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ORIGINAL ARTICLE

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The African swine fever virus isolate Belgium 2018/1 shows high virulence in European wild boar

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Abstract

African swine fever (ASF) is one of the most important and complex viral diseases in domestic pigs and wild boar. Over the last decade, the disease has spread to several European and Asian countries and is now one of the major threats to profitable pig production worldwide. One of the more recently affected western countries is Belgium. To date, only wild boar are affected in a rather defined area in the Luxembourg region close to France, Luxembourg and Germany. While detailed sequence analyses were recently performed, biological characterization was still pending. Here, we report on the experimental inoculation of four sub-adult wild boar to further characterize the virus and its distribution in different tissues. After oronasal inoculation with the virus strain 'Belgium 2018/1', all animals developed an acute and severe disease course with typical pathomorphological and histopathological lesions. Organs and blood samples were positive in qPCR, haemadsorption test and antigen lateral flow devices (LFD). Virus and viral genome were also detected in genitals and accessory sex glands of two boars. There were no antibodies detectable in commercial antibody ELISAs, antibody LFDs and indirect immunoperoxidase tests. Thus, the genotype II ASF virus isolate 'Belgium 2018/1' showed a highly virulent phenotype in European wild boar similar to parental viruses like Armenia 2007 and other previously characterized ASFV strains. The study also provided a large set of well-characterized sample materials for test validation and assay harmonization.

KEYWORDS

African swine fever virus, Belgium, histopathology, viral distribution, virulence, wild boar

1 | INTRODUCTION

African swine fever (ASF) is one of the most important viral diseases in domestic pigs and wild boar. Over the last decade, the disease has spread to several European and Asian countries and is now one of the major threats to profitable pig production worldwide. One of the more recently affected western countries is Belgium. To date, only wild boar (*n* = 829 confirmed positive

cases as of 18 November 2019) are affected in a rather defined area in the Luxembourg region close to France, Luxembourg and Germany. While detailed sequence analyses for the Belgium ASF virus (ASFV) were recently performed (Forth et al., 2019), biological characterization and production of standardized reference materials were still pending.

Here, we report on the experimental inoculation of four subadult wild boar (1–2 years of age) to further characterize the virus, its distribution in different tissues including organs relevant for semen production (male genitals and accessory sex glands), and to produce standardized sample materials.

2 | MATERIAL AND METHODS

2.1 | Experimental design

The study comprised four ASFV and ASFV antibody negative subadult European wild boar, two males and two females. The animals were kept in the quarantine stables at the Friedrich-Loeffler-Institut (FLI) and after an acclimatization phase, the wild boar were inoculated oro-nasally with 2 ml virus suspension containing $2 \times 10^{5.5}$ haemadsorbing units (HAU) ASFV 'Belgium 2018/1'. Upon inoculation, clinical parameters of all animals were assessed daily based on a harmonized scoring system as previously described (Pietschmann et al., 2015). The sum of points was recorded as the clinical score (CS).

Blood samples were collected prior to inoculation and at the day of euthanasia. Animals reaching the humane endpoint of 15 score points or that were suffering unacceptably without reaching the endpoint were euthanized through intracardial injection of sodium pentobarbital (Release, WDT) after immobilization with tiletamine/zolazepam (Zoletil®, Virbac).

Necropsy was performed on all animals and tissue samples (lung, liver, kidney, lymph nodes, tonsil, spleen, bone marrow, salivary gland, brain, male genitals and accessory sex glands), blood (EDTA, native blood), and swab samples were collected.

2.2 | Cells

All virus titrations and haemadsorption tests were carried out using peripheral blood mononuclear cell (PBMC)-derived macrophages. PBMCs were obtained and treated as previously described (Pietschmann et al., 2015).

2.3 | Virus

The inoculum, ASFV 'Belgium 2018/1', was isolated by the Belgian national reference laboratory for ASF at Sciensano from a wild boar carcass found in the Belgian municipality Etalle (Luxembourg region). The isolate belongs to genotype II and is closely related to strains circulating in Eastern Europe (Forth et al., 2019) and beyond.

