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

Towards licensing of a vaccine candidate against African swine fever and recent advances in surveillance and diagnosis of the disease in Germany

von Paul Jörg Deutschmann

aus Jena

München 2023

Aus dem Veterinärwissenschaftlichen Department der Tierärztlichen Fakultät

der Ludwig-Maximilians-Universität München

Lehrstuhl für Virologie

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

Angefertigt am Institut für Virusdiagnostik des Friedrich-Loeffler-Instituts,

Bundesforschungsinstitut für Tiergesundheit, Insel Riems

Mentor: Prof. Dr. Martin Beer

Gedruckt mit der Genehmigung der Tierärztlichen Fakultät

der Ludwig-Maximilians-Universität München

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

Berichterstatter: Univ.-Prof. Dr. Dr. h.c. Gerd Sutter

Korreferent/en: Priv.-Doz. Dr. Matthias Eddicks

Tag der Promotion: 11. Februar 2023

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

Folgende wissenschaftliche Arbeiten sind in dieser Dissertationsschrift enthalten:

-Deutschmann P., T. Carrau , J. Sehl-Ewert, Jan H. Forth, M. Beer and S. Blome (2022). "Taking a promising vaccine candidate further: Efficacy of ASFV-G-ΔMGF after intramuscular vaccination of domestic pigs and oral vaccination of wild boar".
Pathogens, doi.org/10.3390/pathogens11090996

-Deutschmann P., J.-H. Forth, J. Sehl-Ewert, T. Carrau, M. Beer and S. Blome (2022). "Safety and genetic stability of African swine fever virus vaccine candidate "ASFV-G-ΔMGF" in an in vivo "reversion to virulence" study".
Submitted to NPJ Vaccines, doi.org/10.21203/rs.3.rs-1922286/v1

-Forth, J.H., S. Calvelage, M. Fischer, J: Hellert, J. Sehl-Ewert, H. Roszyk, P. Deutschmann, A. Reichold, M. Lange, H.-H. Thulke, C. Sauter-Louis, D. Höper, M. Beer, S. Blome. (2022) "African swine fever virus – variants on the rise".
Emerging Microbes & Infections, doi.org/10.1101/2022.09.07.506908

-Sehl-Ewert, J., P. Deutschmann, A. Breithaupt, S. Blome. (2022) "Pathology of African Swine Fever in Wild Boar Carcasses Naturally Infected with German Virus Variants".
Pathogens, doi.org/10.3390/pathogens11111386

-Pikalo, J., P. Deutschmann, M. Fischer, H. Roszyk, M. Beer and S. Blome (2021). "African Swine Fever Laboratory Diagnosis-Lessons Learned from Recent Animal Trials".
Pathogens, doi.org/10.3390/pathogens10020177

-Deutschmann P., J. Pikalo, M. Beer and S. Blome (2021). "Lateral flow assays for the detection of African swine fever virus antigen are not fit for field diagnosis of wild boar carcasses".
Transboundary and Emerging Diseases, doi.org/10.1111/tbed.14248

Weitere Arbeiten, die nicht in der Dissertationsschrift enthalten sind:

-Roszyk H, Franzke K, Breithaupt A, Deutschmann P, Pikalo J, Carrau T, Blome S, Sehl-Ewert J. (2021) “The Role of Male Reproductive Organs in the Transmission of African Swine Fever-Implications for Transmission”.
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-Pikalo J, Schoder ME, Sehl-Ewert J, Breithaupt A, Cay AB, Lhoëst C, van Campe W, Mostin L, Deutschmann P, Roszyk H, Beer M, Blome S, Tignon M. (2021) “Towards Efficient Early Warning: Pathobiology of African Swine Fever Virus "Belgium 2018/1" in Domestic Pigs of Different Age Classes”.
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-Tanneberger F, Abd El Wahed A, Fischer M, Deutschmann P, Roszyk H, Carrau T, Blome S, Truyen U. (2022) “Efficacy of Liming Forest Soil in the Context of African Swine Fever Virus”.
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-Pikalo J, Carrau T, Deutschmann P, Fischer M, Schlottau K, Beer M, Blome S. (2022) “Performance Characteristics of Real-Time PCRs for African Swine Fever Virus Genome Detection-Comparison of Twelve Kits to an OIE-Recommended Method”.
Viruses, doi.org/10.3390/v14020220

Contents

1. Abbreviations

ASF	African swine fever
ASFV	African swine fever virus
ASP	Afrikanische Schweinepest
ASPV	Virus der Afrikanischen Schweinepest
BAR	Barnim
CSF	Classical swine fever
CSFV	Classical swine fever virus
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme linked immunodsorbent assay
EMA	European Medical Agency
EU	European Union
FLI	Friedrich-Loeffler-Institut
LAMP	loop-mediated isothermal amplification
LAV	live attenuated vaccine
MGF	multi gene family
MOL	Märkisch-Oderland
NRL	National Reference Laboratory
PCR	polymerase chain reaction
PCVAD	Porcine circovirus associated disease
PRRS	Porcine reproductive and respiratory syndrome
qPCR	real-time polymerase chain reaction
RNA	ribonucleic acid
SARS-CoV2	Severe acute respiratory syndrome coronavirus type 2
SPN	Spree-Neiße
UM	Uckermark
VICH	Veterinary International Conference on Harmonization
WOAH	World Organization for Animal Health

2. Introduction

African swine fever (ASF) has evolved into a major infectious threat to domestic and wild suid populations. It is caused by ASF virus (ASFV), a large and complex DNA virus, and often leads to high lethality in domestic pigs and wild suids outside Africa. The disease is notifiable according to the World Organization for Animal Health (WOAH) and in accordance with that, strict measures are applied in the European Union for ASF prevention and outbreak containment. Originally native to sub-Saharan Africa, a genotype II strain of ASFV was introduced to Georgia in 2007. A panzootic spread of ASFV through large parts of Europe, Asia and recently to the Americas followed that killed millions of pigs in the past years. The virus reached Germany in September 2020 and still could not be eradicated from the wild boar population since then, with several introductions into pig farms on the record. Successful eradication was reported from other European countries after punctual entries, but Germany is experiencing a frontline introduction along the eastern border. The recent history of the German epizootic indicates that under these circumstances, eradication may not be successful with the available measures, i.e. fencing, carcass search and removal, and reduction of the wild boar population through hunting and trapping. Consequently, we need to optimize the strategies already at hand and close the big gap in the fight against ASFV, the lack of a safe and efficacious vaccine for licensing.

Oral immunization could aid the protection of wild boar populations, which act as the reservoir for ASFV in Europe, and protect domestic pigs by lowering the infectious pressure in the environment. A small number of promising vaccine candidates has emerged after first proof-of-concept experiments, one of them is “ASFV-G-ΔMGF”. In two studies, we have taken the latter live attenuated vaccine candidate beyond the proof-of-concept phase and towards commercial licensing, assessing the efficacy upon intramuscular immunization of domestic pigs and oral vaccination of wild boar, exploring prospects to scale up vaccine production and investigating safety in an *in vivo* reversion to virulence study.

Integral part of all control actions, with or without the use of vaccines, is reliable surveillance to inform on disease dynamics and success of control measures. To this end, virus strains involved in the epidemic must be characterized. Therefore, in one of the studies included in this work,

ASFV isolates from Germany were genomically characterized, and the identified variants of ASFV-Germany were clustered to geographic areas. Indications for possible differences in virulence were investigated in a pathological study with infected wild boar carcasses from the field.

And finally, in the European wild boar transmission cycle of ASFV, contact to carcasses plays an important role. Removal of succumbed animals is therefore key for effective disease control and reliable methods for early detection are required. The same applies to the pig sector, where timely implementation of control measures is of utmost importance to avoid further spread of the disease after introductions. Therefore, the prospects of a lateral flow device for ASFV point of care detection in carcasses were evaluated, and the suitability of different sample matrices for ASFV laboratory diagnosis was investigated, with a focus on reliability, early detection and non-invasive sampling.

3. Review of Literature

3.1 Virus taxonomy, morphology, and genome

African swine fever virus (ASFV) belongs to the genus *Asfivirus* in the *Asfarviridae* family (Alonso et al., 2018), which is included in the order of *Asfuvirales* in the class of *Pokkesviricetes*. It is the only DNA virus that is classified as an arbovirus (Sanchez-Vizcaino, Mur, Bastos, & Penrith, 2015) due to the involvement of *Ornithodoros* ticks as competent vectors within the sylvatic cycle (Mary Louise Penrith & Kivaria, 2022). The virion shows a round shape with icosahedral symmetry (T=189-217) and a quite large size of 175 – 215 nm in diameter. The viral particle comprises several layers: genomic DNA and associated proteins form the nucleoid in the 70-100 nm wide core. The core is surrounded by the core shell, which represents the inner protein layer with a T = 19 capsid. Outwards, these structures are covered by the inner and outer capsid, which are each enveloped by a lipid membrane (inner and outer membrane). The outer envelope is acquired by the host cell after budding (Andres, Charro, Matamoros, Dillard, & Abrescia, 2020; Salas & Andrés, 2012).

The viral genome consists of linear double stranded DNA and is about 170 to 193 kilobase pairs (kbp) long. It contains 151 to 167 open reading frames (ORFs) with a conserved region of about 125 kbp in the center of the genome and variable ends (L. K. D. A. G. C. Dixon, Christopher L. Netherton, Chris Upton, 2013). Among these variable ends, the genome encodes for five so-called multi gene families (MGFs). Fifty-two structural proteins and more than one hundred non-structural proteins are reported (Salas & Andrés, 2012). Many non-structural proteins are required for virus replication. In addition, functions within the complex viral evasion of host immune responses - such as type I interferon and cell death regulation - are described (Reis, Netherton, & Dixon, 2017). Still, for about half of the genes of ASFV, nothing is known about their function (Alejo, Matamoros, Guerra, & Andres, 2018). The p72 is the major structural capsid protein and provides a basis for the differentiation between all 24 ASFV genotypes (Bastos et al., 2003; Quembo, Jori, Vosloo, & Heath, 2018). The encoding gene B646L is highly conserved, making p72 an often-used target for diagnostic purposes (D. P. King et al., 2003; Pastor, Arias, & Escribano, 1990). A protein that is part of the outer envelope is CD2v (encoded by the EP402R gene) (Rodriguez, Yanez, Almazan, Vinuela, & Rodriguez, 1993), which is also the only protein

found on the surface of extracellular virions (Alejo et al., 2018). CD2v, along with pEP153R, are not essential for intracellular replication, but are required for the binding of infected monocytes to erythrocytes in the blood (hemadsorption) (Borca et al., 1998; Rodriguez et al., 1993). This phenomenon is probably relevant for pathogenesis in the vertebrate hosts and replication in the soft tick (R. J. Rowlands, Duarte, Boinas, Hutchings, & Dixon, 2009). Modifications in the genes encoding for CD2v and pEP153R can lead to non-hemadsorbing ASFV strains, which have naturally occurred in the field (Gallardo et al., 2019).

Viral proteins can be divided by their time of appearance after infection of the host cell. The p30 protein, for instance, is found abundantly at an early phase of the infection, leading to strong antibody response to this highly immunogenic protein (Afonso et al., 1992). Other examples of early proteins are p15 and p22 (Alejo et al., 2018). The previously described p72 as well as p54, a structural protein with an essential role in morphogenesis, are examples for proteins of the later phases (Y. Wang et al., 2021). Protein p72 is also one of the highly immunogenic proteins within ASFV replication and therefore often targeted in indirect diagnostic systems (Liu et al., 2019).

Primary replication takes place in the cells of the mononuclear-phagocytic system, which are entered through clathrin-mediated and dynamin-dependent endocytosis or macropinocytosis (Galindo et al., 2015; Hernaez & Alonso, 2010; Sanchez et al., 2012).

3.2 Clinical presentation, transmission, and pathogenesis

African swine fever can result in variable clinical signs and disease courses. While some strains lead to mild or even clinically inapparent forms, most isolates are virulent and cause a disease that is usually deadly for Eurasian wild boar and domestic pigs (*S. scrofa* and *S. scrofa domesticus*). In contrast, in pig species native to sub-Saharan Africa, e.g., warthogs (genus *Phacochoerus*) and bushpigs (*Potamochoerus larvatus*), the disease is usually clinically inapparent, but viremia is reported (Montgomery, 1921; Oura, Powell, Anderson, & Parkhouse, 1998; Thomson, 1985). African swine fever is characterized primarily by the occurrence of fever (hence the name) and can lead to a broad range of clinical signs, usually beginning with a reduction in liveliness and appetite. In an acute course, after an incubation time of usually 2 to 7 days (J. Pikalo, Zani, Huhr,

Beer, & Blome, 2019; Sanchez-Cordon et al., 2019), anorexia and apathia are observed in most animals and clinical signs worsen throughout the disease course. Respiratory distress, cyanosis around the ears or eyes, insecurities in gait and diarrhoea are also frequently observed. In general, the number and severity of clinical signs tends to increase until death at 7 to 10 days after infection with highly virulent strains. The disease cannot be clinically differentiated from Classical swine fever (CSF), which is interesting because the latter is caused by a non-related RNA virus of the species *Pestivirus C* of the genus *Pestivirus*, family *Flaviviridae (Simmonds et al., 2017)*. Most known ASFV strains, including the genotype II strains involved in the current panzootic, lead to the previously described acute form (Blome, Gabriel, Dietze, Breithaupt, & Beer, 2012; Gabriel et al., 2011; Guinat, Gubbins, et al., 2016; Pietschmann et al., 2015; J. Pikalo et al., 2020). However, highly virulent strains can cause death in a peracute course without any clinical signs (Blome, Gabriel, & Beer, 2013). On the other hand, chronic infections are reported, especially after mild or primarily inapparent infections (Sun et al., 2021). Clinically, these are usually characterized by lameness, reduced weight gain and reproduction rates and increased susceptibility to bacterial infections (Gallardo et al., 2015). No influence of age or sex of pigs is proven for the outcome of infections with highly virulent strains, however, indications for an influence of the hygienic status of animals were recently reported (Radulovic et al., 2022).

In the absence of *Ornithodoros* ticks as in Europe, the virus normally enters the body via the oral or the oronasal route. This route of infection proved to be significantly less efficient but also more variable than parenteral transmission. While McVicar (1984) assumes about 10^4 infectious units are needed for an oronasal infection, other studies showed that significantly lower doses (even below 10) can be sufficient (Pietschmann et al., 2015), especially when the virus is ingested in liquids (Niederwerder et al., 2019). There are indications that age and health status of the animal have an impact on this otherwise statistical event of successful infection. As to be expected for an arbovirus, blood was proven an especially effective agent for transmission (Guinat, Gogin, et al., 2016; J. Pikalo et al., 2019). Further, infected animals shed the virus via all body secretions and saliva, nasal discharge, urine and feces can also play a role in transmission, however less effectively due to significantly lower viral load than in blood (Gabriel et al., 2011; Petrov, Forth, Zani, Beer, & Blome, 2018; Pietschmann et al., 2015).

For the highly virulent ASFV strains circulating in wild boar populations of Europe, infection is sustained by the so-called boar-habitat infection cycle (Chenais, Ståhl, Guberti, & Depner, 2018; Probst, Globig, Knoll, Conraths, & Depner, 2017). The disease usually leads to a rather quick death of infected animals, and carcasses can be infectious for a very long time under favourable conditions due to the high tenacity of the virus. Contact of wild boar to succumbed conspecifics can lead to infection of novel individuals and maintenance of the infectious cycle. This model of disease dynamics theorizes the occurrence of carcasses as an important factor for transmission, however it is known that direct contact of naive with diseased individuals can be sufficient for transmission. The exact role of the different infection routes in the European wild boar scenario is not known (Chenais et al., 2019).

After infection, primary replication takes place in lymphatic tissues of the pharynx and the nose, in the tonsil and in regional lymph nodes (Greig, 1972). Viremia then leads to generalization with a particular high replication in organs with high presence of monocytes or lymphocytes, as in the spleen (J. Pikalo et al., 2019). In blood, 90% of the virus is associated with erythrocytes for hemadsorbing strains (McVicar, 1984), forming an ideal transport vessel for this in its historically endemic area arthropod-borne virus. High viral presence in blood, loss of lymphocytes and the excessive activation of proinflammatory signal pathways ("cytokine storm") are postulated to ultimately lead to hemolysis and impaired hemostatis in infected animals, contributing to the clinical picture of a hemorraghic fever (Basler, 2017; Gomez-Villamandos, Bautista, Sanchez-Cordon, & Carrasco, 2013; Karalyan et al., 2012). This is also mirrored by pathological lesions observed after acute courses of the disease. Enlarged and hemorrhagically activated lymph nodes, often with marbled or even ebony appearance, are a key finding. This is often complemented by petechia in kidneys, in the urinary bladder, the epicardium, pericardium, pleura or the gastric mucosae (J. Pikalo et al., 2020; Sanchez-Vizcaino, Mur, Gomez-Villamandos, & Carrasco, 2015; Sehl et al., 2020). Another typical finding that is less frequently observed under experimental conditions is an enlarged spleen with rounded edges (splenomegaly) (Montgomery, 1921). However, especially peracute disease courses can also produce sparse macroscopic lesions, often limited to findings in the lymph nodes with no further observations. In chronically infected animals, arthritis, necrotic lesions in skin and tonsil, as well as pleural or pericardial

adhesions are often observed. Here, bacterial secondary infections play a role in the manifestation of lesions (Moulton & Coggins, 1968; Pan, Moulton, & Hess, 1975).

Due to the unspecific clinical course, a number of differential diagnoses must be considered, among them CSF, Aujezky's disease, Porcine reproductive and respiratory syndrome (PRRS), Porcine circovirus associated disease (PCVAD), bacterial septicaemias and poisoning, for instance with mercury (OIE, 2021).

3.3 Laboratory Diagnosis

The variable clinical course stresses laboratory investigation as the only reliable option for the diagnosis of ASF. The full set of accredited diagnostic methods is presented in the compilation of methods on the national level (FLI, ("Afrikanische Schweinepest: Amtliche Methode und Falldefinition," 2021)), by the EU reference laboratory on EU level (detailed standard operating procedures provided at asf-referencelab.info/asf/en/procedures-diagnosis/sops, last visited August 27th 2022), and in the WOAH Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (OIE, 2021) on the international level.

In Germany, confirmatory diagnosis performed by the National Reference Laboratory (NRL) for ASF is routinely based on genome detection by real-time PCR (qPCR) and different options are available. The WOAH recommends multiple qPCR protocols, among them the assay published by D. P. King et al. (2003), which detects the highly conserved B646L gene encoding for p72. Another is the protocol published by Tignon et al. (2011). Both assays are accredited in the German NRL and are used along with licensed and accredited qPCR kits (13 commercial qPCR kits for ASFV genome detection are currently licensed in Germany). The use of a WOAH-recommended assay or a licensed commercial kit is mandatory for diagnostic purposes in Germany. In detail, a licensed kit has to be used in accordance with §11 of the German Animal Health Act (Gesetz zur Vorbeugung vor und Bekämpfung von Tierseuchen, TierGesG). To comply with international requirements, WOAH-recommended assays are added e.g. for primary outbreaks. Recently, a study has shown that all 12 of the commercial kits licensed at the time in Germany and the WOAH-recommended protocols are comparable in terms of sensitivity, safety and precision (Jutta Pikalo

et al., 2022). Therefore, a broad set of proven diagnostic tools for ASFV genome detection are available and can be applied based on technical preferences and requirements on internal controls.

African swine fever virus is the only known pig pathogen to cause hemadsorption in infected macrophage cultures (Malmquist, 1960; Sierra et al., 1991), hence virus isolation and confirmation of infectivity can be routinely based on this phenomenon (OIE, 2021). For non-hemadsorbing strains, immunoflourecent staining of the p72 antigen is a suitable alternative (Carrascosa, Bustos, & de Leon, 2011). Field strains are usually unable to grow on routinely used immortalized cell lines without lengthy adaptation that leads to major changes in the viral genome and therefore primary macrophages must be used for virus cultivation. This necessity represents a major pitfall for diagnostic laboratories, as the ongoing production of primary cell lines is labor-intensive and complicates standardization between experiments. Only very recently, immortalized cell lines promising equal characteristics for virus cultivation have been reported and commercialized (Masujin et al., 2021; Portugal, Goatley, Husmann, Zuckermann, & Dixon, 2020).

For serological detection of ASFV, three enzyme linked immunodsorbent assays (ELISAs) are licensed in Germany: the p72 specific INGEZIM PPA COMPAC® (Ingenasa) (Pastor et al., 1990), the p32, p62 and p72 specific ID Screen® African Swine Fever Indirect ELISA (IDvet) and the ID Screen® African Swine Fever Competition (IDVet) for p32 antibody detection. For confirmatory purposes, the immunoperoxidase assay can be performed (standard protocol SOP/CISA/ASF/IPT/1 provided by the European Reference laboratory for ASF with modifications regarding cell and virus type, asf-referencelab.info/asf/images/ficherosasf/PROTOCOLOS-EN/2021_UPDATE/SOP-ASF-IPT-1_2021.pdf, visited June 26th 2022). Another confirmatory method is immunoblotting (Cubillos et al., 2013).

In addition to these laboratory-dependent methods, a number of point-of-care options for ASFV diagnosis have arisen, aiming primarily on regions with lesser developed diagnostic infrastructure. Lateral flow devices for antibody and antigen detection are published and commercialized for pen-side conditions, using fresh EDTA-blood samples (Sastre, Gallardo, et al., 2016; Sastre, Perez,

et al., 2016). In addition, a number of portable PCR devices for mobile genome detection have recently been described (Yang Wang et al., 2021; Zurita et al., 2022).

3.4 Distribution, Control and Surveillance

The historically endemic area of ASF was sub-Saharan Africa. While genotype I strains spread to North Africa, Europe, the Caribbean and Brazil in the mid of the 20th century, by the beginning of the 21st century, eradication in the Americas and mainland Europe was successful and the presence of ASF was limited to Africa with the exception of Sardinia (L. K. Dixon, Stahl, Jori, Vial, & Pfeiffer, 2020). Here, genotype I strains were endemic for more than 43 years and seropositive animals are still found, however the last virus detection dates back to 2019, and the island is under consideration for declaration of ASF eradication (Cappai et al., 2022; Loi et al., 2019).

In 2007, an ASF strain belonging to genotype II, which was circulating in Mozambique, Madagascar, and Zambia beforehand, was introduced into Georgia (R. J. M. Rowlands, V.; Heath, L.; Hutchings, G.; Oura, C.; Vosloo, W.; Dwarka, R.; Onashvili, T.; Albina, E.; Dixon, L.K., 2008). Subsequently, the disease spread successively through Europe and Asia, reaching the major pork producer China in 2018 (X. Zhou et al., 2018) and eventually Germany in 2020 (Sauter-Louis et al., 2020) and as a result globally caused the death of millions of pigs with enormous economic losses for producers. Recently, ASF reached the Americas in the Dominican Republic and Haiti (Gonzales et al., 2021), the virus now being present in 4 out of 5 continents. The disease has an enormous economic significance for pig farmers, but at the same time affects wild pig populations, causing suffering and even threatening some particular rare species in Asia with extinction, e.g. the Bornean bearded pig (*Sus barbatus*) (Ewers, Nathan, & Lee, 2021; Luskin et al., 2021).

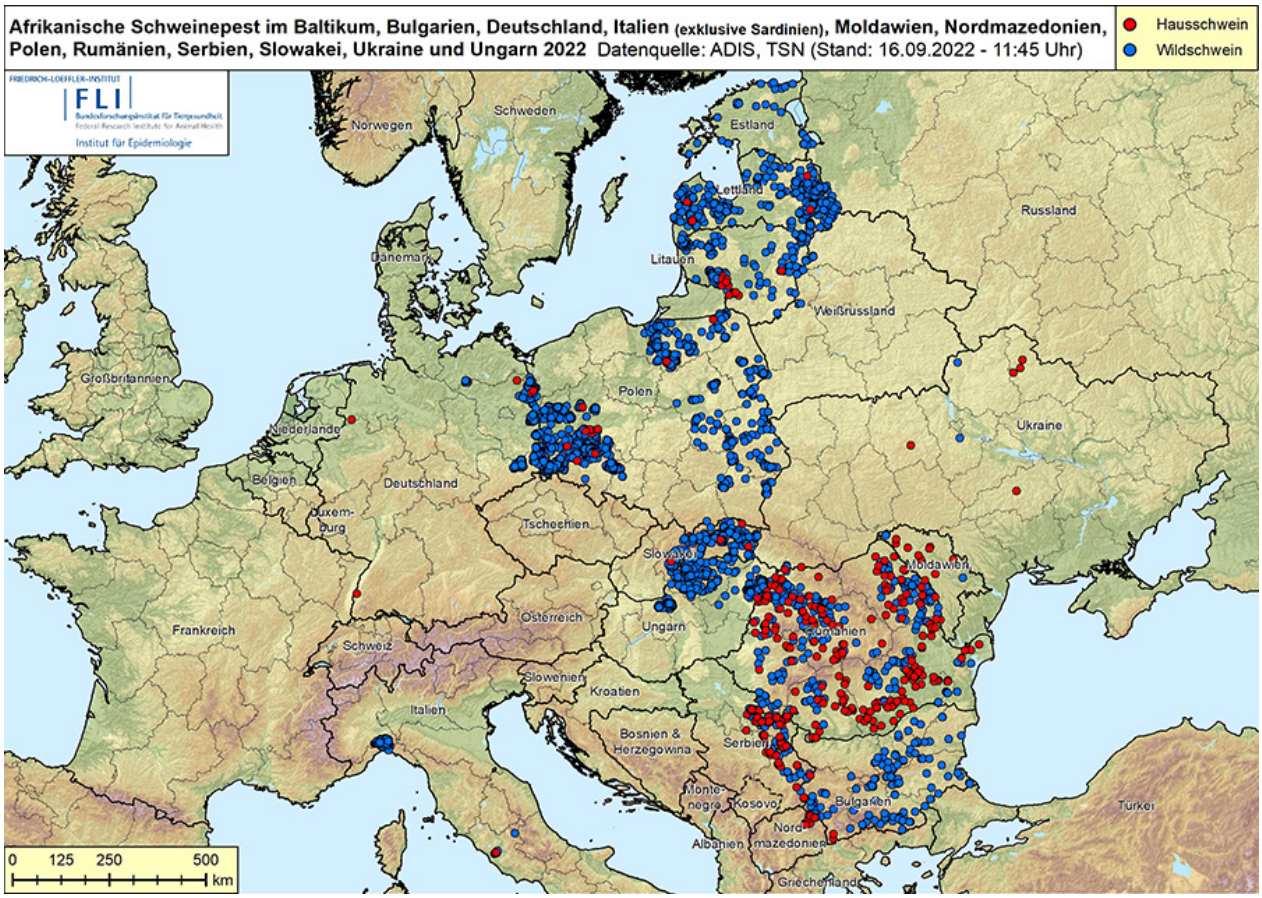

Figure 1: Current distribution of ASFV in Europe, source: fli.de/de/aktuelles/tierseuchengeschehen/afrikanische-schweinepest/karten-zur-afrikanischen-schweinepest/ (visited September 18th, 2022)

Viruses of the recent genotype II panzootic were first reported in the European Union from the Baltic states and Poland in 2014 (see Table 1) and have successively spread westwards since then (Sauter-Louis et al., 2021), with one of the most recent introductions into mainland Italy (Iscaro et al., 2022). The current spread of the disease is depicted in Figure 1. The Czech Republic (Semerád, 2019) and Belgium (Claeyes, 2020) are examples of countries that have managed to eradicate the disease after a punctual entry into wild boar populations, however, countries with (ongoing) frontline introductions into wild boar as Germany have not been able to bring the epizootic to a halt. Internationally, losses in domestic pigs were especially high in Asia (You et al., 2021), but many European countries report a high infection pressure from the wild boar population that constantly threatens to cause spillovers to the domestic pig sector (Sauter-Louis

et al., 2021). More than 4200 cases are confirmed from Germany in five federal states as of now. Most of these cases affect wild boar, but introductions to pig farms have occurred several times.

Country	Genotype II ASFV first report	Current status	Reference
Lithuania	January 2014	affected	State Food and Veterinary Service (2014)
Poland	February 2014	affected	Woźniakowski et al. (2016)
Latvia	June 2014	affected	Oļševskis et al. (2016)
Estonia	September 2014	affected	Nurmoja et al. (2017)
Czech Republic	June 2017	resolved	Semerád (2019)
Hungary	April 2018	affected	EFSA et al. (2018)
Romania	May 2018	affected	EFSA et al. (2018)
Bulgaria	August 2018	affected	Laura Zani et al. (2019)
Belgium	September 2018	resolved	Claeyes (2020)
Slovak Republic	August 2019	affected	EFSA et al. (2020)
Germany	September 2020	affected	Sauter-Louis et al. (2020)
Italy (mainland)	January 2022	affected	Iscaro et al. (2022)

Table 1: List of EU countries that are currently affected or have been affected by ASFV during the recent genotype II panzootic with the first report of ASFV and their status, adapted from Sauter-Louis et al. (2021)

The notifiable disease is fought with preventive measures as increased standards of hygiene (biosecurity) and restrictions in pork trade, and outbreaks lead to culling of entire herds. From a German perspective, ASF is notifiable on the national and international level, and a mandatory legal framework regulates disease control in domestic pigs and wild boar (EU Regulation 2016/429 with its delegated legal acts and, as of now, the German Swine Fever Ordinance / Schweinepest-Verordnung). The implementation of measures is organized by ASF crisis centers on national and regional levels. In Germany, the responsibility for animal disease control lies with the respective federal state.

In recent years, surveillance could be deepened by characterization of the circulating virus strains by genomic analyses. Molecular characterization based on distinct genomic markers can aid in epidemiolocal investigations and, if functional genes are affected, provide indications for possible phenotypic effects. However, ASFV has several repair polymerases and its genome is proven to be highly stable. Consequently, the viruses from the recent Eurasian panzootic were shown to have very little variation with previously no indications for phenotypic differences that might play

a role for epidemiology (J. H. Forth, Forth, Blome, Hoper, & Beer, 2020; J. H. Forth, Forth, Vaclavek, et al., 2020). In 2018, an attenuated phenotype was shown for an Estonian ASFV isolate from 2014 (L. Zani et al., 2018), but the attenuated virus was not re-isolated in the subsequent years, possibly indicating a selective disadvantage in the field. Mutations with phenotypic effects appear to be rare and recently there was only one additional report from Europe that was fully characterized (Gallardo et al., 2019). However, in 2019, a mutation was identified in the viruses circulating in western Poland, affecting the O174L gene, which encodes for the DNA repair polymerase X (Mazur-Panasiuk, Walczak, Juszkiewicz, & Woźniakowski, 2020; Mazur-Panasiuk & Wozniakowski, 2019). A possible mutagenic function is described for Polymerase X (Showalter, Byeon, Su, & Tsai, 2001). Early genomic analyses from Germany in 2020 showed that all German ASFV isolates from wild boar share the same genomic markers in the O174L gene (Sauter-Louis et al., 2020), which aided in the epidemiolocal investigation of virus introduction, but stresses the need for in-depth genomic and phenotypic characterization of the German isolates due to the hypothesized mutagenic function of the gene.

3.5 Vaccines

Given the enormous economic significance of ASF, vaccine research intensified throughout the past years and in June 2022, the first commercialized ASFV vaccine was released in Vietnam (https://link.gov.vn/lkSgZsxV, visited 26th June 2022). All other countries including those of the EU still lack a licenced vaccine.

First attempts to create vaccines in Europe reach back to the 1960s, where field trials were eventually aborted due to observations of chronic forms of disease induced by live-attenuated vaccines (LAV) administered then (Petisca, 1965). This serves as a reminder for the need of proper safety characterization of ASFV vaccines until today (Gavier-Widen, Stahl, & Dixon, 2020).

Different approaches for ASFV vaccine development have been pursued until today (as shown in Figure 2), but in general, vaccine design is still hampered by the lack of fully understanding the complex virus-host interactions (Cadenas-Fernández et al., 2021; Muñoz-Pérez, Jurado, & Sánchez-Vizcaíno, 2021). Until today, it was not possible to identify single protective proteins that

could be targeted by a rational design for subunit vaccines or inactivated formulas. In fact, seroconversion is reported for protected animals that survived infection, but no complete virus neutralization can be achieved, so the presence of antibodies alone cannot be correlated with protection. Consequently, all classically inactivated formulas were unsuccessful (Revilla, Perez-Nunez, & Richt, 2018).

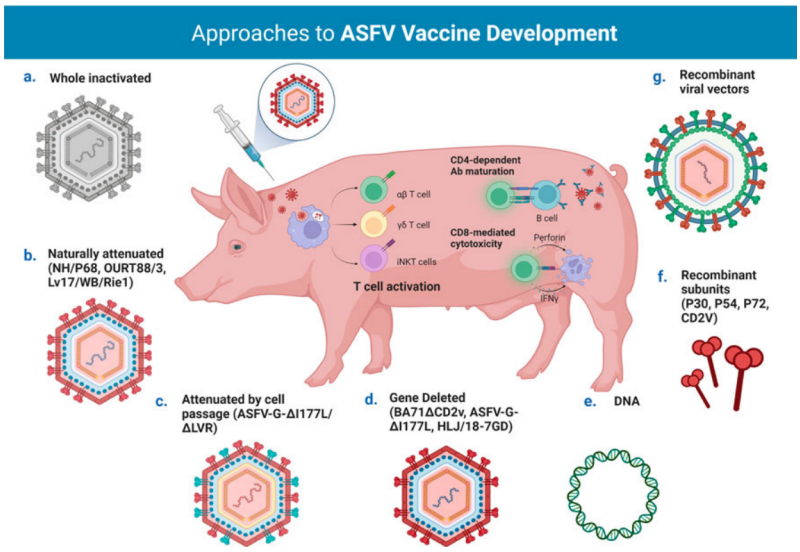

Figure 2: Overview of the different approaches for ASFV vaccine development, obtained from Urbano and Ferreira (2022)

Development of RNA or DNA based vaccines is also hampered by this pitfall and these approaches could not yet induce full protection (Argilaguet et al., 2012; Sunwoo et al., 2019). For vectored and subunit vaccines, advances were achieved throughout the past years, but even with the best result published yet, animals survived (protection from fatal outcome) but were not protected from severe disease (Goatley et al., 2020). Protection against challenge infection after the survival of infection with attenuated field strains has been reported several times (Detray, 1957; K. King et al., 2011; M. L. Penrith, 2009), and the suitability of attenuated field strains as vaccines is still under discussion. However, high levels of residual replication are observed for these candidates (Barasona et al., 2019; Gallardo et al., 2019), which must be regarded with caution especially in the context of possible induction of chronic infections.

Meanwhile, a number of genetically modified LAVs were reported to induce full-protection against highly virulent homologous challenge (Borca et al., 2020; Chen et al., 2020; Douglas P Gladue & Borca, 2022; D. P. Gladue et al., 2021; O'Donnell, Holinka, Gladue, et al., 2015; O'Donnell et al., 2017), and when comparing the different vaccine approaches, genetically modified LAVs represent the most promising group. Here, genes encoding known virulence factors are rationally targeted for deletion, creating artificially attenuated vaccine prototypes which, after inoculation, ideally do not cause disease but protect from subsequent virulent challenge. E.g., the multi gene families (MGFs), a set of multiple genes with repetitive sequences within the ASFV genome, are involved in interferon regulation, and deletions in these genomic regions are reported to produce fully attenuated vaccine prototypes, as for “ASFV-G-ΔMGF” and “HLJ/18-7GD” (Chen et al., 2020; Douglas P Gladue & Borca, 2022; O'Donnell, Holinka, Gladue, et al., 2015; O'Donnell et al., 2016). Problems were reported for some protective vaccine candidates that were incompletely attenuated in high doses (O'Donnell, Holinka, Krug, et al., 2015), allowed residual challenge virus replication (Teklue et al., 2020) or high levels of vaccine virus replication (Douglas P Gladue & Borca, 2022; D. P. Gladue et al., 2021). Close-to-sterile immunity and only low to moderate vaccine virus replication without clinical reaction of animals to the vaccine inoculation were reported for “ASFV-G-Δ177L” (Borca et al., 2020) and “ASFV-G-ΔMGF” (O'Donnell et al., 2016), as well as the genetically similar candidate “HLJ/18-7GD” (Chen et al., 2020). Most of the auspicious vaccine candidates are still in a proof-of-concept phase or results are not disclosed due to ongoing commercialization procedures, therefore in-depth data on safety and efficacy as demanded by the Veterinary International Conference on Harmonization (VICH) is not available to the public. An exception is “ASFV-G-Δ177L” (Borca et al., 2020), where full efficacy and comprehensive safety characterization (Tran et al., 2022) led to the commercialization in Vietnam for intramuscular use in domestic pigs. For Europe, the requirements of a vaccine are regulated by the European Medical Agency (EMA) and licensing is evaluated by an expert panel. So far, none of the candidates have been submitted for licensing with EMA yet.

In general, future vaccination strategies will probably vary depending on the region and mode of application. In western Europe, biosecurity in domestic pig holdings is generally high and trade restrictions in vaccinated pork are to be expected (Sauter-Louis et al., 2021). Meanwhile, the abundant wild boar population serves as a reservoir for the virus. Likely, vaccination strategies

will therefore focus on wild boar. Experiences from CSF eradication in Germany raise hopes that a bait-based vaccination campaign could be effective (Blome, Franzke, & Beer, 2020; Blome, Moss, Reimann, Konig, & Beer, 2017). Safety and efficacy by oral administration are two key characteristics that remain to be experimentally shown for most of the LAVs with promising intramuscular efficacy, before prospects for future commercialization can be evaluated. For "ASFV-G-Δ177L", oronasal efficacy was tested (Borca, Ramirez-Medina, et al., 2021), but this administration route cannot easily be transferred to the oral uptake by baits, since the mucosal contact without inclusion of the nasal cavity is drastically reduced, with possible detrimental effects on vaccine uptake and efficiency. Therefore, the suitability for administration by baits remains to be shown also for this candidate.

In addition, to allow creation and propagation of a standardized master seed virus and in contrast to the propagation in primary macrophages from a donor pig that is neither safe nor practical, genetic stability in an immortalized cell line has to be proven. This from a regulatory point of view essential data is lacking for all auspicious genetically modified LAVs except the "ASFV-G-Δ177L", however here the adaption to the immortalized cell line went along with a modification of the virus in the left variable region of the genome (Borca, Rai, et al., 2021).

4. Objectives

I. Towards market authorization of an ASFV vaccine candidate

a) Efficacy studies in domestic pigs and wild boar

A licensed vaccine against ASF is still missing in Europe, but promising vaccine candidates exist. To put one of these candidates on the long path to licensure, we tested the efficacy of vaccine candidate “ASFV-G-ΔMGF” upon intramuscular immunization of domestic pigs and oral immunization of wild boar. Furthermore, possible effects of vaccine virus production in an immortalized cell line were investigated.

b) Reversion to virulence study

Safety of live attenuated vaccines is crucial for the licensing process. For this reason, we subjected the vaccine candidate “ASFV-G-ΔMGF” to an in vivo reversion to virulence study in accordance with the *International Cooperation on Harmonisation of Technical Requirements for Registration of Veterinary Medicinal Products* guidelines.

II. Characterization of ASFV strains in Germany

The primary aim of this study part was to characterize the ASFV strains circulating in Germany by whole genome sequencing. Surprisingly, the analyses revealed small but distinct differences among the strains from different regions and thus, those variants were followed-up by tailored Sanger sequencing approaches. A pathological study with wild boar carcasses from the affected German regions was carried out to assess phenotypic variability.

III. Recent advances in ASFV diagnosis

Building on sample collections from animal trials over the last years, comparative studies on the suitability of different diagnostic workflows in ASFV detection were carried out. Along with routine sample matrices, alternative sample matrices and workflows were evaluated.

Moreover, we tested a commercial lateral flow assay for its suitability for passive surveillance in general and carcass testing in particular.

5. Results

The publications are grouped according to their topic.

