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Review

Standardization of pathological investigations in the framework of experimental ASFV infections

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ABSTRACT

African swine fever is still one of the major viral diseases of swine for which a commercial vaccine is lacking. For the design and development of such preventive products, researchers involved in African swine fever virus (ASFV) vaccinology need standardized challenge protocols and well characterized clinical, pathological and immunological responses of inbreed and outbreed pigs to different viral strains and vaccine-like products. The different approaches used should be assessed by immunologist, virologist and pathologist expertise. The main objectives of this guideline are to (1) briefly contextualize the clinical and pathological ASFV presentations focusing on points that are critical for pathogenesis, (2) provide recommendations concerning the analysis of clinical, gross and microscopic observations and (3) standardize the pathological report, the terminology employed and the evaluation of the severity of the lesions between the ASFV research groups for comparing inter-group data. The presented guidelines establish new approaches to integrate such relevant pathological data with virological and immunological testing, giving support to the global interpretation of the findings in the future experiments of ASFV-related vaccinology and immunology.

Contents

Abbreviations: ASFV, African Swine Fever Virus; TNF, tissue necrosis factor; IL, interleukin; OIE, Office International des Epizooties; FELASA, Federation of Laboratory Animal Science Associations; WAHID, World Animal Health Information Database.

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1. Introduction

African swine fever virus (ASFV) is the only known DNA arbovirus, belonging to the Asfarviridae family, genus *Asfivirus*. The virus causes African swine fever (ASF), a highly contagious disease affecting domestic pigs and both European and American wild pigs (Dixon et al., 2004), all belonging to the *Sus scrofa* species. Conversely, soft ticks from the *Ornithodoros* genus and African wild pigs such as warthogs (*Phacochoerus africanus*), bushpigs (*Potamochoerus* spp.) and Giant Forest hogs (*Hylochoerus meinertzhangeni*) are usually inapparently infected, acting as natural ASFV reservoirs in Africa (Parker et al., 1969; Thomson et al., 1980; Anderson et al., 1998; OIE WAHID, 2010). In consequence, ASFV has been endemic for decades in sub-Saharan Africa, mainly maintained in a sylvatic cycle between ticks of the *Ornithodoros* genus and wild and/or domestic pigs. Up to now, Europe has been considered free from ASF since mid 1990s, with the exception of the Sardinia island, where it still remains endemic in spite of all the efforts invested in eradicating ASFV. ASFV is currently circulating in Africa with more strength than ever, thus helping to explain the recent exportation of the virus to Georgia and the subsequent spread of the disease to Armenia, Azerbaijan and Russia, creating a growing risk of becoming endemic in the Caucasus area (Sánchez-Vizcaíno et al., 2012; OIE WAHID, 2010). The new expansion wave demonstrates the importance of updating the control and eradication measures for this disease (Costard et al., 2009; Cheneau et al., 1999).

There is no vaccine available against ASF and disease control is currently based on stamping out procedures (OIE WAHID, 2010), including a rapid and accurate diagnosis of the disease and slaughter of infected animals (Oura and Arias, 2008).

The search for an efficient and safe vaccine against ASFV has proven to be difficult, mainly due to the complexity of the virus. Vaccine trials with conventional vaccines based on inactivated viruses gave non-satisfactory results (Mebus, 1988). Up to now, only conventionally attenuated viruses have yielded good results in terms of protection against the homologous viruses, although they are far from being usable mainly due to biosafety reasons (Moulton and Coggins, 1968). Vaccines based on targeted disruption of specific virulence markers by recombination might open new avenues for developing safe and efficient vaccines against ASFV (Chapman et al., 2008). Finally, immunization with subunit vaccines based on several antigenic proteins have given some hope for the future (Ruíz-Gonzalvo et al., 1996; Gómez-Puertas et al., 1998), albeit the intrinsic mechanisms involved in protection still remain controversial. An ideal vaccine against ASFV should be able to induce both humoral and cellular responses since both ASFV-specific antibodies (Onisk et al., 1994) and T-cells (Oura et al., 2005) can play important roles in protection.

The different approaches for vaccine development and ASFV strain characterization should be assessed by a multidisciplinary approach, including research in immunology, virology and pathology. The main objectives of this guideline are to (1) briefly contextualize the clinical and pathological ASFV presentations focusing on points that are critical for pathogenesis, (2) provide recommendations concerning the analysis of clinical, gross and microscopic observations and, (3) standardize the pathological report, the terminology employed and the lesional severity evaluation between ASFV research groups for comparing inter-group data.

2. Pathogenesis of ASFV

Mononuclear phagocytic cells are the main target for viral replication (Mebus, 1988; Fernández et al., 1992a), although in late phases of the disease, ASFV can replicate in endothelial cells (Fernández et al., 1992b), megakaryocytes (Edwards et al., 1984), platelets (Gómez-Villamandos et al., 1996), neutrophils (Carrasco et al., 1996), hepatocytes (Fernández et al., 1992b), and lymphocytes (Ballester et al., 2010). Infected macrophages can release inflammatory mediators like TNF-alpha, IL-1alpha and IL-6 (Gómez-Villamandos et al., 2003; Carrasco et al., 2002; Salguero et al., 2002), which in turn cause severe injury to endothelial cells. This damage may provoke an increase in vascular permeability and haemostatic alterations, leading to disseminated intravascular coagulation (DIC), coagulation factors consumption, fibrinolysis activation and, subsequently, extensive haemorrhage (Villeda et al., 1993a,b, 1995). Other authors have suggested that such damage can be caused by direct endothelial ASFV infection (Gómez-Villamandos et al., 1995; Vallee et al., 2001), although such viral replication has only been described in late infection phases, after the onset of acute haemorrhagic lesions. There is no evident endothelial damage and DIC in the subacute form of ASF, suggesting that observed haemorrhages are associated with severe angiectasia and an increase in vascular permeability.

The acute form of ASFV infection causes extensive lymphatic lesions characterized mainly by apoptosis of infected and non-infected lymphocytes (Gómez-Villamandos et al., 1995; Carrasco et al., 1996; Ramiro-Ibáñez et al., 1996; Oura et al., 1998a). African wild pigs develop milder lymphocyte apoptosis, which do not end up with ASFV-associated lesions. Domestic pigs are not capable to control ASFV-related lymphatic apoptosis, causing impairment of the immune system and contributing to haemorrhagic lesions. Different monokines of infected or activated macrophages have been linked to this apoptosis (Oura et al., 1998b,c; Salguero et al., 2005), since the apoptosis rate is higher in diffuse lymphoid tissue (macrophage-rich area).

