic and easy sampling approach with quick-drying swabs could facilitate this.

In this study, we further evaluated the use of dry-blood swabs for the detection of ASFV antibody and genome with samples from animal trials and diagnostic submissions (blood, bone, and organs) from Estonia. Compared to serum samples, dried blood swabs yielded 93.1% (95% confidence interval: [83.3, 98.1]) sensitivity and 100% [95.9, 100.0] specificity in a commercial ASFV antibody ELISA. Similarly, the swabs gave a sensitivity of 98.9% [93.4, 100.0] and a specificity of 98.1% [90.1, 100.0] for genome detection by a standard ASFV p72 qPCR when compared to EDTA blood. The same swabs were tested in a VP72-antibody lateral flow device, with a sensitivity of 94.7% [85.4, 98.9] and specificity of 96.1% [89.0, 99.2] compared to the serum ELISA. When GenoTube samples tested in ELISA and LFD were compared, the sensitivity was 96.3 % [87.3, 99.5] and the specificity was 93.8% [86.0, 97.9].

This study demonstrates reliable detection of ASFV antibody and genome from swabs. A field test of the swabs with decomposed wild boar carcasses in an endemic area in Estonia also gave promising results. Thus, this technique is a practical approach for surveillance of ASF in both free and endemic areas.

**Keywords**: African swine fever, early warning, passive surveillance, antibody detection, genome detection, diagnostics, forensic swab

## 1. Introduction

African swine fever (ASF) is an often-fatal hemorrhagic disease of domestic swine, feral swine, and wild boar (all *Sus scrofa*) caused by a double-stranded DNA virus, ASF virus (ASFV). African swine fever affects swine of all ages producing a wide range of clinical signs. Since 2007, ASF has spread to the Caucasus region, the Russian Federation, and neighboring Eastern European countries such as Estonia, Latvia, Lithuania, Poland, Ukraine, Moldova (Costard et al., 2009, Costard et al., 2015, European Food Safety et al., 2017), and the Czech Republic (OIE, 2017). In most regions, both domestic pigs and wild boar were affected and, especially in the Baltic States, long-term persistence of the disease in affected wild boar populations was observed (Olsevskis et al., 2016, Guinat et al., 2016). Contact to wild boar plays a key role in the introduction into the domestic pig population and subsequent spread .

The introduction of ASF to naïve wild boar populations is associated with high morbidity and mortality (Costard et al., 2013). Passive surveillance of wild boar and syndromic surveillance of pig mortality are considered the most effective strategies for a timely detection of disease introduction and the control of ASF spread (Guinat et al., 2017). Sampling of fallen wild boar to obtain blood or bone specimens is crucial for surveillance, but it requires great effort by hunters or other untrained personnel. Therefore, a simple sampling tool that is easy to handle and transport could facilitate the collection of samples by hunters, foresters and veterinary services.

Alternative methods for collecting field samples that are stable in warm climates have been described. Braae et al. (2013) investigated the use of FTA cards for blood collection and qPCR testing under field conditions in Tanzania. In later work by Randriamparany et al. (2016) and Michaud et al. (2007), ASFV diagnosis and characterization were successfully performed with dried blood on filter paper, for both experimental and field samples and over extended periods of time. Randriamparany et al. (2016) also demonstrated the suitability of dried blood for antibody detection. No problems with specificity were encountered.

Along the same lines as the FTA cards and filter papers, Petrov et al. (2014) showed that dry-blood swabs are an easy method to test carcasses for ASFV and CSFV genomes. The advantage is that the swab is already combined with a shipment-suitable receptacle, and no further equipment is needed. Swabs give the user a greater separation from the sample, allowing for quicker and easier handling and storage. Among the different materials and products tested, GenoTubes (Thermo Fisher Scientific) were the optimum in terms of ease of handling and

sample stability. The suitability of these swabs for ASFV antibody detection by ELISA was demonstrated in a proof-of-concept study (Blome et al., 2014).

Quick-drying swabs decrease the growth of opportunistic organisms and preserve sample DNA or RNA without refrigeration (Costa et al., 2014). In previous studies, sample material in GenoTubes remained usable for genome and antibody detection for more than a week (unpublished results show stability over several month), and gave good results with accredited qPCR and ELISA methods (Blome et al., 2014, Petrov et al., 2014).

The objectives of the present study were to demonstrate the suitability of the swab sampling method for routine ASFV genome and antibody detection. To this means, blood and other routine sample matrices were compared to swabs dipped into EDTA blood. In the majority of cases, paired samples of the same animal were tested to ensure comparability. In addition to the blood and serum that had been previously tested (Blome, 2014), organ and bone marrow samples were included in this comparative study to broaden the scope. Furthermore, the analyses were extended to a lateral flow device (LFD) for antibody detection, which could provide a useful and easy tool for point-of-care tests.

## 2. Materials and methods

### *2.1 Swine Studies and Sample Origin*

Paired EDTA and serum samples from experimentally infected animals at the Friedrich-Loeffler-Institut (FLI) were used for the study (see Table 1). The same EDTA blood sample was used for routine PCR testing and generation of dried-blood swabs. Animals used in this study were infected with either ASFV genotype I, II or IX, with some surviving long enough to mount an antibody response. We also included older multi-vaccinated sows for specificity assessment as they can often have false positive reactions. Swine used for these studies were kept in accordance with Directive 2010/63/EU. None of the animal experiments were specifically performed for this study. The animal experiments were approved by the competent authority (LALLF Rostock, Germany) under reference numbers 7221.3-1-059/16, 7221.3-1-021/15, and 7221.3-2-023/15. The swabs were directly dipped into vials of thawed whole blood or serum from animal trials that had been stored at -80°C. The swabs were then stored at room temperature to mimic the time in transit from the field to the laboratory: for 8 days prior to processing for DNA extraction, for 10 days prior to processing for ELISA, and for 2 months prior to testing with LFD. After storage, small pieces (2.5 mm in diameter for PCR, 5 mm in diameter

for ELISA) were excised from the dried-blood (or serum) swabs with sterile scissors and processed.

**Table 1:** Summary of samples used from the FLI.

| | | Sample type | | |
|---|---|---|---|---|
| **Virus** | **Animal** | **EDTA Blood** | **Serum** | **Total** |
| ASFV Estonia 2014 (genotype II) | Minipigs | 24 | 24 | 48 |
| ASFV Netherlands 1986 (genotype I) | domestic pigs | 78 | 78 | 156 |
| ASFV Estonia 2014 (genotype II) | domestic pigs | 10 | 10 | 20 |
| ASFV Kenya 05 (genotype IX) | domestic pigs | 3 | 3 | 6 |
| ASFV Estonia 2014 (genotype II) | wild boar | 19 | 19 | 38 |
| ASFV-naive | Sows | 0 | 8 | 8 |
| ASFV-naive | Piglets | 0 | 5 | 5 |
| **Total** | | 134 | 147 | **281** |

*2.2 Additional sampling in Estonia*

A short-term scientific mission was conducted at the Estonian Veterinary and Food Laboratory (VFL), the Estonian National Reference Laboratory for ASF and CSF, in collaboration with the Estonian University of Life Sciences. Forty-two swabs were dipped in various field samples from the VFL collection. These samples were collected from previously archived blood (29), bone marrow (5), and other organs (8) stored at -80°C. All samples were left under the biosafety cabinet until samples were completely thawed to obtain a swab sample. The swabs were then stored at room temperature for 72 hours prior to processing for routine diagnostics. All 42 samples were tested by PCR, while only 25 were tested by ELISA. The processing time differed from the studies conducted at the FLI due to the nature of the short-term scientific mission.

For a small pilot study during this scientific mission, two decomposed wild boar carcasses (Figure 1) in a forest near Vihula, Lääne-Viru County, Estonia (GPS Coordinates 59,5100153; 25,8536846), were sampled with GenoTube swabs. The time of death was not determined; however both carcasses were severely decomposed. For routine diagnostics, bone marrow was also collected (see Table 2).

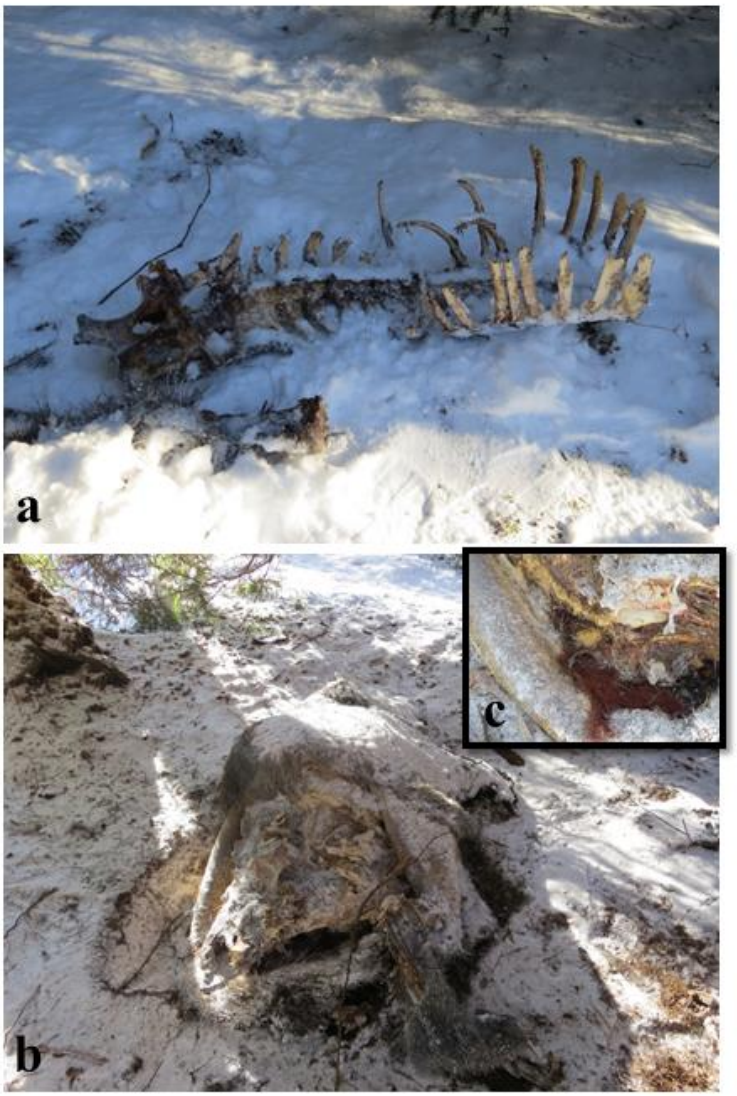


**Figure 1.** Wild boar carcass sampled in an Estonian forest. **(a)** The first carcass was severely decomposed. The remaining body cavity was swabbed, and bone marrow from the humerus was submitted to the lab for routine diagnostics. **(b)** In the second carcass, organs and tissues were still present, and **(c)** it was possible to soak the GenoTube in some blood-tinged liquid.

**Table 2:** Summary of samples tested at VFL

| Sample/Assay | EDTA Blood | Organ | Bone Marrow | Total |
|---|---|---|---|---|
| GenoTube Ingezim ELISA | 18 | 3 | 4 | 25 |
| GenoTube Tignon PCR | 29 | 5 | 8 | 42 |

*2.3 Pathogen detection*

For qPCR, viral nucleic acid was extracted using the Qiagen® MagAttract Virus Mini M48 kit (Qiagen, Hilden, Germany) and the KingFisher® extraction platform (Thermo Fisher Scientific, Vantaa, Finland). The nucleic acid extraction was performed with 75 µl of whole blood or a ~5-$mm^2$ piece of a GenoTube swab that had been dipped in whole blood (Figure 2) and later soaked and incubated in 200 µl of phosphate-buffered saline (PBS). At the FLI, GenoTube pieces were macerated with a metal bead in a 2 ml microcentrifuge tube with 200 µl PBS, mixing for 3 min at 30 Hz in a Qiagen TissueLyser II. Subsequently, qPCR was performed according to the King et al. protocol (King et al., 2003) and an EGFP internal control (Hoffmann et al., 2006), with results recorded as quantification cycle ($C_q$) values. At the VFL, a slightly modified protocol was used. GenoTube pieces were soaked in 200 µl PBS and the tubes were vortexed thoroughly. We used 140 µl of starting material for the automated extraction with a Qiagen QIAcube platform and QIAamp Viral RNA Mini Kit (Qiagen). Thereafter, qPCR was performed on all Estonian samples according to the protocol published by Tignon et al. (Tignon et al., 2011) using swine β-actin as an endogenous control.


**Figure 2.** GenoTube after dipping in EDTA-treated whole blood (a). Trimming the foam of the swab for testing in multiple assays (b). Pieces of foam cut for DNA extraction (5 $mm^2$) (c).

*2.4 ELISA antibody detection*

To detect antibodies against ASFV, the commercial ID Screen® African Swine Fever Indirect ELISA (IDvet, Grabels, France) which detects antibodies against the viral proteins p32, p62 and p72 was used at the FLI. To this end, GenoTube samples were incubated in 200 µl of the ELISA sample dilution buffer shaking at room temperature overnight in a 96 deep-well plate. The protocol was completed as described by the manufacturer for testing filter paper punches.

At the VFL in Estonia, a p72 antibody ELISA (Ingezim PPA Compac, INGENASA, Madrid, Spain) was performed according to the manufacturer's instructions with a slight modification using

a smaller buffer volume. The GenoTube was vortexed in individual tubes with 100 µl of the sample diluent-buffer from the ELISA kit.

*2.5 Lateral flow device for antibody detection*

Antibodies against p72 were detected using the commercial INGEZIM PPA CROM lateral flow device (INGENASA, Madrid, Spain). A 5-$mm^2$ GenoTube sample was cut from the same GenoTube used previously. Samples were then incubated in 200 µl ELISA dilution buffer for 20 minutes shaking at room temperature. Subsequently, 50 µl were pipetted on the LFD and incubated for one minute, then 3 drops of running buffer were added. Results were read within 10 minutes of incubation at room temperature.

*2.6 Calculations for linear regression*

For each comparison, a simple linear model (Montgomery et al., 2015) was fitted with R (https://www.r-project.org/). The calculations were completed using "lm" from package "stats" and "geom_smooth" from package "ggplot2".To test associations between results obtained with different assays, Pearson's product moment correlation coefficient (r) (Lewis, 2009) was calculated and evaluated against a t distribution with n−2 degrees of freedom.

*2.7 Estimation of sensitivity, specificity, and agreement between tests*

Sensitivity and specificity were calculated for the ELISA (values between 30-39 percent positive, PP) and LFD with results in the doubtful range counted as positive, since a doubtful result would alert a diagnostic lab to run further tests.

Sensitivity was calculated as True Positive / (True Positive + False Negative).

Specificity was calculated as True Negative / (True Negative + False Positive).

The confidence intervals were also calculated with R using the exact binomial test with the “binom” package also described by (Clopper and Pearson, 1934).

## 3. Results

*3.1 Pathogen detection*

At the FLI, results from the GenoTubes and the EDTA blood had minor qualitative differences (see a summary of qualitative and quantitative results in supplementary table 1). In qPCR, GenoTubes swabs had lower viral genome loads compared to the original EDTA blood

(difference in $C_q$ value up to 13), and the $r^2$ was 0.917 (Figure 3a). The qPCR sensitivity was 98.8% (one false negative result; [93.4, 100.0]), while the specificity was 98.1% (one false positive result from a true negative animal with a very high $C_q$ value, i.e. >40; [90.1, 100.0]). However, samples tested at VFL had a sensitivity of 85.7% [71.5, 99.6], an $r^2$ of 0.665, while $C_q$ values differed by up to 12 between the original sample and GenoTube (Figure 3b). Specificity was not determined since only positive samples were tested. A summary of these results is provided in supplementary table 2.

The two GenoTube swabs sampled from the almost completely decomposed and frozen carcasses (Figure 1) yielded positive results in the qPCR with $C_q$ values of 37.5 (carcass A) and 37.4 (carcass B) when swabbing bones moistened with water. In both cases the $C_q$ values of the corresponding bone marrow samples were 28.2 and 28.5, respectively.

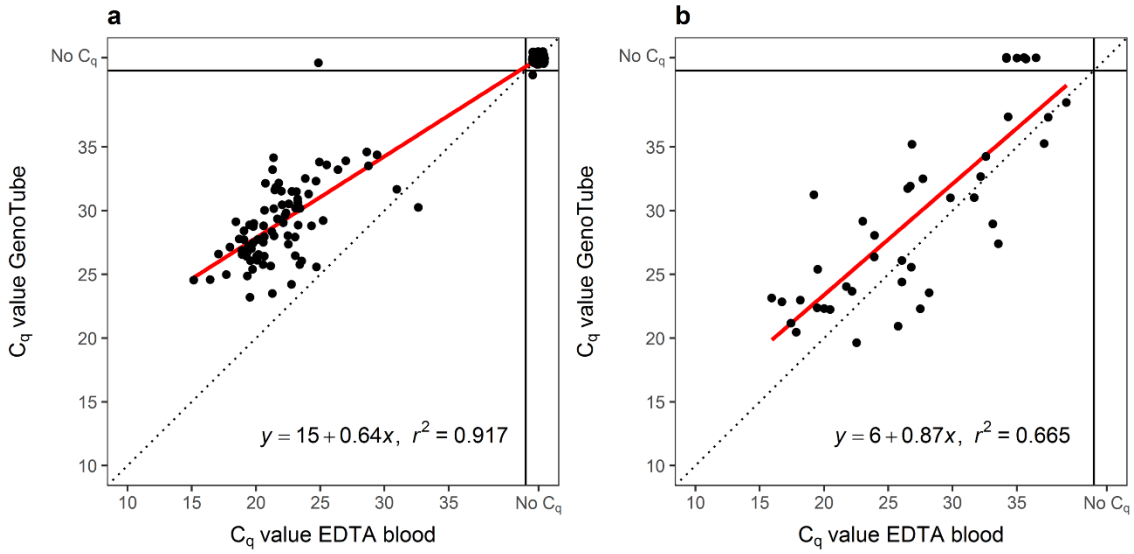


**Figure 3.** $C_q$ values for GenoTubes dipped in whole blood compared to the whole blood itself after automated nucleic acid extraction at the FLI (a) and at VFL (b). The coefficient of determination ($r^2$), the linear equation and the regression line are shown. Equality is marked with a black dotted line.

*3.2 Antibody detection*

In comparative studies with the IDvet antibody ELISA, serum and GenoTube swabs dipped in whole blood had similar results (see a summary of results in supplementary table 1). The PP values of positive samples tested at the FLI ranged from 41 to 120 for the GenoTube swab samples and from 43 to 121 for the serum samples (Figure 4). There was a tight correlation

($r^2$=0.969) between the serum sample and the blood-dipped GenoTube swab. Sensitivity was 93.1% with 100% specificity when comparing GenoTube to serum samples tested with the ELISA at the FLI (Table 3, top). In Estonia, the ELISA (Ingezim PPA Compac ELISA) results had a sensitivity of 83.3% and a specificity of 100% (Table 3, bottom, supplementary table S2).

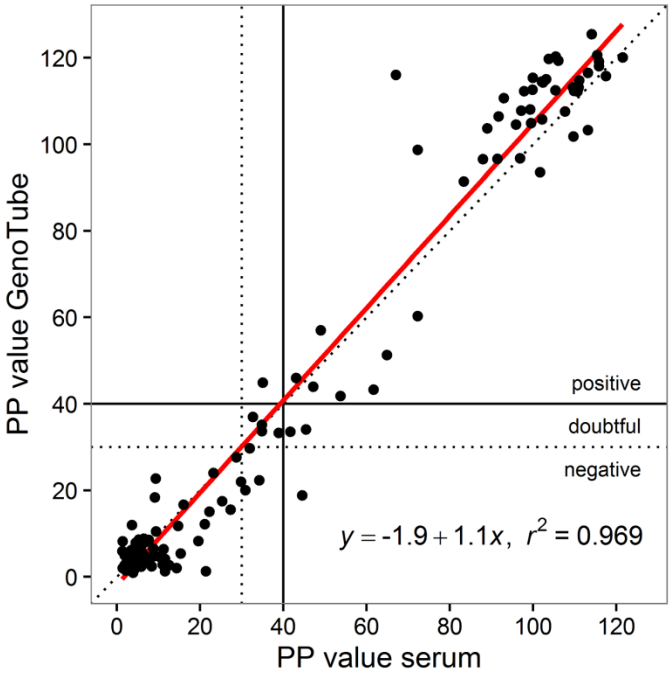


**Figure 4.** GenoTubes dipped in whole blood compared to the corresponding serum samples in the IDvet antibody ELISA. The coefficient of determination ($r^2$), the linear equation and the regression line (red) are shown. Equality is marked with a black dotted line. The black solid line represents the positive cut-off of the ELISA on the x and y axis. Above the dotted line on the x and y axis, results are considered doubtful, while below this line, results are considered negative.

**Table 3:** IDvet and Ingezim ELISA of serum and corresponding whole-blood GenoTube samples at FLI (top) and the VFL (bottom). Sensitivity and specificity were calculated with doubtful results (PP values ≥30) considered positive. Confidence intervals (95%) for the estimates are presented in square brackets.

| **FLI** | | **Serum IDvet ELISA** | | **Total** |
|---|---|---|---|---|
| | | Pos | Neg | |
| **Geno Tube** | Pos | 54 | 0 | 54 |
| | Neg | 4 | 89 | 93 |
| **Total** | | 58 | 89 | 147 |
| **Sensitivity** | | 93.1% [83.3, 98.1] | | |
| **Specificity** | | 100% [95.9 ,100.0] | | |

| **VFL** | | **Serum Ingezim ELISA** | | **Total** |
|---|---|---|---|---|
| | | Pos | Neg | |
| **Geno Tube** | Pos | 15 | 0 | 15 |
| | Neg | 3 | 7 | 10 |
| **Total** | | 18 | 7 | 25 |
| **Sensitivity** | | 83.3% [58.6, 96.4] | | |
| **Specificity** | | 100% [59.0, 100.0] | | |

*3.3 Antibody detection by lateral flow device*

A total of 134 whole blood-dipped GenoTube samples were tested with the Ingezim LFD and compared to parallel serum samples tested in the IDvet ELISA, resulting in 94.7% sensitivity and 96.1% specificity (See Table 4, top and Figure 5). When we compared the GenoTubes samples tested by ELISA or LFD, 96.2% sensitivity and 93.8% specificity were found (See Table 4, bottom). Three GenoTube samples which were negative by LFD were positive by the serum ELISA. Two of these samples were false negative by the GenoTube LFD compared to the ELISA performed with the GenoTube samples.

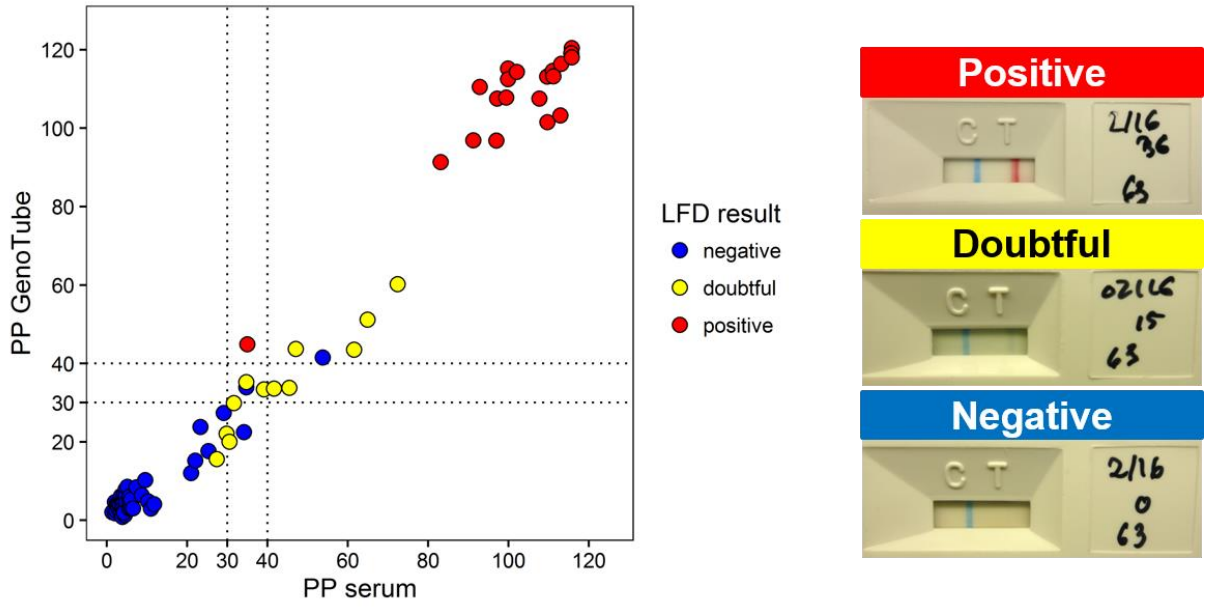


**Figure 5**. Antibody ELISA and lateral flow assay. Percent positive (PP) values from the ELISA are shown for the whole-blood GenoTube samples and the corresponding serum sample. Red dots represent samples that were positive in the LFD, yellows were doubtful and blue dots were negative. See examples of LFDs on right.

**Table 4.** Results for GenoTube samples tested with a lateral flow device compared to IDvet ELISA results for the same GenoTube sample (top) and the corresponding serum (bottom). Sensitivity and specificity were calculated with doubtful results considered positive. Confidence intervals (95%) for the estimates are presented in square brackets.

| | | **GenoTube IDvet ELISA** | | **Total** |
|---|---|---|---|---|
| | | Pos | Neg | |
| **GenoTube LFD** | Pos | 52 | 5 | 57 |
| | Neg | 2 | 75 | 77 |
| **Total** | | 54 | 80 | 134 |
| **Sensitivity** | | 96.3% [87.3, 99.5] | | |
| **Specificity** | | 93.8% [86.0, 97.9] | | |

| | | **Serum IDvet ELISA** | | **Total** |
|---|---|---|---|---|
| | | Pos | Neg | |
| **GenoTube LFD** | Pos | 54 | 3 | 57 |
| | Neg | 3 | 74 | 77 |
| **Total** | | 57 | 77 | 134 |
| **Sensitivity** | | 94.7% [85.4, 98.9] | | |
| **Specificity** | | 96.1% [89.0, 99.2] | | |

## 4. Discussion

Currently only a few reports describe the use of GenoTubes for infectious disease detection (Blome et al., 2014, Sattler et al., 2015, Petrov et al., 2014, Barros et al., 1986). Apart from African and classical swine fever, these reports have demonstrated the value of GenoTubes for oral sampling of swine for porcine reproductive and respiratory syndrome virus and the detection of *Brachyspira hampsonii* in feces (Costa et al., 2014). However, oral and fecal swabs may be less suitable for ASF testing as these matrices contain much lower genome loads (Blome et al., 2013). Whole blood or tissues are preferable because they yield much higher genome loads. Moreover, the Commission Implementing Decision (2014/709/EU, 2015) requires the collection of blood samples of all hunted wild boar in endemic areas. In Germany, which currently remains disease-free but is at risk, sampling of fallen wild boar and roadkill is

mandatory for early disease detection (Regulation on swine fever monitoring, SchwPestMonV, 2016). In order to achieve this goal, we must rely on the cooperation of hunters or foresters to collect tissue, bones or blood in tubes. Since this is inconvenient, a swab could increase their compliance because it is easy to handle and transport. The use of GenoTubes or quick-drying swab minimizes the risk of carryover between carcasses because the length of the swab allows sampling while limiting direct contact of the hunters with bodily fluids, thereby increasing sterility and overall sample quality. At a price of €2 per swab, they provide excellent preservation and safe transport. The GenoTube swabs are made of foam that can be conveniently cut into multiple pieces to run multiple assays from one swab. Since cutting methods are already applied to organ samples tested in the laboratory, this could easily be adapted to swabs. It is important to note that swabs can be directly dipped in AVL or ELISA buffer without cutting to run one assay, if cutting is inconvenient (see Petrov et al., 2014). FTA cards have also shown promising results (Uttenthal et al., 2013, Braae et al., 2015, Michaud et al., 2007, Randriamparany et al., 2016), however, a trained person must handle the samples as it is difficult to avoid contamination during close contact with bloody carcasses and additional transport devices are needed. In studies by (Petrov et al., 2014), FTA cards gave similar qualitative results when compared to the GenoTube swab.

Our studies have demonstrated that qualitative results obtained with GenoTube material were consistent with the original sample used in routine diagnostics. This work demonstrated a tight correlation ($r^2$=0.917) of $C_q$ values from the GenoTube samples and paired EDTA blood (sensitivity 98.8% and specificity 98.1%). One false positive result had a very low genome load and was considered doubtful. One false negative qPCR result, attributed to poor handling, was repeated twice more from two separate cuts of the GenoTube, resulting in $C_q$ values of 28.5 each time. Although initially negative in GenoTube qPCR, separate ELISA and LFD GenoTube pieces were positive for antibodies. Since positive antibody results would require further tests, routine diagnostics would not have missed the infected animal from the sample taken with GenoTubes. The GenoTube swab samples when compared to the IDvet serum ELISA had a tight correlation of $r^2$=0.969. On several occasions, $C_q$ values were higher on GenoTube samples which is probably due to a dilution effect of the swab in PBS. The six false negative results from Estonian GenoTubes can be explained by poor sample quality and the low initial genome load ($C_q$ value > 34) of the original samples, but differences in extraction could also be partly responsible. From these samples, one out of six would have been identified as

infected by the detection of antibodies (sensitivity was 83.3% and specificity was 100% with the Ingezim PPA Compac ELISA).

