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# African swine fever virus infection in domestic pigs and wild boar - pathobiology and optimized diagnostic workflows

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You have your way. I have my way.
As for the right way, the correct way, and the only way,
it does not exist.

- Friedrich Nietzsche -

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# Contents

# I. List of Abbreviation

| | |
|---|---|
| AI | Artificial insemination |
| ASF | African swine fever |
| ASFV | African swine fever virus |
| AQP3 | Aquaporin 3 |
| CREB | cAMP response element-binding protein |
| Cq | Cycle quantification value |
| C8B | Complement C8 beta chain |
| DNA | Deoxyribonucleic acid |
| DOK3 | Docking protein 3 |
| Ds | double stranded |
| EDTA | Ethylenediaminetetraacetic acid |
| ELISA | Enzyme-linked immunosorbent assay |
| ER | Endoplasmic reticulum |
| EU | European Union |
| GFP | Green fluorescent protein |
| GPR82 | G protein-coupled receptor 82 |
| GSTA1 | Glutathione S-transferase A1 |
| HAU | Hemadsorbing units 50 |
| HPX | Hemopexin |
| ICTV | International committee on taxonomy of viruses |
| IHC | Immunohistochemistry |
| IFN | Interferon |
| IL | Interleukin |
| ISH | In situ hybridisation |
| ITGAD | Integrin subunit alpha D |
| JPT2 | Jupiter Microtubule Associated Homolog 2 |
| kbp | Kilo base pair |
| NF-κB | Nuclear factor 'kappa-light-chain-enhancer' of activated B-cells |
| ORF | Open reading frame |
| qPCR | Quantitative polymerase chain reaction |

List of Abbreviation

| | |
|---|---|
| RBP4 | Retinol binding protein 4 |
| RNA | Ribonucleic acid |
| RT-qPCR | Quantitative reverse transcription polymerase chain reaction |
| Ss | Single stranded |
| STAT 1 | Signal transducer and activator of transcription 1 |
| TEM | Transmission electron microscopy |
| TNF-α | Tumor necrosis factor-alpha |
| TSG 6 | Tumor necrosis factor-inducible gene 6 protein |
| uPA | Urokinase-type plasminogen activator |
| uPAR | Urokinase receptor |
| WSL | Wild suid lung |

# II. Introduction

African swine fever is a notifiable animal disease that is now spread worldwide and is often fatal. The viral disease, which is caused by a complex DNA virus of the *Asfarviridae* family, affects both domestic and wild suids and is so far neither preventable by a vaccine nor treatable.

Against the background of the introduction of African swine fever into the European Union including Central Europe, the systems of early detection and diagnostics had to be reconsidered and optimized in the past years and months. In this context, a virus strain from Belgium was characterized in detail in this work. The focus was on the pathobiological characteristics in domestic pigs of different age classes, knowledge which is crucial for early detection.

Early clinical detection has to be flanked by reliable laboratory diagnostic methods. Here, the choice of matrices is important for success and practicability. Taking this into account, various traditional and alternative matrices from animal experiments with viruses of different genotypes were tested and compared with regard to their technical suitability, comparability and ease of use.

Detailed knowledge of the direct and indirect transmission routes of the disease is also of great importance for animal disease control and risk assessment. In order to derive implications for the venereal transmissibility, one additional study turned to the pathobiology of ASFV in the male reproductive tract. Both domestic boars and wild boar could be included in this assessment.

The studies of this thesis thus span an arc from the early detection of ASFV-related disease on a clinical and laboratory diagnostic level to the assessment of possible transmission risks and their mitigation.

# III. Review of Literature

## Taxonomy & Origin

The taxonomic families *Asfarviridae*, *Ascoviridae, Iridoviridae, Marseilleviridae, Mimiviridae, Phycodnaviridae, Pithoviridae* and *Poxviridae* are referred to a group of nucleocytoplasmic large DNA viruses (NCLDV) [1]. Given the hypothesis, that eukaryotic ssDNA viruses apparently evolved via a fusion of genes from prokaryotic rolling circle-replicating plasmids and positive-strand RNA viruses, the origin of NCLDV possibly lays back to the *Tectiviridae* that represent icosahedral tailless ssDNA bacteriophages. This approval is stressed by an evolutionary connection between the virophages of *Mimiviridae* and large eukaryotic dsDNA transposons of the Polinton and Maverick group. Polintons are supposed to be the evolutionary intermediates between bacterial tectiviruses and several groups of eukaryotic dsDNA viruses [2].

NCLDV are now classified in the phylum *Nucleocytoviricota* (ICTV 2020) and constitute an apparently monophyletic group with the existence of a common ancestor that was a virus with genomic complexity. Nevertheless, the majority of group core genes are unlikely to be monophyletic but have been independently edited, comprising evolutionary mechanisms as gene loss and xenologous gene displacement. Moreover, the acquisition of genes from eukaryotes, bacteria, bacteriophages, and links to other large DNA viruses as herpesviruses and baculoviruses have been reported [3]. In line, NCDLV show a broad host tropism that include phagotrophic protists, plants, insects, mammals and reptiles and its members share several biological features [4].

As a sole member within the *Asfarviridae* family, African swine fever virus (ASFV) is classified in the single genus *Asfivirus* and linked to the order *Asfuvirales* (ICTV 2020). The evolution of ASFV remains unsolved, as the paucity of ASFV near-neighbours in nature makes conjecture its origin difficult [5]. So far, abalone asfa-like virus has been designated as the closest ancestor, though this relation remains distant [6, 7]. ASFV is the only known DNA arbovirus [8].

It shows high adaption to its natural hosts, i.e. *Ornithodoros* ticks and indigenous African wild suids [8]. Strengthening the hypothesis of virus-host-coevolution within this ancient sylvatic cycle, endogenous viral elements have been discovered in the genomes of *Ornithodoros moubata* and *porcinus* integrated around 1.46 million years ago [9]. As well, other ASFV-like sequences have been identified in human serum and sewage [10], in freshwater [11] and marine environments [12]. Thus, the upcoming detection of ASFV-like sequences in genomic and

metagenomic data from ASFV vectors and wildlife hosts is anticipated [5]. Despite representing a DNA virus and therefore driven by a lower mutation rate than RNA viruses, specific ASFV gene sequences have been detected to mutate as fast as RNA viruses [5]. Mechanisms to generate ASFV variability include single nucleotide mutations, insertions and deletions, gene duplication and recombination [5, 13], whereas indels are more involved than point mutations driving ASFV diversity [14].

## Morphology & Constitution

The ASFV virion displays a large enveloped icosahedron with an average diameter of 200 nm [15]. Multiple, concentric layers constitute the complex structure of the particle (figure 1): a pleomorphic outer lipid membrane, an icosahedral outer protein capsid, an icosahedral internal lipid membrane, an icosahedral inner protein capsid and the core shell enclosing the nucleoid, that harbours the central DNA [4].

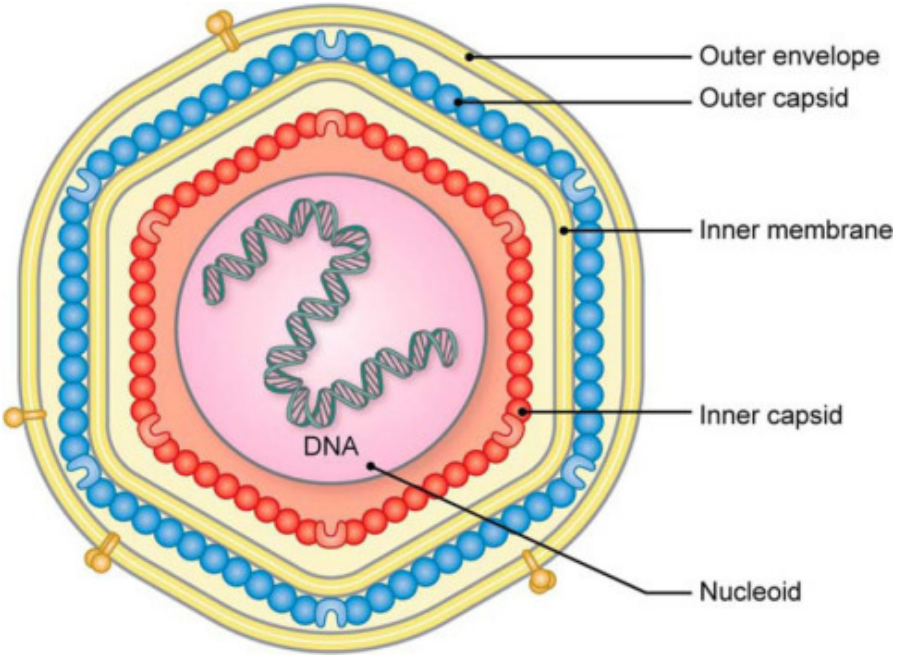


Figure 1. Schematic structure of the ASFV virion; created by Gallagher and Harris [16]

At least 54 viral proteins are known to structure the extracellular virion and to be packaged into it [17]. In accordance, the transcripts of genes encoding proteins in estimated total count involved in capsid formation are indicated to be more abundant than those required in fundamental viral processes [18].

The outer envelope depicts a membrane-lipid bilayer [19]. The localization of the viral protein CD2v (gene EP153R) is attributable to the external membrane [4, 20]. This transmembrane

glycoprotein resembles the T-lymphocyte surface adhesion receptor CD2 and is expressed at a late stage of infection. Its functions are linked to the hemadsorption phenomena to ASFV-infected cells and extracellular virions and to the inhibition of bystander lymphocyte proliferation. Confocal microscopy displayed its localization not only at the outer envelope, but also more abundant in the intracellular space [21-24]. Furthermore, CD2v activates NF-κB, which in turn induces IFN signalling and apoptosis in swine lymphocytes and macrophages [25]. In the ASFV tick vector, present CD2v enhances replication [26].

The outer capsid (T=277) is organized in pseudo-hexameric capsomers, that are constituted by homotrimers of the major capsid protein p72 (gene B646L) and unite in 20 trisymmetrons and 12 pentasymmetrons, complexly stabilized by four suggestive minor capsid proteins H240R, M1249L, p17 (gene D117L) and p49 (gene B438L). Each of the 12 icosahedron-vertices are accessorized with copies of a penton protein [4, 27, 28]. The viral protein pE120R binds to the major capsid protein, as previously demonstrated by co-immunoprecipitation assays and therefore plays a role in virus egress via mediation of the transport of ASFV particles from the assembly sites to the plasma membrane but it is not mandatory for virus infectivity [29].

The inner envelope of intracellular virus consists of a single lipid bilayer [30]. At present, seven known transmembrane viral proteins, more precisely p17, p54 (gene E183L), p12 (gene O61R), p24 (gene KP177L), pH108R, pE199L, and pE248R, have been identified in this layer [4, 20]. The viral proteins p12 and p24 are involved in virus attachment [31-33]. The viral protein p17 is one of the major structuring and stabilizing proteins inhibiting cell proliferation [34]. Moreover, the protein p54 has been referred to virus entry, recruitment of envelope precursors to assembly sites, virus viability and the induction of apoptosis [17, 35-37]. Both pE199L and pE248R are reported to also facilitate viral entry [38].

The components of the inner capsid (T=19) are mature products derived by proteolytic cleavage of viral polyproteins pp62 (gene CP530R) into p35, p15, and p8 and pp220 (gene CP2475L) into p5, p14, p34, p37, and p150. It is also assembled by pseudo-hexameric capsomers [4, 39].

Last, the inner capsid confines the core shell associated with the nucleoprotein p10 (gene K78R) and pA104R [40, 41] and the innermost nucleoid with the transcriptional machinery that makes ASFV nearly autonomous from host transcription. Also, the ASFV virion primary contains several enzymes and factors needed for early mRNA transcription and processing and further advantageous enzymes including for host evasion [42, 43]. In fact, the existence of components of the base excision repair system have been reported, involving a type X DNA polymerase (gene

O174L), AP endonuclease (gene E296R) and dUTPase (gene E165R) [20].

There are many more structural proteins, whose exact location and function remain inconclusive, among them the phosphoprotein p30 (gene CP204L). This viral protein is e.g. highly immunogenic due to pre-replicative expression [44].

## Genome & Life Cycle

The ASFV genome resembles that of other NCLDV and has been compared to the genome of poxviruses in particular. It consists of a linear double-stranded DNA molecule with covalently closed terminal hairpins and inverted repeats. Depending on the viral strain, the genome has a size of 165-194 kbp [45]. Representative genome organization of ASFV “Georgia 2007/1” is depictured in figure 2. The ASF viral genome encodes for 151-167 open reading frames (ORFs) resulting in roughly hundred non-structural proteins, besides the structural proteins [20]. The genome comprises a relatively conserved core of replication-associated genes that have structural or enzymatic functions facilitating nearly autonomous cytoplasmic replication. Within this region, hot spot variable and intergenic regions are included [5]. Irrespective of virulence, at least 125 ORFs are conserved between all genomes of ASFV isolates (Dixon, Chapman et al. 2013). Terminally, there are left and right variable regions, that are predominated by multigene families [5]. At least 17 genes have been identified that encode for virion-structuring proteins [13].

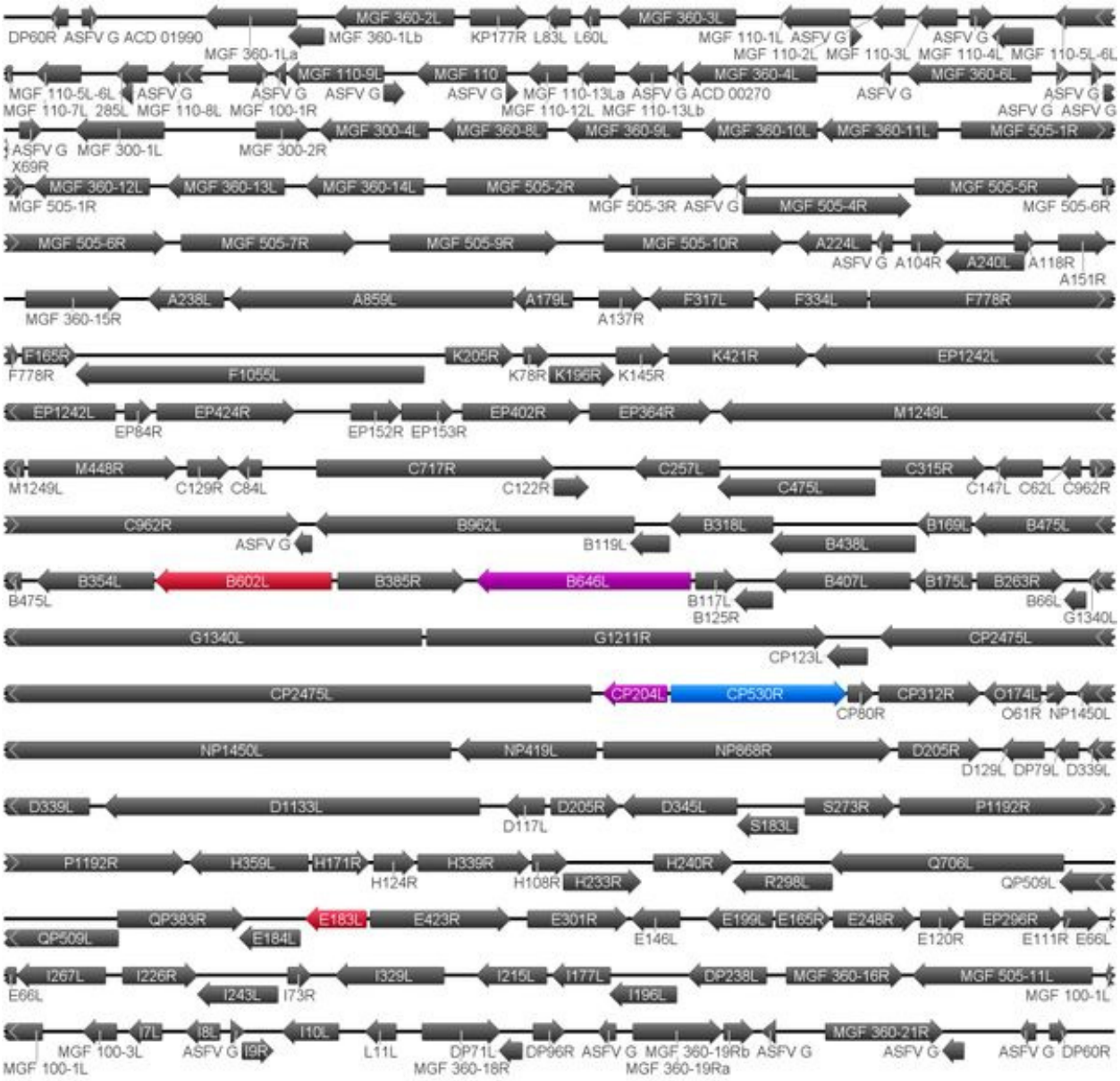


Figure 2. Genome organization of ASFV "Georgia 2007/1. Direct and indirect diagnostic targets as used in Germany are marked in color.

*Purple*: B646L (p72) and CP204L (p30) are used for direct and indirect diagnostics/ genotyping *Red*: B602L and E183L are used for genotyping *blue*: CP530R (pp62) is used for indirect diagnostics

Currently, ASFV isolates are discriminated into 24 genotypes by partial nucleotide sequencing of the variable C-terminal end of the B646L-gene that encodes the major capsid protein p72 [45-48]. Today, most of the 24 genotypes have been linked to outbreaks in Sub-Saharan-Africa. ASFV of Genotype I dominates in Central and West Africa, while highest genetic diversity is found in the Southern and Eastern parts of Africa. Some genotypes in Sub-Saharan-Africa are country-specific, others have transboundary distribution [49]. While Southern and Eastern African

isolates show high genetic diversity, the pandemic strains belonging to genotype II are highly related [13, 50]. However, the analysis of the p72-genotyping does not always provide adequate typing resolution or fails in discrimination of different biological phenotypes. Therefore, the additional assessment of the E183L-, CP205L- and B602L- genes have been approached [5, 51]. Discrimination of ASFV strains of genotype II circulating in Eastern and Central Europe included the analysis of single nucleotide polymorphisms within the K145R, MGF 505-5R genes and tandem repeats within the O174L gene and intergenic region between I73R and I329L genes [52-54]. Analysis of whole genome sequences of current field strains are limited and therefore its extension is needed to unravel more genetic markers [50, 55].The main target cells of ASFV represent mononuclear phagocytes of the myeloid lineage as macrophages, monocytes and dendritic cells [56]. One ASFV life cycle is completed within 18-24 hours and comprises the stages attachment, entry, uncoating, transcription, replication, assembly and release by budding or cell lysis [57]. Figure 3 depictures on ASFV life cycle schematically.

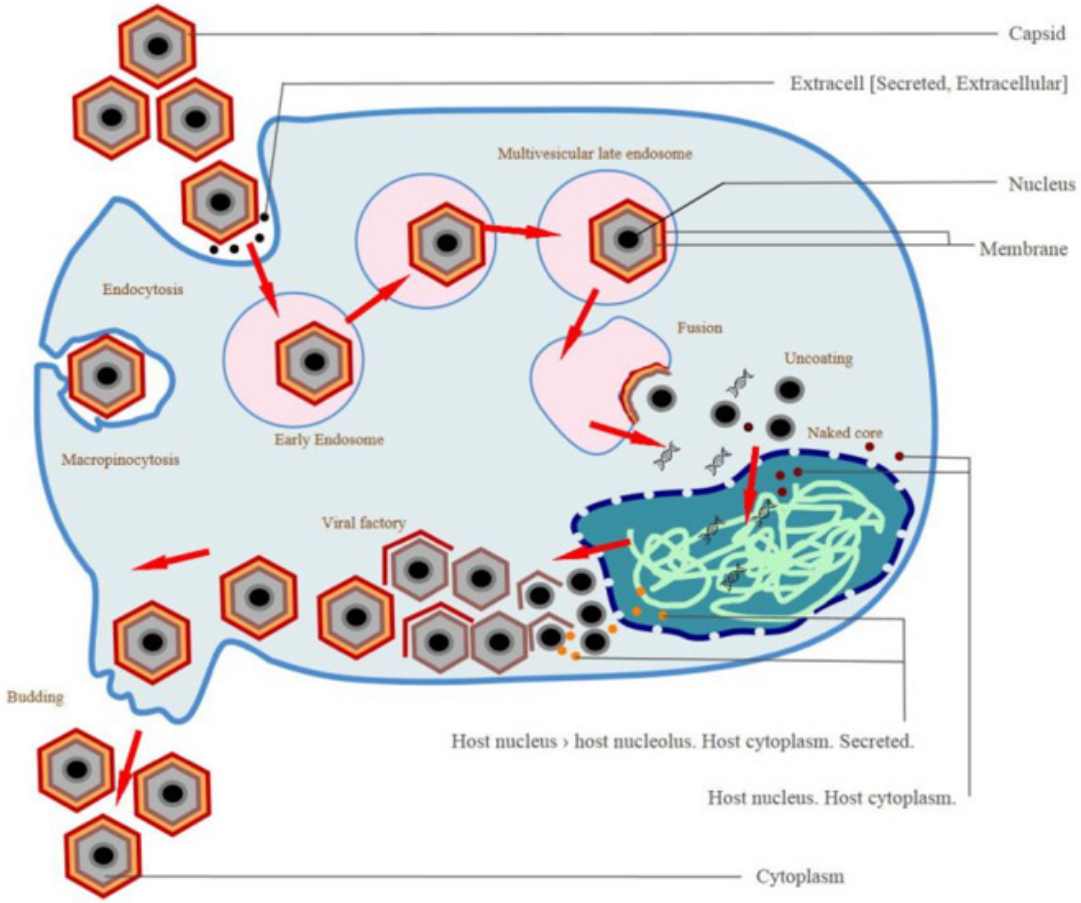


Figure 3. Schematic life cycle of ASFV. Figure obtained from Zhu and Meng [58].

After attachment to the cellular plasma membrane, ASFV is known to be internalized by dynamin- and clathrin-mediated endocytosis, possibly engaging yet unknown receptors. Constitutive

macropinocytosis and actin-dependent pathways also ensure virus entry [59]. After uptake, incoming particles undergo a stepwise, pH-driven capsid disassembly and disruption of the outer membrane, since being transported from early to late endosomes or macropinosomes. Via fusion with the endosomal membrane, cores and DNA are ejected into the cytosol [60].

Subsequently, immediate early and early genes are expressed as provided by the virion-packaged transcriptional machinery before the onset of DNA replication, followed by intermediate and late gene expression [61] (Figure 2). Despite a replicative nuclear phase [62], ASFV exclusively replicates and is assembled in cytoplasmic, perinuclear factories aligned to the microtubule organizing center [63, 64]. They are designated as viral factories and can be depictable via DAPI staining in immunofluorescence microscopy at hour 16 post infection (K. Pannhorst, personal communication). Cellular organelles and proteins, such as the ER, mitochondria and vimentin, are high-jacked in its proximity facilitating progeny assembly [65]. DNA replication intermediates consisting of head-to-head concatemers. Replication is thought to be initiated with the introduction of a single strand nick near to one terminus. Subsequently, DNA is synthesized by a DNA polymerase towards one terminus [13]. For efficient replication in porcine macrophages, ASFV thymidine kinase is required [66].