3. Clinical and pathological presentations of ASFV

ASFV causes persistent chronic infection without clinical signs in African wild pigs and ticks (genus *Ornithodoros*) (Heuschele and Coggings, 1969; Parker et al., 1969). However, in domestic pigs (*Sus scrofa domesticus*) and wild boars (*Sus scrofa ferus*), ASFV can cause diverse clinical syndromes, ranging from hyperacute or acute infection associated with mortalities up to 100% to subclinical or unapparent infections, depending on viral strain virulence, exposure or doses (Kleiboeker, 2002). Infections with moderate/low virulence strains can cause from 30 to 60% of mortality to no mortality at all. Clinically recovered animals may be persistently infected

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Table 1
Clinical evaluation scoring system.

Concept	Severity (scoring)		
	Mild (1)	Moderate (2)	Severe (3)
Fever			
	Between 39.5° and 40.5°	Between >40.5° and 41° (more than 4 days)	>41° (more than 3 days)
Body condition[a]			
Vertebrae	Only detectable with firm pressure	Prominent	Prominent and with a notable acute angle along the entire vertebral column
Ribs	Ribcage not visible but still noticeable with firm pressure	Ribcage apparent; certain difficulty in detecting the ribs individually	Individual ribs very prominent
Pelvic bones	Only detectable with firm pressure	Obvious	Very prominent
Behaviour			
	Decreased activity, mild to moderate clumsiness	Decreased external stimuli response	Markedly decreased or lack of external stimuli response, immobile, prostration
Skin			
	Body cyanotic areas (<10%); minimal multifocal cutaneous necrosis and/or haemorrhages	Body cyanotic areas (11–25%); mild to moderate multifocal cutaneous necrosis and/or haemorrhages	Body cyanotic areas (>25%); moderate to marked multifocal cutaneous necrosis and/or haemorrhages
Digestive system			
	Faeces around the anus	Faeces covering posterior gluteus	Faeces covering posterior gluteus with blood or extensive mucus
Respiratory system			
	Mild dyspnoea (<25% of variation in respiratory normal rate[b]: bradypnea or tachypnea)	Moderate to marked dyspnoea (>25% of variation in respiratory normal rate[b]: bradypnea or tachypnea)	Moderate to marked dyspnoea (>25% of variation in respiratory normal rate[b]: bradypnea or tachypnea) with blood traces around the nostrils

[a] Body condition assessed following recommendations of Zimmerman et al. (2012).
[b] Respiratory normal rate in pigs: weaning pig = 25 to 40 breathing per minute; adult pig = 13–18 breathing per minute with slight variations concerning to farrowing, neonates, etc. Zimmerman et al. (2012).

for a long time (Wilkinson et al., 1981; Villeda et al., 1993a; Leitao et al., 2001). All ASFV strains show high morbidity because of the high efficiency of viral transmission. Incubation period varies from 2 to 5 days in experimental oronasal infections to 5–7 days in natural infected cases in acute or subacute clinical presentations (Mebus, 1988).

Highly virulent strains may cause hyperacute clinical forms, in which pigs die between days 2 and 4 after the infection without clinical signs but fever (Konno et al., 1972; Mebus and Dardiri, 1979). In acute clinical forms, death may occur between days 12 and 14 after the infection, associated with anorexia, fever, nasal haemorrhages, cutaneous erythemas and cyanosis, melena and, in some cases, diarrhoea (Kleiboeker, 2002). The main observed lesions are pulmonary oedema, haemorrhagic splenomegaly, haemorrhagic lymph nodes with marked size increase and renal petechiae (Mebus, 1988; Carrasco et al., 1996, 1997). In the subacute form of ASF, caused by moderately virulent strains, death happens between days 15 and 20 after the infection causing milder clinical signs and lesions than acute forms. However, this clinical form is characterized by extensive haemorrhages in lymph nodes, kidneys and spleen, with diffuse organ enlargement (Mebus, 1988). The chronic form of ASF is characterized by respiratory clinical signs and lesions, although in some cases both can be minimal or absent (Mebus, 1988; Gómez-Villamandos et al., 2003). Fibrinous pleuritis, pleural adhesions, pneumonia and reticuloendothelial hyperplasia of lymph nodes are noted in the chronic form. Fibrinous pericarditis and cutaneous necrosis are common as well (Moulton and Coggins, 1968). A number of these lesions in the chronic form are attributable to bacterial secondary infections.

4. Methodology and suggested guidelines

4.1. Facilities requirements

ASF is a highly contagious disease included in the list of notifiable diseases to the World Organization for Animal Health (OIE WAHID, 2010). Because of this fact and the risk of iatrogenic introduction in European countries (OIE WAHID, 2010), high biological containing facilities are required to work with this pathogen. It is recommended to refer applicable legislation and or guidelines regarding containment conditions.

4.2. Animal model considerations

The use of standardized experimental animal models that follow recommendations of the Federation of Laboratory Animal Science Associations (FELASA) regarding animal welfare, management and biological particularities (Nicklas, 2008; Voipio et al., 2008) should be carefully considered in all designed protocols.

In general, neither inbreed nor outbreed pigs used for ASFV research are phenotyped and/or genotyped. This may lead to a high degree of variability in haematological parameters, clinical chemistry, organometry, anatomy and histology. To avoid this variability and minimize its impact on data interpretation, this guide will refer to experimental infections performed with immunologically mature outbreed pigs (>8 weeks old pigs) and recommends the inclusion of a sufficient amount of negative controls (no infected) in each experiment.

4.3. Clinical evaluation

4.3.1. Assessment of clinical evaluation

All clinical parameters measured are grouped and scored (from 0 to 3) as shown in the Table 1. All the relevant information of the researcher (personal identification, name of the research group, project reference), procedure (date, number issued by the local official administration) and other data concerning the individual animal (number of pen, study group, rectal temperature at different time points of the experimental period, body weight at the beginning of the experiment, body weight at necropsy, and clinical observations) should be clearly stated for each pig. Average daily gain should be calculated and included in study reports.