The dilution of the GenoTube samples in buffer had little effect on the analysis of antibodies with the ELISA as the PP values between paired samples were tightly correlated. The LFD GenoTube compared to the GenoTube ELISA resulted in only two false negatives. The false negative results are likely due to a decreased sensitivity of the LFD device compared to the ELISA. It is also possible that a smaller swab piece was used, and possibly less sample was on that swab piece used for the LFD. In addition, samples of poor quality (hemolysed or clotted blood) submitted to the VFL showed reliable results in LFD. Studies by (Gallardo et al., 2009) also showed that samples stored at 37°C for one month had little effect on the qualitative ELISA result. While (Petrov et al., 2014) demonstrated that samples stored at 37°C for 7 days gave the same qualitative results. This demonstrates the utility and robustness of this sampling strategy combined with a point-of-care test. Even when the LFD qualitative results were compared to the serum ELISA, only 3 samples were false negative.

It is important to mention that each reference lab predominantly uses one of the two accredited qPCRs (King et al., 2003, Tignon et al., 2011) and one of the different ELISAs commercially available (IDScreen ASFV Indirect ELISA by IDvet and Ingezim PPA Compac by Ingenesa). Using multiple assays with same dry swab demonstrates the versatility of these samples and the ease of adapting them in different reference laboratories (see comparison of different PCRs in supplementary table 3).

Since lab samples are hardly comparable to undefined body liquids obtained from decomposed carcasses, the study was extended to the field to test the applicability of GenoTubes. From decomposed carcasses (skeletons), the only available sample is often bone. Their sampling and processing is cumbersome especially since decomposition occurs rapidly in summer. Comparing qPCR from bone marrow to GenoTube samples obtained by swabbing the rotten bodies had higher $C_q$ values, but remained detectable in our qPCR assay. This preliminary data underlines the versatility of the GenoTube.

Under this premise, it is conceivable to expand the use of these swabs while culling swine during an outbreak or sampling hunted wild boar. A sufficient number of samples must be taken during the culling while considering the same risks and obstacles as previously mentioned (2002/60/EG, Article 5, 2002). In outbreaks involving large farms, $CO_2$ is often the preferred culling method (Estonian-Swine-Fever-Code, 2017), leaving masses of unopened

carcasses. The GenoTubes could facilitate this process by swabbing the eye's medial canthus or other source of peripheral blood.

In summary, of the 178 samples from diverse sources and varying quality, only five cases (2.8 %) would not have been identified as infected using the GenoTubes. Fast-drying swabs are therefore an excellent alternative for ASF detection. They have several advantages from easy handling to long-term storage and the ability to cut and use one swab for multiple diagnostic tests. Another key feature of this swab is the diversity of samples it may be used with, including organs and bone marrow from fallen wild boar. In conclusion, these swabs are a practical, inexpensive, and straightforward approach for passive surveillance of ASFV in the deceased wild boar.

**Acknowledgments**

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**Declaration of Conflicting Interests**

None to declare.

## Supplementary Information

**Supplementary table 1:** Overview on samples tested at the FLI

| | | Antibody detection ELISA (PP value) | | | | | Pathogen detection qPCR ($c_q$ value) | |
|---|---|---|---|---|---|---|---|---|
| **inoculum** | **days post inoculation, pig ID** | **serum** | | **genotube** | | **LFD** | **blood** | **genotube** |
| ASFV Estonia 2014 (genotype II) | 0dpi , # 61 | 5 | negative | 4 | negative | negative | no cq | no cq |
| | 0dpi , # 62 | 3 | negative | 5 | negative | negative | no cq | no cq |
| | 0dpi , # 63 | 3 | negative | 12 | negative | negative | no cq | no cq |
| | 0dpi , # 65 | 2 | negative | 2 | negative | negative | no cq | no cq |
| | 0dpi , # 66 | 2 | negative | 3 | negative | negative | no cq | no cq |
| | 0dpi , # 67 | 3 | negative | 2 | negative | negative | no cq | no cq |
| | 0dpi , # 68 | 3 | negative | 2 | negative | negative | no cq | no cq |
| | 0dpi , # 69 | 3 | negative | 2 | negative | negative | no cq | no cq |
| | 0dpi , # 70 | 4 | negative | 2 | negative | negative | no cq | no cq |
| | 0dpi , # 71 | 8 | negative | 4 | negative | negative | no cq | no cq |
| | 0dpi , # 72 | 3 | negative | 2 | negative | negative | no cq | no cq |
| | 15dpi , # 61 | 9 | negative | 18 | negative | negative | 27 | 34 |
| | 15dpi , # 63 | 20 | negative | 8 | negative | negative | 17 | 26 |
| | 15dpi , # 67 | 4 | negative | 6 | negative | negative | 19 | 28 |
| | 15dpi , # 68 | 89 | positive | 104 | positive | positive | 18 | 27 |
| | 36dpi , # 61 | 105 | positive | 120 | positive | doubtful | 28 | 35 |
| | 36dpi , # 62 | 121 | positive | 120 | positive | positive | 23 | 31 |
| | 36dpi , # 63 | 117 | positive | 116 | positive | positive | 21 | 34 |
| | 36dpi , # 64 | 111 | positive | 112 | positive | positive | 25 | 34 |
| | 36dpi , # 65 | 110 | positive | 112 | positive | positive | 23 | 30 |
| | 36dpi , # 66 | 103 | positive | 115 | positive | positive | 24 | 31 |
| | 36dpi , # 68 | 104 | positive | 120 | positive | positive | 21 | 33 |
| | 36dpi , # 70 | 105 | positive | 113 | positive | positive | 21 | 32 |
| | 36dpi , # 71 | 67 | positive | 116 | positive | positive | 21 | 30 |
| ASFV Netherlands 1986 (genotype I) domestic pigs | 0dpi , # 63 | 2 | negative | 2 | negative | negative | no cq | no cq |
| | 0dpi , # 64 | 6 | negative | 5 | negative | negative | no cq | no cq |
| | 0dpi , # 65 | 2 | negative | 5 | negative | negative | no cq | no cq |
| | 0dpi , # 66 | 5 | negative | 9 | negative | negative | no cq | no cq |
| | 0dpi , # 67 | 3 | negative | 4 | negative | negative | no cq | no cq |
| | 0dpi , # 68 | 3 | negative | 4 | negative | negative | no cq | no cq |
| | 0dpi , # 69 | 4 | negative | 1 | negative | negative | no cq | no cq |
| | 0dpi , # 70 | 6 | negative | 5 | negative | negative | no cq | no cq |
| | 0dpi , # 71 | 3 | negative | 2 | negative | negative | no cq | no cq |
| | 0dpi , # 73 | 5 | negative | 8 | negative | negative | no cq | no cq |
| | 0dpi , # 74 | 8 | negative | 8 | negative | negative | no cq | no cq |
| | 0dpi , # 75 | 4 | negative | 4 | negative | negative | no cq | no cq |
| | 0dpi , # 76 | 4 | negative | 7 | negative | negative | no cq | ≥40 |
| | 0dpi , # 77 | 3 | negative | 3 | negative | negative | no cq | no cq |
| | 0dpi , # 78 | 5 | negative | 5 | negative | negative | no cq | no cq |
| | 0dpi , # 79 | 6 | negative | 3 | negative | negative | no cq | no cq |
| | 0dpi , # 80 | 10 | negative | 5 | negative | negative | no cq | no cq |
| | 0dpi , # 81 | 4 | negative | 4 | negative | negative | no cq | no cq |
| | 0dpi , # 82 | 12 | negative | 4 | negative | negative | no cq | no cq |
| | 0dpi , # 83 | 5 | negative | 6 | negative | negative | no cq | no cq |
| | 0dpi , # 84 | 3 | negative | 4 | negative | negative | no cq | no cq |
| | 0dpi , # 85 | 3 | negative | 4 | negative | negative | no cq | no cq |
| | 0dpi , # 86 | 4 | negative | 3 | negative | negative | no cq | no cq |
| | 0dpi , # 87 | 9 | negative | 10 | negative | negative | no cq | no cq |
| | 0dpi , # 88 | 5 | negative | 6 | negative | negative | no cq | no cq |
| | 0dpi , # 89 | 5 | negative | 3 | negative | negative | no cq | no cq |
| | 0dpi , # 90 | 3 | negative | 6 | negative | negative | no cq | no cq |
| | 0dpi , # 91 | 9 | negative | 7 | negative | negative | no cq | no cq |
| | 0dpi , # 92 | 4 | negative | 2 | negative | negative | no cq | no cq |
| | 0dpi , # 93 | 11 | negative | 3 | negative | negative | no cq | no cq |
| | 10dpi , # 64 | 35 | doubtful | 34 | doubtful | negative | 19 | 26 |
| | 10dpi , # 65 | 23 | negative | 24 | negative | negative | 21 | 28 |
| | 10dpi , # 66 | 54 | positive | 42 | positive | negative | 19 | 26 |
| | 10dpi , # 67 | 27 | negative | 16 | negative | doubtful | 20 | 26 |
| | 10dpi , # 68 | 4 | negative | 2 | negative | negative | 25 | 29 |
| | 10dpi , # 69 | 2 | negative | 2 | negative | negative | no cq | no cq |
| | 10dpi , # 70 | 35 | doubtful | 35 | doubtful | doubtful | 21 | 23 |
| | 10dpi , # 71 | 1 | negative | 2 | negative | negative | no cq | no cq |
| | 10dpi , # 73 | 32 | doubtful | 30 | negative | doubtful | 19 | 25 |
| | 10dpi , # 75 | 46 | positive | 34 | doubtful | doubtful | 18 | 25 |
| | 10dpi , # 76 | 34 | doubtful | 22 | negative | negative | 19 | 27 |

| | | Antibody detection ELISA (PP value) | | | | | Pathogen detection qPCR ($c_q$ value) | |
|---|---|---|---|---|---|---|---|---|
| **inoculum** | **days post inoculation, pig ID** | **serum** | | **genotube** | | **LFD** | **blood** | **genotube** |
| | 10dpi , # 77 | 72 | positive | 60 | positive | doubtful | 20 | 28 |
| | 10dpi , # 78 | 4 | negative | 1 | negative | negative | 23 | 25 |
| | 10dpi , # 79 | 47 | positive | 44 | positive | doubtful | 19 | 26 |
| | 10dpi , # 80 | 22 | negative | 15 | negative | negative | 19 | 29 |
| | 10dpi , # 81 | 31 | doubtful | 20 | negative | doubtful | 20 | 27 |
| | 10dpi , # 82 | 30 | negative | 22 | negative | doubtful | 19 | 27 |
| | 10dpi , # 83 | 25 | negative | 17 | negative | negative | 19 | 28 |
| | 10dpi , # 84 | 4 | negative | 5 | negative | negative | 19 | 24 |
| | 10dpi , # 85 | 42 | positive | 34 | doubtful | doubtful | 20 | 28 |
| | 10dpi , # 86 | 21 | negative | 12 | negative | negative | 20 | 26 |
| | 10dpi , # 87 | 62 | positive | 43 | positive | doubtful | 20 | 27 |
| | 10dpi , # 88 | 6 | negative | 3 | negative | negative | no cq | no cq |
| | 10dpi , # 89 | 4 | negative | 3 | negative | negative | 20 | 27 |
| | 10dpi , # 90 | 65 | positive | 51 | positive | doubtful | 21 | 26 |
| | 10dpi , # 91 | 29 | negative | 28 | negative | negative | 20 | 27 |
| | 10dpi , # 93 | 39 | doubtful | 33 | doubtful | doubtful | 19 | 27 |
| | 29dpi , # 63 | 116 | positive | 121 | positive | positive | 31 | 32 |
| | 29dpi , # 64 | 99 | positive | 108 | positive | positive | 22 | 28 |
| | 29dpi , # 66 | 110 | positive | 102 | positive | positive | 24 | 25 |
| | 29dpi , # 67 | 97 | positive | 97 | positive | positive | 23 | 29 |
| | 29dpi , # 68 | 102 | positive | 94 | positive | doubtful | 23 | 27 |
| | 29dpi , # 69 | 83 | positive | 91 | positive | positive | 23 | 28 |
| | 29dpi , # 73 | 116 | positive | 118 | positive | positive | 22 | 28 |
| | 29dpi , # 74 | 116 | positive | 119 | positive | positive | 25 | 29 |
| | 29dpi , # 77 | 113 | positive | 116 | positive | positive | 23 | 26 |
| | 29dpi , # 78 | 109 | positive | 113 | positive | positive | 29 | 33 |
| | 29dpi , # 80 | 91 | positive | 97 | positive | positive | 23 | 30 |
| | 29dpi , # 82 | 113 | positive | 103 | positive | positive | 24 | 29 |
| | 29dpi , # 84 | 100 | positive | 113 | positive | positive | 23 | 30 |
| | 29dpi , # 85 | 108 | positive | 108 | positive | positive | 29 | 34 |
| | 29dpi , # 86 | 111 | positive | 115 | positive | positive | 33 | 30 |
| | 29dpi , # 87 | 100 | positive | 115 | positive | positive | 20 | 26 |
| | 29dpi , # 88 | 35 | doubtful | 45 | positive | positive | 23 | 28 |
| | 29dpi , # 90 | 111 | positive | 113 | positive | positive | 22 | 29 |
| | 29dpi , # 91 | 97 | positive | 108 | positive | positive | 22 | 31 |
| | 29dpi , # 92 | 93 | positive | 111 | positive | positive | 22 | 30 |
| | 29dpi , # 93 | 102 | positive | 115 | positive | positive | 23 | 31 |
| ASFV Estonia 2014 (genotype II) domestic pigs | 14 dpi , # 60 | 72 | positive | 99 | positive | positive | 23 | 27 |
| | 21 dpi , # 100 | 96 | positive | 104 | positive | positive | 23 | 32 |
| | 21dpi, # 60 | no serum available | | | | | 24 | 32 |
| | 21 dpi , # 97 | 92 | positive | 107 | positive | positive | 21 | 29 |
| | 21 dpi , # 98 | 88 | positive | 97 | positive | positive | 20 | 28 |
| | 21 dpi , # 99 | 106 | positive | 119 | positive | positive | 23 | 32 |
| | 28 dpi , # 100 | 100 | positive | 105 | positive | positive | 26 | 33 |
| | 28 dpi , # 60 | 98 | positive | 112 | positive | positive | 25 | 32 |
| | 28 dpi , # 97 | 102 | positive | 114 | positive | positive | 25 | 34 |
| | 28 dpi , # 98 | 102 | positive | 106 | positive | positive | 23 | 30 |
| | 28 dpi, # 99 | 114 | positive | 125 | positive | positive | 25 | [illegible] |
| ASFV Kenya 05 (genotype IX) domestic pigs | 10 dpi , # 10 | 5 | negative | 5 | negative | negative | 16 | 24 |
| | 10 dpi , # 8 | 3 | negative | 5 | negative | negative | 19 | 27 |
| | 11 dpi , # 6 | 4 | negative | 4 | negative | negative | 15 | 25 |
| ASFV Estonia 2014 (genotype II) wild boar | 0dpi , # 11 | 12 | negative | 1 | negative | negative | no cq | no cq |
| | 0dpi , # 12 | 12 | negative | 3 | negative | negative | no cq | no cq |
| | 0dpi , # 14 | 14 | negative | 2 | negative | negative | no cq | no cq |
| | 0dpi , # 15 | 21 | negative | 1 | negative | negative | no cq | no cq |
| | 0dpi , # 16 | 6 | negative | 9 | negative | negative | no cq | no cq |
| | 0dpi , # 17 | 9 | negative | 2 | negative | negative | no cq | no cq |
| | 0dpi , # 18 | 2 | negative | 3 | negative | negative | no cq | no cq |
| | 0dpi , # 19 | 6 | negative | 2 | negative | negative | no cq | no cq |
| | 0dpi , # 20 | 2 | negative | 3 | negative | negative | no cq | no cq |
| | 10dpi , # 11 | 16 | negative | 17 | negative | doubtful | 21 | 32 |
| | 10dpi , # 14 | 33 | doubtful | 37 | doubtful | doubtful | 20 | 29 |
| | 10dpi , # 15 | 9 | negative | 23 | negative | negative | 22 | 32 |
| | 10dpi , # 18 | 7 | negative | 8 | negative | negative | 20 | 26 |
| | endday , # 12 | 6 | negative | 8 | negative | negative | 20 | 29 |
| | endday , # 13 | 15 | negative | 12 | negative | negative | 20 | 29 |
| | endday , # 14 | 49 | positive | 57 | positive | positive | 21 | 32 |
| | endday , # 16 | 2 | negative | 6 | negative | negative | 22 | 28 |
| | endday , # 17 | 43 | positive | 46 | positive | positive | 22 | 30 |
| | endday , # 20 | 4 | negative | 3 | negative | negative | 19 | 29 |

| | | Antibody detection ELISA (PP value) | | | | | Pathogen detection qPCR ($c_q$ value) | |
|---|---|---|---|---|---|---|---|---|
| **inoculum** | **days post inoculation, pig ID** | **serum** | | **genotube** | | **LFD** | **blood** | **genotube** |
| ASFV naïve piglets* | endday, # 143 | 2 | negative | 2 | negative | | no cq | no cq |
| | endday, # 26 | 2 | negative | 2 | negative | | no cq | no cq |
| | endday, # 30 | 2 | negative | 2 | negative | | no cq | no cq |
| | endday, # 83 | 1 | negative | 6 | negative | | no cq | no cq |
| | endday, # 84 | 1 | negative | 8 | negative | | no cq | no cq |
| ASFV naïve sows* | intra partum, # 4423 | 15 | negative | 5 | negative | | no cq | no cq |
| | endday, # 1871 | 9 | negative | 5 | negative | | no cq | no cq |
| | intra partum, # 1871 | 2 | negative | 5 | negative | | no cq | no cq |
| | intra partum, # 4249 | 4 | negative | 3 | negative | | no cq | no cq |
| | endday, # 4423 | 11 | negative | 6 | negative | | no cq | no cq |
| | endday, # 4454 | 6 | negative | 5 | negative | | no cq | no cq |
| | endday, # 6150 | 44 | positive | 19 | negative | | no cq | no cq |
| | intra partum, # 5150 | 7 | negative | 4 | negative | | no cq | no cq |

*negative samples, mainly used to look for false positive with antibody test, retested, negative, retested new cut piece, result was 28.5

**Supplementary table 2:** Overview on samples tested at the FLI

| | | | | pathogen detection (qPCR) routine diagnostic | | pathogen detection (qPCR) genotube | | | | antibody detection ELISA (% inhibition) | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | **County** | **Species** | **Sample type** | **automated extraction** | | **manual extraction** | | **automated extraction** | | **serum** | **genotube** |
| 1 | Tartu | wild boar | organsuspension | positive | 37 | positive | 31 | positive | 37 | x | x |
| 2 | Põlva | wild boar | organs | positive | 28 | positive | 19 | positive | 22 | x | x |
| 3 | Põlva | wild boar | organs | positive | 26 | positive | 18 | positive | 26 | x | x |
| 4 | Saaremaa | wild boar | organs | positive | 26 | positive | 19 | positive | 21 | x | x |
| 5 | Saaremaa | wild boar | organs | positive | 33 | positive | 25 | positive | 27 | x | x |
| 6 | Jõgeva | wild boar | organsuspension | positive | 16 | positive | 18 | positive | 23 | x | x |
| 7 | Lääne | wild boar | organs | positive | 23 | positive | 19 | positive | 20 | x | x |
| 8 | Saaremaa | wild boar | bone marrow | positive | 24 | positive | 22 | positive | 28 | x | x |
| 9 | Harju | wild boar | blood | positive | 19 | positive | 23 | positive | 25 | negative | x |
| 10 | Saaremaa | wild boar | blood | positive | 22 | positive | 21 | positive | 24 | positive | negative |
| 11 | Saaremaa | wild boar | blood | positive | 27 | positive | 24 | positive | 26 | positive | negative |
| 12 | Saaremaa | wild boar | blood | positive | 27 | positive | 31 | positive | 32 | positive | negative |
| 13 | Saaremaa | wild boar | blood | positive | 27 | positive | 35 | positive | 35 | positive | positive |
| 14 | Saaremaa | wild boar | blood | positive | 22 | positive | 25 | positive | 24 | positive | positive |
| 15 | Saaremaa | wild boar | blood | positive | 23 | positive | 23 | positive | 29 | positive | positive |
| 16 | Rapla | wild boar | blood | positive | 26 | positive | 23 | positive | 24 | positive | positive |
| 17 | Saaremaa | wild boar | organs | positive | 18 | positive | 17 | positive | 21 | x | x |
| 18 | Saaremaa | wild boar | blood | positive | 32 | positive | 29 | positive | 31 | positive | positive |
| 19 | Tartu | wild boar | blood | positive | 37 | positive | 34 | positive | 35 | positive | positive |
| 20 | Pärnu | wild boar | blood | positive | 39 | positive | 34 | positive | 39 | negative | x |
| 21 | Saaremaa | wild boar | bone marrow | positive | 28 | positive | 28 | positive | 32 | x | x |
| 22 | Saaremaa | wild boar | bone marrow | positive | 35 | positive | 30 | negative | no cq | x | x |
| 23 | Saaremaa | wild boar | blood | positive | 18 | positive | 21 | positive | 23 | negative | x |
| 24 | Lääne-Viru | wild boar | bone marrow | positive | 19 | positive | 28 | positive | 31 | x | x |
| 25 | Rapla | wild boar | blood | positive | 36 | positive | 36 | negative | no cq | positive | positive |
| 26 | Rapla | wild boar | blood | positive | 24 | positive | 24 | positive | 26 | positive | doubtful. |
| 27 | Pärnu | wild boar | blood | positive | 20 | positive | 19 | positive | 22 | negative | negative |
| 28 | Pärnu | wild boar | blood | positive | 20 | positive | 19 | positive | 22 | negative | negative |
| 29 | Lääne | wild boar | blood | positive | 34 | positive | 34 | positive | 37 | positive | positive |
| 30 | Pärnu | wild boar | blood | positive | 36 | negative | no cq | negative | no cq | negative | x |
| 31 | Saaremaa | wild boar | blood | positive | 33 | positive | 34 | positive | 34 | positive | positive |
| 32 | Pärnu | wild boar | blood | positive | 36 | negative | no cq | negative | no cq | negative | negative |
| 33 | Saaremaa | wild boar | bone marrow | positive | 30 | positive | 26 | positive | 31 | x | x |
| 34 | Lääne | wild boar | blood | positive | 27 | positive | 27 | positive | 32 | positive | doubtful |
| 35 | Lääne | wild boar | blood | positive | 34 | positive | 39 | negative | no cq | negative | negative |
| 36 | Lääne | wild boar | blood | positive | 34 | positive | 37 | negative | no cq | negative | negative |
| 37 | Lääne | wild boar | blood | positive | 17 | positive | 17 | positive | 21 | negative | negative |
| 38 | Lääne | wild boar | blood | positive | 17 | positive | 19 | positive | 23 | negative | negative |
| 39 | Rapla | wild boar | blood | positive | 32 | positive | 29 | positive | 33 | positive | positive |
| 40 | Saaremaa | wild boar | blood | positive | 28 | positive | 18 | positive | 24 | positive | doubtful |
| 41 | Saaremaa | wild boar | blood | positive | 33 | positive | 26 | positive | 29 | positive | positive |
| 42 | Lääne-Viru | wild boar | blood | positive | 19 | positive | 21 | positive | 22 | positive | positive |

**Supplementary table 3:** Overview on samples tested at the FLI

| inoculum | days post inoculation, pig ID | ASF System 1 (King et al. 2005) | ASF System 2 (Tignon et al. 2011) | Qiagen Virotype | Ingenasa UPL* | 1st Run ASF System 1 (King et al. 2005) | 1st Run ASF System 1 (King et al. 2005) |
|---|---|---|---|---|---|---|---|
| | | GenoTube | GenoTube | GenoTube | GenoTube | GenoTube | original blood |
| ASFV Netherlands 1986 (genotype I) domestic pigs | 29dpi , # 78 | 33 | 32 | 29 | 40 | 33 | 29 |
| | 29dpi , # 80 | 30 | 29 | 27 | 35 | 30 | 23 |
| | 29dpi , # 82 | 29 | 29 | 26 | 33 | 29 | 24 |
| | 29dpi , # 84 | 29 | 30 | 27 | 34 | 30 | 23 |
| | 29dpi , # 85 | 36 | 35 | 35 | 44 | 34 | 29 |
| ASFV Kenya 05 (genotype IX) domestic pigs | 10 dpi , # 8 | 27 | 26 | 24 | 30 | 27 | 19 |
| | 11 dpi , # 6 | 25 | 25 | 22 | 28 | 25 | 15 |
| | 10 dpi , # 10 | 24 | 24 | 21 | 26 | 24 | 16 |
| ASFV naïve sows | naive sow | no cq | no cq | no cq | no cq | no cq | no cq |
| | naive sow | no cq | no cq | no cq | no cq | no cq | no cq |

* run with 2 µl of DNA template as prescribed by manufacturer

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## 4.2 Biological characterization of African swine fever virus genotype II strains from north-eastern Estonia in European wild boar

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**Summary**

Due to its impact on animal health and pig industry, African swine fever (ASF) is regarded as one of the most important viral diseases of pigs. Following the ongoing epidemic in the Trans-Caucasian countries and the Russian Federation, African swine fever virus was introduced into the Estonian wild boar population in 2014. Epidemiological investigations suggested two different introductions into the southern and the north-eastern part of Estonia. Interestingly, outbreak characteristics varied considerably between the affected regions. While high mortality and mainly virus positive animals were observed in the southern region, mortality was low in the north-eastern area. In the latter, clinically healthy, antibody positive animals were found in the hunting bag and detection of virus was rare. Two hypotheses could explain the different behavior in the north-east: (i) the frequency of antibody detections combined with the low mortality is the tail of an older, so far undetected epidemic wave coming from the east or (ii) the virus in this region is attenuated and leads to a less severe clinical outcome. To explore the possibility of virus attenuation, a re-isolated ASFV strain from the north-eastern Ida-Viru region was biologically characterized in European wild boar.

Oronasal inoculation led to an acute and severe disease course in all animals with typical pathomorphological lesions. However, one animal recovered completely and was subsequently commingled with three sentinels of the same age class to assess disease transmission. By the end of the trial at 96 days post initial inoculation, all animals were completely healthy and neither virus nor viral genomes were detected in the sentinels or the survivor. The survivor however showed high antibody levels.

Concluding, the ASFV strain from north-eastern Estonia was still highly virulent but nevertheless, one animal recovered completely. Under the experimental conditions, no transmission occurred from the survivor to susceptible sentinel pigs.

**Keywords**: African swine fever virus, Estonia, wild boar, infection experiments, virulence

## 1. Introduction

African swine fever (ASF) is one of the most important and complex notifiable disease of domestic and wild pigs. It is caused by the eponymous virus which belongs to the genus *Asfivirus* within the *Asfarviridae* family (Takamatsu, 2011). Depending on host and virus factors, the disease can run acute, sub-acute and chronic courses. The former is especially linked to highly virulent virus strains and is characterized by severe clinical signs including high fever, general depression, anorexia, gastrointestinal signs, neurological disorders, and hemorrhagic lesions in the final stage of the disease (EFSA Scientific Report, 2009). In general, the disease course does not differ when comparing European wild boar and domestic pigs (Blome et al., 2013, Gabriel et al., 2011)

In 2007, a highly virulent genotype II ASF virus (ASFV) was introduced into Georgia and subsequently into several Trans-Caucasian countries, the Russian Federation, and in 2014, into the European Union (OIE WAHID, visited September 18$^{th}$ 2016). Among the currently affected countries is Estonia. Estonian authorities reported the first outbreaks in wild boar in September 2014, and in this year, a total of 41 ASF cases in wild boar were found in four different counties out of fifteen. In the first four months of 2015, 52 new wild boar cases were reported from four previously infected counties in the southern (three affected counties) and north-eastern part (Ida-Viru county) of the country (see figure 1). By December 2015, the number of ASF cases in wild boar had risen to 723, and 11 counties were affected almost all over the territory of Estonia. Apart from the wild boar population, 18 ASF outbreaks were reported from the domestic pig sector in 2015. Interestingly, outbreak characteristics varied considerably between the southern introduction and the north-eastern introduction. While high mortality (up to 16 dead animals found in one place) and mainly virus positive animals were observed in the southern affected region, mortality was low in the north-eastern outbreak area. In the latter, clinically healthy, antibody positive animals were found in the hunting bag and detection of virus or viral genome was rare. In order to explain the different behavior of the virus in the north-east, two hypotheses were phrased: (i) the frequency of antibody detections combined with the low mortality is the manifestation of an older, so far undetected epidemic wave coming from the east, i.e. we see its tail represented by surviving animals or (ii) the virus in this region is attenuated and leads to less severe courses. An attenuated virus could significantly complicate disease detection and may facilitate long-term endemicity.

To test hypothesis (i), we made an attempt to re-isolate the virus from PCR-positive organ samples from the Ida-Viru region. While isolation in macrophage cultures failed, the virus could be re-isolated by animal passage. Subsequently, the resulting virus was biologically characterized in terms of disease course, virology and serology in ten young wild boar at the Friedrich-Loeffler-Institut (FLI), Isle of Riems, Germany.