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

5.1 Taking a Promising Vaccine Candidate Further: Efficacy of ASFV-G-ΔMGF after Intramuscular Vaccination of Domestic Pigs and Oral Vaccination of Wild Boar

Paul Deutschmann [1], Tessa Carrau [1], Julia Sehl-Ewert [2], Jan H. Forth [1], Elisenda Viaplana [3], Jose Carlos Mancera [4], Alicia Urniza [3], Martin Beer [1] and Sandra Blome [1]

[1] Institute of Diagnostic Virology, Friedrich-Loeffler-Institut, Suedufer 10, 17493 Greifswald – Insel Riems, Germany

[2] Department of Experimental Animal Facilities and Biorisk Management, Friedrich-Loeffler-Institut, Suedufer 10, 17493 Greifswald – Insel Riems, Germany

[3] Zoetis Manufacturing and Research Spain, Finca la Riba, Carretera de Camprodon s/n, 17813 L'Hos-talnou de Bianya, Girona, Spain

[4] Zoetis Belgium, Mercuriusstraat 20, 1930 Zaventem, Belgium

Pathogens

2022

doi.org/10.3390/pathogens11090996

pathogens

MDPI

Article

Taking a Promising Vaccine Candidate Further: Efficacy of ASFV-G-ΔMGF after Intramuscular Vaccination of Domestic Pigs and Oral Vaccination of Wild Boar

Paul Deutschmann [1], Tessa Carrau [1], Julia Sehl-Ewert [2], Jan Hendrik Forth [1], Elisenda Viaplana [3], Jose Carlos Mancera [4], Alicia Urniza [3], Martin Beer [1] and Sandra Blome [1,*]

[1] Institute of Diagnostic Virology, Friedrich-Loeffler-Institut, Suedufer 10, Insel Riems, 17493 Greifswald, Germany
[2] Department of Experimental Animal Facilities and Biorisk Management, Friedrich-Loeffler-Institut, Suedufer 10, Insel Riems, 17493 Greifswald, Germany
[3] Zoetis Manufacturing and Research Spain, Finca la Riba, Carretera de Camprodon s/n, 17813 L'Hostalnou de Bianya, Girona, Spain
[4] Zoetis Belgium, Mercuriusstraat 20, 1930 Zaventem, Belgium
* Correspondence: sandra.blome@fli.de

Abstract: African swine fever (ASF) is a pandemic threat to the global pig industry and wild suids. A safe and efficacious vaccine could monumentally assist in disease eradication. In the past years, promising live attenuated vaccine candidates emerged in proof-of-concept experiments, among which was "ASFV-G-ΔMGF". In our study, we tested the vaccine candidate in three animal experiments intramuscularly in domestic pigs and orally in wild boar. Further, a macrophage-grown vaccine virus and a virus grown on permanent cells could be employed. Irrespective of the production system of the vaccine virus, a two-dose intramuscular immunization could induce close-to-sterile immunity with full clinical protection against challenge infection. After oral immunization, 50% of the vaccinees seroconverted and all responders were completely protected against subsequent challenge. All nonresponders developed ASF upon challenge with two acute lethal infections and two mild and transient courses. The latter results show a lower efficiency after oral administration that would have to be taken into consideration when designing vaccination-based control measures. Overall, our findings confirm that "ASFV-G-ΔMGF" is a most promising vaccine candidate that could find its way into well-organized and controlled immunization campaigns. Further research is needed to characterize safety aspects and define possible improvements of oral efficiency.

Keywords: African swine fever; vaccination; efficacy; domestic pigs; wild boar; oral vaccine; intramuscular vaccine

Citation: Deutschmann, P.; Carrau, T.; Sehl-Ewert, J.; Forth, J.H.; Viaplana, E.; Mancera, J.C.; Urniza, A.; Beer, M.; Blome, S. Taking a Promising Vaccine Candidate Further: Efficacy of ASFV-G-ΔMGF after Intramuscular Vaccination of Domestic Pigs and Oral Vaccination of Wild Boar. *Pathogens* **2022**, *11*, 996. https://doi.org/10.3390/pathogens11090996

Academic Editor: Marta Martínez-Avilés

Received: 1 August 2022
Accepted: 29 August 2022
Published: 31 August 2022

1. Introduction

African swine fever (ASF), caused by the African swine fever virus (ASFV), is a notifiable disease of pigs that has become a tangible pandemic threat to domestic and wild pigs [1]. Currently, more than 35 countries in five world regions (Africa, Asia, Europe, Oceania, and the Americas) are affected and the disease continues to spread (OIE situation report, visited online at African swine fever OIE—World Organisation for Animal Health; 23 April 2022). There is presently no licensed vaccine or treatment option for the disease, which can present with the clinical signs of a viral haemorrhagic fever and very high lethality [2].

While the classical veterinary hygiene measures, i.e., culling of affected farms, establishment of restriction zones and movement bans, can be successfully implemented for industrial pig farms, a spread of the disease in regions with small, family-owned farms and

lack of financial compensation for losses can hardly be stopped with the possibilities available so far [3]. The same applies to outbreaks of ASF in wild boar populations, especially if the disease is introduced over a broader front rather than punctually.

The challenges we are facing in the context of the current ASFV pandemic are difficult to solve without the help of a vaccine, and thus a safe and efficacious vaccine for both parenteral and oral bait application could provide the additional tool that is still missing to regain the upper hand over the disease [4]. Among the most promising ASF vaccine candidates that have shown high potential in pilot studies are genetically engineered live attenuated vaccines, i.e., deletion mutants created through homologous recombination [5–7]. One of the first reported vaccine viruses with rational deletions was "ASFV-G-Δ9GL" [8]. This virus showed residual virulence that could be reduced by an additional deletion of the UK gene [9]. Another deletion mutant that was investigated in more detail was "BA71ΔCD2". This vaccine candidate was able to induce strong humoral and cellular responses, conferred solid protection against the homologous virus (BA71) in a dose-dependent manner, and held some promise for cross-protection [10]. Another mutant virus with deletions of the CD2 and UK genes offered full protection against homologous challenge, but allowed residual replication of the challenge virus [11]. Recently, "ASFV-G-ΔA137R" was reported to induce full protection against homologous challenge, but the vaccine-virus-induced viremia was medium to high [12]. Close-to-sterile protection against homologous challenge, and at the same time, low-to-moderate vaccine-virus-induced viremia were achieved with "ASFV-G-ΔI177L" [13] and "ASFV-G-ΔMGF" [14]. The latter of these particularly promising recombinant vaccines harbors deletions in the multigene families 360 and 505. The same modifications in the MGF regions with additional deletion in the CD2 gene from a Chinese backbone virus also offered full attenuation and protection [15].

Here, we analyzed "ASFV-G-ΔMGF" further and investigated its efficacy in three independent animal trials. Two trials were designed to assess safety and efficacy of a double intramuscular vaccination scheme of domestic pigs. In the first trial, a macrophage-grown virus was used, while in the second, the vaccine virus master seed was grown on a commercial permanent cell line (subject to patent restrictions). The third trial was performed as a proof-of-concept study with a single oral immunization of wild boar using the cell-culture-grown vaccine virus. Oronasal challenge infection of the domestic animals was performed with the highly virulent ASFV strain "Armenia 2008"; the wild boar trial included an oronasal challenge infection with the recent ASFV strain "Germany 2020". All animals were monitored for clinical signs and were investigated using accredited routine virological and serological methods.

2. Results

2.1. Clinical Signs and Pathological Lesions

2.1.1. Domestic Pig Trial A

"ASFV-G-ΔMGF", Intramuscular

The five vaccinees remained completely healthy after both immunizations and after challenge infection, with only one deviation observed. Pig #20 had a febrile body temperature of 40.5 °C on 9 days post-challenge (dpc); however, it remained free of clinical signs and recovered the next day (see Figure 1). No pathomorphological abnormalities were found except for mild pulmonary consolidation in pig #17 and a variable dark reddening of renal and hepatogastric lymph nodes.

Challenge Controls

Following infection with ASFV "Armenia08", the five control animals developed fever up to 41.3 °C (see Figure 1), accompanied by anorexia, reduced liveliness, and reddening of the skin. Animals were euthanized according to our moderate humane endpoint between 6 and 8 dpc with a maximum clinical score (CS) of 5.5. Typical lesions associated with an ASFV infection were identified during necropsy: severely enlarged

haemorrhagic lymph nodes in all animals, extensive gallbladder wall edema and marked alveolar pulmonary edema (#8, #9). Myo- and endocardial haemorrhages were observed in pig #8. Mild ascites and kidney haemorrhages were present in animal #9.

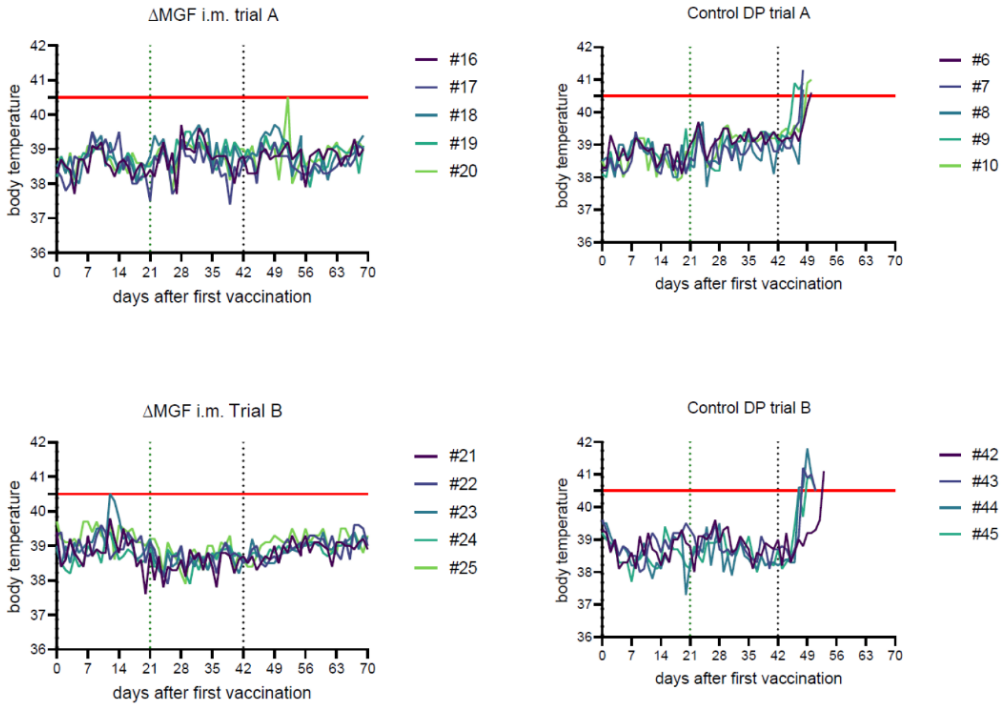

Figure 1. Daily body temperatures of animals recorded throughout the course of each respective animal trail. Individuals are depicted in different colors. The red line marks the threshold for fever at 40.5 °C. The green dotted line marks boost vaccination at 21 dpv; the black dotted line marks the challenge infection at 42 dpv.

2.1.2. Domestic Pig Trial B

"ASFV-G-ΔMGF", Intramuscular

All five animals remained healthy. Only one pig (#23) displayed an elevated body temperature of 40.5 °C on 12 days post-vaccination (dpv) (see Figure 1) without any other clinical abnormalities. No other clinical signs or febrile temperatures were observed after immunizations or challenge infection. Postmortem examination revealed no macroscopic abnormalities except for variable dark reddening of tracheobronchial, renal, and hepato-gastric lymph nodes in animals #22, #23, and #24.

Challenge Controls

The four control pigs displayed fever up to 41.8 °C, clinically reflected by anorexia and reduced liveliness, and were euthanized between 8 and 11 dpc at a maximum CS of 5. Gross lesions included haemorrhagic enlarged lymph nodes in all animals, petechiae on kidneys and / or urinary bladder (#43, #45), and in some cases pulmonary consolidation (#43, #44). One control animal (#45) had developed severe haemoperitoneum.

2.1.3. Wild Boar Trial

"ASFV-G-ΔMGF", Oral

All eight wild boar remained healthy after immunization, and no clinical signs were observed. Following challenge infection, animals #62 and #67 displayed reduced appetite and liveliness beginning at 5 dpc. Clinical signs worsened during the following days and

were eventually accompanied by vomitus and reddened skin and eyes. On 8 dpc, #62 reached a not-yet-critical CS of 6.5, but died during the day in a peracute convulsive seizure within minutes, before the animal could be released by euthanasia. Animal #67 reached the humane endpoint with 12 cumulative CS points and was euthanized at 9 dpc, when it showed severe apathy and anorexia, labored breathing, and reddened ears. Wild boars #61, 63, and 65 developed slightly reduced appetite and liveliness between 5 and 9 dpi (maximum CS of 2); however, they recovered thereafter. Animal #61 reached one CS point for reduced liveliness again on 13 dpc, but was clinically inapparent thereafter. #64 and 68 did not show clinical abnormalities. Macroscopically, in animals #62 and 67, which died or were euthanized, mild-to-severe haemorrhages were present in the kidneys, in various lymph nodes and in the gastrointestinal tract. Serosanguinous peritoneal and thoracic effusion was present in animals #62 and 67, respectively. In addition, severe alveolar edema was found in animal #62, whereas animal #67 revealed an enlarged, friable spleen and pinpoint haemorrhages in the urinary bladder. In contrast, wild boar #61, 63, 64, 65, 66, and 68 mainly showed very mild lesions, including reddening of the hepatogastric and renal lymph nodes. Animals #61, 64, and 65, moreover had mild-to-moderate enlarged friable spleens. Focal mild pulmonary consolidation was observed in pigs #63 and 64. Multifocal pinpoint haemorrhages were detected in the lung of animal #66.

Control Group

The four control WB showed an onset of reduced liveliness and apathy at 4 or 5 dpc, worsening and reaching the humane endpoints at 7 dpc, when they were completely anorectic and apathic (cumulative CS between 4 and 7). Animals #72 and 69 showed reddening of the skin around the ears. Labored breathing was observed in animals #69 and 71.

All pigs revealed multifocal pinpoint haemorrhages in the kidney and large intestine. Up-to-severe haemorrhages were observed in multiple lymph nodes. All pigs except #69 showed haemorrhagic and necrotic areas in the liver. Mild haemorrhages of the urinary bladder and diffuse pancreatic necrosis were observed in #70 and 72, respectively.

2.2. *Genetic Characterization of the Vaccine Virus*

Next-generation sequencing of the vaccine virus grown on the permanent cell line yielded 1.7 M 150 bp ASFV reads, resulting in a full genome with an average depth of 1394 per nucleotide. In comparison to the original "ASFV-G-ΔMGF" sequence, only two point mutations were found: one silent in the B438L gene at position 98378 (A→G) and one in B438L at position 98378 (C→G), which leads to an amino acid replacement alanine → proline with unknown consequences.

2.3. *ASFV Genome Detection*

2.3.1. Domestic Pig Trial A

"ASFV-G-ΔMGF", Intramuscular

Two individual blood samples (animals #18 and #19, respectively) were positive prior to challenge infection on 7 and 21 dpv, (3 and 200 gc, see Table 1). After challenge infection, traces of ASFV genomes were detected in animal #16 on 4 dpc, and in animal #20 at 10 and 14 dpc (<1 genome copy (gc)). All other samples were PCR-negative. One blood sample taken after challenge infection was positive for challenge virus genomes in the differentiating PCR (#10, 10 dpc); the other samples with only traces of genome were not detected in the gel-based PCR system. Swab samples taken throughout the trial were negative for ASFV genomes (see Supplementary Table S3).

All organ samples taken from animals #16 to #19 were negative in the qPCR. Weak positive results were obtained for animals #20 with 10 gc detected in the popliteal lymph node and viral genome traces in a lung sample (see Table 2).

Table 1. Genome detection from blood samples in genome copies/5 µL.

Trial	Group	Pig #	d0	d7	d14	d21	d28	d35	d0pc	d4pc	d7pc	d10pc	d14pc	d21pc	Necr.
DP A	MGF i.m. B	16	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
		17	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
		18	n.d.	2.8×10^{0}	n.d.	n.d.	n.d.	n.d.	n.d.	2.0×10^{-1}	n.d.	n.d.	n.d.	n.d.	n.d.
		19	n.d.	n.d.	n.d.	2.0×10^{2}	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
		20	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	3.7×10^{-1}	3.2×10^{-2}	n.d.	n.d.
	control A	6	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	4.8×10^{3}	†			5.8×10^{5}
		7	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	†				9.5×10^{4}
		8	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	†				3.5×10^{4}
		9	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	5.1×10^{4}	†				1.1×10^{6}
		10	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.3×10^{5}	†			2.7×10^{5}
DP B	MGF i.m. B	21	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
		22	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
		23	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
		24	n.d.	n.d.	1.0×10^{0}	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
		25	n.d.	3.5×10^{0}	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	control B	42	n.d.						n.d.	n.d.	n.d.	1.1×10^{3}	†		7.4×10^{4}
		43	n.d.						n.d.	n.d.	4.9×10^{3}	†			1.5×10^{5}
		44	n.d.						n.d.	9.7×10^{0}	3.7×10^{4}	†			1.0×10^{5}
		45	n.d.						n.d.	4.5×10^{4}	6.2×10^{4}	†			1.5×10^{5}
WB	MGF oral	61	n.d.			9.7×10^{1}									n.d.
		62	n.d.			n.d.									9.2×10^{4}
		63	n.d.			3.3×10^{1}									n.d.
		64	n.d.			n.d.									n.d.
		65	n.d.			1.2×10^{0}									n.d.
		66	n.d.			n.d.									n.d.
		67	n.d.			n.d.									2.7×10^{5}
		68	n.d.			n.d.									n.d.
	control WB	69							n.d.						4.4×10^{5}
		70							n.d.						4.1×10^{5}
		71							n.d.						1.9×10^{5}
		72							n.d.						2.8×10^{5}

DP: domestic pig, WB: wild boar, necr.: necropsy, † animal already euthanized.

Challenge Control

The first genome-positive samples appeared matching with the onset of fever on 4 dpc (see Figure 1 and Table 1). Upon necropsy (humane endpoints between 6 and 8 dpc), all control animals were positive for ASFV genomes in tissues and blood samples (up to 1.1×10^{6} gc, see Table 2 and Supplementary Table S2).

2.3.2. Domestic Pig Trial B

"ASFV-G-ΔMGF", Intramuscular

Upon immunization, two individual blood samples gave weak positive results in qPCR on 7 and 14 dpv, respectively (<4 gc, see Table 1). After challenge infection and until the end of the trial at 28 dpc, all blood and swab samples remained negative for ASFV genomes. The complete panel of tissue samples was negative for ASFV genomes (see Table 2).

Challenge Control

Positive results from control animals in qPCR and virus isolation emerged a short time before the onset of fever as early as 4 dpc (see Figure 1 and Table 1). At day 7 pc, all pigs were positive in blood and swabs for ASFV genomes. When the humane endpoint was

reached at 8 to 11 dpc, all blood and tissue samples (see Tables 1 and 2) were positive for viral genomes (up to 1.5×10^5 gc).

Table 2. Genome detection from tissue samples in genome copies/5 µL.

Trial	Group	Animal #	Lung	Spleen	Kidney	Liver	Hep. Ln.	Popl. Ln.	Mand. Ln.	Tonsil
DP A	MGF i.m. A	16	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.		
		17	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.		
		18	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.		
		19	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.		
		20	1.1×10^0	n.d.	n.d.	n.d.	n.d.	8.7×10^1		
	control A	6	9.4×10^2	1.3×10^3	5.3×10^0	1.2×10^3	2.0×10^1	6.4×10^1		
		7	1.8×10^2	7.3×10^2	1.3×10^0	8.8×10^1	9.4×10^{-1}	n.d.		
		8	4.0×10^1	1.6×10^3	1.8×10^0	3.3×10^2	4.9×10^{-1}	4.5×10^0		
		9	1.4×10^3	1.5×10^3	6.7×10^1	2.4×10^3	6.4×10^2	9.0×10^2		
		10	2.1×10^2	8.9×10^2	6.2×10^0	2.4×10^2	3.8×10^1	9.5×10^1		
DP B	MGF i.m. B	21	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.		
		22	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.		
		23	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.		
		24	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.		
		25	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.		
	control B	42	8.3×10^2	9.3×10^2	9.8×10^0	5.3×10^2	9.0×10^0	3.0×10^0		
		43	8.8×10^1	5.0×10^2	1.2×10^1	6.5×10^2	5.0×10^1	7.0×10^1		
		44	3.6×10^2	6.9×10^2	1.8×10^1	4.8×10^2	6.7×10^2	3.6×10^2		
		45	3.2×10^2	1.1×10^3	2.8×10^1	2.4×10^3	5.1×10^2	2.1×10^2		
WB	MGF oral	61	n.d.	n.d.	n.d.	n.d.	1.2×10^0		2.0×10^0	9.2×10^0
		62	1.6×10^4	3.5×10^4	3.0×10^3	7.7×10^3	1.1×10^5		4.0×10^3	6.1×10^4
		63	n.d.	n.d.	n.d.	n.d.	n.d.		n.d.	n.d.
		64	n.d.	1.6×10^{-1}	n.d.	n.d.	n.d.		n.d.	n.d.
		65	n.d.	n.d.	n.d.	n.d.	n.d.		n.d.	n.d.
		66	n.d.	n.d.	2.7×10^{-1}	n.d.	n.d.		n.d.	5.1×10^{-1}
		67	1.0×10^4	7.5×10^4	2.7×10^3	2.1×10^4	1.1×10^4		2.9×10^3	1.3×10^4
	control WB	68	n.d.	n.d.	n.d.	n.d.	n.d.		n.d.	n.d.
		69	4.4×10^4	1.0×10^5	3.4×10^3	3.9×10^4	4.1×10^4		1.4×10^4	2.4×10^4
		70	1.3×10^4	7.0×10^4	2.4×10^3	1.6×10^4	2.7×10^4		1.1×10^4	1.9×10^4
		71	6.8×10^3	5.4×10^4	1.1×10^3	2.4×10^4	1.2×10^4		1.4×10^3	4.7×10^2
		72	1.3×10^4	5.6×10^4	1.6×10^3	5.4×10^4	1.8×10^4		1.1×10^3	1.9×10^4

2.3.3. Wild Boar Trial

"ASFV-G-ΔMGF", Oral

At 21 dpv, viral genomes were detected in the blood of animals #61, 63, and 65 (<96 gc, see Table 1); the other animals were negative. At necropsy, animals #62 and 67 were highly ASFV genome-positive in all tissues and blood (up to 10^5 gc). Traces of genomes (<10 gc) were found in some organs of #61, 66, and 64 (see Table 2). These corresponding qPCR-positive samples of #61, 66, and 64 were tested in gel-based differentiating PCR, but no ASFV genomes could be detected in this system. Animals #63, 65, and 68 were qPCR-negative in the complete panel of samples taken at necropsy.

Controls

All control animals were highly positive for ASFV genomes in the complete sample set upon necropsy (up to 4×10^5 gc, see Tables 1 and 2).

2.4. Detection of ASFV-Specific Antibodies

2.4.1. Domestic Pig Trial A

The first pigs were positive in the p72 antibody ELISA in the "ASFV-G-ΔMGF"-vaccinated group on 14 dpv, with four out of five animals giving positive results and one animal having a doubtful ASF antibody result on 21 dpv. All animals remained positive from 28 dpv onwards. Control pigs were negative for ASFV specific antibodies throughout the trial (see Figure 2).

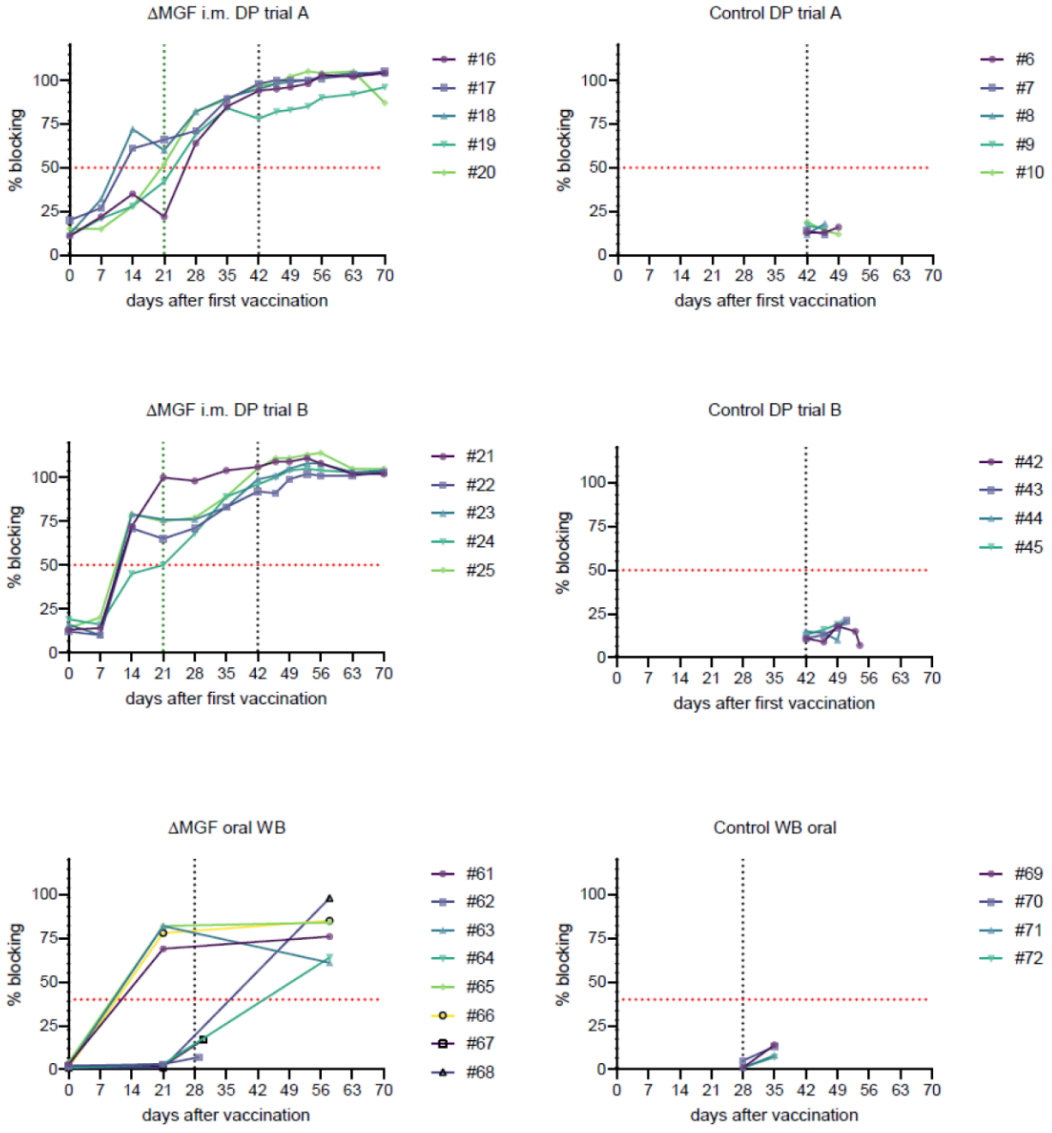

Figure 2. Percentual blocking in the ELISA systems deployed for samples from the respective vaccine trials after vaccinations and challenge infection. Red dotted line marks the threshold for positivity. Individuals are depicted in different colors. The green dotted line marks boost vaccination at 21 dpv (DP/domestic pigs); the black dotted line marks the challenge infection at 42 dpv (DP) or 28 dpv (wild boar/WB), respectively.

2.4.2. Domestic Pig Trial B

Four out of five animals were positive at 14 dpv, along with one doubtful result. From 21 dpv until the end of the trial at 28 dpc, all animals remained positive. The controls remained negative (see Figure 2).

2.4.3. Wild Boar Trial

Four out of eight vaccinated WB were positive in the p32, p64, and p72 specific antibody ELISA on 21 dpv (#61, 63, 65, and 66); the other animals remained seronegative before challenge infection (see Figure 2). Animals #62 and 67 were still negative when they died/were euthanized. Animals #64 and 68 had seroconverted until 28 dpc. The seronegative status of #64 and 68 at 21 dpv was confirmed by additionally testing the samples in the INGEZIM PPA COMPAC ELISA and in highly sensitive IPT (data not further shown).

3. Discussion

With the pandemic spread of ASFV, vaccine research was intensified and promising candidates emerged. Looking at proof-of-concept data of fully efficacious vaccine candidates, one may assume that a licensable vaccine might be closer than it appears [7]. The type of vaccine that is needed may differ depending on the affected region and the disease scenario. While a vaccine for use in domestic pigs could be suitable for epidemiolocal scenarios with frequent introductions into domestic pig backyard holdings in Europe, Africa, or Asia, for central Europe, the epidemiological situation focuses on wild boar. Vaccination of free-ranging wildlife calls for a bait-based formulation; consequently, an oral vaccine is needed here, similar to the successfully used preparation against classical swine fever [16]. Oral vaccine formulations require a live vaccine approach, because only replicative virus is taken up through intact mucosa. In the end, both intramuscular and oral vaccine-delivery options are conceivable for different field-use scenarios for an ASFV vaccine candidate.

Consequently, we took one of the most promising candidates, "ASFV-G-ΔMGF" [14], further confirmed the previously shown full efficacy for intramuscular vaccination, and assessed the possibility of using the vaccine candidate for oral administration. Going beyond this aspect, we examined effects of passaging of the vaccine virus on a permanent cell line.

In both domestic pigs and wild boar, the prototype vaccine was completely attenuated and innocuous and did not cause any traceable harm to the animals. No differences in efficacy or attenuation between the macrophage-derived vaccine and the vaccine derived from a permanent cell line were observed in either domestic pig study. Genetic characterization of the permanent cell-line-derived vaccine yielded only two point mutations, one silent and one without known consequences. Differences in vaccination efficiency, i.e., induction of vaccine virus replication and subsequent host responses, between the oral and the intramuscular application were apparent, however. These differences reflect the situation with field virus strains where oral infection is much less efficient [17].

In the intramuscularly immunized domestic pigs, full clinical protection was observed in all vaccinees and no challenge virus replication was detectable in 9/10 pigs. Only in a single animal were traces of challenge virus replication observed, and macroscopic lesions were largely absent in both groups. Traces of vaccine virus replication were observed in 4/10 animals. The reddening of lymph nodes, which were present in 7 out of 10 animals, most likely indicates previous virus-induced haemorrhage in the lymph node itself or drainage of a bleeding in the tributary area [18,19]. Our data suggest that both vaccine and challenge virus were eliminated by the end of both trials, as not even traces of viral genome were found in any tissues or blood in nine out of ten animals. Thus, vaccine candidate "ASFV-G-ΔMGF" showed a reproducible efficacy after intramuscular immunization against challenge infection with a homologous ASFV strain of genotype II. Since the results in our harmonized experimental setup between DP trial A and B are very similar, the minor genetic adaptions that were found in the permanent cell-line-derived vaccine are likely

without any consequences for vaccine safety and efficacy. With our study design comprising two vaccinations, we exceeded the already promising results achieved by O'Donnell and Holinka [14] in their experimental setup with a single vaccination. Unlike in the preceding study, we observed no febrile reactions with clear correlation to vaccination or challenge infection and detection rate of both vaccine and challenge virus in blood samples was much lower in our study. Comparison of our results suggests that while a single dose of immunization is sufficient to achieve clinical protection by intramuscular immunization, adding a boost immunization could reduce viremia, thereby contributing to optimized safety prospects of the vaccine candidate.

In the wild boar orally immunized with "ASFV-G-ΔMGF", all responders were protected, but not all animals responded to oral immunization, as evidenced by the lack of seroconversion. In detail, only 50% of the animals had seroconverted after 21 dpi. These animals specifically showed a high level of protection after challenge infection. Only transiently reduced liveliness and appetite were observed in animals #61, 63, and 65 (maximum of two cumulative cs points). Seronegative animals #62 and 67, however, displayed signs of severe disease and died or were euthanized. At necropsy, both animals showed characteristic lesions for ASF.

Interestingly, seronegative animals #64 and 68 survived challenge infection without displaying obvious clinical signs, but both had seroconverted upon necropsy. This could be due to biological variability, slight attenuation of the German field ASFV strain used in this trial, or vaccine-induced protection. While the presence of antibodies cannot be seen as a correlate for protection, the authors know of no ASFV vaccine candidate to induce protection without seroconversion, and thus, vaccine-induced protection is rather unlikely. In this pilot trial, other correlates could not be evaluated (e.g., T-cell or interferon responses). Principal functionality of our challenge model can be shown by the fast and severe onset of ASFV in the control animals, who received the identical virus by the same route. Still, oronasal application does hold certain insecurities due to possible differences in virus uptake. The animal's behavior, individual levels of proteases in the saliva, and the susceptibility of the mucosa influences the efficacy and renders the system more error-prone [17,20].

It seems likely that variability in virulence phenotype of the German ASFV field strain led to the observed differences. In conclusion, the experiment has shown that a single oral dose of "ASFV-G-ΔMGF" can induce full protection against field virus challenge in at least 50% of vaccinated animals. Compared to the recently evaluated vaccine candidate "ASFV Lv17/WB/Rie1" that was reported to induce antibodies in 10/12 animals and protection against lethal infection in 11/12 animals [21], the vaccination efficiency of "ASFV-G-ΔMGF" was lower. However, while vaccine virus shedding and chronic lesions could be observed for the non-haemadsorbing candidate "ASFV Lv17/WB/Rie1" [22], indications for a chronic disease course, which would be a major safety concern, were not observed for the "ASFV-G-ΔMGF" vaccine candidate.

While a higher efficiency of oral immunization would be desirable for the latter, the pandemic situation may not allow us to wait for the perfect vaccine and benefit–risk analyses are needed, but chronic disease courses caused by vaccines must be ruled out. Improvement of efficiency for "ASFV-G-ΔMGF" may be reached if more than one bait dose is taken up by the animals, an event that may be inevitable in a natural application when animals have repetitive access to baits, as in the classical swine fever example [23]. Moreover, virus delivery could offer opportunities of optimization. A conceivable approach could be a more viscous medium in the bait to delay swallowing of the virus suspension, preventing quick inactivation in the stomach.

To date, there is only one report of another ASFV prototype vaccine of the same kind where full protection was achieved against lethal challenge by oronasal immunization with "ASFV-G-ΔI177L" [13,24]. Here, all animals were protected; however, the inoculation route differs. Oronasal inoculation offers the vaccine virus suspension an increased contact surface to mucosae, possibly enhancing virus uptake. While the success reported here is

exciting, it remains a rather artificial immunization route, since wildlife will most likely take up baits by feeding on them, only offering contact to the oral mucosa.

Here, we report that "ASFV-G-ΔMGF" is genetically stable after cultivation on a permanent cell line, a major benefit for future commercialization, as it can allow production of large quantities of vaccine virus. The same was recently shown for "ASFV-G-ΔI177L" [25], underlining that both vaccine candidates are highly auspicious.

There are additional prototype vaccines that successfully protect animals intramuscularly. One example is genetically similar candidate "HLJ/18-7GD" [15]. This deletion mutant, based on modifications in the same six MGF-genes plus the extra deletion in the CD2v gene, could also induce a full clinical protection after challenge infection with the virulent backbone strain "HLJ/18". Little to no residual viral replication was detected and complete attenuation in pigs was observed. While direct comparison is hindered by different experimental setups, both candidates showed an equally good intramuscular efficacy, and an oral vaccination study could be auspicious for this candidate.

In order to bring any vaccine closer to licensing, now that we have identified a few efficacious prototypes, more insight into safety characteristics is needed, especially with the prospect of releasing infectious vaccine viruses in the field.

For "ASFV-G-ΔMGF", we have proven a measurable replication in the pig, although to a rather limited extent and with no detection of virus shedding. Additional safety trials are needed as a basis for thorough benefit–risk analyses. For this, research and legal authorities should now work together to define the most relevant knowledge gaps and to concentrate further research on these urgent open questions. In the EU, European Pharmacopoeia defines clear requirements for vaccines, and further studies should specifically address these regulatory aspects to speed up the search for a candidate that is suitable for licensing and could thus eventually be available for use.

4. Materials and Methods

4.1. Experimental Settings and Animals

The complete study comprised three animal experiments, domestic pig (DP) trials A and B, and a wild boar (WB) trial. Domestic pigs were 6–8-week-old crossbred animals bought from the same commercial farm, but from different groups and born approximately 5 months apart. The wild boar enrolled in the study were approximately 6 months old and obtained from two game parks in Brandenburg and Mecklenburg–Western Pomerania, Germany. In DP trial A, 5 vaccinees (numbered #16–20, see Table 2) and 5 control animals (#6–10) were kept; DP trial B consisted of 5 vaccinees (#21–25) and 4 controls (#42–45); and the WB experiment comprised 8 vaccinees (#61–68) and 4 controls (#69–72). All animals were randomly allocated to groups. All animals were moved to the high-containment facilities of the Friedrich-Loeffler-Institut (FLI) and were kept under appropriate containment and animal welfare conditions. Upon arrival, individuals were ear-tagged and the absence of ASFV-related antibodies and genome was confirmed at the start of each trial. Pigs and wild boar were fed a commercial pig feed appropriate for their age, mixed with hay cobs, and had ad libitum access to water. A sufficient acclimatization phase was ensured before the start of each trial.

In both DP trials, animals received two intramuscular vaccinations with 1 mL of virus suspension, respectively. A dose of roughly 10^4 HAD_{50} was administered at both vaccinations in DP trial A and roughly 10^3 HAD_{50} in DP trial B (see Supplementary Table S1 for back titrations). Boost was performed 21 days after the first dose. Challenge infection followed on 42 dpv. Immunizations were administered deep into the muscle of the right neck with a 2 mL syringe with 20 G cannula.

The WB received 2 mL of virus solution orally at 10^5 HAD_{50} with a 5 mL syringe, placed on top of the tongue (see Supplementary Table S1 for back titrations). Challenge infection was conducted at 28 dpv. Oronasal application of the challenge virus suspension was conducted by delivering 0.5 mL into each nasal orifice and 1 mL into the oral cavity using a 3 mL syringe for both the DP and WB trial. All animals were monitored for 28 dpc.

Upon first vaccination until the end of trial, clinical parameters were monitored as previously described [26]. Further, the rectal body temperature of each domestic pig was recorded daily. For the WB, temperature recording was not possible due to the need for immobilization for such procedures. Fever was defined as a body temperature above 40.5° C. Clinical parameters were liveliness, skin alterations, posture, ocular irritations, breathing, gait, feed intake, and defecation. They were assigned to points according to the severity of findings with a range between 0 (asymptomatic) and 3 points (severe). The sum of points was recorded as a cumulative CS, and under consideration of body temperatures, used to define humane endpoints. A moderate humane endpoint was applied in both trials at a CS of $\geq$10 points or in case of unjustifiable sufferings according to the assessment of the responsible veterinarian. Moreover, domestic pigs had to be put down when they displayed fever for three consecutive days accompanied by any other clinical sign, or for four days without accompaniment of other clinical signs.

During the trials, levels of viremia and serological parameters, as well as shedding for the DPs, were investigated. For this purpose, DPs were sampled on 0, 7, 14, 21, 28, 35, and 42 dpv, and on 4, 7, 10, 14, and 21 dpc, collecting EDTA blood and native blood for serum preparation from the jugular vein along with deep oropharyngeal swabs. For the WB, sampling was reduced due to the high susceptibility to stress and the need for immobilization before handling. They were sampled on 0 and 21 dpv (vaccinees) and 0 dpc (controls) as well as upon necropsy.

When animals reached the humane endpoint or the end of the trial, they were put in deep anaesthesia with a combination of tiletamine/zolazepam (Zoletil®, Virbac, Carros, France), xylazine (Xylazin 20 mg/mL, Serumwerk Bernburg, Bernburg, Germany) and ketamine (Ketamin 10%, medistar, Houston, TX, USA) and killed by exsanguination.

All animals underwent full necropsy and were macroscopically scored based on a standardized protocol [27] with slight modifications. EDTA blood and native blood for serum preparation were collected in addition to a panel of organ samples (see Table 2).

Compliance with EU Directive 2010/63/EC and institutional guidelines was assured. Trials were approved by the competent authority (Landesamt für Landwirtschaft, Lebensmittelsicherheit und Fischerei Mecklenburg-Vorpommern) under reference numbers LALLF 7221.3-1.1-003/20 and -035-21).

4.2. Cells

In the framework of the reported trials, virus cultivation, re-isolation, and titrations were conducted on peripheral blood mononuclear cell (PBMC)-derived macrophages. PBMCs were produced from blood of a healthy donor pig as previously described [20].

4.3. Vaccine and Challenge Viruses

4.3.1. Vaccine viruses

The vaccine virus master seed "ASFV-G-ΔMGF" [14] was provided by Zoetis Manufacturing & Research; S.L. Virus in DP trial A originated from passage in PBMC-derived macrophages. For DP trial B and the WB trial, virus was passaged once in a commercial permanent cell line (subject to patent restrictions) and provided ready-to-use by Zoetis. The virus originating from a permanent cell line was characterized by next-generation sequencing to reveal possible genetic modifications inflicted by cell-culture passage. To this means, DNA was sent to and sequenced by Eurofins Genomics. This service included preparation of a 450 bp DNA sequencing library using a modified version of the NEBNext Ultra™ II FS DNA Library Prep Kit for Illumina and sequencing on an Illumina NovaSeq 6000 with S4 flowcell, XP workflow and in PE150 mode (Illumina, San Diego, CA, USA).

4.3.2. Challenge Virus

The highly virulent ASFV "Armenia 2008" strain used for the DP trials was obtained from the German National Reference Laboratory (NRL) for ASF (FLI, Insel Riems, Germany) and was administered to the animals as macrophage cell-culture supernatant. Like the

mutual backbone virus of the deletion mutants in this trial, this well-characterized strain belongs to genotype II and represents field strains of the current epidemic. It shares almost 100% identity with "Georgia07" and is also highly virulent [28].