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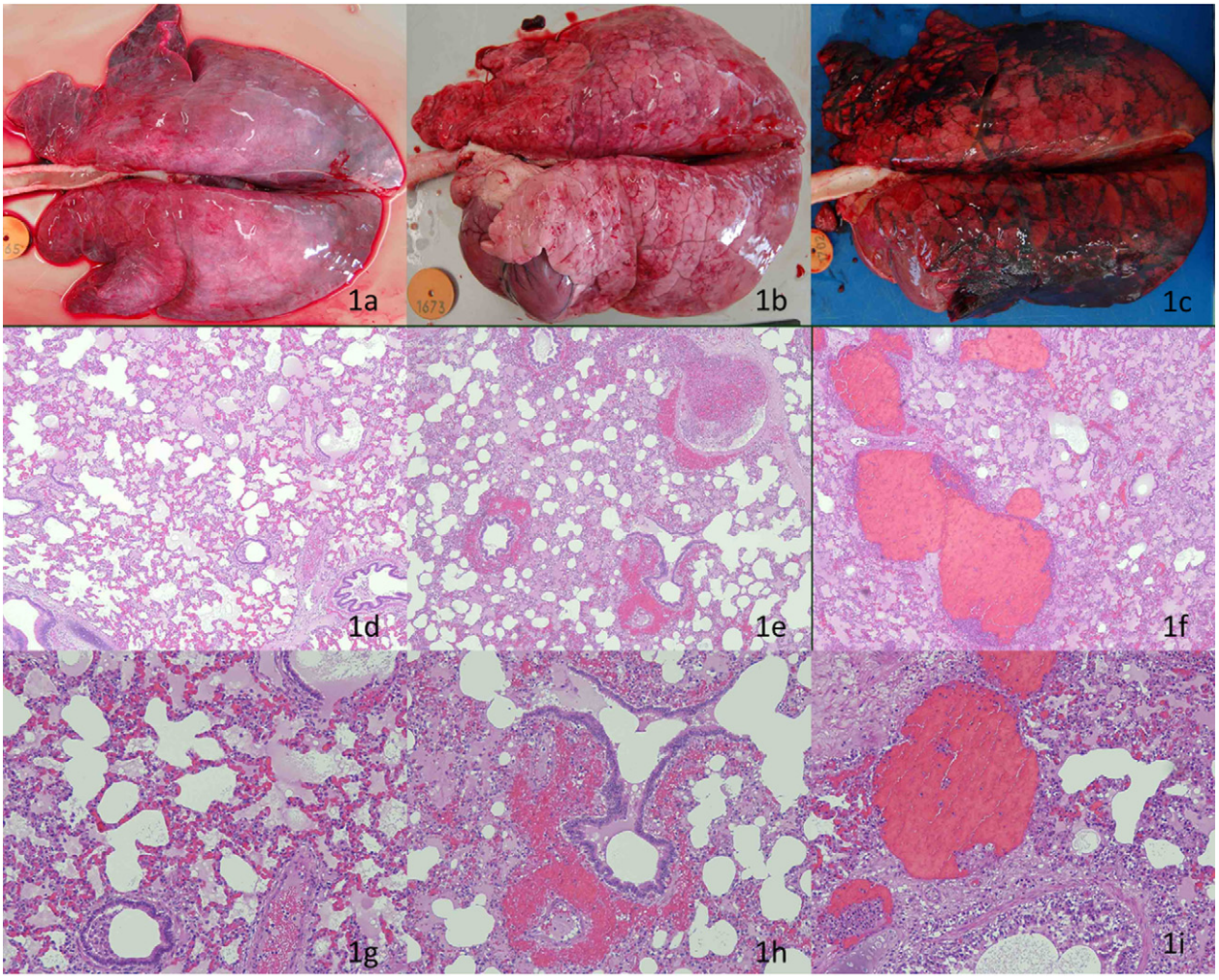

Fig. 1. Reference figures for gross and histopathological pulmonary scores. Lack of pulmonary collapse, oedema and congestion and/or haemorrhage are given on pictures (a), (b) and (c) (gross). Histopathologic findings including oedema, congestion and/or haemorrhage and inflammatory infiltrates are presented on pictures (d), (e), (f), and their respective insets (g), (h) and (i) (haematoxylin and eosin 4 μm paraffin sections; original magnification 10×; insets 20×). Evaluated parameters, lesional severity, scoring and description for gross and histological pulmonary evaluation are given on Table 3. All organs and tissues are from challenged-ASFV pigs.

4.3.2. Endpoint criteria

The endpoint criteria are based on the likely loss of acceptable welfare level for the animal. The endpoint criteria are based in a proposed two-tier evaluation of the clinical scoring as shown in the Table 2. When more than one parameter in the clinical evaluation (Table 1) is scored 3, all 3s are automatically converted to 4. This criterion is an attempt of considering the overall clinical status of the animal when the worst scoring criteria are coincident in the same animal. In the authors' experience, more than one 3s in the proposed clinical scoring means a quick and severe decrease of animal welfare. In these cases, the loss of general welfare should be evaluated carefully and, if it is considered unacceptable, the euthanasia should be applied as soon as possible. Once the clinical evaluation is done, the qualitative endpoint criteria should be applied if it is necessary (Table 2).

Table 2
Endpoint criteria.

Criteria	Decission
Quantitative	
From 0 to 9	No action. From 6 onwards extreme precautions
From 10 to 18	Euthanasia
Qualitative	
	Any animal showing incapacitating prostration should be euthanized immediately
	Any animal showing acute respiratory failure symptoms should be euthanized immediately
	Any animal showing profuse rectal bleeding symptoms should be euthanized immediately

4.3.3. Assessment of clinical pathology

Considering that many ASF-related projects are involved in vaccine research, it is proposed that any evaluation of pathology should include haematological and clinical biochemistry analyses, as described in regulatory procedures for veterinary drugs (EMA, 2008). The clinical pathological scope and analyzed test should be assessed by a pathologist to complete swine health status within each experiment. Considering the ASFV pathogenesis, bone marrow cytology to differentiate between lymphoid, myeloid and erythroid elements should be performed to complete histopathology and/or haematology results. Such data should be included in final pathological reports.