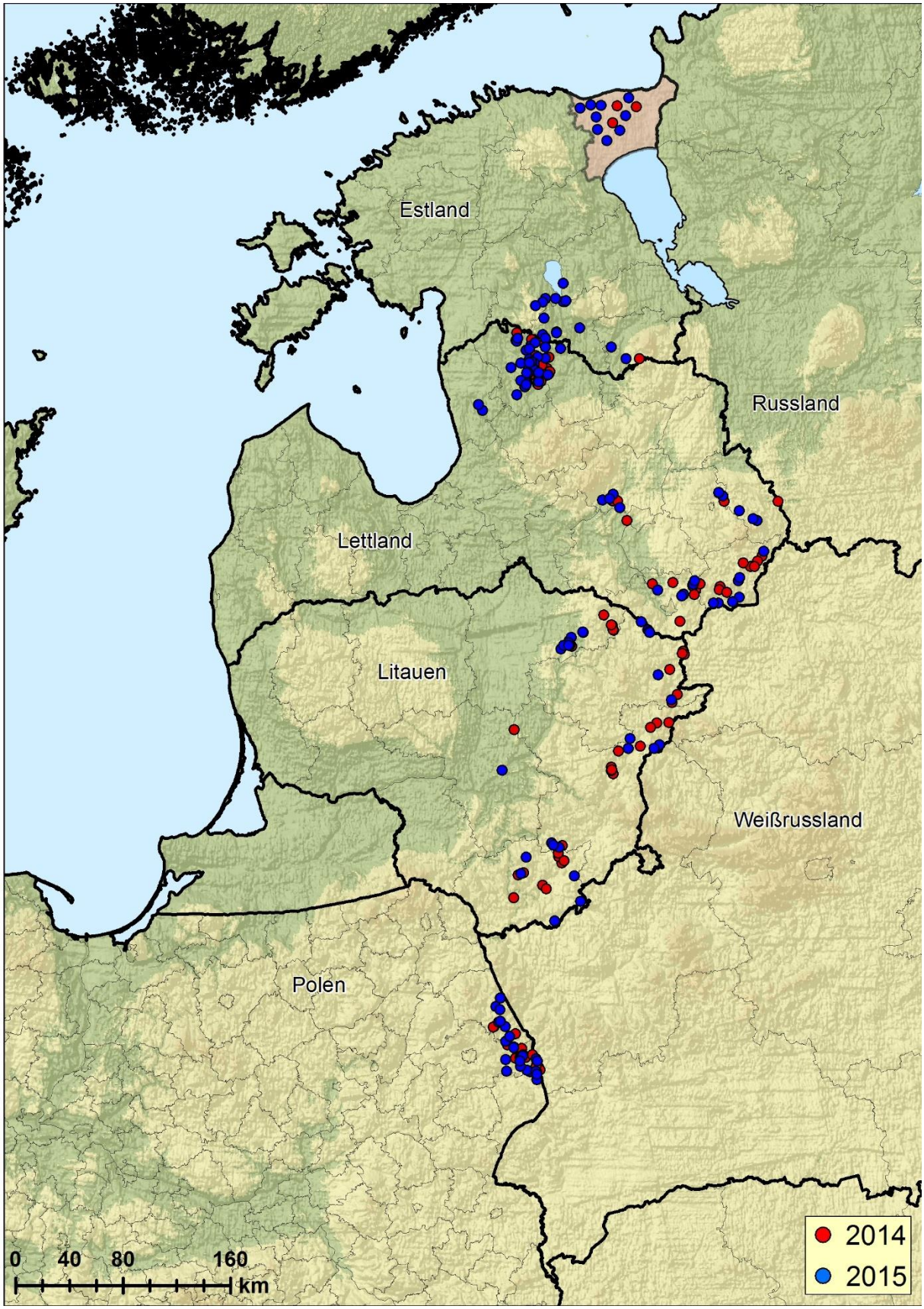


**Figure 1:** ASF cases in Estonia from September 2014 to end of April 2015.

## 2. Materials and Methods

### *2.1 Experimental design*

To re-isolate the causative ASFV strain from weak PCR-positive organ samples from Ida-Viru, three young wild boar were intramuscularly inoculated with an organ homogenate in standard cell culture medium (no viral growth in macrophage cultures). Upon onset of clinical signs and confirmation of infection by real-time PCR (qPCR), the animals were euthanized and standardized blood and organ samples were collected during necropsy. A pooled spleen suspension with a titer of $10^{4.5}$ hemadsorbing units (HAU) per ml was subsequently used for the trial detailed below.

The main study included a total of ten European wild boar from the breeding unit at the FLI aged approximately four month at the start of the trial. The animals were moved from the FLI quarantine stables into the high containment facilities (L3+) where they were kept in one pig pen. All animals were individually ear-tagged with numbers #11 to #20. Over the course of the trial, the animals were fed a commercial pig food with corn and hay-cob supplement and had access to water *ad libitum.* After an acclimatization phase, the wild boar were inoculated oronasally with 2 ml of the above mentioned spleen suspension. Clinical parameters of all animals were assessed daily based on a harmonized scoring system as previously described (Pietschmann et al., 2015). In brief, parameters anorexia, recumbency, joint lesions, breathing, ocular discharge, digestive findings, and neurological disorders were assigned points according to the severity of findings. The sum of the points was recorded as the clinical score (CS) that was also used to define humane endpoints. Over the course of the trial, levels of viremia, virus distribution, virus shedding, and antibody responses were assessed. For this purpose, blood samples were collected along with oropharyngeal and fecal swabs at days 0, 4, 7, and 10 post inoculation (dpi), and at the day of necropsy. Animals reaching the humane endpoint or that were suffering unacceptably without reaching the endpoint were euthanized through intracardial injection of embutramide (T61, Merck) after deep anesthesia with tiletamine/zolazepam (Zoletil®, Virbac). Necropsy was performed on all animals, and at the same time, tissue samples (lymph nodes, spleen, tonsil, salivary gland, lung, and liver), blood (EDTA, serum) and swab samples were collected for reference purposes.

At the end of the initial trial, one wild boar (#19) had recovered completely. To assess virus transmission to susceptible animals, the survivor was commingled with three sentinel wild boar (#1, #2, #3) from day 50 post initial inoculation. The sentinels were roughly the same age

and were purchased from a game park in Mecklenburg Western-Pomerania. The trial ended at 96 dpi. At this day, the remaining animals were euthanized and subjected to necropsy as described above.

In all trial parts, 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 authority under reference number 7221.3-2-023/15.

*2.2 Cells*

Blood for the preparation of Peripheral Blood Mononuclear Cells (PBMC)-derived macrophages was collected from healthy domestic donor pigs. In brief, PBMCs were obtained from EDTA anticoagulated blood using Pancoll Animal density gradient medium (PAN Biotech, Aidenbach, Germany). PBMCs were grown in RPMI-1640 cell culture medium with 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and 10 % fetal calf serum (FCS) at 37 °C in a humidified atmosphere containing 5 % $CO_2$. The medium was supplied with amphotericin B, streptomycin and penicillin to avoid bacterial and fungal growth. To facilitate maturation of macrophages, GM-CSF (granulocyte macrophage colony-stimulating factor, Biomol, Hamburg, Germany) was added to the cell culture medium at 2 ng/ml.

*2.3 Laboratory investigations*

*2.3.1 Processing of samples*

Oropharyngeal swabs were soaked in 1 ml of medium (EMEM without addition of FCS), vortexed for approximately 15 seconds, incubated for one hour at room temperature, and decanted in microcentrifuge tubes. Serum samples, which were obtained from native blood by centrifugation at 2500 x g for 20 minutes at 20°C, were aliquoted and stored at -80 °C until further use. Tissue samples of tonsil, spleen, salivary gland, liver, lung, and lymph nodes were collected at necropsy and stored at -80 °C. For qPCR and virus isolation (hemadsorption tests), tissue samples were homogenized in 1 ml phosphate-buffered saline (PBS) using a TissueLyser II (QIAGEN® GmbH).

*2.3.2 Virus detection*

For qPCR, viral nucleic acid was extracted, using the QIAamp® RNA Viral Mini Kit (Qiagen) or the NucleoMagVet-Kit (MACHEREY-NAGEL) and the KingFisher® extraction platform (Thermo

Scientific). Both extraction methods were slightly modified through the addition of an internal control DNA. The nucleic acid extraction was performed with 75 µl of whole blood and 150 µl of organ homogenate and swab material. Subsequently, qPCR was performed according to the protocol published by King et al. (2003) with slight modifications. For confirmatory reason, the virotype ASFV PCR Kit (Qiagen) was employed according to the manufacturer's instructions. Results of both qPCRs were recorded as quantification cycle (cq) values.

To detect ASFV in serum and tissue samples, a hemadsorption test (HAT) was carried out using PBMC-derived macrophages according to slightly modified standard procedures (Carrascosa et al., 2011). In brief, isolated PBMCs were seeded into a 96 well microplate with a density of app. 1.9 x $10^6$ cells/ml. After 16-24 hours, non-adherent cells were removed and cell culture medium containing GM-CSF was replenished. The culture was then incubated for 24 to 48 hours to allow initial maturation of macrophages. Subsequently, 20 µl of serum samples and 30 µl of organ homogenate were added to each well. Tests were performed in duplicates. When using organ homogenates, cells were washed after 2 hours adsorption time using luke-warm PBS, whereas serum was left on the cells until the evaluation of the test. After 24 hours of incubation 20 µl of homologues 1 % erythrocyte suspension was added to each well. For readout, cultures were analyzed for hemadsorption phenomena over a period of two days. Virus back titration was performed by endpoint titration of the diluted spleen suspensions. In this case, the PBMC preparation was seeded into 96-well microplates, the test volume was 100 µl per dilution step and 20 µl of a 1 % homologous erythrocyte suspension was added. These samples were tested in quadruplicate.

*2.3.3 Antibody detection*

For the detection of antibodies against African swine fever virus, two commercial enzyme-linked immunosorbent assays (ELISA) were carried out following the manufacturer's instructions (Ingezim PPA COMPAC, Ingenasa; ID SCREEN African swine fever virus INDIRECT, IDvet). The Ingezim PPA ELISA detects antibodies directed against p72 in a competitive format. The ID SCREEN is an indirect ELISA using antigens p32, p62 and p72. All serum samples were tested in duplicate.

All data were recorded and evaluated using Microsoft Excel 2010 (Microsoft Deutschland GmbH) and SigmaPlot for Windows version 11.0 (Systat Software, Inc.).

## 3. Results

### *3.1 Clinical course and pathomorphological findings*

Following oronasal inoculation, all animals developed severe, unspecific clinical signs starting from 4 to 6 dpi including general depression, lack of appetite, huddling and respiratory distress. Three animals reacted with some delay, namely animals #17, #18, and #19. These animals were still very active and interested in food at day 4, and showed only mild signs on day 7. Between days 7 and 13, all but one animal (#19) showed worsening clinical signs with dyspnea and ataxia, and were euthanized in a moribund state or died over night spontaneously (#16). Wild boar #19 showed decreasing severity of clinical signs starting app. 14 dpi and completely recovered over the following week.

During necropsy, typical ASF lesions of varying severity were observed in all animals that succumbed to infection (for exemplary findings see figure 2). Lesions ranged from slight lung edema and ebony colored gastro-hepatic lymph nodes to multiple hemorrhages in several organs, hemorrhagic and edematous lymph nodes in all parts of the body, and severe lung edema. Sporadic findings included gall bladder edema, renal infarction, gastritis and arthritis. Severity of lesions increased with time in the experiment.

After commingling of the survivor with three sentinels, no clinical signs were observed and all animals stayed in good health until the end of the trial at day 96. No ASF related lesions were observed during necropsy.

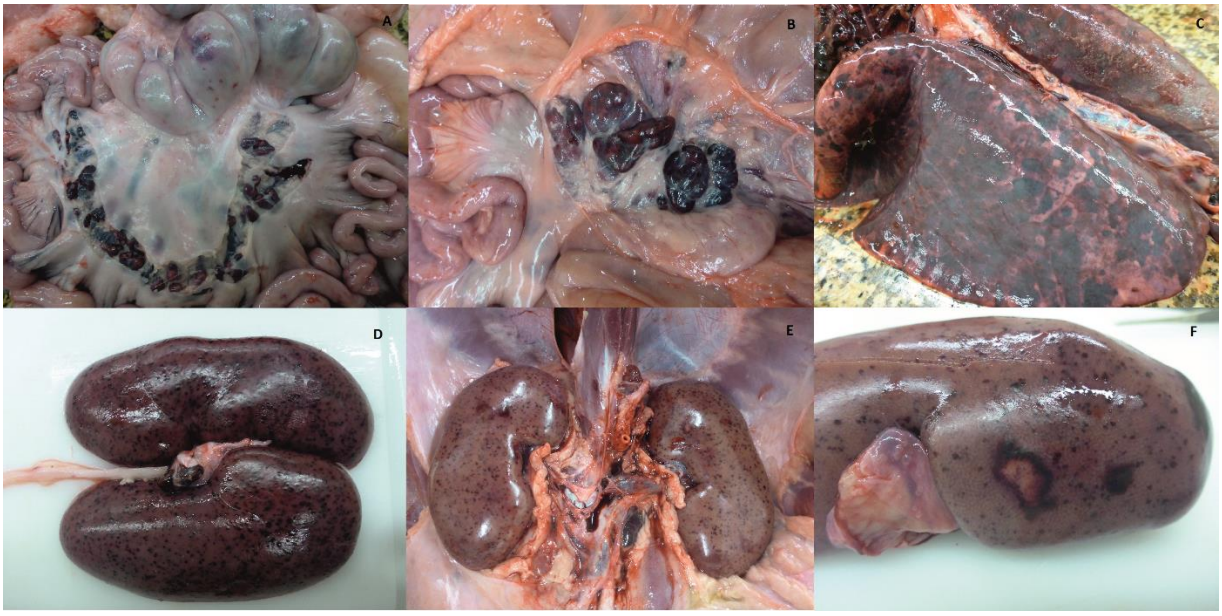


**Figure 2:** Examples of gross pathological findings during necropsy of acute-lethally infected wild boar upon infection with the ASFV strain from north-eastern Estonia. A) hemorrhagic intestinal lymph nodes and striate bleedings in the gut. B) ebony colored, hemorrhagic lymph nodes in the gastro-hepatic area. C) lung edema, fibrinous pleuritis and hemorrhages, D) and E) petechiae in the kidney, F) kidney petechiae and infarction

*3.2 Detection of virus and viral genome*

At 4 dpi, seven out of ten animals were positive in qPCR from EDTA blood with cq-values below 30 (see figure 3A), and two additional animals were weak positive (cq 34 and 41). Animal #17 was still negative at this time. In oropharyngeal swabs, five animals were found positive by qPCR with moderate to low viral loads (cq 28 to 38, see figure 3B). Here, animal #17 was among the weak positives (cq 37), but the two other animals with a low genome load in the blood and with almost no clinical signs were negative (see figure 3B). The qPCR from fecal swabs also yielded five but not completely congruent positive results (see figure 3C). Again, viral loads were low (cq values ranging from 31 to 45). Hemadsorption tests from serum were positive for all but animals #17 and #18. At 7 dpi, all available blood and swab samples were positive in qPCR with moderate to high genome loads in blood (cq 25-29, see figure 3A), and moderate to low genome loads in swabs (cq 30 to 37, see figures 3B and 3C). Here, only five hemadsorption tests were clearly positive, but the positive results included samples from animals #17 and #18. The remaining animals were all strong positive in qPCR from blood at 10 dpi (see figure 3A), but only one oropharyngeal swab (#19) was very weak positive (cq 41, see

figure 3B). Hemadsorption tests from sera were positive for all animals. Spleen, tonsil, lung, salivary gland, and lymph node samples taken during necropsy of animals that succumbed to infection were all positive by qPCR (see table 1), and all spleen samples reacted positive in hemadsorption tests.

Samples taken from the survivor and the sentinels during necropsy at 96 dpi were all negative for ASF virus and viral genome in two independent qPCR systems (see table 1). Among the samples were nine lymph nodes from all over the body (mandibular, parotideal, lung-associated, renal, gastro-hepatic, intestinal from the large and small intestines, inguinal, popliteal).

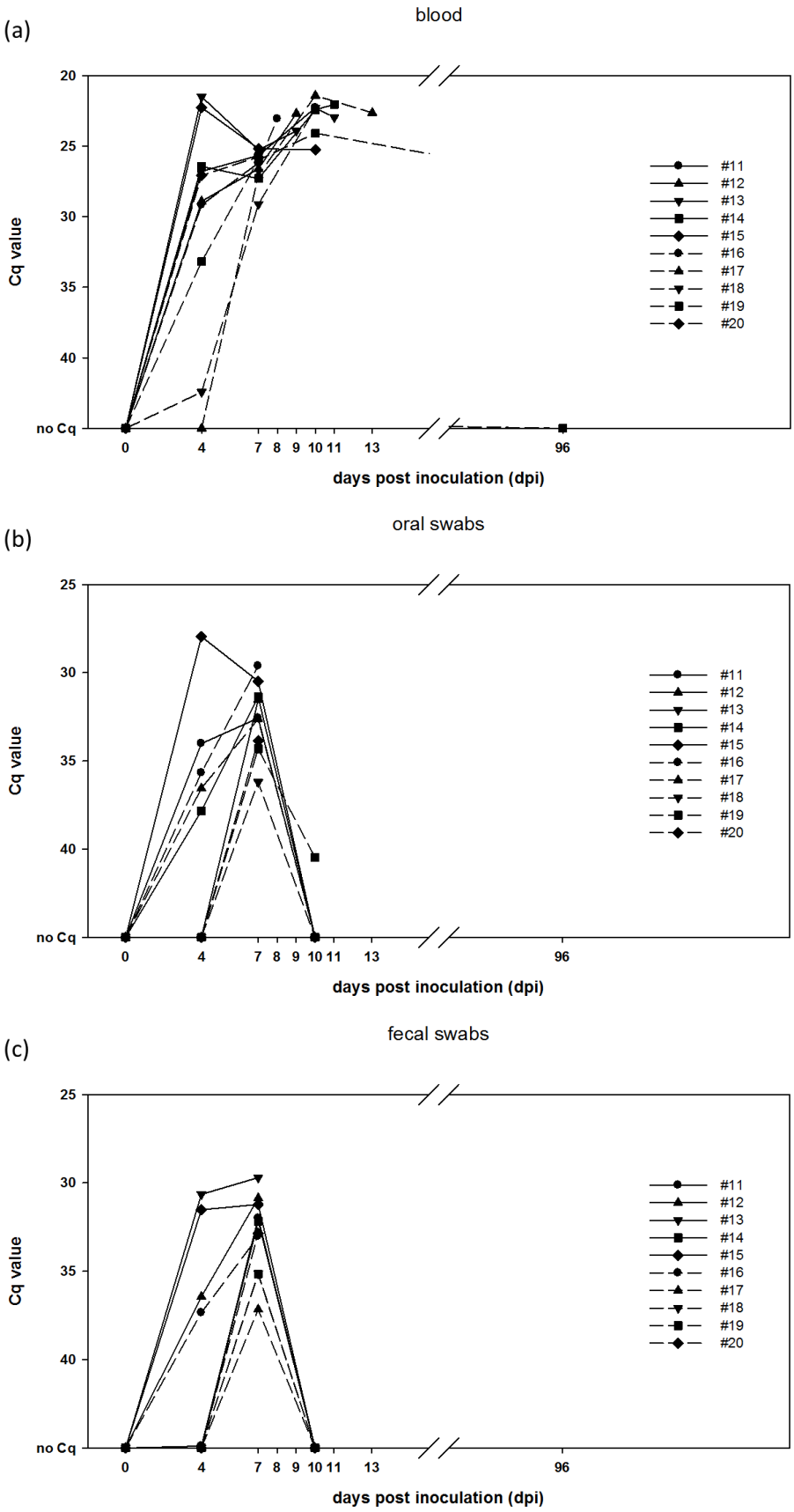


**Figure 3:** Genome detection by qPCR in blood (A), oropharyngeal (B) and fecal swabs (C). Results are depicted as cycle quantification (cq) values.

*3.3 Detection of antibodies against ASFV*

First positive reactions were seen in both ELISA systems between days 9 and 13 post inoculation (see figure 4 A and B). At 10 dpi, #19 was found positive in both test systems, #14 showed doubtful reactions in the Ingezim PPA and positive reactions in the ID SCREEN African swine fever virus. An additional doubtful result for the serum of animal #11 was found in the Ingezim PPA (see figure 4B). At the respective end day, only animal #19 (96 dpi) showed high antibody levels in the Ingezim PPA ELISA (see figure 4B). However, several animals were close to the cut-off (see figure 4B). In contrast, three animals were found positive (#14, 11 dpi; #17, 13 dpi; #19, 96 dpi) and one doubtful (#13, 9 dpi) in the ID SCREEN African swine fever virus (see figure 4A).

## 4. Discussion

African swine fever is no longer an exotic disease in several eastern European countries. Since the introduction into the EU in 2014, ASF has spread continuously despite enormous efforts towards controlling the disease. The causative virus strains are of genotype II and showed high virulence for both domestic pigs and European wild boar under experimental conditions (Blome et al., 2012, Gabriel et al., 2011, Pietschmann et al., 2015, Gallardo et al., 2015, Guinat et al., 2014). This would mean that introduction into a free area would be expected to lead to obvious clinical signs and mortality.

While mortality and virus positive animals were observed in Southern Estonia, this outbreak behavior was missing in the north-eastern outbreak area. One explanation could be local virus attenuation.

In an attempt to understand the different outbreak characteristics and to investigate the virulence of the local viral variants, an animal trial was conducted with a re-isolated ASFV strain from Ida-Viru.

In a nutshell, the ASFV strain from north-eastern Estonia was still highly virulent for young wild boar, but nevertheless, one animal recovered completely. In direct comparison with previous studies (Pietschmann et al., 2015, Tauscher et al., 2015, Blome et al., 2012, Gabriel et al., 2011), genome loads seemed to be slightly lower and detectable antibody responses were observed more often. However, as only cq values but not exact genome copy numbers could be compared, it cannot be ruled out that the differences were only due to variability of PCR

machines and extraction methods. The course of infection, and the pathomorphological signs did not differ for the animal that succumbed to infection. The virological data suggest that at least one animal (#17) got infected later. This confirms that oral infection is error prone and needs a quite high dose. It was reported previously that for oral infection, virus titers $>10^4$ HAU are usually necessary and that the ratio of viral titers needed for infection of a susceptible animal via the intramuscular/intravenous inoculation versus the oral/nasal route is 1 : 140.000 with less than 1 HAU for the parenteral route (McVicar, 1984). The high dose needed for oral infection, and the moderate contagiosity of ASF without blood contact could be part of the explanation why the epidemic in eastern Europe spreads rather slowly.

The survival of one animal gave us the opportunity to study the long-term fate of recovered animals and their potential of transmitting the virus on a limited scale. So far, solid data are missing regarding this issue and are needed to estimate the long-term effects of ASF in the wild boar population. It was suggested that survivors will become virus carriers (Sanchez-Vizcaino et al., 2012), and thus contribute to the long-term persistence of ASF in a region. At least under our experimental conditions, the single survivor was able to eliminate the virus, and it did not transmit to sentinels, even under conditions with slight hierarchical fights upon introduction of the new animals. Consequently, a carrier state is not inescapably for all surviving animals.

Hence, we did not find a clear explanation for the different disease dynamics in north-eastern Estonia. Additional data on viral sequences, viral behavior upon animal passaging and epidemiological drivers are needed.

**Table 1:** Disease course, viral genome and antibody detection upon oronasal (o.n.) inoculation of ten wild boar with an ASFV strain from north-eastern Estonia (ASFV EE). The sentinel animals were commingled with the surviving animal #19 from 50 to 96 days post inoculation (dpi). Genome detection in organs is presented as cycle quantification value (cq).

| Animal | Inoculation | course | end day | viraemia (qPCR) | Antibody detection | virus detection in organs | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Tonsil | SalGland | MandLn | Lung | Spleen | LnPar | LnLu | LnGaHep | LnSInt | LnLInt | LnKd | LnIng | LnPopl |
| **11** | ASFV EE o.n. | AL | 10 | 4 - 10 dpi (ED) | doubtful at 10 dpi | 31 | 28 | 27 | 26 | 30 | | | | | | | | |
| **12** | ASFV EE o.n. | AL | 9 | 4 - 9 dpi (ED) | no antibodies | 35 | 33 | 28 | 30 | 29 | | | | | | | | |
| **13** | ASFV EE o.n. | AL | 9 | 4 - 9 dpi (ED) | doubtful at 9 dpi | 26 | 24 | 29 | 20 | 27 | | | | | | | | |
| **14** | ASFV EE o.n. | AL | 11 | 4 - 11 dpi (ED) | from 10 dpi | 35 | 36 | 36 | 29 | 31 | | | | | | | | |
| **15** | ASFV EE o.n. | AL | 10 | 4 - 10 dpi (ED) | no antibodies | 29 | 30 | 25 | 31 | 28 | | | | | | | | |
| **16** | ASFV EE o.n. | AL | 8 | 4 - 8 dpi (ED) | no antibodies | 29 | 33 | 28 | 28 | 27 | | | | | | | | |
| **17** | ASFV EE o.n. | AL | 13 | 7 - 13 dpi (ED) | at 13 dpi | 27 | 33 | 30 | 24 | 30 | | | | | | | | |
| **18** | ASFV EE o.n. | AL | 10 | 4 - 10 dpi (ED) | no antibodies | 31 | 32 | 29 | 30 | 28 | | | | | | | | |
| **19** | ASFV EE o.n. | AT | 96 | transient* | from 10 dpi | no cq | no cq | no cq | no cq | no cq | no cq | no cq | no cq | no cq | no cq | no cq | no cq | no cq |
| **20** | ASFV EE o.n. | AL | 7 | 4 - 7 dpi (ED) | no antibodies | 25 | 29 | 23 | 24 | 21 | | | | | | | | |
| **1** | Sentinel | na | 96 | negative | no antibodies | no cq | no cq | no cq | no cq | no cq | no cq | no cq | no cq | no cq | no cq | no cq | no cq | no cq |
| **2** | Sentinel | na | 96 | negative | no antibodies | no cq | no cq | no cq | no cq | no cq | no cq | no cq | no cq | no cq | no cq | no cq | no cq | no cq |
| **3** | Sentinel | na | 96 | negative | no antibodies | no cq | no cq | no cq | no cq | no cq | no cq | no cq | no cq | no cq | no cq | no cq | no cq | no cq |

*** detected positive at 4, 7 and 10 dpi; negative 96 dpi (no other samples); end day = ED; acute lethal = AL; acute transient = AT; not applicable = n.a.; antibody = Ab; salivary gland = SalGland; mandibular lymphnodes = MandLn; parotideal lymphnodes = LnPar; lung lymphnodes = LnLu; gastro-hepatic lymphnodes = LnGaHep; lymphnodes from the small intestine area = LnSInt; lymphnodes from the large intestine area = LnLInt; renal lymphnodes = LnKd; inguinal lymphnodes (LnIng); popliteal lymphnode = LnPopl**

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## 4.3 Deletion at the 5'-end of Estonian ASFV strains associated with an attenuated phenotype

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**Abstract**

African swine fever (ASF) was introduced into the Eastern European Union in 2014 and led to considerable mortality among wild boar. In contrast, unexpected high antibody prevalence was reported in hunted wild boar in north-eastern Estonia. One of the causative virus strains was recently characterized. While it still showed rather high virulence in the majority of experimentally infected animals, one animal survived and recovered completely. Here, we report on the follow-up characterization of the isolate obtained from the survivor in the acute phase of infection. As a first step, three in vivo experiments were performed with different types of pigs: twelve minipigs (trial A), five domestic pigs (trial B), and five wild boar (trial C) were inoculated. 75% of the minipigs and all domestic pigs recovered after an acute course of disease. However, all wild boar succumbed to infection within 17 days. Representative samples were sequenced using NGS-technologies, and whole-genomes were compared to ASFV “Georgia 2007/1”. The alignments indicated a deletion of 14560 base pairs at the 5’ end, and genome reorganization by duplication. The characteristic deletion was confirmed in all trial samples and local field samples. In conclusion, an ASFV variant was found in Estonia that showed reduced virulence.

**Keywords:** African swine fever virus, Estonia, attenuation, in vivo characterization, next-generation sequencing

## 1. Introduction

In 2014, African swine fever virus (ASFV) was introduced into Poland and the Baltic European Union (EU) member states Latvia, Lithuania and Estonia. Since then, slow but constant spread of this notifiable disease has been observed [1]. With regard to outbreak characteristics, detection of fallen animals and virus prevails. However, in some regions, a different pattern in cause of the epidemic has been observed [1]. In the follow-up of those observations, we recently reported an animal experiment that aimed at the biological characterization of an ASFV strain from north-eastern Estonia, where an unexpectedly high ASFV-antibody prevalence was found in hunted healthy animals [2]. In this previous animal trial, ten wild boar were inoculated with the above mentioned ASFV strain to evaluate if the clinical course of the disease differed from infections with the so far known highly virulent Caucasian strains [3,4,5,6]. In brief, nine out of ten animals succumbed to the infection showing typical lesions. The surviving wild boar recovered completely and was slaughtered in good health status 96 days post infection (dpi). Comingling of the survivor with three sentinel wild boar from 50 dpi did not lead to disease transmission. Taken together, the virus showed still considerable virulence and lethality, but one animal recovered and could represent one of the antibody positive wild boar found in the hunting bags of north-eastern Estonia. These results left us with several unanswered questions, including: Is the survival of one animal within the normal range of clinical courses of a highly virulent ASFV strain or is it an indication for true attenuation? Could a further animal passage lead to a more attenuated phenotype? If there is attenuation, what is the genetic basis?

To address these questions and to further characterize the virus isolated from the surviving boar, three additional animal trials were performed to characterize the virus with different pig types. Since the survival rates and clinical courses were rather variable in the different trials, representative samples from each trial were full-genome sequenced using next-generation sequencing technologies and the resulting sequences were compared to ASFV "Georgia 2007/1" (FR682468.1). In order to confirm the circulation of the variant strain, Estonian field samples were screened for the mentioned mutation by *real-time* quantitative polymerase chain reaction (qPCR).

## 2. Results

### *2.1 Clinical course and pathomorphological findings*

In all trials, the animals showed unspecific clinical signs during the first 10 days after oronasal inoculation.

In trial A (12 minipigs), all animals developed transient high fever (up to 41°C on day 7 pi). The minipigs also showed transient anorexia and lethargy. One minipig (#69) was found dead the day after blood sampling (8 dpi). Necropsy revealed a mild pericarditis and atelectasis in the left lung. Two animals (#72 and #67) had to be euthanized due to severe respiratory distress (8 dpi and 15 dpi). Animal #72 showed lung edema and several hemorrhagic lymph nodes in necropsy. The other nine minipigs recovered completely and were slaughtered in good health at 36 dpi. The post-mortem examination revealed that two of the recovered minipig sows (#61 and #70) were pregnant around the 45$^{th}$ day of gestation according to the size of the fetuses. The fetuses did not show any pathological findings indicative for ASF or any other disease while one of the pregnant sows (#61) presented a pericarditis. None of the other recovered pigs did have any visible lesions.