For the animal trial, culture supernatant was prepared with a final titre of approximately $1 \times 10^{5.5}$ HAU per ml. The titre was confirmed by back titration of the inoculum.

2.4 | Processing of samples

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. Tissues 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) before virus isolations (haemadsorption tests) and qPCRs were performed.

For histopathological investigation, tissue samples were fixed in 4% neutral buffered formaldehyde and embedded in paraffin.

2.5 | Pathogen detection

For the detection of viral antigen, a commercial lateral flow device (INgezim ASFCROM Antigeno 11.ASF.K42, Ingenasa) was used on blood samples according to the manufacturer's instructions. Detection of viral genome was done employing different real-time PCR (qPCR) systems. For qPCR, viral nucleic acids were extracted using the NucleoMag Vet Kit (Machery-Nagel) on the KingFisher® extraction platform (Thermo Scientific). To provide additional validation data of routinely used PCR assays with this recent ASFV strain, the following protocols were used: King et al. (2003) and Tignon et al. (2011) with slight modifications. In addition, a commercial qPCR kit was employed (virotype ASFV, Indical Bioscience) according to the manufacturer's instructions. All PCRs were performed using a C1000™ thermal cycler with the CFX96™ Real-Time System (Biorad). Results of all three qPCRs were recorded as quantification cycle (Cq) values.

To detect ASFV in serum and tissue samples, a haemadsorption test (HAT) was performed using PBMC-derived macrophages according to slightly modified standard procedures (Carrascosa, Bustos, & Leon, 2011).

2.6 | Antibody detection

Sera were tested in three commercially available antibody ELISAs. In detail, ASFV p72-specific antibodies were detected using the INGEZIM PPA COMPAC ELISA (Ingenasa), p32-specific antibodies were detected using the ID Screen ASF Competition (IDVet), and antibodies against p32, p62 and p72 were detected in the IDScreen ASF Indirect (IDVet) Kit. All tests were carried out according to the manufacturer's instructions.

In addition, all serum samples were tested in the indirect immunoperoxidase test according to the standard protocols provided by the European Reference Laboratory for ASF, and using a commercial lateral flow device for rapid detection (INgezim PPA CROM Anticuerpo 11.PPA.K.41).

2.7 | Histopathology

Formalin-fixed, paraffin-embedded tissues were cut at 3 µm and stained with haematoxylin and eosin (HE) for light microscopical examination. Nomenclature of lesions and scoring was based on Galindo-Cardiel et al. (2013): no lesion (0), mild (1), moderate (2), severe (3). Immunohistochemistry was performed using a rabbit polyclonal antibody against the major capsid protein p72 of ASFV (dilution 1:1,600 in TBS at 4°C, overnight) according to standardized procedures of avidin–biotin–peroxidase complex method (ABC, Vectastain Elite ABC Kit). A bright red intracytoplasmatic antigen labelling was produced with the 3-amino-9-ethylcarbazole substrate (AEC, Dako). The sections were counterstained with Mayer's haematoxylin. The extent of viral antigen labelling was scored on a 0–3 scoring scale: no antigen (0), focal to oligofocal (1), multifocal (2), coalescing/diffuse (3).

3 | RESULTS

3.1 | Clinical findings

Following oronasal inoculation, all animals developed severe, unspecific clinical signs starting from day four post-inoculation (dpi). The signs included general depression, lack of appetite and mobility, hunched-up back, ataxia and respiratory distress (see Figure S1). One animal showed a slight delay in the onset of clinical signs. This animal was still very active on 4 dpi. The animals that had shown clinical signs from 4 dpi displayed worsening clinical signs with dyspnoea and ataxia and were euthanized at 8 dpi. The remaining wild boar was euthanized 10 dpi showing severe dyspnoea.