4.4. Gross evaluation of suggested reference organs

This guideline proposes a basic gross report to standardize pathological records to be used in assays of ASFV pathogenesis (Annex 1). The pathologist should perform a standard, schematic

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Table 3
Gross and histopathological pulmonary scoring.

Concept	Severity (scoring)		
	Mild (1)	Moderate (2)	Severe (3)
Macroscopy			
Collapse	Mild lack of collapse with no ribs impressions	Moderate lack of collapse with mild or scarce ribs impressions	Moderate to severe lack of collapse with apparent ribs impressions
Oedema	Scarce or no presence of foamy material in trachea and minimal distension of interlobular walls	Mild to moderate presence of foamy material in trachea and moderate distension of interlobular walls	Marked presence of foamy material in trachea and intense distension of interlobular walls
Congestion/haemorrhage	Mild congestion or active hyperaemia, diffuse or patchy distributed in parenchyma. No haemorrhages	Multifocal to coalescent randomly distributed petechiae and purpurae. Variable degree of congestion or active hyperaemia	Multifocal to coalescent random and interlobulillar distributed extensive haemorrhages (ecchymoses). Variable degree of congestion or active hyperaemia
Pneumonia[a]	Minimal to mild cranio-ventral (uni/bilateral) consolidation (bronchopneumonia)	Moderate cranio-ventral (uni/bilateral) consolidation (bronchopneumonia)	Marked cranio-ventral (uni/bilateral) consolidation (bronchopneumonia)
Reference figures	Fig. 1a	Fig. 1b	Fig. 1c
Histopathology			
Oedema	Mild intra-alveolar and interstitial proteinaceous oedema (<15% alveolar spaces and interstitial walls)	Moderate intra-alveolar and interstitial proteinaceous oedema (15–30% alveolar spaces and interstitial walls)	Marked diffuse intra-alveolar and interstitial proteinaceous oedema (>30% alveolar spaces and interstitial walls)
Congestion/haemorrhage	Capillary hyperaemia with minimal to mild multifocal vasculopathy[b] and mild diffuse capillary hyperaemia	Mild to moderate angiectasia with mild to moderate multifocal vasculopathy,[b] patchy perivascular and peribronchiolar haemorrhages and mild to moderate diffuse capillary hyperaemia	Marked angiectasia with moderate diffuse vasculopathy,[b] mild multifocal vascular necrosis and patchy perivascular and peribronchiolar haemorrhages
Inflammatory infiltrates	Minimal to mild multifocal increase of inflammatory infiltrates (mononuclear) in alveolar walls, alveolar spaces and peribronchiolar areas	Moderate multifocal to coalescing increase of inflammatory infiltrates (mononuclear) in alveolar walls, alveolar spaces and peribronchiolar areas	Marked diffuse increase of inflammatory infiltrates (mononuclear) in alveolar walls, alveolar spaces and peribronchiolar areas
Reference pictures	Fig. 1d, g	Fig. 1e, h	Fig. 1f, i

[a] Pneumonia is a variable finding in the ASFV-challenged pigs, considered as a secondary bacterial complication. Reference pictures do not show this change.
[b] Vasculopathy is defined as prominent diffuse endothelial activation (cellular hypertrophy, rounded nuclei) and variable smooth myocyte vacuolation of tunica media of small and medium vessels, without intramural inflammatory infiltrations.

and complete necropsy following general procedures (Segalés and Domingo, 2003).

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4.4.1. Collection and weighing of organs

Complete ex-sanguination of euthanized pigs should be performed to minimize the variations of organ weights. The extra recommendations and variations to a general necropsy protocol include:

- Organ/total body weight ratio calculation.
- Weight should be recorded in metric units (International Unit System).
- Pig should be weighted before exsanguination.
- Heart should be separated from lungs and weight before opening cardiac cavities.
- Both kidneys should be dissected from the ureter and weight with capsule.
- Liver should be weighted without gastro-hepatic lymph node.
- Lymphoid organs (thymus, spleen, draining lymph nodes of proposed organs) should be weighted after removal from all adhered non-lymphoid tissue.

Organ-to-body weight ratios represent good screening tools to identify the pathological effects related to infection. These ratios are considered more useful when body weight is affected as well (Michael et al., 2007). Alterations of spleen and thymus weights (considered in conjunction with histopathological evaluation) are likely to be more reliable indicators than peripheral lymph nodes for systemic immunomodulation assessment (Haley et al., 2005; Michael et al., 2007).

4.4.2. Standardization of gross evaluation

It is proposed to establish a lesion classification into four categories (use of a numerical score for statistical analysis): none (0), mild (1), moderate (2) and severe (3). Reference pictures and gross evaluation parameters considered as main criteria for lesion intensity evaluation are shown for lung (Fig. 1; Table 3), liver (Fig. 2; Table 4), kidney (Fig. 3; Table 5), and spleen/lymph node (Fig. 4; Table 6). Based on the authors' experience, kidney may show two different gross lesion patterns, which should be considered in the macroscopic evaluation of ASFV-challenged pigs. Based on the lesional distribution observed, the proposed nomenclatures are medullo-cortical and cortico-medullar patterns (Fig. 3; Table 5).

A careful characterization and classification of the ASF pathological presentation in the studied pigs is recommended (Kleiboeker, 2002). The observation of muco-dermal surfaces and cardiovascular, muscular, reproductive, endocrine, nervous and gastrointestinal systems should be evaluated systematically. All additional findings observed during the gross evaluation should be described and sampled.

4.4.3. Considerations about the use of the preliminary pathological report

Two facts concerning pathology should take into account. First, majority of pathological techniques (in situ hybridization, immunohistochemistry, special staining procedures) are expensive and time-consuming. Second, the preliminary pathological report (clinical signs, haematology, clinical chemistry and gross pathological data) may already give clues on potential vaccine efficiency in the group of ASFV-challenged pigs. Therefore, it is suggested that histopathology and some other special techniques should only be performed when the preliminary pathological assessment in the vaccinated, ASFV-challenged group of pigs offer significant differences with either both controls (positive and negative).

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Table 4
Gross and histopathological hepatic scoring.