The ASFV assembly process follows a strictly coordinated procedure [42].

At first, ER cisternae-derived membrane fragments are assembled displaying precursors of the inner viral envelope implicating its putative origin from two collapsed ER cisternae [67]. Subsequently, the capsid and the core shell are generated simultaneously on the convex and concave side of viral membranes. At last, formation of the nucleoid occurs. Then, mature intracellular particles are transported via the reorganized cytoskeleton to the cellular plasma membrane and budded during virus egress via filopodia-like projections [42, 68].

Apoptosis of the infected cell is likely initiated via the mitochondrial or extrinsic pathway [69]. Before the onset of replication, ASFV is sensed to activate caspase 3. Nevertheless, to ensure maximal release of progeny, it also encodes two anti-apoptotic proteins within A179L and A224L [70]. However, released intracellular virus is also infectious, as the outer envelope is not necessary [6]. Also, both nucleoid-carrying and empty ghost particles occur to be released from cell [71].

## Transmission

As ASFV is competent of infecting all members of the *Suidae* family and the non-vertebrate argasid vector *Ornithodoros* [8], four intersecting cycles can be distinguished that play a key role in the transmission: sylvatic, tick-pig, domestic and wild-boar habitat [72].

In its original endemic niche in Southern and Eastern Africa, ASFV is transmitted in an ancient sylvatic cycle among African wild suids and soft ticks of genus *Ornithodoros*, which harbor in burrows [73]. Warthogs of the genus *Phacochoerus* represent the most significant part in the maintenance of ASF [74]. This cycle is not accompanied by overt disease or mortality in warthogs and bush pigs and would probably go unnoticed, acting as virus reservoirs [75]. Adults do not present with viremia, but partly virus can be found in various lymphoid tissues [76]. Neonatal warthogs become infected in the first weeks of life and develop high viremia sufficient to transmit the virus to naive ticks. In affected regions, almost all warthogs are exposed to ASFV in this stage and develop antibodies [77]. After infection via the red blood cell fraction, ASFV replicates primary in the tick gut, but is secondary distributed to other compartments, as the reproductive tract, and shed by saliva, coxal fluid, and Malpighian excrement [78] and can persist in the tick for up to five years [79]. The soft tick vector is competent to maintain the infection within an exclusive tick-cycle by transstadial, transovarial and transsexual transmission [80-83]. Successful infection of *Ornithodoros* highly depends on the ASFV strain due to molecular interactions designated as virus-tick-pairs [9, 84, 85].

Spill overs from the sylvatic cycle to domestic pigs are relatively rare and most transmission within the African domestic pig population occurs, even in East Africa, from pig to pig [13]. In West African countries, the ancient sylvatic cycle is not involved [84].

However, any introduction of the disease into the domestic pig sector or wild boar habitat leads to a severe systemic disease that can resemble a viral hemorrhagic fever with exceptionally high lethality. Once the disease has left the sylvatic cycle, the competent arthropod vector is no longer required to sustain infection chains and the disease is transmitted directly and indirectly among susceptible suids [86].

It has been shown that the oronasal route of infection is much less effective when compared to the parenteral routes. In detail, roughly $10^4$ infectious doses 50% and with that 140000 times more virus is needed to infect an animal via the oronasal route with high probability [87]. However, more recent study also showed that low doses may be effective in weak animals [88] or when consuming repeatedly liquid feeding [89]. Indirect routes include the feed-to-pig and

fomites-to-pig transmission since ASFV can persist for months in pork, fat and skin and in different types of pork products and for days to weeks in high titers in blood, faeces and urine excreted in the environment by infected pigs [86, 90, 91]. Humans handling contaminated items are main factors responsible for recent long-distance jumps of ASFV transmission [92]. Furthermore, transmission via the aerosol route was described [93]. Little is known about the possibility of venereal transmissibility. However, the reisolation of ASFV in sperm of an experimentally infected boar and subsequent transmission to a recipient female succeeded [94]. In addition, viral genome was detected in male gonads [95]. Transplacental transmission from sow to progeny was reported to be unlikely [96].

Within the wild boar-habitat cycle, three natural factors facilitate direct disease transmission: wild boar social structure, short duration of low concentration virus shedding orally and excrementally and high lethality combined with indirect transmission through infected carcasses. However, wild boar movements only play a minor role in ASF dynamics at a monthly scale [97].

## Disease in *Sus Scrofa*

ASF presents as a severe but unspecific disease that resembles a hemorrhagic fever occupying obligate pathogenicity in *Sus scrofa*. These signs show a vast variance involving virus and host factors [98, 99].

Following oronasal infection, ASFV primary replicates in mononuclear phagocytic cells in the pharyngeal mucosa of tonsils and proximal lymph nodes. Subsequently, it is systemically disseminated via viremia associated to erythrocytes and leukocytes to secondary organs, where it can be detected by day 2 post infection with clinical onset concomitantly [100]. Consequently, massive destruction of affected organs in line with vascular and lymphoid lesions follows, as a large proportion of B and T lymphocytes and macrophages succumbs to cell death caused by a cytokine storm upon activation [101].

Preliminary, transcriptional studies of porcine macrophages infected with a virulent isolate, indicated that the vast majority of analyzed genes did not alter their expression at all, implicating the absence of a general “switch off” of host transcription [102]. Briefly outlined, these genes increased in expression include proinflammatory cytokines such as IL-6, TNF-α, IFN-β, and chemokines of the CC and CXC groups, that in turn activate gene expression of cellular signaling pathways such as Jak2, STAT 1, CREB and phosphoinositide 3-kinase adaptor [102]. As a consequence, recruitment and activation of inflammatory cells favor virus replication. Moreover, transcript levels mediating anti-inflammatory factors and genes encoding several receptors and

proteins, such as cryopyrin and TSG-6, were altered detected possibly suppressing the proinflammatory response. In line, genes encoding cell surface and secreted proteins were upregulated, such as syndecan and galectin 3. Syndecan has been linked to reduce cell invasiveness by binding to IL-8 and collagen [102]. On the one hand, active galectin 3 has anti-apoptotic properties, on the other hand extracellular galectin 3 has been linked to induce lymphocyte bystander apoptosis. Then, proteins involved in antigenic presentation in conjunction with major histocompatibility complex class II presented to be increasingly expressed. Also, the uPAR gene was upregulated and uPA and uPAR harbor pleiotropic functions in inflammation, fibrinolysis and tissue repair [102]. In particular, TNF-α has been linked to ASFV pathogenesis causing intravascular coagulation, apoptosis, and shock [103].

Vascular endothelial cells have been demonstrated to be susceptible to ASFV with similar tropism as for macrophages. In response to infection, the normal inflammatory reaction was inactivated, inhibiting surface expression of important molecules in cell activation, as E-selectin, the MHC class I, and transcription of proinflammatory cytokines, as IL-6 and IL-8. In contrast, the thrombotic state was increased. Eventually, infection resulted in apoptosis [104]. *In vivo*, the association between viral replication and vascular and lymphoid lesions failed. Invasion of virus coincided with an increasing monocyte-macrophage count and with vascular and lymphoid lesions that are possibly induced by the release of cytokines subsequently. In general, the production of TNF-α, IL-1α, IL-1β and IL-6, coincides with the onset of fever, vascular changes and changes in lymphoid structures [105].

Playing a minor role, upon day 5-7 post infection, several other cell types have been demonstrated to be targeted by transmission electron microscopy: hepatocytes, capillary endothelia, smooth muscle cells, reticular cells, epithelia (collector renal duct and tonsillar), fibroblasts, pericytes, ito cells, glomerular mesangial cells, megakaryocytes, neutrophils and lymphocytes [100]. As they are infected while primary target cells are still viable in line with proliferation of macrophages, it hints to a more complex way of infection, possibly also favored by the intracellular release of cytokines [106].

In ASFV naive areas, the peracute form is usually reported and presented with high fever, cutaneous hyperemia and death (100% lethality) 1-4 days after clinical onset [107]. The acute form in domestic pigs commonly shows high fever, anorexia, respiratory and gastrointestinal signs (constipation and diarrhea), cyanosis and central nervous disorders (ataxia and seizures) resulting in high lethality (lethality 90-100%) within 7-10 days post infection. Wild boar acutely

infected show depression and reduced liveliness, dyspnea, hind leg paresis and seizures, resembling courses in domestic pigs [108].. Subacute forms include clinical features similar but less severe to those observed in acutely infected animals with more prominent vascular changes, fluctuating fever, painful walking, abortion and respiratory distress, resulting in death (30-70% lethality) within 7-22 days post infection. Slightly visible, clinical signs aligned to the chronic form include lymphadenopathy, swollen joints, phased fever and reddened and necrotic skin lasting over a month, potentially causing death (lethality <30%) [6].

ASF is characterized by thrombocytopenia and leukopenia (neutropenia, lymphopenia, neutrophilia) and changes in red blood cell counts such as erythrocytopenia or the decrease in hemoglobin c, increased monocyte count or monocytopenia. In general, these changes lead to a state of immunodeficiency [109]. Time-course analysis of acute phase protein concentration in serum of ASFV infected animals revealed that pig Major Acute-phase Protein, haptoglobin and apolipoprotein A-I response coincided with clinical onset. The haptoglobin and pig Major Acute-phase Protein peaks greatly increased in the acute phase serum while the apolipoprotein A-I decreased. There was also a slight decrease in fetuin. C-reactive protein concentration started to rise upon day 6 post infection. In case of albumin, a non-significant decrease was observed upon day 5 post infection [110].

Pathological signs vary according to the variance of clinical courses [108]. Hemorrhage of several organs and edema are widely seen and more severe in acute and subacute forms that are especially linked to organs not harboring a fixed vascular macrophage population, particularly gastrohepatic and renal lymph nodes, but also intestine, kidney and lungs [100]. Moreover, thrombocytopenia, petechial bleedings, and apparently increased vascular permeability with extravasation of blood components are characteristic [99]. Thrombocytopenia is commonly apparent upon day 3 post infection and possibly caused by destruction of bone marrow megakaryocytes and the consumption of peripheral platelets due to the formation of microthrombi [111]. Splenomegaly is a further characteristic feature, especially in the late stage of infection showing a hyperemic red pulp with a destructive character filled with erythrocytes, platelet thrombi and cell debris [101]. In the lung, secondary virus replication affects pulmonary intravascular, interstitial and alveolar macrophages, but also fibroblasts and neutrophils [112]. Infected and non-infected pulmonary intravascular macrophages show signs of secretory activation of proinflammatory cytokines inducing endothelial leakage that results in edema and alveolar hemorrhages [101]. The liver of infected animals present marked congestion including

infection of Kupffer cells and hepatocytes. Malfunction of liver and the anorectic state may contribute to multifocal, hypo-oncotic edema [101].

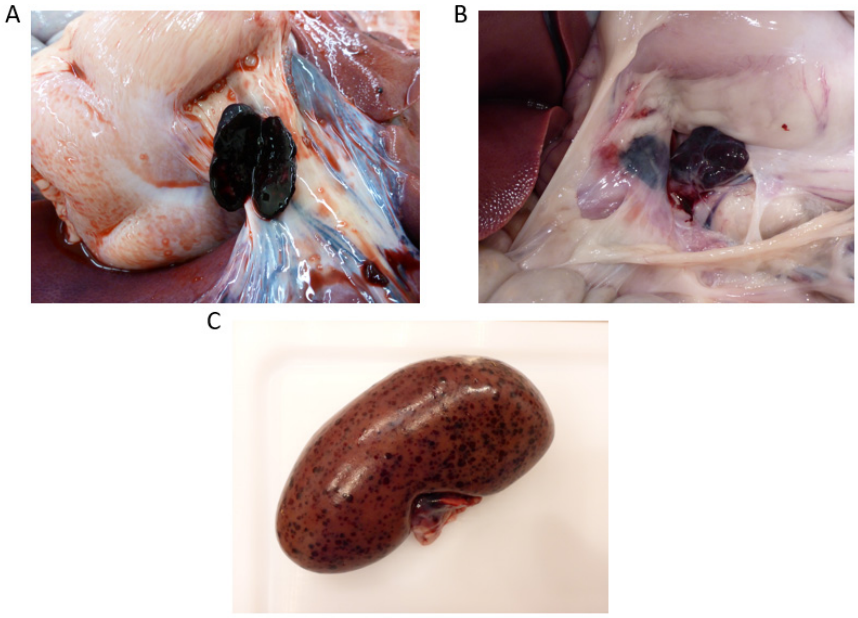


Figure 4. Representative lesions in domestic pigs upon infection with a highly virulent ASFV strain during the acute stage. A and B represent hemorrhagic *Lnn. gastrohepatici*, while C depictures petechiae in the cortex of a kidney (FLI 2022).

As recently reviewed, (per)acute courses are depicted without gross lesions while acute stages include splenomegaly, multifocal lymphadenitis and lesions at mucosa or serosa of further organs as epicardium, urinary bladder and intestine. Animals suffering from a subacute stage commonly present hydropericardium, ascites, multifocal edema particularly in the gall bladder or perirenal fat, hemorrhagic lymphadenitis, petechiae in the kidney and multifocal pneumonia. Chronical courses are depicted with multifocal necrosis in skin and arthritis, growth retardation emaciation, respiratory distress and abortion [101].

European wild boar presents a similar pathological pattern. Gross pathology of wild boar infected intramuscularly and oronasally has resembled lesions in domestic pigs, including lymphadenopathy, hemorrhages in the lymph nodes, splenomegaly, and petechiae in several organs. Once, more specifically described in wild boar, have been extensive hemorrhages in vesical mucosa and necrotic foci in the pancreas [113, 114].

## Diagnosis & Laboratory Workflows

In the absence of a unique diagnostic clinical feature, ASF must be considered in diseases presenting other nonspecific systemic conditions as bacterial septicemias (erysipelas and acute salmonellosis), poisoning, Aujeszky's disease, porcine reproductive and respiratory syndrome, porcine dermatitis and nephropathy syndrome and classical swine fever. Each unspecific disease resembling a hemorrhagic fever has to be examined for ASF (FAO manual). ASF laboratory diagnosis is regulated in recommendations and legal requirements. Methods and protocols can be found in the OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (OIE, 2019) or, for the European Union, on the website of the European Union Reference Laboratory for ASF (https://asf-referencelab.info/asf/en/procedures-diagnosis/sops) [108]. The diagnostic workflow in the German national reference laboratory employs two real-time PCRs (qPCR), based on the detection of a fragment of the B646L-gene. These assays were published by King et al. [115] and Tignon et al. [116] and are used with slight modifications concerning the internal controls. Successful virus isolation on peripheral blood monocytes is routinely detected by either positive hemadsorption phenomena, cytopathic effects or immunofluorescence staining of the p72-antigen [117].

At present, 12 commercially available test systems are licensed for ASF diagnosis. These assays show comparable sensitivity, specificity and precision, with minor variation, when compared to a recommended qPCR method [116], in detail these are Virella ASFV seqc real-time PCR kit, VetMaxTM ASFV Detection kit, ViroReal® Kit ASF Virus, Kylt ASF, Virotype ASFV PCR kit, Virotype ASFV 2.0 PCR kit, ID GeneTM African Swine Fever Duplex, Real PCR ASFV DNA Test, VetAlert ASF PCR Test Kit, INgene q PPA, Adiavet ASFV Fast Time, and ID GeneTM African Swine Fever Triplex [118].

For indirect, serological detection of ASF, three types of enzyme linked immunoadsorbent assays (ELISA) are currently commercially available in Germany. INGEZIM PPA COMPAC (Ingenasa) bases on the detection of p72. The ID Screen® African Swine Fever Indirect ELISA (IDvet) uses an antigen mixture of p72, p62 and p32. In addition, based on the indirect detection of p32, the competitive ELISA, ID Screen African Swine Fever Competition (IDVet) has been released. More semiquantitative approaches comprise the indirect immunoperoxidase test. The targeted protein-encoding genes are depictured in figure 2.

Besides PBMC-derived cultures, further methods to study ASFV include the establishment of primary cultures from pulmonary-, bone marrow- and renal derived macrophages [119, 120].

Primary cultures of porcine aortic endothelial cells and bushpig endothelial cells are also susceptible to ASFV [104].

However, *in vitro* ASFV-studies approaching porcine explants or organoids are not published yet. Recent approaches resulted in the creation of several immortalized macrophage cell lines, such as IPAM [121, 122]. According to continuous cell lines, wild suid lung (WSL) cells have been established, that can be infected with adapted ASFV isolates for studies within a porcine background [119]. Interspatial, ASFV has been adapted to growth in chick embryo, baby hamster and monkey kidney cells [123]. Monkey derived cultures further expanded to ASFV growth in different established monkey cell lines like Vero, MS and CV [119].

## Distribution, Significance & Control

ASF was once regarded an exotic disease restricted to the Kenyan region upon its first description in 1921 [124]. Genotype I spread through Central and Western Africa and from there caused the first trans-continental introductions of ASFV to Portugal in 1957 and 1960, and subsequently spread to other European countries, including the Italian island of Sardinia, and also to the Caribbean and Brazil [13]. This epidemic was successfully eradicated, but the disease remains endemic in the island of Sardinia until today [125].

In 2007, ASFV genotype II was introduced to Georgia from South-East Africa. From there, this genotype gradually spread to Eastern European countries [126], circulating within the wild boar population sporadically causing outbreaks in the domestic pig population [127].

The actual global disease situation as published by OIE-WAHIS 07/03/2022 is depictured in figure 5.

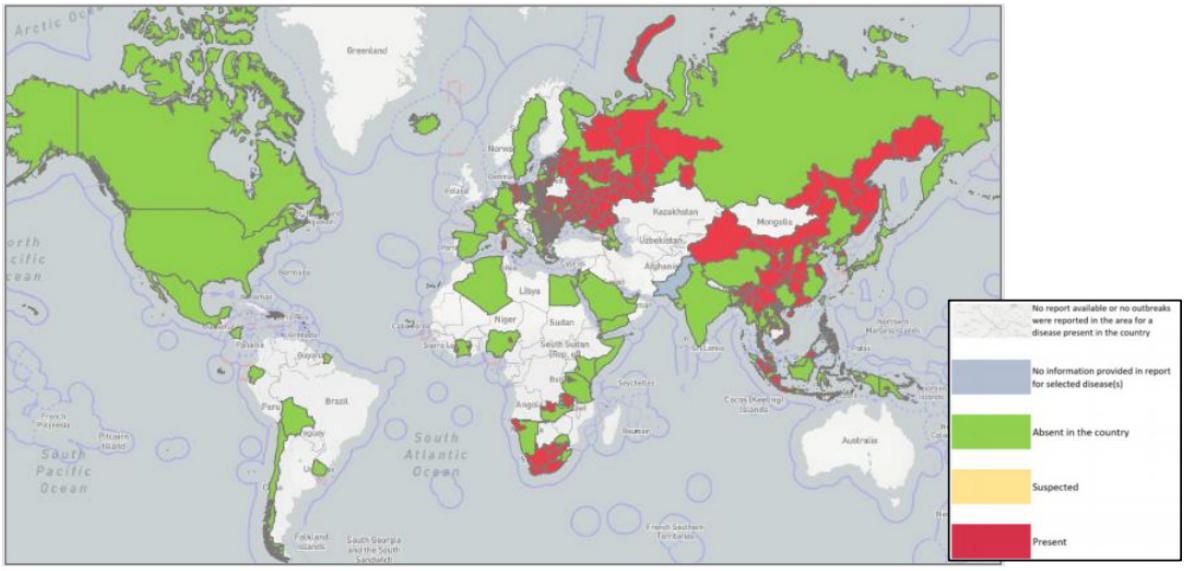


Figure 5. Global map depicturing reported ASF outbreaks from 2020- 04/03/2022. *Red:* Present Y*ellow*: Suspected *Green:* Absent in the country *Grey*: No data available in report *White*: No report available or no outbreak documented in the area for a disease present in a country. Source: OIE-WAHIS 07/03/2022 – ASF Situation Report 7

In 2014, the first occurrence of ASF was reported in the European Union and since then, numerous EU countries have been progressively affected and outbreaks were reported in 16 countries from 2020 to 2022 . Meanwhile, Belgium and the Czech Republic managed to eradicate the disease.

In 2018, the virus reached Asia and since then, 16 countries got infected. Oceana was reported to be affected in 2019 and the Americas, in detail the Dominican Republic and Haiti, got involved in 2021 after almost 40 years of viral absence (OIE WAHIS 2022, visited 12.03.2022). From 2016 – 2020, 32 different African countries all falling under South Africa reported ASFV to the OIE [49]. In 2020, the OIE notified the presence of ASFV in five different world regions in 33 countries, affecting more than 1.000.000 pigs and more than 29.000 wild boar with more than 1.600.000 animal losses (OIE WAHIS 2022, visited 12.03.2022).

Indeed, ASFV has a tremendous impact at sanitary and socioeconomic level as the disease is a significant global source of animal suffering and losses. With regard to its economic impact, the livestock and food industry, trade and tourism are most vulnerable. In case of an affected origin, the agriculture is impaired regarding the feed demand, the export of pork and corresponding products decreases, new suppliers have to be found affecting the trade and prices, the consumer´s demand towards pork products might fall and at last, rural tourism and ecotourism

are negatively impacted [128]. Therefore, the worst scenario came true when ASFV affected China, the world largest pig producer [129].

Due to these transboundary consequences, ASF control and eradication are strictly controlled by legislation. Briefly outlined, at EU level this includes special control measures laying down in the Regulation EU 2016/429 (Animal Health law) and its Commission Implementing Regulation (EU) 2021/605 in addition to rules for the prevention and control of certain listed diseases in Commission Delegated Regulation (EU) 2020/687. In Annex I to Commission Implementing Regulation (EU) 2021/605, member states that are affected by ASF are divided into restricted zones I, II, III according to the epidemiological situation. With respect to the evolution of ASF latest specific zone measures are based on Commission Implementing Regulation (EU) 2022/205 (European Commission).

# IV. Objectives

I. Pathobiological studies on the virulence of ASFV “Belgium 2018/1” in domestic pigs of different age classes

The first case of ASF in wild boar was documented in Belgium in September 2018. The virus strain involved in this case, ASFV “Belgium 2018/1”, has shown a high virulence in wild boar. In order to further characterize its pathobiology, subadult and weaner pigs were infected experimentally and evaluated in terms of clinical courses, replication kinetics, antibody production and pathological lesions as well as their corresponding tissue-viral load.

II. Comparative studies on the suitability of different diagnostic workflows in ASFV detection

In the absence of available vaccines or treatment options, early and efficient ASFV detection is mandatory. A sample set from experimental studies was used comprising EDTA blood, serum and different tissues obtained from domestic pigs and/ or wild boar acutely infected with ASFV “Estonia 2014”, “Belgium 2018/1”, “CHZT 90/1”, “MFUE 6/1”, “RSA W1/99”, “KAB 6/2” or “SUM 14/1”. Thus, common diagnostic workflows for both passive and active surveillance were compared and evaluated including the designation of best suited matrices and operations.