The challenge virus for the WB trial, ASFV "Germany 2020" (genotype II, German variant IV, ASFV/GER/2020/WB/IV_SN) was isolated from a wild boar carcass found in Saxony, Germany, in 2020. It was previously characterized at the NRL and showed high virulence in domestic pigs. The strain that shares >99% identity with ASFV "Armenia 2008" was chosen to represent the current situation in the field. The virus was passaged once in domestic pigs in a preceding animal trial at the NRL. It was administered as sea sand homogenate of infected spleen tissue in RPMI-1640 cell-culture medium. Viruses used for challenge infections were back-titrated to roughly 10^5 HAD_{50} (DP trial B) or 10^4 HAD_{50} (DP trial A and WB trial, see Supplementary Table S1).

4.4. Laboratory Investigations

4.4.1. Processing of Samples

Serum was obtained from native blood through centrifugation at 2500× *g* for 15 min at 20 °C. Swabs were soaked in 1 mL of RPMI-1640 cell-culture medium at 20 °C for one hour, then vortexed thoroughly and aliquoted. Tissue samples were homogenized for nucleic acid extraction with a metal bead in 1 mL phosphate-buffered saline (PBS) at 30 Hz for 3 min using a TissueLyser II (Qiagen). All samples were stored at −80 °C until further use.

4.4.2. Virus Detection

For qPCR, viral nucleic acids were extracted from blood and tissue samples using the NucleoMag Vet Kit (Machery-Nagel) on the KingFisher® extraction platform (Thermo Scientific) or the manual QIAamp® RNA Viral Mini Kit (Qiagen. The qPCR was conducted employing the protocol published by King et al. [29] or with a commercial qPCR (virotype 2.0 ASFV, Indical Bioscience, Leipzig, Germany). All PCRs were performed on C1000™ thermal cyclers with the CFX96™ Real-Time System (Biorad). Results were recorded as quantification cycle (cq) and genome copy (gc) values, calculated by applying an ASFV in-house full-genome standard. For differentiation between vaccine and challenge viruses, tailored PCR targeting a deletion site was used. Samples positive for ASFV genomes in qPCR after challenge infection were thus further investigated. Primers used for detection of "ASFV-G-ΔMGF" amplified a 422 bp region deleted within the MGF505-1R-Gene (primers: forward, 5 = GAGGATGATTTGCCCTTCACTCA = 3; reverse, = 5CGCCAC-TAGTAAACATTGTTCTATCT = 3) [14]. Amplicons were then determined by 2% agarose gel electrophoresis.

For titrations, haemadsorption test (HAT) was used under slightly modified standard procedures, as recently described [30]. Titers were calculated in accordance to method published by Spearman and Kärber [31,32].

4.4.3. Antibody Detection

For the detection of ASFV-specific antibodies, two commercially available ELISA systems were used according to the manufacturer's instructions. In the DP trials, sera were tested in the OIE-recommended p72 antibody-specific INGEZIM PPA COMPAC (Ingenasa). In the WB trial, samples were screened in the p32, p64, and p72-antibody-specific IDScreen ASF Indirect (IDVet) Kit according to the manufacturer's instructions. The application of the IDScreen ASF Indirect kit for the WB trial was due to limitations in sample availability with the more stress-prone wild boar. INGEZIM PPA COMPAC requires serum, while plasma can be used for the IDScreen ASF Indirect assay.

For confirmatory reasons, doubtful samples were additionally tested in the indirect immunoperoxidase test (IPT) according to the standard protocols provided by the European Reference Laboratory for ASF.

5. Conclusions

"ASFV-G-ΔMGF" is fully efficacious when administered intramuscularly, and likewise, all responders to oral immunization are protected. Passaging of the vaccine virus on a permanent cell line did not result in any alterations of characteristics, providing a basis for possible commercialization of this promising candidate. However, efficiency of oral immunization has room for improvement as only 50% of the animals seroconverted. Very limited vaccine virus replication in swine and no virus shedding were observed. Future research should now focus on safety aspects to provide a basis for evaluation by regulatory authorities of this highly promising candidate.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/pathogens11090996/s1, Table S1: back titration of viruses used for inoculations; Table S2: blocking percentages from sera; Table S3: genome copies detected in swabs by qPCR.

Author Contributions: Conceptualization, P.D., E.V., J.C.M., A.U., M.B. and S.B.; methodology, P.D., T.C., M.B. and S.B.; formal analysis, P.D., T.C., J.S.-E., J.H.F. and S.B.; investigation, P.D., T.C., J.S.-E. and J.H.F.; resources, M.B. and S.B.; data curation, P.D., J.H.F. and S.B.; writing—original draft preparation, P.D. and S.B.; writing—review and editing, S.B., E.V., J.C.M., A.U. and M.B.; visualization, P.D., T.C. and S.B.; supervision, S.B. and M.B.; project administration, S.B., E.V., J.C.M., A.U. and M.B.; funding acquisition, S.B. and M.B. All authors have read and agreed to the published version of the manuscript.

Funding: The study received funding by Zoetis (Grant Number: Ri-0781).

Institutional Review Board Statement: All applicable animal welfare regulations including EU Directive 2010/63/EC were taken into consideration for the animal experiments. The animal experiments were externally approved by the competent authority (Landesamt für Landwirtschaft, Lebensmittelsicherheit und Fischerei (LALLF) Mecklenburg-Vorpommern) under reference numbers 7221.3-2-003/20 and 7221.3-2-035-21.

Data Availability Statement: All data to support the findings described in the text are included in the main text or in the supplementary materials. Additional data are available from the corresponding author upon reasonable request.

Acknowledgments: The authors would like to thank Ulrike Kleinert, Robin Brandt, and Christian Loth for their technical support, and further, the animal caretakers Matthias Jahn, Steffen Brenz, Domenique Lux, Stefanie Marzahl, Felix Zimak, Rene Siewert, and Patrice Mary for their help in the stable.

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

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5.2 Safety and genetic stability of African swine fever virus vaccine candidate “ASFV-G-ΔMGF” in an in vivo “reversion to virulence” study

Paul Deutschmann[1], Jan-Hendrik Forth[1], Julia Sehl-Ewert[1], Tessa Carrau[1], Elisenda Viaplana[2], Jose Carlos Mancera[3], Alicia Urniza[2], Martin Beer[1], Sandra Blome[1]

[1]Friedrich-Loeffler-Institut, Suedufer 10, 17493 Greifswald – Insel Riems, Germany

[2]Zoetis Manufacturing and Research Spain, Finca la Riba, Carretera de Camprodon s/n, 17813 L'Hostalnou de Bianya, Girona, Spain

[3]Zoetis Belgium, Mercuriusstraat 20, 1930 Zaventem, Belgium

Submitted to NPJ Vaccines

Preprint, 2022

doi.org/10.21203/rs.3.rs-1922286/v1

Safety and genetic stability of African swine fever virus vaccine candidate “ASFV-G-ΔMGF” in an in vivo “reversion to virulence” study

Sandra Blome (✉ sandra.blome@fli.de)
Friedrich Loeffler Institute
Paul Deutschmann
Friedrich-Loeffler-Institut
Jan-Hendrik Forth
Friedrich-Loeffler-Institut
Julia Sehl-Ewert
Friedrich-Loeffler-Institut
Tessa Carrau
Friedrich-Loeffler-Institut
Elisenda Viaplana
Zoetis Manufacturing and Research Spain
Jose Mancera
Zoetis Belgium
Alicia Umiza
Zoetis Manufacturing and Research Spain
Martin Beer
Friedrich-Loeffler-Institute https://orcid.org/0000-0002-0598-5254

Article

Keywords: African swine fever, vaccine, reversion to virulence, genetic adaptation

Posted Date: August 17th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1922286/v1

Abstract

African swine fever (ASF) has gained panzootic dimensions and commercial vaccines are still unavailable. Recently, a series of live attenuated vaccines has raised hope for an efficacious and safe vaccine, among them “ASFV-G-ΔMGF”. We tested the latter in a in vivo reversion to virulence study in accordance with International Cooperation on Harmonisation of Technical Requirements for Registration of Veterinary Medicinal Products guidelines. Upon forced animal passaging, a virus variant emerged that was associated with transient fever and an increased replication and shedding. However, all animals were healthy upon completion of the study and reversion to significant virulence was not observed. The genomic changes involved deletions and reorganizations in the terminal regions of the genome. While our study underscores that in-depth safety characterization is needed for live ASF vaccines, one should still conduct a thorough benefit risk analysis considering all safety and efficacy aspects when assessing their use in disease control.

1. Introduction

African swine fever (ASF) has recently spread in panzootic dimensions, exerting an immense pressure on the global pig industry and at the same time endangering entire populations of rare wild pig species (Luskin, Meijaard et al. 2021). The disease is caused by ASF virus (ASFV), a member of the genus *Asfivirus* within the family *Asfarviridae* (Alonso, Borca et al. 2018). Outside its sylvatic cycle in sub-Saharan Africa, the disease is characterized by a haemorrhagic fever with high lethality in domestic and wild suids, which represent the only susceptible mammals (Bosch, Barasona et al. 2020). Following an introduction into Georgia in 2007, ASF spread successively through eastern and central Europe, most of Asia, and recently to the Caribbean (Dixon, Stahl et al. 2020, Gonzales, Moreno et al. 2021, Sauter-Louis, Conraths et al. 2021). Without vaccines, the available control measures have failed to eliminate the disease in most countries affected by ASF (Dixon, Stahl et al. 2020). Thus, the call for a safe and efficacious vaccine is louder than ever, and research efforts to find solutions have recently intensified. Still, only a few attempts have produced successful vaccine candidates that have gone beyond proof-of-concept studies. To date, live attenuated vaccines (LAV) are the most promising concept, since complete protection against lethal field strains have only been shown with this group of vaccines (Muñoz-Pérez, Jurado et al. 2021). While reports in peer-reviewed publications of the first efficacious LAV prototypes raise hope for a licensable product on the horizon, there are still significant concerns with their safety (Bosch-Camós, Lopez et al. 2020). In particular, the inevitable ability of live vaccines viruses to replicate may result in in genetic mutations and adaption in target tissues.

In the present study, we evaluated vaccine candidate “ASFV-G-ΔMGF” (O'Donnell, Holinka et al. 2015), from here on called “ΔMGF”), a genetically modified LAV that has shown a most promising efficacy profile, in a standard *in vivo* reversion to virulence study in naïve weaner pigs. In short, the vaccine virus was passaged five times in domestic pigs in accordance with *VICH (International Cooperation on Harmonisation of Technical Requirements for Registration of Veterinary Medicinal Products) guideline 41*

for the examination of live veterinary vaccines in target animals for absence of reversion to virulence (reference number EMA/CVMP/VICH/1052/2004).

2. Methods

2.1 Experimental design

The VICH guideline 41 states that the study is to be carried out using the master seed at the maximum release titer expected in the recommended dose for five serial passages in target animals. The time interval between inoculation of the animals and harvest for each passage must be justified based upon the characteristics of the test organism. Moreover, the most sensitive class, age, sex and serological status of animals should be used. At least two animals are to be used for the first four groups and a minimum of eight for the fifth group. The initial administration and subsequent passages shall be carried out using a recommended route of administration or natural route of infection that is the most likely to lead to reversion to or increase in virulence and result in recovery of the organism following replication in the animal. Passage inocula should be collected and prepared from the most likely source of spread of the organism.

The above-mentioned recommendations were implemented as follows: Dose and route of inoculation were chosen to maximize the chance of reversion to or increase in virulence, representing a worst-case scenario of vaccine virus transmission. Considering this, undiluted master seed virus (MSV) was used and day 7 was set as timepoint for organ and blood collection for passaging. At this point, recovery of vaccine virus had the highest chance based on our experience from previous studies. Along the same lines, an intramuscular transfer of material was chosen to maximize the chance of infection given the experience that the parenteral route is much more efficient than an oral or oro-nasal inoculation (Guinat, Gogin et al. 2016). Given the low detection rate in previous trials, the study was performed in groups of ten, 6-10-week-old weaned naïve pigs which were obtained from the breeding unit of the Friedrich-Loeffler-Institute (FLI) in Mariensee, Germany, and moved to the high containment facilities of the FLI on the Isle of Riems, Germany. Before inoculation, blood of each pig was collected in an EDTA tube for reference purposes. At the respective day of study completion, a full pathological examination was performed based on the modified protocol published by Galindo-Cardiel (Galindo-Cardiel, Ballester et al. 2013) as previously described (Sehl, Pikalo et al. 2020). Blood as well as tissue samples including spleen, lung, liver, tonsil, kidney, salivary gland and gastro-hepatic and mandibular lymph nodes were collected upon necropsy. Pigs of the first four passages were observed for seven days post inoculation (dpi). Passage five was observed for 21 dpi, and oral, nasal and rectal swabs were collected weekly to trace vaccine virus shedding during the last passage. The animal experiment was approved by the State Office for Agriculture, Food Safety and Fishery in Mecklenburg-Western Pomerania (LALFF M-V) under reference number 7221.3-1-020/21.

2.2 Cells and titrations

Titrations and virus isolations were performed on porcine peripheral blood mononuclear cell (PBMC) derived macrophages obtained as previously described (Fischer, Mohnke et al. 2020). For titrations, cells were seeded in 96-well culture plates (Primaria; Corning) with 100 µl / well at a density of 5x10⁶ cells/ml and cultivated as described before (Fischer, Pikalo et al. 2021). 100 µl of the respective samples diluted in cell culture medium at factors 10^{-1} to 10^{-8} was added to each well for end point titration. 20 µl of a 1% suspension of erythrocytes from the same donor pig in PBS was added after 24 h. For determination of titers, infected wells were read 48 and 72 h post infection using the hemadsorption as read-out. The HAD_{50} was calculated according to the method by Kaerber (1931).

2.3 Passaging of the virus

A pure pre-master master seed virus grown on primary swine macrophages was prepared by USDA-ARS at the Plum Island Animal Disease Center and transferred to Zoetis. Master seed virus grown on a proprietary commercial permanent cell line was subsequently prepared by Zoetis and provided to the FLI ready to use.

Five groups of ten pigs each were inoculated intramuscularly with 1 mL of the respective virus suspension into the right side of the neck using 2 mL syringes with 21G cannulas.

Group one received the “ΔMGF” MSV at a dose of 1.75 x 10^6 HAD_{50}/ml. Organs were sampled after each passage and screened by qPCR for the presence of ASFV genome. For subsequent passages, tissues with the highest genome loads were selected, pooled at equal proportions, and homogenized in PBS at 20 Hz for 30 seconds in a grinding jar set compatible with the TissueLyser II (QIAGEN) to receive a 1 % tissue suspension. A total of 1.5 g of tissue was weighed and suspended in 13.5 mL of PBS for preparation of each inoculate. After centrifugation at 3500 rpm for 5 minutes, supernatants were obtained and administered to the following passage group. Tissue homogenate from passage one was back titrated to $10^{4.25}$ HAD_{50}/mL. The subsequent inoculates contained $10^{2.25}$ (P2), 10^4 (P3) and $10^{5.75}$ HAD_{50}/mL (P4). An overview of the study design and the organs chosen for passaging is provided in Fig. 1.

2.4 Laboratory investigations

2.4.1 Preparation of samples and qPCR

Tissue samples were homogenized in 1 mL phosphate buffered saline (PBS) with a metal bead on a TissueLyser II (QIAGEN) at 30 Hz for 3 minutes, then centrifuged at 14000 rpm for 5 minutes. Swab samples were soaked in medium for 1h, then thoroughly vortexed and aliquoted. All samples were stored at -80°C or immediately processed.

DNA was extracted using the NucleoMag Vet Kit (Machery-Nagel) on the KingFisher® extraction platform (Thermo Scientific). qPCR for the detection of ASFV genome was either conducted according to the protocol published by King, Reid et al. (2003) or with commercial virotype 2.0 ASFV (Indical Bioscience)

on C1000™ thermal cyclers with the CFX96™ Real-Time System (Biorad). An in-house ASFV full genome standard was employed for calculation of genome copies and harmonization between runs.

2.4.2 Whole-genome sequencing

For whole-genome sequencing, a minimum of 100 ng of DNA was sent to and sequenced by Eurofins Genomics. This service included preparation of a 450 bp DNA sequencing library using a modified version of the NEBNext Ultra™ II FS DNA Library Prep Kit for Illumina and sequencing on an Illumina NovaSeq 6000 with S4 flowcell, XP workflow and in PE150 mode (Illumina).

2.4.3 Data analysis

The sequence data received from Eurofins Genomics was quality trimmed and mapped against a previously produced MSV whole-genome sequence as reference using Newbler 3.0 (Roche) with default parameters. Mapped reads were extracted and assembled using SPAdes 3.13 in the mode of error correction prior to assembly and standard parameters. The resulting contigs were mapped against the ASFV MSV reference sequence and the contigs was curated and assembled manually in Geneious Prime. For validation of the assembly and mean coverage determination, all reads were mapped against the final contig again using Newbler 3.0 with default parameters.

2.4.4 Tailored qPCR

For the identification of the novel ASFV variant, two qPCRs were designed using Geneious Prime (Biomatters) spanning the reorganization site at the 5’ end (probes directly positioned at the reorganization site) with one specifically recognizing the ΔMGF MSV (FAM-labeled) and one recognizing the ΔMGFnV (HEX-labeled) (see Table 1 and supplementary table 3).

Table 1
List of modified genes found in the ΔMGFnV genome

Gene	Type	Minimum	Maximum	Function
MGF 360-1La CDS	Deletion	1,63	2,463	Unknown
MGF 360-1Lb CDS	Deletion	2,391	2,711	Unknown
MGF 360-2L CDS	Deletion	2,797	3,885	Unknown
KP177R CDS	Deletion	4,026	4,559	P22, structural protein, transmembrane domain
L83L CDS	Deletion	4,696	4,941	Early gene, located in cytoplasm, interacts with IL1B, non-essential
L60L CDS	Deletion	5,042	5,2	Unknown
MGF 360-3L CDS	Deletion	5,359	6,429	Unknown
MGF 110-1L CDS	Deletion	6,822	7,466	Early gene. Membrane protein. Might play a role in virus cell tropism, and may be required for efficient virus replication in macrophages (inferred from homology).
ASFV G ACD 00090 CDS	Deletion	7,465	7,578	Unknown
MGF 110-2L CDS	Deletion	7,646	7,96	Early gene. Might play a role in virus cell tropism, and may be required for efficient virus replication in macrophages (inferred from homology).
MGF 110-3L CDS	Deletion	8,057	8,431	Unknown
ASFV G ACD 00120 CDS	Deletion	8,542	8,766	Unknown
MGF 110-4L CDS	Deletion	8,745	9,119	Early gene, causes the redistribution of lumenal ER protein to an enlarged ERGIC compartment. Glycosylated.
MGF 110-5L-6L CDS	Deletion	9,308	9,925	Early gene, might play a role in virus cell tropism, and may be required for efficient virus replication in macrophages (inferred from homology).

Gene	Type	Minimum	Maximum	Function
MGF 110-7L CDS	Deletion	10,132	10,545	Glycosylated, might plays a role in virus cell tropism, and may be required for efficient virus replication in macrophages (inferred from homology).
285L CDS	Deletion	10,86	11,144	Transmembrane domain.
DP60R CDS	Deletion	232	396	Transmembrane domain, glycosylated.
ASFV G ACD 01990 CDS	Deletion	517	663	Unknown
ASFV G ACD 01980 CDS	Duplication	1,354	1,548	Unknwon
MGF 360-21R CDS	Duplication	1,537	2,607	Might play a role in virus cell tropism, and may be required for efficient virus replication in macrophages (inferred from homology).
ASFV G ACD 01960 CDS	Duplication	3,053	3,184	Unknown
MGF 360-19Rb CDS	Duplication	3,253	3,522	Might play a role in virus cell tropism, and may be required for efficient virus replication in macrophages (inferred from homology).
MGF 360-19Ra CDS	Duplication	3,536	4,345	
ASFV G ACD 01940 CDS	Duplication	4,498	4,656	Unknown
DP96R CDS	Duplication	4,956	5,246	Unknown
DP71L CDS	Duplication	5,345	5,557	Interacts with the host phosphatase PP1 catalytic subunit (PPP1CB) and recruits it to dephosphorylate EIF2S1/eIF2alpha and therefore restores the host translation that has been shut-down by the host. Also inhibits the EIF2S1/eIF2alpha-ATF4-DDIT3/CHOP pathway.
MGF 360-18R CDS	Duplication	5,54	6,253	Might play a role in virus cell tropism, and may be required for efficient virus replication in macrophages (inferred from homology).
L11L CDS	Duplication	6,483	6,764	Membrane protein.

Gene	Type	Minimum	Maximum	Function
I10L CDS	Duplication	7,003	7,515	Structural protein, in virion, Transmembrane protein. In viral envelope.
I9R CDS	Duplication	7,591	7,881	Transmembrane protein
ASFV G ACD 01870 CDS	Duplication	7,844	7,981	Unknown
I8L CDS	Duplication	8,076	8,387	Non-essential. Unknown.
hypthetical CDS	Duplication	8,434	8,541	Unknown
I7L CDS	Duplication	8,601	8,909	Transmembrane protein
MGF 100-3L CDS	Duplication	9,008	9,316	Might play a role in virus cell tropism, and may be required for efficient virus replication in macrophages (inferred from homology).
MGF 100-1L CDS	Duplication	9,681	10,106	Unknown
MGF 505-11L CDS	Duplication	10,225	11,853	Might play a role in virus cell tropism, and may be required for efficient virus replication in macrophages (inferred from homology).
MGF 360-16R CDS	Duplication	11,933	12,991	Might play a role in virus cell tropism, and may be required for efficient virus replication in macrophages (inferred from homology).
DP238L CDS	Duplication	13,168	13,884	Unknown
I196L CDS	Duplication	13,978	14,586	Late gene. Unknown.
I177L CDS	Duplication	14,579	15,112	Late gene. Single pass membrane protein. Gylcosylated.
I215L CDS	Duplication	15,153	15,791	Early and late gene. Accepts ubiquitin from the E1 complex and catalyzes its covalent attachment to other proteins. Performs the second step in the ubiquitination reaction that targets specifically a protein for degradation via the proteasome. By controlling the ubiquitination status of specific host proteins, the virus may target them to degradation and thereby optimize the viral replication. Knockdown impairs viral infection, with lower number of synthesized viral genomes and lower viral progeny.
I329L CDS	Duplication	16,08	17,069	Late gene. Single-pass type I membrane protein. Highly glycosylated.

Gene	Type	Minimum	Maximum	Function
I73R CDS	Duplication	17,279	17,497	Unknown
I243L CDS	Duplication	17,581	18,312	Late gene. Transcription factor S-II-related protein
DP60R CDS	Duplication	416	580	Transmembrane domain, glycosylated
ASFV G ACD 01990 CDS	Duplication	701	847	Unknown

2.4.5 Growth kinetics

The novel virus variant ΔMGFnV was isolated from the blood of pig #22 of the fifth passage and cultivated on macrophages in T25 cell culture flasks (Primaria; Corning) obtained as described above. Absence of the MSV virus was assured by tailored qPCR. Growth kinetics were conducted on macrophages. To this end, T25 flasks were infected at a multiplicity of infection of 0.1 with either the ΔMGF MSV, the ΔMGFnV or both viruses simultaneously. After two hours of incubation, medium supernatants were removed, cells were rinsed once with PBS- and flasks were resuspended with cell culture medium. 300 µl of supernatant were removed at -2 hours post infection (hpi, before adding the virus solution), 0 hpi (after incubation), and 4, 8, 12, 24, 48 and 72 hpi and immediately stored at -80°C. Samples were analyzed by the commercial ASFV real-time PCR system virotype 2.0 (Indical Bioscience GmbH) and tailored qPCR for differentiation between ΔMGF MSV and ΔMGFnV replication, and by titration on macrophages using the methods described above.

3. Results

3.1 Clinical Observations

No clinical abnormalities or fever were observed during the first animal passage (see Fig. 1). In the second animal passage, however, three pigs displayed a transient rise in body temperatures to up to 40.4°C. Beginning from passage three and in both subsequent passages, high fever to a maximum of 42.1°C (pig #31, P4, 6 dpv, supplementary Fig. 1) was observed in numerous animals with a peak at around five and six dpi. In passage five, a body temperature of 41° C or above was recorded in nine out of ten animals for at least one day. Elevated body temperatures were clinically mirrored by mild to moderate signs of anorexia and apathy, which were scored to a maximum of three cumulative clinical score points in a single animal in passage four (animal #31, 6 dpi). All other clinical observations between passages three and five were very mild and resulted in only one cumulative clinical score point. In passage five, with an extended observation period of 21 dpi, normalization of body temperatures and the subsequent disappearance of clinical abnormalities were observed after the fever peak. Animals were clinically healthy at the end of the 21-day observation period of passage five, with the exception of animal #1110,

which showed transient lameness (not related to ASFV) on 14 and 15 dpi, recovering thereafter. One animal (#21) displayed a complete loss of sensory and motoric function of the front right leg immediately after inoculation. Treatment of the clinically diagnosed nerval lesion with dexamethasone did not result in any improvement of the lameness, so the animal was euthanized for ethical reasons on 7 dpi.

3.2 Pathological findings

All pigs were subjected to detailed pathological examination. Overall, very few lesions were detectable in all passages, i.e. slightly enlarged lymph nodes and pulmonary consolidation. No correlation or significant difference was observed when comparing lesions with passage level or time point after inoculation, i.e. 7 versus 21 days (data not further shown).

3.3 Laboratory investigations

3.3.1 Detection of ASFV genomes

qPCR screening of the samples taken from the first passage yielded less than $7.2x10^1$ ASFV genome copies (gc) per 5 µl template from the entire sample set (shown in Fig. 3). In two pigs, vaccine virus could not be detected. In passage two, no more than $5x10^1$ gc were detected in a single sample and in five pigs, vaccine virus was not detected at all. In the third passage, however, all pigs were positive in at least one sample and up to $1.8x10^3$ gc were quantified. All pigs were positive for ASFV genome on passage four with a maximum $2.6x10^3$ gc in a sample. In passage five, again, all animals were positive for ASFV genome, however with slightly lower genome loads in the different tissues but comparable loads in blood to the previous passages (prolonged monitoring phase of 21 dpi, see Fig. 4). In pig #21, up to $2.6x10^3$ gc were detected (euthanized on 7 dpi).

Investigation of different swab samples showed low viral genome loads in seven out of ten animals in at least one type of swab on seven dpi (maximum of 4 gc in a sample). On 14 dpi, six out of nine animals were positive and three out of nine were positive 21 dpi. Interestingly, higher genome loads were detected from swabs at 21 dpi than in the weeks before (see supplementary table 1).

3.3.2 Whole-genome sequencing

In two samples of passage four, an ASFV variant was detected characterized by a large deletion at the 5'-end of the genome. This deletion of 11197 bp leads to the loss of 18 previously annotated ASFV genes (see Fig. 2 and Table 1 for detailed and functional findings). Interestingly, the deletion is accompanied by a duplication of 18592 bp from the 3'-end of the genome which are bound to the 5'-end in reverse complementary orientation leading to the duplication of 29 genes.

The new virus variant that evolved during *in vivo* passaging was named "ΔMGFnV".

3.3.3 Screening for the novel virus variant ΔMGFnV

Using tailored qPCR, the emergence of the novel virus variant ΔMGFnV was tracked back to pig #1081 of passage one, the spleen of which was part of the organ pool for subsequent passaging (see supplementary table 3). In this animal, a mixed infection of the MSV and ΔMGFnV was observed, while in six other pigs only the wild-type MSV was detected. In the second passage, the ΔMGFnV was detected in two pigs (#1080 monoinfection, #1073 mixed infection with MSV), while three pigs were positive for just the MSV. In passage three, all positive pigs harbored the ΔMGFnV, one individual (#1082) as a monoinfection and nine other animals as mixed infection with the MSV. The picture remained similar after the fourth passage: All pigs were positive for ΔMGFnV, one of which by monoinfection and nine out of ten were also infected with the MSV. In passage five, ΔMGFnV was detected as a monoinfection in four pigs, while the other six animals were coinfected with both the MSV and the variant.

3.3.4 Comparative growth kinetics

The purity of the respective ΔMGF and ΔMGFnV isolates as well as the presence of both isolates in the coinfected cell culture was confirmed by tailored qPCR. After incubation of the virus suspensions (0 hpi), titers between $10^{2.75}$ (MSV + nV, nv) and 10^{3} HAD_{50}/ml (MSV) were detected from supernatants in HAT (see supplementary table 4). Increase of titers developed uniformly with a maximum logarithmic deviation of 0.5 at a single point in time. Viruses grew up to $10^{6.75}$ HAD_{50}/ml (MSV and nV monoinfecton) and 10^{7} HAD_{50}/ml (coinfection).

When testing samples from the kinetics in the tailored qPCR, for the FAM channel (detection of ΔMGFnV, a mean deviation of 2.9 % was recorded from ASFV genome detection in virotype 2.0. For the HEX channel (nV detection), mean deviation from virotype 2.0 was 8.55% (shown in supplementary table 4). Variant ΔMGFnV reached cq values of roughly 17 in mono- and coinfection at 48 and 72 hpi, while growth of the nV variant yielded cq values of roughly 19 (monoinfection) or 21 (coinfection), at the respective times. Considering the beforementioned deviations in the tailored qPCR, growth kinetics of both isolates developed quite uniformly.

4. Discussion

As the global spread of ASF continues, the situation for pig holders and nature conservationists has never been as tense as it is now. Millions of pigs are at risk, representing the livelihoods of farmers, and entire species of certain wild suids, e.g. bearded pigs, that are now threatened with extinction (Ewers, Nathan et al. 2021). Against this background, a vaccine is urgently needed to complement available control measures. In this context, we may not be in the position to wait for a perfect vaccine candidate and should rather stress the application of a practicable solution as fast as reasonably possible. On the other hand, we cannot afford to compromise on vaccine safety, or as Gavier-Widen, Stahl et al. (2020) put it, allow hasty solutions. Experiences in Spain and Portugal from the last century using attenuated field isolates (Petisca 1965), and possibly very recently in China (https://www.reuters.com/article/us-china-swinefever-vaccines-insight-idUSKBN29R00X, visited May 15th 2022), show us that premature field testing of live vaccines can cause prolonged forms of ASF with extended shedding and delayed clinical

characteristics, often below the detection limit. The use of vaccine viruses with unacceptable residual virulence or that revert to virulence can lead to an iatrogenic, self-sustaining infection cycle with increasing virulence. This would further complicate eradication efforts and these scenarios must be avoided under all circumstances.

Consequently, we took one of the few fully efficacious and possibly licensable vaccine candidates, “ΔMGF”, and examined its safety profile in terms of genetic stability and reversion to virulence under a worst-case scenario.

In our traditional reversion to virulence study over five animal passages, we observed the occurrence of a virus variant in one animal of passage one that subsequently overgrew the wild-type MSV. This variant was genetically characterized by a large deletion at the 5’-end of the genome and an accompanied duplication at the 3’-end. Clinically, the variant was associated with a slightly increased virulence, e.g. induction of a short episode of fever in most animals in the later passages. However, all animals even in the final 5th passage were clinically inapparent at the end of the experiment, showed no evidence of incipient, chronic infection, and showed little or no vaccine virus in the tissues tested.

For further characterization of the “ΔMGFnV”, we conducted comparative *in vitro* growth kinetics of both viruses in primary macrophages, revealing no indications for a significant advantage in *in vitro* replication of the variant virus in this setup. Clear limits in explanatory power should be considered, however, since modifications in the MGF regions are known to have effects on interferon expression (Wang, Kang et al. 2021), and the full consequences of such changes are probably only observable *in vivo*. The underlying factors causing the *in vivo* replication advantages of ΔMGFnV remain therefore unanswered, stressing that many of these questions can only be addressed by further animal experiments due to the highly complex virus-host interactions of ASFV.

Whether our findings showing genetic changes and a slight rise in virulence after *in vivo* passaging disqualify the vaccine candidate is a matter of critical debate, since the mode of transmission is highly artificial (selecting particularly positive samples for further passaging and intramuscular injection of tissue homogenates), and there is no evidence of reversion to the original levels of high virulence of ASFV “Georgia07”. In this context, it could be discussed whether a brief period of fever can be tolerable for an efficacious and therefore otherwise practicable first generation ASFV vaccine. A prerequisite for this assumption would be that the novel variant is genetically stable and does not mark the beginning of a maintained process of further genetic adaption, only mirrored by this first mutation.

The mutant detection qPCR described here was also used retrospectively for representative samples from several efficacy tests with the ΔMGF vaccine candidate, with clearly negative results (data not further shown).

It should be noted, however, that in this case a viral variant with altered geno- and phenotypic properties has already emerged in the first passage, i.e. after application of the MSV. This phenomenon did not occur in any of the previous studies, but it is relevant because it could also happen in the field during

intensive use. It is also remarkable that the mechanism of a large deletion complemented by reorganization of genomic regions has been observed for an ASFV strain under natural circumstances before (Zani, Forth et al. 2018). We may have unraveled a common mechanism of ASFV for genetic adaption when a certain selection pressure is applied.

For a fact-based benefit-risk assessment, further studies with the evolved virus variant are needed, which should address excretion, long-term effects, and transmission to naive contact animals.

In general, our study confirms that even the most promising ASF live vaccine candidates require very comprehensive safety testing (Gavier-Widen et al., 2020). However, it also provides a first indication of what an attenuated ASF vaccine virus would need to do to increase its replication efficacy in the animal or to compensate for deletions in the MGF region. This knowledge can be deepened and used to devise strategies to make these changes even more difficult for the virus.

If field application is considered after benefit-risk-assessment, one should apply genetic tools to differentiate infected from vaccinated animals (DIVA). The PCR described here could aid such approaches. Moreover, conditional licensing under controlled conditions could be a solution to obtain field data for final decisions on the use of the vaccine to complement national control measures.

Declarations

Acknowledgements

The authors would like to thank Ulrike Kleinert, Robin Brandt and Christian Loth for their excellent technical support and further the animal caretakers Matthias Jahn, Steffen Brenz and Domenique Lux for their help in the stable.

Author Contributions

Conceptualization, P.D., E.V., J.C.M., A.U., M.B., and S.B.; methodology, P.D., T.C., J.S.-E., M.B. and S.B..; formal analysis, P.D., T.C., J.S.-E., J.-H.F., and S.B.; investigation, P.D., T.C., J.S.-E. and J.-H.F.; resources, M.B. and S.B..; data curation, P.D. and S.B.; writing—original draft preparation, P.D. and S.B.; writing—review and editing, S.B., E.V., J.C.M., A.U. and M.B.; visualization, P.D., J.-H.F and S.B.; supervision, S.B. and M.B.; project administration, S.B., E.V., J.C.M., A.U. and M.B. funding acquisition, S.B. and M.B. All authors have read and agreed to the published version of the manuscript.

Competing Interests statement

Elisenda Viaplana, Jose Carlos Mancera and Alicia Urniza are employed by Zoetis. Zoetis has a commercial license for the vaccine candidate described in the manuscript. The overall project, coordinated by Sandra Blome at the FLI, received funding by Zoetis, including the salary of Paul Deutschmann. All other authors declare no competing interests.

Ethical Statement

All applicable animal welfare regulations including EU Directive 2010/63/EC complied with for the animal experiment. The animal experiment was approved by the competent authority (Landesamt für Landwirtschaft, Lebensmittelsicherheit und Fischerei (LALLF) Mecklenburg-Vorpommern) under reference number 7221.3-2-020/20.

Data Availability Statement

All relevant data to support the findings described in the text are included in the main text or in the supplementary materials. Additional data is available from the corresponding author upon reasonable request.

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Figures

Figure 1

Overview of the passaging groups and the study design. Tissues used from the respective animals for further passaging are depicted as organs in the center. Clinical and virological results throughout the study are visualized on the right side. Created with BioRender.com

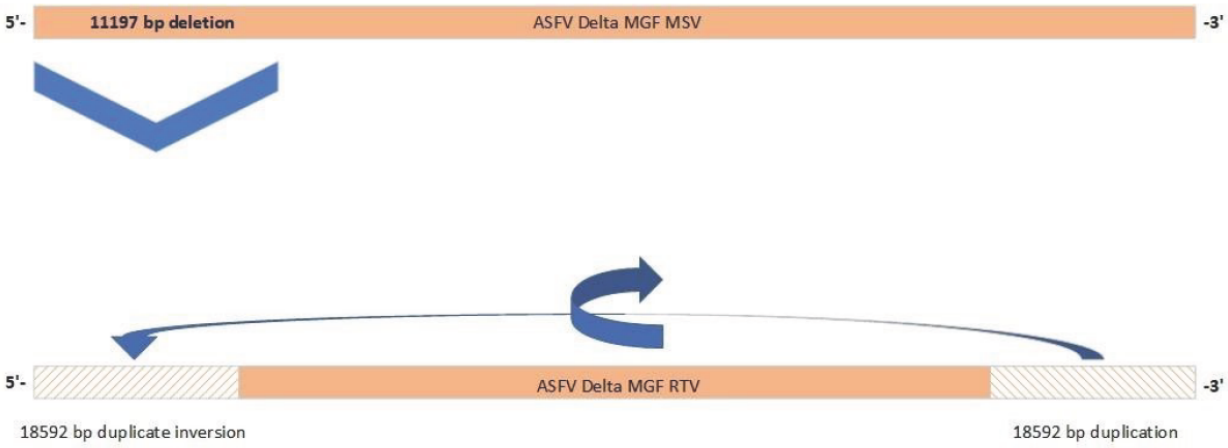

Figure 2

Schematic overview of the mutation characterized by duplicate inversion identified in the novel variant. The genome of "DMGF" with the deletion is depicted on top and the genome of the "DMGFnv" with the duplicate inversion mutation is represented at the bottom.