In trial B (5 domestic pigs), four out of five pigs started showing mild clinical signs such as lethargy, reduced feed intake and increased body temperature 4 to 6 dpi. Animal #98 reacted slightly later on day 10 pi and started with unspecific clinical signs like the other pigs. The second week after inoculation, the animals showed more severe clinical signs with transient high fever (see Fig. 1), reduced liveliness and responsiveness, and transient anorexia. At 17 dpi, animal #97 showed short-term (less than 24 hours) cyanosis on the acra (ears, mouth and tail). After this acute phase, all pigs recovered. From 19 dpi on, no clinical signs were observed, apart from animal #98 with a single body temperature peak at 29 dpi. Apart from one pig (#99) with fibrinous pericarditis, the post-mortem examination did not reveal any pathological findings indicative for an ASFV infection.

In trial C (5 wild boar), the animals developed unspecific clinical signs such as reduced feed intake and lethargy at 3-4 dpi. One female adult wild boar (#1) was found dead the morning after blood sampling (8 dpi). The next day, the second adult female (#3) was found dead and the male adult wild boar (#8) was euthanized due to severe respiratory distress. None of the animals had been near the humane endpoint the previous evening preceding death or euthanasia. The piglets survived until day 16 dpi (#81) and 17 dpi (#82) when they were euthanized reaching the humane endpoint. Necropsy revealed typical findings for an acute

ASFV infection such as lung edema, hemorrhagic lymph nodes and petechiae in the renal cortex.

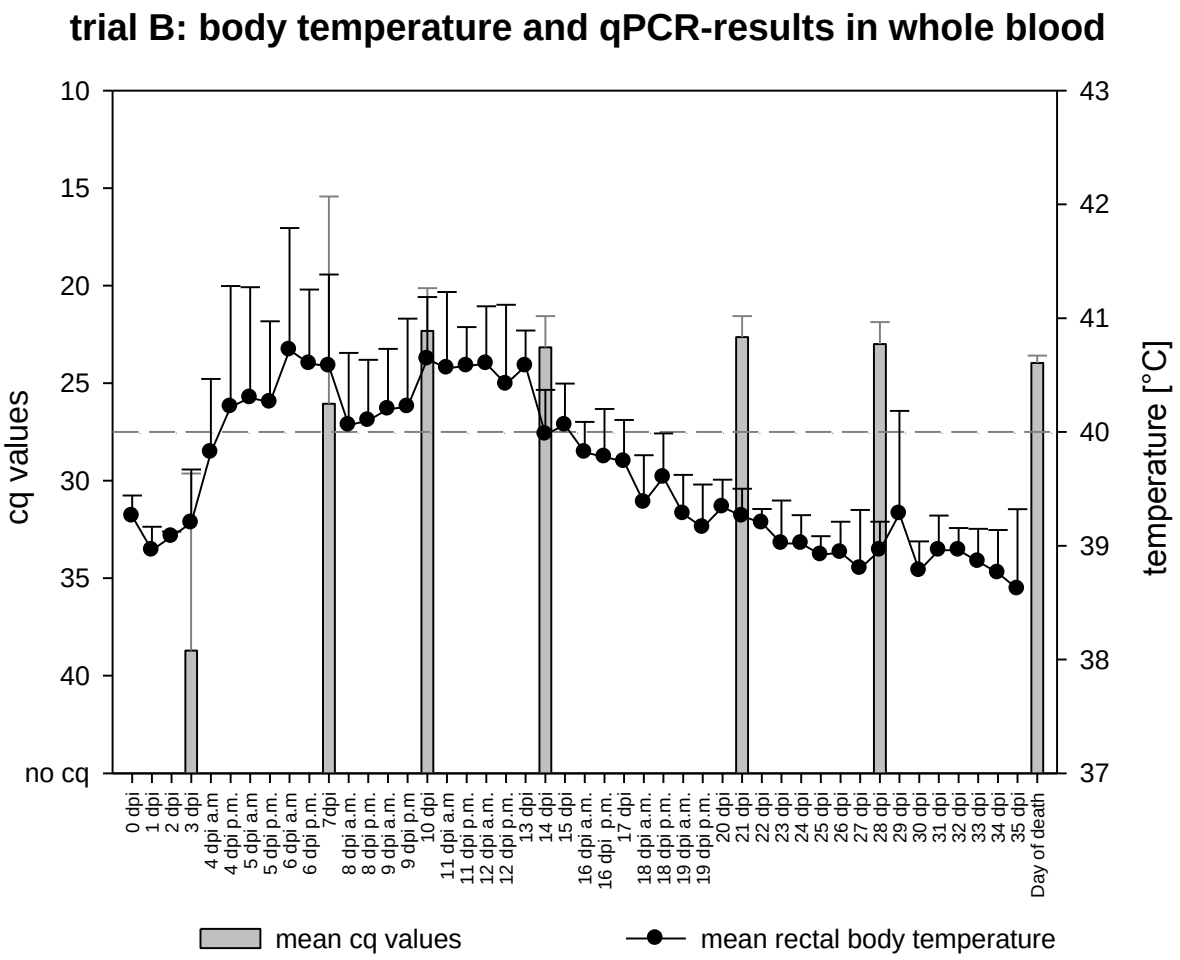


**Fig. 1: Trial B; body temperature and qPCR results in whole blood**; grey bars indicate the mean cq-values at the sampling days; mean rectal body temperature is graphed as line and scatter plot; medium-dashed line represents the fever-cutoff (40°C); in case of "dpi a.m. / p.m." rectal temperature was assessed twice a day, upper standard deviation is shown in error bars

*2.2 Detection of virus and viral genome*

In trial A, the first animals started yielding positive qPCR results in whole blood samples from 7 dpi on. All animals were positive for ASFV genome in qPCR at day 15 pi and the recovering minipigs showed stable genome loads until the end of the trial (see Fig. 2). In organ pools of the fetuses of two pregnant sows (#61 and #70), no ASFV genome was detectable by qPCR while the sows showed positive qPCR results in whole blood on the day of necropsy. Regarding

the tissue samples, minipigs that died during the acute phase of the disease yielded much higher viral genome loads in most organs compared to the recovered minipigs (see supplementary Tab 1). The oropharyngeal and fecal swabs showed single weakly (quantitation cycle (cq) value >35) or moderately (cq value >22) positive qPCR results during the acute phase of the disease.

Results of the hemadsorption test of the sera were corresponding to the samples with detectable virus genome at day 7 and 15 pi. At the day of necropsy, serum samples of the animals that died during the acute phase reacted positive in the hemadsorption test, while the sera of the recovered pigs were negative. The tissue samples of the recovered minipigs showed positive results in lungs or tonsils while all other organs were negative for virus isolation (see supplementary Tab. 2).

In trial B, on 3 dpi two animals started with positive results in whole blood tested in qPCR. On 7 dpi, all pigs but animal #98 were tested positive in whole blood by qPCR and from 10 dpi on, ASFV genome was detectable in the whole blood of all pigs until the end of the trial (see Fig. 1). At the day of necropsy (36 dpi), samples of spleen and tonsils of all pigs except animal #100 showed weak positive results in qPCR. The tissue samples of the different lymph nodes and the salivary gland showed sporadic weak positive qPCR results in different pigs, whereas no ASFV genome was detectable in lung tissues (see supplementary Tab. 1). The oropharyngeal and fecal swabs showed single weakly (cq values > 35) positive qPCR results during the acute phase of the disease (see supplementary Fig. 1a+b).

The hemadsorption test of the serum from 3 dpi reflects these results with one clearly positive result from animal #99 and a doubtful result of animal #97. On 7 dpi, all pigs with positive qPCR results in whole blood showed clearly positive hemadsorption phenomena in serum samples. The serum of animals #97 and #100 reacted positive in the hemadsorption test while the serum of animal #60 was negative. From animals #98 and #99 no serum samples were taken due to their critical health status during the acute phase of the disease. Two weeks after the inoculation, serum of all pigs reacted positive in the hemadsorption test. Subsequently, at 21 dpi the serum of two pigs (#99, #100) showed negative results in the hemadsorption test. At 28 dpi, again animal #100 was the only animal reacting positive for hemadsorption using serum, and only three tissue samples were positive for virus isolation (see supplementary Tab. 2).

In trial C, on 7 dpi all wild boar were highly positive for viral genome in qPCR reaching a mean cq value of 17 in whole blood. The tissue samples of the three adult wild boar were all tested positive in qPCR. The tissue samples of the two piglets showed overall lower genome loads and some organs of animal #81 were tested negative for viral genome in the qPCR assays (see supplementary Tab. 1). Homogenized spleen samples showed high titers ($10^{4.5}$-$10^{5.5}$ hemadsorbing units (HAU)/mL) for the three adult wild boar. The two piglets yielded lower titers around $10^{2}$-$10^{3}$ HAU/mL in their spleen suspensions. The same distribution was seen in the hemadsorption assay of lung and tonsil tissue: titers from $10^{3.25}$ -$10^{4.25}$ HAU/mL in the three adult wild boar while the same tissues were negative for hemadsorption in the two piglets (see supplementary Tab. 2). The blood samples from the day of necropsy showed high titers from $10^{5.25}$ HAU/mL (#1) to $10^{7.25}$ HAU/mL (#8).

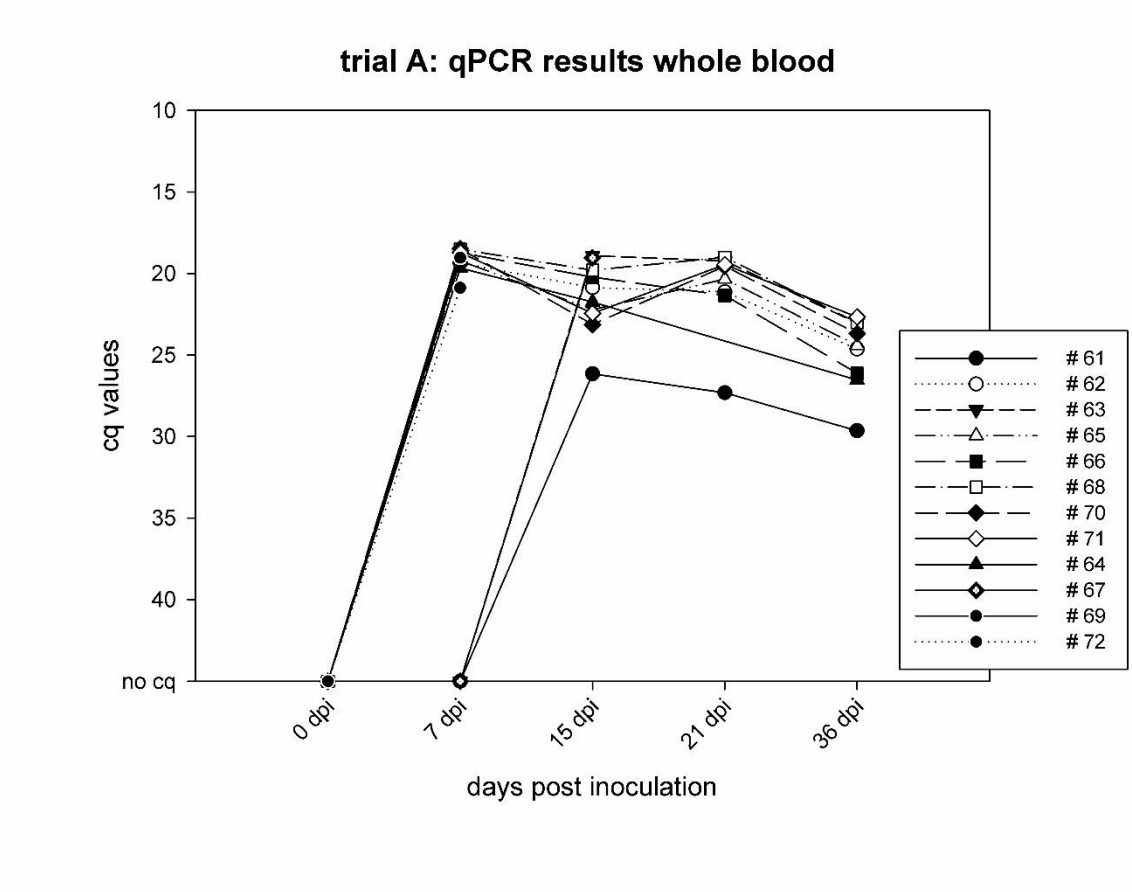


**Fig. 2: trial A; qPCR results whole blood** cq values graphed as line and scatter plot

*2.3 Detection of antibodies against ASFV*

In trial A, 15 dpi four minipigs were tested positive for antibodies against ASFV and at 21 dpi all but one of the recovered minipigs showed positive enzyme-linked immunosorbent assay (ELISA) results. At the necropsy on the end of the trial, all nine minipigs that survived the acute phase of the disease were still positive for ASFV-specific antibodies (see Fig 3).

The pigs of trial B showed the first positive ELISA results on 10 dpi and two weeks after the inoculation four out of five pigs were tested clearly positive for antibodies against ASFV. From 21 dpi until the end of the trial at 36 dpi, all animals were tested positive for antibodies (Fig. 3).

The wild boar in trial C showed negative ELISA results in samples from 7 dpi. The sera taken from the two piglets at the necropsy showed positive results (see Fig. 3), while the sera from the adult wild boar were still negative for antibodies at their endpoints.

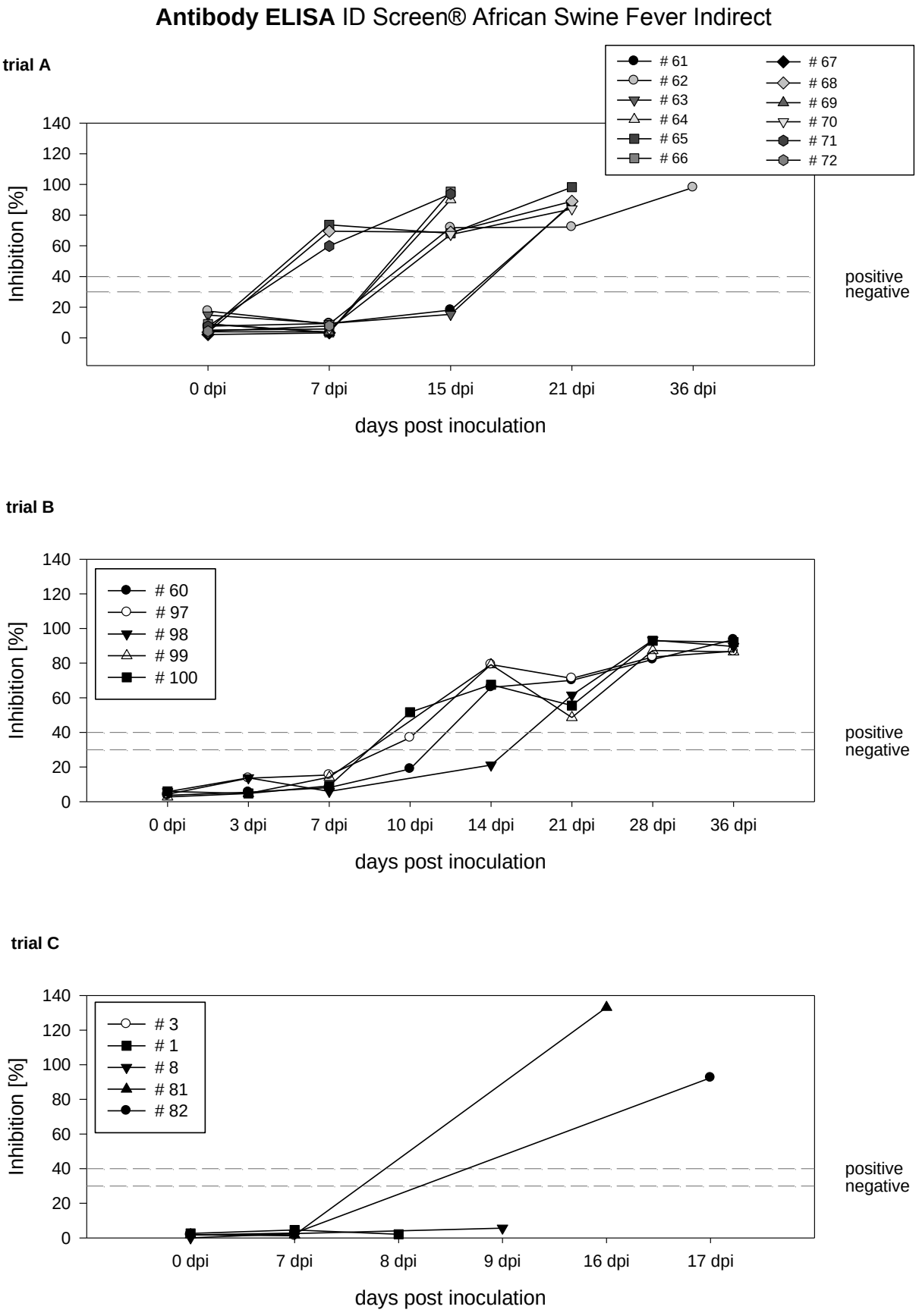


**Fig. 3 antibody response trial A-C** ELISA results in [%] inhibition graphed as line and scatter plots

*2.4 Next-generation sequencing (NGS)*

For four out of five samples (inocula for animal trials and trial samples), the full ASFV-genome sequence could be assembled. The genomes comprise 182,446 base pairs (bp) with an overall sequence identity of 99.99 % among themselves. In comparison to the reference genome ASFV “Georgia 2007/1” (FR682468.1), which comprises 189,344 bp, the first 14,560 bp at the 5’ end are missing. This deletion results in the loss of 26 complete genes including I83L, I60L and KP177R as well as members of the MGF110 (1L-14L), MGF360 (1L-3L), and the partial MGF110 13L gene. Furthermore, 7271 bp from the 3’ end were found to be inversely bound at the 5’ end leading to the duplication of 10 complete genes including members of the MGF360 (18R and 21R) and L11L as well as one partial gene I10L (Fig. 4). In comparison to FR682468.1, the sequence identity of the core genome of ~175 kB amounts to 99.9 %, thereby not considering the very different 5’ end and a 344 bp longer tail at the 3’-end.

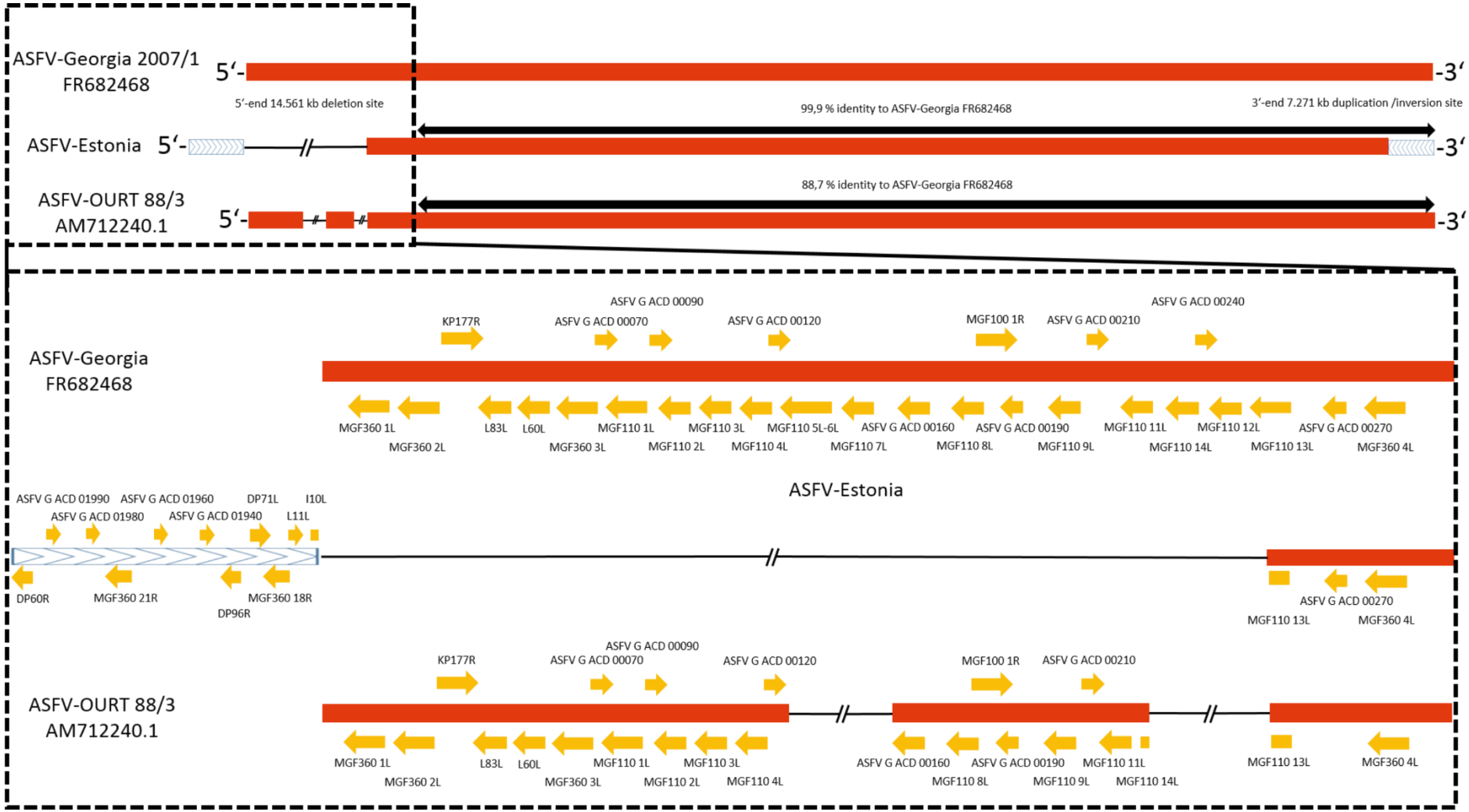


**Fig. 4: deletion and reorganization site** overview of the ASFV Estonia reorganization sites and comparison with ASFV Georgia07 and the natural attenuated ASFV OURT 88/3

*2.5 Confirmation of deletion site and field sample screening*

Representative samples from all animal experiments including the initial wild boar trial [2] and the original Estonian field sample were examined by tailored PCR and Sanger sequencing. The deletion site was confirmed in all samples (see supplementary Fig. 2).

Sixty-one Estonian field samples from 2014 were screened for the characteristic deletion site and three samples were tested positive for the mutation by qPCR. All three samples were from Ida-Viru county in north-eastern Estonia and one of them was the original field sample used in the first trial (see map in Fig. 5). The other 58 samples were tested positive for the ASFV wild-type sequence.

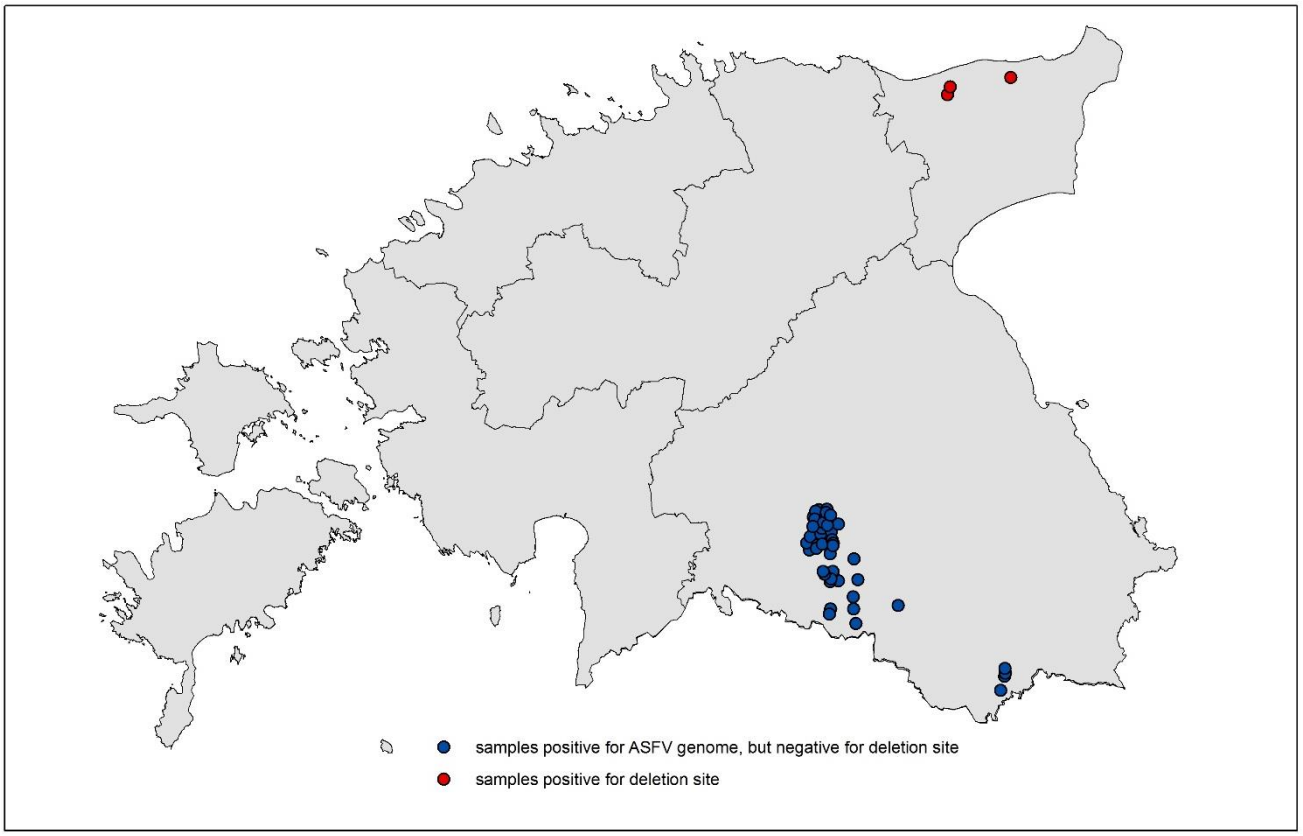


**Fig. 5: map of Estonia including the results of the field sample screening**

The screening included 61 original field samples from 2014 provided by Estonian Veterinary and Food Laboratory.

## 3. Discussion

Compared to what is so far known about the virulence of ASFV genotype II in both domestic pigs and European wild boar under experimental conditions [3-7] , the north-eastern Estonian strain re-isolated from a surviving animal during acute infection showed a clearly attenuated phenotype in trials A and B. After all pigs developed acute clinical disease, these trials ended with survival rates between 75% and 100% (see Fig. 6 and Fig. 7).

The deaths of three minipigs in trial A were not clearly linked to ASFV infection and could as well be consequence of their high stress sensitivity and the invasive sampling procedures during the acute phase of the disease. The use of minipigs with potbelly pig ancestry for ASF trials with blood sampling has to be reassessed for animal welfare reasons. In our experience, stress responses were much less pronounced in domestic pigs and even in (tame) wild boar. In both trials, all recovering animals showed seroconversion that was detectable by all routine diagnostic methods. The first antibody responses were detected at day 10 pi (trial B) and 15 dpi (trial A), respectively. This matches the results of previous studies[2,7]. However, there are not many reports about the time point of seroconversion of ASFV genotype II infected animals because up to now in most trials the animals died before the development of an antibody response. The fecal and oral shedding of ASFV genome was quite low compared to the genome load in blood samples and limited to the acute phase of the disease. This agrees with results of previous experiments [3,7]. With regard to gastrointestinal signs in general, only slight obstipation was observed in the febrile phase of infection, and macroscopically no blood admixture was seen. Based on the observed detection frequency and the low viral genome load, the suitability of fecal and oral swab samples for reliable and timely detection of the disease has to be questioned. The qPCR-negative results of the minipig fetuses indicate that transplacental transmission did not occur over the whole study period. Given the estimated stage of gestation (roughly 45 days), the mothers were inoculated in very early pregnancy and did not transmit the virus over 36 days of infection. This is in line with unpublished field observations but not with a case report from Nigeria [8]. However, under our experimental conditions, all fetuses remained negative and did not show any negative effects related to the infection of the mothers.

The outcome of trial C is in contrast to the other trials. However, it reflects more or less the disease course in the initial wild boar trial [2] in which all but one wild boar succumbed to the infection. This could lead to the assumption that wild boar are more susceptible to infection

with this ASFV strain than domestic pig breeds which is not in accordance with the literature [5] and is also only partially in line with the field observations that showed several apparently healthy, but sero-positive wild boar in the hunting bag of north-eastern Estonia. The slightly higher inoculation dose in trial C is not a sufficient explanation for the higher mortality either, since previous studies [5] did not reveal a measurable dose dependency. However, the piglets survived at least until dpi 16 and 17. Therefore, it could be discussed if they are more resistant compared to the adult wild boar, which is inconsistent with former studies on ASFV Armenia [9] but fits with the observation that the detection of antibodies was more likely in the young age class [1]. In general, a negative influence of the necessary immobilization of the adult animals during the acute phase of the disease has to be taken in consideration. However, this had no influence in previous trials but harmonized experiments targeting the direct comparison of the clinical course together with the assessment of immunological parameters in domestic pigs and wild boar, infected with the variant strain, would be required to finally clarify this issue.

The attenuated disease course shown especially in trials A and B can be associated with the results of the full-genome sequencing.

Among the 26 genes missing from the viral genome, thirteen belong to the multigene family MGF110 (1L-14L) [10,11] and three to the MGF360 (1L-3L). While the specific functions of these genes are unknown [12], it was shown that members of the MGF110 carry C-Terminal KDEL endoplasmic reticulum retention motifs and might be involved in preparing the ER for viral morphogenesis [13]. Although the respective MGF360 1L-3L genes are not characterized and their function is also unknown, other MGF360 members were found to be important for ASFV replication in ticks [14], macrophages [15,16] as well as domestic pigs [17,18,19,20].