III. Pathobiological studies on the importance of male reproductive organs in ASF

Boars and boar semen represent an important avenue in the wide-spread transmission of several infectious diseases. To test the possibility of venereal transmissibility of ASFV, studies were carried out on the detectability of the virus in male reproductive organs and accessory sex glands. The studies with the ASFV isolates “Germany 2020”, “KAB 6/2” and “SUM 14/11” included sexually mature domestic boars and adolescent wild boar. Viral distribution patterns in the male genitals were investigated by virological and molecular techniques as well as immunohistochemistry and *in situ* hybridization. Supplementing, transmission electron microscopy was employed to depict *in vitro* ASFV- spermatozoa interactions.

# V. Results

The publications included into this thesis are grouped according to their topic. The reference section of each manuscript is presented in the style of the respective journal and is not included at the end of this document. The numeration of figures and tables corresponds to the published form of each manuscript.

# Towards Efficient Early Warning: Pathobiology of African Swine Fever Virus “Belgium 2018/1” in Domestic Pigs of Different Age Classes

Jutta Pikalo [1], Marie-Eve Schoder [2], Julia Sehl-Ewert [1], Angele Breithaupt [1], Ann Brigitte Cay [2], Coline Lhoëst [2], Willem van Campe [2], Laurent Mostin [2], Paul Deutschmann [1], Hanna Roszyk [1], Martin Beer [1], Sandra Blome [1],* and Marylène Tignon [2,] *

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*Article*

# Towards Efficient Early Warning: Pathobiology of African Swine Fever Virus "Belgium 2018/1" in Domestic Pigs of Different Age Classes

**Jutta Pikalo [1], Marie-Eve Schoder [2], Julia Sehl-Ewert [1], Angele Breithaupt [1], Ann Brigitte Cay [2], Coline Lhoëst [2], Willem van Campe [2], Laurent Mostin [2], Paul Deutschmann [1], Hanna Roszyk [1], Martin Beer [1], Sandra Blome [1,*] and Marylène Tignon [2,*]**

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**Simple Summary:** African swine fever (ASF) is a devastating viral disease of both wild boar and domestic pigs. Historically, the disease was mainly found in Sub-Saharan Africa. However, after the introduction of ASF into Georgia in 2007, the fatal disease spread to many European and Asian countries. In the absence of vaccines or treatment options, early detection of disease incursions is of paramount importance to limit the impact on animal health and pig industry. Thus, the biological characteristics of circulating virus strains must be known and communicated to practitioners and official veterinarians. Along these lines, the ASFV strain found in Belgium in 2018 was further characterized for its disease course in young and subadult domestic pigs. In general, clinical and pathological findings were in line with previous experiments utilizing highly virulent ASFV genotype II strains. However, in one of our experimental infections, four out of eight subadult domestic pigs showed milder signs and recovered, which was unexpected and points to an age dependency of clinical signs that could impact the early recognition of ASF incursions. We hope that communication of the available data will help practical and official veterinarians in the field to detect ASF as early as possible and thus minimize its impact.

**Abstract:** African swine fever (ASF) is one of the most important and devastating viral diseases in wild boar and domestic pigs worldwide. In the absence of vaccines or treatment options, early clinical detection is crucial and requires a sound knowledge of disease characteristics. To provide practitioners and state veterinarians with detailed information, the objective of the present study was to characterize the ASF virus (ASFV) isolate "Belgium 2018/1" in subadult and weaning domestic pigs. To this end, two animal trials were performed. Trial A included eight subadult domestic pigs and trial B five weaner pigs. In general, clinical signs and pathological lesions were in line with previous studies utilizing highly virulent ASF genotype II viruses. However, in trial A, four subadult domestic pigs survived and recovered, pointing to an age-dependent outcome. The long-term fate of these survivors remains under discussion and would need further investigation.

**Keywords:** African swine fever virus; Belgium; virulence; clinical course; domestic pigs

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## 1. Introduction

African swine fever (ASF) is a highly contagious and devastating disease of *Suidae*. The causative agent, African swine fever virus (ASFV), is a large double-stranded DNA virus, which belongs to the genus *Asfivirus* in the *Asfarviridae* family [1].

African swine fever has been endemic in many Sub-Saharan African countries and in Sardinia for many decades. However, after the introduction into Georgia in 2007, the disease spread to numerous eastern European countries and reached the European Union (EU) in 2014 with the first outbreaks in wild boar in the Baltic States and Poland [1,2]. In September 2018, the first case of ASF in wild boar was documented in Belgium [3]. The ASFV isolate associated with this outbreak, "Belgium 2018/1" [1,4], belongs to p72 genotype II and has shown high virulence in European wild boar [5]. Thus, it was comparable to other genotype II strains that are circulating in Europe and Asia, showing almost 100% lethality in animals of all age classes and sexes [6]. Clinical signs associated with such an infection include high fever, depression, inappetence, and respiratory distress [2,7]. Under experimental conditions, the animals showed the first clinical signs starting at 3–5 days post infection (dpi) [6], and animals developing an acute lethal disease course died within 7–13 dpi. Pathomorphological changes included enlarged, haemorrhagic lymph nodes; reddening of tonsils; congestion of spleen or splenomegaly; petechiae in different organs such as the kidney, colon, or urinary bladder; and lung and gall bladder wall edema [7]. Experimentally, the clinical course of ASF mostly has been studied in younger pigs (8–12 weeks), but there are some indications that the clinical course of ASF could be age-dependent under certain conditions [8].

In the absence of vaccines or other treatment options, early clinical detection is of paramount importance and requires detailed knowledge of clinical signs and pathological changes. Thus, biological strain characterization is important to inform farmers, practitioners, and state veterinarians involved in disease control [9].

Here, we report on the experimental inoculation of eight subadult domestic pigs and five weaner pigs for further characterization of the ASFV strain "Belgium 2018/1" and assessment of the influence of age on the clinical course and survival rate.

## 2. Materials and Methods

### *2.1. Experimental Design*

The study comprised two animal experiments (trials A and B), that were performed to assess virulence and pathogenesis of genotype II ASFV from Belgium (ASFV strain "Belgium 2018/1"), and to collect suitable reference materials. Trial A was performed at Sciensano, Brussels, Belgium and trial B was carried out at the Friedrich-Loeffler-Institut (FLI), Greifswald-Insel Riems, Germany.

### *2.2. Animal Trials*

#### 2.2.1. Trial A

The study comprised eight ASFV and ASFV antibody negative domestic pigs (Hypor hybrid × Pietrain breed) from a conventional farm weighing about 20 kg (10 weeks old). They were housed in a group at Sciensano in Animal Safety Level 3 facilities on slatted floors with water and food ad libitum for the duration of the experiment. The experiment was authorized by the Ethical Commission of Sciensano under N°20190614-01 and approved by the Biosafety commission. Upon arrival, the animals were randomly marked with ear tags starting from 1 to 8.

As these animals were part of another trial, each animal received an intramuscular injection of 1 mL of saline solution (mock injection) after one week acclimation. A second mock administration was performed in the same manner 23 days after the first injection.

At 49 days post mock injection and day 0 for this experiment, the subadult animals (18 weeks of age, weighing between 60 and 80 kg) were inoculated nasally using a small nebulizer (1-mm spray opening) fixed on a syringe to drip the infectious dose into the nostrils. Each animal received 4 mL virus suspension (2 mL per nostril) containing $1 \times 10^{4.3}$ hemadsorbing units (HAU)/mL of ASFV "Belgium 2018/1". The inoculum was cultivated on PBMC following standard procedures (EURL protocol: https://asf-referencelab.info/asf/images/ficherosasf/PROTOCOLOS-EN/SOP-ASF-VI-1REV2018.pdf (accessed on 28 August 2021). Following the initial protocol that had

been drafted assuming acute disease courses, the experiment was terminated at 18 dpi. Due to the lack of high containment capacities and organizational reasons, the study could not be extended beyond 18 dpi.

Upon inoculation, body temperature and clinical parameters of all animals were assessed daily based on a harmonized scoring system as previously described [2]. Evaluated parameters are anorexia, recumbence, skin hemorrhage/cyanosis, swelling, breathing/coughing, ocular discharge, digestive trouble, and temperature. Based on the severity of the clinical signs, zero to three score points were awarded per parameter. The sum of points was recorded as the clinical score (CS). Temperatures higher than 40.5 °C were considered as severe fever, whereas temperatures higher than 39.7 °C (average +3 SD) were considered as mild fever. Animals reaching the humane endpoint of three subsequent days with severe fever (>40.5 °C), 9 score points, or were suffering unacceptably without reaching the endpoint score, were killed by electrocution and exsanguination.

Blood and serum samples were collected prior to inoculation, 3, 7, 10, 14, and 18 dpi at the day of euthanasia. Necropsy was performed on all animals, and at the same time, tissue samples (lymph nodes, spleen, tonsil, lung, liver, and kidney) and blood (EDTA, serum) were collected.

#### 2.2.2. Trial B

The study comprised five ASFV and ASFV antibody negative domestic weaner pigs (German Landrace × Large White) from a conventional farm weighing 20 to 25 kg. They were kept in the high containment facility (L3+) of the FLI. The animal experiment was approved by the competent authority (Landesamt für Landwirtschaft, Lebensmittlsicherheit und Fischerei (LALLF) Mecklenburg-Vorpommern, Rostock, Germany) under reference number LALLF 7221.3-2-011/19. Upon arrival, all animals were ear-tagged individually with numbers from 16 to 20. Over the course of the trial, the animals were fed a commercial pig feed with hay cob supplement and had access to water ad libitum. The animals were kept in one group and received species specific stable enrichment.

After an acclimatization phase, the animals were inoculated oro-nasally with 2 mL virus suspension containing $2 \times 10^{4.6}$ HAU/mL ASFV "Belgium 2018/1". The experiment was carried out until 9 days post-infection, when all animals had reached the humane endpoint as defined above. Upon inoculation, clinical scoring was performed as described for trial A. The same endpoint definitions were applied. Animals reaching the humane endpoint were euthanized through intracardial injection of embutramide (T61, Merck, Darmstadt, Germany) after deep sedation with tiletamine/zolazepam (Zoletil®, Virbac, Carros, France), ketamine (Ketamin 10%, Medistar, Ascheberg, Germany) and xylazine (Xylavet® 20 mg/mL, CP-Pharma, Burgdorf, Germany) or ketamine (Ketamin 10%, Medistar, Ascheberg, Germany) and azaperone (Stresnil™ 40 mg/mL, Elanco, Bad Homburg, Germany). Blood samples were collected prior to inoculation and at the day of euthanasia. At necropsy tissue samples (lymph nodes, spleen, tonsil, lung, liver, and kidney) and blood (EDTA and serum) were collected from all animals.

### *2.3. Virus Inoculum*

#### 2.3.1. Trial A

The inoculum, ASFV "Belgium 2018/1", was isolated by the Belgian national reference laboratory (NRL) for ASF at Sciensano from a wild boar carcass found in the Belgian municipality Etalle (Luxembourg region) [3]. The isolate belongs to genotype II and is closely related to strains circulating in eastern Europe [4] and beyond.

For the animal trial, cell culture supernatant was prepared on porcine peripheral blood monocytic cells (PBMCs) with a final titer of approximately $1 \times 10^{4.3}$ HAU/mL. The titer was confirmed by an end-point back titration of the inoculum and calculated using the Reed and Muench method [10]. Titers were expressed as the amount of virus causing hemadsorption in 50% of infected cultures (HAU 50/mL).

2.3.2. Trial B

The inoculum, ASFV "Belgium 2018/1", was shipped from the Belgian NRL for ASF at Sciensano to the NRL for ASF in Germany at the FLI. For animal trial B, culture supernatant was prepared with a final titre of approximately $1 \times 10^{4.6}$ HAU/mL. The titre was confirmed by an end-point back titration of the inoculum and calculated as described in trial A.

*2.4. Cells for Virus Titration*

2.4.1. Trial A

All virus titrations and haemadsorption tests were carried out using PBMC-derived macrophages according to the protocol of the European Union Reference Laboratory for ASF in which harvesting of the PBMC by buffy coat had been replaced by separation on SepMate™ PBMC Isolation Tubes (STEMCELL Technologies, Vancouver, BC, Canada) with Ficoll-Paque™ PLUS Media (GE Healthcare, Chicago, IL, USA).

2.4.2. Trial B

All virus titrations and hemadsorption tests were carried out using PBMC-derived macrophages. PBMCs were obtained and treated as previously described [11].

*2.5. Pathology*

2.5.1. Trial A

Full autopsy was performed on all subadult domestic pigs infected with the ASFV strain "Belgium 2018/1". Pigs were investigated macroscopically, and all lesions documented.

2.5.2. Trial B

Full autopsy was performed on all domestic weaner pigs infected with the ASFV strain "Belgium 2018/1". Pigs were evaluated macroscopically according to a scoring system published by Galindo-Cardiel et al. [12] with slight modifications [6].

*2.6. Processing of Samples*

2.6.1. Trial A

Serum samples were obtained from native blood through centrifugation at 2.500× *g* for 20 min at 20 °C. Aliquots were stored at −80 °C until further use.

Tissue samples were collected during necropsy and stored at −80 °C. Fragments of about 100 mg tissue were homogenized in 1 mL phosphate-buffered saline (PBS) with 2 metal beads using a TissueLyser II (Qiagen® GmbH, Hilden, Germany) for 2 × 2 min at 25 Hz before nucleic acid extraction.

2.6.2. Trial B

Serum samples were obtained from native blood through centrifugation at 2.500× *g* for 20 min. Aliquots were stored at −80 °C until further use.

Tissue samples were cut into pea-sized fragments during necropsy and were stored at −80 °C for future use. One fragment was homogenized in 1 mL phosphate-buffered saline (PBS) with a metal bead using a TissueLyser II (Qiagen® GmbH, Hilden, Germany) for 3 min at 30 Hz before virus isolations (haemadsorption tests) and qPCRs were performed.

*2.7. Pathogen Detection—Nucleic Acid Extraction and Real-Time PCR*

2.7.1. Trial A

Detection of viral genome was done in blood, serum, and tissues using real-time PCR (qPCR). For qPCR, viral nucleic acids were extracted from blood and serum using the IndiMag Pathogen Kit (Indical Bioscience, Leipzig, Germany) on the Indimag48® extraction platform (Indical Bioscience, Leipzig, Germany) and for tissue, the High Pure Viral Nucleic Acid Kit (Roche Applied Science, Penzberg, Germany) was used. All qPCRs were performed using the primers and probes for ASFV and endogenous gene beta-actin published by Tignon et al. [13] in AgPath-ID™ One-Step RT-PCR Master mix (Applied

Biosystems, Foster City, USA) as described in Schoder et al. [14]. All PCRs were performed using a LC480® cycler (Roche, Basel, Switzerland). Results of the qPCR were recorded as quantification cycle (Cq) values. Using a dilution series of an in-house ASFV DNA standard, the genome copies in the respective samples were determined. For generation of the ASFV standard, p72 gene from Lisbon60 strain (genotype 1) was amplified and cloned in pCR2.1 (Invitrogen, Carlsbad, CA, USA) for further multiplication in competent *E. coli*. The plasmid was extracted with Plasmid Plus Maxi Kit (Qiagen, Hilden, Germany) and linearized by restriction with BamHI. Subsequently, the DNA concentration was determined by spectrophotometry using a Nanodrop 2000 c (Thermo Fisher Scientific, Waltham, MA, USA) and the exact number of DNA molecules was calculated using an online tool (http://www.molbiol.edu.ru/eng/scripts/0107.html (accessed on 25 March 2020).

#### 2.7.2. Trial B

Prior to real-time PCR analysis, viral nucleic acids from all samples were extracted using the NucleoMag VET kit (Macherey-Nagel, Düren, Germany) on the automated KingFisher 96 flex platform (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's recommendations. Subsequently, nucleic acids were analyzed using the qPCR protocols published by King et al. [15] and Tignon et al. [13] on a Biorad CFX real-time cycler (Bio-Rad Laboratories, Hercules, CA, USA). For each qPCR, a quantification cycle (Cq) value was determined. Using a dilution series of the same standard as described in trial A, which was provided by Sciensano (Belgium), the genome copies in the respective samples were determined.

### *2.8. Antibody Detection*

#### 2.8.1. Trial A

Sera were tested in three commercially available antibody ELISAs. In detail, ASFV p72-specific antibodies were detected using the INGEZIM PPA COMPAC ELISA (Ingenasa, Madrid, Spain), the ID Screen ASF competition ELISA (IDVet, Grabels, France) for antibodies against p32, and the ID Screen® African Swine Fever Indirect (IDVet, Grabels, France) for antibodies against p32, p62 and p72. The tests were carried out according to the manufacturer's instructions.

In addition, serum samples were tested in the indirect immunoperoxidase test according to the standard protocols provided by the European Union Reference Laboratory for ASF with slight modifications regarding the virus strain (Lisbon60 ASFV strain adapted on Vero cells). Over the study period, results were recorded in a qualitative way (positive/negative). Sera taken upon necropsy were end-point titrated in log2 steps to obtain semi-quantitative antibody titers.

#### 2.8.2. Trial B

Serum samples were tested as described for trial A. Slight modifications concerned the virus strain. Here, a cell culture adapted variant of genotype II ASFV "Armenia 2008" was used for the indirect immunoperoxidase test.

### *2.9. 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 graph creation.

## 3. Results

### *3.1. Clinical Findings*

#### 3.1.1. Trial A

Following nasal inoculation, all animals developed unspecific clinical signs starting from day 4 post inoculation (pi) (see Figure 1). The signs included general depression,

lack of appetite and unwillingness to stand up, reduced mobility, tremor, reddened skin, cyanosis on the ears and snout, hunched-up back, and respiratory distress. The highest score was reached at 8 and 9 dpi with a maximum of 7 points.

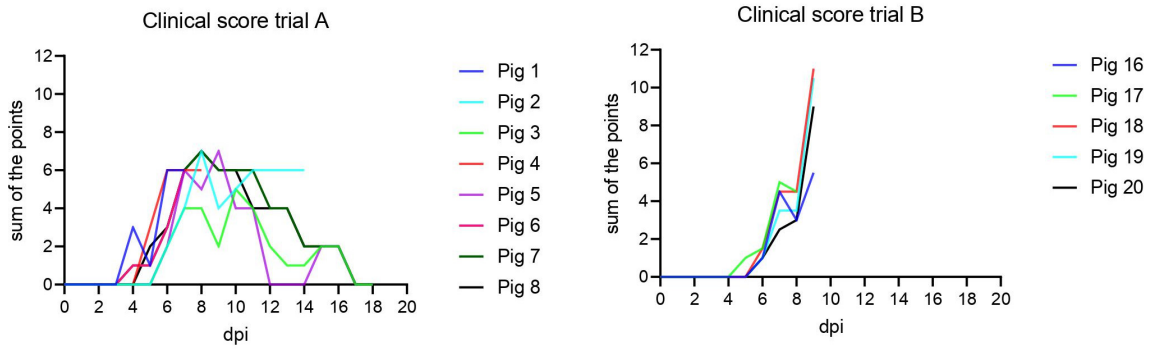


**Figure 1.** Clinical curve of trials A and B compared. In trial A, the maximum score of the animals was a sum of 7 points at 8 and 9 dpi compared to trial B, where animals reached scores of up to 11 points at 9 dpi.

The onset of fever was observed as early as 4 dpi and the climax of the illness was observed around 8–9 dpi (see Figure 2). The peak of fever was observed at 7 and 8 dpi with the highest number of severe feverish animals. Afterwards the intensity of the fever decreased but remained present in a mild form until 14 dpi. No temperature increase was observed during the infection period for animal #3 despite the presence of other clinical signs. The average number of days with fever was as follows: for severe fever 2.75 +/− 2.25 and for mild fever 5.5 +/− 3.85.

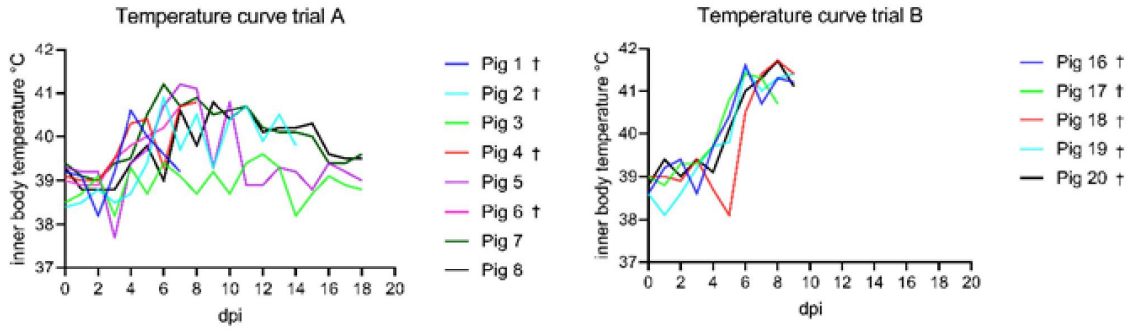


**Figure 2.** Temperature curves of trials A and B compared. In general, subadult animals of trial A showed lower temperatures compared to the weaner group of Trial B. The maximum temperature recorded in trial A was 41.2 °C at 7 dpi. In trial B, the highest temperature was 41.7 °C at 8 dpi. Animals that showed an acute lethal disease course and reached the humane end point (or died) are marked with a cross.

Ethical euthanasia was performed between 7 and 14 dpi on animals with clinical scores between 6 and 7 and showing persistent severe fever (3 subsequent days) and/or very poor reactivity. Starting from 12 dpi, the remaining animals started to recover. At the end of the experiment (18 dpi), the surviving pigs, four out of eight, presented no more clinical signs.

3.1.2. Trial B

Following oronasal inoculation, all animals developed severe, unspecific clinical signs starting from day five pi (see Figure 1). The signs included general depression, lack of appetite and mobility, hunched-up back, ataxia, and respiratory distress. The onset of fever

was observed at 5 dpi (see Figure 2). On 9 dpi, all animals reached the humane endpoint except one (pig 17), which died overnight. The clinical scores ranged from 5.5 up to 11.

The survival curve from both animal trials is presented in Figure 3.

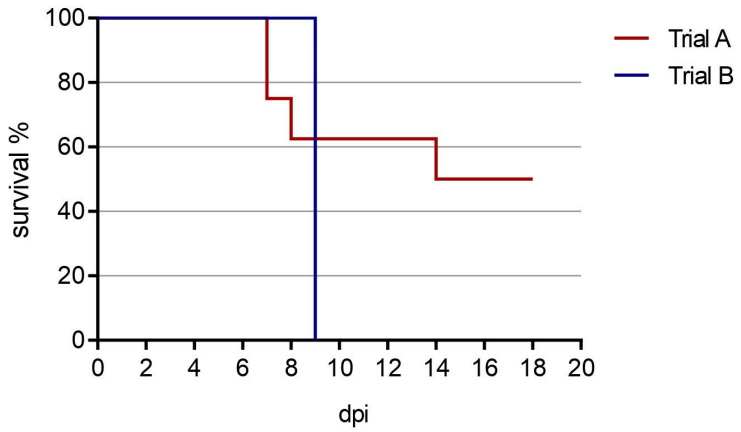


**Figure 3.** Survival curve from both animal trials. In trial A, four out of eight pigs survived until the end of the experiment at 18 dpi. In trial B, all animals were euthanized by 9 dpi (reaching the humane endpoint). One animal died spontaneously in the night from 8 to 9 dpi.