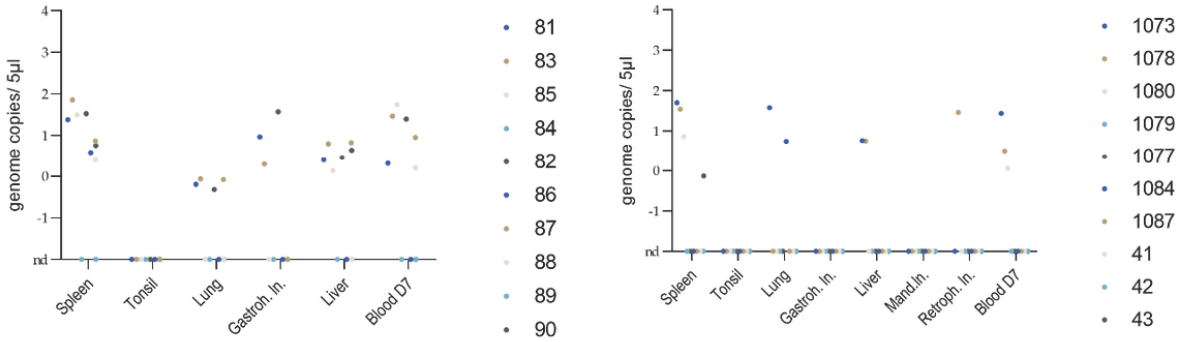

Figure 3

Genome load in the sample sets in passage 1 (top left), 2 (top right), 3 (bottom left) and 4 (bottom right) after 7 days of observation. Individual blots mark tissue samples from respective animals in each passage.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Supplementsmergedpdf.pdf

Annex to 4.2 “Safety and genetic stability of African swine fever virus vaccine candidate “ASFV-G-ΔMGF” in an in vivo “reversion to virulence” study”:

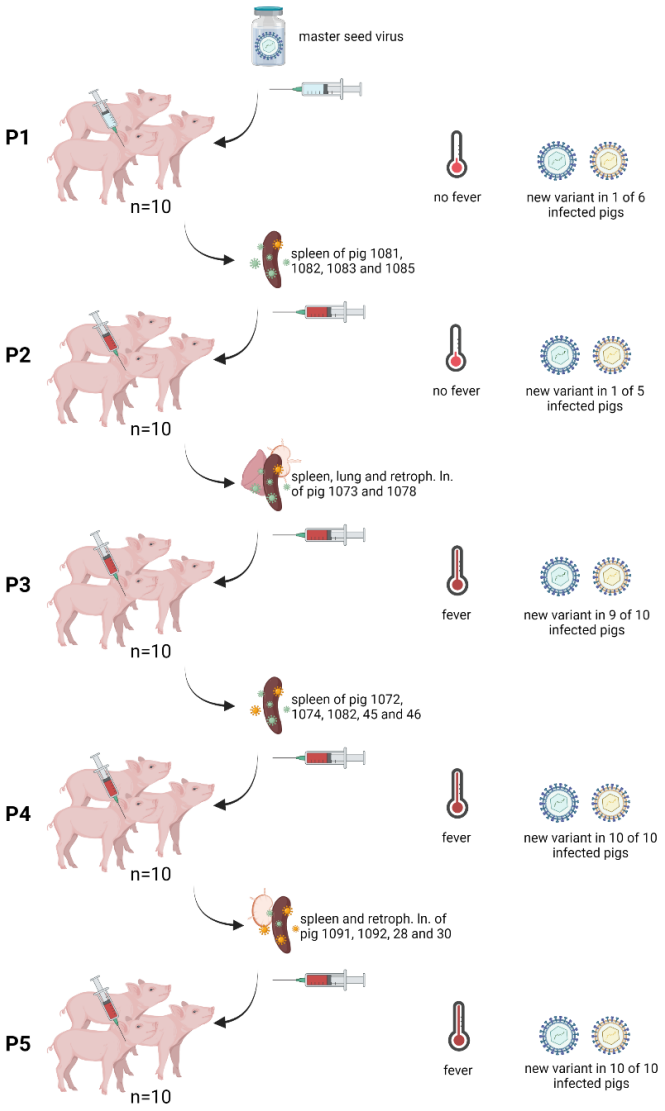

Figure 3: Overview of the passaging groups and the study design. Tissues used from the respective animals for further passaging are depicted as organs in the center. Clinical and virological results throughout the study are visualized on the right side. Created with BioRender.com

5.3 African swine fever virus – variants on the rise

Jan H. Forth[1], Sten Calvelage[1], Melina Fischer[1], Jan Hellert[2], Julia Sehl-Ewert[1], Hanna Roszyk[1], Paul Deutschmann[1], Adam Reichold[3], Martin Lange[3], Hans-Herman Thulke[3], Carola Sauter-Louis[1], Dirk Höper[1], Svitlana Mandyhraf[4], Maryna Sapachova[4], Martin Beer[1], Sandra Blome[1]

[1] Friedrich-Loeffler-Institut, Federal Research Institute for Animal Health, Greifswald, Insel Riems – Germany

[2] Centre for Structural Systems Biology (CSSB), Leibniz-Institut für Experimentelle Virologie (HPI), Hamburg, Germany

[3] Helmholtz Centre for Environmental Research GmbH - UFZ, Dept Ecological Modelling, PG Ecological Epidemiology, Leipzig - Germany

[4] Institute of Veterinary Medicine of the National Academy of Agrarian Science of Ukraine, Kiev, Ukraine

Emerging Microbes & Infections

2023

doi.org/10.1101/2022.09.07.506908

Emerging Microbes & Infections
2023, VOL. 12, e2146537 (13 pages)
https://doi.org/10.1080/22221751.2022.2146537

RESEARCH ARTICLE

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African swine fever virus – variants on the rise

Jan H. Forth[a]*, Sten Calvelage[a]*, Melina Fischer[a], Jan Hellert[b], Julia Sehl-Ewert[c], Hanna Roszyk[a], Paul Deutschmann[a], Adam Reichold[d], Martin Lange[d], Hans-Hermann Thulke[d], Carola Sauter-Louis[e], Dirk Höper[a], Svitlana Mandyhra[f], Maryna Sapachova[f], Martin Beer[a]† and Sandra Blome[a]†

[a]Institute of Diagnostic Virology, Friedrich-Loeffler-Institut, Greifswald, Germany; [b]Centre for Structural System Biology (CSSB), Leibnitz-Institut für Virologie, Hamburg, Germany; [c]Department of Experimental Animal Facilities and Biorisk Management, Friedrich-Loeffler-Institut, Greifswald, Germany; [d]Department of Ecological Modelling, Helmholtz Centre for Environmental Research, Leipzig, Germany; [e]Institute of Epidemiology, Friedrich-Loeffler-Institut, Greifswald, Germany; [f]State Scientific and Research Institute of Laboratory Diagnostics and Veterinary and Sanitary Expertise (SSRILDVSE), Kiev, Ukraine

ABSTRACT

African swine fever virus (ASFV), a large and complex DNA-virus circulating between soft ticks and indigenous suids in sub-Saharan Africa, has made its way into swine populations from Europe to Asia. This virus, causing a severe haemorrhagic disease (African swine fever) with very high lethality rates in wild boar and domestic pigs, has demonstrated a remarkably high genetic stability for over 10 years. Consequently, analyses into virus evolution and molecular epidemiology often struggled to provide the genetic basis to trace outbreaks while few resources have been dedicated to genomic surveillance on whole-genome level. During its recent incursion into Germany in 2020, ASFV has unexpectedly diverged into five clearly distinguishable linages with at least ten different variants characterized by high-impact mutations never identified before. Noticeably, all new variants share a frameshift mutation in the 3′ end of the DNA polymerase PolX gene O174L, suggesting a causative role as possible mutator gene. Although epidemiological modelling supported the influence of increased mutation rates, it remains unknown how fast virus evolution might progress under these circumstances. Moreover, a tailored Sanger sequencing approach allowed us, for the first time, to trace variants with genomic epidemiology to regional clusters. In conclusion, our findings suggest that this new factor has the potential to dramatically influence the course of the ASFV pandemic with unknown outcome. Therefore, our work highlights the importance of genomic surveillance of ASFV on whole-genome level, the need for high-quality sequences and calls for a closer monitoring of future phenotypic changes of ASFV.

ARTICLE HISTORY Received 7 September 2022; Revised 7 November 2022; Accepted 8 November 2022

KEYWORDS African swine fever virus; genomic epidemiology; variants; whole-genome sequencing; epidemiological modelling

Introduction

It is widely accepted today that most virus populations consist of a variety of genetic variants rather than one clonal virus. The emergence of these virus variants is driven by the virus specific mutation rate [1], which depends on multiple factors including mode of replication, fidelity of polymerases, the availability of repair mechanisms, as well as selection. Together, these two factors are responsible for the speed with which evolution progresses (evolutionary rate), demonstrated by the emergence of new virus variants [2]. While some viruses evolve very fast and new variants develop quickly, impressively demonstrated during the recent SARS-CoV2 pandemic [3], other viruses demonstrate a high degree of genetic stability and evolve very slowly. One example for the latter is the African swine fever virus (ASFV) [4,5].

This large and complex DNA virus has been first described in Kenya in 1921 [6], where it is transmitted in an ancient sylvatic cycle between warthogs and soft ticks of the genus Ornithodoros [7,8]. In 2007 the virus was translocated to Eurasia and since then spreads in wild boar and domestic pig populations. While the African warthogs remain largely asymptomatic after infection [9], the virus is highly lethal to domestic pigs [10,11] and Eurasian wild boar [11–13]. Although distantly related viruses have been identified in amoebae and some degree of similarity to irido- and poxviruses has been shown, no closely related viruses are known today [5]. Therefore, ASFV was only recently grouped into the phylum Nucleocytoviricota and, because it is the only known member of its family Asfarviridae and the genus Asfivirus [5], is still considered a mystery in modern virology.

CONTACT Sandra Blome sandra.blome@fli.de Institute of Diagnostic Virology, Friedrich-Loeffler-Institut, Federal Research Institute for Animal Health, Suedufer 10, 17493, Greifswald – Insel Riems, Germany
*Both authors contributed equally.
†Both senior authors contributed equally.
Supplemental data for this article can be accessed online at https://doi.org/10.1080/22221751.2022.2146537.

The ASFV genome, a single molecule of covalently closed double-stranded DNA with a size of up to 190 kbp [14], has a remarkably high genetic stability. Modern virus strains show a very high degree of nucleotide sequence identity to viral elements integrated in the soft tick genome dated to at least 1.46 million years [15]. This observation is supported by recent analyses of the ASFV strain introduced into Georgia in 2007. Despite over ten years of epidemic circulation, the virus strain has accumulated only very few mutations overall and even less affecting viral genes [16,17].

When ASFV was introduced into the wild boar population of eastern Germany in 2020 [18], whole genome sequencing revealed an ASFV strain similar to the strains known to circulate in western Poland including a mutation within the O174L gene, coding for ASFV DNA repair polymerase X [19,20]. This insertion of a tandem repeat was utilized together with other mutations in K145R, MGF 505-5R and the intergenic region between 173R and I329L as genetic marker to trace outbreak clusters in affected Polish counties [21]. While this discovery was no doubt interesting, no evidence for differences in the virus phenotype were observed at that point. What came as a surprise was the subsequent detection of numerous ASFV variants in Germany characterised by high impact mutations that have never been described before affecting known ASFV open reading frames (ORFs). While some of the changes affect regions of the viral genome that could be linked to potential immune modulators or virulence factors, the influence of most mutations remains unknown. Therefore, we wanted to (i) investigate in more detail what might underlie this new genetic variability, (ii) utilize this newly emerged genetic variance for molecular epidemiology, and (iii) identify consequences of our findings at the epidemiological level using models.

The present manuscript summarizes the canon of all these investigations and suggests that the previously described mutation in the O174L gene coding for ASFV polymerase X has led to an increased mutation rate and thus higher evolutionary rate culminating in the emergence of the viral variants.

Material and methods

DNA extraction

For routine diagnostics

Field samples were extracted using the QIAamp® Viral RNA Mini kit (Qiagen, Hilden, Germany) or the NucleoMagVet kit (Macherey-Nagel, Düren, Germany) on a KingFisher® extraction platform (Thermo-Fisher-Scientific, Waltham, USA) according to the manufacturer's instructions.

For next-generation sequencing

DNA was extracted from field samples using the NucleoMagVet kit (Macherey-Nagel) according to the manufacturer's instructions. The extracted DNA was stored at – 20°C until analysis. Prior to next-generation sequencing (NGS) library preparation, DNA from the samples was quantified using a Nanodrop spectrophotometer (ThermoFisher Scientific).

For Sanger sequencing

DNA was extracted from field samples using the QIAamp® Viral RNA Mini kit (Qiagen) according to the manufacturer's instructions.

PCR

Routine ASFV diagnostics

Field samples were analysed by an OIE listed ASFV specific qPCR [22] including a heterologous internal control [23] and by the commercial virotype ASFV 2.0 kit (Indical Biosciences). The latter included both a heterologous and an endogenous internal control and was carried out according to the manufacturer's instructions. All analyses were done on a Bio-Rad C1000^{TM} thermal cycler (BIO-RAD, Hercules, USA), with the CFX96^{TM} Real-Time System of the same manufacturer.

Sequencing

Sample selection

To identify samples suitable for shotgun sequencing, e.g. samples with a favourable ratio between host and viral genome, ASFV positive samples showing a difference of at least 5 Cq values between the ASFV target and the house-keeping gene beta actin as host genome representative (used as internal control) were chosen from the pool of routine diagnostic samples and DNA samples received from the Ukraine stored at – 20° at the FLI.

iSeq 100 and MiSeq sequencing

The sequencing instrument was chosen based on the in-house instrument availability and expected proportion of viral reads in the datasets as estimated from the Cq differences between virus and host genes (see above).

For Illumina iSeq 100 sequencing, DNA sequencing libraries were produced using the GeneRead DNA Library I Core Kit (Qiagen) and Netflex Dual-index DNA Barcodes (Perkin Elmer, Waltham, USA) according to the manufacturer's instructions. Prior to sequencing, libraries were analysed on a Bionalayzer2100 (Agilent, Santa Clara, USA) using the High Sensitivity DNA Analysis kit (Agilent) and quantified using the KAPA Library Quantification Kit for Illumina® Platforms (Roche, Basel, Switzerland). iSeq

100 sequencing was performed according to the manufacturer's instructions in 150 bp paired-end mode using an iSeq 100 i1 Reagent v2 (300-cycle) kit (Illumina). For the Illumina MiSeq, sample preparation was performed as described for the iSeq100. Final libraries were sequenced on the MiSeq using the Reagent Kit v2 or v3 (Illumina) according to the manufacturer's instructions.

NovaSeq 6000 sequencing

Due to the considerable size of the ASFV genome and an unfavorable virus/host-ratio detected for most of the investigated samples, sequencing efforts were scaled up to consistently reach ASFV read numbers necessary for high-quality whole-genome sequencing. Since repeated runs on the smaller Illumina platforms (iSeq 100, MiSeq) drive the costs for a single ASFV whole-genome and are also time-consuming, a commercial sequencing service was utilized running on an Illumina NovaSeq 6000 platform for a more cost-effective approach. Following DNA extraction as described before, a minimum of 100 ng of DNA was sent to and sequenced by Eurofins Genomics. This service included preparation of a 450 bp DNA sequencing library using a modified version of the NEBNext Ultra™ II FS DNA Library Prep Kit for Illumina and sequencing on an Illumina NovaSeq 6000 with S4 flowcell, XP workflow and in PE150 mode (Illumina).

Sanger sequencing

Marker identification and genetic typing of 834 positive tested field samples was realized by PCR and Sanger sequencing of ten target ASFV genome regions. To this end, conventional PCR was performed using region specific primer pairs (Supplementary Table 2) and the Phusion Green Hot Start II High Fidelity PCR Master Mix (Thermo Fisher Scientific) according to the manufacturer's instructions in a 25 µl reaction on a C1000 Thermo Cycler (Biorad, Hercules, USA). Subsequently, PCR reactions were sent to and analysed by Microsynth Seqlab GmbH (Göttingen, Germany) or Eurofins Genomics (Ebersberg, Germany). The service included PCR clean-up and Sanger sequencing.

Data analysis

Next-generation sequencing

NGS data from German field samples was analysed by mapping all reads against the ASFV Germany 2020/1 genome sequence (LR899193) [18] as reference using Newbler 3.0 (Roche) with default parameters including adapter and quality trimming. Subsequently, mapped reads were extracted and assembled using SPAdes 3.13 [24] in the mode of error correction prior to assembly with default parameters and automatically chosen K-mer length. Assembled contigs were assessed in Geneious Prime® 2021.0.1 and manually modified where necessary (especially in G/C homopolymer regions). For validation, all reads were mapped to the assembled contig using Newbler 3.0 and the sequence was corrected manually when necessary. For detection of novel ASFV variants, ASFV whole-genome sequences were aligned with the ASFV Germany 2020/1 genome sequence (LR899193) [18] as reference using MAFFT v7.450 [25] in Geneious Prime. The obtained 22 whole-genome sequences were submitted to the European Nucleotide Archive (ENA) under the project accession PRJEB55796.

Criteria for the selection of ASFV whole-genome sequences from public databases for sequence comparison

Sequences were downloaded from the International Nucleotide Sequence Database Collaboration (INSDC) databases. To reduce the rate of calling false positive mutations due to sequencing errors, sequences were chosen due to the availability of quality parameters such as a mean coverage per nucleotide of at least 40 and aligned using MAFFT v7.450 [25] in Geneious (Supplementary Table 3). Furthermore, due to the inaccuracy of modern sequencing platforms to correctly call the number of G/C nucleotides in homopolymer stretches and frequent sequence artefacts due to low coverage at the genome ends, the extensive G/C homopolymer-regions at the 5'-end as well as the ITR regions (genome position <1379 and >189207) of the ASFV genome were excluded from the analysis.

Sanger sequencing

The received data from Sanger sequencing was analysed in Geneious Prime by alignment with the ASFV Germany 2020/1 genome sequence (LR899193) [18] as reference using MAFFT v7.450 [25].

Epidemiological modelling

We investigate data from model simulations using the software SwiFCo-rs (for technical documentation see https://ecoepi.eu/ASFWB/). The model links individual animal behaviour to the spatio-temporal structure of wild boar population over thousands of square kilometres. Hence, individual level knowledge about infection, transmission and virus genome drives the observable outcome at the landscape or population level. The model was verified, validated, and applied with different problems of ASFV epidemiology [26]. The model is developed in the Rust language and used as Python library. The latter is available from the authors upon reasonable request.

The model compiles (i) an ecological component detailing processes and mechanisms related to the

ecology, sociology and behaviour of wild boar in natural free-roaming populations of the species Sus scrofa; (ii) an epidemiological ASF component reflecting individual disease course characteristics and transmission pathways including direct contact on different social scales and environmental transmission caused by ground contamination or contacts to carcasses of succumbed infected host animals; and (iii) a pseudo-genetics component manipulating inheritance of code patterns with every successful infection between two wild boar individuals. The model is stochastic in relation to all three components and parametrised using reported distributions from literature including variability and uncertainty [27].

The basic principle of transmission relates to the number of adjacent/in contact animals and carcasses using event probabilities, i.e. each infectious object provides a chance of transmission to every susceptible animal sufficiently close. The wild boar-ASF-system comprises three modes of potential transmission, i.e. between live animals of the same social group (within group transmission), between live animals of different groups (between group transmission) and between carcasses of animals succumbed to the infection and live animals (carcass-mediated transmission). Parametrisation of the modes of transmission integrates multiple sources [28–30].

The model runs on habitat maps reversely calibrated to generate spring population density according to European density models [31] and covering about 200 km to the West and East of the German Polish border. Dynamic visualisations of model runs are available from https://ecoepi.eu/ASFWB/VAR. All model runs were performed on the same geographical landscape. The infection was released in the north-eastern part of the simulation landscape. Simulated spread generated westwards and southwards waves with continuous approach towards the Polish-German border.

Variant dynamics were determined by the parameter mutation probability. Whenever a transmission event occurred, the newly infected animal either inherits the variant of the source of the infection or is assigned a completely new variant not yet attached to any other individual. The variants are modelled as opaque identifiers without a genetic code. This avoids having to describe how and where a variant changed the genetic information.

The output measure per simulation was the spatial distribution of variants, and the number of variants that covered more than 100 km² by varying the rate at which new variants stochastically occur. Furthermore, we estimated the probability distribution to detect exactly one out of three samples and at least 10 variants from 50 samples selected from the infectious carcasses on the German side in the first year since arrival of the simulated epidemic at the border.

Results

Whole genome sequencing reveals ten distinct ASFV variants in Germany

Whole-genome sequencing was successful for 22 ASFV positive field samples representing different areas of disease introduction. They comprised of either EDTA-blood, blood-swabs or bone marrow. Whole-genome sequences (WGS) were successfully assembled with mean coverages per nucleotide varying from 21.4–943.4 (Supplementary Table S1). These German ASFV sequences show a very high overall nucleotide sequence identity to other available ASFV GTII WGS from the INSDC databases and clearly belong to P72 GTII (Supplementary Figure S1(A)). However, through alignment with the first ASFV WGS from Germany (LR899193.1) [18], five lineages with a total of ten variants were identified based on single nucleotide variations (SNV) as well as insertions or deletions (indels) of one or two nucleotides (Table 1, Figure 1 and Supplementary Figure S1). Lineages were defined as groups of ASFV genomes that share at least one common mutation relative to the LR899193.1 reference sequence (which was set as a lineage of its own), while variants were demarcated by unique mutations or combinations of mutations. In order to facilitate the differentiation between lineages and variants, a nomenclature based on Roman numerals for lineages (I-V) and an appendix of Arabic numerals representing individual variants was introduced as shown in Figure 1.

ASFV variants in Germany are characterized by 13 novel mutations affecting annotated open Reading frames (ORFs)

When compared to the first German ASFV WGS LR899193.1 [18], the 22 WGS of German ASFV presented here are characterized by 17 novel mutation sites of which 13 affect annotated ORFs. These mutations affect the five multigene family (MGF) genes MGF110-14L, MGF360-10L, MGF505-4R, MGF360-15R, and MGF100-3L as well as the genes DP60R, ASFV G ACD 00190 CDS, ASFV G ACD 01990 CDS, A240L, K196R, NP868R, D339L, and E199L (Table 1).

Of these 13 ORF-affecting mutations, two synonymous (in K196R and MGF110-14L) (Figure 1 and Table 1) and three non-synonymous mutations (in NP868R, D339L, and E199L) are classified as low-impact mutations (LI mutations). The remaining eight mutations, which lead to truncations of the affected ORFs are classified as high-impact mutations (HI mutations). Of the eight HI mutations, six indels lead to frameshifts resulting in truncations

Table 1. Genetic differences in German ASFV variants compared to ASFV Georgia2007/1 (FR682468.2).

							Variants								
Position*	Gene	Function	Polymorphism type	Change	Effect	Amino acid change	I.1* Cov 2082	II.1* Cov 943	II.2* Cov 117,7	III.1* Cov 960	III.2* Cov 140	IV.1* Cov 36	IV.2* Cov 119	IV.3* Cov 129	IV.4* Cov 279,9	V.1* Cov 31,3
477	DP60R CDS	Unknown	Insertion (tandem repeat)	(T)9 -> (T)10	Frame Shift	None				1		1				
1.418	None	None	SNP (transversion)	A -> T	None	None										1
6.783	None	None	Deletion (tandem repeat)	(T)9 -> (T)8	None	None	1	1	1	1	1	1	1	1	1	1
7.012	MGF 110-1L CDS	Unknown	Deletion (tandem repeat)	(G)5 -> (G)4	Frame Shift	None									1	
7.059	MGF 110-1L CDS	Unknown	SNP (transition)	C -> T	Truncation	None	1	1	1	1	1	1	1	1	1	1
10.668	MGF 110-7L CDS	Unknown	SNP (transition)	G -> A	None	None	1	1	1	1	1	1	1	1	1	1
12.578	ASFV G ACD 00190 CDS	Unknown	Deletion (tandem repeat)	(A)10 -> (A)9	Frame Shift	None							1	1		
13.809	MGF 110-10-L - MGF110-14L fusion CDS	Unknown	SNP (transversion)	A -> T	None	None									1	
22.898	None		Deletion (tandem repeat)	(T)9 -> (T)8	None	None	1	1	1	1	1					1
27.197	MGF 360-10L CDS	Unknown	SNP (transition)	G -> A	Truncation	None				1	1					
37.027	MGF 505-4R CDS	Unknown	Deletion	-A	Frame Shift	None				1	1					
39.306	MGF 505-5R CDS	Unknown	SNP (transition)	G -> A	Substitution	V -> I	1	1	1	1	1	1	1	1	1	1
44.576	MGF 505-9R CDS	Unknown	SNP (transition)	A -> G	Substitution	K -> E	1	1	1	1	1	1	1	1	1	1
49.192	A240L CDS	Thymidinylat kinase	Insertion (tandem repeat)	(AT)2 -> (AT)3	Frame Shift	None		1	1							
50.906	MGF 360-15R CDS	Unknown	Deletion	-C	Frame Shift	None							1	1		
50.922	MGF 360-15R CDS	Unknown	SNP (transition)	C -> T	Truncation	None						1	1	1	1	
64.395	K205R CDS	Unknown	SNP (transition)	G -> A	None	None	1	1	1	1	1	1	1	1	1	1
65.259	K196R CDS	Thymidine kinase	SNP (transition)	C -> T	None	None								1		
66.152	K145R CDS	Unknown	SNP (transversion)	C -> A	Substitution	S -> Y	1	1	1	1	1	1	1	1	1	1
129.288	O174L CDS	Unknown	Insertion	+TTTTTCAGTAGTGA	Frame Shift	None	1	1	1	1	1	1	1	1	1	1
129.982	None		SNP (transversion)	A -> T	None	None			1							
134.514	NP419L CDS	Unknown	SNP (transition)	T -> C	Substitution	N -> S	1	1	1	1	1	1	1	1	1	1
136.845	NP868R CDS	Guanylyltransferase	SNP (transversion)	C -> A	Substitution	L -> I					1					
140.696	D339L CDS	RNA polymerase subunit 7	SNP (transition)	G -> A	Substitution	L -> F							1	1		
167.068	E199L CDS	Transmembrane protein/ innere core	SNP (transition)	C -> T	Substitution	E -> K										1
170.862	I267L CDS	Unknown	SNP (transversion)	T -> A	Substitution	I -> F	1	1	1	1	1	1	1	1	1	1
173.018	None	None	Deletion (tandem repeat)	(T)10 -> (T)9	None	None	1	1	1	1	1	1	1	1	1	1
173.382	None	None	Insertion (tandem repeat)	(TATATAGGAA)2 -> (TATATAGGAA)3	None	None	1	1	1	1	1	1	1	1	1	1
176.636	None		Deletion (tandem repeat)	(A)10 -> (A)9	None	None			1							
181.407	MGF 100-3L CDS	Unknown	Deletion (tandem repeat)	(A)6 -> (A)5	Frame Shift	None				1	1					
187.978	None	Insertion (tandem repeat)	(A)9 -> (A)10	None	None	None						1	1	1	1	
189.780	ASFV G ACD 01990 CDS	Deletion (tandem repeat)	(T)9 -> (T)8	Frame Shift	None	None				1						
190.116	DP60R CDS	Insertion (tandem repeat)	(A)9 -> (A)10	Frame Shift	None	None				1	1	1	1	1	1	1

* in reference to ASFV Georgia2007/1 (FR682468.2).

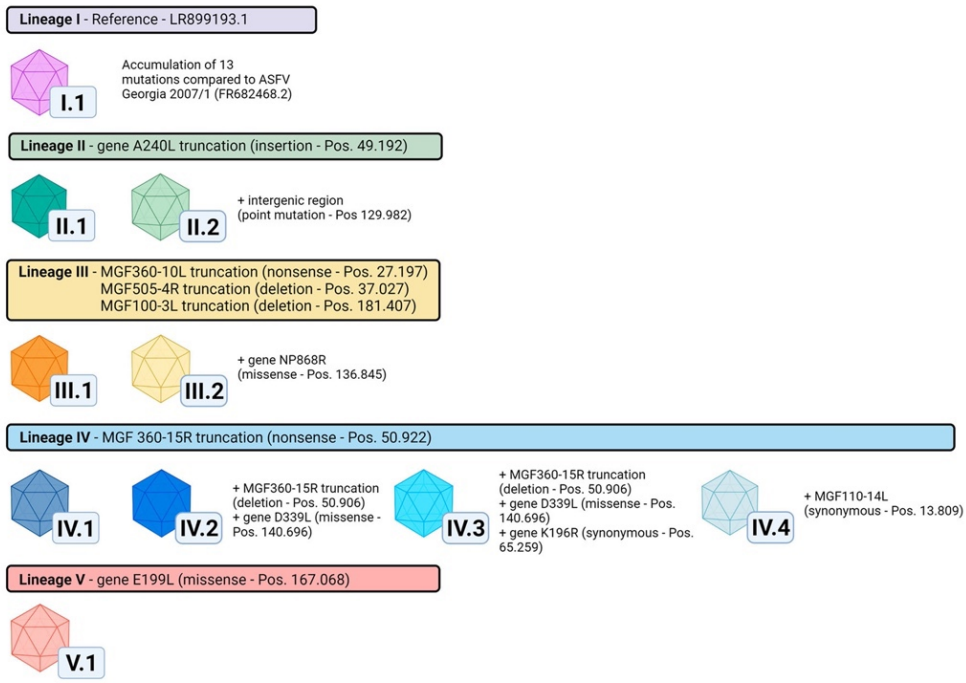

Figure 1. ASFV variants and lineages in Germany. Lineages are indicated by coloured header together with identified marker mutations in comparison to the German ASFV sequence LR899193 (set as reference lineage I). Variants are characterised by insertions/deletions in homopolymer and non-homopolymer regions as well as synonymous, missense and nonsense mutations found in annotated genes and intergenomic regions. Mutations used to discriminate variants are stated together with their gene positions relative to ASFV Georgia 2007/1 (FR682468.2). A complete list of all mutations identified in this study can be found in Table 1. In total, five lineages and ten variants could be discriminated based on this system. Figure created with Biorender.com.

(MGF505-4R, A240L, MGF360-15R, MGF100-3L, ASFV G ACD 00190 CDS and ASFV G ACD 01990 CDS) and two nonsense mutations lead to truncation (MGF360-10L and MGF360-15R)HI mutation.

Stochastic emergence of geographic clusters of variants

We ran epidemiological model simulations starting with few infected wild boars at the location where the first cases were confirmed in Western Poland in 2019, 200 km distant to previous virus circulation. Figure 2 illustrates the geographic emergence of spatial clusters of variants. The randomly emerging variants (different colours in Figure 2) formed spatially separated clusters on the German side of the border. Figure 2(a–c) illustrates the temporal development of variant clusters in a single simulation run. The further the spread of the infection branches geographically the more individual variant clusters emerge. In Figure 2(d–f) we show the variant map at the end of three different simulation runs using identical model parameters.

More systematic, using model output of 100 runs, Figure 3 shows the counts of variants that formed a minimum cluster size of 100 km² dependent on the parameter *mutation probability*, describing the rate of variant emergence per new animal infection (animal passage). The cluster size of 100 km² was used to reflect the cluster dimensions found in Germany while excluding containment measures. The rate of variant emergence per animal passage that resulted in at least 10 variants with cluster size of 100 km² was at 1.15% (Figure 3). The spatial clustering of variants in the model output does suggest such relationship between the variants found in the field.

Mutation sites can be used as markers for genomic epidemiology of ASFV in Germany

The 22 newly generated WGS as well as the previously published ASFV Germany 2020/1 genome sequence (LR899193) were used as template for PCR primer design to amplify ten different mutation-regions as genetic markers (Supplementary Table 2) selected to cover the complete range of ASFV variation circulating in Germany to this time point. In total 834 field samples were successfully assigned to one of the ten variants (Figure 1). When geographically displayed, a clear spatial clustering was detected (Figure 4). Variants of lineages I and II, i.e. I.1, II.1 and II.2 were found in the Brandenburg districts of Oder-Spree

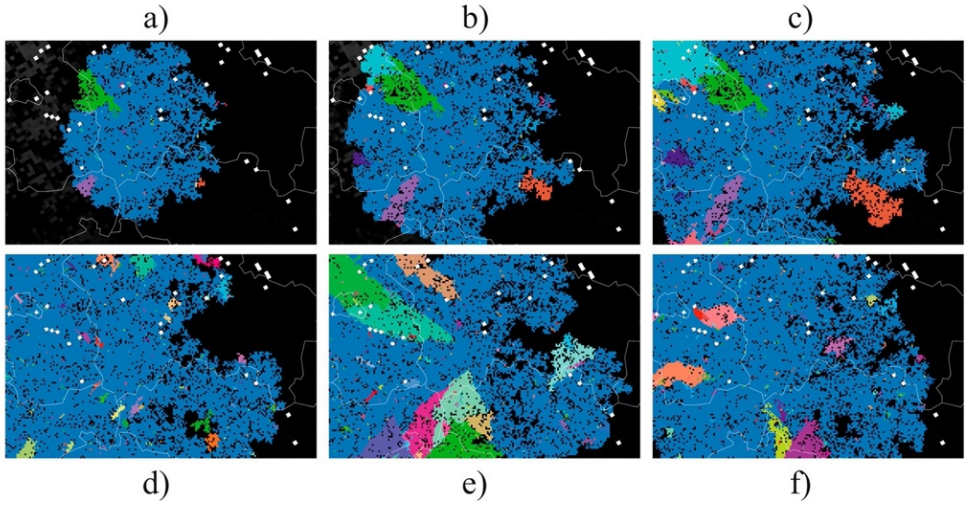

Figure 2. Model output as spatial snapshots with different variants differently coloured, either showing the dynamic development of infection distribution (a–c) or mapping the stochastic variability of the final distribution (d–f). Pixels represent social groups of individual wild boar and lines are administrative borders.

(see Figure 4, LOS) and its neighbouring districts Spree-Neiße (SPN, Variant I.1 - northern part) and Dahme-Spreewald (LDS, Variant II.1), whereas variants of lineage III were detected in the more northern districts of Brandenburg. In detail, cases of variant III.1 were detected in Märkisch-Oderland (MOL), Barnim (BAR) and Uckermark (UM) while Variant III.2 was detected in MOL, Frankfurt (Oder) (FF) and LOS. Variants of the lineage IV were found in the southern areas like SPN (Variant IV.1 – southern part) and, so far, represent the sole variants detected in the federal state of Saxony (Görlitz – GR, Variant IV.1, IV.2, IV.3, IV.4). The distribution of variant V.1 spans closely to the Polish border from FF to LOS. Notably, with the exception of Dahme-Spreewald (LDS), all involved German districts share a border with Poland.

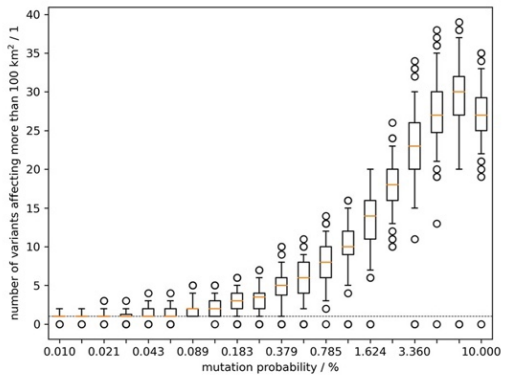

Figure 3. Model outcome of the number of emerging variants which affected at least 100 km^2 of wild boar habitat. Box whisker plots summarise 100 model runs per value of the mutation parameter.

Variants analyses suggest local spill-over from wild to domestic hosts

Three outbreaks in domestic pigs occurred within the study period (Figure 4(A,B)). Using whole-genome sequencing, all three outbreak strains could be assigned to variants circulating in the immediate vicinity of the outbreak farms. In detail, variant III.1 was found in two domestic pig outbreaks in the district MOL while variant IV.1 was found in SPN. In all three cases, the variant was first detected in wild boar, hence an introduction from the local wild boar population is likely.

Compared to worldwide ASFV GTII whole-genome sequences ASFV Germany shows excessive high-impact mutations

We evaluated if the findings in Germany indicate a novel and different situation regarding the frequency of high-impact mutations. Altogether, 35 international ASFV WGS were compared (Supplementary Table 3). We chose 21 publicly available ASFV WGS originating from eastern and western Europe (including the first German sequence LR899193), Russia and Asia from 2007 to 2020 due to (i) availability and geographic distribution and (ii) available sequence quality parameters (e.g. mean coverage per nucleotide >50) (Supplementary Table 3). Moreover, we added five WGS generated from samples of domestic pigs collected in the Ukraine in 2017-2018. Finally, we included nine WGS from Germany that represented the range of viral variants found in Germany to this date. The 35 sequences were examined for their genetic variance relative to the sequence of ASFV

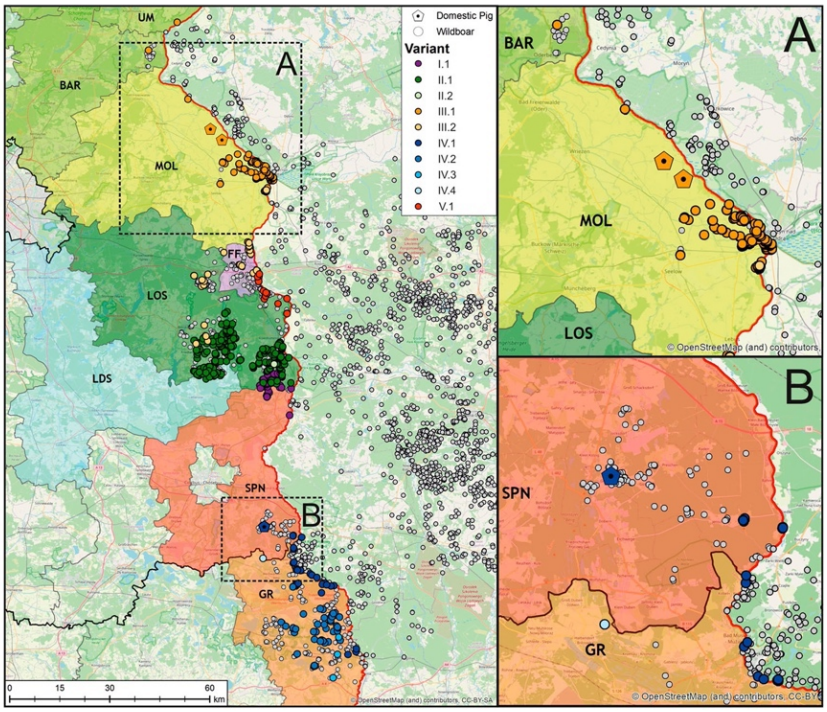

Figure 4. Geographic distribution of viral variants detected in the federal states of Saxony and Brandenburg along the Polish border (left). Confirmed ASFV cases in wild boars from 10 September 2020 until 12 August 2021 are depicted as circles (white), whereas outbreaks in domestic pigs are shown as pentagons ($n = 3$, areas A and B). In order to facilitate the visualization of spatial ASFV clusters, variants confirmed by Sanger sequencing ($n = 834$) were coloured according to their assignment to one of the five lineages.

introduced into Georgia in 2007 [32]. Due to frequent issues in sequencing the inverted terminal repeat regions and resulting variations in sequence length, only genome positions (in regard to ASFV Georgia 2007/1) from 1379–189207 were included.

In total, 131 variations were detected including 34 indels and 97 nucleotide substitutions (Supplementary Table 3). From 96 mutations affecting annotated ORFs, 81 are non-synonymous LI mutations and 15 are HI mutations leading to ORF truncation by non-sense mutation or frameshift. From these 15 HI mutations, nine can be detected in German ASFV sequences; of these, eight are exclusively detected in recent sequences from Germany and one is shared with other GTII sequences (Figure 5). Thus, of all HI mutations recorded in 35ASFV GTII WGS over 14 years 53% (8/15) specifically occur in ten German ASFV sequences of samples collected in about one year.

Analysis of ASFV WGS sequences from the Ukraine in a comparable spatiotemporal scenario shows genetic variability but only few high-impact mutations

To compare the situation in Germany with another country with similar geographical and temporal distribution of ASFV outbreaks, we analysed samples collected in 2017/2018 from domestic pig outbreaks in northern Ukraine by whole-genome sequencing. In total, WGS from five samples were successfully assembled with mean coverages per nucleotide ranging from 68.5-209.8. When aligned with the ASFV-

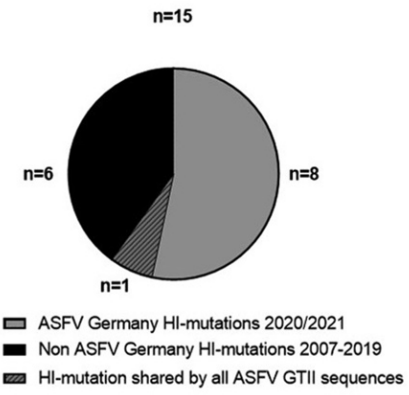

Figure 5. High-impact mutations in ASFV whole-genome sequences in comparison with the ASFV Georgia 2007/1 sequence (FR682468.2). Number of HI mutations in the ASFV WGS from Germany vs HI mutations in 5 WGS from the Ukraine and 20 publicly available WGS

Georgia2007/1 genome sequence (FR682468.2) [32] as reference, 32 mutations were detected of which ten are LI mutations and two are HI mutations leading to the truncation of annotated genes (MGF300-4L and ASFV G ACD 00270 CDS) (Supplementary Table S1 and S4). While compared with the German ASFV variants, the total number of mutations is comparable (31 mutations in sequences of samples from Germany, 32 mutations in sequences of samples from the Ukraine) but the number of novel HI mutations is higher (eight for Germany and two for the Ukraine) (Supplementary Table S1 and S4). The different ratios of HI:LI mutations (8:23 vs. 2:30) contradict the assumption that both ratios reflect similar mutation dynamics (Fisher's exact test 0.043, $p < 0.045$).

Plausibility of difference in number of variants in separated virus populations

We tested on a model setup (1 variant out of 3 analysed samples. vs. 10 variants out of 50 analysed samples) whether the number of variants detected in sequencing data from the Baltics (low sample number example) was compatible with the number found in Germany (large sample number example) under the assumption that the mutation rate did not change between these settings. Figure 6 combines the model predictions for one variant detected from 3 genetically determined samples, called $P(v = 1|s = 3)$, with those of 10 variants out of 50 samples, called $P(v \geq 10|s = 50)$. The former captures the available data of the Baltics where no variants were detected and 3 WGS of the virus were assembled (blue distribution), the latter captures the situation in Germany where 10 variants were detected in 50 genome sequences of viruses sampled during the first year after entry (orange distribution).

The two sample outcomes (1/3 & 10/50) give an estimate of the situation in the past and thus we are interested in the probability of their joint occurrence in the model setting. The probability of both sequence sampling outcomes together was factually zero for large ranges of variant emergence rate (Figure 6(A)). The eligible range of positive variants' emergence rate is very narrow around 2%. However, even there the probability of joint observations of both sequencing data is only about 5% in median (Figure 6(B)). Therefore, the model data suggested that the two sequencing scenarios more likely result from virus populations with different variant emergence rates.