Further deleted genes include MGF100 1R, L83L, L60L and KP177R. While for the first three, no function is known [12], the KP177R genes encodes for the early membrane protein P22 [21]. The mechanism by which this major genome re-organisation occurred remains unclear. Nonetheless, the 5' end deletion as well as the duplication and inverse binding of ~7kb from the 3' to the 5' end could be explained by a false separation of head-to-tail concatamers during viral DNA replication [22].

Whether the duplication of ten genes including DP71L and DP96R, two genes which were previously reported as important for virulence [12,23,24] and the uncharacterised genes MGF360

18R, MGF360 21R, L11L and DP60R as well as the partial duplication of the I10L gene, which codes for a P22 homologue, has an effect on virulence remains to be investigated.

A direct comparison of the ASFV Estonia strain with the naturally attenuated ASFV strain OURT 88/3 reveals some similarities (see figure 4) but also major differences. While both strains lack members of the MGF110 (4L-7L and 12-13L) 25,26, the major deletions are at different positions. While the main changes of the Estonian strain are at the true 5'-end of the coding sequence, the major deletion of OURT88/3 is further downstream concerning e.g. members of the MGFs 306 and 505. Yet, one can speculate that the shared deletions are already part of the attenuation process.

It could be hypothesized that large-scale mutations could occur more often but an ASFV strain that has an attenuated phenotype with lower mortality rates in swine, in the absence of a reservoir vector, will probably vanish due to the animals clearing the virus before it is transmitted via bloody excretions or the dead animal's carcass. The low case number and the limited geographical distribution of the sub-genotype in 2014 can substantiate this hypothesis: all three samples positive for the deletion site are located in Ida-Viru county, around 200 kilometers from the outbreak in southern Estonia (see map in Fig. 5). At the same time, two of the wild boar infected with the sub-genotype were tested positive for ASFV-specific antibodies, a fact that fully supports the theory of an attenuated phenotype.

The question whether the variant strain occurred in the local wild boar population by spontaneous mutation or was introduced from somewhere else, remains unanswered and needs further investigation.

However, the presence of attenuated phenotypes, leads to new challenges regarding surveillance of wild boar population as well as domestic pig farms. The observed inconspicuous clinical signs and low mortality of the animals in the first two trials could easily go unnoticed under common pig farm conditions. Thus, farmers and veterinarians should be sensitized to suspect ASF not only if severe signs are observed. The occurrence of almost silent infections with mild and unspecific signs carries the risk of undetected disease spread and surveillance should be adapted accordingly. In this context, it might be reasonable to sample not only wild boar carcasses in ASF-free areas, but also to test hunted wild boar. Otherwise the occurrence of such an attenuated ASFV-subtype could be missed.

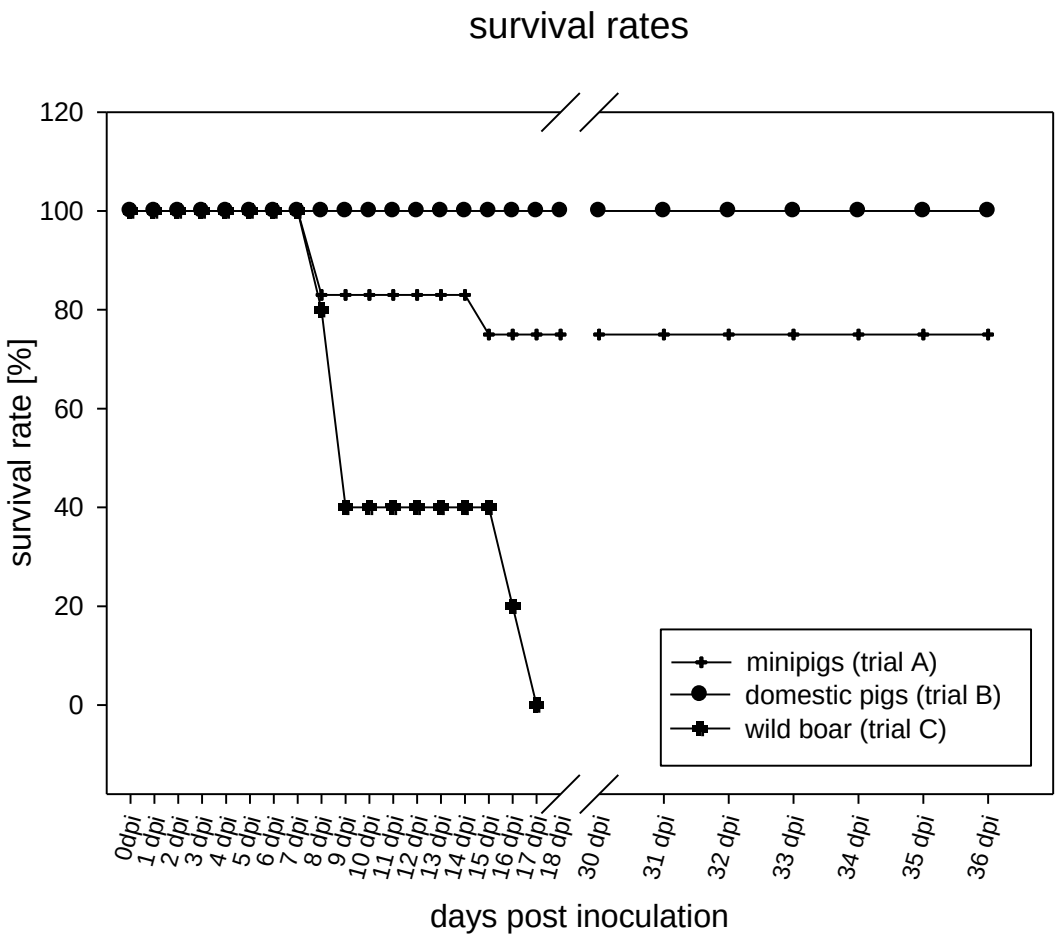


**Fig. 6: survival rates trial A-C** survival rates of the inoculated animals in [%] graphed as line and scatter plot

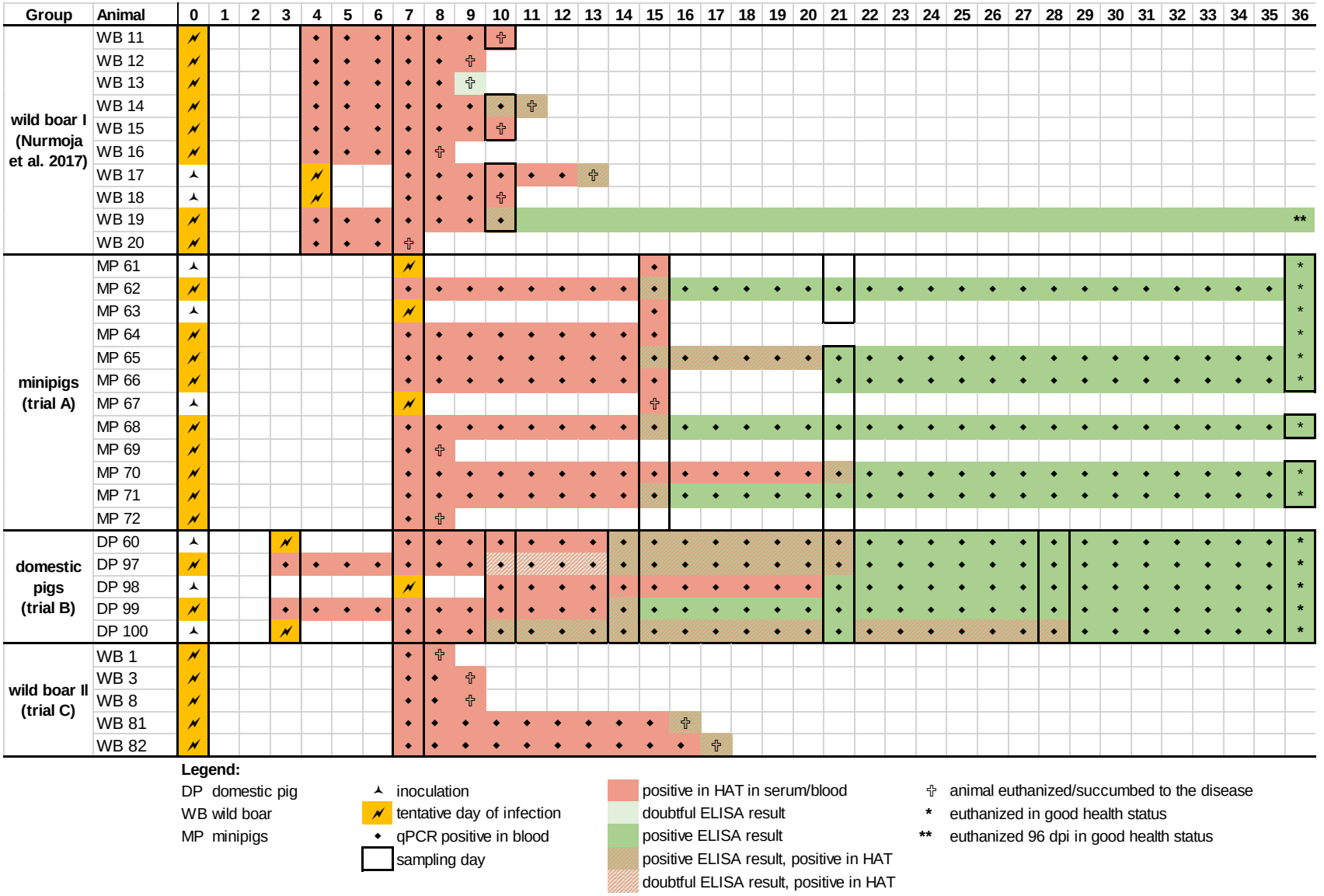


**Fig. 7: overview of antibody response, disease course and viremia** data between sampling days has been assumed

## 4. Materials and Methods

### *4.1 Experimental design*

The study comprised three animal experiments (trials A, B and C) that were carried out to collect suitable reference materials and to assess virulence and pathogenesis characteristics of genotype II ASFV from Estonia upon animal passaging. To this end, an ASFV-positive blood suspension was prepared that contained ethylenediaminetetraacetic acid (EDTA)-treated blood samples from a wild boar with acute-transient ASF [2] diluted in phosphate-buffered saline (PBS) to a final titer of app. $10^5$ HAU per mL. The suspension was used to inoculate the pigs in trial A and B.

Trial A comprised 12 sub-adult minipigs of both sexes from the breeding unit at the Friedrich-Loeffler-Institut (FLI) aged approximately six months at the start of the trial. For the experiment, the animals were moved from the FLI quarantine stables into the high containment facilities (L3+) where they were kept together in one pen. All animals were individually ear-tagged with numbers #61 to #72. Over the course of the trial, the animals were fed a commercial pig food with hay cob supplement and had access to water *ad libitum.* After an acclimatization phase, the minipigs were oronasally inoculated with 2 mL of the above-mentioned blood suspension using a single-use syringe without needle. Clinical parameters of all animals were assessed daily based on a harmonized scoring system as previously described [5]. In brief, parameters like temperature (assessed only on sampling days), anorexia, recumbency, skin alterations (cyanosis, hemorrhages, necrosis), joint lesions, breathing, ocular discharge, digestion, and neurological disorders were assigned points according to the severity of findings. The sum of the points was recorded as the clinical score (CS) that was also used to define humane endpoints. Over the course of the trial, levels of viremia, virus distribution, virus shedding, and antibody responses were assessed. For this purpose, blood samples were collected along with oropharyngeal and fecal swabs at days 0, 7, 15, 21 dpi and at the end of the trial (36 dpi). Animals reaching the humane endpoint or that were suffering unacceptably without reaching the endpoint were euthanized through intracardial injection of embutramide (T61, Merck) after deep sedation with tiletamine/zolazepam (Zoletil®, Virbac). Necropsy was performed on all animals, and at the same time, tissue samples (lymph nodes, spleen, tonsil, salivary gland and lung), blood (EDTA, serum) and swab samples were collected for reference purposes.

Trial B comprised five commercial domestic pigs aged approximately six months at the start of the trial. All animals were individually ear-tagged upon arrival with numbers #60 and #97 to #100. After an acclimatization phase under the husbandry conditions detailed above, the animals were oronasally inoculated with 2 mL of the above-mentioned blood suspension. As in trial A, clinical monitoring took place on a daily basis, and levels of viremia, virus distribution, virus shedding, and antibody responses were assessed.

For this purpose, blood samples were collected along with oropharyngeal and fecal swabs at 0, 3, 7, 10, 14, 21, 28 dpi and at the end of the trial at 36 dpi. All animals were slaughtered 36 dpi (exsanguination after electro-stunning) and necropsy was performed. Again, tissue samples (lymph nodes, spleen, tonsil, salivary gland and lung), blood (EDTA, serum) and swab samples were collected for reference purposes.

Trial C comprised five wild boar from the breeding unit at the FLI of different sexes and ages (three adult wild boar around two years old and two piglets app. 6 months old). The wild boar were immobilized with an intramuscular injection of tiletamine/zolazepam (Zoletil®, Virbac) and moved to the high-containment facilities. They were individually eartagged (#1, #3, #8, #81, and #82) and oronasally infected with 2 mL blood suspension (titer of app. $10^{6.5}$ HAU/ mL) from trial B (a mixture of blood samples from different pigs).

As in trials A and B, clinical monitoring took place on a daily basis, apart from the rectal body temperature assessment, due to working safety conditions. Levels of viremia, virus shedding, and antibody responses were assessed. For this purpose, blood samples were collected along with oropharyngeal and fecal swabs at day 0 and day 7 pi. Since it was necessary to immobilize the wild boar to take blood samples, the sampling time points were reduced to a minimum. Animals reaching the humane endpoint or that were suffering unacceptably without reaching the endpoint were euthanized through exsanguination after deep sedation with tiletamine/zolazepam (Zoletil®, Virbac). Necropsy was performed on all animals and tissue samples (lymph nodes, spleen, tonsil, salivary gland and lung) and blood (EDTA, serum) were collected for reference purposes.

In all trial parts, 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 authority (Landesamt für Landwirtschaft, Lebensmittelsicherheit und Fischerei (LALLF) Mecklenburg-Vorpommern) under reference number LALLF 7221.3-2-023/15.

*4.2 Cells*

Blood for the preparation of Peripheral Blood Mononuclear Cells (PBMC)-derived macrophages was collected from healthy domestic donor pigs that are routinely kept at the FLI. In brief, PBMCs were obtained from EDTA-treated blood using Pancoll Animal density gradient medium (PAN Biotech). PBMCs were grown in RPMI-1640 cell culture medium with 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and 10 % fetal calf serum (FCS) at 37 °C in a humidified atmosphere containing 5 % $CO_2$. The medium was supplied with amphotericin B, streptomycin and penicillin to avoid bacterial and fungal growth. To facilitate maturation of macrophages, granulocyte macrophage colony-stimulating factor (GM-CSF) was added to the cell culture medium at 2 ng/mL.

*4.3 Laboratory investigations*

*4.3.1 Processing of samples*

Oropharyngeal swabs were soaked in 1 mL of medium (EMEM without addition of FCS), vortexed for app. 15 seconds, incubated for one hour at room temperature, and decanted in microcentrifuge tubes. Serum samples, which were obtained from native blood by centrifugation at 2500 x g for 20 minutes at 20°C, were aliquoted and stored at -80 °C until further use. Tissue samples of tonsil, spleen, salivary gland, lung, and lymph nodes were collected at necropsy and stored at -80 °C. For qPCR and virus isolation (hemadsorption tests), tissue samples were homogenized with a metal bead in 1 mL phosphate-buffered saline (PBS) using a TissueLyser II (Qiagen).

*4.3.2 Virus detection*

For qPCR, viral nucleic acid was extracted using the QIAamp® RNA Viral Mini Kit (Qiagen) or the NucleoMagVet-Kit (Macherey-Nagel) and the KingFisher® extraction platform (Thermo Scientific). Both extraction methods were slightly modified through the addition of an internal control DNA. The nucleic acid extraction was performed with 75 µl of whole blood and 150 µl of organ homogenate and swab material. Subsequently, qPCR was performed according to the protocol published by King et al. [29] with slight modifications. For confirmation, the virotype ASFV PCR Kit (Qiagen) was employed according to the manufacturer's instructions. Results of both qPCRs were recorded as quantification cycle (cq) values.

To detect ASFV in serum and tissue samples, a hemadsorption test (HAT) was carried out using PBMC-derived macrophages according to slightly modified standard procedures [30]. In brief, isolated PBMCs were seeded into a 96-well microplate at a density of 1.9 x $10^6$ cells/mL, 100 µL per well. After 16-24 hours, non-adherent cells were removed and cell culture medium containing GM-CSF was replenished. The culture was then incubated for 24 to 48 hours to allow initial maturation of macrophages. Subsequently, 20 µl of serum samples and 30 µl of organ homogenate were added to each well. Tests were performed in duplicates. When using organ homogenates, cells were washed after 2 hours adsorption time using lukewarm PBS, whereas serum was left on the cells until the evaluation of the test. After 24 hours of incubation 20 µl of homologues 1 % erythrocyte suspension was added to each well. For readout, cultures were analyzed for hemadsorption phenomena over a period of two days. Doubtful results were confirmed by an additional passage. Virus titration was performed by endpoint titration of the diluted blood suspensions. In this case, the PBMC preparation was seeded into 96-well microplates, the test volume was 100 µl per dilution step and 20 µl of a 1 % homologous erythrocyte suspension was added. These samples were tested in quadruplicate.

*4.3.3 Antibody detection*

For the detection of antibodies against African swine fever virus, two commercial ELISA kits were carried out following the manufacturer's instructions (Ingezim PPA COMPAC, Ingenasa; ID SCREEN African swine fever virus INDIRECT, IDvet). The Ingezim PPA ELISA detects antibodies directed against p72 in a competitive format. The ID SCREEN is an indirect ELISA using antigens p32, p62 and p72. All serum samples were tested in duplicate.

*4.4 Full-genome sequencing*

*4.4.1 Sample preparation*

The material for the full-genome sequencing was gained by salting-out of viral DNA [31] after propagation of the virus on PBMC-derived macrophages to avoid high loads of swine related DNA. Therefore, the protocol was slightly modified regarding a shortened incubation time (60 minutes at 37°C) and the addition of RNase A (10 mg/ml) in the first step instead of proteinase K (10 mg/mL), which was added in the next step and incubated for 60 minutes at 56°C.

*4.4.2 Sequencing*

For full genome sequences, 500 ng of input material were fragmented using Covaris M220 Focused-ultrasonicator™ (Covaris), and ligated to suitable Illumina adapters (NEXTflex-96™ DNA Barcodes, BiooScientific) using a SPRI-TE library system (Beckman Coulter) with SPRIworks Fragment Library Cartridges II (for Roche FLX DNA sequencer; Beckman Coulter). Size exclusion was performed manually with AMPure XP magnetic beads in two steps for a final size distribution of 500 - 600 bp long fragments. After quality control of the libraries on a Bioanalyzer 2100 (Agilent Technologies), the libraries were quantified using using Kapa Library Quantification Kit for Illumina platforms (Kapa Biosystems), pooled and sequenced on a MiSeq instrument (Illumina) with MiSeq reagent Kit v3 in 2x300bp PE mode (Illumina). For data analysis, the reads were mapped against the nearest reference genome (Newbler v3.0, Roche). All mapped reads were extracted and de novo assembled (Newbler v3.0, Roche). Since this approach delivered three or more contigs, the software ContigGraph (unpublished) was used to determine the connections of single contigs for manual assembly of the full genome. Afterwards the whole data set was mapped against the full genome (Newbler v3.0, Roche).

*4.4.3 Sanger sequencing, PCR and qPCR screening*

All nucleic acids for PCR and qPCR were extracted using the High Pure DNA Template Preparation Kit (Roche) or the QIAamp® RNA Viral Mini Kit (Qiagen) according to the manufacturer's instructions. For classical PCR, Phusion Green Hot Start II High-Fidelity PCR Mastermix (Thermo-Scientific) and for qPCR, QuantiTect Multiplex PCR NoROX Kit (Qiagen), were used according to the manufacturer's instructions.

For classical PCR and sanger sequencing, primers were designed to amplify either the reorganised or the wild type sequence by placing them overlapping the reorganisation site.

For qPCR screening, tailored primers were designed amplifying short DNA-fragments of the reorganisation site with an additional Taqman-probe inside the target fragments. The tested field samples were provided by the Estonian Veterinary and Food Laboratory. The panel of 61 samples contained blood, spleen and bone marrow specimens from Estonian wild boar collected during the outbreak situation in 2014.

All primers and probes were designed using Geneious v. 10.0.9 (supplementary table).

All data were recorded and evaluated using Microsoft Excel 2010 (Microsoft Deutschland GmbH) and SigmaPlot for Windows version 11.0 (Systat Software, Inc.)

*4.4.4Data availability*

All sequence data was uploaded to the European Nucleotide Archive (EMBL-EBI) under the study accession number PRJEB24381.

**Acknowledgements**

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**Author contributions**

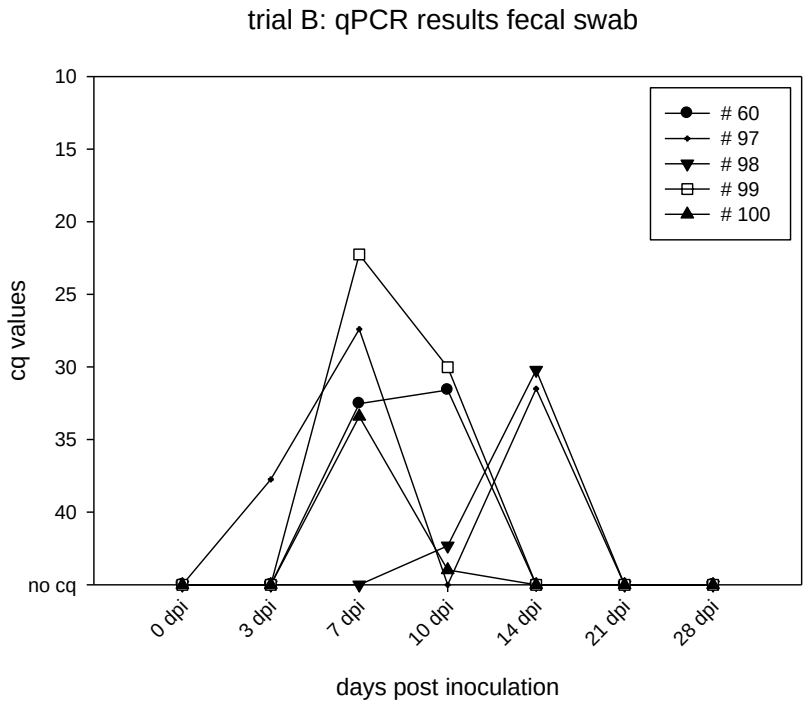
B.M. and B.S. designed the experiments. Z.L., B.S., L.S., B.C., H.J. and C.J. carried out the animal trials and collected clinical samples. Sample analyses were performed by Z.L., F.JH.. Z.L. and F.JH. analysed the data. The manuscript was written by Z.L., F. JH., B.S. and B.M.. S-L. C. created the map. F.L. performed NGS and analyzed the obtained data under supervision of H.D. N.I. and V.A. supported with their expertise.

All authors took part in discussion and interpretation of results. All authors read, advised and approved the final manuscript.

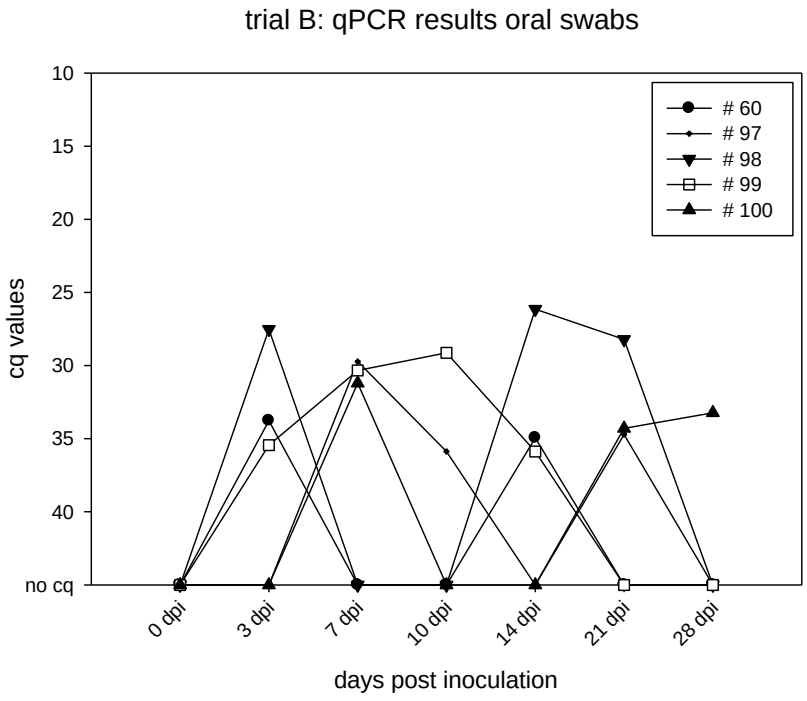
**Competing financial interests**

All authors declare, that they have no competing interests.

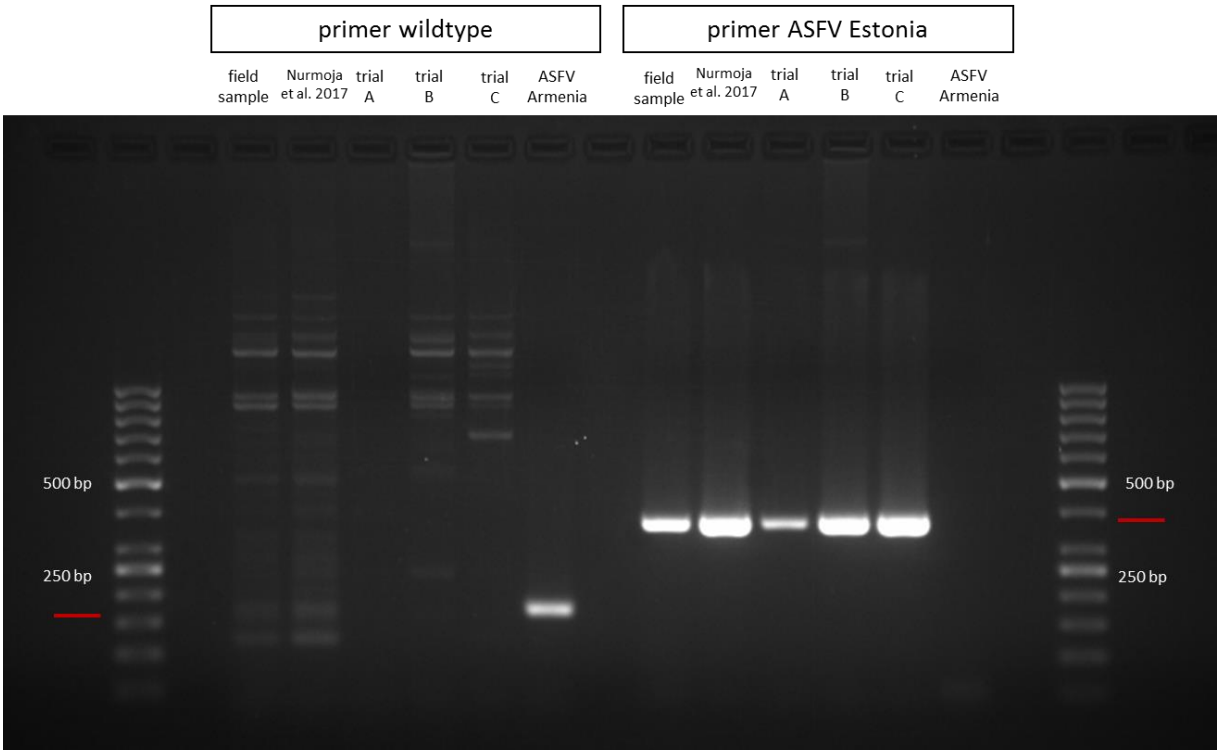
**Supplementary figures and tables**




**supplementary Fig. 1a: Trial B; qPCR results of fecal swabs** cq values graphed as line and scatter plot




**supplementary Fig. 1b: Trial B; qPCR results of oral swabs** cq values graphed as line and scatter plot




**supplementary Fig. 2: PCR products of the screening for the deletion site.** The amplicon length of the wildtype primers was 172bp and the amplicon length of the deletion site primers was 375bp. Representative trial samples of the trials were chosen and ran on one single gel exposed as a whole.