### *3.2. Pathomorphological Findings*

#### 3.2.1. Trial A

Animals were euthanized either during the experiment for ethical reasons (severe fever for more than 3 consecutive days or high clinical score or poor health condition) or at the end of the experiment (18 dpi). At necropsy, various pathological patterns were observed: from asymptomatic to typical ASF lesions of varying severity: generalized hemorrhagic lymphadenopathy especially of the gastrohepatic lymph nodes, congestion of the spleen, and multiple hemorrhages in several organs, particularly in the kidneys (Table 1).

**Table 1.** Overview of pathological findings per animal from trial A.

| Animal | Day of Euthanasia | Cause | Major Pathological Observations |
|---|---|---|---|
| 1 | 7 dpi | humane endpoint | ▪ Hemorrhagic lymph nodes |
| 2 | 14 dpi | humane endpoint | ▪ Hemorrhagic lymph nodes<br>▪ Renal petechiae<br>▪ Congestion of spleen |
| 3 | 18 dpi | end of experiment | ▪ No evident lesion |
| 4 | 8 dpi | humane endpoint | ▪ Hemorrhagic lymph nodes<br>▪ Renal petechiae<br>▪ Congestion of spleen |
| 5 | 18 dpi | end of experiment | ▪ No evident lesion |
| 6 | 7 dpi | humane endpoint | ▪ Hemorrhagic lymph nodes<br>▪ Renal petechiae<br>▪ Congestion of spleen |
| 7 | 18 dpi | end of experiment | ▪ No evident lesion |
| 8 | 18 dpi | end of experiment | ▪ No evident lesion |

3.2.2. Trial B

Five ASFV “Belgium 2018/1” infected domestic pigs reached the humane endpoint at 9 dpi (n = 4) or died spontaneously (n = 1) and were submitted to necropsy. At gross pathologic investigation, all infected pigs revealed typical lesions indicative of ASF. All pigs showed severely hemorrhagic enlarged lymph nodes with the gastrohepatic and renal lymph nodes mainly affected. Renal petechiae were present in all pigs and were mainly confined to the renal cortex and to a lesser extent to the medulla. Four out of five pigs had ascites. Marked sanguineous effusion was present in two pigs, while the other two showed accumulation of serous fluid. Likewise, rather mild, serous to sanguineous pleural effusion was present in three animals. Bruises of variable size appeared in four pigs. In individual cases, mild to moderate perirenal and gall bladder wall edema, mucosal petechiae in the urinary bladder, bilateral cyanosis of the ears, mild multifocal pulmonary consolidation, and alveolar edema were observed (Table 2).

**Table 2.** Overview of pathological findings per animal from trial B.

| Animal | Day of Euthanasia | Cause | Major Pathological Observations |
|---|---|---|---|
| 16 | 9 dpi | humane endpoint | ■ Multiple bruises<br>■ Pulmonary consolidation<br>■ Hydroperitoneum<br>■ Renal petechiae<br>■ Enlarged, hemorrhagic lymph nodes |
| 17 | 9 dpi | died acutely over night | ■ Single bruise<br>■ Subcutaneous hematoma on chest<br>■ Pulmonary alveolar edema<br>■ Renal petechiae<br>■ Enlarged, hemorrhagic lymph nodes |
| 18 | 9 dpi | humane endpoint | ■ Cyanosis of ears<br>■ Hydrothorax<br>■ Hydroperitoneum<br>■ Pulmonary consolidation<br>■ Renal petechiae<br>■ Mucosal petechiae in urinary bladder<br>■ Enlarged, hemorrhagic lymph nodes |
| 19 | 9 dpi | humane endpoint | ■ Cyanosis of ears<br>■ Multifocal bruises<br>■ Hemothorax<br>■ Hemoperitoneum<br>■ Perirenal edema<br>■ Renal petechiae<br>■ Enlarged, hemorrhagic lymph nodes |
| 20 | 9 dpi | humane endpoint | ■ Single bruise<br>■ Hydrothorax<br>■ Pulmonary consolidation<br>■ Hemoperitoneum<br>■ Gall bladder wall edema<br>■ Renal petechiae<br>■ Enlarged, hemorrhagic lymph nodes |

*3.3. Pathogen Detection*

3.3.1. Trial A

Prior to infection, all animals were tested negative for ASFV nucleic acids by qPCR in blood samples. After infection, the presence of the virus was first detected in blood samples of 3 animals collected at 3 dpi. All remaining animals were detected ASFV PCR

positive at the following sampling time point (7 dpi), except one animal (#3) which became positive 10 dpi. Once detected positive, the animals remained positive until death by ethical euthanasia or at the end of the experiment (18 dpi).

When detected at 3 dpi, the virus load was low (<25 copies/run). However, at 7 dpi and until 14 dpi, the virus load in blood was at a maximum with $1.29 \times 10^3$ to $6.80 \times 10^4$ copies/reaction (Table 3).

**Table 3.** Trial A: real-time PCR results of blood from 0 to 18 dpi. Genome detection in blood is presented as genome copies per reaction (5 µL). nd: not detected; NA: due to technical and organizational problems samples could not be taken and analyzed. The color intensity indicates the relative level of viral loads (darker colors indicating higher loads, from light yellow to deep red).

| Animal | 0 dpi | 3 dpi | 7 dpi | 10 dpi | 14 dpi | 18 dpi |
|---|---|---|---|---|---|---|
| 1 | nd | 0.3 | 29,200 | | | |
| 2 | nd | nd | 1270 | NA | 4260 | |
| 3 | nd | nd | nd | 19 | 15 | 8 |
| 4 | nd | 0.7 | 15,100 | | | |
| 5 | nd | nd | 202 | 1290 | 353 | 284 |
| 6 | nd | 22 | 68,000 | | | |
| 7 | nd | nd | 401 | 1660 | 525 | 127 |
| 8 | nd | nd | 2810 | 2810 | 870 | 20 |

At the end of the trial, viral genome was detected in blood, serum, and all tissue samples (spleen, lung, lymph nodes, tonsil, kidney, and liver). Highest loads of viral genomes (>$1 \times 10^3$ to $1 \times 10^4$ copies/reaction) were found in blood, serum, and tissues from animals presenting severe clinical signs, whereas low viral genome loads (<500 copies/reaction) were detected in the blood of animals that survived the infection (Table 4).

**Table 4.** Trial A: real-time PCR results of blood, serum, and organ samples at the day of euthanasia. Genome detection in blood, serum, and organs are presented as genome copies per reaction (5 µL). The color intensity indicates the relative level of viral loads (darker colors indicating higher loads, from light yellow to deep red). nd: not detected.

| Animal | Day of Euthanasia | Blood | Serum | Spleen | Tonsil | Lymph Node | Lung | Liver | Kidney |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 7 dpi | 29,200 | 1140 | 37,100 | 21,000 | 33,300 | 20,100 | 13,200 | 734 |
| 2 | 14 dpi | 4260 | 595 | 4520 | 855 | 6600 | 820 | 2630 | 120 |
| 3 | 18 dpi | 8 | nd | 4 | 57 | 43 | 479 | 2 | 1 |
| 4 | 8 dpi | 15,100 | 1000 | 18,700 | 13,100 | 8760 | 9540 | 30,600 | 894 |
| 5 | 18 dpi | 248 | 0.2 | 5 | 514 | 132 | 5 | nd | 2 |
| 6 | 7 dpi | 68,000 | 15,200 | 43,000 | 56,700 | 37,000 | 59,600 | 224,000 | 13,800 |
| 7 | 18 dpi | 127 | 0.1 | 3 | 423 | 319 | 8 | 2 | 1 |
| 8 | 18 dpi | 20 | 0.02 | 10 | 102 | 7 | 6 | 4 | 3 |

#### 3.3.2. Trial B

Prior to inoculation, all animals were tested negative for ASFV, ASFV antigen, and viral nucleic acids.

At the end of the trial, viral genome was detected in blood and all tissue samples (spleen, lung, lymph nodes, tonsil, kidney, and liver). Highest loads of viral genome were found especially in blood, serum, and spleen (Table 5).

### *3.4. Antibody Detection*

#### 3.4.1. Trial A

Prior to inoculation, all animals were tested negative for ASFV antibodies (Ab) by ELISA tests.

After infection, seroconversion against ASFV p72 was demonstrated using the INGEZIM PPA COMPAC ELISA (Ingenasa, Madrid, Spain) in the five still remaining animals

at 10 dpi (two positive and three doubtful results) (Figure 4). With the ID Screen ASF Competition ELISA (IDVet, Grabels, France), which detects p32, all remaining animals were positive from 10 dpi, except one which already showed a questionable result at 7 dpi. By testing with the ID Screen ASF Indirect ELISA (IDVet, Grabels, France), three animals showed a questionable result at 10 dpi, one was negative, and one showed a positive antibody result from 10 dpi onwards. From 14 dpi and onwards, all four remaining animals were seropositive in all three ELISA assays.

**Table 5.** Trial B: real-time PCR results of blood, serum and organ samples at the day of euthanasia. Genome detection in blood, serum, and organs are presented as genome copies per run (5 µL). The color depth indicates the level of the viral load. From white to red indicates from low to high viral load.

| Animal | Day of Euthanasia | Blood | Serum | Spleen | Tonsil | Lymph Node | Lung | Liver | Kidney |
|---|---|---|---|---|---|---|---|---|---|
| 16 | 9 dpi | 10,200 | 2320 | 4510 | 164 | 42 | 122 | 359 | 49 |
| 17 | 9 dpi | 66,400 | 15,800 | 15,400 | 8560 | 9770 | 4520 | 48,600 | 3780 |
| 18 | 9 dpi | 103,000 | 22,500 | 14,000 | 1900 | 589 | 5810 | 22,000 | 707 |
| 19 | 9 dpi | 61,900 | 13,900 | 4610 | 3360 | 587 | 552 | 4040 | 500 |
| 20 | 9 dpi | 78,000 | 27,100 | 23,000 | 18,900 | 3120 | 5680 | 11,000 | 987 |

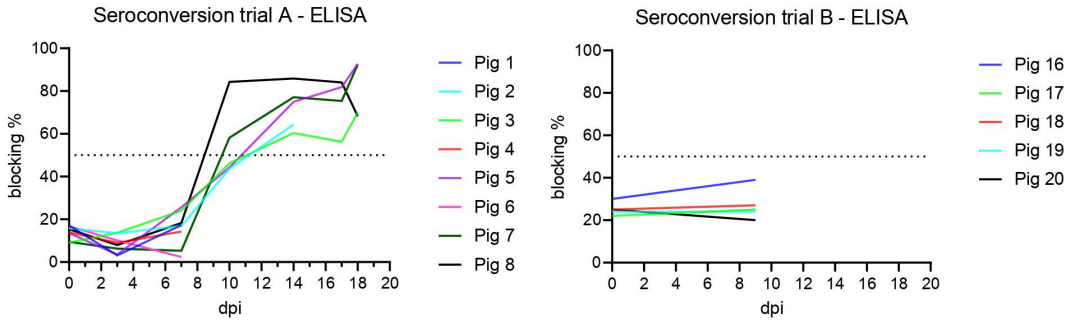


**Figure 4.** Seroconversion of the subadult animals from trial A detected on 10 dpi with INGEZIM PPA COMPAC ELISA (Ingenasa, Madrid, Spain). Weaner pigs from trial B showed no ELISA detectible seroconversion at 9 dpi. The ELISA cutoff is 50% blocking which is indicated as a dotted line in both graphs.

With the confirmatory indirect immunoperoxidase test, seroconversion was observed starting from 7 dpi (qualitative result). At the end of the experiment (18 dpi), the surviving animals presented antibody titers between 2560 and 5120 (semi-quantitative result).

#### 3.4.2. Trial B

No antibodies were detected in the sera prior to inoculation or at the end of the trial on 9 dpi with all ELISAs (Figure 4). With the indirect immunoperoxidase test, however, the animals showed semi-quantitative antibody titers between 160 and 620 at 9 dpi.

## 4. Discussion

African swine fever virus has entered the European Union and many countries in eastern Europe and Asia, threatening animal health and agriculture alike. In the absence of a licensed vaccine or other effective treatment options, prevention through farm biosafety and the earliest possible detection of outbreaks are of utmost importance. Early detection is only assured if clinical signs are recognized promptly and interpreted correctly by farmers and practitioners. Thus, well-trained livestock farmers and veterinarians in practice and subsequently the competent authorities play a crucial role here. Basic data to inform these key persons can be obtained from animal experimental work, such as that carried out in the National Reference Laboratories (NRLs) as part of their tasks. These studies are not

infrequently conducted to generate and archive relevant and well-characterized sample materials for validation and harmonization of diagnostic methods at the national level (Regulation (EU) 2017/625, Article 101), but provide space to map issues of pathogenesis and virulence of locally relevant viral variants. In this context, the present study was conducted in close cooperation with the Belgian and the German NRLs with the aim to further characterize the ASF virus isolate "Belgium 2018/1" in domestic pigs. Thereby, the focus was laid on the clinical courses and pathomorphological changes in different age classes of animals. While various studies with genotype II strains have been conducted in weaner pigs, studies with older animals are scarce. Although there are indications that no age dependency is involved in highly virulent strains [16], different courses have been described for moderately virulent strains [8]. In the study published by Post et al. [8], age had a marked effect on disease outcome, while the inoculation dose was secondary. The latter is consistent with studies describing similar, often lethal, courses with different doses of highly virulent virus strains [11].

In our combined study, inoculation of weaner pigs did not hold any surprise. After an incubation period of five days, which is in line with previous findings [17,18], all young animals developed an acute lethal disease course with high fever, general depression, anorexia, ataxia, and respiratory distress. All animals reached the humane endpoint or had died acutely by 9 dpi. It is noteworthy that again the clinical signs were severe but rather unspecific, leading to many differential diagnoses that could be relevant in the field. Necropsy findings were in line with previous studies using ASFV "Armenia 2008" [6]. Viral genome was found in organs, blood, and serum of all animals irrespective of the disease course. In line with previous studies, blood, spleen, and liver showed the highest copy numbers in the majority of animals. However, genome loads reflected the clinical course and timepoint of sampling. While viral genome loads in spleen were roughly 10,000 genome copies per run for animals sampled between days 7 and 9, less than 10 genome copies were found in recovering pigs at 18 dpi.

The inoculation of the older pigs took a different, rather unexpected turn. Although the Belgian virus hardly differs at the sequence level from the highly virulent strains from Armenia and Georgia [4], which showed rather age-independent courses in previous studies [16], only a 50% lethality was recorded in the present study until the end of the experiment at 18 dpi. Clinical signs were milder and pathomorphological findings reflected the clinical course (severe to absent lesions). It must be mentioned, however, that the capacity-related observation period of 18 days limits a reliable and complete statement on the final clinical outcome. Previous studies have shown intermittent and recurring viremia after 18 dpi [2]. In addition, it cannot be excluded from the clinical scoring and virus detection that at least one animal (#3) got infected only by contact (not following inoculation) and thus was slightly delayed. Nevertheless, the lack of findings in the pathological-anatomical examinations and low genome loads in organs indicate that the remaining animals, including animal #3, were true survivors or showed only a sub-clinical infection due to the lack of lesions. Survivors were rarely seen when using highly virulent ASFV strains of genotype II; however, they were reported. As an example, Gallardo et al. [2] reported on the survival of one out of eight pigs inoculated intramuscularly with the Lithuanian ASFV strain "LT14/1490". The respective pig showed weak and intermittent peaks of viremia, and viral DNA could be detected in tissues at 38 dpi. However, no seroconversion was observed, which was different in our study where all survivors seroconverted. Animals surviving the acute phase were also reported by Walczak et al. [9]. In this study, two animals inoculated with the Polish ASFV "Pol18_28298_O111" strain (pig one got 1000 HAU and pig two got 500 HAU) developed chronic disease courses after a delayed incubation period (pig one 12 dpi: clearly visible clinical signs, like joint swelling and minor breathing disorders, moderate fever, and constant low virus load value in blood but without pathological lesions. Pig two 20 dpi: showed only moderate fever and enlarged submandibular lymph nodes) but had to be euthanized 24 and 32 dpi, respectively. While it is obvious that animals may survive, the long-term fate and epidemiological role

of these survivors are still discussed controversially and need further long-term studies to allow final conclusions. While survivors may eventually recover completely, longer term virus excretion will impact on transmission dynamics. In a previous long-term study with a moderately virulent genotype I strain, virus was isolated from recovering pigs up to day 63 post infection [19].

Apart from age, application route and dose, the general health and immune status, genetic background (hybrid breed), and concomitant infections could impact on the clinical outcome and thus explain our observations. Indeed, due to the size of the sub-adult pigs, inoculation had to happen in standing position rather than in dorsal recumbency, which was used for the weaner pigs. This could have influenced the amount of virus that reached the tonsils. Adding to that, the dose is reduced when compared to body weight. However, as mentioned earlier, several studies showed severe courses upon low-dose infection and did not report a significant impact of the viral dose on the final outcome [2,9,11]. Whether an even lower dose would have led to more survivors or just less infected pigs could be debated. Considering the study reported by Pietschmann et al. [11], we could rather expect lower infection rates. The route itself can also play a role in the efficiency of infection. While trial A was conducted with nasal inoculation, trial B was done with oro-nasal inoculation. In this context, Howey et al. [20] reported on the variable efficiency of intranasopharyngeal (INP) and intraoropharyngeal (IOP) inoculations, especially using lower doses ($10^2$ HAU). In this study, oropharyngeal infection was less efficient. However, infection was confirmed in all animals of our study upon either nasal or oral infection. Given that the full range of clinical outcomes and swift seroconversion in survivors were seen, a dose-related impact cannot be excluded but does not seem likely. Considering that both studies employed clinically healthy, commercial pigs, the impact of the general health status seems rather small.

With regard to the genetic background, differences in susceptibility are seen rather frequently in indigenous pig breeds in Africa [21,22]. Yet, our study involved only widely used pig breeds of Europe that may not differ markedly in their susceptibility, even if a different hybrid breed was used in trial A than in trial B. As genotyping of pigs was not carried out, no final conclusion on this aspect can be drawn.

Recently, the gut microbiota was discussed as an important factor for ASF susceptibility. Interestingly, fecal microbiota transplantation from warthogs to domestic pigs resulted in higher resistance of the latter [23]. While we cannot rule out such factors, we do not assume a high impact of the gut microbiota in our study.

The variability of clinical signs and outcomes in our study points to both a certain age-dependency and biological variability that has to be taken into account when communicating clinical signs and typical disease outcomes.

## 5. Conclusions

Taken together, we saw a variable clinical picture when considering all age classes of animals that could cause problems in the clinical evaluation of ASF under field conditions and in early warning scenarios. The final fate of the here observed survivors could not be addressed under the limited time scheme of our study and should be part of further long-term studies with older pigs.

The outcome of our study highlights the need for swift and reliable laboratory diagnosis, even when only mild to moderate clinical signs are detected. Easy and fast genome detection by qPCR did not pose any problems even with the surviving animals.

We hope that communication of the available data will help practical and official veterinarians in the field to detect ASF as early as possible and thus minimize its impact. The study outcome clearly underlines once again that clinical courses can be highly variable and non-specific. For this reason, exclusion diagnostics of ASF by sensitive qPCR methods should be routine, especially in light of the current situation with ASF outbreaks in several countries including Germany.

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**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) under reference number 7221.3-2-011/19. Experiment at Sciensano has been authorized by the Ethical Commission of Sciensano under N°20190614-01 and approved by the Biosafety commission.

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

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

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

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

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**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



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## 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.

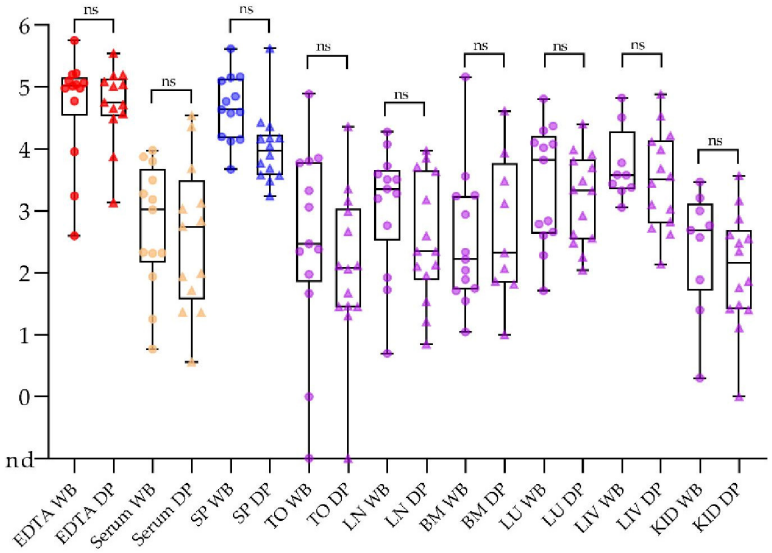


**Figure 1.** Comparison of sample matrices taken from wild boar (WB; dots) and domestic pigs (DP; triangles). The qPCR results are depicted as $\log_{10}$ genome copy numbers per run. Abbreviations: nd = not detected; SP = spleen, TO = tonsil, LN = lymph node, BM = bone marrow, LU = lung, LIV = liver, 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 $4.2 \times 10^5$ 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%.

*Pathogens* **2021**, *10*, 177 

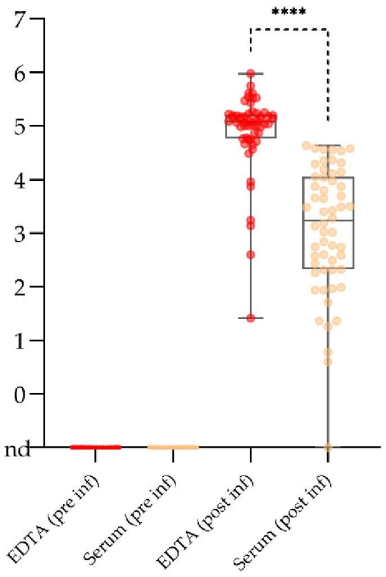


**Figure 2.** Overall comparison of $\log_{10}$ genome copy numbers in EDTA blood and serum (prior to infection = pre inf; post infection = post inf). The boundaries of the boxes indicate the 25th and 75th percentiles, the line within the box marks the median. Whisker boundaries indicate minimum and 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.