3.2 | Pathomorphological and histopathological findings

At necropsy, all animals presented with typical ASF lesions of varying severity: up to severe pulmonary haemorrhage and oedema, generalized haemorrhagic lymphadenopathy especially of the gastrohepatic lymph nodes, moderate hyperaemic splenomegaly and multiple haemorrhages in several organs, particularly in the kidneys. Histopathologically, congestion as well as haemorrhage was confirmed in most organs tested. Up to severe lymphoid depletion due to apoptosis of lymphocytes and macrophages was present in lymphatic tissues. Inflammatory changes were detected up to moderately in the lung, liver, tonsil, brain and male reproductive tract. Immunohistochemistry revealed ASFV-labelled monocytes/macrophages in all organs tested. In addition, single endothelial cells and organ-specific parenchymal cells were observed to carry viral antigen in several tissues. Viral antigen was also detected in male genitals. Details are shown in Table 1. Representative histopathology and immunohistochemistry findings are displayed in Figure 1 and Figures S1–S3.

3.3 | Pathogen detection

Prior to inoculation, all animals were tested negative for ASF virus, antigen and nucleic acid. At the end of the trial, viral genome was detected in all tissue samples including the male genitals and accessory sex glands irrespective of the PCR assay used. The same was true for blood and swab samples. High loads of viral genome were found especially in blood and spleen. Virus detection by HAT corresponded to these results (see Tables S1 and S2). Viral antigen was detected by lateral flow devices in all blood samples collected at the end of the trial (8 and 10 dpi, respectively).

3.4 | Antibody detection

No antibodies were detected in the sera prior to inoculation or at the end of the trial.

4 | DISCUSSION

To further characterize the ASFV strain introduced into Belgium in 2018, an experimental infection was carried out in European wild boar. In a nutshell, the genotype II ASF isolate 'Belgium 2018/1' showed a highly virulent phenotype in European wild boar. In this respect, it did not differ from previously characterized Georgia strain-related ASFV, and clinical signs and lesions were in line with previous findings (reviewed by Pikalo, Zani, Hühr, Beer, & Blome, 2019).

In our study, pathological investigation was conducted in European wild boar. Generally, typical gross lesions induced by ASFV could be found in the investigated wild boar as demonstrated previously (reviewed in Sánchez-Cordón et al., 2019). Histologically, severe haemorrhages were especially present in the lungs, lymph nodes, spleen and the kidneys. Lymphoid depletion most likely caused by secretory activation of ASFV infected macrophages (reviewed in Goméz-Villamandos, Bautista, Sanchez-Cordon, & Carrasco, 2013) was prominent in the lymphatic tissues. Further, inflammatory changes were detectable in the lung, liver, tonsil and brain, as well as in the male reproductive tract, characterized by either acute, mainly granulocytic infiltrates in tissues affected by necrosis or mainly mononuclear infiltrates. Immunohistochemistry of the male gonads revealed ASFV positively labelled cells identified as macrophages, endothelial cells and peritubular fibroblasts based on the cellular phenotype.

TABLE 1 Histopathology and immunohistochemistry results of tissues investigated from four African swine fever virus Belgium 2018/1 infected wild boars