**supplementary Tab. 1: qPCR results of tissue samples of trial A-C** cq values detected in different tissue samples; higher and lower genome loads highlighted

| | pig ID | spleen | tonsil | lung | salivary gland | mandibular lymph node | day of death |
|---|---|---|---|---|---|---|---|
| trial A | # 61 | no cq | no cq | no cq | no cq | no cq | dpi 36 |
| | # 62 | no cq | no cq | 33 | 36 | no cq | dpi 36 |
| | # 63 | 36 | no cq | no cq | no cq | 45 | dpi 36 |
| | # 64 | no cq | 44 | no cq | no cq | no cq | dpi 36 |
| | # 65 | 29 | 31 | 34 | no cq | no cq | dpi 36 |
| | # 66 | no cq | 34 | no cq | no cq | no cq | dpi 36 |
| | # 67 | 23 | 25 | 27 | 26 | 26 | dpi 15 |
| | # 68 | no cq | no cq | no cq | 35 | 45 | dpi 36 |
| | # 69 | 25 | 26 | 28 | 33 | 28 | dpi 8 |
| | # 70 | 37 | 33 | no cq | 37 | no cq | dpi 36 |
| | # 71 | 30 | 31 | no cq | 37 | 43 | dpi 36 |
| | # 72 | 23 | 26 | 26 | 33 | 28 | dpi 8 |
| trial B | # 60 | 35 | 30 | no cq | no cq | no cq | dpi 36 |
| | # 97 | 44 | 36 | no cq | no cq | no cq | dpi 36 |
| | # 98 | 34 | 35 | no cq | 38 | no cq | dpi 36 |
| | # 99 | 36 | 43 | no cq | no cq | 34 | dpi 36 |
| | # 100 | no cq | no cq | no cq | no cq | no cq | dpi 36 |
| trial C | # 1 | 21 | 29 | 24 | 25 | 26 | dpi 8 |
| | # 3 | 18 | 24 | 27 | 29 | 25 | dpi 9 |
| | # 8 | 17 | 25 | 18 | 29 | 25 | dpi 9 |
| | # 81 | 35 | 40 | 34 | no cq | 33 | dpi 16 |
| | # 82 | 31 | 30 | 27 | 36 | 29 | dpi 17 |

low genome load
high genome load

died during the first 20 dpi
died during the first 10 dpi

**supplementary Tab. 2: virus isolation results of tissue samples** pos + indicates weakly positive, pos ++ strongly positive virus isolation results; neg marks negative virus isolation results

| | Pig ID | spleen | tonsil | lung | salivary gland | mandibular lymph node | day of death |
|---|---|---|---|---|---|---|---|
| trial A | # 61 | pos ++ | | | | | dpi 36 |
| | # 62 | neg | | neg | neg | | dpi 36 |
| | # 63 | pos ++ | | | | neg | dpi 36 |
| | # 64 | neg | neg | | | | dpi 36 |
| | # 65 | neg | pos ++ | neg | | | dpi 36 |
| | # 66 | pos + | pos ++ | | | | dpi 36 |
| | # 67 | pos ++ | pos ++ | pos ++ | neg | | dpi 15 |
| | # 68 | pos ++ | | | neg | neg | dpi 36 |
| | # 69 | pos ++ | pos ++ | pos ++ | pos ++ | pos ++ | dpi 8 |
| | # 70 | pos ++ | pos ++ | | neg | | dpi 36 |
| | # 71 | pos + | neg | | neg | | dpi 36 |
| | # 72 | pos ++ | neg | pos + | pos ++ | pos ++ | dpi 8 |
| trial B | # 60 | neg | neg | | | | dpi 36 |
| | # 97 | pos + | neg | | | | dpi 36 |
| | # 98 | pos ++ | pos ++ | | neg | | dpi 36 |
| | # 99 | neg | neg | | | neg | dpi 36 |
| | # 100 | | | | | | dpi 36 |
| trial C | # 1 | pos ++ | pos ++ | pos ++ | pos ++ | pos ++ | dpi 8 |
| | # 3 | pos ++ | pos ++ | pos ++ | pos ++ | pos ++ | dpi 9 |
| | # 8 | pos ++ | pos ++ | pos ++ | pos + | pos ++ | dpi 9 |
| | # 81 | pos + | neg | neg | neg | pos + | dpi 16 |
| | # 82 | pos + | neg | neg | neg | pos + | dpi 17 |

| | |
|---|---|
| | died during the first 20 dpi |
| | died during the first 10 dpi |
| | not done due to negative qPCR result |

| | |
|---|---|
| neg | negative result |
| | weakly positive result |
| | strongly positive result |

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## 4.4 No evidence for long-term carrier status of pigs after African swine fever virus infection

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**Summary**

This study targeted the assessment of a potential ASF virus (ASFV) carrier state of 30 pigs in total which were allowed to recover from infection with ASFV "Netherlands'86" prior exposure to six healthy sentinel pigs for more than two months. Throughout the whole trial, blood and swab samples were subjected to routine virological and serological investigations. At the end of the trial, necropsy of all animals was performed and viral persistence and distribution assessed.

Upon infection, a wide range of clinical and pathomorphological signs were observed. After an initial acute phase in all experimentally inoculated pigs, 66.6% recovered completely and seroconverted. However, viral genome was detectable in blood samples for up to 91 days. Lethal outcomes were observed in 33.3% of the pigs with both acute and prolonged courses. No ASFV transmission occurred over the whole in-contact phase from survivors to sentinels. Similarly, infectious ASFV was not detected in any of the tissue samples from ASFV convalescent and in-contact pigs. These findings indicate that the suggested role of ASFV survivors is overestimated and has to be reconsidered thoroughly for future risk assessments.

**Keywords:** African swine fever, long-term persistence, carrier state, transmission, virus shedding

## 1. Introduction

African swine fever (ASF) is one of the important diseases of pigs worldwide and is notifiable to the World Organization for Animal Health (OIE). The causative agent is African swine fever virus (ASFV), a large double-stranded DNA virus of the genus *Asfivirus* within the *Asfarviridae* family (Takamatsu, 2011). It is the only known DNA virus which can be transmitted by arthropods (ARBO-virus = arthropod borne virus), in this case, soft ticks of the *Ornithodoros* genus (Penrith, 2009).

The disease is endemically present in several countries of Sub-Saharan Africa and on Sardinia. Moreover, an unresolved disease cluster is found in Eastern Europe and the Caucasus region. Following the introduction of ASF into Georgia in 2007, the disease has spread into several Trans-Caucasian countries, the Russian Federation, Belarus, and Ukraine. In 2014, the disease reached the Eastern Borders of the European Union and as of today, the Baltic Member States, Czech Republic, and Poland are affected, especially in the wild boar population. Most recent outbreaks affected also Moldova and Romania (OIE WAHID interface, visited online 11.11.2017).

The causative virus strains in Eastern Europe and the Caucasus region are of genotype II and showed a high virulence for both domestic pigs and European wild boar under experimental conditions (Blome et al., 2012, Gabriel et al., 2011, Nurmoja et al., 2017, Pietschmann et al., 2015, Gallardo et al., 2015a, Mur et al., 2014, Guinat et al., 2016). Given the high lethality in all age classes, disease dynamics could have shown self-limitation after introduction into the wild boar population. This behaviour was seen previously on Sardinia (Laddomada et al., 1994) and in Spain (Perez et al., 1998), but up to now not in the Baltic Member states. Explosive spread based on both the high tenacity and contagiosity could have been an alternative option. In the end, neither happened (Depner et al., 2016), and at present, numerous new cases are reported from a quite stable geographical region every week, an endemic cycle was apparently established within the affected wild boar populations, and further spread can be seen. The latter is evidenced by the most recent cases that were reported from Czech Republic (Animal Disease Notification System of the EU at https://ec.europa.eu/food/animals/animal-diseases/not-system_en ,visited December 13$^{th}$ 2017). The affected region in Zlin county is about 500 km away from the next reported case in Ukraine and thus the disease jumped a long distance.

The factors leading to a long-term perpetuation of the disease and the overall dynamics are far from being understood. Amongst the factors that were discussed for the observed long-term persistence of the virus in a region was a possible carrier status of recovered animals (Arias and Sánchez-Vizcaíno, 2008). In the past and also very recently, clinically recovered pigs were suggested to play a crucial role in disease persistence as silent carriers (Allaway et al., 1995, Boinas et al., 2004, Sanchez-Vizcaino et al., 2012). In 2015, a possible transmission was shown from animals surviving infection with a low virulent ASFV strain (Gallardo et al., 2015b). However, reports targeting the assessment of an ASFV carrier status are very rare and often highly contradictory. A limited data corpus is mainly seen due to the fact that long-term experiments with surviving animals are expensive and difficult to perform. Nevertheless, we could show very recently that a wild boar surviving the infection with an Estonian ASFV strain did not transmit the virus to commingled sentinel pigs after clinical recovery, and was able to eliminate the virus (Nurmoja et al., 2017). However, also this report is still only anecdotal and does not allow reliable risk assessment.

Therefore, in the presented study, long-term infection and the possible carrier status of recovered pigs was reassessed with a larger number of animals and for longer time frames. Due to the fact that a considerable number of recovered animals could not be expected from an infection with the Eastern European genotype II isolates, a moderately virulent ASFV isolate from the Netherlands (Netherlands’86) was used as a model virus. In the initial outbreak that occurred near The Hague in 1986, this ASFV strain caused 19% mortality within a farm over a period of 21 days (Terpstra and Wensvoort, 1986). This virus was already used for transmission and pathogenesis studies (de Carvalho Ferreira et al., 2013, de Carvalho Ferreira et al., 2012, de Carvalho Ferreira et al., 2014) and thus, important background data were available.

## 2. Methods

*2.1 Experimental design:*

The long-term animal trial consisted of two steps: at first, the experimental infection of pigs (potential „carrier“-pigs/ „c-pigs“) with moderately ASFV „Netherlands’86“ and secondly, the exposure of healthy pigs („sentinel“-pigs/ „s-pigs“) to the surviving c-pigs. In total, 36 mature cross-bred domestic pigs of about five to six month of age were employed, 30 served as c-pigs for initial inoculation and six as in-contact s-pigs which were commingled after about three month post infection (99 dpi).

All animals were individually ear-tagged upon arrival in the facilities of the Friedrich-Loeffler-Institut (FLI). All applicable animal welfare regulations, including EU Directive 2010/63/EC and institutional guidelines, were taken into consideration. The animal experiment was conducted in the high containment facilities of the FLI and was approved by the competent authority under reference number LALLF 7221.3 – 1 – 021/15.

At the beginning, all c-pigs were divided into three groups of ten animals each which were kept in separate pens of one stable unit. Animals suffering from acute lameness during the acclimatization phase were treated with Metapyrin 500 mg/ml (Medistar, Arzneimittelvertrieb GmbH, Ascheberg, Germany) for three consecutive days.

After acclimatization, each c-pig was oro-nasally inoculated with 2 ml cell culture supernatant containing 2 x $10^4$ hemadsorption units (HAU) of ASFV “Netherlands’86”, kindly provided by W.L.A. Loeffen, Central Veterinary Institute (CVI), Virology Department, AB Lelystad, The Netherlands. Prior to infection, the absence of ASFV and related antibodies was confirmed. Over the course of the trial, rectal body temperatures and clinical signs were recorded daily. Fever was defined as a body temperature ≥ 40.0°C for at least two consecutive days. The evaluation of clinical signs was based on the adapted scoring system by Mittelholzer et al. (2000) comprising liveliness, bearing, breathing, gait, skin, eyes, faeces and feed intake as vital parameters (reaching from 0 (asymptomatic) to 3 points each (severe)). The sum of score points was documented as clinical score (CS). End points were defined as a CS ≥ 14 or in cases of unjustifiable sufferings according to assessment by the responsible veterinarian. For euthanasia embutramide (T61, Merck) was injected intracardial after deep anaesthesia with tiletamine/zolazepam (Zoletil®, Virbac) and Xylazin (Rompun® 2% Bayer HealthCare, Leverkusen, Germany).

In order to assess levels of viremia, virus shedding and immune responses, blood samples (EDTA blood and sera) were taken from the jugular vein prior to infection and at days 3, 7, 10, 14, 20, 29, 42, 48, 63 and 91 post infection (dpi). Along with that, swab samples were collected comprising fecal and two kinds of oral cotton swabs (common saliva swabs routinely used for superficial oral application and in addition, cotton swabs („salivettes“) which were sampled from the deeper oropharynx. From 105 to 126 dpi, swabs were taken on a weekly base.

At 99 dpi, the surviving c-pigs (19) were remixed (to receive equal distribution) and six s-pigs were commingled (two per group) which were also shown to be free of ASFV and related antibodies prior to exposure. Evaluation of clinical parameters and sample collections were conducted similarly until 28 days upon exposure, at 105, 112, 119 and 126 dpi. Afterwards, experimental measures focused on clinical observations and pathomorphological investigations when necessary.

The animal trial ended at day 164/ 165 post infection of the c-pigs. The remaining pigs where slaughtered (electro-stunning and exsanguination).

Necropsy was performed on all animals. Thereby, the following tissue samples were collected: tonsil, salivary gland, spleen, lung and the mandibular lymph node. From clinical convalescent c-pigs and from all s-pigs additional lymph nodes were sampled: pulmonary, inguinal, jejunal, colical, gastrohepatic, renal, parotideal and popliteal. Further samples comprised the submandibular lymph node and the ovary in several cases. In addition, spleen samples from eleven fetuses were gathered from one sow (s-pig) which was unexpectedly found pregnant during necropsy.

*2.2 Cells and Viruses*

Primary macrophages derived from peripheral blood mononuclear cells (PBMCs) were used for virological and serological issues. For generation, EDTA-blood obtained from donor pigs was subjected to density gradient centrifugation using Pancoll animal, density 1.077 g/ml (PAN-Biotech GmbH, Aidenbach, Germany) and buffered ammonium chloride solution (155 mM $NH_4Cl$, 10 mM $KHCO_3$, 1 mM EDTA/pH 7.4) for lysis of remaining erythrocytes. Cells were cultured in Ham's F12 / IMDM (1:1) cell culture media including 10% fetal calve serum (FCS) as well as antibiotics/ antimycotics (Anti-Anti, Amphotericin B) at 37°C in a humidified atmosphere containing 5% $CO_2$. In order to facilitate maturation of macrophages GM-CSF

(granulocyte macrophage colony-stimulating factor, Biomol, Hamburg, Germany) was added at 2 ng/ml.

The used ASFV strain "Netherlands´86" could be assigned to genotype I and was originally isolated during an outbreak in The Netherlands in 1986 (Terpstra and Wensvoort, 1986). Virus containing cell culture supernatant was diluted with cell culture medium to obtain a titer of 2 x $10^4$ HAU for experimental inoculation per pig. The administered dose was verified by end point virus titration on PBMCs.

2.3 *Laboratory investigations*

*2.3.1 Processing of samples*

All swabs, were soaked with 1 ml serum-free cell culture medium and incubated for 1 hour at room temperature upon intense vortexing/ squeezing. Thereafter, standard oral and fecal swabs were decanted into micro-centrifuge tubes while salivettes were centrifuged for 1 min at 1000 *g* with subsequent decantation.

EDTA-blood samples were subjected to nucleic acid extractions immediately. Serum was obtained from native blood samples through centrifugation for 20 min at 2031 *g* at room temperature and was stored at -70°C until further usage. Tissue samples were aliquoted and stored at -70°C as well.

*2.3.2 Virus detection*

Viral DNA was extracted from all swab-, EDTA blood-, and tissue samples automatically on the King Fisher 96 Flex instrument (Thermo Scientific).

For swabs and EDTA-blood the MagAttract® Virus Mini M48 Kit (QIAGEN GmbH, Hilden, Germany) was employed according to the manufacturer's instructions. Tissue samples were extracted with the NucleoMag® VET kit for Viral RNA / DNA isolation from MACHEREY-NAGEL (Düren, North Rhine-Westphalia, Germany) according to manufacturer's instructions upon homogenization in 1 ml serum-free cell culture medium using a TissueLyser II (QIAGEN® GmbH).

All nucleic acids were subjected to ASFV-specific real-time PCR (qPCR) according to (King et al., 2003), performed with a Bio-Rad CFX 96 Real-Time Detection Systems (Bio-Rad, Hercules, CA, USA). Organ samples from reconvalescent pigs were additionally subjected to the commercial virotype ASFV real-time PCR (Qiagen Leipzig). In order to exclude other

pathogens, whole blood samples from ten selected animals were screened for Porcine reproductive and respiratory syndrome virus (PRRSV), Aujeszkey's disease virus (Suid herpesvirus 1 (SuHV1)), Foot-and-mouth disease virus (FMDV),Porcine circovirus type 2 (PCV2), Classical swine fever virus (CSFV), Porcine Epidemic Diarrhea Virus (PEDV) and Influenza A Virus (IAV) by (RT-) qPCR (Wernike et al., 2013, with slight modifications).

For detection of viable ASFV in serum and tissue samples, the hemadsorption test (HAT) was employed as previously descried (Pietschmann et al., 2015) on the basis of standard procedures (Carrascosa et al., 2011). In brief, 100 µl of sera/ tissue samples were added in duplicates to 200 µl PBMC derived macrophages seeded in 48-well microplates (5 x $10^6$ cells/µl) which were allowed to mature for three days. After 24 h, homologue erythrocytes were added in a 1 % dilution in sterile phosphate-buffered saline (PBS) (40 µl/ well). Subsequently, cultures were analysed for hemadsorption phenomena over a period of two to four days.

Similarly, the HAT was used for read-out of virus back-titration which was performed by end point titration of the diluted inoculation virus. For this purpose, 100 µl per virus dilution step and 20 µl of a 1 % homologue erythrocyte dilution were added to mature PBMC derived macrophages seeded in 96 well microplates (100 µl/well). All virus dilution steps were tested in quadruplicates.

### *2.3.3 Immune responses*

#### *2.3.3.1 Serology*

The INGEZIM PPA COMPAC ELISA (Ingenasa) was used for the detection of ASFV p73-specific antibodies in all serum samples. The test was carried out according to the manufacturer's protocol.

In addition, selected serum samples were screened for their neutralizing capacities against different test viruses (ASFV „Netherlands'86" and „Armenia'08") by neutralization assays (NPLA; neutralization peroxidase-linked antibody assay) on mature PBMC-derived macrophages. Each test virus was employed in two inoculation titers verified by back titrations, „Netherlands'86" was used at 100 and 400 HAU, „Armenia'08" at 10 and 100 HAU respectively. Sera were chosen from six pigs showing highly varying clinical courses, antibody ELISA -, qPCR - and HAT - results, ranging from acute -lethal in combination with negative or weak-positive ELISA-results, and highly positive virus detection-results to acute-transient pigs,

strongly positive in the ELISA-test and negative in all virus detection assays. All employed serum samples were heat-inactivated for 2 h at 56 °C prior to usage. For read - out, the HAT test was employed as described above.

## 3. Results

### *3.1. Clinical and pathological findings*

The (potential „carrier"-pigs/ „c-pigs") showed a wide range of clinical signs and courses upon experimental infection, ranging from acute-lethal over prolonged-lethal to complete clinical convalescence. An overview comprising the different disease courses, time points of death, and predominating clinical and pathological findings is given in table 1 and supporting information table 1.

In brief, all inoculated pigs developed clinical signs including fever. However, some animals were more likely infected through contact to sick pen-mates (see supporting information table 1) as they developed fever at a later time point and stayed negative in diagnostic tests for a prolonged period of time. Detailed information concerning the clinical score (CS) and fever development as well as the mortality are illustrated in supporting information fig. 1. In the majority of cases, first raises in CS and body temperatures were detected from four to six days post infection (dpi), and the latest from 24 dpi. The individual onset, duration and extent of fever are depicted in fig. 1 and supporting information table 1. During this acute phase, general depression, conjunctivitis, loss of appetite, reddened skin - especially on ears and around the eyes - huddling, obstipation as well as deficiencies in bearing and gait were predominating, accompanied by moderate to high fever up to 41.8 °C (see supporting information fig. 1). One animal developed central - nervous disorders five days after onset of fever and reached the humane endpoint. The acute phase lasted on average eight to ten days per animal (ranging from 4 – 19 days) with maximum CS ranging from 7 to 14 (see supporting information fig. 1). Thereby, three animals succumbed to infection, two on day 16 and one at 17 dpi. These courses are regarded as acute-lethal, and the major pathomorphological findings comprised hemorrhages in several organs, swollen and hemorrhagic peripheral lymph nodes (especially gastrohepatic and renal), petechiae in kidneys and lung edema.

Afterwards, an overall decline of clinical symptoms and fever was observed and only re-emerged sporadically in five pigs at later disease stages. Parallel to the clinical recovery, many pigs developed petechiae-like lesions and cyanotic areas which were either punctiform

(especially on ears or anogenital regions) or extensive and map-like (almost covering the whole body). These signs were observed for a couple of days from 13 dpi and a peak at 19 dpi (40.7 % affected pigs). The temporary recovery after the acute fever-phase was noticed in all but two of the remaining pigs. Those animals (assigned as „chronic-like“ in table 1) showed an early onset of clinical signs upon inoculation (four to five dpi). Despite of variable fever durations both showed clinical signs over a long period. Predominating signs comprised anorexia, wasting, gastro-intestinal signs (vomiting, bloody diarrhea, severe weight loss, growth retardation and polyarthritis, either associated with cyanotic joints or necrotic skin ulcers on the limbs). While one of them recovered from disease starting from 34 dpi, the other had to be euthanized at 28 dpi (#76). During necropsy of animal #76, several hemorrhagic, enlarged and absceding lymph nodes, interstitial pneumonia and ascites were found. At necropsy (128 dpi), the recovered animal (#66) showed an absceding mandibular lymph node, renal infarction (old scar, right), and edema of the periarticular tissues of the right tarsal joint. Subsequently to the phase of temporary recovery, a second CS-peak occurred (reaching maximum CS ranging from 5.5 to 14) between 21 to 34 dpi. During that time, six pigs (#83, 65, 70, 63, 86, 74) succumbed to infection within one to three days after a sudden onset of respiratory signs (severe tachypnea and dyspnea), a pronounced icterus (affecting the whole body surface and mucosal membranes), mostly in absence of fever (assigned as „prolonged-lethal“ course in table 1 and supporting information table 1). Most animals (54.2 %) were affected on day 27. A severe lung edema with a strong alveolar focus including alveolar hemorrhages, an extensive icterus as well as effusions in diverse body cavities were major pathological findings. Afterwards, all remaining c-pigs recovered from infection and showed no pathomorphological changes upon necropsy.

The timely correlation between CS and fever development along with the onset of skin petechiae/cyanosis and respiratory signs is displayed in supporting information fig. 2.

With regard to the („sentinel“-pigs/ „s-pigs“), no clinically or pathomorphological changes could be observed.

During the long-term trial, three C- pigs and one s-pig had to be euthanized due to unrelated causes, i.e. limb injuries (arthritis and tendovaginitis) or lesions from hierarchical fights (one c-pig at 86 dpi, two c-pigs at 128 dpi, one s-pig at 135 dpi) revealing either none or unspecific pathological signs during necropsy.

**Table 1: Overview about clinical courses, signs and pathomorphological lesions.**

Clinical courses upon experimental infection including the respective number of pigs ("no.") and the time point of death ("† dpi"). Pigs were assigned to different clinical courses according to their clinical and pathomorphological signs given in this table. The complement „1x" indicates that one of all pigs assigned to a clinical course had died at the mentioned time point ("† dpi"). „Early virus clearaence" is defined as negative ASFV-specific qPCR results prior 91 dpi and „late virus clearaence" after 91 dpi respectively.

| clinical course | | no. | † dpi | clinical signs | pathomorphological signs |
|---|---|---|---|---|---|
| *acute-lethal* | | 3 | 16 - 17 | early onset of fever, general depression, bearing/ gait dysfunctions, conjunctivitis, huddling, anorexia, reddened skin, central-nervous disorders (1x) | "typical"/ ASFV-related (hemorrhages in several organs, swollen and hemorrhagic peripheral lymph nodes, petechiae in kidneys, lung edema) |
| *prolonged-lethal* | | 5 | 24 - 32 | sudden onset of severe respiratory disorders (tachypnoe/ dyspnoe) leading to death within 1-3 days; intense icterus (whole skin, mucosal membranes) | alveolar lung edema and alveolar hemorrhages, thoracal/ pericardial/ abdominal effusions, icterus |
| *chronic-like (transient or lethal)* | | 2 | 28 (1x) | long-lasting clinical signs, recurrent fever, anorexia, vomiting, diarrhea, loss of body weight, growth retardation, polyarthritis, cyanotic joints and ears, skin ulcers | several hemorrhagic, enlarged and absceding lymph nodes, interstitial pneumonia, ascites |
| | | | (1x) | long-lasting clinical signs, recurrent fever, loss of body weight, vomiting, lameness, cyanotic skin areas | absceding mandibular lymph node, renal infarction (old scar, right side), periarthritis |
| *acute-transient* | *early virus clearance* | 9 | 86 (1x), 128 (1x) | complete clinical convalescence after an acute phase (1x severe limb injuries/ lameness, 1x injuries due to hierarchical fights) | none or variable unspecific signs, not indicative for ASFV |
| | *late virus clearance* | 10 | | complete clinical convalescence after an acute phase | |

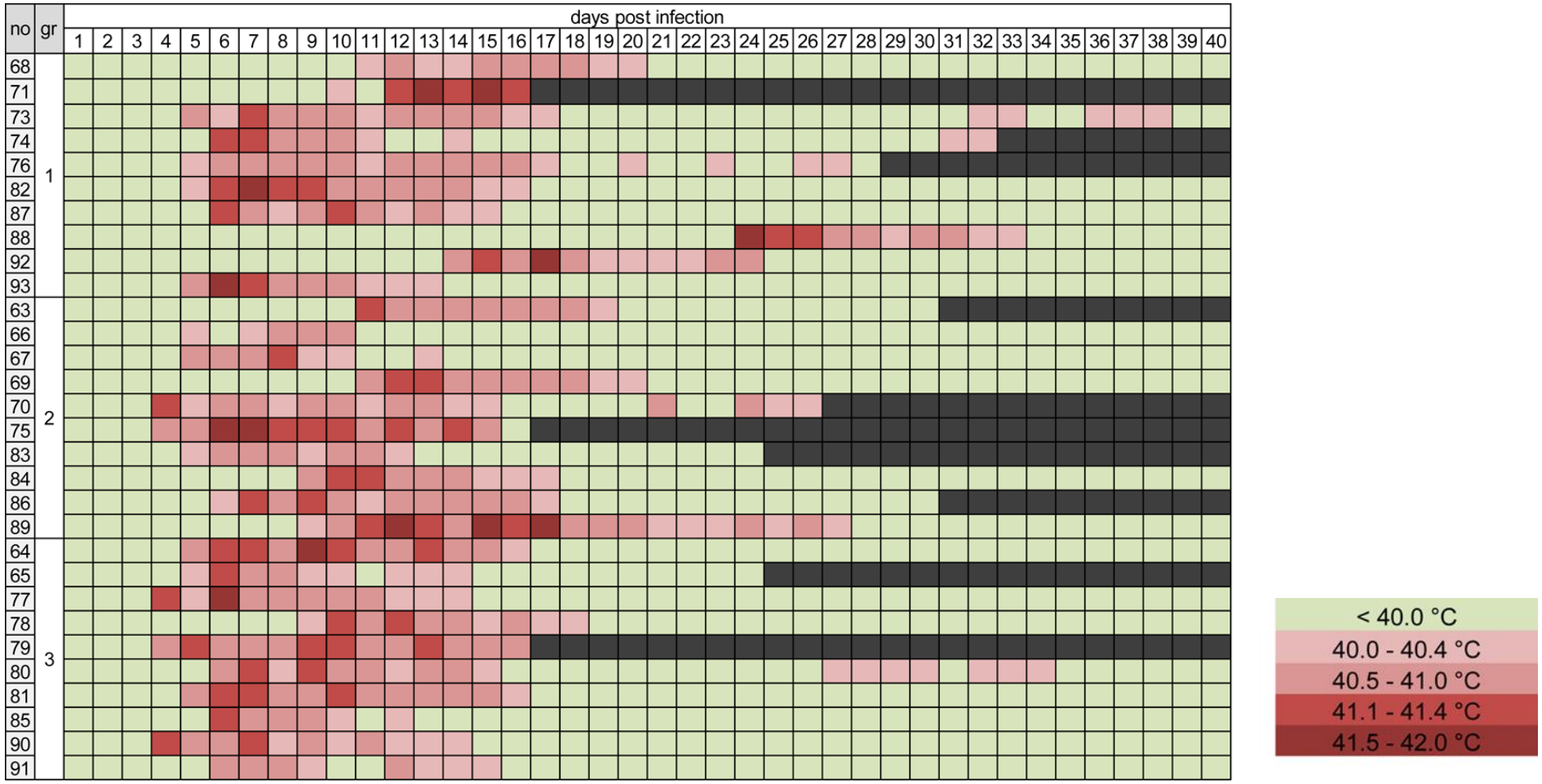



**Fig. 1. Development of rectal body temperatures from 0 to 40 dpi per animal ("no") and per group ("gr").**

No fever (< 40°C) is marked green, increasing body temperatures are marked in corresponding red intensity and deaths of pigs are marked in grey.

*3.2 Pathogen detection*

Prior to inoculation, all animals were tested negative in the ASFV specific qPCR and hemadsorption test (HAT). The back titration of the challenge virus verified the administered titer of 2 x $10^4$ hemadsorbing units (HAU) per pig.

ASFV genome and viable virus was found in blood samples from all experimentally infected animals over the course of the trial. First individual positive qPCR results in EDTA blood samples mainly correlated to the onset of clinical signs/fever, apart from seven cases in which ASFV genome was detected prior to the clinical onset (see supporting information table 1). The course of ASFV genome detection in whole blood samples by qPCR over the first 91 days is illustrated in fig. 2 along with the percentage of positive animals at each time point. First positive results were detected between 3 and 21 dpi. Starting from day three, the number of qPCR positive animals increased steadily until at 29 dpi all pigs were found positive. Highest genome loads were detected at 10 and 14 dpi by reaching maximum Cq values of 18 to 19. On day 29, a Cq decrease up to six was detected. At 42 dpi, first negative qPCR results were recorded in six pigs, seven at 48 dpi and ten at 63 dpi respectively. After 91 dpi, 52 % c-pigs were still positive qPCR results with decreased genome loads (Cq 28 - 30). The duration of detection is depicted in table 1.

In general, first ASFV detections by HAT in sera were in accordance with whole blood qPCR results (see fig. 3), apart from four exceptions in which positive HATs were detected earlier and one case showing a positive qPCR result prior to the first positive HAT reaction. Most positive HAT results were observed at 14 dpi when 28 out of 30 pigs were found positive. In contrast to the qPCR, first negative HAT results occurred at 29 dpi (two pigs). Subsequently, a rapid decline of positive HAT samples was observed until all surviving pigs were found negative from day 63 on (see fig. 3).