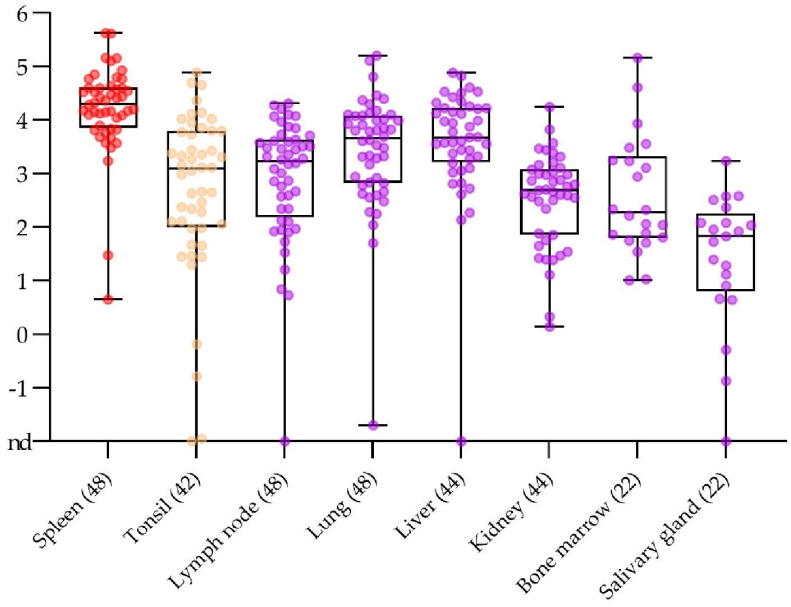


**Figure 3.** Comparison of $\log_{10}$ genome copy numbers per run in different organs over the entire data set. The numbers in brackets indicate the number of animals included for the respective matrix. All samples are individually depicted together with the box plot. The boundaries of the boxes indicate 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 $1.8 \times 10^6$ per run. Comparative data are depicted in Figure 4.

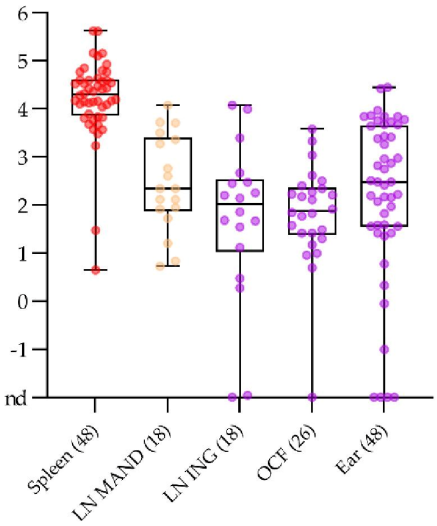


**Figure 4.** Comparison of $\log_{10}$ genome copy numbers per run in spleen, inguinal lymph nodes (LN ING), and mandibular lymph nodes (LN MAND), ocular fluids (OCF), and ear punches (ear). All samples are individually depicted together with the box plot. The boundaries of the boxes indicate the 25th 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 $3 \times 10^4$ with a mean of $3.5 \times 10^3$). 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 $3.8 \times 10^2$). 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).

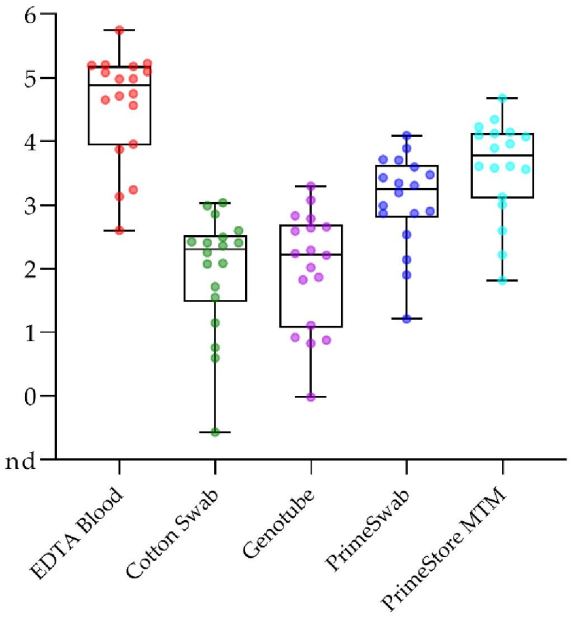


**Figure 5.** Comparison of genome copy numbers in different swab options and EDTA blood as comparator. Samples were taken from wild boar (WB) and domestic pigs (DP) over the entire time of the experiment. All samples are individually depicted together with the box plot. The boundaries of the boxes indicate 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, $1.5 \times 10^5$ 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 ($5.6 \times 10^4$ 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 $2 \times 10^5$ 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 $2 \times 10^5$ 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.

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

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# The Role of Male Reproductive Organs in the Transmission of African Swine Fever—Implications for Transmission

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# The Role of Male Reproductive Organs in the Transmission of African Swine Fever—Implications for Transmission

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**Abstract:** African swine fever (ASF) has evolved from an exotic animal disease to a threat to global pig production. An important avenue for the wide-spread transmission of animal diseases is their dissemination through boar semen used for artificial insemination. In this context, we investigated the role of male reproductive organs in the transmission of ASF. Mature domestic boars and adolescent wild boars, inoculated with different ASF virus strains, were investigated by means of virological and pathological methods. Additionally, electron microscopy was employed to investigate in vitro inoculated sperm. The viral genome, antigens and the infectious virus could be found in all gonadal tissues and accessory sex glands. The viral antigen and viral mRNAs were mainly found in mononuclear cells of the respective tissues. However, some other cell types, including Leydig, endothelial and stromal cells, were also found positive. Using RNAScope, p72 mRNA could be found in scattered halo cells of the epididymal duct epithelium, which could point to the disruption of the barrier. No direct infection of spermatozoa was observed by immunohistochemistry, or electron microscopy. Taken together, our results strengthen the assumption that ASFV can be transmitted via boar semen. Future studies are needed to explore the excretion dynamics and transmission efficiency.

**Keywords:** African swine fever; male reproductive tract; pathogenesis; virus detection; histopathology; venereal transmission



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## 1. Introduction

African swine fever (ASF) is one of the most complex viral diseases affecting livestock and has tremendous socio-economic impact [1]. This impact, and its potential for transboundary spread, has led to its inclusion in the list of notifiable diseases. Over the last 14 years, ASF has evolved from an exotic animal disease to a threat to global pig production and endangered wild suids, now affecting Europe, Asia, Oceania, and first countries in the Americas, in addition to Africa (https://www.oie.int/en/disease/african-swine-fever/, accessed on 15 October 2021).

The roots of ASF lie in sub-Saharan Africa, where ASF virus (ASFV), the sole member of the *Asfarviridae* virus family, is transmitted in an ancient sylvatic cycle among warthogs and the soft ticks of genus *Ornithodoros* [2]. This cycle is not accompanied by overt disease or mortality in warthogs, and would probably go unnoticed. However, any introduction of the disease into the domestic pig sector via ticks or fomites leads to a severe systemic disease that can resemble a viral hemorrhagic fever, with exceptionally high lethality (over 90% of infected animals die). Once the disease has left the sylvatic cycle, the competent arthropod vector is no longer required to sustain infection chains, and the disease is transmitted directly and indirectly among susceptible suids [3].

An important avenue for the wide-spread transmission of animal diseases is the dissemination of viruses through boars and boar semen, as artificial insemination is now practiced on a vast majority of all sows inseminated in countries with industrialized pig production [4]. Thus, the impact of contaminated semen multiplies, with the widespread distribution of semen from centralized boar studs [5].

Little is known regarding the involvement of the male genitals in ASFV infection and its potential transmission via semen. Thacker et al. [5] cite that ASFV was isolated from semen from an experimentally infected viraemic boar, and that transmission occurred to a recipient female. This is in line with our previous study, that demonstrated the viral genome and virus in all relevant gonadal tissues of wild boar infected with a Belgian ASFV strain [6]. Immunohistochemistry of the male gonads revealed ASFV positively labelled cells identified as macrophages, endothelial cells and peritubular fibroblasts based on the cellular phenotype. Inflammatory changes and vasculitis/vasculopathy were observed.

To further elaborate on these data, we took advantage of the opportunity provided by a study to characterize two African ASFV isolates in sexually mature boars. Moreover, we could investigate samples taken from adolescent wild boar in an experiment with a German ASFV strain from Saxony.

We turned to the detectability of ASFV in the reproductive organs and accessory sex glands. In addition to the virological and molecular detection of the virus and its genome, we investigated the distribution of the virus by immunohistochemistry and in situ hybridization.

Furthermore, in vitro inoculation of commercial boar semen was carried out followed by electron microscopic investigations to establish whether ASFV is found in the spermatozoa themselves.

## 2. Materials and Methods

### *2.1. Experimental Design*

#### 2.1.1. Trial A

This study part included six seven-month-old intact domestic crossbred boars, kept in the high-containment animal facilities at the Friedrich-Loeffler-Institut (FLI), Greifswald-Insel Riems, Germany. In the animal experiment, all applicable animal welfare regulations including EU Directive 2010/63/EC and institutional guidelines were taken into consideration. The animal experiment was approved by the competent authority (Landesamt für Landwirtschaft, Lebensmittelsicherheit und Fischerei (LALLF) Mecklenburg-Vorpommern) under reference number 7221.3-2-011/19. The choice of virus strains reflected the main purpose of this study, i.e., reference material acquisition and characterization of virus strains.

The individually ear-tagged boars were randomly divided into two separate quarantine pens and received seven days to acclimate upon arrival. Subsequently, each group (n = 3) was inoculated intramuscularly, with approximately $1 \times 10^3$ hemadsorbing units 50% ($HAU_{50}$), of either ASFV isolate "KAB 6/2" or "SUM 14/11", respectively (for details see Section 2.2). Over the study period of eight days, all animals were monitored daily, based on a modified clinical scoring system that assigns severity points to common alterations, e.g., reduced liveliness or feed intake [7]. The clinical assessment was complemented by the recording of rectal body temperature profiles. An elevated temperature was defined as a body temperature $\geq$40.0 °C. A cumulative clinical score of 15 score points or unjustifiable suffering, as assessed by a veterinarian, were set as humane endpoints. Upon termination of the study, the animals were sacrificed by exsanguination in deep anesthesia with a combination of tiletamine/ zolazepam (Zoletil®, Virbac), xylazine (Xylazin 20 mg/mL, medistar, Ascheberg, Germany), and ketamine (Ketamin 100 mg/mL, cp-pharma, Burgdorf, Germany). Subsequently, all animals were subjected to a full necropsy, and macroscopic findings were assessed following the protocol by Galindo-Cardiel [8], with slight modifications [9]. Blood and the following tissues were sampled for subsequent investigations and reference material acquisition: spleen; lung; lymph nodes; liver; kidney; skin; tonsil; testis; epididymis; epididymal sperm; vesicular

gland; bulbourethral gland, and prostate. Epididymal sperm was obtained by flushing the ducts of the cauda epididymidis with phosphate-buffered saline (PBS) after dissecting the spermatic cord, according to a modified protocol as previously published [10].

#### 2.1.2. Trial B

Eight adolescent male wild boar, aged roughly five months upon enrolment in the study, were investigated using virological techniques. Given the uniform result, only three representative animals underwent subsequent histopathology and immunohistochemistry. The wild boar originated from the breeding unit at the FLI, and were individually ear-tagged upon transfer to the high-containment facility. The animals were part of the mock-inoculated group in a vaccination/challenge trial. The initial animal experiment was approved by the competent authority (Landesamt für Landwirtschaft, Lebensmittelsicherheit und Fischerei (LALLF) Mecklenburg-Vorpommern) under reference number 7221.3-1-035/21.

After acclimation, the animals were immobilized with tiletamine/ zolazepam (Zoletil®, Virbac), administered via blowing pipe, to conduct blood sampling and oro-nasal inoculation with 3 mL of a spleen suspension containing a highly virulent ASFV strain isolated in Germany (Saxony 2020, for details see Section 2.2). Clinical scoring and necropsy were conducted as described above; however, rectal body temperatures were only assessed at the endpoint. Given the younger age of the animals, epididymal sperm could not be obtained.

### *2.2. Viruses*

Two Zambian ASFV isolates [11], kindly provided by Dr Christopher Netherton (the Pirbright Institute, Pirbright, UK), were used in the framework of trial A. The ASFV isolate "KAB 6/2" was initially obtained from an Ornithodoros soft tick collected from warthog burrows in Central Kafue National Park, Zambia, which, according to its capsid protein p72 encoding region, belongs to genotype (gt) XI. The ASFV isolate "SUM 14/11" was extracted from a soft tick originating from Sumbu National Park, Zambia, and is considered as gt XIII [12]. Virus stocks were grown and titrated on porcine peripheral monocytic cell (PBMC)-derived macrophages, as previously described [13]. For inoculation purposes, virus suspensions were diluted in phosphate-buffered saline (PBS) to a titer of $10^3$ $HAU_{50}$ per mL. The actual virus doses applied were determined by back titration of the inocula.

The ASFV strain "Germany 2020" was isolated from the spleen of a wild boar piglet found dead in the Federal State of Saxony, District of Goerlitz, municipality Krauschwitz. The virus belongs to gt II (German variant VI, ASFV/GER/2020/WB/IV_SN) and shows very high identity with other epidemic strains in Europe and Asia. The virus was administered at a dose of $10^4$ $HAU_{50}$ per mL.

For in vitro inoculation of sperm, the recombinant and wild boar lung cell-adapted strain ASFV "ArmeniaΔ285LGFPhuCD4" was used, as recently described [14].

### *2.3. Cells*

For virus isolation and titration, PBMCs were derived from the EDTA blood of healthy donor pigs and isolated by overlaying on Ficoll-Paque density gradient medium (GE Healthcare Life Sciences, Uppsala, Sweden). The erythrocyte fraction was removed and stored at +4 °C for subsequent use in hemadsorption tests. Following further washing and centrifugation steps of the PBMC fraction, cell count was set to $5 \times 10^6$ cells per ml. Cells were suspended in RPMI 1460 medium supplemented with 10 % bovine serum, 0.01% β-mercaptoethanol, penicillin, streptomycin and amphotericin B (Gibco TM Antibiotic-Antimycotic, Thermo Fisher Scientific, Bleiswijk, The Netherlands). Finally, 96-well plates ($5 \times 10^5$ cells/well) and 24-well plates ($2.5 \times 10^6$ cells/well) were prepared. After an incubation period at a humidified atmosphere of 5% $CO_2$ and 37 °C for 24 h, culture medium was renewed and granulocyte macrophage colony-stimulating factor (GM-CSF) was added at 2 ng/mL. Following another overnight incubation, cells were used for downstream laboratory tests.

### *2.4. Processing of Samples*

For the purpose of the presented study, samples of the blood, spleen, testis, epididymis, epididymal semen, vesicular gland, prostate, and bulbourethral gland were processed in triplicates regarding Trial A. In the case of Trial B, equal sampling proceeded while samples were processed uniquely for qPCR and RT-qPCR, and duplicated for virus isolation.

All tissue samples were cut into lentil-size pieces (~100 mg). The pieces were homogenized in 2 mL reaction tubes, each containing a 5 mm metal bead and PBS (1000 µL) using a TissueLyser II (Qiagen) at 30 Hz for 3 min. Epididymal sperm was diluted 1:1 in PBS for usage in the quantitative polymerase chain reaction (qPCR). EDTA blood was aliquoted and, along with homogenates, stored at −20 °C until further use. For further histopathological processing, tissues were fixed in 10% neutral-buffered formalin for at least three weeks.

### *2.5. Pathogen Detection*

#### 2.5.1. Quantitative Polymerase Chain Reaction (qPCR)

Nucleic acids were extracted from EDTA blood, diluted epididymal sperm and homogenate supernatants (100 µL per sample) using the NucleoMag® VET kit (Macherey-Nagel, Düren, Germany) on the King Fisher 96 flex platform (Thermo Fischer Scientific) according to the manufacturer's instructions. To validate the PCR reaction, a universal heterologous internal control DNA [15] was added to all samples and co-extracted. Subsequently, viral nucleic acids (p72 encoding region) were detected following the qPCR protocol published by King et al. [16]. All qPCRs were performed on a Bio-Rad C1000TM thermal cycler with the CFX96TM Real-Time System (Bio-Rad, Hercules, CA, USA).

Results of qPCR were initially recorded as quantification cycle (Cq) values. Using a dilution series of a full ASFV DNA standard, the genome copies in the respective samples were estimated [17].

#### 2.5.2. Reverse Transcription qPCR (RT-qPCR)

With the aim to detect viral p72 mRNA, RT-qPCR was performed following stringent RNA extraction using TRIzol pre-treatment, in combination with the above detailed magnetic bead-based automatic extraction [18]. To eliminate residual DNA, the extracted samples were treated with DNase employing the TURBO DNA-free™ kit (Thermo Scientific) according to the manufacturer's instructions. Subsequently, RT-qPCR was performed using the QuantiTect® Probe RT-PCR kit (Qiagen, Hilden, Germany) with the primers and probes designed by Tignon et al. [19]. To control for DNA-contamination, each extracted sample also underwent qPCR simultaneously. Positive results in RT-qPCR were only recorded if the qPCR gave a negative result.

#### 2.5.3. Detection of Infectious Virus (Virus Isolation)

To detect the infectious virus in the blood, spleen, male reproductive tissues, and epididymal sperm, virus isolation and hemadsorption tests were carried out according to slightly modified standard procedures [20] on mature PBMC cultures. To this means, EDTA blood and epididymal sperm were diluted 1:10 in PBS. All other samples were used as homogenates, as described above. In a first blind passage, the PBMC cultures were inoculated in duplicate with 200 µL of the respective materials per well, on a 24-well plate. Following an adsorption time of 2 h at 37 °C, in an incubator with a humidified atmosphere (5% $CO_2$), cells were gently washed with lukewarm PBS and the medium was renewed. Following incubation (72 h, 37 °C, 5% $CO_2$), the plates underwent a freeze/thaw cycle. The resulting culture supernatants were subjected to a routine hemadsorption test, as previously described [21]. Following the same protocols, endpoint virus titrations were carried out for culture supernatants from epididymal sperm.

### *2.6. Histopathology*

Formalin-fixed tissue was trimmed, embedded in paraffin wax, and cut at 2–3 µm thick slices. Tissue sections were further processed for the detection of viral antigens by immunohistochemistry (IHC), for the detection of viral mRNA by RNAScope in situ hybridization (ISH), and for pathomorphological analysis.

#### 2.6.1. Immunohistochemistry (IHC) and Semi-Quantitative Scoring of Viral Antigens

Viral antigen detection was performed following the protocol published recently [9], using an in-house rabbit polyclonal primary antibody against the major capsid protein p72 of ASFV. Cells were evaluated positive when showing a fine granular cytoplasmic signal. Histological specimens were analyzed using a Zeiss AXIO Scope A1 microscope with four objectives, allowing magnification between 2.5× and 40×. For immunohistochemical analysis, the area with the highest viral antigen load was selected and scored per high power field, as follows: no antigen (0); 1–3 positive cells (1); 4–15 cells (2); and ≥16 cells (3).

#### 2.6.2. RNAScope In Situ Hybridization (ISH)

In order to detect the replication of the virus beyond immunohistochemistry, RNA in situ hybridization (ISH) was performed on tissues obtained from the domestic boars with RNAScope 2–5 HD Reagent Kit-Red (ACD, Advanced Cell Diagnostics, Newark, CA, USA), according to the manufacturer's instructions. RNAScope® probes were designed by ACD against p72 mRNA. A positive probe expressing the housekeeping gene peptidylprolyl isomerase B (cyclophilin B, ppib) and a negative bacterial probe expressing dihydrodipicolinate reductase (DapB) were used to verify the sensitive detection of the target mRNA. To eventually compare results after immunohistochemistry and RNAScope in situ hybridization, tissue sections were scored accordingly on a 0–3 scale, as described above.

#### 2.6.3. Dual RNAScope ISH and Immunofluorescence

To identify ASFV-infected cells, double labeling with RNAScope ISH combined with immunofluorescence staining was performed. RNAScope ISH was followed by incubation with a rabbit polyclonal anti-Iba-1 antibody (dilution 1:500, incubation overnight) (FUJIFILM Wako, Madison, WI, USA). Sections were then treated with a goat anti-rabbit secondary antibody Alexa 488 (Invitrogen, Thermo Fisher Scientific) (dilution 1:1000, incubation 1 h). Cellular nuclei were visualized with Hoechst (dilution 1:5000, incubation 15 min) and sections were mounted with Aquatex (MerckKGaA, Darmstadt, Germany).

#### 2.6.4. Pathomorphology and Semi-Quantitative Scoring of Lesions

Pathomorphological changes were assessed on hematoxylin and eosin-stained tissue sections, following a standard protocol as described earlier [9]. Pathohistological changes scored on a 0–3 scale (normal (0); mild (1); moderate (2); severe (3)), are shown in Table 1.

### *2.7. Transmission Electron Microscopy*

For transmission electron microscopy (TEM) analysis, $1 \times 10^8$ sperm cells suspended in Tyrode's albumin lactate pyruvate media [22] were inoculated with ASFV "ArmeniaΔ285L-GFPhuCD4" [14], gt II (MOI 5). Sperm cells were incubated with the virus suspension (48 h, 37°C, 5% $CO_2$, humidified atmosphere). A negative sperm cell culture control was treated similarly to verify appropriate handling and culture conditions.

Subsequently, inoculated sperm cells were washed three times in 1× PBS by centrifugation at 134× *g*, 10 min, 23 °C. The resulting pellet was treated with fixing solution (2.5% glutaraldehyde buffered in 0.1 M sodium cacodylate (pH 7.2), 300 mosmol, Serva Electrophoresis, Heidelberg, Germany) for at least 2 h at 4 °C, and embedded in 1.8% low-melting agarose (Biozym). Small pieces were postfixed in 1.0% aqueous OsO4, and stained *en bloc* with uranyl acetate. After stepwise dehydration in ethanol, cells were cleared in propylene oxide, infiltrated with Glycid Ether 100 (Serva Electrophoresis), and polymerized at 60 °C for 3 days. 60–70 nm ultrathin sections were prepared with an ultramicrotome

(UC7, Leica Microsystems, Wetzlar, Germany) and collected on EM grids (300 mesh, Plano). Finally, the sections were counterstained with uranyl acetate and lead citrate and analyzed with a Tecnai-Spirit TEM (FEI) at an accelerating voltage of 80 kV.

**Table 1.** List of microscopical criteria evaluated and scored in organs of the male reproductive tract.

| | Testis | Epididymis | Prostate | Vesicular Gland | Bulbourethral Gland |
|---|---|---|---|---|---|
| Vasculitis/vasculopathy | X | X | X | X | X |
| Inflammatory infiltration of fibromuscular stroma | X | X | X | X | X |
| Interstitial single cell apoptosis/necrosis | X | X | X | X | X |
| Destruction of (tubuloalveolar) glands | - | - | X | X | X |
| Destruction of seminiferous tubules/epididymal ducts | X | X | - | - | - |
| Presence/absence of luminal spermatozoa * | X | X | - | - | - |

* Findings were only described as present/not present.