Concept	Severity (scoring)		
	Mild (1)	Moderate (2)	Severe (3)
Macroscopy			
Hepatopathy	Minimal or mild diffuse lobular pattern with multifocal intraparenchymatous colour changes	Mild diffuse lobular pattern with multifocal intraparenchymatous colour changes and multifocal punctiform redness areas	Moderate diffuse lobular pattern with extensive intraparenchymatous colour changes and coalescent punctiform redness areas
Gallbladder	Mild to moderate oedema affecting cystic duct and/or common hepatic duct	Mild to moderate diffuse peribiliar haemorrhage with moderate oedema affecting common bile duct and vascular hilus	Severe diffuse peribiliar haemorrhage with extensive oedema affecting common bile duct and vascular hilus
Reference pictures	Fig. 2a	Fig. 2b	Fig. 2c
Histopathology			
Blood vessels	Mild portal angiectasia with mild multifocal centrolobulillar sinusoidal dilation and minimal to mild hypertrophy of cholangiocytes	Moderate portal angiectasia with mild multifocal centrolobular sinusoidal dilation and minimal to mild hypertrophy and hyperplasia of bile ducts	Severe portal angiectasia with, moderate multifocal centrolobular sinusoidal dilation and moderate hypertrophy and hyperplasia of bile ducts. Mild multifocal fibrin thrombi (occasional)
Hepatitis[a]	Mild diffuse hepatocellular degeneration with occasional apoptotic hepatocytes, mild multifocal hepatocyte pyknosis, anisokaryosis and Kupffer cell hypertrophy. Mild periportal mononuclear inflammatory infiltrates with general increase of intrasinusoidal cellularity	Moderate diffuse hepatocellular degeneration with scarce apoptotic hepatocytes, moderate multifocal hepatocyte pyknosis, anisokaryosis and Kupffer cell hypertrophy. Moderate periportal mononuclear inflammatory infiltrates with general increase of intrasinusoidal cellularity	Severe diffuse hepatocellular degeneration with common apoptotic hepatocytes, moderate multifocal hepatocyte pyknosis, anisokaryosis and Kupffer cell hypertrophy. Severe periportal mononuclear inflammatory infiltrates with general increase of intrasinusoidal cellularity
Gallbladder	Mild submucosal and peribiliar oedema with minimal angiectasia and haemorrhage. Minimal or mild epithelial changes	Moderate submucosal and peribiliar oedema with mild angiectasia and mild haemorrhage. Mild to moderate ischaemic mucosal necrosis	Severe submucosal and peribiliar oedema with moderate to severe angiectasia and moderate to extensive haemorrhage. Extensive ischaemic mucosal necrosis
Reference pictures	Fig. 2d, g	Fig. 2e, h	Fig. 2f, i

[a] Based on lesional description of viral hepatitis found in Crawford and Liu, 2010.

Table 5
Gross and histopathological renal scoring.

Concept	Severity (scoring)		
	Mild (1)	Moderate (2)	Severe (3)
Macroscopy			
Haemorrhages[a] (medullo-cortical pattern)	Mild multifocal cortical and medullar petechiae with multifocal vascular angiectasia	Moderate multifocal cortical (petechiae) and medullar (purpurae) haemorrhages with moderate pelvic dilation	Marked diffuse cortical and medullar haemorrhages with diffuse general renal darkness, marked pelvic dilation and extensive subcapsular haemorrhages
Haemorrhages[a] (cortico-medullar pattern)	Minimal to mild multifocal cortical and medullar petechiae	Moderate multifocal cortical and medullar haemorrhages (petechiae) with preponderance of cortical affectation	Marked multifocal cortical and medullar haemorrhages (petechiae) with preponderance of cortical affectation with/without multifocal moderate pelvic purpurae
Reference pictures	Figs. 3a, 4a	Figs. 3b, 4b	Figs. 3c, 4c
Histopathology			
Congestion/haemorrhage[a] (medulla)	Mild multifocal medullar angiectasia with minimal or mild multifocal (perivascular or interstitial) petechiae and oedema	Moderate multifocal medullar angiectasia with mild or moderate multifocal (perivascular or interstitial) petechiae and oedema, and variable (absent to mild) multifocal disseminated intravascular coagulation	Marked multifocal medullar angiectasia with intense multifocal (perivascular or interstitial) haemorrhages and minimal or mild disseminated intravascular coagulation
Congestion/haemorrhage[a] (cortex)	Mild multifocal cortical angiectasia with minimal or mild multifocal (perivascular or interstitial) petechiae and oedema	Moderate multifocal cortical angiectasia with mild or moderate multifocal (perivascular or interstitial) petechiae and oedema, and variable (absent to mild) multifocal intraglomerular disseminated intravascular coagulation	Marked multifocal cortical angiectasia with intense multifocal (perivascular or interstitial) haemorrhages and minimal or mild intraglomerular disseminated intravascular coagulation
Necrosis	Minimal to mild multifocal segmental tubular necrosis with minimal to mild multifocal (intratubular) hyaline cast	Moderate multifocal segmental tubular necrosis with mild multifocal (intratubular, glomerular) hyaline cast	Marked multifocal (extensive) segmental tubular necrosis with moderate multifocal (intratubular, glomerular) hyaline cast
Renal inflammation[b]	Mild multifocal interstitial (perivascular, peritubular) mononuclear inflammation	Moderate multifocal interstitial (perivascular, peritubular) mononuclear inflammation	Marked multifocal (extensive) interstitial (perivascular, peritubular) mononuclear inflammation
Reference pictures	Fig. 3d, g, j	Fig. 3e, h, k	Fig. 3f, i, l

[a] The presence of vascular congestion and perivascular or interstitial haemorrhages defines both patterns observed in the ASFV-challenged pigs.
[b] Renal inflammation is a variable finding that includes different but closely related findings; chronic interstitial nephritis, cortical scars or acute renal infarcts. Such specific renal changes should be noted in the "observations" section of the final pathological report.