A mutation in the ASFV polymerase X (O174L gene) might act as mutator and contribute to the increased number of ASFV variants

Since the simulation cannot identify the reason for the difference in variants' emergence rate, we

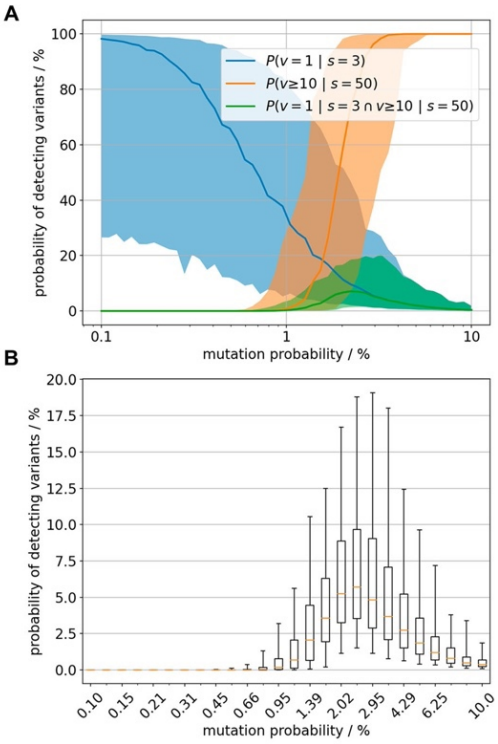

Figure 6. (A) Likelihood of observing 1 variant out of 3 sequenced samples (blue) and 10 out of 50 sequenced samples (orange) shown by the median value (bold line) and the 90% credibility interval (shaded area). The probabilities are estimated for varying rate of variants' emergence (x-axis, log scaled). The green graph represents the joint distribution i.e. the probability to observe both sample outcomes with constant variants' emergence rate. (B) Distributional details of the green graph i.e the joint probability.

surveyed the German ASFV WGS for mutations that might act as mutators, i.e. mutations, that could increase the viral mutation rate. Alignment of the German WGS together with available GT II ASFV WGS (including the Georgia 2007/1 genome sequence FR682468.2) revealed that a previously described HI mutation is present in all German and three Polish ASFV WGS (MT847620.1, MT847622.1 and MT847623.1). A 14 bp tandem duplication of the bases 129,275–129,288 (relative to ASFV Georgia 2007/1 (FR682468.2) [32]) leads to a frameshift and truncation of the O174L gene (Figure 7(A), Table 1 and Supplementary Table 3) [18–21]. This gene encodes the DNA polymerase X (PolX), a well-characterised enzyme involved in base-excision repair [33–36]. The frameshift results in a truncation by seven amino acid residues from the C-terminal end (R168-L174) as well as an additional substitution of eight residues preceding the truncation, four of which lie within the last α-helix of the enzyme, called αF (Figure 7(B)). Although the conformation of this

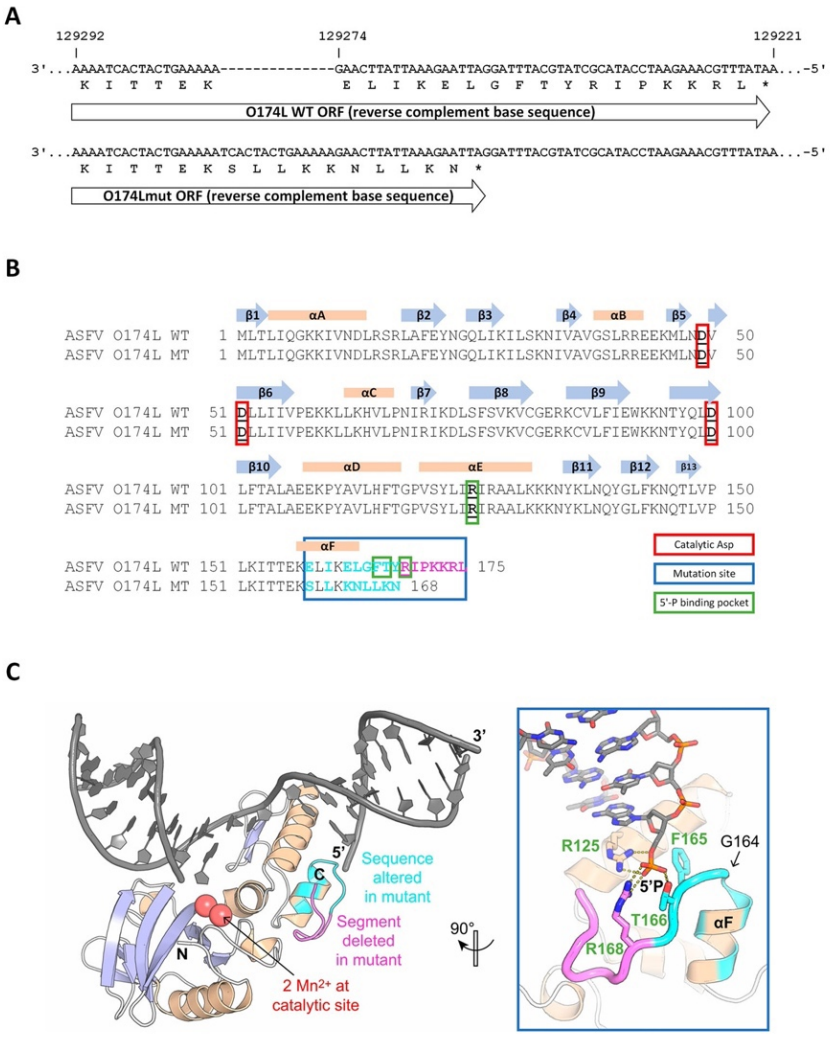

Figure 7. Comparison of O174L wildtype and mutant nucleotide and protein sequence and the effects of observed mutations on the wild-type ASFV PolX protein structure. Alignment of ASFV O174L wildtype and mutant nucleotide sequence (A) and protein sequence (B) including structural information from the literature [34]. Catalytic sites (red box), mutation site (blue box), amino acids forming the 5′-binding pocket (green box) and altered amino acids (magenta letters) are highlighted. The nucleotide alignment was done using MAFFT v7.4506 and the protein alignment using Clustal W in Geneious. (C) X-ray structure of wild-type ASFV PolX in complex with nicked DNA (PDB accession: 5HRI) [34]. Positions with altered sequence in the mutant are coloured in cyan and positions that are missing in the mutant are coloured in magenta. The illustration was prepared with PyMol (Schrödinger, Inc.).

αF helix (residues 156-163) is likely preserved in the mutant owing to the conservative nature of its four amino acid substitutions, the terminal peptide connected to this helix (residues 164-167) likely adopts a different conformation in the mutant. This assumption is based on the substitution of the helix-breaking glycine-164 residue of the wild type for a helix-stabilizing leucine in the mutant (Figure 7(B,C)).

In the wild-type enzyme, the C-terminal region including the αF helix forms part of a positively charged pocket composed of residues R125, T166, and R168 that bind the negatively charged 5'-phosphate end of DNA substrates at single-strand breaks that are introduced into the repair sites by the viral apurinic/apyrimidinic (AP) endonuclease [34]. Whereas R125 remains unaffected by the mutation, the other positively charged residue of the pocket, R168, is lost in the deletion. It is however possible that the substitutions E162 K and T166 K, which introduce two new positive charges, compensate for this loss (Figure 7(A,B)). Therefore, the mutant PolX enzyme may still be overall functional, whereas its kinetic and thermodynamic parameters, or its substrate specificity, are likely affected.

Discussion

Despite the extremely high genetic stability of the ASFV genome, the existence of genetic variation is not surprising and has been documented in previous studies [16,21,37]. However, the results we present in this study on ASFV variants in Germany are unexpected and show an extraordinary development that has not been described before. Within one year of ASFV spread in German wild boar, several geographical clusters have been formed that can be assigned to genetically distinct and, so far, undescribed virus sub-populations. The herein presented results give evidence for at least five lineages with ten variants differing from the ASFV strain first introduced into Germany in September 2020.

Epidemiological simulation of the spread and inheritance of virus variants illustrates the clustered occurrence of stochastic, geographically distinct variants in a wild boar population without any selection forces. The newly identified characteristic mutation sites were used as genetic markers to enable genomic epidemiology for the different ASFV outbreak strains in Germany. This allowed us to show the geographical distribution and to track the spread of the different ASFV variants in Germany. Using this technique, we were furthermore able to directly connect the ASFV strains responsible for three outbreaks in domestic pigs to the strains circulating in the wild boar population in the same area. Therefore, for the first time since the spread of ASFV GTII in Europe and Asia the transmission pathway between wild and domestic suids was unravelled and spread of ASFV variants could be differentiated in space and time. However, it also highlights the fact that the continuous generation of ASFV WGS is essential, and the only basis on which molecular epidemiology with genetic markers can be performed.

ASFV whole-genome sequencing is laborious and technically challenging, but we were able to generate 22 German and 5 Ukrainian ASFV WGS using Illumina-based sequencing techniques allowing for single-base resolution and single nucleotide variant identification. The Illumina technology is well suited for ASFV whole-genome sequencing, but the correct calling of G/C homopolymer regions and sequencing the inverted terminal repeats is still error-prone and these regions are therefore excluded from the analyses. To validate the results and rule out sequencing or bioinformatic artefacts, all identified mutation sites in German ASFV sequences have in addition been validated by PCR amplification and Sanger sequencing confirming the whole-genome sequencing results. Therefore, all analyses concerning variant detection and genomic epidemiology are based on validated and confirmed sequencing data.

Interestingly, variants of the lineages III and IV show genetic variations within four MGF genes i.e. MGF360-10L, MGF360-15R, MGF100-3L and MGF505-4R, while variant II only shows a variation in ASFVs thymidylate-kinase (A240L), an enzyme involved in nucleotide metabolism [14]. Although no function is known for any of the affected MGF genes and corresponding proteins, other ASFV MGF360 and MGF505 genes were shown to be involved in virulence and pathogenicity for example interfering with the hosts interferon response [14,38–40].

The main question remains why this huge increase of ASFV genetic variety was first reported in Germany and could not be detected before. It can be argued that the worldwide number of sequenced samples, especially due to the high efforts needed to generate high-quality ASFV WGS, was not sufficient to cover the extent of ASFV GTII genomic diversity circulating in suids over the past decades. However, the comparison of the German WGS with five Ukrainian and 20 publicly available high-quality ASFV WGS from all over the world draws a different picture. Despite a general tendency seen in all ASFV GTII sequences to accumulate point mutations over time (either synonymous or non-synonymous), a dramatic increase in the detection of high-impact mutations leading to a genetic frameshift or truncation can be observed in the German ASFV sequences. Our presented results do not comply with the hypothesis of equal mutation dynamics in the German virus population and strains previously observed. Moreover, it is tempting to argue that the conservation of HI mutations offers an evolutionary advantage over the wildtype virus since virus variants defined by mutations with a negative or even neutral impact would not be able to prevail and spread like the formation of variant clusters in Germany suggests.

The comparison of different variant-sample ratios from different virus populations does not give reliable support to assume that the dynamics of variant generation is constant across affected wild boar populations in Europe. Accelerated variant generation dynamics was suggested when comparing very early (Baltics) and recent (Germany) genomic survey data. Under the assumption that there is an inevitable link between a generally increased mutation activity and the number of emerging variants, the WGS from Germany in comparison with WGS from other regions in Eastern Europe indeed suggest an increased mutation rate in the ASFV affected region in Germany and the directly connected region of Western Poland.

However, the increased identification of HI mutations in the Polish-German border region may be due to certain selection pressure (Figure 2).; alternatively, in the other regions the rate of variant emergence may be just underestimated due to limits in producing ASFV WGS. To address these uncertainties more, high-quality ASFV WGS are needed, especially

from Western Poland, where extreme high numbers of ASFV cases have been reported.

The increased mutation rates among German ASFV variants can likely be linked to the HI mutation in the ASFV DNA PolX gene (O174L), which is shared by all ASFV WGS from Germany as well as the available sequences from Poland [20,21]. As reported in previous studies, ASFV PolX is a repair polymerase that participates in viral base excision repair, to exchange single damaged nucleotides [33–35]. It therefore seems reasonable to hypothesize that the frameshift mutation in the C-terminus of PolX has a negative effect on its repair activity, thus leading to increased accumulation of mutations in the viral genome. However, despite its function as a repair polymerase, even the wild-type enzyme introduces an unusually high number of errors in its DNA substrates, which has already in the past led to speculations that wild-type PolX might be a strategic mutagenase [41]. This raises the question whether the increased mutation rate is indeed caused by a reduction or perhaps even a gain of activity in the mutated PolX enzyme. While the exact fidelity – i.e. the frequency with which wild-type PolX introduces wrong nucleotides – is still under debate, it is clear that errors are strongly biased towards dG:dGTP misincorporation [41,42]. If such dG:dGTP misincorporation was the reason for the accelerated evolution in German ASFV variants, we would expect to observe a high frequency of dG → dC and dC → dG mutations. Yet, no such mutations are found in our dataset (Table 1). This observation goes in line with the previous finding that experimental mutation of the 5'-phosphate binding pocket of PolX, which is also impacted by the frameshift mutation in the German variants, has an even stronger negative effect on dG:dGTP misincorporation efficiency than on Watson–Crick-paired incorporation [34]. It is therefore plausible that the higher mutation rate in German ASFV variants is, at least in part, the result of overall reduced enzymatic activity rather than increased dG:dGTP misincorporation efficiency of the reparative polymerase PolX.

Conclusion

In conclusion, we report here the emergence of distinct ASFV variants that point to a higher sequence variability of ASFV in strains observed at the German-Polish border. We identified a frameshift mutation in the O174L gene/ PolX that affects the 5' binding pocket of the enzyme as plausible cause. The resulting ASFV variants allow, on the upside, for the first time a meaningful genomic ASFV epidemiology. On the downside, the accelerated occurrence of viral variants has the potential to result in ASFV variants with novel features which might in the future dramatically influence the course of the ASFV epizootic with unknown outcome.

Acknowledgements

The authors gratefully thank Patrick Zitzow, Ulrike Kleinert and Robin Brandt for excellent technical assistance. Olha Chechet and Mykola Sushko for the selection of representative samples and providing sample material.

Disclosure statement

No potential conflict of interest was reported by the author (s).

Funding

This work was supported by the German Federal Foreign Office in the project "Strengthening biosecurity in dealing with proliferation-critical animal disease pathogens in Ukraine" (VetBioSi); and the FLI ASF research network.

ORCID

Martin Beer http://orcid.org/0000-0002-0598-5254

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5.4 Pathology of African Swine Fever in Wild Boar Carcasses Naturally Infected with German Virus Variants

Julia Sehl-Ewert[1], Paul Deutschmann[2], Angele Breithaupt[1], Sandra Blome[2]

[1]Department of Experimental Animal Facilities and Biorisk Management

[2]Institute of Diagnostic Virology

Friedrich-Loeffler-Institut, Südufer 10, 17493 Greifswald-Insel Riems Germany

Pathogens

2022

doi.org/10.3390/pathogens11111386

pathogens

MDPI

Article

Pathology of African Swine Fever in Wild Boar Carcasses Naturally Infected with German Virus Variants

Julia Sehl-Ewert [1,*], Paul Deutschmann [2], Angele Breithaupt [1] and Sandra Blome [2]

[1] Department of Experimental Animal Facilities and Biorisk Management, Friedrich-Loeffler-Institut, Südufer 10, 17493 Greifswald-Insel Riems, Germany
[2] Institute of Diagnostic Virology, Friedrich-Loeffler-Institut, Südufer 10, 17493 Greifswald-Insel Riems, Germany
* Correspondence: julia.sehl-ewert@fli.de

Abstract: In 2020, African swine fever (ASF) was first identified in German wild boar, reaching a case number of about 4400 to date. Upon experimental infection, pathology is well documented; however, data on field infections are scarce in domestic pigs and not available from wild boar, respectively. Although the ASF viral genome is considered exceptionally stable, a total of five lineages with 10 distinct virus variants of genotype II have emerged in Eastern Germany. To investigate the pathology in naturally infected wild boar and to evaluate virus variants II, III and IV for their virulence, wild boar carcasses were obtained from three different outbreak areas. The carcasses underwent virological and pathomorphological investigation. The animals revealed characteristic ASF lesions of the highest severity accompanied by bacterial infections in several cases. In particular, wild boar infected with variant IV from Spree-Neiße (SN) district showed lower viral genome loads and total viral antigen scores, but simultaneously tended to reveal more chronic lesions. Our findings indicate a protracted course of the disease at least after infection with variant IV, but need confirmation under standardized experimental conditions. There is a strong need to monitor differences in the virulence among variants to identify potential attenuation that might complicate diagnosis. In addition, veterinarians, hunters and farmers need to be made aware of less acute courses of ASF to consider this as an important differential to chronic classical swine fever.

Keywords: ASFV; pathology; Germany; virus variant; wild boar; natural infection

Citation: Sehl-Ewert, J.; Deutschmann, P.; Breithaupt, A.; Blome, S. Pathology of African Swine Fever in Wild Boar Carcasses Naturally Infected with German Virus Variants. *Pathogens* **2022**, *11*, 1386. https://doi.org/10.3390/pathogens11111386

Academic Editors: Lauro Velazquez-Salinas, Gisselle N. Medina and Elizabeth Ramirez-Medina

Received: 27 September 2022
Accepted: 17 November 2022
Published: 20 November 2022

1. Introduction

Since its first occurrence in Georgia in 2007, African swine fever (ASF) has continuously spread from the Trans-Caucasian region to Russia, and, in 2014, further to countries of Europe [1]. In September 2020, the disease was confirmed for the first time in a wild boar found in the Spree-Neiße (SN) district in Eastern Germany close to the German–Polish border [2]. To date, more than 4400 cases in German wild boar in the Eastern federal states Brandenburg, Saxony and Mecklenburg-Western Pomerania as well as seven outbreaks in domestic pig holdings located in Brandenburg, Mecklenburg-Western Pomerania, Baden-Wurttemberg and Lower Saxony have been officially identified (https://tsis.fli.de/Reports/Info.aspx, accessed on 9 September 2022).

African swine fever, which is caused by the large, enveloped, double-stranded DNA African swine fever virus (ASFV), can occur as acute, subacute, chronic and subclinical disease courses depending on the virulence of the virus strain as well as on the age and immunological background of the animals [3]. In European countries except Sardinia, highly virulent virus strains of genotype II are prevalent in domestic and wild pigs typically causing acute-lethal disease similar to a hemorrhagic fever [4–6]. Genotype II strains were also identified in the German federal states of Brandenburg and Saxony including the outbreak areas Märkisch-Oderland (MOL), Oder-Spree (LOS), Spree-Neiße (SN) and

Görlitz, in which, surprisingly, five lineages (I-V) including a total of ten viral variants (I, II, II.1, III, III.1, IV, IV.1, IV.2, IV.3, V) have emerged due to single nucleotide variations, insertions and deletions affecting different genes including five multigene families [7]. More specifically, variants III and IV comprise genetic variations in four multigene family (MGF) genes MGF360-10L, MGF360-15R, MGF100-3L and MGF505-4R while variant II shows variation only in the A240L gene coding for the ASFV thymidylate kinase. Whereas the functions of these genes are largely unknown, ASFV MGF360 and MGF505 have been associated with the virulence and pathogenicity of the virus [8,9]. Geographic mapping showed that variant II was predominantly spread in the outbreak area LOS, variant III in MOL and variant IV in the southern part of the outbreak area SN as well as in the federal state of Saxony.

To date, macroscopic pathological records of varying depths of detail largely exist only for experimentally ASFV-infected domestic pigs [10–16] and less frequently for wild boar [4,17–20], which is mainly due to the limited access to wild boar and the associated difficulties to keep them under experimental conditions. Moreover, histopathological data obtained from animal experiments are much less available, but gained importance in the last few years [16,18,21]. Very recently, the first three reports were published concerning naturally ASFV-infected domestic pigs from an outbreak in Vietnam, reporting on the clinical and pathological findings of succumbing and surviving pigs [21,22] and describing ASF-associated age-related lesions [23]. In contrast, descriptions of pathological findings of wild boar that succumb to infection under field conditions are completely missing although this animal species is of great relevance in the maintenance and spread of ASFV in Europe. Hence, the diversity and dimensions of ASFV-associated lesions in the field are only very sparsely represented urging more thorough investigations.

Based on this, we aimed to perform pathological examination of wild boar carcasses infected with ASFV to gain more profound knowledge of the pathology of the animals succumbing to ASF under natural conditions. We took the opportunity to analyze whether three different variations of the emerging virus variants in Germany may have an impact on the virulence of ASFV and the severity and duration of the disease. For this purpose, detailed pathological and molecular virological investigations were performed on wild boar carcasses infected with variants II, III and IV found in LOS, MOL and SN, respectively.

2. Material and Methods

2.1. Study Design

In accordance with the Animal Disease Crisis Unit of the federal states of Brandenburg and Saxony, sixteen wild boar carcasses were obtained from different outbreak areas (n = 7 from Landkreis Oder-Spree (LOS), n = 5 from Märkisch-Oderland (MOL), n = 4 from Spree-Neiße (SN)) between February and March 2021 where ASF virus variants II, III and IV have emerged as published previously [7]. Following legal requirements, the carcasses were tested positive for ASFV by the federal state laboratories of Brandenburg and Saxony. ASF diagnosis was confirmed by the national reference laboratory for ASF. The carcasses were transported to the Friedrich-Loeffler-Institut in compliance with national animal disease and hygiene regulations. The wild boar carcasses were examined in pathological and virological detail. Bacteriologic investigations of secondary bacterial infections were not performed for biosecurity reasons. Details on the cadaver material including location of origin, detection of virus variant, age, sex, weight and preservation status are given in Table 1.

Table 1. Summary presentation of examined wild boar carcasses from three different German outbreak areas LOS, MOL and SN.

No	Origin	Virus Variant	Age (Year)	Sex	Weight (kg)	Stages of Decomposition *	Found Dead/Shot	Anomalies/Comments
1	LOS	II	<1	female	10	fresh stage	dead	Brachygnathia superior
2	LOS	II	<1	female	30	fresh stage	dead	/
3	LOS	II	>2	female	62	bloat stage	dead	/
4	LOS	II	<1	female	40	bloat stage	dead	/
5	LOS	II	<1	female	31	fresh stage	dead	/
6	LOS	II	<1	male	37	bloat stage	dead	/
7	LOS	II	<1	female	27	fresh stage	dead	/
8	MOL	III	<1	female	22	fresh stage	dead	/
9	MOL	III	<1	female	28	fresh stage	dead	/
10	MOL	III	<1	female	36	fresh stage	dead	/
11	MOL	III	<1	female	38	bloat stage	dead	/
12	MOL	III	<1	female	36	bloat stage	shot	Lung not available
13	SN	IV	<1	male	36	fresh stage	dead	/
14	SN	IV	<1	male	30	bloat stage	dead	Scavenger feeding marks (thorax)
15	SN	IV	<1	female	31	fresh stage	dead	/
16	SN	IV	>2	female	75	bloat stage	dead	/

* Stages of decomposition were classified as reviewed by Brooks [24] with further modifications. Fresh stage: no bloating, no discoloration. Bloat stage: bloating, gray to green discoloration of organs.

2.1.1. Pathological Examination

Necropsy

Full necropsies were performed on the wild boar carcasses (n = 16). The organ lesions were scored from 0 to 3 (0 = normal, 1 = mild, 2 = moderate, 3 = severe; unless not otherwise stated) as recently published [25] with the additional modifications shown in Table 2. Tissues samples including the popliteal lymph node, spleen, lung, kidney, liver, heart, brain (cerebellum and cerebrum) and adrenal gland were taken from wild boar and fixed in 10% neutral-buffered formalin for at least 3 weeks.

Table 2. Assessment of gross pathological criteria in ASFV-infected wild boar.

Organ	Macroscopic Finding	Annotations
Lymph node (popliteal)	Enlargement	/
	Hemorrhage	
Lung	Alveolar edema	/
	Interstitial edema	
	Hemorrhage	
	Collapse	
	Consolidation	
	Thoracic effusion	
	Pleuropneumonia	

Table 2. *Cont.*

Organ	Macroscopic Finding	Annotations
Kidney *	Hemorrhage	Assessment of size (petechia, ecchymosis) and distributional pattern (focal (n = 1), oligofocal (n ≤ 20), multifocal (n ≥ 20))
	Pelvic dilation	/
	Pelvic hemorrhage	
Liver and gall bladder *	Congestion	/
	Gall bladder wall hemorrhage/edema	
Spleen *	Determination of relative spleen weight	/
Pancreas	Hemorrhage/edema	/
	Necrosis	
Abdominal cavity *	Peritonitis	/
	Ascitis	
Urinary bladder	Hemorrhage	/
Bone marrow	Hemorrhage	/
Heart	Hemorrhage	Describing localization: endocardial, myocardial, epicardial
	Pericardial effusion	/
	Pericarditis	
Tonsils	Hemorrhage	/
	Necrosis	
Brain	Hemorrhage	/
Adrenal gland		
Genitals		
Skin		
Larynx		

* Further lesions were described.

Histopathology and Immunohistochemistry

The tissue samples were embedded in paraffin wax and cut at 2–3 µm slices. Hematoxylin-eosin (HE) staining was performed to examine the main macroscopic lesions in more histological detail. To visualize viral antigens, anti-ASFV p72 immunohistochemistry was conducted on the respective organs as described earlier [17,18]. In brief, sections were treated with an in-house rabbit polyclonal primary antibody against the major capsid protein p72 of ASFV (diluted in TBS 1:1600, 1 h), followed by incubation with a secondary, biotinylated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA, USA; diluted in TBS in 1:200, 30 min). Positive antigen detection was visualized by the Avidin–Biotin Complex (ABC) method providing horseradish peroxidase that converted the added chromogen 3-amino-9-ethylcarbazole (AEC) into insoluble red-colored deposits at the reaction site. As negative control, consecutive sections were labeled with an irrelevant antibody (M protein of Influenza A virus, ATCC clone Hb64). An ASF positive control slide was included in each run.

Histopathology including Semiquantitative Antigen Scoring

The slides were scanned using a Hamamatsu S60 scanner and evaluated using NDPview.2 plus software (Version 2.8.24, Hamamatsu Photonics, K.K. Japan). While the histopathological lesions obtained on HE-stained sections were described only qualitatively

(present/absent) due to autolysis-related limited assessability, the viral antigen content in the respective organ was determined on a semiquantitative scoring scale as previously published [18]. The most affected area (420 × 260 µm) per sample sections was scored with score 0 (no antigen), score 1 (1–3 positive cells), score 2 (4–15 cells) or score 3 (>16 cells). Cells with fine granular cytoplasmic labeling were considered positive whereas chromogen aggregations without cellular association were not counted.

2.1.2. Detection of ASFV Genome

To determine the viral genome load, the tissue samples were homogenized in 1 mL of phosphate buffered saline with a metal bead using a TissueLyzer II (Qiagen GmbH, Hilden, Germany). Viral nucleic acids were extracted from blood and homogenized spleen, lung, liver, kidney, popliteal lymph node and brain with the NucleoMag Vet Kit (Machery-Nagel, Düren, Germany) on the KingFisher extraction platform (Thermo Scientific, Waltham, MA, USA). Quantitative real-time PCR (qPCR) was conducted according to the protocol published by King et al. [26] with an in-house full virus standard for determination of genome loads on a C1000 thermal cycler with the CFX96 Real-Time System (Biorad, Hercules, CA, USA).

2.1.3. Detection of Anti-ASFV Antibodies

For investigation of ASFV-specific antibodies, an accredited indirect immunoperoxidase test (IPT) was applied according to the standard protocol SOP/CISA/ASF/IPT/1 provided by the European Reference laboratory for ASF with modifications regarding cell and virus type (https://asf-referencelab.info/asf/images/ficherosasf/PROTOCOLOS-EN/2021_UPDATE/SOP-ASF-IPT-1_2021.pdf, accessed on 4 April 2022). As sample material, plasma was obtained from EDTA blood by centrifugation at 18.000 g-force for 10 min from German wild boar carcasses and domestic pigs infected with ASFV "Estonia 2014" from a previous trial for comparison. Titers were determined semiquantitatively by endpoint dilution from 1:40 to 1:12,800.

2.1.4. Statistical Analysis

Using GraphPad Prism (Version 8.4.2), statistical analysis was conducted to determine overall group differences in terms of viral genome load, viral antigen amount, macroscopic lesion scores and antibody titers. For this purpose, the non-parametric Kruskal–Wallis test with post hoc Dunn's test was performed. A p value ≤ 0.05 was considered significant.

3. Results

3.1. Pathogen Detection in Blood and Tissues

Full necropsies were performed on all wild boar obtained from the outbreak areas LOS, MOL and SN to determine the amount of viral genome and antigen. The results are shown in Figure 1 and details are given in Supplementary Tables S1 and S2.

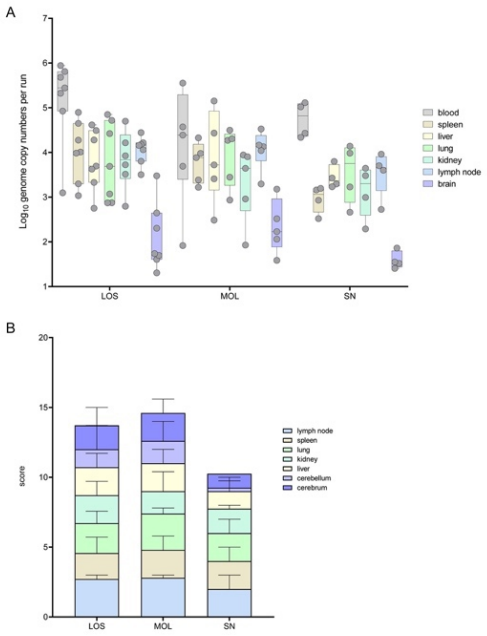

Figure 1. Pathogen detection in blood and tissue samples of ASFV-infected wild boar carcasses from LOS, MOL and SN. (**A**) Box plot presenting the individual viral genome load in blood and organ samples. (**B**) Corresponding stacked bar diagram showing the median viral antigen score with range per organ. Organs were scored on a scale from 0 to 3 based on the number of positively labeled cells in the most-affected tissue area per high power field.

Viral genome could be found in all the samples of the infected wild boar. In general, the highest viral genome loads were detected in blood samples, varying between 1×10^2 and 9×10^5 genome copies (gc)/5 µL nucleic acid. In general, genome loads in most organ samples were roughly one logarithmic step lower than the corresponding blood samples. A lower mean viral genome load was detected in wild boar found in SN when compared to animals from LOS and MOL (Figure 1A).

The viral antigen score of selected tissue sections reflected the results obtained by qPCR. Consistent with the lower number of viral genome copies, wild boar from SN also reached lower viral antigen scores (Figure 1B). Details on immunohistochemistry are included in the histopathological evaluation of organ systems in the following section.

3.2. Pathological Assessment of Organ Systems

All carcasses were scored macroscopically based on a standardized scoring system [25] with further modifications as indicated in Table 2. Histopathological alterations were reported only as present/absent due to the reduced number of well-preserved available tissues. A summary of all macroscopical and histopathological ASF-associated [27] and bacterial-induced or background alterations [28–31] including immunohistochemistry results are shown in Table 3.

Table 3. Summary of macroscopical and microscopical lesions in ASF-infected wild boar carcasses. Pathological findings are listed as primary lesions, characteristically associated with ASF [27] and as lesions, usually induced by bacteria or as common background lesions [28–31].

Organs/Tissues	Gross Pathology	Histopathology	Immunohistochemistry
Immune system	**Primary lesions associated with ASF** Lymph nodes: • Hemorrhagic lymph-adenopathy Spleen: • Increased spleen weight Bone marrow: • Hemorrhages	**Primary lesions associated with ASF** Lymph nodes: • Lymphoid depletion • Thrombosis • Necrosis of interfollicular, paracortical areas and medullary chords Spleen: • Lymphoid depletion • Apoptosis/necrosis of myelomonocytic cells Bone marrow: • N/A	Lymph nodes: • Positive, macrophages Spleen: • Positive, macrophages Bone marrow: • N/A
Respiratory system	**Primary lesions associated with ASF** Lung: • Alveolar edema • Hemorrhages • Consolidation • Loss of collapse Nose: • Hemorrhages **Lesions, usually induced by bacteria or common background lesions** Lung: • Fibrous pleuropneumonia	**Primary lesions associated with ASF** Lung: • Alveolar edema • Hemorrhages • Necrotizing interstitial pneumonia Nose: • N/A **Lesions, usually induced by bacteria or common background lesions** Lung: • Fibrino-suppurative/necrotizing bronchopneumonia	Lung: • Positive, alveolar/interstitial macrophages Nose: • N/A
Cardiovascular system	**Primary lesions associated with ASF** Heart: • Hemorrhages (epi-, myo-, endocardial) **Lesions, usually induced by bacteria or common back-ground lesions** Heart: • Fibrous pericarditis	**Primary lesions associated with ASF** Heart: • Hemorrhages Mononuclear infiltration (endo-/subendocardial)	Heart: • Positive, macrophages

Table 3. *Cont.*

Organs/Tissues	Gross Pathology	Histopathology	Immunohistochemistry
Urinary system	**Primary lesions associated with ASF** Kidney: • Hemorrhages (cortical, medullary, pelvic) • Perirenal edema and hemorrhages Urinary bladder: • Hemorrhages (mucosal, serosal, transmural)	**Primary lesions associated with ASF** Kidney: • Hemorrhages (interstitial, glomerular) • Vascular thrombosis Urinary bladder: • N/A **Lesions, usually induced by bacteria or common background lesions** Kidney: • Non-suppurative tubulointerstitial nephritis • Tubular necrosis	Kidney: • Positive, macrophages Urinary bladder: • N/A Urinary bladder: • N/A
Gastrointestinal system/abdominal cavity	**Primary lesions associated with ASF** Liver: • Congestion • Hemorrhages (subcapsular) Gall bladder: • Wall edema • Wall hemorrhages Stomach: • Hemorrhagic gastritis Small intestine: • Hemorrhages (serosal, mucosal) Large intestine: • Hemorrhages (serosal, mucosal) Abdominal cavity: • Hemorrhagic ascites **Lesions, usually induced by bacteria or common back-ground lesions** Stomach: • Ulcerative gastritis Abdominal cavity: • Fibrous peritonitis	**Primary lesions associated with ASF** Liver: • Apoptosis/necrosis of Kupffer cells and hepatocytes Gall bladder: • N/A Stomach: • N/A Intestine: • N/A **Lesions, usually induced by bacteria or common back-ground lesions** Liver: • Mixed-cellular sinusoidal and periportal infiltration	Liver: • Positive, Kupffer cells Gall bladder/stomach/intestine: • N/A
Nervous system	**Primary lesions associated with ASF** Brain: • Hemorrhages	**Primary lesions associated with ASF** Brain: • Hemorrhages • Non-suppurative meningitis (cerebral, cerebelar) • Non-suppurative encephalitis (cerebral, cerebellar) • Non-suppurative plexus choroiditis	Brain: • Positive, macrophages

Table 3. *Cont.*

Organs/Tissues	Gross Pathology	Histopathology	Immunohistochemistry
Endocrine system	**Primary lesions associated with ASF** Adrenal gland: • Hemorrhages Pancreas: • Hemorrhages • Edema	**Primary lesions associated with ASF** Adrenal gland: • Hemorrhages (cortical, medullary) • Sinusoidal thrombosis • Mixed-cellular infiltration (medullary Pancreas: • N/A	Adrenal gland: • Positive, macrophages Pancreas: • N/A
Reproductive system	**Primary lesions associated with ASF** Testicle (spermatic chord): • Hemorrhages Vestibulum: • Hemorrhages	• N/A	• N/A

The overall score obtained upon macroscopical evaluation turned out to be the opposite when compared to the viral genome load and antigen score. Therefore, wild boar from SN tended to show a higher total score when compared to wild boar from LOS and MOL (Figure 2). Individual animal scores given for macroscopical findings are included in Supplementary Table S3.

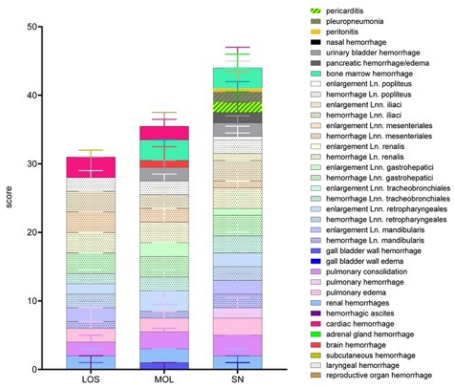

Figure 2. Summary of scoring results following macroscopical investigation of ASFV-infected wild boar carcasses from LOS, MOL and SN. Stacked bar diagram showing the total gross lesion score, which is composed of individual scores given for macroscopical findings shown on the right. Lesions were scored on a scale from 0 to 3. Bars indicate the median with range.

In the following gross and histopathological findings, the different organ systems will be described.

3.3. Immune System

3.3.1. Lymph Nodes

Gross Pathology

In general, hemorrhagic lymphadenopathy was present in all animals irrespective of the outbreak area (Figure 3). Details are given in Supplementary File S1 and Table S3.

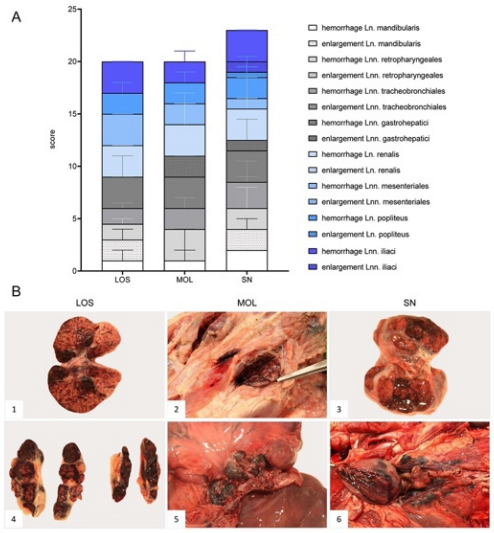

Figure 3. Representative macroscopical findings of lymph nodes in ASFV-infected wild boar carcasses from German outbreak areas. (**A**) Stacked bar diagram showing the total gross lesion score given for enlargement and hemorrhages of various lymph nodes evaluated on a scale from 0 to 3. Bars indicate the median with range per finding. (**B**) Lymph nodes (Ln. mandibularis ((**B1**)–(**B3**)), Ln. renalis (**B4**), Lnn. gastrohepatici (**B5**), Lnn. iliaci (**B6**)) revealed hemorrhages of varying degree.

Histopathology

The popliteal lymph node was examined in more histological detail as demonstrated in Figure 4. The findings were characterized by lymphoid depletion, hemorrhages (Figure 4A,B), necrosis (Figure 4B) and vascular thrombosis (Figure 4C). Animals showed p72 positively labeled cells morphologically consistent with macrophages (Figure 4D). Details for individual animals are given in Table S2.

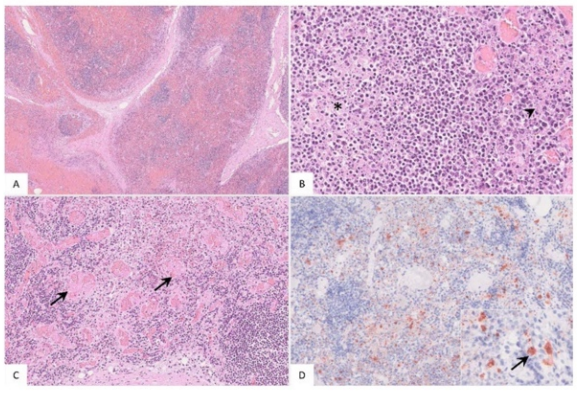

Figure 4. Pathohistological findings of the popliteal lymph node in German ASFV-infected wild boar carcasses. (**A**) Diffuse lymphoid depletion and hemorrhage affected the follicles, paracortex and medullary chords thereby effacing the physiological lymph node architecture, HE stain. (**B**) A lymphoid follicle with lymphoid depletion (asterisk) was surrounded by necrosis (arrowhead), HE. (**C**) Numerous vessels were occluded by fibrin thrombi (arrows) throughout the lymph node, HE. (**D**) A large number of viral-antigen-positive cells are shown, which were morphologically consistent with macrophages (inlay), anti-p72 immunohistochemistry, ABC method.

3.3.2. Spleen

Gross Pathology

Macroscopic assessment of the spleen was limited due to poor preservation. Therefore, the spleen was evaluated by determination of the relative spleen weight based on a recent publication in domestic pigs [32] shown in Figure S1.

High relative spleen weight values were observed in all wild boar irrespective of the district of origin. Median values reached 0.81 (LOS), 0.69 (MOL) and 0.97 (SN).

Histopathology

Briefly, histological examination of the spleen revealed congestion and hemorrhage with lymphoid depletion in all wild boar (Figure S2A,B). Immunopositive cells were detected, phenotypically consistent with macrophages (Figure S2C,D). Individual histopathological results are summarized in Table S2.

3.3.3. Bone Marrow

Gross Pathology

Pathological changes in the femoral bone marrow included hemorrhages in all groups (Figure 5). All scores given for each individual animal can be found in Table S3.

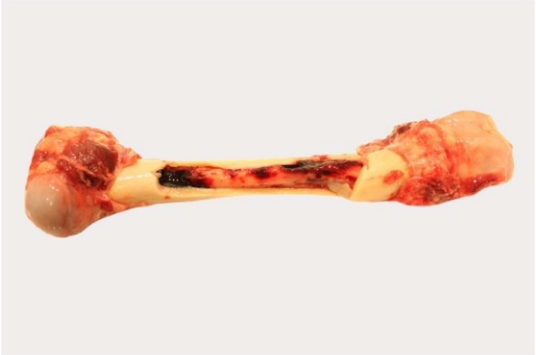

Figure 5. Gross pathology of the bone marrow of naturally ASFV-infected wild boar carcasses from German outbreak areas. Bone marrow hemorrhages, if present, were severe throughout.

Histopathological examination was not performed, because in the majority of animals, progression from red to yellow marrow had already occurred.

3.4. Respiratory System

3.4.1. Lung

Gross Pathology

ASF-associated macroscopic findings of the lung were up to severe pulmonary edema, incomplete collapse with foci of consolidation and congestion as well as hemorrhages (Figure 6B, 1–4). In individual wild boar, fibrous pleuropneumonia, likely as a consequence of bacterial infection, was detected (Figure 6B, 5–6). Furthermore, isolated verminous pneumonia was present in wild boar from all districts. Details on macroscopic lung scores of all animals are summarized in Table S3 and described in Supplementary File S1.