## 3. Results

### *3.1. Clinial Signs and Gross Pathology*

#### 3.1.1. Trial A

Upon intramuscular inoculation with $10^3$ $HAU_{50}$ of ASFV strain "KAB 6/2" (gt XI), boars presented with clinical signs and lesions characteristic of severe acute infection with highly virulent ASFV. The clinical signs included pyrexia (>40.0 °C), anorexia, lethargy, respiratory distress, and conjunctivitis. The clinical scores (CS) rapidly increased from day 3 pi to final scores of 7.5 (#61), 16.5 (#51) and 19 points (#48) (see Table S1). Boar #48 suffered from marked pulmonary failure leading to sudden death on day 7 pi. Pig #51 showed swiftly deteriorating general health and was euthanized reaching the humane endpoint. Post-mortem examination revealed accumulation of serosanguinous fluid in abdominal and thoracic cavities of all pigs, focal subcapsular hemorrhages in the kidneys of two pigs, and moderate gall bladder wall edema in a single boar.

In ASFV "SUM 14/11" (gt XIII)-infected animals, non-specific clinical signs were observed starting on day 4 pi, including pyrexia (>40.0 °C), reduced feed intake, lethargy, respiratory distress, conjunctivitis as well as intradermal hemorrhages. On day 8 pi, which has been determined as the end of the experiment, all boars reached a moderate final CS of 6 points (see Table S1).

At necropsy, gross lesions were slightly more pronounced in ASFV-"SUM 14/11"-inoculated pigs than in the ASFV-"KAB 6/2"-inoculated animals, even though the pigs showed milder clinical signs. All ASFV"SUM 14/11"-inoculated boars revealed a mild, diffuse reddening of the scrotum. Serosanguinous ascites were present in all pigs, whereas focal hemorrhages in the kidney only occurred in two animals. In contrast to ASFV "KAB 6/2", hemorrhages in the lymph nodes were present to a variable extent, mainly affecting the hepatogastric and renal nodes. Gross lung lesions included lack of retraction or focal atelectasis, which was present in two pigs.

#### 3.1.2. Trial B

Eight adolescent wild boar were inoculated oro-nasally with $10^4$ $HAU_{50}$ per ml of ASFV "Germany 2020" (gt II). The animals showed reduced feed intake and lack of liveliness from 4 dpi. Their condition worsened over the next three days, and all animals were sacrificed at 7 dpi. At that point, the animals had a CS of 4 to 6 score points with anorexia, pyrexia (>40.5 °C), slight ataxia, and depression (see Table S2).

Macroscopic lesions included moderate hemorrhages mainly in the renal and hepatogastric lymph nodes, renal petechiae and mild to moderate pulmonary consolidation.

### *3.2. Pathogen Detection*

#### 3.2.1. Detection and Quantification of Viral DNA and mRNA

To detect the ASFV genome in the male reproductive tract of domestic pigs and wild boar, the testis, epididymis and accessory sex glands were analyzed by qPCR and RT-qPCR,

and compared to blood and spleen samples (see Figure 1). Tables 2 and 3 summarize results from qPCR and RT-q PCR (Cq values) from domestic pigs and wild boar, respectively. The corresponding genome copy numbers for ASFV "KAB 6/2" and "SUM 14/11" are shown in Table S3. Copy numbers regarding ASFV "Germany 2020" are depicted in Table S4.

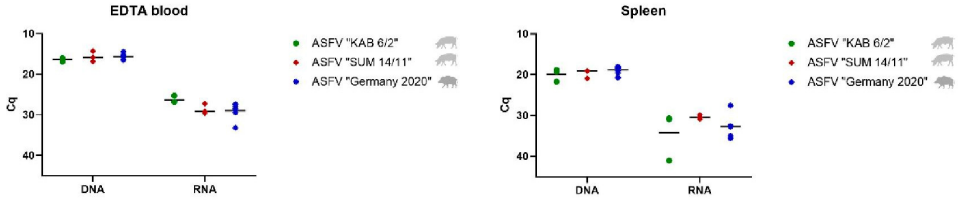


**Figure 1.** Viral genome loads in EDTA blood and spleen represented by Cq values in qPCR and RT-qPCR. Boars infected with ASFV "KAB 6/2" are depicted by green dots, ASFV-"SUM 14/11"-infected animals by red squares, and ASFV-"Germany 2020"-infected wild boar by blue stars. The mean Cq value per group is represented by a black line.

**Table 2.** Summarized results of qPCR, RT-qPCR and virus isolation of organ samples of ASFV "KAB 6/2"- and "SUM 14/11"-infected domestic pigs.

| **Inoculum** | | **ASFV "KAB 6/2"** | | | **ASFV "SUM 14/11"** | | |
|---|---|---|---|---|---|---|---|
| Animal ID | | #48 | #51 | #61 | #53 | #58 | #60 |
| Age (months) | | | 7 | | | 7 | |
| Necropsy (days post inoculation) | | | 7 | | | 8 | |
| Final clinical score points | | 19 | 16.5 | 7.5 | 6 | 6 | 6 |
| Blood | DNA Cq value | 16.86 | 16.01 | 16.13 | 16.80 | 15.86 | 14.28 |
| | mRNA Cq value | 25.26 | 26.75 | 26.79 | 29.57 | 29.08 | 27.23 |
| | Infectivity | +++ | +++ | +++ | +++ | +++ | +++ |
| Spleen | DNA Cq value | 21.69 | 18.8 | 19.18 | 20.88 | 19.07 | 19.08 |
| | mRNA Cq value | 41.01 | 30.91 | 30.66 | 30.89 | 30.39 | 29.86 |
| | Infectivity | +++ | +++ | +++ | +++ | +++ | +++ |
| Testis | DNA Cq value | 18.65 | 16.41 | 16.05 | 18.25 | 16.98 | 17.03 |
| | mRNA Cq value | - | 31.14 | 30.86 | 30.52 | 29.02 | 29.40 |
| | Infectivity | - | - | +++ | +++ | +++ | +++ |
| Epididymis | DNA Cq value | 19.55 | 18.74 | 19.29 | 21.90 | 18.25 | 17.43 |
| | mRNA Cq value | 41.18 | 31.22 | 32.62 | 31.84 | 30.95 | 30.34 |
| | Infectivity | - | +++ | +++ | ++ | +++ | +++ |
| Epididymal sperm | DNA Cq value | 25.64 | 25.49 | 25.32 | 22.21 | 27.12 | 24.74 |
| | mRNA Cq value | 31.59 | 30.32 | 30.44 | 31.10 | 41.40 | 35.01 |
| | Infectivity | +++ | +++ | +++ | +++ | +++ | +++ |
| Prostate gland | DNA Cq value | 26.70 | 27.77 | 26.6 | 28.30 | 28.19 | 26.21 |
| | mRNA Cq value | - | - | - | - | - | - |
| | Infectivity | - | + | +++ | +++ | + | - |
| Vesicular gland | DNA Cq value | 28.90 | 28.99 | 28.71 | 30.19 | 28.74 | 26.97 |
| | mRNA Cq value | - | - | - | - | - | - |
| | Infectivity | +++ | +++ | +++ | +++ | +++ | + |
| Bulbourethral gland | DNA Cq value | 27.70 | 29.97 | 27.74 | 28.47 | 26.36 | 25.35 |
| | mRNA Cq value | - | - | - | - | - | - |
| | Infectivity | +++ | +++ | ++ | ++ | +++ | - |

Cq values are given as mean values of three biological replicates of each animal. +++ = tested positive in 3 of 3 replicates; ++ = tested positive in 2 of 3 replicates; + = tested positive in 1 of 3 replicates; - = tested negative.

**Table 3.** Summarized results obtained after qPCR, RT-qPCR and virus isolation of ASFV "Germany 2020"-infected wild boar.

| **Inoculum** | | **ASFV "Germany 2020"** | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Animal ID | | #10 | #11 | #13 | #14 | #15 | #17 | #18 | #19 |
| Age (months) | | 6 | | | | | | | |
| Necropsy (days post inoculation) | | 7 | | | | | | | |
| Final clinical score points | | 5 | 4 | 5 | 6 | 5 | 5 | 5.5 | 5 |
| EDTA Blood | DNA Cq value | 14.43 | 15.15 | 16.41 | 16.46 | 15.60 | 15.50 | 15.72 | 15.42 |
| | mRNA Cq value | 33.16 | 28.93 | 29.43 | 28.52 | 27.38 | 28.93 | 27.95 | 27.41 |
| | Infectivity | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ |
| Spleen | DNA Cq value | 18.10 | 18.40 | 18.00 | 18.38 | 19.59 | 18.29 | 20.70 | 18.96 |
| | mRNA Cq value | 27.54 | 34.94 | 32.71 | 32.50 | 35.53 | 32.66 | 32.43 | 32.59 |
| | Infectivity | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ |
| Testis | DNA Cq value | 17.12 | 17.49 | 17.07 | 19.16 | 17.36 | 18.50 | 17.88 | 17.58 |
| | mRNA Cq value | 35.06 | 34.66 | 32.13 | 32.23 | 30.33 | 33.71 | 34.01 | 31.52 |
| | Infectivity | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ |
| Epididymis | DNA Cq value | 23.86 | 23.22 | 25.53 | 23.97 | 22.21 | 25.27 | 25.86 | 24.94 |
| | mRNA Cq value | 34.45 | 37.43 | - | 35.17 | - | 37.57 | - | 33.82 |
| | Infectivity | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ |
| Prostate gland | DNA Cq value | 26.48 | 25.17 | 27.29 | 27.91 | 25.96 | 28.63 | 29.11 | 29.18 |
| | mRNA Cq value | - | 35.70 | - | - | - | - | - | - |
| | Infectivity | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ |
| Vesicular gland | DNA Cq value | 27.13 | 25.99 | 27.64 | 28.98 | 26.82 | 27.88 | 26.86 | 28.37 |
| | mRNA Cq value | 36.97 | - | 29.47 | 32.23 | - | - | - | - |
| | Infectivity | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ |
| Bulbourethral gland | DNA Cq value | 30.65 | 26.92 | 30.19 | 31.45 | 30.27 | 29.81 | 30.55 | 28.63 |
| | mRNA Cq value | 37.66 | - | - | - | - | - | - | - |
| | Infectivity | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ |

Cq values are given unique per boar. ++ = tested positive in 2 of 2 replicates; + = tested positive in 1 of 2 replicates.

### Blood and Spleen

In blood samples, the mean Cq values of 16.33 (ASFV "KAB 6/2"), 15.65 (ASFV "SUM 14/11"), and 15.59 (ASFV "Germany 2020") for viral DNA, and 26.27 (ASFV "KAB 6/2"), 28.62 (ASFV "SUM 14/11") and 28.96 (ASFV "Germany 2020") for viral mRNA were detected on days 7 or 8 pi, respectively. In the spleen, viral DNA loads were comparably high in ASFV "KAB 6/2" (mean Cq 19.89) and ASFV "SUM 14/11" (mean Cq 19.68) -infected domestic boars and ASFV "Germany 2020" (mean Cq 18.80) -infected wild boar, whereas viral mRNA amounts were lower with mean Cq values of 34.19 (ASFV "KAB 6/2"), 30.38 (ASFV "SUM 14/11") and 32.61 (ASFV "Germany 2020"), respectively.

### Testis and Epididymis

Compared with blood and spleen, viral genome loads were equally high in testicular samples of all animals independent of the ASFV isolate with mean Cq values of 17.03 (ASFV "KAB 6/2"), 17.42 (ASFV "SUM 14/11"), and 17.77 (ASFV "Germany 2020") (Figure 2). However, viral mRNA loads were lower than the corresponding DNA values with mean Cq values of 35.67 in ASFV "KAB 6/2"- (no detection in animal #48), 29.65 in ASFV "SUM 11/14"- and 32.96 in ASFV "Germany 2020"-infected pigs.

As shown in the testis, high amounts of viral DNA were also detected in the epididymis with mean Cq values of 19.19 for ASFV "KAB 6/2" and 19.23 for ASFV "SUM 14/11", and a slightly lower value of 24.36 for ASFV "Germany 2020" (Figure 2). When compared to the DNA values, lower values were detected for viral mRNA with mean Cq values of 35.01 (ASFV "KAB 6/2"), 31.04 (ASFV "SUM 14/11") and 39.18 (ASFV "Germany 2020").

In epididymal samples of boar #48 (ASFV "KAB 6/2"), as well as in the epididymides of wild boar #13, #15 and #18 (ASFV "Germany 2020"), viral mRNA was not detectable.

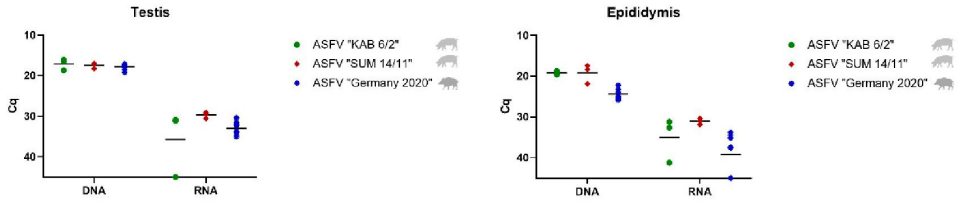


**Figure 2.** Detection of viral DNA and mRNA in testicular and epididymal samples of mature domestic pigs infected with ASFV "KAB 6/2" (green dots), ASFV "SUM 14/11" (red squares) and prepubescent wild boar infected with ASFV "Germany 2020" (blue stars). The mean Cq value per group is depicted by a black line.

Prostate, Vesicular and Bulbourethral Gland

Unlike the testis and epididymis, only low to moderate amounts of viral DNA were found in the accessory sex glands (Figure 3). More specifically, in prostate samples, mean DNA Cq values reached 27.02 (ASFV "KAB 6/2"), 27.57 (ASFV "SUM 14/11"), and 27.47 (ASFV "Germany 2020"). Similar amounts were present in the bulbourethral gland with mean Cq values of 28.47 (ASFV "KAB 6/2"), 26.73 (ASFV "SUM 14/11"), and 29.81 (ASFV "Germany 2020"), followed by the vesicular gland with 28.87 (ASFV "KAB 6/2"), 28.63 (ASFV "SUM 14/11"), and 27.46 (ASFV "Germany 2020"). While detection of viral mRNA failed in all accessory sex glands of ASFV "KAB 6/2" and "SUM 14/11" infected pigs, it was successful in "Germany 2020"-infected wild boar, with a high mean Cq value of 40.46 for boars #10, #13 and #14 in the vesicular gland, as well as in the prostate gland (wild boar #11) and bulbourethral gland (wild boar #10).

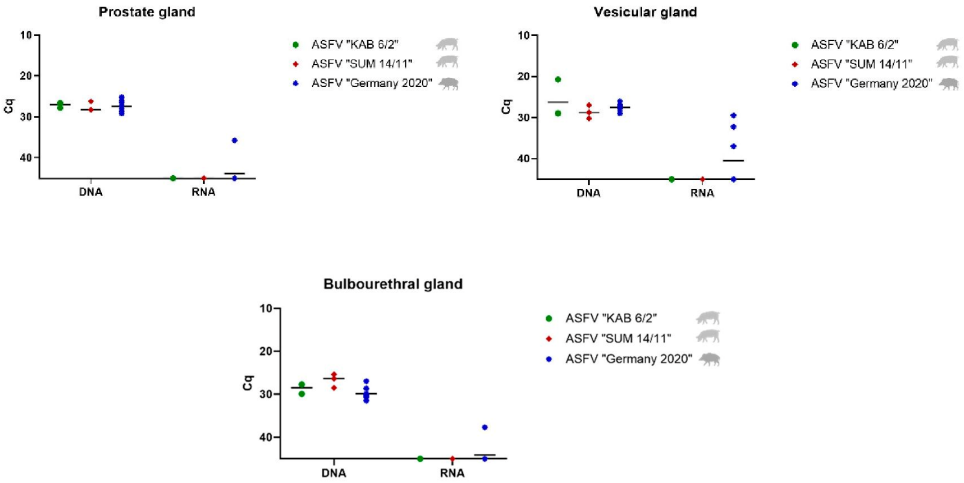


**Figure 3.** Viral genome and mRNA load in the prostate, vesicular, and bulbourethral glands in ASFV "KAB 6/2"- (green dots) and ASFV "SUM 14/11"-infected domestic pigs (red squares) and ASFV "Germany 2020"-infected wild boar (blue stars). The mean Cq value per group is displayed by a black line.

3.2.2. Detection of Infectious Virus

To test for the presence of infectious ASFV, hemadsorption tests were performed on blood, spleen, testis, epididymis, and accessory sex glands (Tables 2 and 3).

Infectious virus could be isolated from the blood and spleen of all boars, irrespective of the virus isolate used. Except for two pigs infected with "KAB 6/2", virus isolation from testicular samples succeeded in all animals infected with the three virus isolates.

Virus isolation was also possible from epididymal samples of 2/3 boars of the ASFV "KAB 6/2" group, and of all boars infected with ASFV "SUM 14/11" and ASFV "Germany 2020".

While infectious virus could be isolated from the vesicular gland of all boars, the prostate gland was positive in all wild boar infected with ASFV "Germany", but only in 2/3 of those with ASFV "KAB 6/2", and 2/3 of those with ASFV "SUM 14/11". Likewise, virus isolation was successful from the bulbourethral gland in 2/3 ASFV "KAB 6/2"-, 2/3 ASFV "SUM 14/11"- and all ASFV "Germany 2020"-infected pigs.

### *3.3. Detection and Quantification of Viral DNA, mRNA and Infectious Virus in Epididymal Sperm*

Epididymal sperm was collected only from mature boars infected with ASFV "KAB 6/2" and "SUM 14/11", and tested for the presence of viral DNA, mRNA and infectious virus (Figure 4, Table 2). In both groups, moderately high amounts of viral DNA and low amounts of viral mRNA were detected (Figure 4). Viral DNA was found with mean Cq values of 25.48 (ASFV "KAB 6/2") and 24.69 (ASFV "SUM 14/11"), whereas viral mRNA was present with Cq values of 30.78 (ASFV "KAB 6/2") and 35.84 (ASFV "SUM 14/11"). All tested epididymal sperm samples were revealed to be infectious by virus isolation (Table 2).

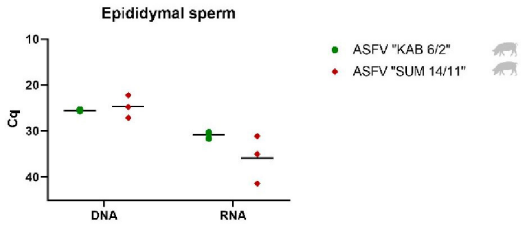


**Figure 4.** Viral DNA and mRNA loads of epididymal sperm samples of domestic pigs infected with ASFV "KAB 6/2" (green dots) and "SUM 14/11" (red squares).

### *3.4. Histopathology*

#### 3.4.1. Distribution of Viral Antigen and mRNA in Male Reproductive Organs

Tissue specimen of all male reproductive tissues (MRT) were stained against the ASFV main capsid protein p72, using IHC (ASFV "KAB 6/2", "SUM 14/11", "Germany 2020") as well as p72 mRNA using RNAScope ISH (ASFV "KAB 6/2", "SUM 14/11"), and scored on a 0–3 scale (Figure 5). In the following, the highest IHC scores obtained for each organ of the MRT will be given. As shown by qPCR, the viral antigen amount obtained by IHC was highest in the testis (score 3) and epididymis (score 3). The accessory sex glands showed few positive signals (prostate, score 1; vesicular gland, score 1; bulbourethral gland, score 1), independent of the ASFV isolate used (Figure 5A). Similar results were obtained by RNAScope ISH tested on tissues from ASFV "KAB 6/2" and "SUM 14/11"-infected boar (Figure 5B). However, in some individuals, the frequency of positive signals after RNAScope ISH was higher compared with IHC. Representative sections of the testis, epididymis, prostate, vesicular and the bulbourethral gland after IHC and RNAScope ISH are illustrated in Figure 5C.

#### 3.4.2. Detection of ASFV-Infected Target Cells in the Male Reproductive Tract

In both the testis and epididymis, as well as in the accessory sex glands, IHC identified large mononuclear cells, phenotypically consistent with macrophages, which were predominantly infected by ASFV. To a lesser extent, Leydig cells as well as stromal cells, including smooth muscle cells, fibrocytes, pericytes, and occasionally endothelial cells, were found positive for the p72 antigen in the testis and epididymis, respectively. With IHC against p72 antigen and RNAScope ISH against p72 mRNA, scattered halo cells of the epididymal duct epithelium showed positive signals (Figure 6A). This was confirmed by the double labelling of sporadically Iba-1 positive ASFV-infected cells within the epididymal epithelia using

RNAScope ISH against ASFV p72 mRNA, and subsequent immunofluorescence against the macrophage-specific protein Iba-1-identifying halo cells, respectively (Figure 6B).

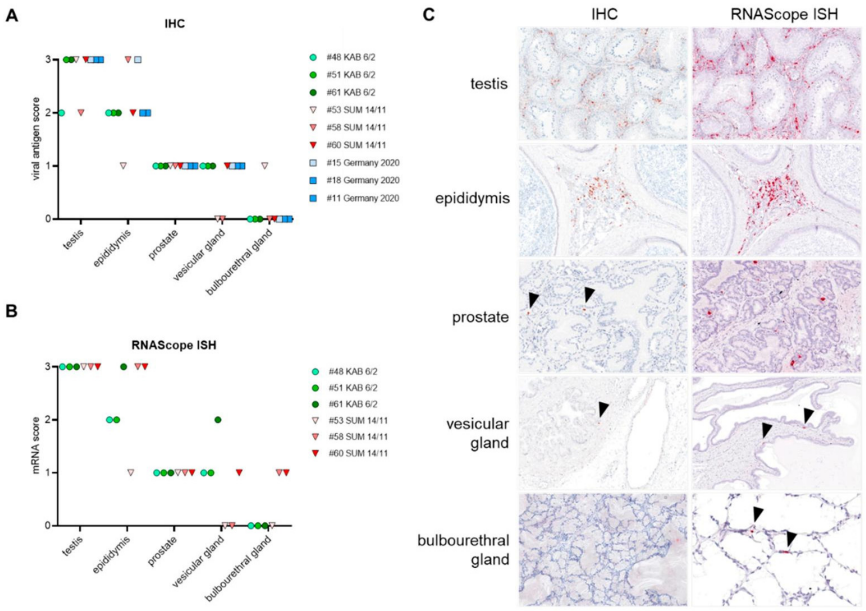


**Figure 5.** Semiquantitative results of: (**A**) immunohistochemistry (IHC); (**B**) RNAScope in situ hybridization (ISH) of male reproductive organs from ASFV "KAB 6/2", "SUM 14/11"and "Germany 2020"-infected pigs; (**C**) representative images showing IHC for anti-p72 capsid protein (red-brown signal) and RNAScope ISH (red signal) for anti p72 mRNA of ASFV-infected male reproductive organs. Arrowheads indicate positive cells in less affected accessory sex glands. Tissue sections were counterstained with Mayer's Hematoxylin.

### 3.4.3. Histopathological Lesions in the Male Reproductive Tract

Tissue sections of the testis, epididymis, prostate, vesicular and bulbourethral gland obtained from domestic pigs infected with ASFV "KAB 6/2" and "SUM 14/11", as well as from wild boar infected with "Germany 2020" on day 7 and 8 pi, respectively, were stained with hematoxylin and eosin, and scored for microscopical lesions on a 0–3 scale.