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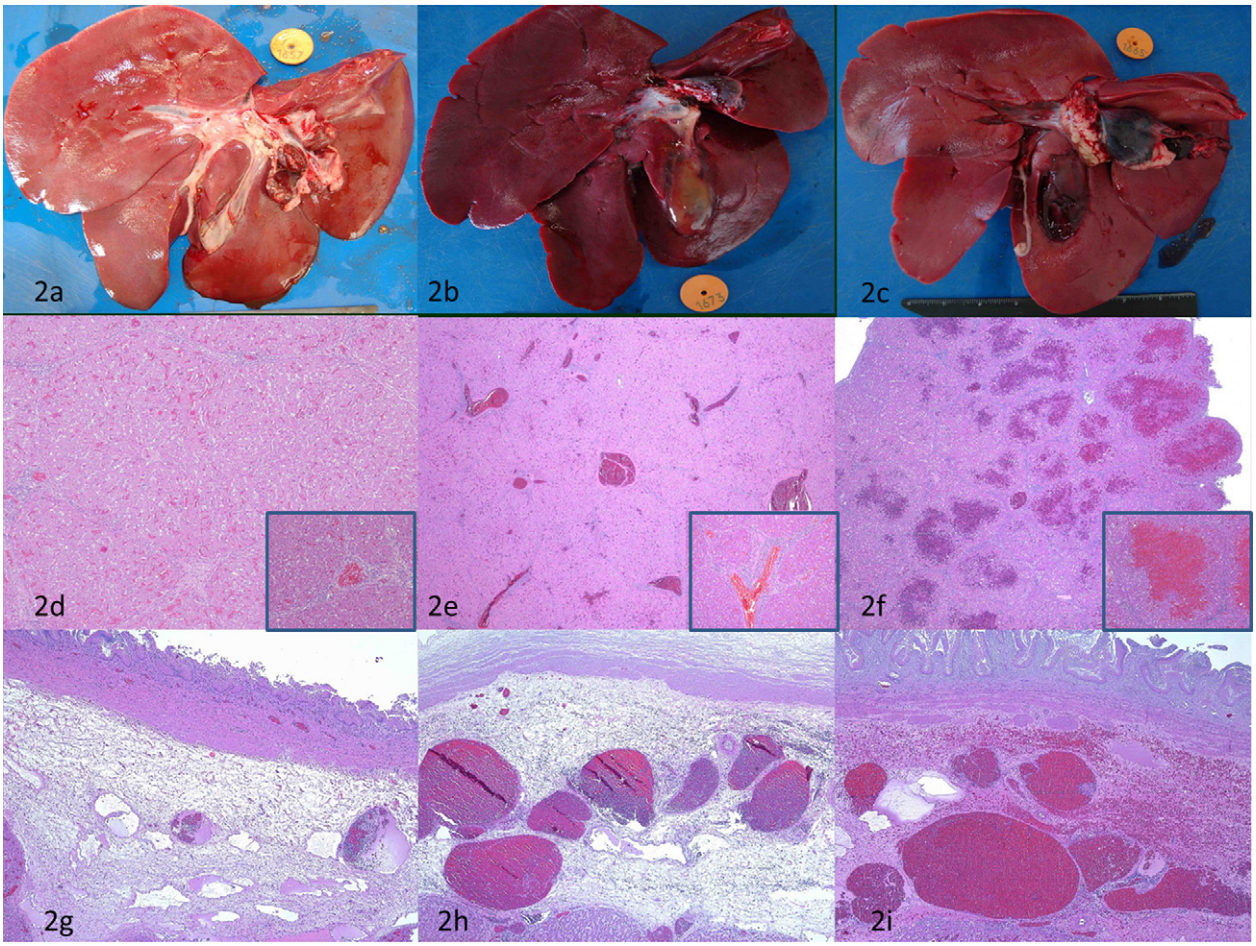

Fig. 2. Reference figures for gross and histopathological hepatic scores. Hepatopathy and gall bladder haemorrhages and oedema are displayed on pictures (a), (b) and (c) (gross). Histopathologic parameters of vasculopathy, hepatitis and gall bladder lesions are presented on pictures (d), (e), (f), (g), (h) and (i) and their respective insets (haematoxilin and eosin 4 μm paraffin sections; original magnification 10×; insets 20×). Evaluated parameters, lesional severity, scoring and description for gross and histological hepatic evaluation are given on Table 4. All organs and tissues are from challenged-ASFV pigs.

Table 6
Gross and histopathological splenic scoring.

Concept	Severity (scoring)		
	Mild (1)	Moderate (2)	Severe (3)
Macroscopy			
Congestion/haemorrhage	Minimal to mild bleeding after sectioning	Moderate bleeding after sectioning	Marked bleeding after sectioning
Necrosis	Multifocal splenic infarcts[a]	Multifocal to coalescent splenic infarcts	Marked extensive splenic infarcts
Reference pictures	Fig. 4a	Fig. 4b	Fig. 4c
Histopathology			
Ratio white pulp/red pulp	>1:4. Mild diffuse lymphoid depletion with minimal to mild hypertrophy of reticulo-endothelial system and mild red pulp hyperplasia	1:5–1:8. Moderate diffuse lymphoid depletion with mild hypertrophy of reticulo-endothelial system and moderate red pulp hyperplasia	<1:9. Severe diffuse lymphoid depletion with moderate hypertrophy of reticulo-endothelial system and moderate red pulp hyperplasia
Necrosis	Multifocal splenic infarcts.[a] Multifocal lytic necrosis of paranodal areas (including reticular system)	Multifocal to coalescent splenic infarcts. Multifocal to coalescent lytic necrosis of paranodal areas (including reticular system), peritrabeculae and sinusal areas	Severe extensive splenic infarcts. Extensive lytic necrosis of reticular and endothelial lymphatic system
Lymphocytolysis	Mild multifocal lymphoid and histiocytic single cell necrosis/apoptosis	Moderate multifocal to coalescent lymphoid and histiocytic single cell necrosis/apoptosis	Severe focally extensive lymphoid and histiocytic single cell necrosis/apoptosis
Vascular damage	Minimal or mild multifocal angiectasia with mild vasculopathy[b] and mild diffuse red pulp congestion	Moderate multifocal angiectasia with mild vasculopathy[b] and moderate diffuse red pulp congestion and variable haemorrhages	Severe multifocal angiectasia with mild vasculopathy[b] and intense diffuse red pulp congestion and massive haemorrhages
Reference pictures	Fig. 4d	Fig. 4e	Fig. 4f

[a] Mild splenic focal necrosis or infarcts were not observed grossly in the reviewed ASFV-vaccinated pigs, but this fact does not rule out such possibility.
[b] Vasculopathy is defined as prominent diffuse endothelial activation (cellular hypertrophy, rounded nuclei) and variable smooth myocyte vacuolation of tunica media of small and medium vessels, without intramural inflammatory infiltrations.