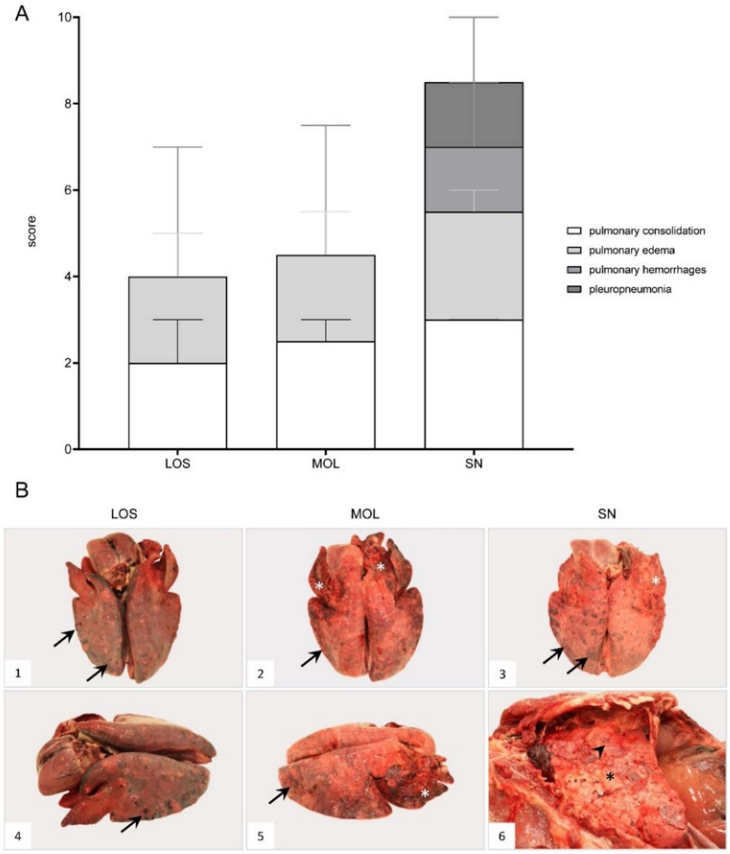

Figure 6. Macroscopical lung lesions of ASFV-infected wild boar carcasses found in German districts. (**A**) Stacked bar diagram demonstrating the median with range of individual scores given for each pathological criterion shown on the right legend. The presence and severity of each finding was scored from 0 to 3. (**B**) All lungs showed consolidated areas of different size (asterisk) and loss of pulmonary collapse ((**B1**)–(**B6**)). Pulmonary hemorrhages of varying severity are demonstrated by arrows ((**B1**)–(**B5**)). Chronic pleuropneumonia, likely due to bacterial infection, is shown in B6 with extensive fibrous pleural adhesions (arrowhead).

Histopathology

Histopathological findings are shown in Figure 7 and Table S2. Pulmonary inflammation either presented as fibrino-suppurative to necrotizing bronchopneumonias, probably due to bacterial infections (Figure 7A–C), or interstitial pneumonia (Figure 7D). Positively labeled cells consistent with intravascular, -alveolar and interstitial macrophages were detected by immunohistochemistry (Figure 7E,F).

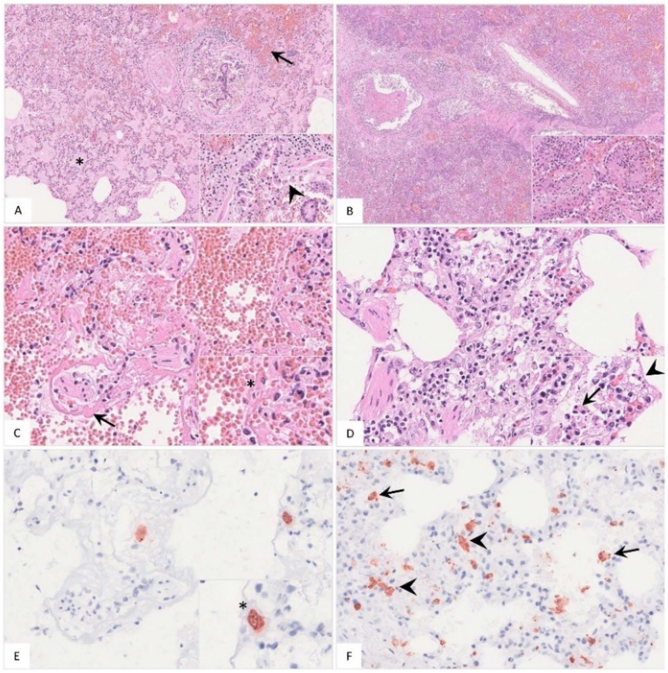

Figure 7. Histopathological findings of lungs in German naturally ASFV-infected wild boar carcasses. (**A**) Alveoli were filled with protein-rich edema fluid (asterisk), erythrocytes (arrow) and fibrin strands. The bronchiolus revealed epithelial necrosis (inlay, arrowhead) and contained cellular debris and erythrocytes. A distended pulmonary vein with fibrin thrombi was present left from the bronchiole, HE. (**B**) In a few animals, severe fibrino-suppurative to necrotizing bronchopneumonia was detected. Alveoli were densely filled with cellular debris, fibrin, viable and degenerate neutrophils, plasma cells, macrophages and lymphocytes as well as erythrocytes (inlay), HE. (**C**) A low number of wild boar showed loss of alveolar epithelium and hyaline membranes (arrow). An intravascular macrophage is indicated by asterisk (inlay), HE. (**D**) Alveolar septa showed epithelial necrosis (inlay, arrowhead), infiltration by necrotic macrophages (inlay, arrow), neutrophils, lymphocytes and plasma cells, HE. (**E**) and (**F**) Immunohistochemistry showed viral antigen-positive cells morphologically consistent with intravascular ((**E**), asterisk, consecutive section of (**C**)) intraalveolar ((**F**), arrow) and interstitial ((**F**), arrowhead) macrophages, anti p72-immunohistochemistry, ABC method.

3.5. Cardiovascular System

3.5.1. Heart

Gross Pathology

Hemorrhages affected wild boar of each group (Figure 8). Bacterial infection led to pericarditis with fibrous adhesions in one animal from SN. Details for individual animals are given in Supplementary File S1 and Table S3.

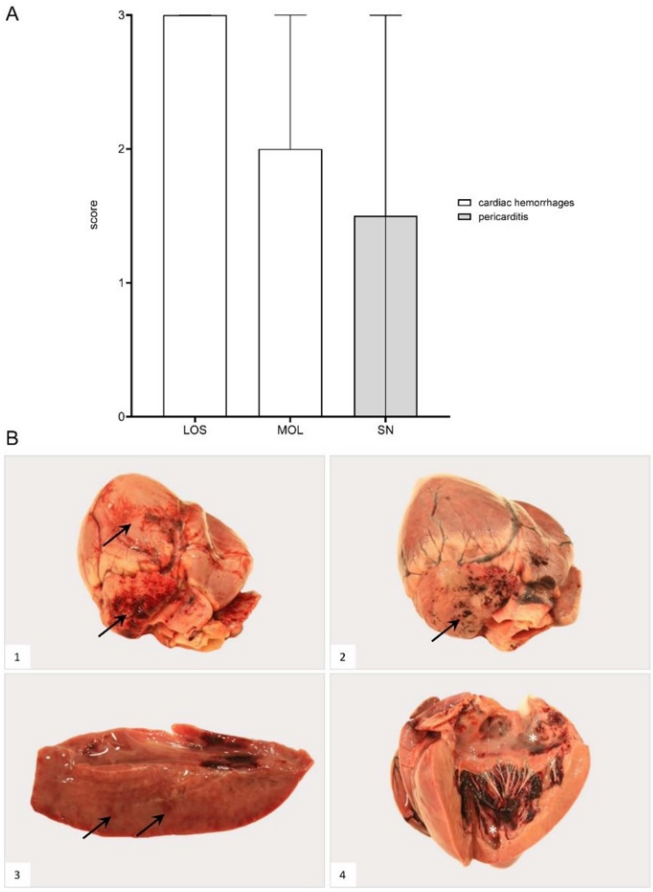

Figure 8. Heart lesions in naturally ASFV-infected wild boar carcasses from German outbreak areas. (**A**) Scoring of the heart included the presence and severity of hemorrhages as well as pericarditis, which were evaluated on a scale from 0 to 3. Bars indicate the median with range. (**B**) Hemorrhagic lesions of different locations and severity of ASFV-infected wild boar are shown. Multifocal paintbrush to coalescing hemorrhages were found in the epicardium (arrow) to a variable extent ((**B1**),(**B2**)). Scant myocardial hemorrhages (arrow) are indicated in (**B3**). Multifocal endocardial hemorrhages (asterisk) are present in (**B4**). The darker blood coagulum had to be differentiated from hemorrhages.

Histopathology

In addition to hemorrhages (Figure 9A,B), in a few animals, there was endocardial and subendocardial infiltration by mononuclear cells (Figure 9C,D). Viral antigen was found in cells mostly morphologically consistent with macrophages (Figure 9E,F). Detailed histopathological evaluation of the heart can be found in Table S2.

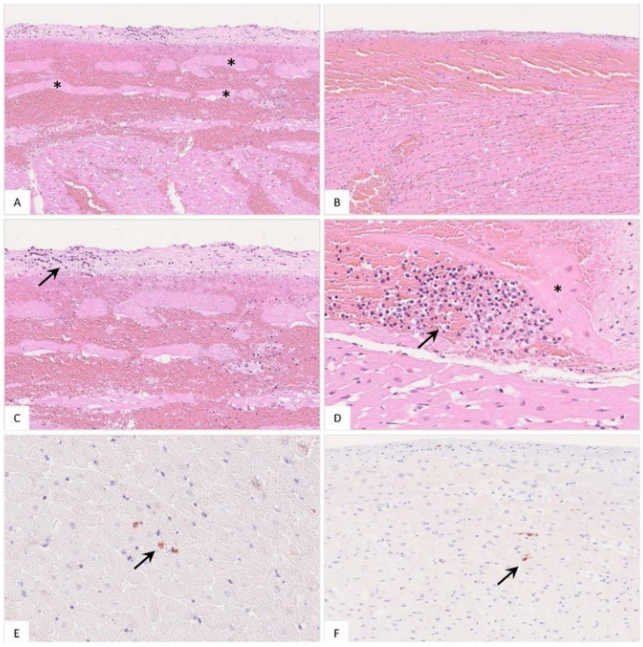

Figure 9. Histopathology of the heart in naturally ASFV-infected wild boar carcasses from Germany. (**A**) Massive hemorrhage involved the endocardium as well as the myocardium, displacing subendocardial Purkinje fibers (asterisk), HE. (**B**) The epicardium was also affected by diffuse hemorrhage radiating into the myocardium, HE. (**C**) Higher magnification from (**A**) shows minimal accumulation of infiltrating mononuclear cells in the endocardium (arrow), HE. (**D**) Subendocardial infiltrates (arrow) were also present between Purkinje fibers (asterisk), HE. (**E**,**F**) Immunohistochemistry of the heart showed only few positive macrophages (arrow), anti-p72 immunohistochemistry, ABC method.

3.6. Urinary System

3.6.1. Kidney

Gross Pathology

Renal and perirenal hemorrhages were present in all wild boar irrespective of the district (Figure 10). Details can be found in Supplementary File S1 and Table S3.

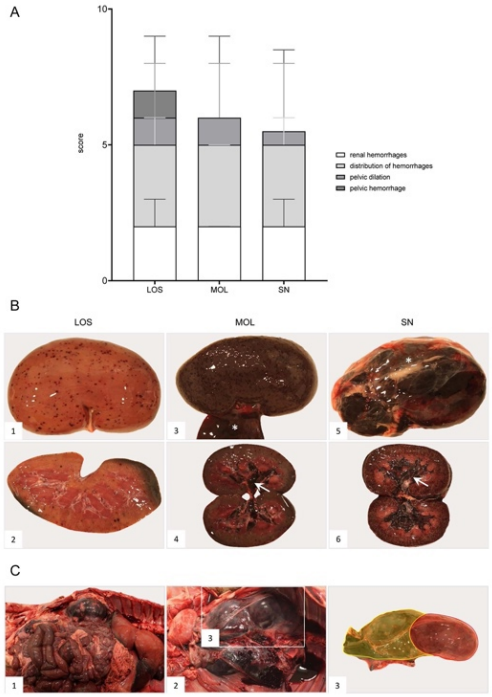

Figure 10. Pathologic changes in kidneys of ASFV-infected German wild boar carcasses from different outbreak areas. (**A**) Stacked bar diagram of gross lesion scoring of pathological criteria listed on the right. Scoring was conducted on a scale from 0 to 3 or from 0 to 4 (distribution pattern of hemorrhages). Individual scores are given as median values with range. (**B**) Hemorrhagic lesions of various size and severity affecting different parts of the organ are shown in ((**B1**)–(**B6**)). Multifocal petechiae with fewer ecchymoses primarily located to the renal cortex are depicted in ((**B1**),(**B2**)). Gray discoloration of the kidney periphery was due to beginning autolysis (**B2**). Mainly affecting the renal cortex (cortico-medullar pattern), diffuse ecchymoses are present in ((**B3**),(**B4**)). Marked dilation and diffuse bleeding into the renal pelvis are depicted in ((**B4**),(**B6**)) (arrows). To a lesser extent, oligofocal petechiae (arrowhead) could be found in the medulla (**B6**). Edema of the perirenal tissue is represented in ((**B3**),(**B5**)) (asterisk). (**C**) Massive hemorrhage resulted in expansion and bulging of the renal capsule ((**C1**),(**C2**)). The hemorrhage further extended into the perirenal and retroperitoneal tissue including the ureter (**C2**). To better distinguish the kidney and the extent of hemorrhage from (**C2**), the kidney was shaded red and the hemorrhage was highlighted in yellow (**C3**).

Histopathology

The histopathological findings included hemorrhages (Figure 11A), glomerular alterations (Figure 11B), non-suppurative tubulointerstitial nephritis, (Figure 11C), tubular epithelial necrosis (Figure 11D) and renal vein thrombosis (Figure 11E). Immunohistochemistry revealed positive cells morphologically consistent with macrophages (Figure 11F). Details on the histopathological findings are shown in Table S2.

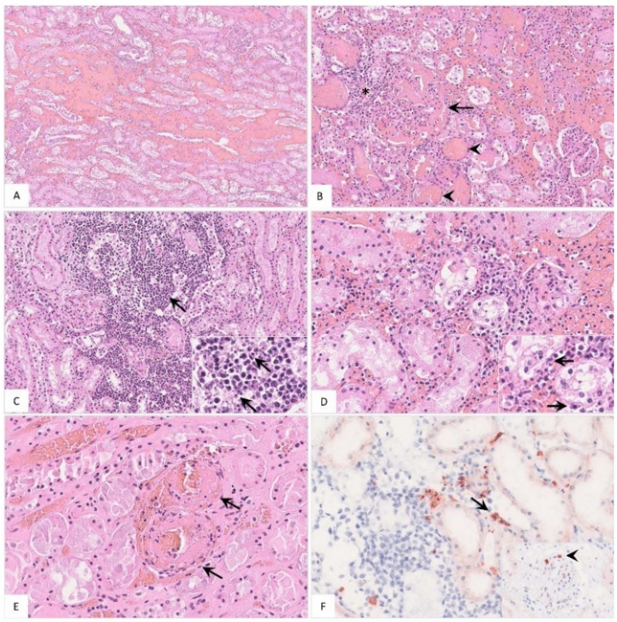

Figure 11. Pathohistological findings of the kidney in naturally ASFV-infected wild boar carcasses. (**A**) Diffuse hemorrhages were present expanding the renal medullary interstitium, HE. (**B**) A glomerulus showed extravasation of fibrin admixed with erythrocytes into the Bowman's space (arrow). There was periglomerular infiltration of partly degenerated mononuclear cells (asterisk). Red blood cell casts were present in several tubules surrounding the glomerulus (arrowhead), HE. (**C**) Extensive mononuclear cell infiltrates accumulated around tubules and glomeruli (arrow) and revealed multiple foci of apoptosis/necrosis (inlay, arrow), HE. (**D**) In some areas, tubulointerstitial nephritis was associated with tubular epithelial apoptosis/necrosis (inlay, arrow), HE. (**E**) Fibrinoid vascular necrosis could be found in varying amounts of renal veins (arrow), HE. (**F**) Representative immunohistochemical image showing moderate numbers of positively labeled macrophages in the renal interstitium (arrow) or glomerular capillaries (inlay, arrowhead), anti-p72 immunohistochemistry, ABC method.

3.6.2. Urinary Bladder

Gross Pathology

The urinary bladder presented with hemorrhages in wild boar of all three groups (Figure 12). Details on the lesions found in the animals as well as individual scores can be found in Supplementary File S1 and Table S3.

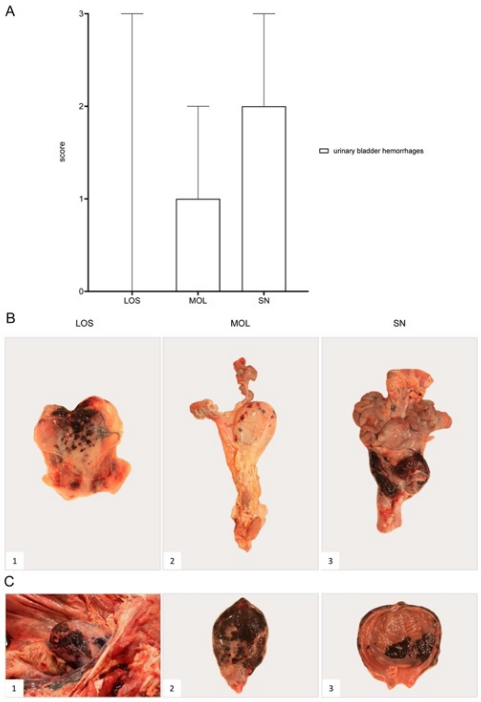

Figure 12. Pathology of the urinary bladder in naturally ASFV-infected wild boar carcasses from German outbreak districts. (**A**) Bar diagram showing hemorrhagic changes of the urinary bladder scored on scale from 0 to 3. Bars indicate the median with range. (**B**) Hemorrhages of varying severity were observed during necropsy. Multifocal-to-coalescing hemorrhages (**B1**) and multiple ecchymoses (**B2**) or severe, diffuse hemorrhage of the urinary bladder radiating into surrounding connective tissue (**B3**) were found. (**C**) Severe hemorrhages were located to the serosa ((**C1**),(**C2**)) as well as to the mucosal surface of the urinary bladder (**C3**).

Histopathological examination was not performed due to poor preservation.

3.7. Gastrointestinal System

3.7.1. Liver and Gall Bladder

Gross Pathology

Due to poor preservation, not all livers could be examined. Hepatic congestion and hemorrhages as well as edema affecting the gall bladder wall were present (Figure S3). Details on lesions are given in Supplementary File S1 and Table S3.

Histopathology

Microscopical lesions of well-preserved livers included apoptosis/necrosis of Kupffer cells (Figure S4A) and hepatocytes (Figure S4B), and sinusoidal and periportal infiltrates (Figure S4C). Immunohistochemistry revealed positive immunolabeling of cells phenotypically consistent with Kupffer cells (Figure S4D). A summary of histopathological observations is included in Table S2.

3.7.2. Stomach and Intestine

Gross Pathology

Due to progressive autolysis, the gastrointestinal tract could be evaluated only in individual animals. Macroscopic findings included hemorrhagic gastritis and hemorrhages in the small and large intestine as indicated in Figure S5. Hemorrhagic ascites was further

detected. Occasionally, gastric ulcers as well as fibrous peritonitis, likely associated with bacterial infection, were also found in animals from SN. Supplementary File S1 and Table S3 provide detailed results.

Histopathological examination was not carried out due to advanced autolysis of the gastrointestinal tract.

3.8. Nervous System

3.8.1. Brain

Gross Pathology

The brain was affected by hemorrhages only occasionally in some animals from MOL as shown in Figure 13A. Both the cerebellum and cerebrum were further evaluated by histopathology since data on respective lesions are sparse.

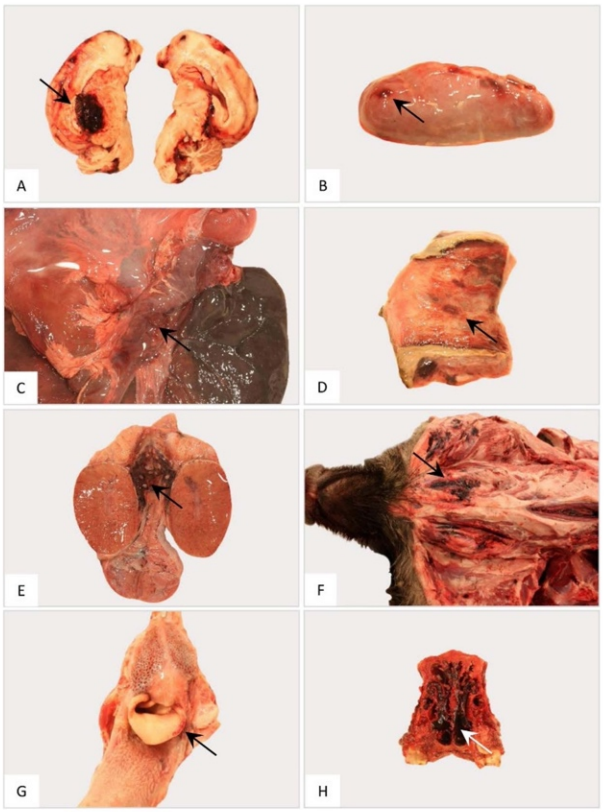

Figure 13. Gross pathology of the nervous, endocrine and reproductive organ systems and other findings in naturally ASFV-infected wild boar carcasses from Germany. Representative lesions included hemorrhages in the cerebrum (**A**), adrenal gland (**B**), pancreas (**C**), vestibulum vaginae (**D**), testis (**E**), subcutaneous tissue (**F**), larynx (**G**) and nasal mucosa (**H**). Arrows indicate hemorrhagic changes in the respective organs.

Histopathology

Microscopical findings of the cerebellum and cerebrum included meningitis, encephalitis and plexus choroiditis as depicted in Figures 14 and 15, respectively. Occasionally, hemorrhage as well as satellitosis and microgliosis were detected. Detailed histopathological results are described in Supplementary File S1 and Table S2.

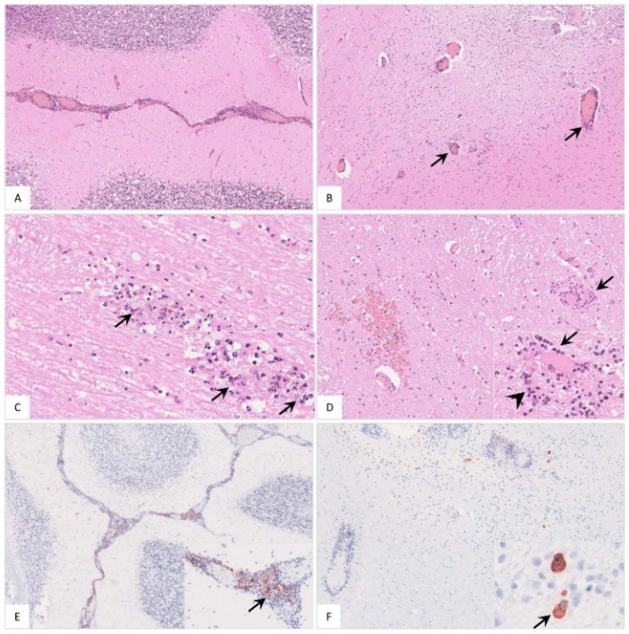

Figure 14. Histopathological findings in the cerebellum of ASFV-infected wild boar carcasses. (**A**) Meningitis was present in affected animals. (**B**) Cerebellar encephalitis was characterized by multifocal perivascular cuffs consisting of mononuclear cell infiltrates. (**C**) Parenchymal mononuclear infiltrates (arrow) showed multifocal apoptosis/necrosis (inlay, arrow). (**D**) Hemorrhage (left), perineural satellitosis (arrow, also see inlay) and microgliosis (inlay, arrowhead) were recognized. (**E**) and (**F**) Cerebellar meninges as well as brain parenchyma revealed positively labeled macrophages of differing amounts (inlays, arrow).

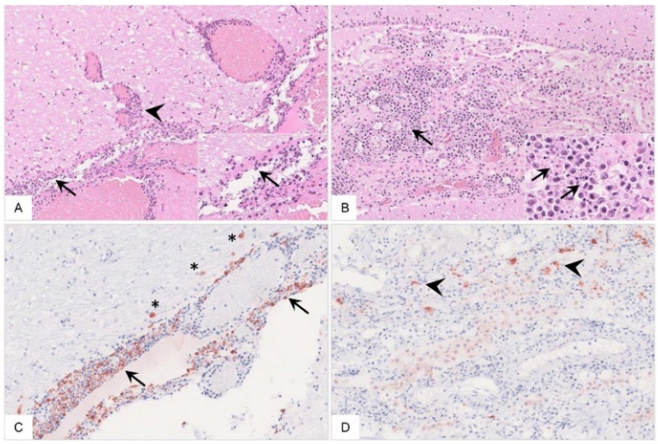

Figure 15. Histopathology of the cerebrum of ASFV-infected wild boar carcasses. (**A**) The meninges (arrow) and adjacent brain parenchyma (arrowhead) were infiltrated by mononuclear cells via Virchow Robin spaces. Mononuclear cells showed multifocal apoptosis/necrosis (inlay, arrow). Meningeal vessels were prominently dilated. (**B**) Mononuclear inflammation was limited to the choroid plexus within ventricles (arrow) with multifocal apoptosis/necrosis of infiltrating cells (inlay, arrow). There was degeneration of only a few plexus epithelial cells. (**C**,**D**) Immunopositive cells were present to variable extents in the meninges (arrow) and brain parenchyma (asterisk) as well as in the choroid plexus epithelium (arrowhead), phenotypically consistent with macrophages.

Immunohistochemical results showed viral antigen-positive cells with macrophage morphology.

3.9. Endocrine System

3.9.1. Adrenal Gland

Gross Pathology

Hemorrhages were observed in the adrenal glands of animals from LOS and SN (Figure 13B).

Histopathology

Histopathology revealed hemorrhages (Figure 16A), sinusoidal thrombosis and necrosis (Figure 16B,C) as well as inflammation (Figure 16D,E). Positively labeled macrophages were detected by immunohistochemistry (Figure 16F). Individual histopathological results are listed in Table S2.

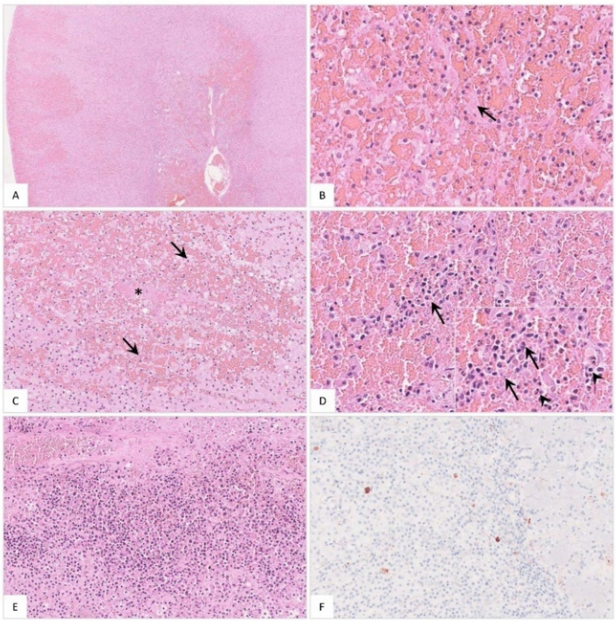

Figure 16. Histopathological findings of the adrenal gland in ASFV-infected wild boar carcasses. (**A**) Overview of the adrenal gland of a deceased wild boar. The adrenal gland showed extensive cortical and medullary hemorrhages. (**B**) Multifocally, fibrin thrombi were visible in the sinusoids (arrow). (**C**) Occasionally, areas of necrosis were present in the cortex (arrow). There was fibrin deposition (asterisk) and massive hemorrhage in the affected location. (**D**) The medulla was markedly expanded by hemorrhage. Infiltrating mononuclear cells as well as a few neutrophilic granulocytes (inlay, arrowhead) accumulated around degenerated cells (inlay, arrow). (**E**) The adrenal medulla was severely infiltrated by mononuclear cells admixed with fewer neutrophils. (**F**) Moderate amounts of antigen-positive macrophages were found in the majority of animals.

3.9.2. Pancreas

Gross Pathology

Pancreatic edema and hemorrhage were detected in animals from SN (Figure 13C). Histopathological examination was not carried out due to advanced autolysis.

3.10. Reproductive System

Occasionally, hemorrhages were found in the vaginal vestibulum in one wild boar from LOS (Figure 13D) and in the spermatic cord in a wild boar from MOL (Figure 13E).

3.11. Occasional Findings

Further hemorrhages were found in the subcutis in animals from LOS (Figure 13F), and in the epiglottis (Figure 13G) and nasal cavity (Figure 13H) in wild boar from SN.

3.12. Antibody Detection against African Swine Fever Virus

All animals were tested for anti-ASF antibodies by IPT as shown in Figure S6. Except for one animal from LOS, all wild boar developed antibodies of different titers between 200 and 800. Higher titers tended to be found in the animals from MOL, titers ranging from 200 to 1600, and in two wild boar from SN having titers of 800 and 3200, respectively. One animal from SN showed a titer of 40. In the fourth wild boar from SN, no test could be performed due to limited sample material.

For comparison, three domestic pigs from a previous study inoculated with the moderately virulent ASFV strain "Estonia 2014" were analyzed for anti-ASFV-specific antibodies. Starting at day 14 pi, all pigs developed antibody titers between 200 and 400. Since one pig had died at day 14 pi, only two animals could be analyzed in the following days. On day 21 pi, titers increased to 800 and 1600. On day 28 pi, titers further increased to 3200 or even remained at the same level of 1600 while on day 35 pi antibodies dropped in one animal to 800, but increased in the other pig to 3200. On day 41 pi, a second increase in the titer to 1600 was noted in one pig whereas in the other one antibodies remained constantly high at 3600.

4. Discussion

Filling the documentation gap on the pathology after ASF field infection, the aim of the present study was to examine ASFV-infected wild boar that succumbed to the disease under natural conditions in both virological and pathomorphological detail. Furthermore, the impact on the virulence of emerging virus variants II, III and IV in the ASF outbreak areas of Eastern Germany was analyzed.

A total of 16 wild boar aged between 0 and 2 years of different sexes were investigated. Despite the different preservation status, the organs of each animal could be examined for ASFV genome load and revealed consistently positive results. While it has to be noted that a direct comparison has to be conducted with great care due to many unknown factors, all animals were found as carcasses in affected regions and that would allow us to assume they reached a similar point of infection, i.e., the terminal phase. At that point, significant differences were not found between animals of different outbreak areas and the three variants, but wild boar from SN tended to show both lower viral genome loads and viral antigen scores compared to animals from LOS and MOL. However, the viral genome load has limited informative value at this point since viral genome can be detected up to 100 days after infection [33] and the time at which the genome load decreases varies greatly between experiments [11,18,19].

In addition to organ-wide detection of viral genome, all wild boar irrespective of the outbreak area and virus variant were diagnosed with characteristic and severe ASF lesions resembling a systemic hemorrhagic disease [6]. While no data exist for wild boar that died of ASF under natural conditions, pathology in domestic pigs has recently been described [21,34,35]. Typically, domestic pigs show comparable lesions such as hemorrhagic lymphadenopathy, splenomegaly, pulmonary consolidation and edema, hemorrhages in the heart and kidneys and hepatomegaly with edema of the gallbladder wall, as well as edematous, hemorrhagic meninges.

While most of the macroscopic findings in this study have been described after experimental infection in wild boar [36], they do not reflect the severity and diversity seen under field conditions. Comparing the three different virus variants, striking, but not significant,

differences were evident. Interestingly, the highest total score for gross pathological changes was given for wild boar from SN infected with variant IV, followed by animals from MOL infected with variant III, which showed an intermediated total score, and wild boar from LOS infected with variant II, had the lowest macroscopical score.

For ASF, four different courses of the disease have been described and include peracute, acute, subacute and chronic stages, which are associated with typical lesions [6]. Petrov et al. [33] moreover specified the subacute stage as chronic-like and differentiated into lethal and transient course after infection with moderately virulent ASFV. Gross pathomorphological changes of the subacute/chronic-like stage include multifocal hemorrhages, edema, lymphadenitis, interstitial pneumonia and ascites [6,27,33] whereas bacterial secondary infections inducing fibrinous polyserositis, chronic pneumonia and necrosis of tonsils, however, without vascular changes, predominate in chronic courses [6]. Lesions in acutely and chronically ASFV-infected domestic pigs were also already presented in detail decades ago [12]. The animals with chronic disease showed comparable lesions as observed in the acutely infected pigs, but additionally revealed chronic changes particularly including pericarditis, pneumonia and lymphadenitis. In the present study, in contrast to the animals from LOS and MOL, although without statistical significance, wild boar from SN more frequently showed lesions most likely associated with bacterial infections indicative for a lethal subacute protracted disease course.

More specifically, chronic inflammatory processes such as fibrous pericarditis, pleuropneumonia and peritonitis were more frequently detected in SN animals. At the same time, wild boar from SN, and to a lesser extent also animals from MOL, tended to show more severe hemorrhages in the urinary bladder and bone marrow, but fewer acute hemorrhages as detected in the hearts of animals from LOS. Detailed pathomorphological investigation of experimentally infected wild boar that succumbed to highly virulent ASFV "Armenia07" infection revealed only mild petechiae of the urinary bladder, variable hemorrhages of the heart and congestion of the bone marrow while extensive hemorrhages or lesions induced by other circulating pathogens were absent [36].

Based on this, and in line with virological and immunohistochemical data, this may indicate that at least wild boar infected with the SN variant experienced a more protracted disease course than pigs from LOS suggesting a slightly decreased virulence of the SN virus variant IV to wild boar that still led to the death of the respective animals. This demonstrates that veterinarians, hunters and farmers need to be aware of less acute courses of ASF, usually attributed to classical swine fever, in order to consider this as important differential diagnosis in each case. However, considering the small number of carcasses and the indefinite sample material, this should be interpreted with caution and must be confirmed experimentally under standardized conditions in any case.

Although the majority of organs could be assessed macroscopically, we had to refrain from a detailed semiquantitative histopathological analysis because autolysis had already progressed too far in some cases, which would have considerably reduced the number of samples for investigation. However, in line with macroscopic findings, histopathology confirmed the severe course of disease in all animals regardless of the outbreak area and the virus variant. Since most wild boar studies focus only on macroscopic pathology, it is even more important to study the histopathology of natural ASF infection in more depth [17,18,36,37].

Most of the histopathological findings obtained in this study are fully comparable with those observed in domestic animals investigated upon outbreaks [21,34]. However, some of the observed lesions have already been described, but are not associated with ASF in the first line. For example, adrenal hemorrhages, which have been described to occur in wild boar upon experimental infection [36], were examined in more histopathological detail and revealed interesting results in the present study. Our findings mirror a condition known as Waterhouse Friderichsen syndrome [38]. It has been correlated with several bacterial and viral diseases and is characterized by severe hemorrhage, necrosis and microvascular thrombosis. Although the pathophysiology is not fully understood, hemorrhages are

explained by a stress-induced release of adrenaline, vasculitis and coagulation disorders including disseminated intravascular coagulation. In line with the latter, microvascular thrombosis could be shown in multiple organs as signs of acute organ injury in wild boar investigated in this study [12].

Of note, histopathology further highlighted the unique finding of localized inflammation of the cerebral choroid plexus, which occurred in wild boar irrespective of the outbreak district, but mainly affected the majority of animals from MOL and SN. So far, there are only minor reports on ASF lesions in the central nervous system [12,34,39], which can occur at all stages of the disease as demonstrated by Moulton and Coggins [12] in acutely and chronically succumbing as well as in surviving pigs after experimental and natural infection. In addition to mononuclear infiltration of meningeal and cerebral vessels, perivascular hemorrhage, occasional vascular thrombosis and neuronal degeneration, necrosis of the choroid plexus epithelium has been described only once in a few acutely infected animals [12]. The naturally infected wild boar presented in this study showed pronounced mononuclear inflammation with massive cell deaths in addition to occasional necrosis of the plexus epithelium, again suggesting a longer disease course, at least in animals from SN.

To further extrapolate how long naturally infected wild boar might have lived with the disease, antibody titers were determined and compared to those of surviving ASFV "Estonia 2014" experimentally infected domestic pigs from a previous trial. In domestic pigs, low antibody titers were detectable from day 14 to a maximum titer of 400, then increased to a max of 3200 by day 28, and remained constantly high until 41 days post infection, at least in one domestic pig. However, the other pig showed a drop from 3200 to 800 on day 35 pi and a second subsequent increase. While it cannot be excluded that a consumption or decay of antibodies occurred, one should also consider measurement inaccuracies of the semiquantitative test when targeting the fluctuant antibody titers. When comparing this to wild boar, which showed titers of at least 200, the majority of animals independent of the outbreak area might have lived with ASF for more than 14 days.

As suspected, based on the pathological data in animals from SN, but also MOL, the course of the disease was probably longer since they tended to show higher titers of max 3200 and 1600, respectively, while wild boar from LOS reached titers of only max 800. Surprisingly, antibody titers showed no clear correlation to the chronicity of lesions observed in several wild boar since one animal from SN with obviously chronic lesions produced only minimal antibody titers. On the one hand, the chronic lesions in this animal could have already existed before and might not necessarily be associated with ASFV infection. On the other hand, as hypothesized above, the antibodies may have declined over time. To date, little is known about the host's immune response against ASFV, but it is of general acceptance that antibodies directed against ASFV are not sufficient for protection against the disease [40]. However, experiments to investigate the dynamics of antibody development in ASF could be useful to draw conclusions on the disease in wildlife.

5. Conclusions

In summary, this is the first study describing the lesion spectrum in wild boar succumbing to ASF after infection with the different virus variants that have emerged within one year in Germany. Virological and pathomorphological data suggest possible differences in the virulence of the variants. At least, wild boar infected with the SN variant IV tended to experience a more protracted but nevertheless lethal disease course compared to animals infected with LOS variant II or the MOL variant III, which is more likely to be classified as intermediate. These findings are particularly important with regard to the spread and continued occurrence of the ASFV in endemic areas. To elucidate the pathogenicity and differences in the virulence and disease dynamics of the emerging virus variants more thoroughly, further experimental studies in wild boar as well as comparative investigations in domestic pigs under late human endpoint conditions are urgently needed. These

studies should also address the impact of protracted disease courses on shedding and thus transmission characteristics.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/pathogens11111386/s1, Table S1: Summary of individual organ genome copy numbers in wild boar; Table S2: Summary list of histopathological changes and immunohistochemistry results in wild boar; Table S3: Summary list of gross lesions scored on a semiquantitative scale in wild boar; Figure S1: Relative spleen weights of naturally ASFV-infected wild boar, Figure S2: Histopathology of the spleen of naturally ASFV-infected wild boar carcasses, Figure S3: Macroscopical findings of the liver in German ASFV-infected wild boar carcasses, Figure S4: Histopathological results detected in the liver of naturally ASFV-infected wild boar carcasses from Germany, Figure S5: Gross pathology of the gastrointestinal tract in naturally ASF-infected wild boar carcasses from German outbreak areas, Figure S6: Antibody titers determined by immunoperoxidase test in German wild boar carcasses compared to experimentally infected domestic pigs on different days pi, File S1: Detailed analysis.

Author Contributions: Conceptualization, J.S.-E. and S.B.; Methodology, J.S.-E., S.B., P.D. and A.B.; Validation, J.S.-E. and S.B.; Formal Analysis, J.S.-E. and P.D.; Investigation, J.S.-E., S.B., P.D. and A.B.; Resources, S.B. and J.S.-E.; Data Curation, J.S.-E. and P.D.; Writing—Original Draft Preparation, J.S.-E. and P.D.; Writing—Review and Editing, J.S.-E., S.B., P.D. and A.B.; Visualization, J.S.-E. and P.D.; Supervision, J.S.-E. and S.B. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Informed Consent Statement: Not applicable.

Data Availability Statement: All data available can be obtained on request from the corresponding authors.

Acknowledgments: We would like to thank the Brandenburg Crisis Center and the veterinary authorities in the districts Märkisch-Oderland, Oder-Spree and Spree-Neiße for their help and the submission of the examined wild boar carcasses. Further, we would like to thank Christian Loth, Silvia Schuparis, Ulrike Kleinert and Robin Brandt for their valuable support during necropsies and in the laboratory.

Conflicts of Interest: The authors declare no conflict of interest.