In general, histopathological changes in the testis and epididymis were slightly more pronounced in ASFV "KAB 6/2"-infected pigs than in the ASFV "SUM 14/11" group. Testicular vessels showed diffuse, prominent endothelial activation and intramural necrotizing inflammation of varying degrees (score 1 to 2) (Figure 7A,B). Ranging from mild to severe, there was extensive apoptosis and/or necrosis of perivascular fibromuscular stromal cells, accompanied by mild lymphohistiocytic infiltration and edema (Figure 7C,D). Likewise, slightly stronger necrotizing vasculitis, perivascular stromal inflammatory infiltration and acute cell loss were detected in the epididymis (Figure 8A–D). However, elongated spermatids were present in both the testis and the epididymis, and none or only minimal infiltration was observed in the seminiferous tubule or epididymal duct epithelia, respectively (Figure 8C). Only minimal changes were detected in the prostate and vesicular gland, comprising occasional single-cell apoptosis/necrosis in the fibromuscular stroma (score 1) and mild, mainly mixed-cellular infiltration (score 1) (data not shown). The bulbourethral gland was unaffected in all animals. Wild boar infected with ASFV "Germany 2020" presented with immature testes, and revealed generally milder lesions compared with "KAB 6/2"- and "SUM 14/11"-infected boar. While all accessory sex glands in wild boar were unaffected, only mild infiltration of the testicular/epididymal fibromuscular

stroma with single-cell apoptosis/necrosis, as well as a mild infiltration of single testicular tubules, were observed.

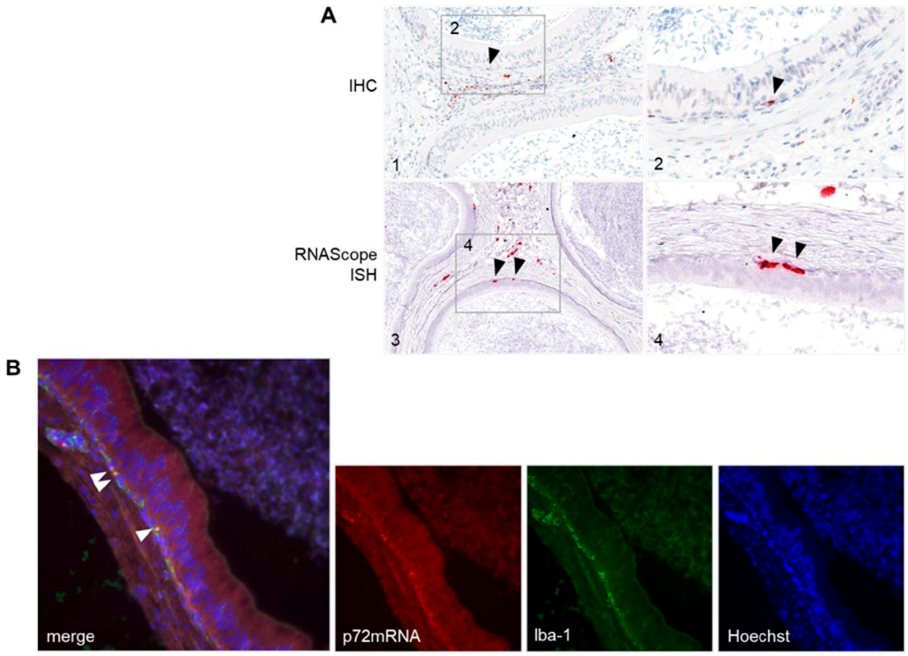


**Figure 6.** (**A**) Immunohistochemistry (IHC) using a rabbit polyclonal antibody against p72 and RNAScope in situ hybridization (ISH) using a probe against p72 mRNA performed on ASFV-infected epididymis-indicating halo cells (black arrows). Tissues were counterstained with Mayer's hematoxylin. (**B**) Dual RNAScope in situ hybridization (ISH) and Iba-1 immunofluorescence using a mRNA probe against p72 and a rabbit polyclonal antibody against Iba-1. White arrowheads indicate double staining of ASFV p72mRNA and Iba-1 expression in epididymal halo cells. Tissue sections were counterstained with Hoechst.

### *3.5. Electron Microscopy of In Vitro Inoculated Sperm*

In vitro inoculated sperm was embedded in epoxy resin and cut in different directions. While no virus particles were found inside boar spermatozoa, envelope-free ASFV was detected in the matrix of previously washed sperm cells and inside non-sperm cells. The pattern of distribution appeared to be random without any affinity towards the spermatozoa (see Figure 9).

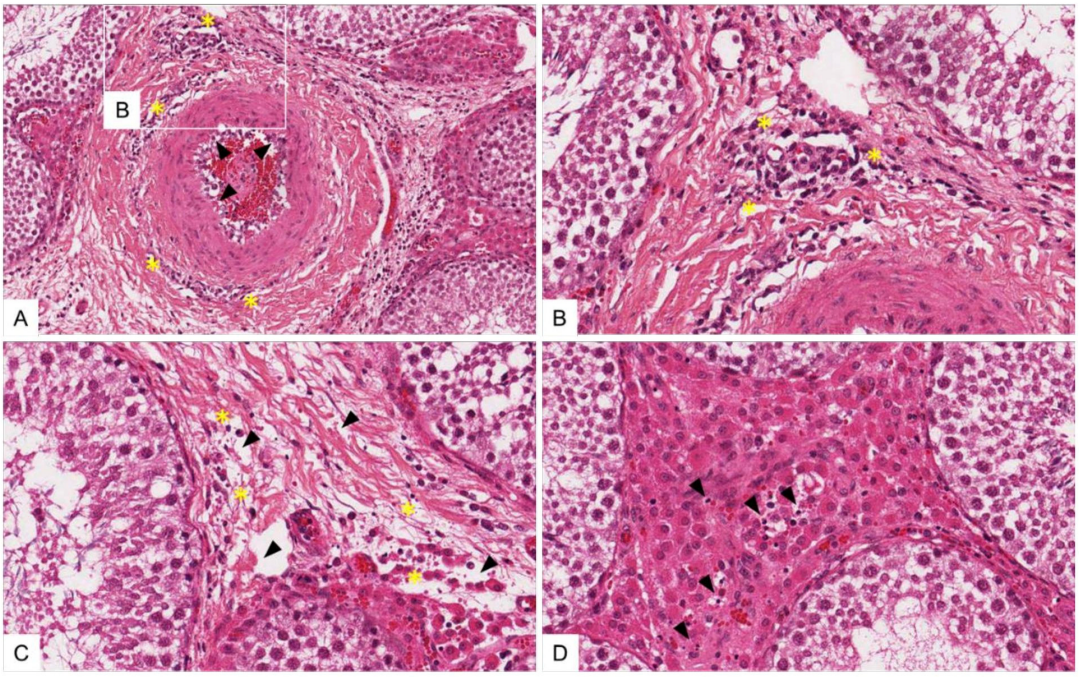


**Figure 7.** Tissue sections of an ASFV-infected swine testis stained with hematoxylin-eosin. (**A**) Testicular artery showing diffusely activated endothelium (arrow). Vasculitis and perivasculitis of small caliber vessels (rectangle above) expand to the tunica adventitia of the artery (asterisk). (**B**) Magnification of (**A**). Small caliber vessels with activated endothelium, and moderate intramural and perivascular infiltration by mononuclear cells admixed with multiple apoptotic/necrotic cells (asterisk). (**C**) Multifocal single-cell apoptosis/necrosis of cells (asterisk) in mildly edematous testicular stroma (arrows) adjacent to an unaffected seminiferous tubule. (**D**) Focal area showing apoptosis/necrosis of multiple cells (arrows) between normal seminiferous tubules.

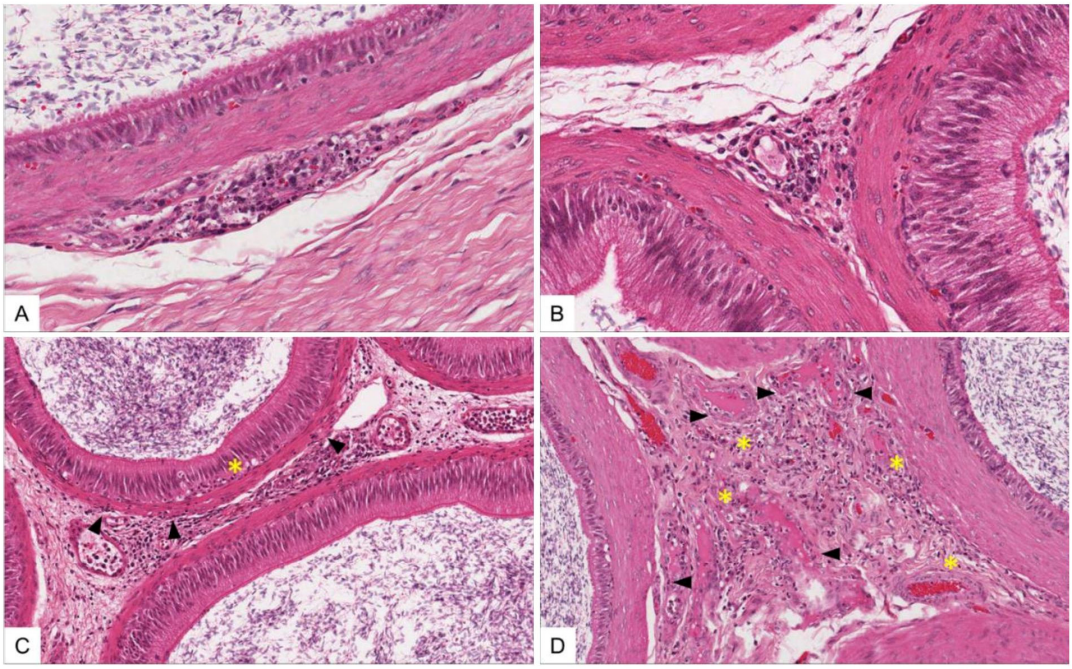


**Figure 8.** Tissue sections of ASFV-infected swine epididymis, hematoxylin and eosin stain. (**A**,**B**) Moderate necrotizing vasculitis and perivasculitis of a small caliber vessel adjacent to unaffected epididymal ducts. (**C**) Fibromuscular epididymal tissue showing multifocal to coalescing necrotizing inflammation. Smooth muscle cells surrounding epididymal ducts and the basal compartment of the duct epithelium are mildly infiltrated by mononuclear cells (arrows) with occasional single cell apoptosis/necrosis (asterisk). (**D**) Moderate multifocal necrotizing vasculitis affecting small caliber vessels (arrows) and diffuse mononuclear infiltration of fibromuscular tissue admixed with abundant apoptotic/necrotic cells (asterisk).

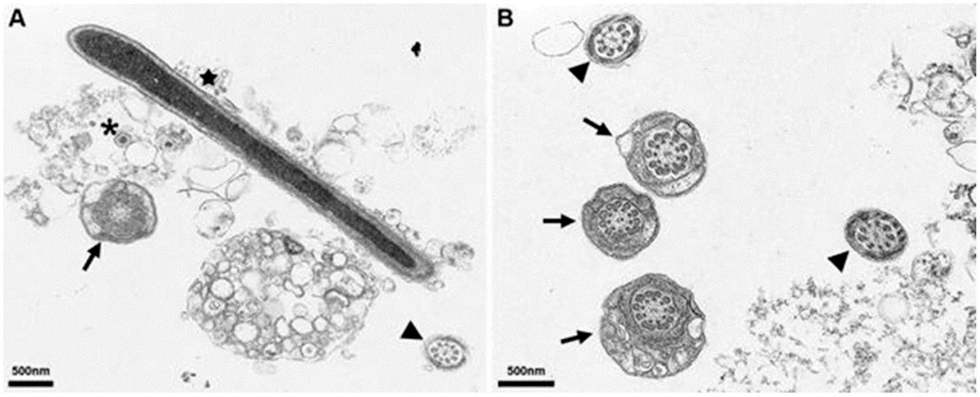


**Figure 9.** EM Micrographs of sperm from boar. (**A**,**B**) show epoxy resin embedded in vitro inoculated sperm cut in different directions (asterisk). Arrows show the cross section of the head and middle part of a sperm cell, arrowheads show the cross section of the flagellum and the star indicates a longitudinal section of the flagellum. The asterisk represents ASFV particles.

## 4. Discussion

African swine fever is currently threatening the global pig population with active outbreaks on four continents; thus, understanding disease dynamics and transmission pathways is key when it comes to risk mitigation and prevention. In a globalized world, artificial insemination and international trade of sperm are a widely used instrument for distributing elicited superior genes into sow herds while minimizing the prevalence of transmissible venereal diseases [23]. Yet, once a pathogen passes the high hygiene and precautionary measurements of boar stations, AI threatens to introduce novel pathogens, including transboundary animal diseases such as ASF, into naïve pig populations [24]. The impact that the detection of a notifiable animal disease can have on boar stations was evident in the outbreak of classical swine fever in the Netherlands in 1997/98. There, two semen collection centers became infected. At that time, it was concluded that all the semen collected from the boars at the stations and distributed in a six-week risk period was potentially contaminated. As a consequence, a total of 1680 pig herds were officially declared CSF suspect [25].

Against this background, we undertook further studies to shed light on the possible transmission via boars and boar semen as a high impact avenue for wide-spread dissemination. We took advantage of ongoing animal trials with virulent ASFV strains of different genotypes. In detail, ASFV isolates "KAB 6/2" (gt XI), "SUM 14/11" (gt XIII), and "Germany 2020" (gt II) were used in mature domestic boars and adolescent wild boars, respectively. The trials ended at the height of viraemia, at days 7 or 8 pi. To obtain a broader picture, classical real-time PCRs to detect viral DNA were supplemented with real-time RT-PCRs to detect viral mRNAs. This was done under the assumption that a pure transport of intact virions or any residual DNA could be distinguished from a possible replication. Moreover, we characterized the localization of viral antigen and viral mRNAs using IHC and RNAScope ISH. Finally, we attempted to inoculate commercial boar sperm in vitro, to elaborate on the susceptibility of spermatozoa.

### *4.1. African Swine Fever Virus Is Found in All Male Reproductive Tissues and Epididymal Sperm*

In line with our previous studies [6,9], and irrespective of the virus strain or host type involved, ASF viral genome and infectious virus were detected in all organs of the male reproductive tract and in epididymal sperm.

In detail, viral DNA loads were detected in the male reproductive organs, i.e., testis and epididymis, that were *on par* with the loads in the spleen as an organ classically associated with ASF [26]. The detection of viral mRNAs was also accomplished in many cases, pointing at least to replication-competent viruses. Somewhat lower genome loads were found in the still nearly inactive epididymides of the prepubescent wild boar, which may reflect the intensity of blood flow, or the somewhat slower disease progression after oral infection. The results were confirmed by p72 antigen and mRNA detection in IHC and RNAScope ISH, respectively. The accessory sex glands showed only moderate loads of viral genomes, on average 1000-fold lower in the prostate than in the testes, and showed very limited mRNA detections, possibly due to early degradation.

Virus isolation was successful for the majority of domestic pig samples and for all wild boar samples tested. The semen samples also allowed virus isolation. Thus, there is some discrepancy with the detection of viral mRNA. However, it should be noted that virus isolation was performed using an amplifying blind passage. Furthermore, since pure mRNA detection was not a priority, we abstained from measures that would have specifically preserved the integrity of the mRNAs (e.g., chemical stabilization or immediate deep freezing), so that degradation during sample processing was likely. Moreover, one cannot exclude the possibility that infectivity was present in a blood-bound manner, and was therefore not associated with replication directly in the tissues.

The detection of ASFV in the male genitals is in line with the observation in human medicine that not only classical venereal disease pathogens, but also agents causing severe systemic diseases, e.g., Zika or Ebola virus, can be found in the male reproductive tract,

including sperm, and can cause persistent infections [27,28]. So far, the putative pathogenesis of these nonclassical viral agents seeding into the male reproductive tract is referred to the testis being an immune-privileged site [29].

### *4.2. Replication Is Linked to Mononuclear Cells of the Respective Tissues*

Immunohistochemistry showed that the viral antigens and mRNA were mainly confined to large mononuclear cells, phenotypically consistent with macrophages. Macrophages represent the largest leucocyte populations in testis and epididymis [30,31] and were shown to be the primary target cells of ASFV [32]. Positive signals in both IHC and RNAScope ISH were also detected in epididymal halo cells. Halo cells are considered intraepithelial immune cells of the epididymal duct, but the exact immunological function of these cells is currently still controversial. Several data indicate that halo cells belong to the mononuclear phagocyte system (MPS) [33,34], while others refer to halo cells as T lymphocytes [35]. However, showing high consistency to our results Serre and Robaire [36] showed that halo cells are positive for CD68, a macrophage-specific protein, and recently it was postulated that these cells belong to the MPS, using RNA sequencing [33]. More specifically, halo cells are suspected to contribute to the immunological part of the blood-epididymis barrier [36–39]. Thus, they can play a crucial role in tissue specific pathogenesis, e.g., by creating proinflammatory environments and facilitating the disruption of barrier integrity.

### *4.3. ASFV Does Not Directly Infect Spermatozoa*

Viral antigens were not detected by IHC or RNAScope ISH on the testicular or epididymal germ cell site. However, virus isolation was positive from epididymal sperm. Moreover, the detection of viral mRNA by RT-qPCR indicated the presence of productively infected cells in epididymal sperm. In part, this discrepancy might be explained by blood contamination, cell-free virus particles and/or few migrating infected cells.

In the present experiment, the animals were killed in the viraemic phase, but the disease would probably have taken an even more severe course. Thus, the question remains open as to how the further course of infection affects the distribution of the virus. The detection of viral antigens in halo cells and the signs of inflammation/vasculitis indicated that the integrity of the blood-epithelial barrier may have already been compromised. Especially in presence of systemic inflammation and viremia, the blood-testis/-epididymis/-deferens barriers depicted an insufficient compartmentalization, likely granting access to virions and infected cells [40]. For this reason, the possibility of long-term persistence of ASFV in the male genital tract should also be the subject of future investigations.

With regard to sperm, our EM analyses of in vitro ASFV-inoculated sperm samples points to a low or non-existent susceptibility of mature spermatozoa. However, non-sperm cells were infected, and cell-free virus particles were obvious. Boar sperm always contains seminal plasma and a small fraction of other cells [41], including blood-derived leukocytes that could act as source of infectivity. As entry sites for blood-derived leukocytes, the rete testis and accessory sex glands, particularly the prostate, are suspected [41–43]. Consequently, ASFV shedding into sperm might be linked to spermatozoa-accompanying cells and fluids. The same pattern is seen with the Porcine reproductive and respiratory syndrome virus (PRRSV), where round cells were identified as a virus source. Christopher Hennings et al. [34] postulated that PRRSV entered boar semen through replication in reproductive tissue macrophages that became infected upon the viraemia-associated systemic distribution of infected monocytes. Inflammatory processes in the male genitals would most probably lead to higher entry into the sperm.

## 5. Conclusions

Based on our results, it must be assumed that transmission of ASFV via the sexual act or artificial insemination might be possible.

African swine fever in the wild boar population is particularly relevant in Europe. It should also not be ignored that male animals that survive the infection participate in the

reproductive process, and that prolonged virus replication in the male reproductive organs, e.g., after initial loss of integrity, may be a problem in maintaining chains of infection.

Since neither the long-term behaviour nor the dose necessary for transmission via sperm are known, only further studies can shed light on these issues.

**Supplementary Materials:** The following are available online at https://www.mdpi.com/article/10.3390/v14010031/s1, Table S1: Clinical scoring of domestic boars infected with ASFV "KAB6/2" and "SUM14/11", Table S2: Clinical scoring of wild boar infected with ASFV "Germany 2020", Table S3: Summary presentation of individual organ Cq values and genome copy numbers of ASFV "KAB6/2" and "SUM14/11" infected domestic boars, Table S4: Summary presentation of individual organ Cq values and genome copy numbers of ASFV "Germany 2020" infected wild boar.

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

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**Institutional Review Board Statement:** The study was conducted according to animal welfare regulations, including EU Directive 2010/63/EC and institutional guidelines, and was approved by the State Office for Agriculture, Food Safety and Fishery in Mecklenburg—Western Pomerania (LALFF M—V) under reference numbers 7221.3-2-011/19 and 7221.3-1-035/21.

**Informed Consent Statement:** Not applicable.

**Data Availability Statement:** The data presented in this study are available on request from the corresponding author.

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

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# VI. Discussion

African swine fever is one of the most important diseases in swine populations. In recent times, it has devastated pork industry – both commercial and backyard- throughout much of the world and is endangering rare pig species in South East Asia [130]. However, despite its global spread and tremendous impact, ASF has not garnered the widespread attention that has e.g., the SARS-Cov-2 pandemic. Outside the rather narrow animal health sector, ASF can justifiably be called a "forgotten pandemic" [131], and knowledge transfer to relevant stakeholders shows room for improvement.

The presented studies set out to provide competent authorities, veterinary services, veterinary practitioners, and other decision makers and stakeholders with science-based background knowledge on the pathobiology, diagnosis, and transmission of ASF.

I. Pathobiological studies on the virulence of ASFV "Belgium 2018/1" in domestic pigs of different age classes

In September 2018, the first case of ASF in wild boar was detected in southern Belgium, more than 1000 km west of the nearest foci in the Czech Republic [132]. Explaining the skipping of 1000 km, it was hypothesized that infected, asymptomatic wild boar could have been imported and released illegally. Alternatively, disposal of contaminated meat products or contaminated equipment was discussed [133]. Belgium managed to eradicate the disease and the event was resolved in March 2020 [134]. Whole-genome sequence analysis of the causative strain ASFV "Belgium 2018/1" revealed attribution to the p72 genotype II with 99.98% overall sequence identity to ASFV "Georgia 2007/1" at nucleotide level and moreover, similar identities to other Georgia 2007-like ASFV strains from other regions while lacking any unique genetic marker [55]. Experimentally, it has shown very high virulence in infected European wild boar [95]. For efficient early warning, knowledge about the disease phenotype of locally relevant ASFV strains is crucial. To further elaborate on the pathobiological characterization of ASFV strain "Belgium 2018/1", the conducted studies included the oronasal or nasal infection of domestic pigs of different age classes, i.e., weaners and subadult pigs. Thereby, the focus was laid on the virulence and expression of clinical and pathomorphological signs.

Clinical features observed in weaner pigs were unspecific but severe, resembling a hemorrhagic fever with high lethality. These observations were in line with characteristics of highly virulent

and well characterized other Georgia 2007-like strains like ASFV “Armenia 08” [135, 136]. More precisely, upon 5 dpi, weaner pigs developed high fever, general depression, anorexia and respiratory distress. All animals were sacrificed at the humane end point on 9 dpi. In line, pathomorphological lesions were indicative for acute ASF, including lymphadenopathy, renal petechiae, ascites, pleural effusion, bruises and punctually, gall bladder wall edema, urinary bladder mucosal petechiae, cyanosis of the ears, multifocal pulmonary consolidation and alveolar edema. Highest viral genome loads were detected in blood, serum and spleen by qPCR.