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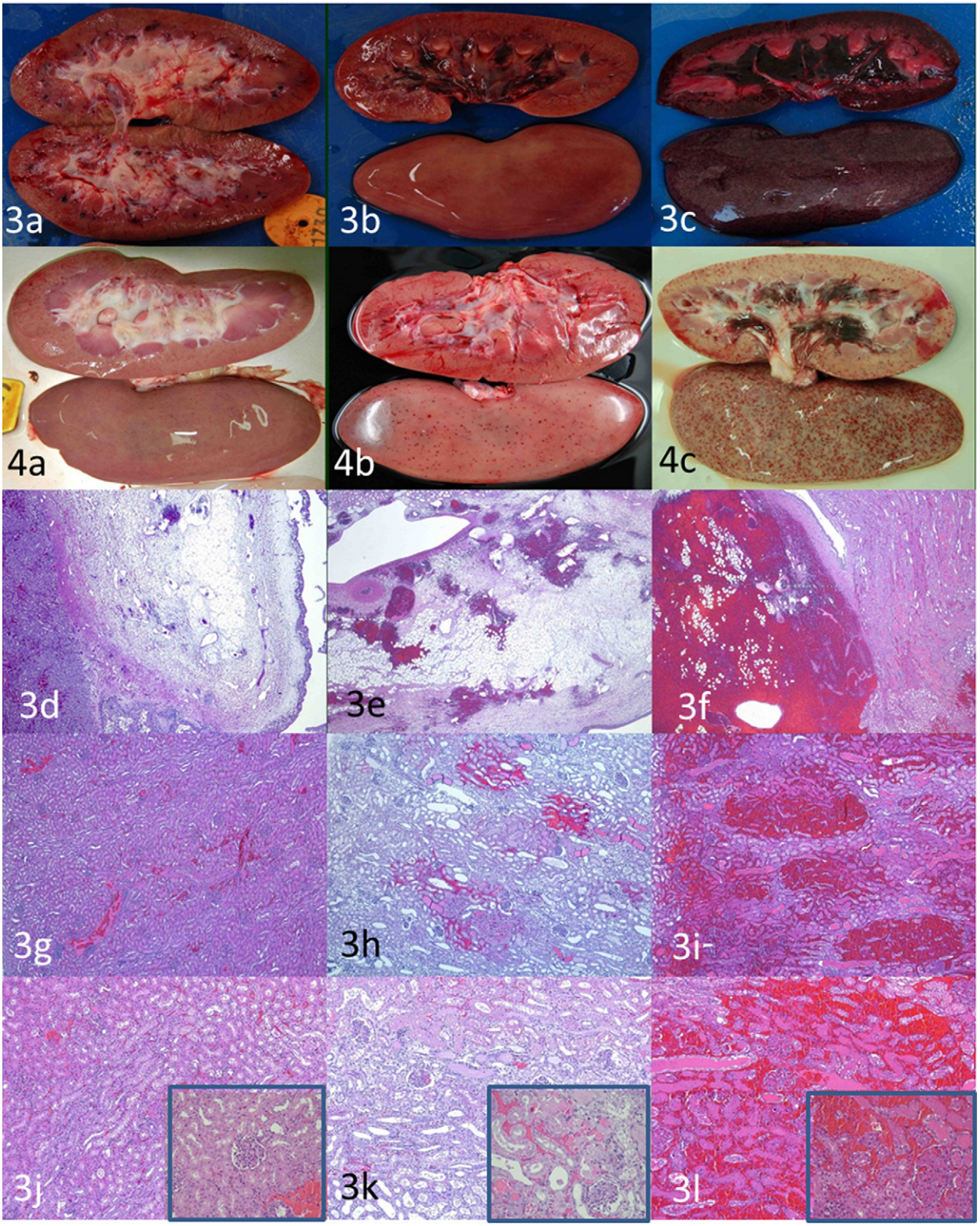

Fig. 3. Reference figures for gross and histopathological renal scores. Renal haemorrhages are displayed on pictures (3a), (4a), (3b), (4b), (3c), and (4c) (gross). Histopathologic findings of congestion and haemorrhages of cortex and medulla, tubular necrosis and inflammatory infiltrates are presented on pictures (3d), (3e), (3f), (3g), (3h), (3i), (3j), (3k), and (3l) and their respective insets (haematoxilin and eosin 4 μm paraffin sections; original magnification 10×; insets 20×). Evaluated parameters, lesional severity, scoring and description for gross and histological renal evaluation are given on Table 5. All organs and tissues are from challenged-ASFV pigs.