Organs	Histopathology based on Galindo-Cardiel et al. (2013): none (0), mild (1), moderate (2), severe (3)		Cellular tropism based on immunohistochemistry none (0), focal to oligofocal (1), multifocal (2), coalescing/diffuse (3)	
Lung	Oedema, alveolar	1–3	Monocytes/ macrophages	2
	Inflammatory infiltrates	1–2		
	Congestion/haemorrhage	2		
	Lymphoid hyperplasia	1		
Liver	Vascular/sinusoidal congestion	1–2	Macrophages, *Kupffer cells*	2
	Blood vessel angiectasia	1–2	Endothelium	1
	Hepatitis with thrombi	1–2	Hepatocytes	1–2
Kidney	Congestion/haemorrhage	1–3	Macrophages	1
	Tubular/glomerular necrosis	1–3	Endothelium	1
			Glomerular cells, tubular epithelium	1
Spleen	Congestion/haemorrhage	1	Macrophages	3
	Lymphocytic/histiocytic apoptosis	1–2		
	Ratio white pulp:red pulp	3		
Lymph nodes	Congestion/haemorrhage	1–2	Macrophages	1–3
	Lymphocytic/histiocytic apoptosis	1–3	Endothelium	1
Tonsil	Congestion/haemorrhage	1–2	Macrophages	1–2
	Lymphocytic/histiocytic apoptosis	1–2	Crypt and mucosal epithelium	1–2
	Crypt necrosis, acute inflammation[a]	1–2		
Testis	Congestion/haemorrhage	1	Macrophages	2–3
	Necrosis/interstitial inflammation	1	Endothelium	1–2
	Vasculopathy/vasculitis	1	Peritubular fibroblasts	1
Epididymis	Congestion/haemorrhage	1–2	Macrophages	2–3
	Necrosis/interstitial inflammation	1	Endothelium	1–2
	Vasculopathy/vasculitis	2	Peritubular fibroblasts	1
			Intraductal apoptotic cells	1
Male accessory sex glands	None	0	Macrophages	1
Brain	Congestion	1	Macrophages/ glial cells	1
	Inflammation	1		
Salivary gland	None	0	Macrophages	1

[a]Commonly observed in wild boar, but immunohistochemistry showed positively stained lesion-associated mucosal epithelium after immunohistochemistry.

Double-labelling strategies to confirm the results, also in other tissues (i.a. for Kupffer cells in the liver) and to identify the specifically affected cell type (i.e. within renal glomeruli), will be addressed in upcoming studies. As shown by others, ASFV is able to infect several other cells than macrophages (reviewed in Blome, Gabriel, & Beer, 2013 and Goméz-Villamandos et al., 2013), but this has yet not been reported for the male reproductive tract. Moreover, we could investigate the distribution of ASFV in male