In contrast, the clinical features assessed for the subadult pigs were milder than for younger pigs. Only half of the inoculated animals had to be sacrificed at the humane endpoint until 18 dpi, the predetermined end of study. In detail, upon 4 dpi animals presented fever, general depression, anorexia, tremor, reddened skin, cyanosis of ears and snout and respiratory distress. Most severe signs were observed on 8 and 9 dpi. However, the clinical score was still moderate. Reaching the humane end point, euthanasia of severely diseased pigs was performed between 7 and 14 dpi. The remaining pigs started to recover upon 12 dpi. At necropsy, various pathological patterns were observed of varying severity comprising generalized hemorrhagic lymphadenopathy, congestion of spleen, multiple hemorrhages in several organs, particularly in kidneys. Highest viral genome loads were detected by qPCR in blood, serum, and tissues from animals with severe clinical signs, whereas those in the blood of survivor animals were low. Identification of seroconversion against p72 and p32 succeeded in the survivor pigs upon 10 dpi by ELISA. Given the early end point of 18 dpi, the seroconversion and low viral genome loads in blood allow the anticipation that the experimental survivors were true survivors.

The observed age-related attenuation in virulence of ASFV “Belgium 2018/1” was unexpected, as before, this observation was described mainly for moderate, but not for highly virulent ASFV strains [137]. Survival of older pigs infected with the moderately virulent ASFV “Netherlands ´86” was linked to physiological blood parameters while mortality was linked to γδT cells and IL-10 [137]. Moreover, survivor pigs were rarely reported under experimental settings using highly virulent strains attributed to genotype II [138, 139].

Besides the factor age, varying clinical courses could be related to application dose and route, and involvement of host factors such as general health, immune status and genetic background [98, 140]. Given the small number in our trials, the outcome could also reflect natural variability rather than true attenuation. So far, measurement of virulence was mainly the ability to replicate in macrophages and the cause of cytopathic effects [141]. Though recently, also reports that one

strain can result in varying clinical courses have been published [109].

In fact, that the Belgium strain is capable of inducing subacute clinical outcomes in naive pigs is of utmost interest, as it may indicate an increased fitness under field conditions. A slightly attenuated phenotype could favor disease persistence in endemic areas and present a positive selection mechanism [127]. Considering the high identity between pandemic strains, this property might be held by further virulent strains of genotype II.

It has been documented, that over time, ASFV might be evolutionary driven to establish prolonged, chronic and inapparent infections in countries outside Africa [142-144]. These changes in virulence were based on mutations of the viral genome and emergences of new, less virulent mutants has high importance for viral survival [96, 142].

However, to further explore agents and host factors that influence the disease phenotype and severity, additional studies are needed which could also help to understand beneficial host reactions that could be triggered by novel vaccine approaches.

To emphasize molecular determinants for virulence and pathogenicity, it is crucial to understand similarities and commonalities between circulating ASFV strains and to characterize host responses [145]. In addition to the genomic comparison [55], proteome and transcriptome analysis point out functional genomics providing information about differences in gene expression programs and host responses to infection. Herein, isolates varying in virulence and hosts differing in subspecies, age or immunological status should be involved [145]. Also, studies conducted *in vitro* and *in vivo* should be compared. For example, studies comprised comparative, transcriptional analysis of ASFV “Georgia 2007/1” that highlighted virulence-specific transcripts belonging to multigene families, including two MGF 100 genes, I7L and I8L and its macrophage response profile [145]. Using RNA-Seq technique, host and viral gene differences between acutely infected, dead and cohabiting asymptomatic pigs infected with a virulent strain of genotype II from an outbreak in Qingyang City were assessed [146]. Briefly outlined, significant differently expressed genes identified in spleen samples were attributable to viral resistance, stimulation of macrophage antiviral response, the inflammatory response, inhibition of viral replication, cytoskeletal involvement and GTPase activity. These results suggest that after viral invasion of the host, it causes significant differential expression of a large number of genes in multiple signaling pathways of host genes, and most of these differentially expressed genes are related to host resistance to the virus as well as tissue damage repair. It was found that some viral genes in cohabiting asymptomatic pigs might integrate into host genes (DP96R, I73R and

L83L) or remain in the tissues of these pigs. Thus, mRNA of these genes may be possible markers to detect survivor pigs. Differential expression of genes attributable to survivor pigs were e.g. upregulation of CD68, GPR82, CD79a, ITGAD, AQP3, DOK3, CXCL13, and JPT2 and downregulation of GC, CCL16, HPX, RBP4, C8B, and GSTA1 [146].

In conclusion, the disease features obtained upon infection with ASFV "Belgium 2018/1" are comparable to these in ASFV "Armenia 08" and therefore mainly attributable to highly virulent ASFV. Remarkably, attenuated virulence in older pigs was assessed. It is noteworthy, that in all animals, the disease resulted in unspecific clinical signs and gross lesions that makes it indistinguishable from other ASFV strains or systemic conditions of different causes. Moreover, against the background of the existence of mitigated courses that can be easily misinterpreted, early and efficient detection is mandatory. For resolving, problems impeding early detection have to be counteracted by training farmers and veterinarians accordingly and promoting exclusion diagnostics.

II. Comparative studies on the suitability of different diagnostic workflows in ASFV detection

Swift and reliable laboratory diagnostic methods are of utmost importance considering the vast variance and imprecision of the clinical signs of ASF in *Sus scrofa*. In consciousness about the progressive spread of ASFV within Central Europe and beyond, sample matrices and test systems used have to be reconsidered as the best choice which is essential for success and practicability. The diagnostic manuals of the OIE and EU recommend diagnostic workflows and provide guidance on sample matrices (OIE Terrestrial Manual 2019). These workflows comprise direct detection as e.g., hemadsorption tests and qPCR, and several immunological diagnostics including ELISAs. Recommended sample matrices to send into the laboratory include anticoagulated (EDTA for PCR, heparin or EDTA for virus isolation), serum and tissues, mainly spleen, lymph nodes, bone marrow, lung, tonsil and kidney (OIE Terrestrial Manual 2019). However, different settings may require flexibility and amendments to the routine sample set.

To approach this, different animal trials with domestic pigs and wild boar were used to compile a set of routine and alternative sample matrices including EDTA blood, serum, different tissues, and a set of blood swabs. The trials were performed with ASFV strains of different genotypes and virulence, i.e., ASFV "Estonia 2014" (genotype II, moderately virulent), "Belgium 2018/1" (genotype II, highly virulent), "CHZT 90/1" (genotype XIX), "MFUE 6/1" (genotype XII), "RSA W1/99" (genotype IV), "KAB 6/2" (genotype XI), and "SUM 14/1" (genotype XIII). Using this unique sample set, common diagnostic workflows for both passive and active surveillance were

compared and evaluated including the designation of best suited matrices and operations.

In general, detection success of matrices obtained from domestic pigs and wild boar was comparable.

For *intra vitam* laboratory diagnosis, serum has been discussed as the most robust matrix for use in virus isolation, automated extraction and ELISA systems. Serum induces lower PCR inhibition than EDTA blood and concomitant detection of genome and antibodies, respectively. Comparison of EDTA and serum in suitability in early, intermediate and late infection phases revealed, significant lower DNA amounts in serum, especially with limitation before clinical onset. When it came to pooling of serum samples, false negative results occurred. Therefore, serum is not indicated for the detection of clinically healthy animals in the incubation period as it might be the case for screening in restriction zones. Upon clinical onset, serum represents a reliable matrix. Serum is suited when it comes to late clinical stages reaching high cq values, but not to a reconvalescent stage.

In blood, the virus is mainly linked to erythrocytes and has shown a slow genome clearance of >90 days [147]. Though, the use of EDTA blood should be improved with regards to PCR inhibition and use of certain extraction methods, as guanidinium thiocyanate extraction [148]. PCR inhibitors may affect amplification by lowering or even blocking the DNA polymerase activity, by interacting with the nucleic acids (i.e., DNA template or primers) and by quenching of fluorescence, leading to failed detection of amplicons. The main amplification inhibitors in human whole blood are hemoglobin, lactoferrin and immunoglobulin G [149, 150]. Anticoagulants, as heparin, may also inhibit the PCR reaction [151]. Hemoglobin disturbs DNA polymerase activity, as shown by great differences in hemoglobin tolerance between different DNA polymerases. The ability to release iron has been suggested to be the reason why hemoglobin and blood inhibit the PCR. IgG was suggested to act on single-stranded DNA, therefore impeding the annealing step [149, 150]. A methodology called pre-PCR processing has been proposed to overcome limitations caused by inhibitors and to achieve optimal detection limits by using an appropriate PCR composition. Thus, Taq DNA polymerases have been generated by site-directed mutagenesis resulting in greater resistance to inhibitors in blood as well as an increased tolerance to high concentrations of DNA-binding dyes. The buffer composition can also be altered to improve polymerization in presence of inhibitors, e.g. applying an elevated pH [152]. As it cannot be guaranteed that the preparations are free of PCR inhibitors, all reactions should be monitored for the presence of inhibitory effects [153]. For monitoring, the

simultaneous detection of GFP [115] or ß-Actin [116] are included into the applied ASFV qPCRs. In addition, the prior dilution of whole blood is included into diagnostic workflows of the German Reference Laboratory.

*Post mortem,* in case of tissue samples, all matrices that are recommended by the diagnostic manuals of the OIE are suitable and reliable with highest loads in spleen, lung and liver as reflected by their composition of ASFV target cells. Tonsils revealed a higher heterogeneity especially in the early clinical phase of infection. This is contradictory, as tonsils present primary replication sites [99] and could be explained by a misfit of fat and tissue and poor homogenization. Moreover, this study failed to apply biological triplicates.

When it comes to passive surveillance, externally accessible samples are better suited with regards to sanitary issues. Therefore, inguinal lymph nodes that can be obtained without opening the body cavities, ocular fluid and ear punches were approached. Inguinal lymph nodes revealed reliable but showed heterogenous results in the early phase. Ocular fluid and ear punches weren't suitable in genome load and practicability though ear punches are a common used matrix to detect bovine viral diarrhea virus [154] and ocular fluid was suitable for the detection of e.g. feline herpesvirus 1 [155]. Furthermore, blood swabs PrimeSwab and PrimeStore ® MTM were regarded and came out suitable. The advantages of PrimeStore ® MTM comprise the inactivation of microbes and covalent conservation and stabilization of DNA and RNA for subsequent molecular techniques, such as next generation sequencing and qPCR. During transport, no cooling is required (EKF-diagnostic GmbH, Barleben). MTM lyses and inactivates biological pathogens and therefore reduces infection risk so that samples can be transported with minimal risk in compliance with transport and customs regulations [156].

In this study, commercial lateral flow devices ("Point of Care" tests) for the detection of antigen and antibodies as a tool for resource-limited settings were also assessed. Antigen lateral flow assays, here LFD INgezim ASF CROM Ag (11.ASF.K42, Ingenasa, Madrid, Spain), targeting blood and serum of clinically diseased pigs (7 dpi) presented almost all samples positive. However, it came to limitations when corresponding genome loads were low, e.g., during the incubation period and reconvalescent phase. Negative antigen LFD results corresponded to genome loads ranging between 6.0 and $5.2^4$ genomic copies per run. The attempt to optimize the outcome for EDTA blood samples through freeze-thawing or dilution in distilled water did not yield better results. Concluding, sensitivity was low and a negative result would need confirmation if ASF is suspected. In line, results targeting blood samples from wild boar carcasses collected under field

conditions in Germany were even worse. Here, a sensitivity of roughly 77% in thawed high-quality samples, and a drastically reduced sensitivity of 12.5% in blood samples of wild boar carcasses was obtained. In contrast, freeze thawing increased the sensitivity to 44%. In contrast, a specificity of 100% was perceived. In summary, the antigen LFA should not be regarded as a substitute for any OIE listed diagnostic method [157].

When analyzing antibody lateral flow assays, here LFD INgezim PPA CROM Ab (11.PPA.K.41, Ingenasa, Madrid), using EDTA blood and serum, results were positive at 10 dpi and comparable to commercially available ELISAs and the indirect immunoperoxidase test. Therefore, antibody lateral flow assays show promising results with samples from recovering animals.

III. Pathobiological studies on the importance of male reproductive organs in ASF

Boars and boar semen represent a widespread avenue for the transmission of several viruses affecting swine, for example of porcine reproductive and respiratory syndrome virus and classical swine fever virus [158], and height of viral shedding was implemented during height of viremia. In a globalized world, artificial insemination (AI) and international trade of sperm are a widely used instrument for distributing elicited superior genes into sow herds while minimizing the prevalence of transmissible venereal diseases [159]. Now practiced for over 40 years, more than 90% of sows have been bred by AI in European countries in the last two decades [160]. Yet, once a pathogen passes the high hygiene and precautionary measurements of boar stations, AI threatens to introduce novel pathogens into naive pig populations. Historically, relevant animal diseases, such as porcine reproductive and respiratory syndrome virus, were transboundary transmitted via AI [161]. Thus, transmission of ASF would multiply when undetectably affecting boar sperm. As particularly in regulation (EU) 2016/429 of the European parliament and of the council regimented, an introduction of ASFV into a boar station would implement vast economic burdens on trade of porcine sperm. Against the background that boar sperm represents a potential multiplying vehicle for ASF transmission, little is known about the shedding of ASFV into boar sperm and subsequent infection of a recipient sow. Initially described, not only the ASFV re-isolation in sperm of an experimentally infected boar, but also the transmission to a recipient female succeeded [94]. In addition, immunohistochemistry of male gonads of subadult wild boar experimentally infected with ASFV “Belgium 2018/1” revealed positively labelled cells identified as macrophages, endothelial cells, peritubular fibroblasts, in line with inflammatory changes and vasculitis/vasculopathy, and viral genome was detected by qPCR [95].

In order to emphasize implications for venereal transmissibility, at first, detailed knowledge

about the distribution pattern of ASFV in the male genitals is crucial. To approach this issue, we studied the experimental infection of eight adolescent wild boar with ASFV “Germany 2020” via the oronasal, and each three adult domestic boars with ASFV “KAB 6/2” and “SUM 14/11” via the intramuscular route. The ASFV strains used reflected the current German field situation and diverse African genotypes. At height of viremia, at day 7 and 8 post infection, the trials ended and subsequently, animals were investigated virologically (qPCR and RT-qPCR) and pathomorphologically (immunohistochemistry and RNAScope ISH). Supplementary, *in vitro* sperm cultures were inoculated with ASFV and underlying interactions were depictured by TEM. Irrespective of the ASFV strain used and subspecies infected, similar pathobiological patterns were observed: Highest viral genome loads were detected first in testis and second in epididymis, with viral genome loads on par with spleen by qPCR. Epididymal sperm showed considerable DNA amounts while accessory sex glands showed minor detection rates and heights. Lower genome loads were detected in subadult wild boar epididymis, possibly due to differences in response to the oronasal inoculation route, or due to yet inactive tissues. ASFV specific RT-qPCR revealed successful mRNA detection and implicated virus replication in testis, epididymis and epididymal sperm in particular. In line, virus isolation presented that the vast majority of male reproductive tissue contained infectious virus. Pathomorphological investigation confirmed that viral antigens and mRNA were linked to large mononuclear cells, phenotypically consistent with macrophages. Macrophages represent the largest leucocyte populations in testis and epididymis [162, 163] and are further recruited via the blood stream in inflammatory conditions [164, 165]. Remarkably, IHC and RNAScope identified epididymal Halo cells to be targeted. This cell type has been linked to the mononuclear phagocyte system as stained positive for CD68, but their exact function remains unsolved. More specifically, Halo cells have an intraepithelial position in the epididymal duct and might contribute to the immunological part of blood-epididymis barrier [166, 167]. The successful, simultaneous detection of ASFV mRNA in investigated tissues hinted to true replication within identified cells rather than passive contamination.

Viral antigens were not detected by IHC or RNAScope ISH on the testicular or epididymal germ cell site. However, virus isolation was positive for epididymal sperm. Moreover, the detection of viral mRNA by RT-qPCR indicated the presence of productively infected cells in epididymal sperm. In part, this discrepancy might be explained by blood contamination, cell-free virus particles and/or few migrating infected cells. The detection of viral antigen in halo cells in line with the signs of inflammation/vasculitis indicated that the integrity of the blood-epididymis barrier may

have already been compromised. As assessed in other viral systemic conditions, especially in presence of systemic inflammation and viremia, the blood-testis/-epididymis/-deferens barriers depicted an insufficient compartmentalization, likely granting access to virions and infected cells [168, 169]. Boar sperm contains seminal plasma and a small fraction of other cells, including blood-derived leucocytes that represent a replication source [170]. As entry sites for white blood cells, accessory sex glands, in particular the prostate gland, and the rete testis were suspected [171, 172]. Consequently, ASFV shedding into sperm might be linked to spermatozoa-accompanying cells and fluids. That is in line with our observations from *in vitro* inoculated sperm cultures. TEM images revealed no virus inside mature spermatozoa, but envelope-free virus in the matrix and virus in non-sperm cells. Though, this approach represented an artificial model excluding spermatogenesis and the role of the envelope with limitation towards extrapolation. Therefore, the resulted shedding pattern was in line with this of other viruses, for example, porcine reproductive and respiratory syndrome virus. Here round cells were identified as a virus source, possibly entering boar sperm via replication in reproductive tissue macrophages that became infected upon the viraemia- associated systemic distribution of infected monocyte [173, 174].

The detection of ASFV in the male genitals is in line with the observation in human medicine that not only classical venereal disease pathogens, but also agents causing severe systemic diseases, e.g., Zika or Ebola virus, can be found in the male reproductive tract, including sperm, and can cause persistent infections [169]. So far, the putative pathogenesis of these nonclassical viral agents seeding into the male reproductive tract is referred to the testis being an immune-privileged site [168].

In conjunction with the above-mentioned possibility of the existence of survivor pigs in endemic areas, it should also not to be ignored, that male survivors might shed and transmit virus within the wild boar population. Such prolonged virus replication after loss of barrier integrity, but also via blood derived leucocytes, might be a problem in maintaining infection chains.

These results implicate for the excretion of ASF into sperm and the possibility of venereal transmission. Therefore, this route should be considered e.g., in future risk assessments.

# VII. Summary

African swine fever represents a global source of animal suffering and losses occupying tremendous socioeconomic burdens. The disease is progressively spreading since its third transcontinental introduction into Georgia in 2007. Nowadays, it has even reached pandemic proportions and entered Central Europe and Asia. As neither efficient treatment nor vaccine are available, early detection in terms of subsequent mitigation of infection chains is of utmost importance. The presented studies set out to provide competent authorities, veterinary services, veterinary practitioners, and other decision makers and stakeholders with science-based background knowledge on the pathobiology, diagnosis, and transmission of ASF.

Thus, the pathobiological characterization of the European strain ASFV “Belgium 2018/1” revealed that clinical and pathomorphological outcome in domestic weaner pigs resembled those upon infection with the highly virulent ASFV “Armenia 08”. Remarkably, older pigs showed milder clinical outcomes and implications for the existence of survivor pigs were found. Consequently, early and efficient detection is mandatory. Clinical, unspecific features need to be communicated to farmers and veterinarians accordingly.

Laboratory diagnostics are mandatory within the diagnosis of ASF. In consciousness about the progressive spread of ASFV within Central Europe and beyond, sample matrices and test systems used were reconsidered as the best choice is essential for success and practicability. This study revealed that the OIE recommended matrices, e.g., blood, serum, spleen, liver and lung in combination with qPCR represent the most reliable diagnostic workflow. Though, some alternative sample matrices, such as lymph nodes and blood swabs, and test systems, e.g., lateral flow assays can be taken into consideration but should be interpreted carefully.

Last, the pathobiological distribution pattern of different ASFV strains was investigated revealing that all parts of the male reproductive tract are affected by ASFV, with highest viral loads in testis and epididymis. Remarkably, epididymal halo cells were identified to be targeted, likely impacting the blood-epididymis barrier. Therefore, we concluded, that excretion of ASFV into boar sperm is possible, especially at height of viremia and might not only play a role in artificial insemination, but also in survivor pigs in endemic areas.

# VIII. Zusammenfassung

Die Afrikanische Schweinepest ist eine weltweite Ursache für Tierleid und -verluste, die eine enorme sozioökonomische Belastung darstellen. Seit ihrer dritten transkontinentalen Einschleppung nach Georgien im Jahr 2007 breitet sich die Krankheit progressiv aus. Inzwischen hat sie sogar pandemische Ausmaße erreicht und ist in Mitteleuropa und Asien aufgetreten. Da weder eine wirksame Behandlung noch ein Impfstoff zur Verfügung stehen, ist eine frühzeitige Erkennung im Hinblick auf eine spätere Abschwächung der Infektionsketten von größter Bedeutung. Die vorgestellten Studien sollen den zuständigen Behörden, Veterinärdiensten, Tierärzten und anderen Entscheidungsträgern und Interessengruppen wissenschaftlich fundiertes Hintergrundwissen über die Pathobiologie, Diagnose und Übertragung der ASP vermitteln.

So ergab die pathobiologische Charakterisierung des europäischen ASFV-Stammes „Belgium 2018/1", dass die klinischen und pathomorphologischen Bilder bei Absatzferkeln denen bei einer Infektion mit dem hochvirulenten ASFV „Armenia 08" ähneln. Bemerkenswerterweise zeigten ältere Schweine abgeschwächte klinische Verläufe, und es wurden Hinweise auf die Existenz von rekonvaleszenten Schweinen gefunden. Folglich ist eine frühzeitige und effiziente Erkennung unerlässlich. Klinische, unspezifische Merkmale müssen den Landwirten und Tierärzten entsprechend mitgeteilt werden.

Die Labordiagnostik ist für die Diagnose der ASP unerlässlich. Im Bewusstsein der fortschreitenden Ausbreitung der ASPV in Mitteleuropa und darüber hinaus wurden die verwendeten Probenmatrizen und Testsysteme überdacht, da die beste Wahl für den Erfolg und die Durchführbarkeit entscheidend ist. Diese Studie ergab, dass die vom OIE empfohlenen Matrices, z. B. Blut, Serum, Milz, Leber und Lunge, in Kombination mit qPCR den zuverlässigsten diagnostischen Arbeitsablauf darstellen. Allerdings können auch alternative Probenmatrizes, wie Lymphknoten, und Testsysteme, z. B. Lateral-Flow-Assays, in Betracht gezogen werden, die jedoch sorgfältig interpretiert werden sollten.

Schließlich wurde das pathobiologische Verteilungsmuster der verschiedenen ASFV-Stämme untersucht, wobei sich herausstellte, dass alle Teile des männlichen Fortpflanzungstrakts von ASFV betroffen sind, wobei die Viruslast in Hoden und Nebenhoden am höchsten ist. Bemerkenswerterweise wurde festgestellt, dass die Halo-Zellen betroffen sind und so die Blut-Nebenhoden-Schranke beeinträchtigen könnten. Schließlich ist eine Ausscheidung von ASPV in

Ebersperma möglich, insbesondere auf dem Höhepunkt der Virämie, und würde eine Rolle nicht nur bei der künstlichen Besamung, sondern auch bei überlebenden Schweinen in endemischen Gebieten spielen.

# IX. References


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