Virus shedding was observed in all kinds of swabs in each experimentally infected pig at different amounts as illustrated in fig. 4. Standard oral cotton swabs (OPF) revealed first weak positive qPCR results on day three (two pigs). At seven and ten dpi most OPFs were found positive with Cq-

values ranging from 30 to 41 (34 to 36 on average). The total number of positive swabs decreased from 14 dpi until on days 21 and 29 only one sample reacted positive. All OPFs were negative 42 dpi (see fig. 4). In the so-called salivettes, the course of ASFV genome detection was generally in line with OPFs by showing a peak of positive results at seven and ten dpi and decreasing numbers starting from 14 dpi, however with more positive results, higher genome loads (maximum Cq 28) and a longer detection period up to 63 dpi (see fig. 4). In fecal swabs, qPCR results were similar, showing a clear peak at ten dpi with Cq values of about 34 and the last positive result at 29 dpi (see fig. 4).

In contrast to the c-pigs, all s-pigs revealed negative qPCR and HAT results in all sample matrices including organs during the whole course of the trial.

With regard to tissue/organ samples, ASFV was only detected in pigs who succumbed to infection (see table 2 for detailed information concerning the genome loads and viral distribution). In brief, high and homogeneous genome loads were found in EDTA blood, spleen, and lungs, whereas rather variable genome loads were detected in tonsils and parotid gland samples. Mandibular lymph nodes showed high genome loads for almost all animals with two exceptions (reproducible negative results). Virus was isolated from spleen samples of all pigs that showed a lethal course of the disease.

All surviving c-pigs and all s-pigs were tested negative in the HAT and the routine PCR (King et al., 2003) including the spleen samples from fetuses of one s-pig. Using the commercial virotype ASFV PCR kit, a few lymph node samples were found positive with Cq values above 35. With one exception, the respective animals were the ones that were most likely infected through contact. None of these findings was accompanied by the detection of replicative virus.

In addition, ten pigs were screened for other pathogens as mentioned in 2.3.2 by qPCR. Those were selected according to the occurrence of petechiae-like lesions and cyanotic areas. No other pathogens apart from ASFV could be detected.

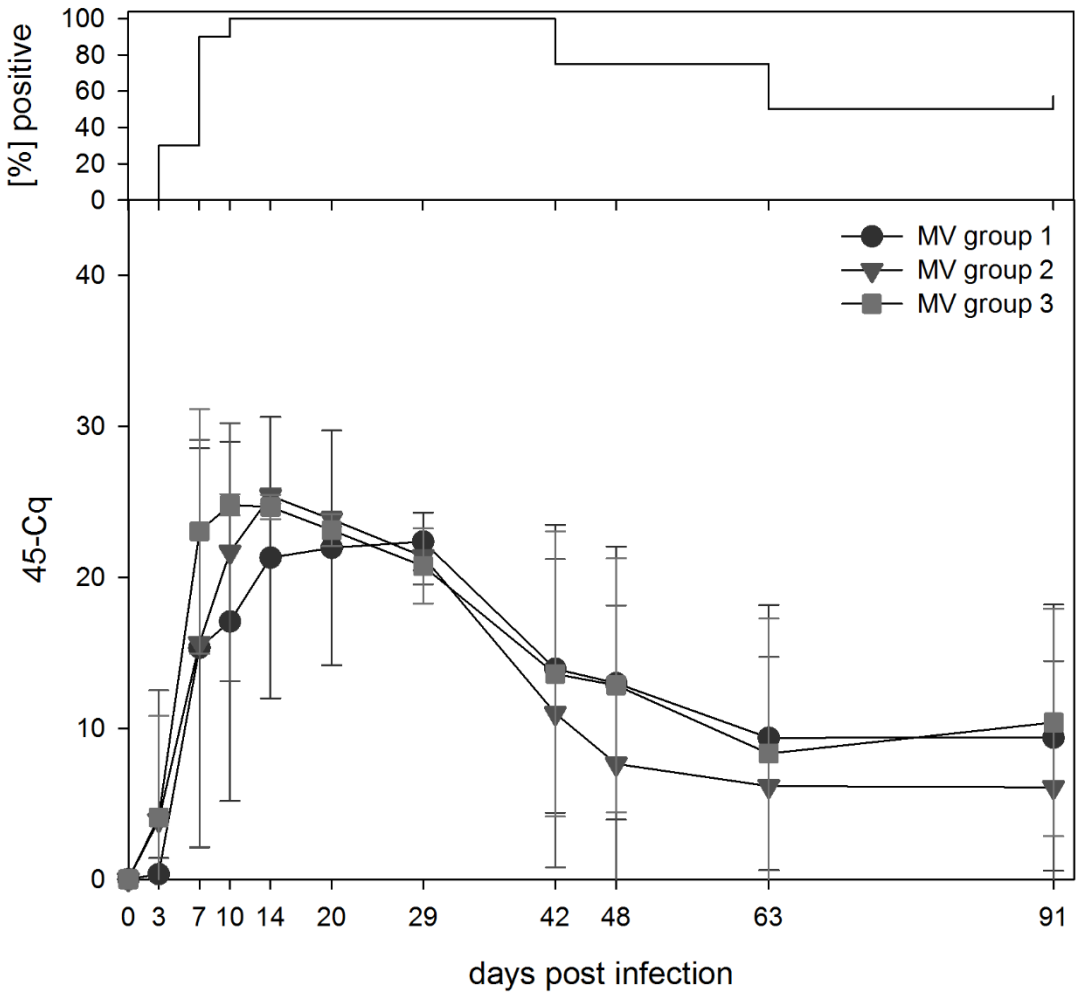



**Fig. 2. Group mean values ("MV") of ASFV specific qPCR results of EDTA blood samples of experimentally infected pigs (c-pigs) from 0 to 91 dpi.** Results are displayed as 45 – Cq. Error bars indicate standard deviations. Presented mean values were generated from all qPCR positive pigs per time point. The total percentage [%] of qPCR positive pigs is illustrated at the top ("[%] positive").

**Table 2: ASFV qPCR results from blood and tissue samples from lethal cases**

Results of ASFV specific qPCR (Cq values) from blood and tissues/ organs collected during necropsy from pigs succumbing to infection during the acute phase (16 – 34 dpi) are shown. Pigs were referred to their individual ear tag numbers ("pig no.") and ordered according to the time points of death ("† dpi"). „ln.mand" = mandibular lymph node

| pig no. | † dpi | blood | tonsil | parotis | spleen | lung | ln.mand. |
|---|---|---|---|---|---|---|---|
| **71** | 16 | 18.50 | 24.80 | 29.30 | 19.42 | 24.73 | 25.04 |
| **75** | 16 | 21.53 | 27.31 | 27.61 | 21.71 | 22.53 | 27.10 |
| **79** | 17 | 23.55 | 41.43 | 34.38 | 23.14 | 26.03 | 28.76 |
| **83** | 24 | 21.82 | 29.38 | 40.69 | 25.92 | 28.25 | 30.97 |
| **65** | 25 | 27.94 | 30.21 | 33.24 | 26.03 | 29.22 | 29.43 |
| **70** | 26 | 24.12 | no Cq | 31.44 | 21.60 | 26.13 | 28.01 |
| **76** | 28 | 22.73 | 43.11 | 32.01 | 24.13 | 27.35 | no Cq |
| **63** | 30 | 27.53 | 31.01 | 40.89 | 27.09 | 30.11 | 29.10 |
| **86** | 30 | 26.66 | 41.98 | 43.14 | 25.29 | 28.24 | no Cq |
| **74** | 34 | 27.27 | 33.91 | 33.05 | 25.80 | 30.21 | 30.49 |

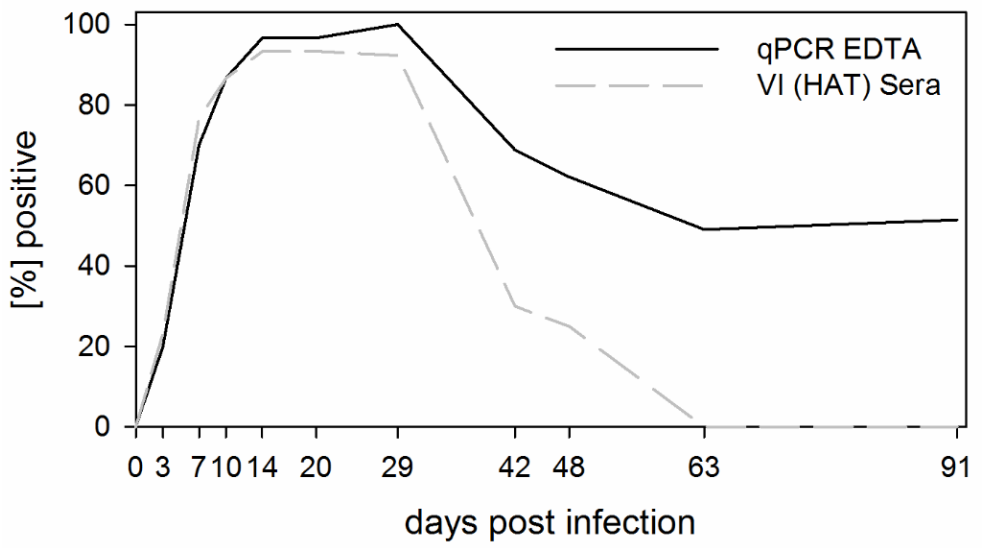



**Fig. 3. Comparison of ASFV genome detection by qPCR and viable ASFV detection by virus isolation ("VI") through hemadsorption test ("HAT").** The total amount of animals reacting positive in the corresponding assay is given as percentage of all surviving pigs at each time point. Both assays were performed on the basis of blood samples: EDTA blood was used for ASFV specific qPCR and sera for HAT respectively.

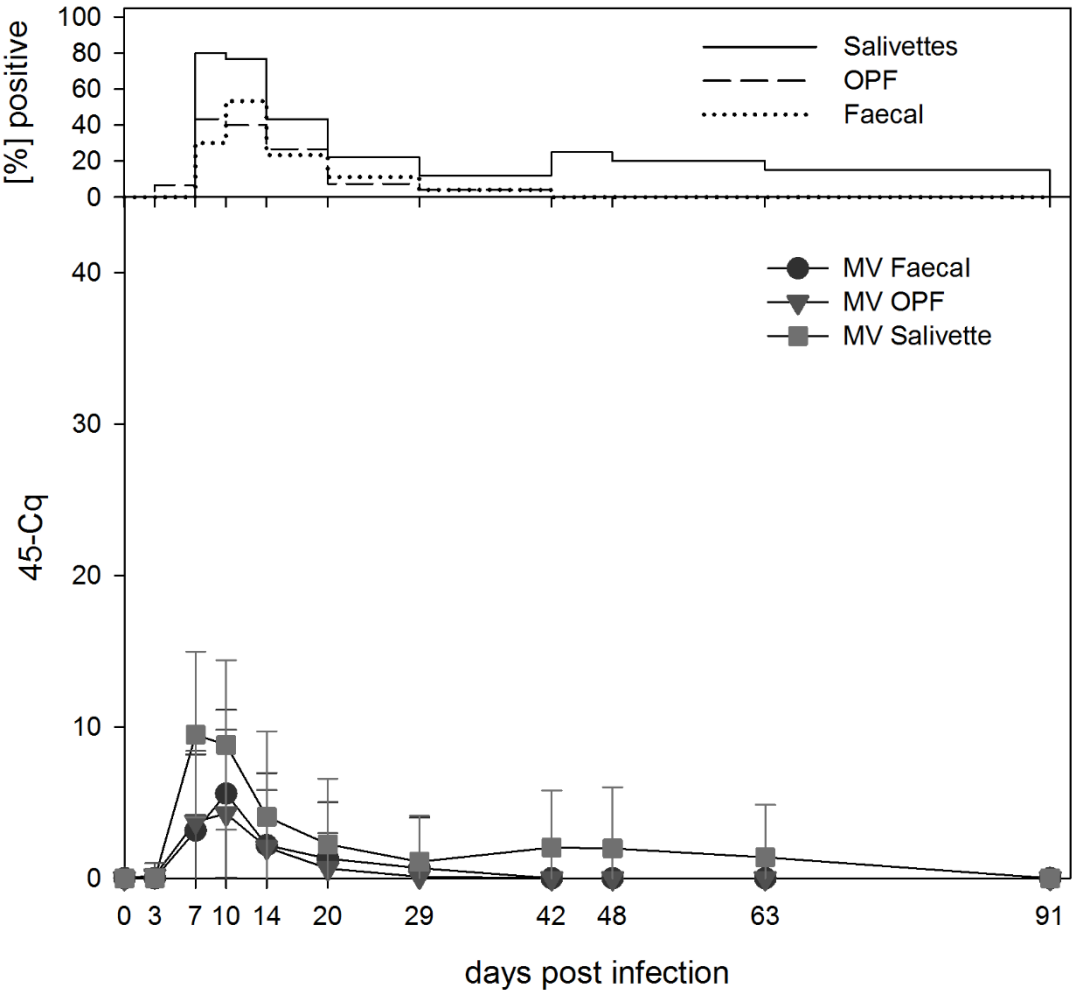



**Fig. 4. Overall mean values ("MV") of ASFV specific qPCR results of swab samples**: faecal swabs ("MV Faecal"), oropharyngeal swabs from the superficial oropharynx ("MV OPF"), oropharyngeal swabs from the deeper oropharynx ("MV Salivette") from all experimentally infected pigs (c-pigs) from 0 to 91 dpi. Results are displayed as 45 – Cq. Error bars indicate standard deviations. Presented mean values were generated from all qPCR positive swabs samples per time point. The total percentage [%] of qPCR positive swab samples from each kind of swab is illustrated at the top ("[%] positive").

*3.3 Immune responses*

*3.3.1 Serology*

After all pigs were shown to be free of ASFV-specific antibodies prior to the trial, ASFV p73-specific antibodies could be detected by ELISA in sera from all but one initially infected pigs four to nine days after the individual onset of clinical symptoms including fever. As illustrated in fig. 5, first doubtful (5) and positive (13) results were observed at ten dpi. Subsequently, a steady increase of positive animals and blocking values was detected (see fig. 5) until all pigs were found positive from 29 dpi on (blocking values 74% - 98%). The quantitative results kept increasing and reached 99 - 100 % in all c-pigs at 63 dpi (see fig.5). All surviving animals were still positive by the end of the trial.

No neutralizing capacities could be revealed for any of the ELISA-positive samples in the employed NPLAs on mature macrophages. The inoculation titers of the selected ASFV strains were confirmed by back titration. However, all HATs revealed equally infected wells in each dilution step in all serum samples. In s-pigs, no ASFV p73-specific antibodies could be detected throughout the whole trial.

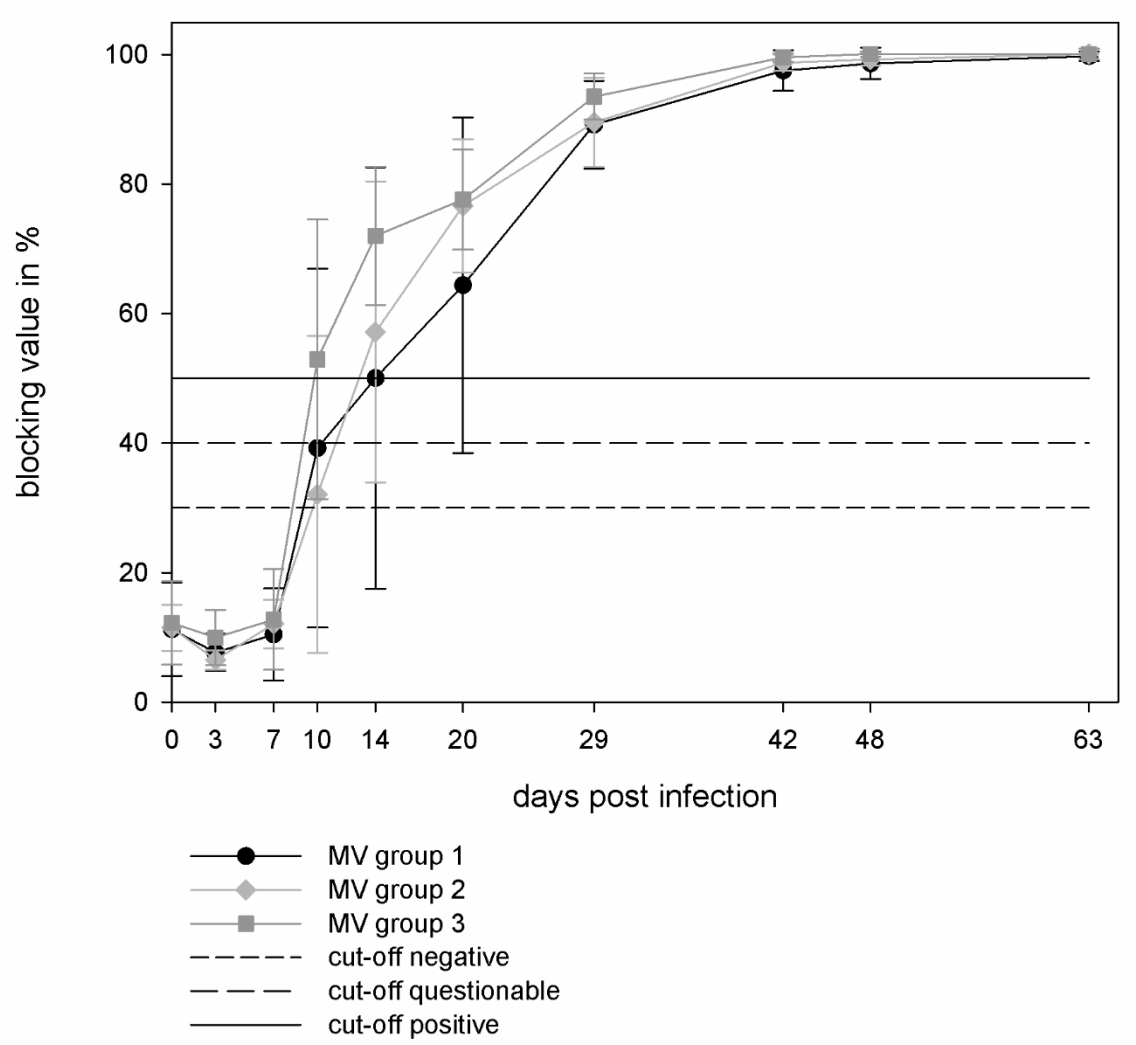



**Fig. 5. Mean values ("MV") of ASFV p73-specific antibody responses in serum samples from all infected groups** using the INGEZIM PPA COMPAC ELISA (Ingenasa). Results are given as blocking values in % from 0 – 63 dpi. ELISA cut-offs are illustrated as straight lines, bars indicate standard deviations.

## 4. Discussion

The possible ASFV carrier status of surviving animals, i.e. persistent or chronic infections that result in constant or remittent shedding and transmission of ASFV by apparently healthy convalescent animals, is most controversially discussed. While it may not play an important role under industrialized settings with a stamping out strategy of affected domestic pig herds, the fate of long-term survivors is of particular importance for wild boar populations where these animals will remain in the population for months or years. Should it hold true that viral persistence/long term chronicity is established in a considerable number of surviving animals, survivors would be a risk factor of utmost importance which could not be easily eliminated.

In the past, it was suggested that, apart from chronically infected pigs, also those which recovered from ASFV infection might further shed and transmit the virus (Wilkinson, 1984) and in this way cause new outbreaks (Bech-Nielsen et al., 1995). This would be of particular importance in areas with longer ASFV persistence for which decreased mortality and thus higher survival rates were reported frequently (Allaway et al., 1995, Fasina et al., 2010, Owolodun et al., 2010, Thomson, 1985).

Against this background, our study was undertaken to add the missing data by using a well-controlled dataset and a considerable number of survivors that resulted from experimental infection of domestic pigs with a moderately virulent ASFV strain of genotype I. Inoculation of 30 subadult fattening pigs resulted in 20 survivors of which 19 were commingled after 99 days with six sentinel pigs.

The trial ended after more than two months with the very clear results that no transmission occurred.

Moreover, all tissue samples taken at necropsy were negative for ASFV in virus isolation tests suggesting that the survivors were able to fully control and eliminate the virus. Routine PCR was also negative, and only very low genome loads could inconsistently be found by a commercial PCR kit.

Taken together, these results do not suggest a major role of survivors as persistently/chronically infected carriers. Given the experience with another moderately virulent ASFV strain from the Dominican Republic reported by Hamdy and Dardiri (Hamdy and Dardiri, 1984) that comingling of sentinels at 120 days post infection and hemadsorption tests gave negative results while transmission through direct injection of pooled blood still resulted in infection of susceptible animals, it cannot be completely excluded that the virus content

was just below the limit of detection in our test systems. However, in an epidemiological situation without tick involvement, direct parenteral inoculation is rather unlikely and for oral infection, virus titers >$10^4$ HAU are usually needed (McVicar, 1984). The ratio of viral titers needed for infection of a susceptible animal via the intramuscular/intravenous inoculation versus the oral/nasal route was reported to be 1 : 140.000 with less than 1 HAU for the parenteral route (McVicar, 1984). Even if we would take the low titers (10 HAU) that were sufficient in our hands to infect weak wild boar piglets by the oro-nasal route, it is still way above the detection limit (Pietschmann et al., 2015).

In terms of valid transfer of results and conclusions to the situation in wild boar, we do not expect major differences between domestic pigs and wild boar based on parallel trials that had very similar outcomes for both pig types (e.g. Pietschmann et al., 2015). Another point of discussion is the influence of strain type and virulence. Here, the data body is rather limited and may need additional input. However, neither the surviving wild boar in a study with a genotype II strain from Estonia (Nurmoja et al., 2017) nor the surviving wild boar of an infection study with a genotype I strain (Cossu et al., 1991, described by Bech-Nielsen et al., 1995) transmitted the virus to commingled sentinels.

A factor that was not covered by our experimental setting is immunosuppression. One could argue that the commingling stress was accompanied by slight immunosuppression, but this was not systematically assessed. Nevertheless, stress and immunosuppression were previously suggested to play a role in reactivation of virus from apparently healthy carriers (Wilkinson, 1984, Hamdy and Dardiri, 1984, Sanchez Botija, 1982) and this should be further studied in future trials.

Despite the above said, clearance took also a long time, i.e. >90 days for viral genome from blood (though viable virus was not detected from 63 dpi) and thus, we could speak of long-term persistence of the virus. In view of the fact that ASFV is considered to be associated to erythrocytes, wrapped safely into their membranes (Bastos et al., 2003, Gallardo et al., 2009), these results might be explained by the estimated maximum life span of porcine erythrocyte of about 65 to 85 days (Liebich, 2003). Also in Bluetongue virus (BTV) infection, erythrocyte association leads to long-term detectability (MacLachlan et al., 1994). Our results are also in line with the study reported by de Carvalho Ferreira et al. (2012) detecting ASFV „Netherlands´86“ genome in blood even after 70 dpi, but in contrast to this study also in oral

swabs over the whole trial. To further investigate the kinetics of virus potentially hidden in erythrocyte membrane folds, HAT could be performed on lysed blood samples in future trials. While differentiation of “long-term persistence”, “persistent infection” or “chronic infection” might be a matter scientific discussion, the long-term impact on affected regions is still not the same. True and live-long persistence could mean an almost uncontrollable situation in wild boar with recovered animals as loose cannon as mentioned above; whereas long-term persistence but final elimination of virus would mean that recovered animals are only a limited problem.

Our results are somewhat in contrast to recent suggestions (de Carvalho Ferreira et al., 2012, Gallardo et al., 2015b), but it has to be kept in mind that our study lasted much longer (165 days) and is in line with long-term detectability of ASFV in blood and deep oropharyngeal swab samples for up to 70 days. The detection of very limited numbers of viral genomes in some lymph node samples may need some further discussion although it was in no case accompanied by virus isolation, and limited to animals that were probably infected at a later time point.

In terms of risk assessment, one has to take the worst case scenario into consideration. Should the wild boar die from whatever other cause, the carcass could be a point of infection for susceptible animals. In this case, we have to assume oral infection rather than parenteral routes. In this case, the above said should be true and only a hypothetic reactivation would yield viral titers sufficient to infect another animal.

Seroconversion and the role and nature of antibodies is also a matter of constant debate. In our study, seroconversion was detected in all animals within four to nine days upon clinical onset except for one pig which had to be euthanized due to central nervous disorders within four days. Despite the fact that ELISA blocking values increased steadily up to 100 %, no neutralizing capacities could be found by neutralization assays on macrophage cultures, neither against the currently circulating „Armenia’08“ strain (genotype II) nor against the homologue isolate. This contrasts observations that transfer of serum antibodies but also colostral antibodies can confer a quite highy level of protection (Schlafer et al., 1984a, Schlafer et al., 1984b, Onisk et al., 1994). Also, studies with attenuated and recombinant viruses (Gomez-Puertas et al., 1995) have shown *in vitro* neutralization (reviewed by Escribano et al. 2013). In this respect, it has to be kept in mind that our assays were done with wild-type virus on macrophage cultures only. This assay looks for complete *in vitro* neutralization in a rather

diagnostic manner and does not evaluate beneficial responses in the host. Yet, in contrast to classical swine fever virus, where the antibodies are able to induce this complete block, ASFV was not blocked to a visible extent.

Generally, our study shows that clinical outcome and measurable antibody production (at least against p73) is not well correlated. In this context, future studies must target not only cellular responses but also reactions against other antigens.

## 5. Conclusion

Taken together, we did not see any evidence of a carrier status in animals surviving infection with a moderately virulent ASFV strain. The long-term detectability of viral genome in blood is not only a risk but also a chance for diagnosis. For at least 90 days, PCR will be able to detect the virus in infected pigs. The observed atypical clinical and pathomorphological lesions elucidate the urgent necessity of laboratory analyses in ASFV diagnosis and the need for awareness among farmers and veterinarians.

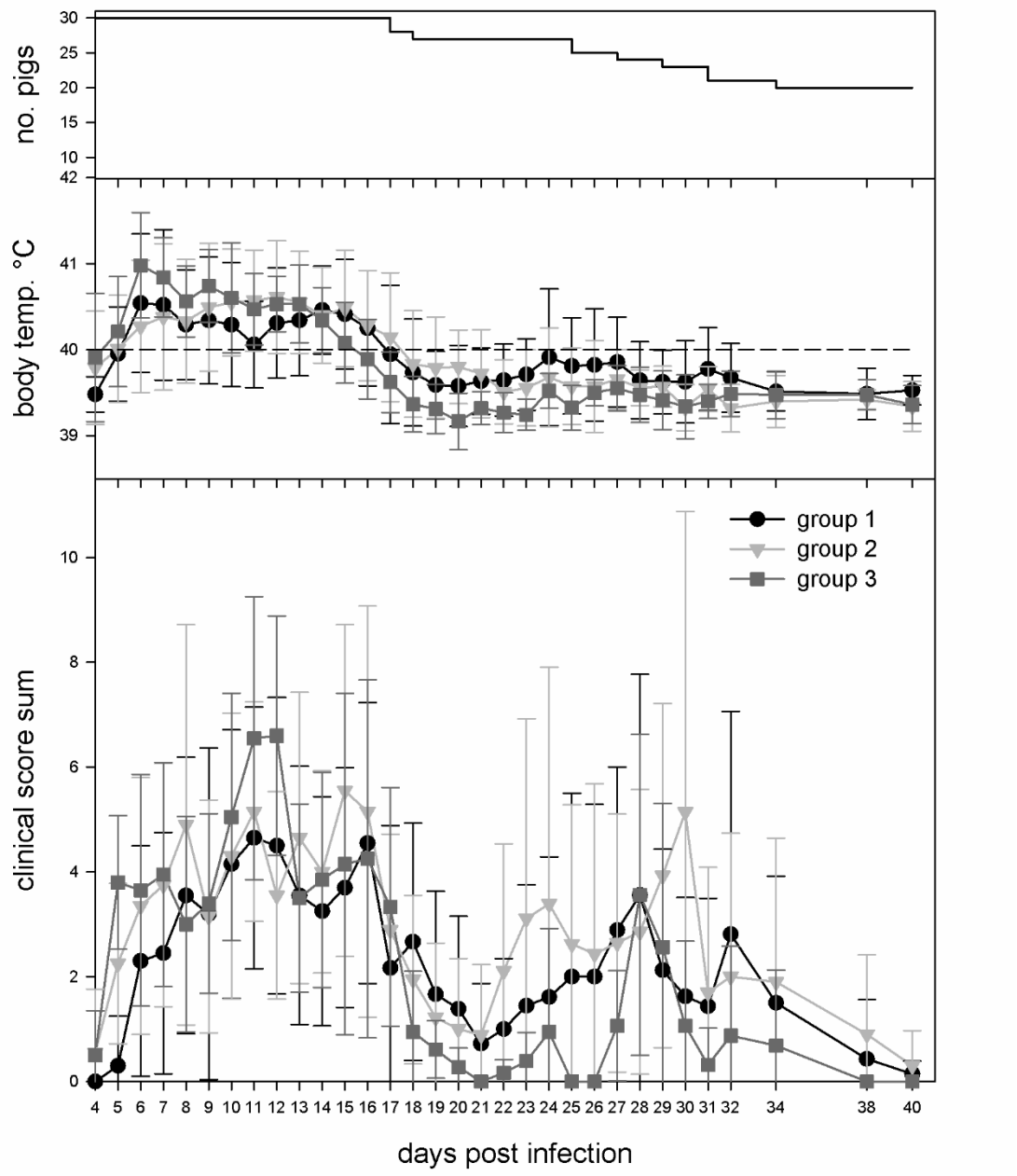
## 6. Conflict of interests

The authors declare no conflict of interest.