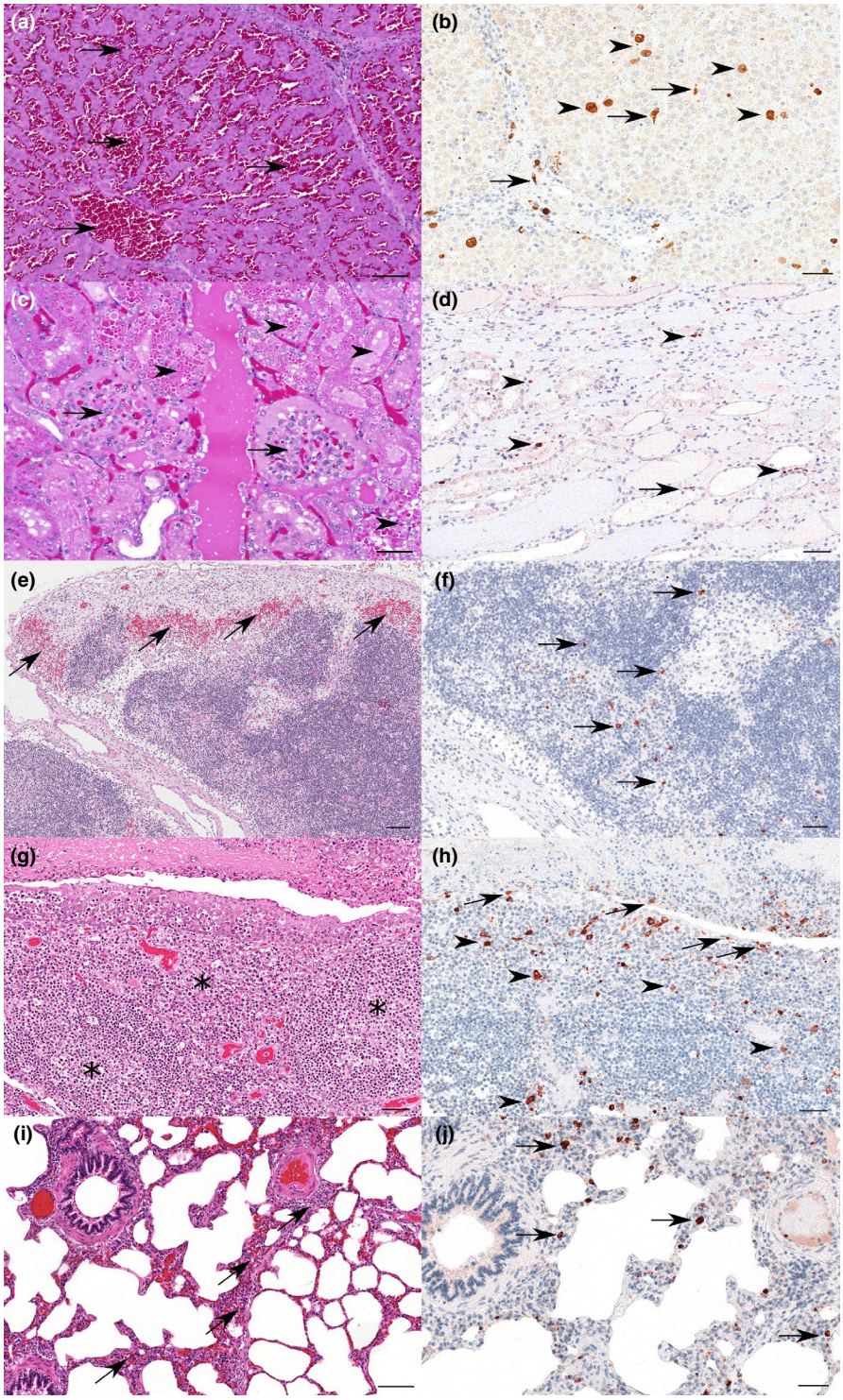

FIGURE 1 Histopathology of different organ tissues from wild boars infected with ASFV Belgium. Haematoxylin and eosin (a, c, e, g, i) and corresponding anti-ASFV p72 immunohistochemistry labelling (b, d, f, h, j) of the liver, kidney, lymph node, tonsil and lung. Liver: (a) severe sinusoidal congestion (arrow), (b) positively labelled Kupffer cells (arrow) and hepatocytes (arrowhead). Kidney: (c) severe glomerular (arrow) and tubular damage (arrowhead), (d) immunolabelled tubular epithelial cells (arrow) and interstitial macrophages (arrowhead). Lymphnode: (e) mild subcapsular haemorrhage (arrow), (f) immunopositive macrophages (arrow). Tonsil: (g) moderate, focally expansive crypt necrosis (asterisk) (h) immunopositive mucosal epithelium (arrow) and macrophages (arrowhead). Lung (i) mild, diffuse, interstitial, lymphohistiocytic inflammation, (j) immunopositive intravascular macrophages (arrow)

genitals and accessory sex glands. The latter is of importance when assessing the risk of ASFV venereal transmission through semen. Given the fact that experimental animals are often either young or neutered, data on the tissues relevant for semen production are sketchy. We demonstrate that viral genome and virus are found in all relevant gonadal tissues. This could suggest shedding through semen. However, histopathological investigations showed viral antigen primarily in macrophages rather than endothelial or stromal cells. For this reason, it cannot be excluded that the virus was only present due to the blood content. In this respect, future studies are needed that address the question of ASFV shedding through semen in particular.

Within the limitations of this small study, lateral flow devices were further assessed as rapid point of care test. In our study, blood samples from all sick animals were tested positive for viral antigen and no false positive reactions occurred with negative samples. The results of the antibody LFDs were in line with all other tests but positive results did not occur.

Finally, the study also provided a large set of well-characterized reference materials that are available for test validation and ring trials.

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ETHICAL APPROVAL

The animal experiment was performed in accordance with the EU Directive 2010/63/EC, and the following institutional guidelines were taken into consideration. The animal experiments were approved by the competent authority (Landesamt für Landwirtschaft, Lebensmittelsicherheit und Fischerei (LALLF) Mecklenburg-Vorpommern) under reference number 7221.3-2-011/19.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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