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5.5 African Swine Fever Laboratory Diagnosis—Lessons Learned from Recent Animal Trials

Jutta Pikalo, Paul Deutschmann, Melina Fischer, Hanna Roszyk, Martin Beer and Sandra Blome

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

Pathogens

2021

doi.org/10.3390/pathogens10020177

pathogens

MDPI

Article

African Swine Fever Laboratory Diagnosis—Lessons Learned from Recent Animal Trials

Jutta Pikalo, Paul Deutschmann, Melina Fischer, Hanna Roszyk, Martin Beer and Sandra Blome *

Institute of Diagnostic Virology, Friedrich-Loeffler-Institut, 17493 Greifswald-Insel Riems, Germany; Jutta.Pikalo@fli.de (J.P.); Paul.Deutschmann@fli.de (P.D.); Melina.Fischer@fli.de (M.F.); Hanna.Roszyk@fli.de (H.R.); martin.beer@fli.de (M.B.)

* Correspondence: Sandra.Blome@fli.de

Abstract: African swine fever virus (ASFV) causes a hemorrhagic disease in pigs with high socio-economic consequences. To lower the impact of disease incursions, early detection is crucial. In the context of experimental animal trials, we evaluated diagnostic workflows for a high sample throughput in active surveillance, alternative sample matrices for passive surveillance, and lateral flow devices (LFD) for rapid testing. We could demonstrate that EDTA blood is significantly better suited for early ASFV detection than serum. Tissues recommended by the respective diagnostic manuals were in general comparable in their performance, with spleen samples giving best results. Superficial lymph nodes, ear punches, and different blood swabs were also evaluated as potential alternatives. In summary, all matrices yielded positive results at the peak of clinical signs and could be fit for purpose in passive surveillance. However, weaknesses were discovered for some matrices when it comes to the early phase of infection or recovery. The antigen LFD showed variable results with best performance in the clinical phase. The antibody LFD was quite comparable with ELISA systems. Concluding, alternative approaches are feasible but have to be embedded in control strategies selecting test methods and sample materials following a "fit-for-purpose" approach.

Keywords: African swine fever virus; laboratory diagnosis; genome detection; antibody detection; sample matrix; blood swabs

Citation: Pikalo, J.; Deutschmann, P.; Fischer, M.; Roszyk, H.; Beer, M.; Blome, S. African Swine Fever Laboratory Diagnosis—Lessons Learned from Recent Animal Trials. *Pathogens* **2021**, *10*, 177. https://doi.org/10.3390/pathogens10020177

Academic Editor: Pedro J. Sánchez-Cordón

Received: 31 December 2020

Accepted: 3 February 2021

Published: 6 February 2021

1. Introduction

African swine fever virus (ASFV), a large, enveloped, double-stranded DNA virus, which belongs to the genus *Asfivirus* within the *Asfarviridae* family, causes an often fatal hemorrhagic disease in domestic pigs and wild boar with high socio-economic consequences worldwide [1]. Over the past decade, the disease has spread to several European and Asian countries and is still moving further, putting pig industry and the connected value chain at stake [2].

For early detection of ASF and timely implementation of control measures, targeted sampling of sick and dead animals, i.e., passive surveillance, is of utmost importance [3,4]. This is particularly crucial because of the fact that the disease is associated with high lethality, but also moderate or even low morbidity and mortality [5]. The latter is linked to contagiosity that can be moderate in wild boar populations or larger domestic pig farms in the absence of parenteral transmission routes by competent vectors [6–8]. The animals to be sampled in passive surveillance are obviously sick or have died, so it can be assumed that a significant viral load is present in several organs and tissues [6]. Direct detection methods have priority to detect the disease. With this in mind, and considering that ASFV is highly stable even in decaying carcasses [9,10], pragmatic approaches for sample collection, suitable sample matrices, and reliable testing can be discussed that could facilitate compliance and thus efficient early warning. Along these lines, several approaches have been assessed in the recent past. Specifically, the applicability of different dry blood swabs [11,12], dried filter papers and FTA cards [13–15], fecal samples [16], oral,

nasal and rectal swabs [17], meat-juice [18], and different rope-based options [19,20] has been assessed. Further matrices such as intraocular fluid, superficial lymph nodes (e.g., inguinal lymph nodes), ear punches following the example of BVDV diagnosis [21], and the like have been discussed.

Apart from passive surveillance, high-throughput active surveillance and monitoring are still needed in affected countries with intensive pig industry and/or high density of wild boar. To this means, random sampling of live animals or the wild boar hunting bag is applied, and healthy animals with a low probability of infection are the large majority. Under these circumstances, low expected virus prevalence is linked to low viral loads, and antibody detection should be included [22]. Here, the choice of the most reliable and resource-saving sample matrices can also be crucial.

In the context of a series of animal experiments with strains of different ASFV genotypes and defined endpoints within the acute phase of ASFV infection, i.e., 4 to 10 days post infection (DPI), we took the opportunity to compare and evaluate diagnostic workflows for both active and passive surveillance. Our focus was primarily on qPCR detection of ASFV genomes. In particular, we investigated the possible limitations of serum as sample matrix for monitoring purposes, compared different organs and tissues of wild boar and domestic pigs for their viral loads, and evaluated alternative sample matrices that could be used in the context of passive surveillance in domestic pigs and wild boar.

Finally, we investigated the performance characteristics of "point-of-care" or "pen-side" diagnostics for both ASFV antigen and antibody detection.

2. Results

2.1. Samples Taken from Domestic Pigs and Wild Boar Are Comparable

Our sample set (see Supplementary Table S1) comprised samples from domestic pigs (n = 37) and European wild boar (n = 16). Therefore, it had to be clarified whether the samples were comparable and thus evaluable together. Taking the post infection data set of all wild boar and the directly corresponding domestic pigs (n = 13 each), none of the tested sample matrices showed significant differences (see Figure 1 and Supplementary Figure S1). All downstream analyses were therefore performed with both wild boar and domestic pigs in one combined data set.

2.2. Serum May Reach Its Limits for Active Surveillance

In the attempt to limit the sampling effort to one matrix with low inhibitory effects in qPCR, high potential for automation, and general suitability for all direct and indirect diagnostic tests, serum was evaluated in detail. One aspect was the comparison with EDTA blood as a standard matrix that is known to contain high viral loads.

At all sampling days, positive and valid qPCR results were obtained for all EDTA blood samples and for all but one serum sample taken from inoculated domestic pigs and wild boar. Control animals remained negative. Thus, EDTA would ensure 100% sensitivity in the given test system, serum reaches only 98%. No problems arose with the internal control system applied (heterologous control). Over the entire comparison, considerably higher genome loads were found in EDTA blood samples at all times and in all animals. The difference was most obvious in the early phase of the ASFV infection (4 DPI) where serum samples contained genome copy numbers as low as 4 or 6 copies per run. In this experimental phase, five out of six animals yielded copy numbers below 100. In the phase of obvious clinical signs, serum contained also higher genome loads but these loads were still much lower than in EDTA blood. The difference was higher again at 10 DPI. The single negative serum originated from an animal that had shown a subclinical disease course upon infection with a genotype IV ASFV strain. All individual results (experimental background and genome copy numbers per run) are depicted in Supplementary Table S1.

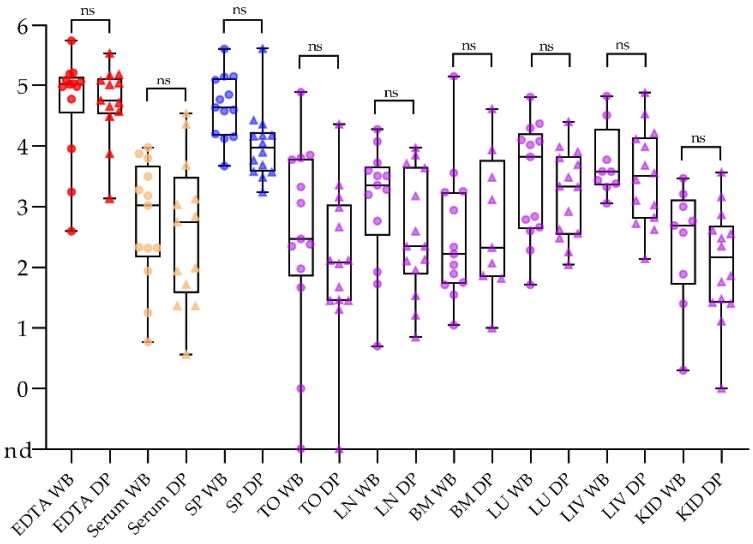

Figure 1. Comparison of sample matrices taken from wild boar (WB; dots) and domestic pigs (DP; triangles). The qPCR results are depicted as $\log_{10}$ genome copy numbers per run. Abbreviations: nd = not detected; SP = spleen, TO = tonsil, LN = lymph node, BM = bone marrow, LU = lung, LIV = liver, KID = kidney, ns = not significant in pairwise comparison.

Comparing the overall genome loads at all time points (53 pairs), EDTA blood showed significantly higher (p value < 0.0001) values post infection (see Figure 2). Especially in the early phase, serum was close to the limit of detection and the mean genome loads in EDTA blood were roughly 200 times higher (see Supplementary Table S1). As no false positive reactions occurred, performance with negative samples was not significantly different (see Figure 2, EDTA and Serum pre inf).

2.3. No Surprise in the Comparison of Routine Post Mortem Sample Matrices

Standard organs for passive and active surveillance, i.e., tonsils, spleen, mandibular lymph nodes, bone marrow, lung, liver, and salivary glands, were analysed and compared for the presence and the amount of ASFV genome (see Figure 3).

Over the whole data set, spleen samples gave consistently positive results with rather high genome loads that reached a maximum of 4.2×10^5 genome copies per run (see Figure 3 and Supplementary Table S1). The genome loads in spleen were significantly higher than in tonsils (p value 0.0016), lymph nodes (p value 0.0007), lung (p value 0.0116), liver (p value 0.0100), and kidneys (p value 0.0007). Not considering the large difference in sample numbers for bone marrow and salivary gland versus spleen (22 vs. 48), pairwise comparison showed no significant difference between bone marrow and spleen (p value 0.0507) but a significant difference between salivary gland and spleen (p value 0.0148). Individual false negative results were observed with samples taken from tonsils, lymph nodes, salivary glands, liver, and kidney. Three out of five false negative results were obtained from one animal. The same animal gave a false negative result using serum (see above). No false positive results were obtained from control animals (see Figure S1). Considering sensitivity (disregarding quantitative differences), spleen, bone marrow, and lung reached 100%. A sensitivity of roughly 98% was reached using tonsils, lymph nodes, kidney, and liver. Resulting from the smaller sample size, sensitivity of salivary gland samples was 95.5%.

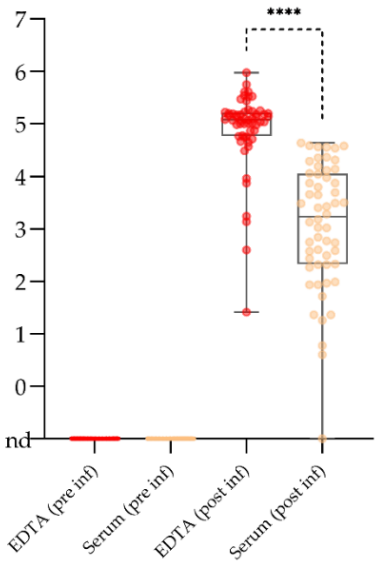

Figure 2. Overall comparison of $\log_{10}$ genome copy numbers in EDTA blood and serum (prior to infection = pre inf; post infection = post inf). The boundaries of the boxes indicate the 25th and 75th percentiles, the line within the box marks the median. Whisker boundaries indicate minimum and maximum values. A paired t-test was performed to test the significance with a resulting **** p-value of < 0.0001 for samples taken post infection. nd = not detected.

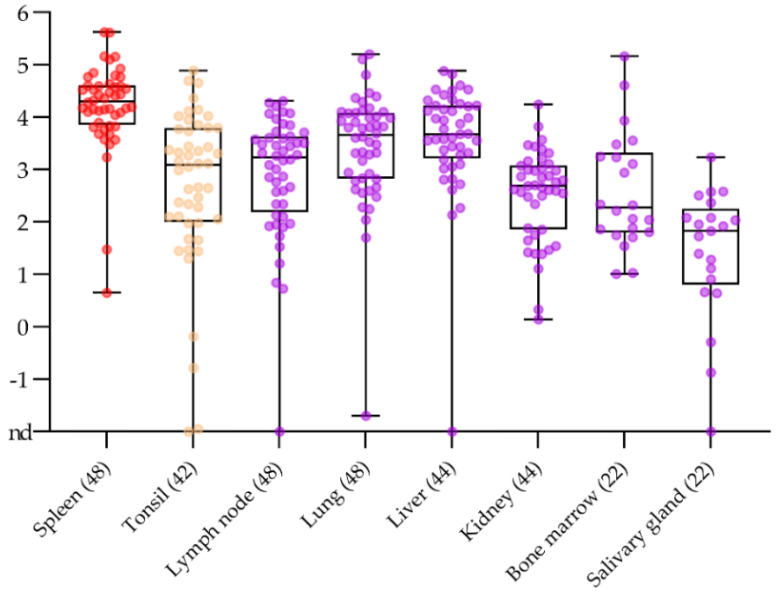

Figure 3. Comparison of $\log_{10}$ genome copy numbers per run in different organs over the entire data set. The numbers in brackets indicate the number of animals included for the respective matrix. All samples are individually depicted together with the box plot. The boundaries of the boxes indicate the 25th and 75th percentiles, the line within the box marks the median. Whisker boundaries indicate minimum and maximum values. Lymph node: mandibular/sub-mandibular lymph node; salivary gland: parotis. nd = not detected.

2.4. Alternative Sample Matrices for Passive Surveillance in Domestic Pigs and Wild Boar

2.4.1. Sampling Fallen Domestic Animals without Opening Body Cavities in the Stable

Superficial lymph nodes, ear punches, and ocular fluids were investigated as sample matrices for fallen domestic animals upon the request of (German) veterinary authorities and practitioners in pig-dense areas.

Among the lymph nodes that are easiest to access without opening the abdominal cavity, or the need to cut deep into the carcass, are inguinal lymph nodes. Their suitability for ASF diagnosis was assessed in comparison with the best choice sample spleen and the mandibular lymph node that could also be taken without opening any body cavities. For this comparison, the data set was restricted to the comparative study with ASFV strain "Estonia 2014" where different lymph nodes had been separated (n = 18 samples per matrix). In summary, all samples gave positive results in qPCR. However, the variability was highest and the genome load lowest for the inguinal lymph node. Values far below one copy (at the detection limit) to roughly 10^4 genome copies per run were observed. For the mandibular lymph node, a rather low variability was observed with a mean copy number of 1.8×10^6 per run. Comparative data are depicted in Figure 4.

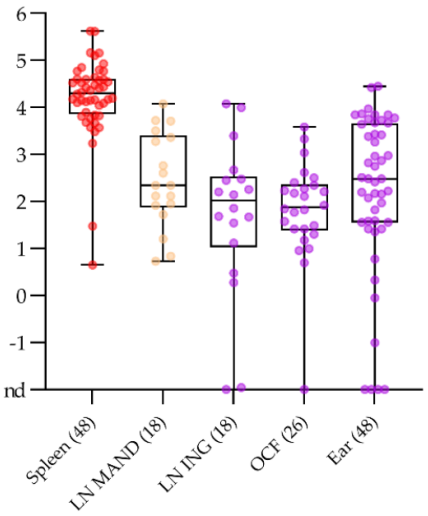

Figure 4. Comparison of $\log_{10}$ genome copy numbers per run in spleen, inguinal lymph nodes (LN ING), and mandibular lymph nodes (LN MAND), ocular fluids (OCF), and ear punches (ear). All samples are individually depicted together with the box plot. The boundaries of the boxes indicate the 25th and 75th percentiles, the line within the box marks the median. Whisker boundaries indicate minimum and maximum values.

With regard to ear punch samples, all 53 animals were included in the comparison, 48 animals post infection and 5 controls. Forty-four out of 48 samples (92%) taken post infection were found positive for viral genome in low to moderate amounts (trace amounts to 3×10^4 with a mean of 3.5×10^3). No false positive reactions occurred in the controls.

Ocular fluids (aqueous humour) was sampled from 26 animals. The sampling was difficult and the final sample matrix was rather undefined material from the interior of the eye than aqueous liquid. However, all but one animal (96%) gave a positive signal with rather low genome loads (mean 3.8×10^2). The negative animal was again the one already described for other sample matrices.

A comparison of the above-mentioned alternative sample matrices with spleen samples is depicted in Figure 4. Spleen showed significantly higher genome loads than any of the tested alternative matrices. A significant difference was also observed between the

mandibular lymph nodes and the ocular fluid (p value 0.0330). No significant differences were seen among the other alternatives.

2.4.2. Blood Swabs Can Still Be Optimized

Continuing previous studies [11,12,23], we compared and evaluated different blood swab options. Along with the previously tested plain COPAN cotton swabs (cotton swab) and GenoTube Livestock Swabs (Genotubes), PrimeSwabs and the inactivating PrimeStore MTM transport buffer were included in the assessment. Comparison was done with EDTA blood as standard matrix, and among the different swab and swab buffer options. For this study part, matched samples were available from the comparative trial with ASFV "Estonia 2014". Taken the entire data set of domestic pig and wild boar samples from this trial, all tested matrices of infected animals gave positive results. However, EDTA blood contained significantly higher viral genome loads (p-value < 0.01). Comparing the different swab options, viral genome loads varied significantly. Plain cotton swabs and Genotubes gave weakest results with several samples that contained only trace amounts or less than 10^2 genome copies per run. Both PrimeSwabs and PrimeStore MTM buffer performed significantly better. Comparing PrimeSwab and PrimeStore MTM buffer directly, the MTM buffer performed best and significantly better than any other swab option, including the PrimeSwab (p-values ranging from 0.02 to 0.003). An overview is presented in Figure 5. No significant differences were observed again between domestic pigs and wild boar (see Supplementary Figure S2). All control animals were tested negative with all swab options (see Supplementary Table S2).

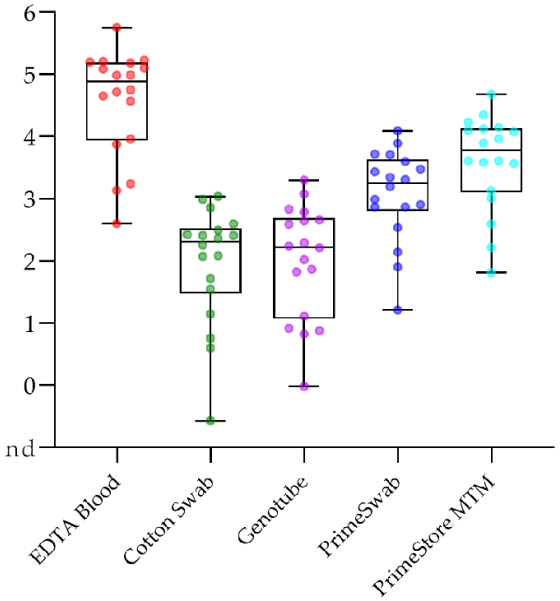

Figure 5. Comparison of genome copy numbers in different swab options and EDTA blood as comparator. Samples were taken from wild boar (WB) and domestic pigs (DP) over the entire time of the experiment. All samples are individually depicted together with the box plot. The boundaries of the boxes indicate the 25th and 75th percentiles, the line within the box marks the median. Whisker boundaries indicate minimum and maximum values. PrimeSwab indicates the swab itself, PrimeStore MTM the accompanying transport buffer.

The direct comparison of the alternative matrices (superficial lymph nodes, ear punches, ocular fluid, and swab options) underlines the good performance of blood swabs and the inactivating transport buffer (see Supplementary Figure S3).

2.5. "Point-of-Care" Tests for Ressource-Limited Settings and as a Tool for Epidemiological Investigations

The presented study included the use of commercial lateral flow devices (LFD) for the detection of ASFV antigen or antibodies in the comparative trial using ASFV "Estonia 2014". To assess both sensitivity and specificity, all samples were incorporated, irrespective of the sampling day (0 to 10) and the anticipated outcome.

2.5.1. Lateral Flow Devices for ASFV Antigen Detection Have Limitations but Yield Positive Results in the Clinical Phase

The antigen LFD was assessed with EDTA blood and serum as sample matrix, and the results were compared with the outcome of standard qPCRs. At 4 DPI, only one domestic pig showed a questionable LFD result using serum and a positive LFD result using EDTA blood. The reactive animal was also the one with the highest genome loads (691 copies per run in serum, 1.5×10^5 copies in EDTA blood). In the phase of overt disease, at 7 DPI, almost all samples gave positive LFD results using either serum or EDTA blood of domestic pigs or wild boar. One domestic animal showed a negative LFD result when using EDTA blood (but a positive result with serum). The negative result was not linked to a significantly lower content of viral genome although it was in the EDTA blood taken that day (5.6×10^4 copies per run). At 10 DPI, all serum samples were found positive with weaker results that corresponded in the majority of cases with lower genome copy numbers in qPCR. When taking EDTA blood as a matrix, two domestic pigs were found negative. These animals were the ones with rather the lowest genome copy numbers. However, these copy numbers were much higher than for positive sera. A summary of visual results and their interpretation is presented in Supplementary Figure S4 and Table S2.

The attempt to optimize the outcome for EDTA blood samples through freeze-thawing or dilution in distilled water did not yield better results.

2.5.2. ASFV Antibody Lateral Flow Devices Show Promising Results with Samples Taken from Recovering Animals

The antibody LFDs were also used with both EDTA blood and serum. The results were compared to three commercial antibody enzyme-linked immunosorbent assays (ELISA) that are routinely used in the laboratory. Moreover, indirect immunoperoxidase tests were used for final confirmation.

All samples taken at 4 DPI and 7 DPI were found negative in all assays applied for antibody detection, including the indirect immunoperoxidase test. At 10 DPI, all three domestic pigs showed positive LFD results when using serum as sample matrix (see Supplementary Figure S5). These results corresponded to positive results in all ELISA assays (see Supplementary Table S3). Two of these animals were also positive when applying EDTA blood. The later results corresponded to positive or questionable results in all ELISAs. The remaining domestic pig showed a negative result with EDTA blood. However, the same animal showed positive or questionable results in the ELISA tests. The questionable results were found in an indirect ELISA format. The wild boar showed a more heterogeneous reactivity. Testing serum, a negative, a weak positive, and a questionable result were obtained. There was also heterogeneity in ELISA results (see Supplementary Table S3) with highest positive rates in competitive formats. Using EDTA blood, two weak positive and one negative result were obtained. Also with this sample matrix, higher heterogeneity was observed in the ELISA, and negative results were obtained in the indirect format (see Supplementary Table S2). The overall results of antibody detection corresponded to the observation that the domestic pigs were already recovering at 10 DPI while the wild boar were still showing signs of disease. The indirect immunoperoxidase test was positive for all animals sampled at day 10 confirming their status as positive.

3. Discussion

Because of its impact on animal health and pig industry, ASF is considered as one of the most important viral diseases of domestic pigs and wild boar. In the absence of

commercial vaccines or treatment options, timely detection and implementation of control measures is of utmost importance [22]. The clinical manifestation of ASFV infection is usually most severe in domestic pigs and Eurasian wild boar [24]. However, most signs are highly unspecific and therefore, laboratory diagnosis is mandatory to confirm any clinical suspicion [25].

Over the past decade, the disease has gone pandemic and has reached not only the world's largest pig producer [26] but also several other countries with considerable pig production in both Asia and Europe. An additional layer of complexity is added through the involvement of wildlife with wild boar as a reservoir in several European countries [27]. Surveillance activities in pig-dense areas can mean tremendous sample numbers and optimization of diagnostic workflows is of utmost importance to direct human and financial resources in a senseful manner, especially in times of other pandemic diseases of high significance that also demand diagnostic resources. In this context, limitation to one single sample matrix for *intra vitam* laboratory diagnosis has been discussed and one of the favored matrices under Central European conditions would be serum. In Germany and other Central European countries, collection of native blood from hunted wild boar has its roots in classical swine fever surveillance and was also applied for domestic pigs. Serum is a rather robust matrix that can be put on automated extraction and ELISA systems, and is suitable for all direct and indirect swine fever tests (both African and classical swine fever). Apart from being suitable for all antibody detection methods, inhibitory effects in qPCR are lower in serum than in anticoagulated blood [28]. Quality can be an issue when sampling is performed by hunters, but this also applies to other sample matrices. Against this background, we tested the suitability in the early, clinical, and later phase of ASFV infection in comparison with EDTA blood. Given the fact that ASFV has usually hemadsorbing capacities and is attached to erythrocytes [29,30], it is not surprising that there is a significantly higher load of viral genome in EDTA bloods samples. Yet, our experience from previous trials showed that serum was comparable in overall diagnostic sensitivity as long as clinically diseased animals were sampled (unpublished data accompanying the study reported by Gabriel et al. [31]). Here, animals in the early, pre-clinical phase, animals showing almost no obvious signs of disease, and animals that were showing first signs of recovery were included. With these samples, serum got to its limits and considering our results, we could not recommend using serum for the screening of apparently healthy animals (e.g. in restriction zones). Especially when planning to use any pooling of samples, false negative results have to be expected. As a consequence, the German official method collection for notifiable diseases was amended regarding the sample matrix for ASF diagnosis in animals without obvious clinical signs or lesions. Taking EDTA blood as the standard matrix may require some optimization regarding PCR inhibition [28] and use of certain extraction methods in larger settings. For passive surveillance, serum is probably fit for purpose. In only one of our samples taken at 7 DPI or later, results got close to the detection limit of the PCR. This one animal was also negative in several other matrices and was only picked up reliably in spleen and blood. It should be also kept in mind that the moderate virulence of some of the virus strains used in our experiments could have influenced assay sensitivity in the early phase. Comparing trials with ASFV "Armenia08" and "Estonia 2014", there is roughly a ten-fold lower genome load in the early phase. An advantage of serum is definitively the suitability for virus isolation. Toxic effects and contamination are seen much less frequently with serum than with organ samples or blood.

Regarding tissue samples, all matrices recommended by the diagnostic manuals of the World Organization for Animal Health (OIE) [32] or the EU [33] gave reliable results with highest viral genome loads in spleen, lung, and liver, as expected for a virus that replicates in myelomonocytic cells including circulating monocytes and tissue macrophages [34,35]. However, endothelial cells [35], megakaryocytes [36], and parenchymal cells like hepatocytes [35] among others, also proved to be permissive for ASFV which is also reflected by the outcome of the tissue comparison. Quite surprisingly, tonsil samples were less

homogeneous, especially in the early phase of the infection. This is contradictory as the tonsil is one of the primary replication sites [37]. It cannot be excluded that the texture of the sample, i.e., the coarse nature, and our decision to test in a diagnostic manner without biological replicates led to poor homogenization and release of less viral nucleic acids for extraction. In this respect, spleen, lung, and liver were easiest to work with. The salivary gland was taken into the set of samples under the assumption that shedding through saliva would be accompanied by the presence of viral genome in the gland tissue. Considering our results of high variability and rather low genome loads, the salivary gland will remain a matrix for scientific studies targeting shedding of ASFV.

The sample matrices described above are routine for veterinary practitioners or pathologists. However, if passive surveillance is the most important tool for early detection of ASF [3,4,7], alternative samples may be better suited, especially for carcasses. In the European Union, the Commission implementing decisions [38] direct the sampling toward fallen animals that occur in a farm. In this context, samples that could be taken without the need to open the body cavities of the carcass would be beneficial in terms of environmental contamination. For this reason, we investigated inguinal lymph nodes, ocular fluid, and ear punches, especially for the domestic pig setting. The inguinal lymph node gave rather reliable results as can be expected from this tissue type. However, variability was high, and in the early phase, genome loads close to the limit of detection were observed. An explanation could be that the virus was not yet distributed to peripheral sites. However, this would not be in line with the antigen detection in popliteal lymph nodes in the same study [39] and thus, sampling error, i.e., inclusion of fatty or connective tissues of the inguinal region, cannot be completely excluded. Ocular fluid was difficult to sample and genome loads were low. In our hands, this matrix was not practicable. Ear punches of clinically diseased animals were positive for viral genome which is in line with recent findings that also show the skin yielded positive results when testing wild boar carcasses [40] or experimentally infected animals [17]. However, the low level of viral genome and the quite difficult handling does not make this matrix an alternative candidate for routine settings.

Over the last years, our group has validated blood swabs as an alternative matrix for passive surveillance, especially in wild boar [11,12,23]. Only recently, the approach was also put to field practice when ASF entered Germany, and it performed well [40]. As optimization is always possible, and new development have been put on the market, we include a new type of swab and transport buffer into our comparison. The PrimeSwab and the accompanying PrimeStore MTM lived to our expectations and performed best in the comparison. This system has been evaluated using both bacterial and viral pathogens, including SARS-CoV-2 and is intriguing because of the safe inactivation of pathogens and preservation of nucleic acids [41–44]. Whether it is worth using this system or its sequels (PrimeStore HCP) instead of simple swab systems, remains the choice of users based on risk assessment, integration into strategies, and financial resources.

In summary, our results add to the data body that alternative sample matrices could be considered. Among the published options that were not further followed up in the presented study are oral fluids, faecal samples, and swabs as well as meat juices. While shedding will depend on the virulence of the isolate [45], most secretions and excretions will be positive for ASFV genomes in the clinical phase [17]. When it comes to antibody detection, oral fluids were shown to work with a slight delay in detection [46] and faeces worked in principle but with high limitations [47]. Meat juice has proven to be a good matrix for the sero-surveillance of bacterial, protozoal, and viral diseases. With certain limitations, this also applies to the detection of ASFV- and ASFV-specific antibodies [18,48].

Our last focus was on the lateral flow assays that could aid diagnosis in resource-limited areas or help with rapid results during epidemiological investigations. In a nutshell, performance of antibody lateral flow devices was again rather comparable to ELISAs and the promising results that are published for similar assays could be confirmed [49]. Nevertheless, antibody detection might not be the most important part for ASFV point-of-care approaches. In the latter context, antigen detection would be the key focus. Our results

showed that clinically diseased animals have a fair chance of being positive for viral antigen in the LFD. However, sensitivity is low and a negative result would need confirmation if signs or epidemiological settings would suggest ASF. Overall, further testing under field conditions is needed to conclude on the acceptability of ASFV antigen LFDs under different conditions.

In summary, routine matrices performed best, but some alternative sample matrices deserve attention and could be part of well-designed surveillance strategies. So far, lateral flow devices for antigen detection require careful use and further investigations.

4. Materials and Methods

4.1. Experimental Design

The study comprised defined sample materials from domestic pigs and wild boar that were collected during an animal experiment where the animals had been oro-nasally inoculated with 2×10^5 hemadsorbing units 50% (HAU) of ASFV "Estonia 2014". This genotype II strain originates from Estonian wild boar [50] and shows moderate virulence [51] with a tendency of more severe disease courses in wild boar. For this reason, the strain was chosen for a comparative study on clinical outcome and pathology that was recently published by Sehl et al. [39]. Another aim of the study was generation of well-defined sample matrices for diagnostic test validation. The samples presented here were taken from nine domestic pigs and nine wild boar that were sequentially euthanized at 4, 7, and 10 DPI. Two domestic pigs and three wild boar were included as negative controls and were euthanized at 0 DPI. The sample set comprised EDTA anticoagulated blood, plasma, serum, spleen, tonsil, mandibular and inguinal lymph nodes, bone marrow, lung, liver, salivary gland, and the ear. The blood samples were additionally used to generate swab samples using different devices, the ear was used to create punches with commercial ear-tag tongs (see below).

To complete the sample set for this study, further samples from different animal experiments were analyzed (see Supplementary Table S1): (1) samples from four wild boar and five domestic pigs that had been oro-nasally inoculated with 2×10^5 HAU of ASFV "Belgium 2018/1". This ASFV strain belongs also to the p72 genotype II showing high virulence in both species. In this study, samples were collected at the humane endpoint before euthanasia (between 8 DPI and 10 DPI). The sample set comprised EDTA anticoagulated blood, plasma, serum, spleen, tonsil, lymph nodes, bone marrow (wild boar only), lung, liver (domestic pigs only), salivary gland (wild boar only), kidney (domestic pigs only), intraocular fluid (domestic pigs only) and the ear. (2) Samples taken from domestic pigs intramuscularly inoculated with different African ASFV isolates that were kindly provided by Dr. Christopher Netherton (The Pirbright Institute, Pirbright, UK), i.e., five animals inoculated with 10 HAU of genotype IV strain "RSA W1/99" (South Africa [52]) and euthanized 8 DPI, five animals inoculated with 10 HAU of genotype XII strain "MFUE 6/1" (Zambia [52]) and euthanized at 7 DPI, five animals inoculated with 10 HAU of genotype XIX strain "CHZT 90/1" (Zimbabwe) and euthanized 7 DPI, three animals inoculated with 1000 HAU of genotype XI strain "KAB 6/2" (Zambia [52]) and sampled 8 DPI, and three animals inoculated with 1000 HAU of genotype XIII strain "SUM 14/11" (Zambia [52]) and sampled 8 DPI. The sample set for these additional animals comprised EDTA anticoagulated blood, serum, spleen, tonsil, lymph nodes, lung, liver, kidney, intraocular fluid (aqueous humour) and the ear. The clinical score which defined human endpoints was determined using the protocol described in Pietschmann et al. 2015 [53] with slight modifications.

All domestic pigs were bought from commercial pig farms and were clinically healthy upon arrival. The wild boar originated from different game parks and were purchased in healthy condition. All animals were tested negative for ASFV- and ASFV-specific antibodies prior to enrolment in the studies.

The initial animal experiments for strain characterization and reference material collection were approved by the competent authority (LALLF, Rostock, Germany) under reference number 7221.3-2-011/19.

4.2. Processing of Samples and Preparation of Swabs

From the first animal trial where the animals (domestic pigs and wild boar) had been inoculated with ASFV "Estonia 2014", samples were taken for both pathogen and antibody detection including preparation of swabs and use with lateral flow devices. Serum was obtained from native blood samples through centrifugation for 20 min at 2031× *g* at room temperature and was stored together with aliquoted EDTA blood samples at −80 °C until further usage. To obtain plasma for confirmatory testing, separate EDTA blood aliquots were centrifuged as described above.

Tissue samples from all animal trials were collected and aliquoted during necropsy and stored at −80°C prior to further use. For the ear punch samples, ears were punched with the FlexoPlus R ear tagging system (Caisley, Bocholt, Germany). In preparation of nucleic acid extraction, all tissue samples were homogenized for 3 min at 30 Hz in 1 mL phosphate-buffered saline (PBS) with a metal bead using a TissueLyser II (Qiagen®GmbH, Hilden, Germany).

Three types of commercial swabs were used to generate blood swabs: (1) plain cotton swabs (Rayon, COPAN, Brescia, Italy), (2) GenoTube Livestock Swabs (Thermo Fisher Scientific, Waltham, MA, USA), and (3) PrimeSwabs (Longhorn, Vaccines and Diagnostics, San Antonio, TX, USA). GenoTube Livestock swabs are equipped with a collection tube that rapidly dries the sample to avoid degradation of nucleic acids. The PrimeSwab is a flocked swab and compatible with PrimeStore Molecular Transport Medium (MTM). This MTM (Longhorn, Vaccines and Diagnostics), is a buffer based on guanidine thiocyanate that provides for virus inactivation and nucleic acid stabilization upon transport and storage.

All swab types were directly dipped into vials of whole blood upon or shortly after sampling. The COPAN cotton swabs (Rayon, COPAN, Brescia, Italy) and the GenoTube Livestock (Thermo Fisher Scientific, Waltham, MA, USA) devices were placed back into their receptacles, and the PrimeSwabs (Longhorn, Vaccines and Diagnostics, San Antonio, TX, USA) were placed into PrimeStore MTM (Longhorn, Vaccines and Diagnostics, San Antonio, TX, USA) tubes. All samples were then stored at room temperature for five days prior to further processing to mimic transfer of samples from the field to the laboratory. After storage, small pieces (2.5 mm in diameter) were excised from all blood swabs with sterile scissors and processed like tissue samples. In addition, the PrimeStore MTM buffers, in which the PrimeSwabs had been submerged, were used for nucleic acid extraction following the protocol for fluid samples.

4.3. Detection of Viral DNA

Blood, serum, swab, and tissue samples were extracted with the NucleoMag®VET kit for Viral RNA/DNA isolation (MACHEREY-NAGEL, Düren, Germany) on a King-Fisher®extraction platform (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. Thereafter, nucleic acids were subjected to the OIE recommended ASFV-specific qPCR according to King et al. [54] with slight modifications. All PCRs were performed using a Bio-Rad C1000^{TM} thermal cycler (BIO-RAD, Hercules, CA, USA), with the CFX96^{TM} Real-Time System of the same manufacturer. Results of qPCR were initially recorded as quantification cycle (cq) values. Using a dilution series of a full-virus ASFV DNA standard, the genome copies in the respective samples were estimated. For generation of the ASFV standard, DNA from an ASFV "Armenia08" macrophage culture supernatant was extracted using the QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations. Subsequently, the DNA concentration was determined by spectrophotometry using a Nanodrop 2000c (Thermo Fisher Scientific, Waltham, MA, USA) and the exact number of DNA molecules was calculated with an online tool (http://www.molbiol.edu.ru/eng/scripts/0107.html). Small standard aliquots were stored at −20 °C and thawed not more than five times. The standard was meant to compare the magnitude of viral DNA content rather than giving exact genome copy numbers.

4.4. Detection of ASFV-Specific Antibodies

Serum samples were tested in commercially available ELISAs for the presence of ASFV p72-specific antibodies using the competitive INGEZIM PPA COMPAC ELISA (Ingenasa, Madrid, Spain), for p32-specific antibodies in the ID Screen ASF Competition ELISA (IDVet, Grabels, France), and for antibodies against p32, p62, and p72 using the ID Screen ASF Indirect (IDVet, Grabels, France) Kit according to the manufacturer´s instructions. All serum samples were tested in duplicate. To obtain a value that could be compared to the antibody LFD result using EDTA blood, this matrix was screened in single runs following the protocols provided for serum or plasma.

For confirmatory purposes, all serum and plasma samples were tested in an indirect immunoperoxidase test according to the standard protocols provided by the European Reference Laboratory for ASF with slight modifications regarding the virus strain (standard operating procedure last accessed at SOPs (asf-referencelab.info) on 30 December 2020).

4.5. Pen-Side Tests

For pen-side antigen detection, the LFD INgezim ASF CROM Ag (11.ASF.K42, Ingenasa, Madrid, Spain) was used with EDTA blood and serum, following the manufacturer's instructions. In an attempt to optimize the outcome for EDTA blood samples with negative LFD result but high viral genome load, freeze-thaw cycles and dilution 1:1 in distilled water were attempted for all samples.

The corresponding LFD INgezim PPA CROM Ab (11.PPA.K.41, Ingenasa, Madrid, Spain) was used on serum and EDTA samples for detection of antibodies against ASFV p72.

4.6. Statistical Analysis

Initial data recording and analyses (comparison of mean values, transformation of values) were done using Microsoft Excel 2010 (Microsoft Germany GmbH, Munich, Germany).

GraphPad Prism 8 (Graphpad Software Inc., San Diego, CA, USA) was used for further statistical analyses and graph creation. Statistically significant differences were investigated by paired (for samples taken from the same animal but investigated by different means) or unpaired t-tests (comparison among animals). Statistical significance was defined as $p < 0.05$ and indicated with an asterisk (*), $p < 0.01$ was indicated with two asterisks (**).

Supplementary Materials: The following are available online at https://www.mdpi.com/2076-0817/10/2/177/s1. Figure S1: Detection of ASFV genome by qPCR in blood and organ samples. Figure S2: Comparison of genome copy numbers in different swabs and swab buffers. Figure S3: Comparison of genome copy numbers in alternative sample matrices and standard samples (spleen and EDTA blood). Figure S4: Impressions of the lateral flow devices for the detection of ASFV antigen. Figure S5: Impressions of the lateral flow devices for the detection of ASFV-specific antibodies. Table S1: Overview of the sample set. Table S2: Comparison of antigen LFD and qPCR for detection of ASFV. Table S3: Comparison of antibody LFD with ELISAs and an indirect immunoperoxidase test.

Author Contributions: Conceptualization, M.B., S.B.; methodology, J.P. and S.B.; formal analysis, J.P. and S.B.; investigation, J.P., P.D., H.R., M.F., and S.B.; resources, M.B. and S.B.; data curation, J.P., P.D., and S.B.; writing—original draft preparation, J.P. and S.B.; writing—review and editing, S.B. and M.B.; visualization, J.P. and S.B.; supervision, S.B. and M.B.; project administration, S.B.; funding acquisition, S.B. All authors have read and agreed to the published version of the manuscript.

Funding: This publication is partly based upon work from COST Action 15116 ASF-STOP supported by the European Cooperation in Science and Technology and was partially funded by the FLI ASF research network.

Institutional Review Board Statement: In the animal experiment, all applicable animal welfare regulations including EU Directive 2010/63/EC were taken into consideration. The animal experiment was externally approved by the competent authority (Landesamt für Landwirtschaft, Lebensmittelsicherheit und Fischerei (LALLF) Mecklenburg-Vorpommern, Rostock, Germany) under reference number 7221.3-2-011/19.