## 7. Acknowledgements

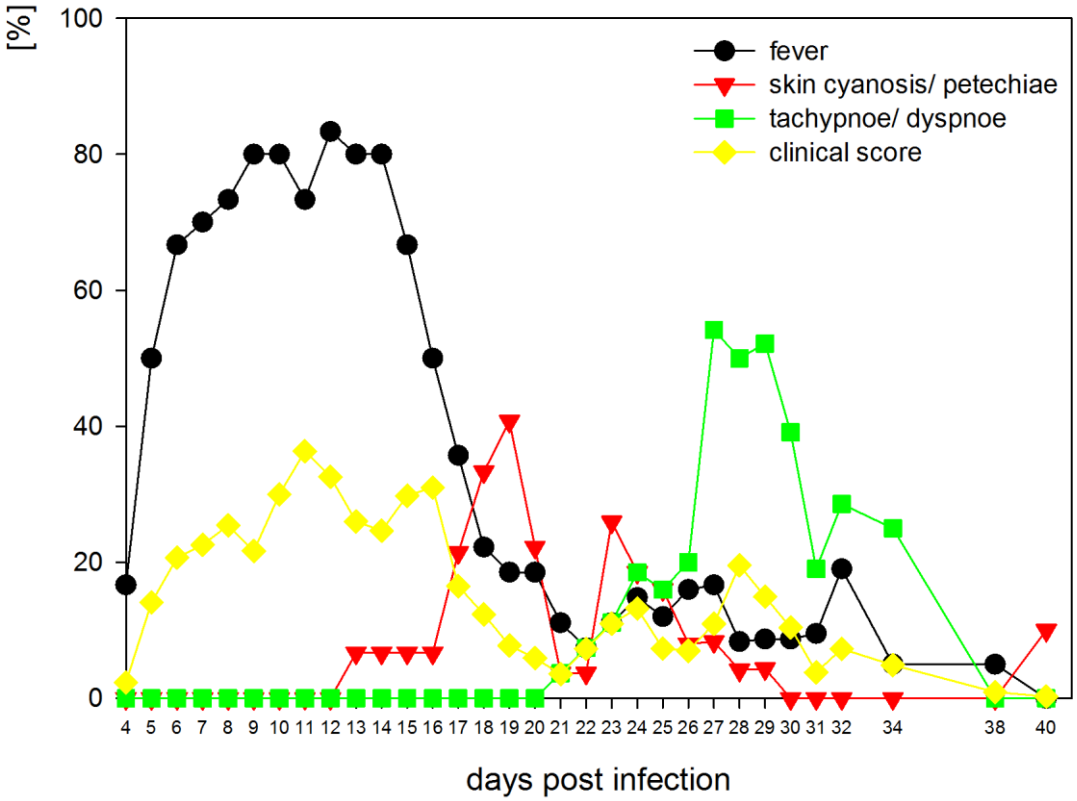
We would like to thank all animal caretakers and technicians involved in this study for their excellent work. Our special thank goes to Dr Bernd Hoffmann for sampling at bottleneck times. This work has been carried out as part of the interdisciplinary ASF Research Network at the FLI.

**Supporting information**




**Supporting information fig. 1.**

Daily summation of clinical parameters of experimentally infected pigs (c-pigs) during the acute phase. Top ("no. pigs"): survival of pigs is shown by presenting the total number at each time point; middle ("body temp. °C"): group mean values (mean value ± standard deviation) of rectal body temperatures; bottom ("clinical score sum"): group mean values (mean value ± standard deviation) of clinical score (CS) sums basing on the scoring system by Mittelholzer (2000). Error bars indicate standard deviations.




**Supporting information fig. 2.**

A summary of all vital parameters from all experimentally infected pigs (c-pigs) during the acute phase (0 – 40 dpi) comprising the onset of "fever" (defined as >40.0°C), sum of clinical score points ("clinical score"), numbers of pigs displaying respiratory findings ("tachypnoea/ dyspnoea") and cyanotic or petechial lesions ("skin cyanosis/ petechiae") respectively at each time point. All parameters are illustrated as percentage values. The "fever" curve illustrates the percentage of pigs displaying fever in correlationship to all living animals per time point (calculated as 100% per time point). The "clinical score" curve was calculated on the basis of a clinical sore sum of 14 which was defined as maximum end point criterion and therefore regarded as 100%. For illustration of occurrence of the clinical signs "tachypnoea/ dyspnoea" and "skin cyanosis/ petechiae" the number of pigs displaying the symptom in question was correlated to the total amount of living pigs at each time point as 100%.

**Supporting information Table 1:**

**Overview over disease courses, time points of death, and predominating clinical and pathological findings** Time points of ASFV positive qPCR results from blood, feces and OPF-swabs, positive virus isolation, detection of ASFV specific antibodies, fever and clinical disease course

| Animal | Course | Fever | Virus detection | Viral genome blood | Viral genome feces | Viral genome OPF | Antibody detection | Final day | Remarks |
|---|---|---|---|---|---|---|---|---|---|
| # 68 | T | 11 - 20 dpi | 10 - 29 dpi | 10 - 91 dpi | 14 - 20 dpi | 7 dpi | from 20 dpi | 164 | CI possible |
| # 71 | AL | 12 - 16 dpi<br>5 - 17 dpi | 14 dpi | 14 - 20 dpi | 14 dpi | 14 dpi | not detected | 16 | |
| # 73 | T | 32 - 33 dpi<br>36 - 38 dpi | 7 - 20 dpi | 7 - 29 dpi | 7 - 10 dpi | 7 - 42 dpi | from 10 dpi | 164 | |
| # 74 | PL | 6 - 11 dpi<br>31 - 32 dpi | from 7 dpi | 7 - 29 dpi | not detected | 10 and 29 dpi | from 10 dpi | 32 | |
| # 76 | PL | 5 - 17 dpi<br>26 - 27 dpi | from 7 dpi | 7 - 29 dpi | 7 - 10 dpi | 7 - 10 dpi<br>20 dpi | doubtful 10 dpi<br>positive from 14 dpi | 28 | chronic-like signs |
| # 82 | T | 5 - 16 dpi | 7 - 29 dpi | 7 - 91 dpi | 7 - 10 dpi | 7 - 10 dpi<br>48 dpi | from 10 dpi | 164 | |
| # 87 | T | 6 - 15 dpi | 7 - 29 dpi | 7 - 91 dpi | 7 - 10 dpi | 7 - 14 dpi<br>48 dpi | from 10 dpi | 164 | |
| # 88 | T | 24 - 33 dpi | 29 - 48 dpi | 29 - 91 | 29 dpi | 29 dpi | from 29 dpi | 164 | CI likely |
| # 92 | T | 14 - 24 dpi | 20 - 42 dpi | 14 - 91 dpi | 20 dpi | 20 and 63 dpi | from 20 dpi | 164 | CI possible |
| # 93 | T | 5 - 13 dpi | 7 - 29 dpi<br>48 dpi | 7 - 48 dpi | 10 dpi | 7 dpi | doubtful 10 dpi<br>positive from 14 dpi | 164 | |
| # 63 | PL | 11 - 19 dpi | from 10 dpi | 10 - 29 dpi | not detected | 7 - 14 dpi | from 20 dpi | 30 | CI possible |
| # 66 | T | 7 - 10 dpi | 3 - 29 dpi | 7 - 29 dpi | 10 - 14 dpi | 7 - 10 dpi | from 10 dpi | 128 | transient chronic-like signs<br>euthanized due to other causes |
| # 67 | T | 5 - 10 dpi | 7 - 29 dpi | 7 - 91 dpi | 10 dpi | 7 - 10 dpi | doubtful 10 dpi<br>positive from 14 dpi | 164 | |
| # 69 | T | 11 - 20 dpi | 14 - 29 dpi | 14 - 29 dpi | 14 dpi | 7 - 14 dpi | positive from 20 dpi | 164 | CI possible |
| # 70 | PL | 4 - 15 dpi<br>24 - 26 dpi | from 3 dpi | 14 - 29 dpi | 7 - 10 dpi<br>20 dpi | 7 - 14 dpi | positive from 10 dpi | 26 | |
| # 75 | AL | 4 - 15 dpi | from 3 dpi | 3 - 20 dpi | 7 - 10 dpi | 10 dpi | doubtful 10 dpi<br>positive from 14 dpi | 16 | |
| # 83 | PL | 5 - 12 dpi | from 7 dpi | 7 - 29 dpi | 7 - 10 dpi | 7 - 10 dpi<br>20 dpi | doubtful 10 dpi<br>positive from 14 dpi | 24 | |
| # 84 | T | 9 - 17 dpi | 3 - 48 dpi | 10 - 91 dpi | 10 dpi | 10 - 14 dpi<br>42 - 48 dpi | from 14 dpi | 164 | CI possible |
| # 86 | PL | 6 - 17 dpi | from 7 dpi | 7 - 29 dpi | not detected | 7 - 14 dpi | from 14 dpi | 30 | |
| # 89 | T | 9 - 27 dpi | 10 - 29 dpi | 10 - 42 dpi | not detected | 7 and 42 dpi | from 14 dpi | 164 | protracted clinical signs, CI possible |
| # 64 | T | 5 - 16 dpi | 7 - 29 dpi<br>48 dpi | 7 - 48 dpi | 10 dpi | 7 - 20 dpi | from 14 dpi | 164 | |
| # 65 | PL | 5 - 14 dpi | from 7 dpi | 7 - 29 dpi | 10 dpi | 7 - 10 dpi | from 10 dpi | 25 | |
| # 77 | T | 4 - 14 dpi | 3 - 48 dpi | 3 - 91 dpi | not detected | 7 - 10 dpi<br>42 dpi | from 10 dpi | 164 | |
| # 78 | T | 9 - 18 dpi | 7 - 29 dpi | 10 - 48 dpi | not detected | 7 - 10 dpi<br>48 dpi | from 14 dpi | 164 | CI possible |
| # 79 | AL | 4 - 16 dpi | from 7 dpi | 3 - 20 dpi | 10 dpi | 7 - 14 dpi | from 10 dpi | 164 | |
| # 80 | T | 6 - 15 dpi<br>27 - 30 dpi<br>32 - 34 dpi | 7 - 29 dpi | 7 - 29 dpi | not detected | 7 dpi | from 10 dpi | 164 | |
| # 81 | T | 5 - 16 dpi | 7 - 29 dpi | 7 - 91 dpi | 7 dpi | 7 - 20 dpi<br>63 dpi | from 10 dpi | 164 | |
| # 85 | T | 6 - 10 dpi | 7 - 20 dpi | 7 - 29 dpi | 7 - 10 dpi | 7 - 14 dpi | from 10 dpi | 128 | euthanized due to other causes |
| # 90 | T | 4 - 14 dpi | 3 - 48 dpi | 3 - 91 dpi | 14 dpi | 7 - 10 dpi<br>42 and 63 dpi | from 10 dpi | 164 | |
| # 91 | T | 6 - 9 dpi<br>12-15 dpi | 3 - 14 dpi<br>29 - 42 dpi | 7 - 91 dpi | 7 - 14 dpi | 7 - 14 dpi | from 14 dpi | 164 | |

PL = prolonged lethal
AL = acute lethal
T = transient
CI = contact infection
dpi = days post infection

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# 5 Discussion

Over the last decade, ASF has changed from an exotic disease of Sub-Saharan Africa to a considerable and tangible threat to pig industry in Central Europe. With its re-introduction into the European Union in 2014, the disease has apparently also found a fertile breeding ground in the abundant wild boar population. Based on previous experience on the Iberian Peninsula and Sardinia, wild boar were so far not considered as major and long-term reservoir for ASFV (Laddomada et al. 1994, Perez et al. 1998), and self-sustaining cycles were not anticipated (Efsa Panel on Animal Health and Welfare 2010). However, disease dynamics were completely different under the North-Eastern European conditions and long-lasting endemic cycles without domestic pig involvement were established in all affected countries once the virus was introduced into the wild boar population. Despite high virulence of the virus strains and considerable mortality among wild boar, these cycles are self-sustained over several years now. This leaves competent authorities, veterinary services and other decision makers at loss for reliable risk assessments and design and implementation of reasonable control measures. The first step for both risk assessment and strategy design is knowledge about critical factors of disease transmission and dynamics. A major part of the presented studies was therefore done to elucidate some of these factors. Among them was the integration of pragmatic sampling schemes into disease surveillance as basis for both diagnosis and epidemiological studies, the in-detail characterization of Estonian ASFV strains and the evaluation of possible carrier states of surviving animals.

## 5.1 Simplified tools for effective surveillance

Early disease detection as prerequisite for timely intervention is the main target in areas that are free of the disease but at risk. For this reason, all efforts have to be directed towards collection of representative and risk-based samples from the susceptible population. With regard to the wild boar population, this means intensification of passive surveillance by encouraging hunters and foresters to sample fallen wild boar and animals that show clinical signs when addressed for hunting. These efforts are currently carried out in all parts of Germany.

As sampling of fallen wild boar is not the main interest and duty of this group of persons, it should be on one hand as easy and convenient as possible, and on the other hand allow a reliable ASFV-detection. Thus, a compromise has to be found that makes sampling as

pragmatic as possible. Up to now, blood and/or organ samples (spleen if possible) were prescribed, or a larger bone for decomposed carcasses (World Organization for Animal Health (OIE) 2017). Taking these samples means often very close contact with the rotting carcass and necessitates opening it. This is both rather disgusting and bears the risk of contamination for the surroundings and the tools that are used to process the sample. In addition, proper collection and transport tubes are necessary. Regarding this issue, colleagues from the Estonian Veterinary and Food Laboratory, where all samples from hunted wild boar in Estonia have to be sent, report from blood samples arriving in recycled glass bottle or misused gloves (I. Nurmoja, personal communication, February 2017). These special "sampling tools" are difficult to transport and handle, and are usually highly contaminated from the outside. To avoid these severe sampling and handling problems, a convenient, clean and stable sampling tool is required. One approach would be sampling on filter papers or so-called FTA cards that allow immobilization and stabilization of nucleic acids. FTA cards dipped in bloody fluids, already showed good performance for pathogen detection even after long-term storage under tropical conditions without cooling (Michaud et al. 2007, Braae et al. 2015), but still with the disadvantages of direct contact with the carcass, need for a transport device and, to a lesser extent, contamination. For this reason, our study continued the validation of fast-drying GenoTube Livestock swabs that had shown very promising results for African swine fever virus genome and antibody detection in proof-of-concept studies(Blome et al. 2014, Petrov et al. 2014). These devices allow transport and storage without refrigeration directly in the swab receptacle. In detail, the presented study targeted further validation of both ASFV antibody and genome detection from these blood swabs and included a proof-of-concept part that employed point-of-care test systems for antibody detection (lateral flow assays) together with the swab. It was confirmed on both experimental samples and field samples of bad quality that reliable diagnosis is possible using the swab samples, and the point-of-care test proved to be highly robust. The use of the latter would need discussion, especially when it comes to the group of people that would be allowed to use it for the detection of a notifiable disease.
However, it has to be added that a disadvantage of the GenoTubes is the relatively high price compared to normal cotton swabs or blood collection tubes. However, if the simplified sampling technique could increase the number of samples within the passive surveillance and lead to an early disease detection, the financial effort would be very quickly relativized.

Taken together, one approach for the above mentioned compromise between practical handling and reliable diagnosis was found. For this reason, it was already included in the "Amtliche Methodensammlung" (Friedrich-Loeffler-Institut 2016) and is mentioned in the "Schweinepest-Monitoringverordnung" (SchwPestMonV 2016).

## 5.2 Understanding disease dynamics in the Baltics through characterization of recent ASFV strains from Estonia

In addition to the overall observation of endemic cycles, some regions showed peculiarities that needed to be explained. One example was the high seroprevalence in the North-Eastern part of Estonia, an EU-country which can serve as a model case especially for ASF-spread in the wild boar population. Positive animals occurred mainly in hunting bags and mortality was almost absent.

The present studies on the characterization of an Estonian ASFV strain isolated from wild boar out of an area with such particular disease dynamics, add to a deeper understanding about the epidemiological behaviour and evolution of the disease in wild boar populations. In the initial trial, the re-isolated strain did not act like a low or moderate one since only a single wild boar survived the acute phase of the infection. However, the survivor allowed to investigate the long-term fate of recovering animals and the transmission risk to commingled sentinel animals. In a nutshell, the obtained data supported the hypothesis, that recovering animals can get rid of infectious virus and already 56 days after the infection, when the animal was probably still positive for viral genome in the blood, the risk of transmission to other swine by direct contact is low. The further characterization of the Estonian strain in different pig breeds led to highly variable disease courses. Nonetheless, even though the animals were tested positive for viral genome in blood until the end of the trials, no viable virus could be isolated in their blood at the day of necropsy 36 days post inoculation. These findings highlight the importance of differentiation between detection of the viral genome and the detection of infectious virus when it comes to risk assessment. The viral particles attached to erythrocytes seem to lead to positive qPCR results over the whole live-span of these red cells (app. 86 days) (Bush et al. 1955), even if the virus is already inactivated. However, this was only a side effect of the performed study and it could still be discussed, that infectious virus hidden in the bone marrow (Carrillo et al. 1994) could be reactivated e.g. in a case of immunosuppression.

Main target of the studies presented here was the final characterization of the Estonian strain. The observed low lethality in domestic pigs and the still high virulence in wild boar associated with a genomic variation gave new insights into virus evolution on the one hand but raised new questions on the other. Literature actually indicates nearly identical disease courses in wild boar and domestic pigs when inoculated with the same ASF strain (Blome et al. 2013, Pietschmann et al. 2015). Studies comparing the immune systems of wild boar and domestic pigs in detail are lacking. The few available data demonstrate only minor immunogenetical differences between the two closely related species (Chen et al. 2013, Frantz et al. 2013). Other studies hint more to the direction of farmed swine being more susceptible to bacterial infections due to a downregulation of Major Histocompatibility Complex (MHC) II genes (Goedbloed et al. 2015). In case of ASF, downregulated MHC could eventually be of advantage for domestic pigs suffering the disease, since it could help to avoid excessive immune reactions leading to immunopathological lesions. After all, this is still highly speculative and needs further investigation. In addition, the trial-related immobilizing of the wild boar for sampling procedures during the acute phase of the disease and the hereby caused stress could have had an impact on the disease course. Thus, future experiments investigating this issue should be run with harmonized conditions. Nonetheless, the attenuated disease course in domestic swine and the field reports of higher survival rates in the area where the investigated strain comes from, (Nurmoja et al. 2017) support the thesis of a lowered virulence of the investigated variant strain. It was detectable in field samples from 2014 but could not be found in samples obtained a few years later. This raises the hypothesis, that attenuation might be an evolutionary drawback for ASFV strains in the absence of a potent arthropod vector. Naturally attenuated strains have been found in areas where soft ticks are part of disease transmission, like OUR T 88/3, isolated from Portuguese soft ticks (*Ornithodoros erraticus*) (Boinas et al. 2004, Sanchez-Cordon et al. 2016). If these potent vectors are not available, the picture might look different: Due to the limited shedding of the virus via saliva and feces (Guinat et al. 2014), and the lack of an competent arthropod vector, the virus seems to be trapped in the infected swine as long as it is alive. This could explain the temporally limited occurrence of the variant strain. For proof of principle, further studies are required and the transmission of the disease by other potential arthropod vectors such as biting midges and hard ticks needs to be excluded. In addition, it would be interesting to screen more field samples of regions in the vicinity to the place where the variant has been re-isolated, to see

where and when it came up for the first time. If the assumption of a disadvantage of attenuated strains holds true for areas lacking the tick-vector, it could be further hypothesized, that genetic variations and deletions altering the pathogenicity, occur more often than it was anticipated. For a more profound insight in the evolution of the complex viral genome of ASFV, further information is required especially in regard to genome-based similarities and differences of relevant ASFV strains. However, the possibility of randomly occurring attenuated strains, has to be taken in consideration regarding outbreaks in pig farms. The mild and unspecific clinical signs observed in the presented trials could easily go unnoticed under farming conditions. Responsible veterinarians and farmers in endemic, but also in still disease-free areas, should be sensitized for this problem and it might be reasonable to intensify surveillance measures and apply early differential diagnostic measures for diseased pigs.

## 5.3 Evaluation of possible carrier states in recovered animals

“Survivors are virus carriers for life” has been stated officially in the Scientific review on ASF of the European Food Safety Authority (EFSA) in 2009. The role of animals surviving the disease has been and is controversially discussed as the neutralizing capacity of ASFV-specific antibodies seems to be limited (Neilan et al. 2004) or at least debatable (Escribano et al. 2013). During the first wave of outbreaks in Europe it was anticipated, that pigs, recovering from the disease, stay persistently or chronically infected and shed the virus for the rest of their life acting as a source for new outbreaks (Bech-Nielsen et al. 1995). When the disease was re-introduced onto the European continent in 2007, the circulating strain was found to be highly virulent leading to mortalities up to 100% (Gabriel et al. 2011, Blome et al. 2012). Therefore, the question addressing the role of recovering animals acting as potential carriers was of minor importance. Nowadays, the picture changed and the disease situation can be regarded as endemic in the Baltic States such as Estonia (Nurmoja et al. 2017) indicating a higher number of wild boar surviving at least the acute phase of the disease. At this point, the question concerning the role of potential carriers is coming up again. If the assumption of “carriers for life” holds true, it creates catastrophic scenarios for the wild boar population in areas like Eastern Europe: Every single surviving wild boar could migrate and lead to new outbreaks by mingling with uninfected sounders. This would help the disease to spread fast and enter new naïve populations easily. During the early outbreaks on the European

continent, scientists argued with the role of warthogs: persistently infected young warthogs bitten by soft ticks maintain the natural wild cycle when infected ticks transmit the disease effectively to other naïve swine (Sanchez-Vizcaino et al. 2015). Epidemiological observations of re-occurring outbreaks in Portugal and Spain without a clear source of infection supported their idea of persistently infected survivors acting as transmission vehicles even in the absence of an arthropod vector (Wilkinson 1984). However, even in early studies (Sanchez-Botija 1982), isolation of infectious virus from tissues of pigs 5 to 10 month after infection failed. Direct injection of blood, obtained from recovered animals 120 days post infection, into sentinels resulted in acute disease courses while direct contact to the survivors at the same time point did not lead to infection (Hamdy et al. 1984). This parenteral infection route is important in areas with potent vectors, but not in Central Europe where soft ticks are lacking. More recent infection experiments with low virulent strains, where sentinels were commingled to recovered pigs 72 days post infection showed that ASF can be transmitted by direct contact up to this time point (Gallardo et al. 2015). Although this is no proof of long-term carrier status of recovered animals, the impact of carrier animals is expected to be high at least up to two months after the infection.

The here presented study on a moderately virulent ASFV strain of genotype I does not give any evidence for a long-term carrier status of recovered pigs. Sentinels commingled with the survivors 99 days post inoculation did not show any signs indicative for an ASFV infection and no viable virus could be isolated from any tissue sample of a surviving pig at the end of the trial, 165 days post inoculation. Therefore, the role of recovering animals acting as carriers seems to be temporarily limited to the first 2-3 months after the infection. This does not exclude that longer carrier states are possible, but it does not seem to be a major outcome. This knowledge helps to understand the epidemiological behaviour of the disease in the European wild boar population and contributes important data for decent risk analyses.

# 6 Outlook

The reliability of dry-swabs as alternative sampling strategy has been proven and the obtained data could contribute to a higher compliance in sampling fallen and hunted wild boar and thus, increase the chance to detect the disease as early as possible. However, the competent authorities need to be convinced about the advantages of the swab sampling and it will take time, until the modified workflows are broadly established as routine diagnostics.

The characterization of the Estonian ASFV strain served as an example for the evolution of the virus and brought many ends together for a general understanding of altered disease dynamics. Nonetheless, further investigation of differences and similarities within the immune response of domestic pigs and wild boar is required. In addition, control measures and awareness campaigns might need adjustment to make sure, farmers and veterinarians are familiar with the variable disease courses that can occur.

The role of recovered animals acting as carriers that contribute to the disease spread might have been overestimated in the past, as the performed study indicates. However, for a final risk assessment, further evaluation of the virus distribution in different kinds of tissues throughout the whole disease course is necessary.

# 6 Summary

African swine fever (ASF) is a notifiable animal disease with large impact on the pig farming sector and the wild boar population. As of March 2018, ASF is present in the Baltic Member States of the European Union, Poland, Czech Republic, and Romania, and it threatens to spread further towards Germany. Against this background, early warning strategies have to be implemented and possible control measures updated. Since an effective vaccine is lacking, a profound understanding of the pathogenesis and epidemiology is of utmost importance to design suitable control measures, especially for wild boar. While profound data is available for the sylvatic cycle of the disease in Africa, disease dynamics among European wild boar are far from being understood and remain to be elucidated. For this reason, the presented studies targeted the characterization of relevant virus strains and the further validation of suitable alternative sampling tools to generate diagnostic data. It was already known, that dry-swabs are suitable for pathogen and antibody detection of Classical and African swine fever but the applicability on field samples of poor quality and the combination with point-of-care test systems needed to be assessed. It could be shown, that dry-swabs minimize the risk of contamination and allow still decent diagnostic results without the need of a cooling device during transport and storage. This simple tool could encourage hunters and foresters to intensify the sampling of fallen wild boar which is urgently required for early disease detection. Furthermore, an Estonian field strain has been characterized in detail. Four animal trials including wild boar and different domestic pig breeds have been carried out for biological characterization. Rather variable disease courses with lethalities ranging from 0% (domestic pigs) to 100% (wild boar) have been observed. Full-genome sequencing revealed a large-scale mutation (14 kilo base pairs) in the re-isolated strain and screening of field samples demonstrated a locally and temporally limited circulation of the strain in north-eastern Estonia. In addition, a long-term animal trial provided data on the controversially discussed role of persistently or chronically infected survivor pigs acting as potential carriers. It could be shown, that swine surviving the acute phase of the disease, can recover completely and eliminate the virus. Under the experimental conditions no transmission to sentinel pigs, commingled 99 days after the inoculation was observed. This data contributed to the deeper understanding of the epidemiological behavior of ASF and could help to optimize disease control.

# 7 Zusammenfassung

Aufgrund ihrer enormen Bedeutung für die Schweinefleischindustrie und die Wildschweinpopulation gehört die Afrikanische Schweinepest (ASP) aktuell zu den gefürchtetsten anzeigepflichtigen Tierseuchen. Die Erkrankung tritt derzeit unter anderem im Baltikum, in Polen, Tschechien und Rumänien auf und eine Ausbreitung nach Deutschland ist zu befürchten. Da kein wirksamer Impfstoff zur Verfügung steht, kommt dem Verständnis der Pathogenese und Epidemiologie eine noch größere Bedeutung zu. Über den sylvatischen Zyklus der Erkrankung in Afrika ist schon relativ viel bekannt, während die Wiedereinschleppung der Tierseuche in die europäische Wildschweinpopulation viele neue Fragen aufwirft. Die vorgestellten Studien umfassen die Charakterisierung von relevanten Virusstämmen und die weiterführende Validierung von alternativen Beprobungsverfahren. Tupferproben waren als brauchbare Alternative für den Nachweis der klassischen und Afrikanischen Schweinepest bereits bekannt, aber die Belastbarkeit der Methode im Einsatz mit schlechter Probenqualität und die Kombination mit Sofortdiagnostika musste noch erprobt werden. Es konnte gezeigt werden, dass die verwendeten Tupfer, die ungekühlt transportiert werden können, eine simple und kontaminationsarme Probennahme erlauben. Dieses vereinfachte Verfahren könnte Jäger und Förster zur intensiveren Beprobung von Fallwild motivieren und so zu einer frühzeitigen Entdeckung der Tierseuche beitragen. Außerdem wurde ein estnisches Isolat des Virus der Afrikanischen Schweinepest im Detail untersucht. Zu diesem Zweck wurden zunächst vier Tierexperimente mit Haus- und Wildschweinen durchgeführt. Die beobachteten Krankheitsverläufe waren mit einer Letalität zwischen 0% (Hausschwein) und 100% (Wildschwein) sehr variabel. Eine Vollgenomsequenzierung zeigte eine große Mutation (14 Kilobasenpaare) im Genom des Re-Isolates. Untersuchte Feldproben konnten das temporär und regional begrenzte Vorkommen des Isolats im Nordosten Estlands belegen. Des Weiteren lieferte ein Langzeittierexperiment wichtige Daten zum Thema der persistent oder chronisch infizierten Schweine, die als potenzielle Überträger der Erkrankung kontrovers diskutiert werden. Es konnte gezeigt werden, dass Schweine, die die akute Phase der Erkrankung überleben, in der Lage sind, sich vollständig zu erholen und das Virus zu eliminieren. Unter experimentellen Bedingungen konnte 99 Tage nach der ASP-Infektion keine Übertragung der Tierseuche auf zugestallte Sentineltiere beobachtet werden. Diese Daten tragen zu einem tieferen Verständnis der ASP-Epidemiologie bei und können helfen die Tierseuchenbekämpfung zu optimieren

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# 9 Abbreviations

ASF African swine fever

ASFV African swine fever virus

CSF Classical swine fever

cq quantitation cycle

DNA deoxyribonucleic acid

dpi days post inoculation

EDTA ethylenediaminetetraacetic acid

EGFP Enhanced Green Fluorescent Protein

ELISA Enzyme-linked Immunosorbent Assay

EU European Union

FCS fetal calf serum

FLI Friedrich-Loeffler-Institut

GM-CSF granulocyte macrophage colony-stimulating factor

HAT hemadsorption test

HAU hemadsorbing units

kbp kilobase pair

LFD Lateral flow device

MGF multigene family

nm nanometer

NRL national reference laboratory

OIE World Organisation for Animal Health

ORF open reading frame

PBMC Peripheral Blood Mononuclear Cells

PBS phosphate buffered saline

PCR polymerase chain reaction

PP percent positive

PRRS Porcine Reproductive and Respiratory Syndrome

qPCR quantitative polymerase chain reaction

RNA ribonucleic acid

VFL Estonian Veterinary and Food Laboratory

VP viral protein

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*Christopher McCandless*

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