Informed Consent Statement: Not applicable

Data Availability Statement: The data that support the findings of this study are available from the corresponding author upon reasonable request.

Acknowledgments: The authors would like to thank all animal caretakers, especially Matthias Jahn, Steffen Brenz, Dominique Lux, and Christian Loth for their dedicated work and help wherever needed. We also acknowledge our technicians Ulrike Kleinert and Robin Brandt who were involved in this study for their excellent work. We would also like to thank Carola Sauter-Louis for advice and help with statistical analyses, and Christopher Netherton for providing the African ASFV isolates. Finally, we acknowledge Jan-Hendrik Forth for helping in the final revision of the manuscript.

Conflicts of Interest: The authors declare no conflict of interest.

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5.6 Lateral flow assays for the detection of African swine fever virus antigen are not fit for field diagnosis of wild boar carcasses

Paul Deutschmann, Jutta Pikalo, Martin Beer, Sandra Blome

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

Transboundary and Emerging Diseases

2021

doi.org/10.1111/tbed.14248

Received: 28 April 2021 | Revised: 3 July 2021 | Accepted: 18 July 2021

DOI: 10.1111/tbed.14248

Transboundary and Emerging Diseases WILEY

ORIGINAL ARTICLE

Lateral flow assays for the detection of African swine fever virus antigen are not fit for field diagnosis of wild boar carcasses

Paul Deutschmann | Jutta Pikalo | Martin Beer | Sandra Blome

Institute of Diagnostic Virology, Friedrich-Loeffler-Institut, Greifswald, Germany

Correspondence
Sandra Blome, Friedrich-Loeffler-Institut, Institute of Diagnostic Virology, Suedufer 10, Insel Riems, 17493 Greifswald, Germany.
Email: sandra.blome@fli.de

Funding information
The study received funding by the FLI-internal ASF Research Network.

Abstract

African swine fever (ASF) is one of the most important viral diseases of domestic pigs and wild boar. Apart from endemic cycles in Africa, ASF is now continuously spreading in Europe and Asia. As ASF leads to severe but unspecific clinical signs and high lethality, early pathogen detection is of utmost importance. Recently, 'point-of-care' (POC) tests, especially immunochromatographic assays, have been intensively discussed for the use in remote areas but also in the context of on-farm epidemiological investigations and wild boar carcass screening. The later topic was the starting point for our present study. In detail, we evaluated the performance of the commercially available INGEZIM ASFV CROM Ag lateral flow assay (Eurofins Technologies Ingenasa) with selected high-quality reference blood samples, and with blood samples from wild boar carcasses collected under field conditions in Germany. While we observed a sensitivity of roughly 77% in freeze-thawed matrices of close to ideal quality, our approach to simulate field conditions in direct testing of blood samples from carcasses without any modification, resulted in a drastically reduced sensitivity of only 12.5% with the given sample set. Freeze thawing increased the sensitivity to roughly 44% which mirrored the overall sensitivity of 49% in the total data set of wild boar carcass samples. A diagnostic specificity of 100% was observed. In summary, the antigen LFA should not be regarded as a substitute for any OIE listed diagnostic method and has very limited use for carcass testing at the point of care. For optimized LFA antigen tests, the sensitivity with field samples must be significantly increased. An improved sensitivity is seen with freeze-thawed samples, which may indicate problems in the accessibility of ASFV antigen that could be overcome, to a certain extent, with assay modifications.

KEYWORDS
African swine fever virus, antigen lateral flow assay, point-of-care test, sensitivity, specificity

1 | INTRODUCTION

African swine fever (ASF) is caused by African swine fever virus (ASFV), a large double-stranded DNA virus, and the sole member of the genus *Asfivirus* within the *Asfarviridae* family (Alonso et al., 2018). African swine fever usually causes an exceptionally high lethality in domestic pigs and Eurasian wild boar and is a notifiable disease according to the World Organization for Animal Health (OIE). Following its introduction to Georgia in 2007, ASFV spread successively through neighboring countries in the Trans-Caucasian region to several parts of Europe and Asia (Dixon et al., 2020). Since the virus reached China in 2018 (Zhou et al., 2018), millions of pigs were culled and effects on the global pork market were severe. Within the European Union, the abundant wild boar population was most severely affected and plays a major role in the epidemic (EFSA et al., 2018). First cases of ASF in Germany in 2020 (Sauter-Louis et al., 2021) sent another shockwave through the pig industry, as trade restrictions on pork took hold even though only wild boar are affected until now. With neither treatment nor a licensed vaccine available to date, strategies to fight the disease have to rely solely on strict sanitary measures, an early and reliable diagnosis, and the culling of affected herds (Blome et al., 2020). For the wild boar situation, fencing, adapted hunting and hunting rest practices, trapping, incentives for carcass search and removal, as well as a general reduction of the wild boar populations have been implemented (Busch et al., 2021; Chenais et al., 2019).

With the effectiveness of disease control measures relying on a timely implementation after an outbreak (Sanchez-Vizcaino et al., 2012), and laboratory analysis being rather resource intensive, questions regarding the utility of point-of-care (POC) assays, possibly even to replace laboratory testing, have arisen. These tests could help diagnosing the disease in remote areas with scarce infrastructure and limited laboratory capacities but also aid epidemiological investigations on outbreak farms during the culling procedure. Moreover, screening of wild boar carcasses prior to their safe removal without the need of sophisticated laboratory diagnosis could save time and resources and was discussed on high level.

One of the assays that could suit these scenarios is the INGEZIM ASFV CROM Ag LFA (Sastre et al., 2016), commercialized by Eurofins Ingenasa. This immunochromatographic assay is designed to detect ASFV antigen in blood samples under field conditions and showed rather promising results in previous studies under laboratory conditions (Pikalo et al., 2020; Pikalo et al., 2021). Taking the question regarding wild boar carcass testing as a starting point, we aimed to assess the applicability with samples of reduced quality, although the assay was not originally designed for this purpose. A total set of 237 blood samples of different origins was therefore investigated. The results of the antigen LFA were compared to OIE listed qPCR diagnosis, and the resulting sensitivity and specificity were evaluated for assessment of the practicability of the on-site test under realistic conditions with real field samples.

2 | MATERIAL AND METHODS

2.1 | Sample origin

Eighty-six EDTA blood samples were obtained from recent animal trials conducted at the Friedrich-Loeffler-Institute (FLI) with domestic pigs, wild boar and minipigs infected with different ASFV strains of genotypes I, II or X (see Table S1). The animal trials for strain characterization and reference material generation were approved by the competent animal welfare authority under reference number 7221.3-2-011/19. Blood was aliquoted and promptly frozen after sampling, allowing high sample quality. Additionally, 11 blood samples of shot wild boar confirmed with ASF from the affected regions in Germany were included in the study. These animals were sampled immediately after death, also ensuring close to ideal matrix quality. Eighty blood samples originated from wild boar carcasses confirmed with ASF during the German outbreak (see Table S2). In addition, 60 negative field samples originating from shot wild boar of the same region were included. These field samples had been sent to the NRL for investigation between September 2020 and April 2021. Blood from carcasses and shot wild boar were mainly taken by local veterinary officers at the point-of-care and sent to the FLI (samples C17–C80 and negative field samples from shot animals), or was obtained during necropsy of the wild boar cadavers directly at the high containment facilities at the FLI (samples C1–C16, see Tables S2 and S3). Carcass-derived blood samples were 'field-like' in various stages of decomposition and impaired by clotting and/or autolysis. Field samples C17–C80, negative wild boar samples and samples H81–H91, which the NRL received prior to the start of the study or in which LFA testing could not immediately be conducted, were stored at −80°C before investigations. Samples C1–C16 could be obtained during necropsy and were tested before, and, for comparison, after freeze-thawing (see Table S3).

2.2 | Rapid tests

The Ingezim® ASFV CROM Ag (Eurofins Technologies Ingenasa) is a double antibody sandwich immunochromatographic assay for the detection of ASFV antigen in blood samples (Sastre et al., 2016). The test procedure was conducted according to the manufacturer's instructions with the exception of also including previously freeze-thawed samples in the study (see Tables S1 and S2). The outcome was interpreted either as positive or negative (see Figure 1). Only valid results were counted (appearance of the control line).

2.3 | Nucleic acid extraction and real-time PCR

Viral nucleic acids were extracted using the QIAamp® RNA Viral Mini Kit (Qiagen) according to the manufacturer's instructions. Subsequently, qPCR was conducted according to the protocol published by

FIGURE 1 Exemplary antigen LFA results. D2 shows a valid positive result, D1 shows a valid negative result

King et al. (2003) with slight modifications (addition of a heterologous internal control DNA), or with the commercial qPCR kits virotype ASFV or virotype ASFV 2.0 (Indical Bioscience) according to the manufacturer's instructions. All qPCR runs were performed on C1000™ thermal cyclers with the CFX96™ Real-Time System (Biorad). Results were recorded as quantification cycle (cq) values.

2.4 | Screening for ASFV-specific antibodies

The ID Screen ASF Indirect ELISA Kit (ID.vet) was used according to the manufacturer's instructions to screen the field samples for ASFV-specific antibodies. The multi-antigen indirect ELISA kit detects antibodies against ASFV p32, p62 and p72 in porcine serum, plasma or blood filter paper samples.

2.5 | Statistical analysis

Results of LFAs were evaluated in comparison to results obtained in qPCR. For this purpose, qPCR was regarded as the standard for pathogen detection. Accordingly, the outcome of the LFA was rated true positive (TP), true negative (TN), false positive (FP) or false negative (FN). Diagnostic sensitivity was calculated as $TP/(TP + FN) \times 100$. Diagnostic specificity was calculated as $TN/(TN + FP) \times 100$. Confidence intervals were calculated of share values.

3 | RESULTS AND DISCUSSION

To obtain a broader picture on assay performance, the study comprised experimental samples of high quality and the targeted blood samples of wild boar carcasses. Of the 97 blood samples of ideal or close to ideal quality (samples from animal trials and samples H81–H91, see Table S1), 79 were positive by qPCR. The INGEZIM ASFV CROM Ag LFA detected 61 positives, resulting in a diagnostic sensitivity of 77.2% [95% confidence interval (68%, 86%)]. No false positives occurred, hence 100% specificity was observed on this dataset. The performance was therefore in line with the study published by Sastre et al. (2016) where field samples of unimpaired quality were detected with roughly 67% sensitivity when compared to an OIE listed qPCR. Specificity was also close to 100%. With the ongoing circulation of ASF in European wild boar, however, virus detection in carcasses as initial test or screening prior to save removal, has become an important issue. Sample quality is then usually reduced due to decomposition effects, an aspect that has not yet been elucidated for the ASFV antigen LFA. All our 80 carcass-derived blood samples were obtained from ASF-positive wild boar and confirmed by qPCR with cq values ranging from 14 to 38 (see Table S2). Here, significant differences were observed between the samples that were previously frozen, and those that were not: in native samples tested without any modifications (C1–C16, $n = 16$, see Table S3), the LFA delivered only two positive results [sensitivity of 12.5% (0%, 25%)]. After freeze-thawing, testing of the same 16 samples in the LFA yielded seven positives [sensitivity of 43.75% (19%, 68%)]. Unexpectedly, one of the samples that had yielded a positive result in the native context was now tested negative. The increase of overall positive results is in accordance with the sensitivity of 48.75% (38%, 60%) we observed in all of the previously freeze-thawed carcass-derived samples (C1–C80), where 39 positives were detected by the LFA (see Table S2). Interestingly, however, we did not observe a better sensitivity after freeze-thawing in EDTA-blood samples of high quality in a previous study by our group (Pikalo et al., 2021). The positive effect of freeze-thawing is probably due to the fact that most of the virus in blood is associated with erythrocytes (Wardley & Wilkinson, 1977), and therefore, the destruction of blood cells during freeze-thawing results in a higher antigen availability for detection in the test, a process especially effective when erythrocytes are bound to clots in samples of reduced quality. No false positive reactions occurred with any sample types.

In our study, the INGEZIM ASFV CROM Ag assay could not deliver reliable results with native blood from carcasses. Particular samples with cq values as low as 15 (C4, see Table S3), indicating a considerable virus load in the carcass, still delivered negative results in the LFA.

While we observed increased sensitivity after erythrocytolysis by freeze-thawing (12.5% vs. 44% sensitivity, samples C1–C16; see Table S3), for the practical implementation of the assay in the field, of course, freezing cannot be an option due to the technical requirements not fitting a point-of-care application. Possible alternatives to freeze-thawing for erythrocytolysis could be the dilution of blood in *aqua dest.* or lysis buffer. On a very limited dataset ($n = 4$) that does not allow for statistical significance, hypotonic lysis seemed to improve the results (3 FN native, 1 FN after water lysis, no FN after freeze-thawing; data not further shown). While both methods could be feasible under field conditions, the effects of this deviation from the manufacturer's instructions on the assay should be elucidated and could be the basis of future optimization of the assay. After all, it must be noted that even with erythrocytolysis through freeze-thawing, we could only achieve a sensitivity of roughly 50% in carcass-derived samples, a value not fit for purpose. Considering the negative impact of immune-complexes, samples were screened for the presence of antibodies. Only seven samples were positive for ASFV-specific antibodies and three delivered doubtful results in the antibody ELISA (see Tables S1 and S2). Eight of these samples were positive and two were false negative in the antigen-specific LFA

(see Tables S1 and S2). While the small number does not allow for evaluation of possible interference, the principal functionality of the test in the presence of antibodies is indicated.

In general, the LFA was more reliable using samples with cq values below 30, indicating a rather high viral load. Of those samples derived from animal trials ($n = 64$), 56 were true positive according to the rapid test, resulting in a sensitivity of 87.5% (79%, 96%) in that group. This goes along with observations in a previous study performed in our group, when the LFA was most sensitive during the clinical phase of ASF, at the peak of viral replication (Pikalo et al., 2021). In the present study, however, it was observed that the influences of clotting and decay in the carcass-derived samples seemed be able to outweigh the effects of higher viral loads, since here no clear correlation even with very low cq values and positive results in the LFA was observed (see Table S2).

Taking into consideration the differences between the highly amplifying qPCR and native antigen detection by LFA, the marked lower sensitivity in the later is to be expected. Still, the possibility for point-of-care testing holds a considerable advantage and on-site assays can provide a valuable additional diagnostic tool under certain circumstances. An acceptable sensitivity of the LFA was confirmed during the clinical phase of the disease, when fresh samples can be obtained from live animals or immediately after death. Here, the application of a rapid test could be of value in domestic pig holdings, when ASF is clinically suspected and live animals can be picked for sampling (given a careful interpretation of negative results in the LFA and still immediate initiation of laboratory diagnosis). Furthermore, epidemiological investigations can benefit from antigen assays for the on-site analysis of infected populations, when weaknesses in sensitivity are considered. However, with a sensitivity of roughly 50%, or even well below when no erythrocytolytic procedure is applied as proposed by the manufacturer's instructions, our findings imply that the LFA has only very limited use for antigen detection in blood from carcasses after extended *post-mortem* intervals. When resources are scarce and prioritization of diagnostic workflows is needed, the high specificity may allow for positive on-site results in the LFA to surrogate a laboratory confirmation. Under field conditions, pre-testing could help directing resources, for example, succession of removal, treatment of surroundings, or disinfection measures. Negative results, however, must always be interpreted with high caution due to the low sensitivity we observed in samples of reduced quality. OIE listed methods such as qPCR remain the only safe and proven methods for the unreserved detection of an ASFV infection. Therefore, the on-site assay should be regarded as a complimentary option rather than a substitute to laboratory diagnosis for carcass testing.

AUTHOR CONTRIBUTIONS

Conceptualization: PD and SB. Data curation: PD and JP. Funding acquisition: SB and MB. Investigation: PD. Methodology: PD, JP and SB. Visualization: PD. Writing – original draft: PD and SB. Writing – review & editing: MB. All authors have read and agreed to the published version of the manuscript.

ACKNOWLEDGEMENTS

The authors would like to thank Ulrike Kleinert and Robin Brandt for their excellent technical assistance.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ETHICS STATEMENT

The authors confirm adherence with the journal's ethical statements as noted in the journal's authors guideline page. Blood samples listed in Table S1 came from animal experiments conducted at the FLI, which were performed in accordance with EU Directive 2010/63/EC and approved by the competent authority (Landesamt für Landwirtschaft, Lebensmittelsicherheit und Fischerei (LALLF) Mecklenburg-Vorpommern).

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID

Paul Deutschmann https://orcid.org/0000-0002-2717-1358
Sandra Blome https://orcid.org/0000-0001-5465-5609

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

How to cite this article: Deutschmann, P., Pikalo, J., Beer, M., & Blome, S. (2022). Lateral flow assays for the detection of African swine fever virus antigen are not fit for field diagnosis of wild boar carcasses. *Transboundary and Emerging Diseases*, *69*, 2344–2348. https://doi.org/10.1111/tbed.14248

6. Discussion

African swine fever is present in Germany since September 2020 (Sauter-Louis et al., 2020). The virus caused suffering and death of thousands of wild boar and domestic pigs and cost millions of Euros for eradication efforts. While in the German domestic pig sector, point incursions have occurred and were contained, the situation in wild boar is characterized by a persistent wave of new cases along the Polish border, with a trend to a westward spread in some regions. In this situation, the available measures to prevent and fight the disease, i.e. increased biosecurity, intensified wild boar hunting, laboratory diagnosis for early detection and, after the introduction, fencing, culling and timely removal of wild boar carcasses within the core restriction zones, were able to limit the further spread of the virus. However, as of yet, complete eradication was only successful in very limited areas in Germany and we have not been able to contain the epizootic from a cross-regional perspective. The same applies to the global ASF situation, since most countries affected by the recent ASFV panzootic have not been able to eliminate the disease. These experiences from the recent years imply that we need new and improved eradication measures for the situation we are facing in wild boar, but also for domestic pig holdings with low biosecurity.

Towards market authorization of an ASFV vaccine candidate

The call for a licensed vaccine against ASFV is louder than ever, rooted in the hope that a vaccine is the missing additional tool to regain the upper hand over the disease (Muñoz-Pérez et al., 2021). Meanwhile, the clock for vaccine development is ticking, as millions of pigs have already died from ASF (L. K. Dixon et al., 2020; You et al., 2021), putting farmers' livelihoods and people's food supply at risk. At the same time, the disease causes suffering for wild boar and threatens some rare wild suid populations with extinction (Ewers et al., 2021; Luskin et al., 2021). In recent time, big advances in ASFV vaccine development were reported, but the path towards licensing in Europe is still long even for the most promising ASF vaccine candidates.

In 2022, Vietnam was the first country to commercially license a vaccine against ASF (Borca et al., 2020). This is exciting news, but for the European situation, the candidate lacks the full extent of comprehensive characterization required by the EMA for consideration of licensing. In addition,

the vaccine is still produced on primary cells, hampering standardization and constituting a potential safety risk. The use of the vaccine was temporarily suspended in August 2022 due to an increased occurrence of deaths in the vaccinated pig population with unclear connection to the vaccine (www.reuters.com/world/asia-pacific/vietnam-suspends-african-swine-fever-vaccine-after-pig-deaths-2022-08-24/, visited August 28th 2022). And finally, the Vietnam-licensed product is designed for intramuscular use in domestic pigs, which may not fit for the German situation. High standards of biosecurity here provide an effective protection of pigs against wide-spread ASF and other diseases, even without vaccination. Trade restrictions on meat from vaccinated pigs are to be expected, so in conclusion immunization strategies against ASF in Germany should probably focus on the wild boar situation.

For wild boar, oral application via baits appears to be the only feasible administration route (Blome et al., 2020) and the availability of an oral vaccine against ASF enables other promising applications. One example is the conservation context in Asia, where we also need to protect the endangered wild pig species from ASF (Ewers et al., 2021). And further, in the backyard farm setting in eastern Europe, oral vaccines could be directly distributed to farmers, forgoing without professional veterinary staff and bypassing a likely bottleneck in regions with a poor infrastructure. Similar prospects were previously discussed for CSF (Dietze, Milicevic, & Depner, 2013).

Classical swine fever was the European example where oral vaccination was successfully used to fight a disease in the wild boar population. The oral vaccine against CSF containing the C-strain (Kaden, Lange, Fischer, & Strebelow, 2000) was genetically safe, induced close to sterile immunity which was maintained over sufficiently long periods of time to eventually assist in the successful eradication of CSF from wild boar in Germany (Blome, Moss, et al., 2017; Rossi et al., 2015). These experiences raise hopes for the success of future ASFV oral vaccination strategies, however we should be cautious to define our expectations on ASFV vaccines for wild boar based on this model. While EMA defines clear requirements on the characteristics of licensable vaccines, a benefit-risk analysis within these requirements is needed to evaluate which candidate could be suitable for commercialization.

“ASFV-GΔMGF” is one of the most promising vaccine candidates and was previously shown to induce full protection with very low residual vaccine or challenge virus replication. In the studies included in this work, we took the vaccine candidate beyond the proof-of-concept phase and towards more profound characterization that will provide a basis for this benefit-risk analysis to consider licensing.

We could confirm the full efficacy of the vaccine after cultivation in primary macrophages for intramuscular application in domestic pigs in an independent experimental setup. This is important since experiences with ASFV have shown that clinical course, and, consequently, responses after challenge infection can vary depending on the experimental conditions and animals (J. Pikalo et al., 2020; Radulovic et al., 2022). Therefore, reproducibility of auspicious results under different experimental setups is key for the early evaluation of promising ASFV vaccine candidates. However, the necessity for cultivation of this live vaccine on primary cells depicts a major pitfall for commercialization in Europe, technically because of the need to up-scale vaccine production, but also because of legislative requirements on good manufacturing practice and standardization. We addressed this issue by administering “ASFV-GΔMGF” after passage in a commercial immortalized cell line in an additional animal experiment, observing the same experimental outcome and high genomic stability, so it can be concluded that large-scale production as a requisite of future commercialization is feasible.

Differences were observed with the oral vaccination in comparison to intramuscular vaccination, however. Our results indicate that the effectiveness of the single-dose oral vaccination is lower than single dose (O'Donnell, Holinka, Gladue, et al., 2015) or two dose intramuscular vaccination (see above). This is somewhat expected and was also seen with oral vaccine candidates against CSF (Feliziani et al., 2014). It is important to underline that the inoculation route was a proof of concept and no baits were applied for oral administration yet, cell culture supernatant was delivered directly to the animals. In our study, all animals with detectable replication of “ASFV-GΔMGF” and seroconversion were fully protected against challenge infection, indicating that efficiency of oral delivery rather than vaccine efficacy should be the issue to address in the design of an oral immunization campaign. Here, characteristics of the vaccine candidate must be integrated into the campaign design. Our results indicate that a single dispense of baits would

probably lead to an insufficient proportion of immune animals within the population and multiple dispenses are necessary. Similar experiences came from the vaccination of wild boar against CSF, as here the best results in the field were achieved with three double vaccinations in spring, summer and autumn (Kaden et al., 2002; Kaden & Lange, 2004). In such a vaccination scheme, possibly unsuccessful single vaccine uptake may be tolerable, and repetitive uptake of baits could in the end likely facilitate a successfully immunized population, a prospect that should be addressed in future studies. At the same time, the issue of vaccine safety after overdose if multiple baits are taken by animals in the field must be addressed. There is, however, no indication that higher doses of "ASFV-GΔMGF" would be harmful (O'Donnell, Holinka, Gladue, et al., 2015). Evaluation and design of vaccination strategies will be possible after the vaccine candidate can be tested with a bait-based formulation under close-to-natural conditions.

The corn-based baits used for CSF vaccination can be a model (Riemser Schweinepestoralvakzine; CEVA Tiergesundheit GmbH, former Riemser Arzneimittel) (Kaden et al., 2000; Rossi et al., 2015), however the suitability for ASFV vaccination remains yet to be elucidated. To begin with, the blister volume is limited to 1.6 mL and capacities for cultivation of future ASFV vaccines will have to show whether titers allowing a sufficient immunization dose in this volume can be achieved.

Another big knowledge gap for future commercialization in Europe is vaccine safety. Here, profound characterization will be a legally and ethically demanded prerequisite for the licensing of any live vaccine candidate. When we tested "ASFV-GΔMGF" in an in vivo reversion to virulence study in accordance with the *International Cooperation on Harmonisation of Technical Requirements for Registration of Veterinary Medicinal Products* guidelines, upon forced animal passaging, a virus variant emerged that was associated with transient fever and an increased replication and shedding. While the emergence of a virus variant seems to underline safety as a critical issue for live ASF vaccines, given the highly artificial and worst-case route of inoculating homogenized tissue supernatants, one should still conduct a thorough benefit-risk analysis considering all safety and efficacy aspects when evaluating these results in the light of future licensing. In terms of virulence, the novel variant of the virus was still nowhere near the highly virulent backbone virus, causing only transient fever and no other clinical signs clearly attributable to the inoculation. Even the novel variant of "ASFV-GΔMGF" may therefore still represent a

feasible vaccine candidate. Nevertheless, further studies should elucidate the possibilities to enhance genetic stability, e.g. by evaluating the likelihood of mutations in different regions of the ASFV genome and integrating these insights in the choice of targets for rational deletion in vaccine design. The fact that we observed genomic deletion in combination with reverse complementary insertion, which was previously described for a field strain of ASFV (L. Zani et al., 2018) is exciting and can help to understand mechanisms of ASFV evolution. In consequence, our findings stress that reversion to virulence studies are also necessary for any other live attenuated ASFV vaccine candidate before field application. In doing so, a worst-case transmission route should be chosen for evaluation of genomic stability to uncover even unlikely mutation events. After all, low likelihood may be compensated by sheer quantity of applications in the field.

An additional important question that remains to be targeted in future studies is duration of immunity (Blome et al., 2020). No concept or vaccination strategy can be developed before we have the insight into the biologically possible duration of vaccine-induced protection in the broader ASF context. The issue of unknown prospects for duration of immunity is stressed by studies reported on other ASFV vaccine candidate (Sánchez-Cordón, Jabbar, Chapman, Dixon, & Montoya, 2020).

In the end, we are getting closer to a licensable vaccine, but many questions are still unanswered. And at the same time, while vaccination represents an exciting opportunity to assist disease eradication, the look over to the CSF situation provides a reminder that even excellent vaccines need to be integrated in an efficient strategy for successful eradication. Several Asian countries like China have been using vaccines against CSF for years and still have not been able to become free from the disease (Blome, Staubach, Henke, Carlson, & Beer, 2017). Reasons may lie in a combination of problems, e.g. with biosecurity, timely diagnosis, a working contact tracing system and, for the vaccines, incomplete cool chains, bad production standards resulting in contamination of vaccines, illegal dilution and a lack of continuity in vaccination policy (B. Zhou, 2019). These still ongoing issues in CSF eradication stress the understatement that even if we have a licensed vaccine against ASF, we must not neglect the other effective measures, at foremost efficient surveillance and diagnosis. A vaccine must not be understood as the single gamechanger, but as a valuable addition to a well-kept toolbox.

Characterization of ASFV strains in Germany

Exact insight into the distribution and the characteristics of the circulating viruses provides a basis for the selection of effective tools for ASF eradication. Looking at the disease dynamics in Germany, a continuity of cases in wild boar was observed since the introduction in 2020. A cause could lie in repetitive virus entries along the German-Polish border, given that western Poland is experiencing an ongoing ASFV situation in wild boar at the same time (Frant et al., 2022). On the German side, a game fence was built to reduce cross-border wild boar movements, but reports of sustained permeability through roads and municipalities exist. Such “frontline” introductions are much harder to contain than single point incursions (Sauter-Louis et al., 2021). The studies included in this work may hint to another relevant factor for local disease dynamics (Jan Hendrik Forth et al., 2022). Five genetic lineages and ten variants of ASFV-Germany were defined based on genetic markers, and, if at least some of these variants are characterized by slightly less virulent phenotypes, as indicated by pathological analysis of wild boar carcasses (Sehl-Ewert, Deutschmann, Breithaupt, & Blome, 2022), our eradication strategy may have been hit on a weak spot. Here, in contrast to the pig sector, daily health surveillance is impossible and early detection relies mainly on the finding of carcasses. Since wild boar usually die within few days after infection with the virulent ASFV field strains and the high viral load in their cadavers represents a high risk to their conspecifics if they establish contact (Fischer, Hühr, Blome, Conraths, & Probst, 2020), the quick removal of the succumbed wild boar, together with population reduction, can provide a major mitigating effect on infection pressure in the ASFV affected region (O'Neill, White, Ruiz-Fons, & Gortázar, 2020). However, for less virulent ASFV strains, animals could survive infection for a longer time (Gallardo et al., 2019; L. Zani et al., 2018) and direct contact between living individuals would gain importance as a transmission factor (M. L. Penrith & Vosloo, 2009). In vivo characterization of the German ASFV strains under standardized experimental conditions is necessary to gain more insight into the virulence and transmission properties of these virus variants. Should the indications for reduced virulence from the field be confirmed, the eradication strategy in wild boar would have to rely even more on population reduction and control.

While the emergence of an increased genomic diversity within the German ASFV strains is a reason for concern, it also offers us a new tool for ASFV surveillance and eradication in Germany.

The clustering of the virus variants to distinct geographical regions allows molecular epidemiology, i.e. the possibility to trace and connect new cases to known ones based on their genomic markers. In Germany, ASFV affected wild boar populations are contained in restriction zones that must allow no passage of animals. With the tool of genomic surveillance, possible local weaknesses in ASFV containment can be rapidly identified and corrected. As the strategy to fight the disease in wild boar differs depending on the federal state and district, we can now provide a feedback on the effectiveness of the containment in the restriction zones, which will help to assess the success of measures. Further, in case of outbreaks in domestic pigs, molecular analysis can help to trace the introduction to regions with affected wild boar populations within Germany, or exclude a domestic origin. E.g., Variant III is distinctly clustered to wild boar populations of the districts of Märkisch-Oderland (MOL), Barnim (BAR) and Uckermark (UM). Variant IV circulates in wild boar in the south of district Spree-Neiße (SPN), as well as in the federal state of Saxony (Jan Hendrik Forth et al., 2022). Consequently, when ASFV introductions into domestic pig holdings in the state of Brandenburg were recorded in 2021, genomic characterization reveiled a virus of German variant III in two farms in MOL and variant IV in a pig holding in southern SPN, suggesting the local wild boar populations as a source. Following the work presented in this thesis, genomic analyses are now routinely applied at the NRL and the insight gained is helping epidemiologists and local authorities in their assessment of the situation. To accelerate the workflows for genomic characterization, tailored molecular assays on the basis of a padlock PCR (using the platform published by Zurita et al. (2022)) and qPCR are currently implemented (publication in preparation).

Recent advances in ASFV diagnosis

A third pillar among the effective measures to fight the ASF panzootic lies in early and efficient diagnosis. Regardless of the pathogenicity or origin of the circulating ASFV strains, succumbed animals represent a major infection risk for the remaining wild boar population (Fischer et al., 2020; O'Neill et al., 2020). Control measures must be implemented as early as possible to successfully contain any outbreak. This stresses the need to continuously establish advances in laboratory methods into the workflows for ASFV diagnosis. Early and precise diagnosis can be a challenge in the wild boar context, where cases emerge in remote forest areas, far away from

laboratory capacities. The same problem applies to backyard farms in Asia, Africa or eastern Europe where access to full laboratory capacity is limited. Here, optimization of diagnostic workflows would effectively aid in disease eradication.

One starting point for optimization would be to take analysis into the field and establish methods for point of care diagnosis, effectively shortening the period between emergence and clarification of a suspected ASF case. A practical approach for on-site diagnosis is demanded by stakeholders and options for molecular and antigen-based detection are published. Auspicious results have been reported using mobile systems for molecular point-of-care diagnosis. Here, assays based on PCR, as well as loop-mediated isothermal amplification (LAMP) are available and have been proven to be sensitive and deliver feasible results for ASFV diagnosis under field conditions (Ceruti et al., 2021; Daigle et al., 2021; Elnagar, Pikalo, Beer, Blome, & Hoffmann, 2021; Yang Wang et al., 2021; Zurita et al., 2022). However, these devices usually require trained and experienced personnel and can hence not be conducted by farmers or other first-line stakeholders. In addition, acquisition costs of equipment and reagents are generally rather high, a drawback especially for the backyard farm application in regions of low socioeconomic standard. From an application-related perspective, lateral flow devices are the promising option due to low costs and easy handling that can also be conducted by untrained personnel, as experiences from the humane medicine sector have shown during the SARS-CoV2 pandemic (Loeffelholz & Tang, 2020). Unfortunately, the results included in this work indicate lateral flow devices for ASFV antigen detection (Sastre, Gallardo, et al., 2016) are not reliable if viral load is not high or the sample matrix is of bad quality. Even for passive surveillance, e.g. to test shot wild boar after hunting, blood clots will likely occur before initiation of testing, which we observed to have detrimental effects upon sensitivity.

Our study has shown practicable sensitivity only when fresh, anticoagulant treated blood containing high viral loads is tested, leaving only the field testing of domestic pigs during peak viremia as an auspicious application, and veterinary professionals would then be required for blood sampling. This appears ineffective for early detection in comparison to the initiation of laboratory testing, which is sensitive during early phases of infection and less error-prone. In

Germany, we have a diagnostic infrastructure for the centralized laboratory detection of ASFV that usually allows clarification of suspected cases within hours after sampling if required.

Until a feasible and reliable approach for ASFV point of care detection is available, the most promising approach to enhance the efficiency of diagnosis would therefore be improved sampling workflows. As a concretization to the guidelines by the WOAH (OIE, 2021) and the FLI compilation of methods ("Afrikanische Schweinepest: Amtliche Methode und Falldefinition," 2021), we have evaluated which sample matrices are best fit for reliable and early ASFV detection using laboratory methods. In the context of point of care sampling of wild boar carcasses, minimal-invasive sampling is desired to reduce the transmission risk by cadavers if the body cavity is not opened, spilling blood and other potentially infectious materials in the surroundings (M. L. Penrith & Vosloo, 2009), but also to reduce the amount of work and time needed to sample carcasses. Since searching for carcasses and subsequent sampling can be extremely laborious, more efficient methods can aid the local authorities in their work for disease containment. We have shown that in a late stage of infection, all tissues with good blood circulation are feasible for ASFV genome detection, but sampling of peripheral organs such as ears may miss early moments of infection. Blood is the major carrier of infectivity during ASFV infection (McVicar, 1984). Making use of that, blood swabs require minimal invasivity in a carcass and represent are a good compromise between high sensitivity even in early stages of infection and minimal risk of environmental contamination.

In conclusion, blood swabs are identified as the ideal option for field sampling of wild boar. For the German situation, laboratory analysis by qPCR enables reliable sensitivity even in early stages of ASFV infection or from decomposed wild boar carcasses. It is usually available within hours with the established diagnostic infrastructure and, considering superior precision, remains the most efficient option for ASFV detection among the currently available methods.

7. Summary

To be successful in eradicating ASF, we should consider every option available to us, optimizing the measures already at hand, developing new techniques and integrating them into an effective strategy. In this light, we have advanced the search for a safe and efficacious vaccine candidate that fulfills the requirements for EMA authorization. With the new insight into promising vaccine candidate “ASFV-GΔMGF”, proving intramuscular efficacy, the concept of oral vaccination and capabilities for cultivation on permanent cells, we are getting closer towards a commercial ASFV vaccine. We have described genetic changes of “ASFV-GΔMGF” in a reversion to virulence study, but no highly virulent phenotype emerged, so the results can help to understand mechanisms of viral evolution and provide a basis for a benefit-risk assessment of the vaccine.

Genomic surveillance allows us to have exact insight into disease dynamics and epidemiological developments. We have described five genetic lineages and ten variants of ASFV-Germany with their associated geographical distributions and by this means enable molecular analysis to aid in epidemiological investigations. Our findings indicate variable virulence of the German ASFV strains in wild boar, and further characterization under standardized experimental conditions will be important to clarify this matter.

Irrespective of other eradication measures, diagnosis remains necessary at the highest possible efficiency. For optimization of the established workflows, we have defined the best suited sample matrices for laboratory diagnosis, pointing out blood swabs as a good compromise between sensitivity and low contamination for field sampling. A great challenge for the future will be how to enable reliable diagnosis also at the point of care and in structurally disadvantaged countries, as we have shown that lateral flow devices are not fit for reliable field diagnosis and laboratory methods are still required for precise results.

After all, we need a combined toolbox of effective measures to regain the upper hand over ASF and eventually eliminate this devastating disease from wild boar and domestic pig populations in Germany and worldwide.

8. Zusammenfassung

Für eine erfolgreiche ASP-Bekämpfung müssen alle uns zur Verfügung stehenden, effektiven Maßnahmen berücktsichtigt werden. Dabei sollte eine Optimierung bereits vorhandener Methoden erfolgen und gleichzeitig neue Ansätze entwickelt und in eine wirksames Gesamtkonzept eingebunden werden. Vor diesem Hintergrund haben wir die Suche nach einem sicheren und wirksamen Impfstoffkandidaten vorangetrieben, der die Anforderungen für eine EMA-Zulassung erfüllt. Mit den neuen Erkenntnissen über den vielversprechenden Impfstoffkandidaten "ASFV-GΔMGF", welche die intramuskuläre Wirksamkeit bestätigen, das Konzept der oralen Immunisierung belegen und Möglichkeiten zur Kultivierung auf permanenten Zellen beleuchten, kommen wir einem kommerziellen ASFV-Impfstoff näher. Wir haben genetische Veränderungen des Vakzine-Kandidaten "ASFV-GΔMGF" in einer Reversion-zu-Virulenz Studie beschrieben, dabei aber keinen hoch virulenten Phänotyp nachgewiesen. Die Ergebnisse können so zum Verständnis der Mechanismen viraler Evolution beitragen und eine Grundlage für eine Nutzen-Risiko-Bewertung des Impfstoffs bieten.

Die Einbeziehung genomischer Daten in die Tierseuchenüberwachung ermöglicht uns einen genaueren Einblick in die Krankheitsdynamik und in epidemiologische Prozesse. Wir haben fünf genetische Linien und zehn Varianten von ASPV-Germany mit der dazugehörigen geografischen Verteilung beschrieben und ermöglichen so molekulare Untersuchen, deren Ergebnisse direkt in epidemiologische Analysen einfließen können. Unsere Erkenntnisse deuten auf eine variable Virulenz der deutschen ASPV-Stämme beim Schwarzwild hin. Weitere Versuche zur Charakterisierung unter standardisierten Bedingungen sollten zur Klärung dieser Frage erfolgen.

Eine möglichst effiziente Diagnostik bleibt dabei ungeachtet anderer wirksamer Bekämpfungsmethoden unverzichtbarer Teil der ASP-Bekämpfung. Zur Optimierung der etablierten Arbeitsabläufe haben wir die am besten geeigneten Probenmatrices für die Labordiagnose definiert, wobei wir Bluttupfer als guten Kompromiss zwischen Sensitivität und geringem Kontaminationsrisiko für die Probenahme im Feld hervorgehoben haben. Eine große Herausforderung für die Zukunft wird darin bestehen, zuverlässige ASPV-Diagnosen auch direkt vor Ort und in strukturell benachteiligten Ländern zu ermöglichen, da wir gezeigt haben, dass

Lateral-Flow-Tests für eine zuverlässige Felddiagnose nicht geeignet sind und für präzise Ergebnisse weiterhin Labormethoden erforderlich sind.

Schlussendlich brauchen wir ein kombiniertes Instrumentarium an wirksamen Maßnahmen, um die ASP erfolgreich zu bekämpfen und diese verheerende Tierseuche in den Wild- und Hausschweinebeständen in Deutschland und weltweit nachhaltig zu tilgen.

9. References

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10. Acknowledgements

Firstly, I would like to thank Prof. Dr. Dr. h.c. Gerd Sutter and the reviewers for their assessment of this work.

My gratitude goes to Prof. Dr. Martin Beer for always listening and for the ongoing encouragement and motivational discussions. Thank you for the opportunity to work at your institute!

I want to express my deepest thanks to PD Dr. Sandra Blome for all the guidance during the last years. She supported me in my scientific and professional developement, promoted my strengths and encouraged me to always go a step futher. Thank you, Sandra, for everything I have learned, for your supervision and for always having an open ear!

I'm no less grateful to my labmates: Tessa Carrau Garreta, Virginia Friedrichs, Ulrike Kleinert and Mila Wendland, I'm so glad that we have become such a great team and are always supporting each other. It is a pleasure to work with you!

My thanks go to the animal caretakers and necropsy staff, in particular and among others to Matthias Jahn, Christian Loth, Steffen Brenz and Domenique Lux for all their help during the last years.

Further, I would like to thank Zoetis Manufacturing and Research, in particular, Alicia Urniza, Elisenda Viaplana and Jose Carlos Mancera for the support in our studies and for providing the funds for my project.

And finally, want to express my heartfelt gratitude to my family and my partner for their continuous love and for always being there for me. The same deep thankfullness goes to all friends who kept supporting me during the past years, no matter if we have got stranded on the same island or if our paths separated.