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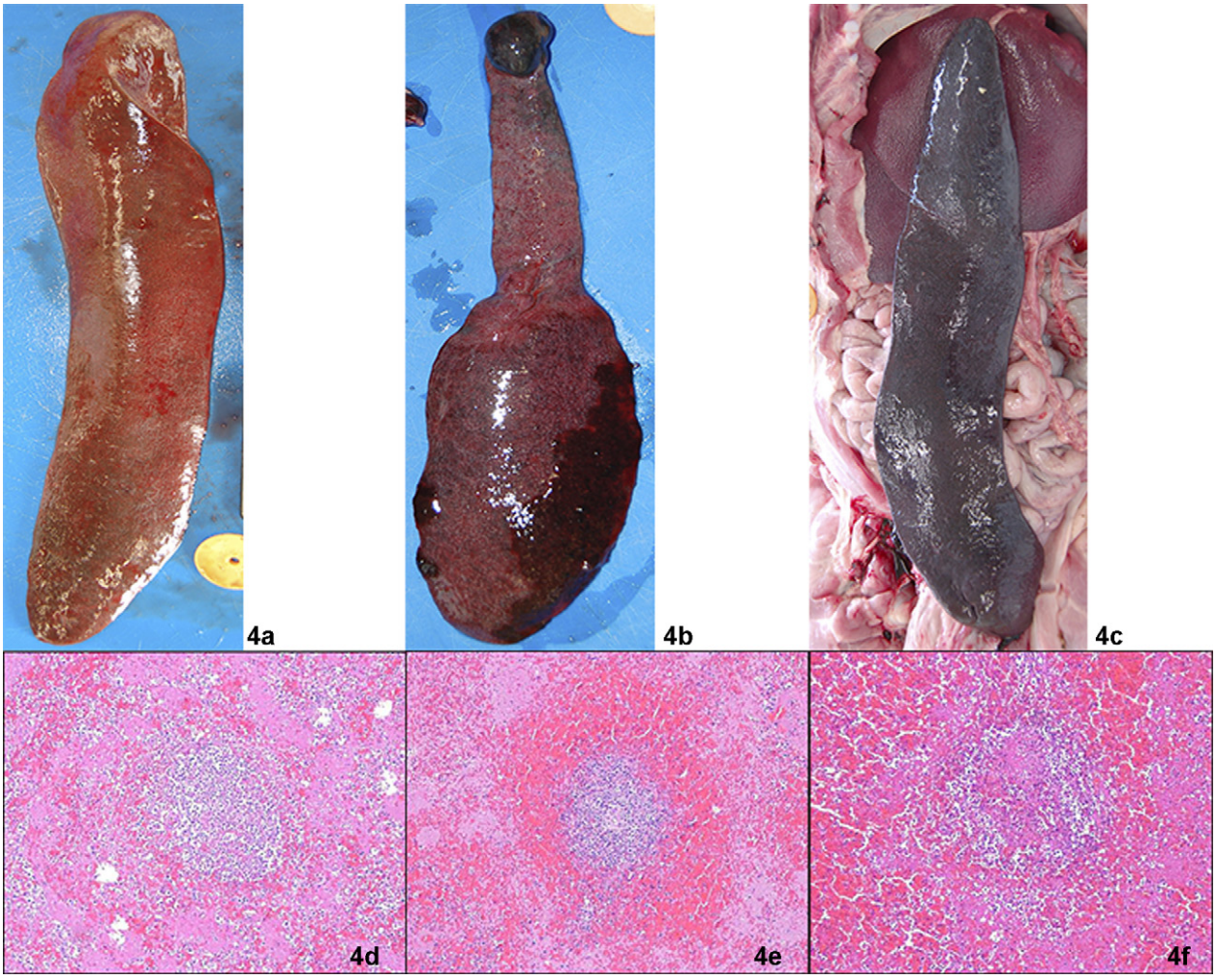

Fig. 4. Reference figures for gross and histopathological splenic scores. Congestion and/or haemorrhages and presence of splenic infarcts are displayed on pictures (a), (b) and (c) (gross). Histopathologic parameters about evaluation of white pulp/red pulp, necrosis, lymphocytolisis and vascular damage are presented on pictures (d), (e) and (f) (haematoxilin and eosin 4 μm paraffin sections; original magnification 20×). Evaluated parameters, lesional severity, scoring and description for gross and histological splenic evaluation are given on Table 6. All organs and tissues are from challenged-ASFV pigs.

4.5. Histopathology

Current histopathological methods could be enhanced by the application of improved techniques, semiquantitative terminology, and additional training (Maronpot, 2006). All tissue compartments should be routinely examined and any abnormalities recorded with consistent terminology, focusing on the assessment of ASFV vaccine development.

4.5.1. Considerations for the histological evaluation

The pathologist should identify and examine all compartments of all sampled tissues, making entries for specific compartments if an abnormality is identified. Normal ranges should be noted as "No apparent lesions (NAL)" or another analogue term. Any abnormality should be noted using appropriate terminology supervised by a trained pathologist. Before starting histological evaluation, non-pathologist researchers should be aware of the standard definitions of the pathological terms used in this guideline.

As a matter of example, the authors recommend applying the three primary points that are emphasized in a reported compartment-based lymphoid tissue histopathological approach: (1) each organ has separate compartments that support its own functions (in the case of lymphoid tissue, specific immune responses), (2) these compartments should be evaluated individually for changes, and (3) descriptive/semiquantitative (i.e., reduced/increased numbers of lymphocytes, or cortical renal angiectasia) rather than interpretative terminology (i.e., lymphoid atrophy/hyperplasia or renal congestion), should be used to characterize changes within these compartments (Kuper et al., 2000; Kuper, 2002; Ruehl-Fehlert et al., 2005). The use of such terms should include appropriate indicators of severity to maximize their descriptive value. In many cases, the indication of the cell type (i.e., lymphocyte, macrophage, mast cell, etc.) modified (i.e., decrease/increase in number) should be included (Table 6).

4.5.2. Standardization of histological evaluation

Reference figures and histopathological evaluation parameters considered as main criteria for lesional severity are shown for lung (Fig. 1; Table 3), liver (Fig. 2; Table 4), kidney (Fig. 3; Table 5), and spleen/lymph node (Fig. 4; Table 6). Other lesions or unexpected finding in any other body system not mentioned before should be considered as an incidental background change, and further investigation is recommended to be performed at the discretion of the researcher(s). Moreover, the pathologist should coordinate the most appropriate terminology used in a study with the virologist and immunologist involved in the project. A model for histological report is suggested in Annex 2.

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5. Discussion

The present guideline proposes a standardized methodology to perform the pathological assessment of the experimental infection outcome.

Pigs involved in ASFV challenges are usually classified as developing a chronic, subacute or acute ASFV presentation following described patterns (Konno et al., 1972; Mebus and Dardiri, 1979; Wilkinson et al., 1981; Mebus, 1988; Villeda et al., 1993a; Gómez-Villamandos et al., 2003; Carrasco et al., 1996, 1997; Leitao et al., 2001; Kleiboeker, 2002). This allows correlating the severity and distribution of the ASF lesions with key parameters measured in parallel, including viraemia and proportion of ASFV-infected cells present in blood, lymphopenia severity and presence of pro-inflammatory molecules in serum (Ramiro-Ibáñez et al., 1996, 1997; Argilaguet et al., 2012). A subsequent pathological analysis needs to have adequate controls because of the high variability of genotypes and phenotypes of ASFV strains, background changes in infected pigs and different aspects linked with the challenge model (ways of administration, dose, vaccine product type, etc.).

Standard pathological scores should be introduced in the future into the ASFV vaccinology experiments for adequate inter-group and inter-experiment comparisons. The suggested approach emphasizes the use of descriptive terms in the recording of findings, as compared to interpretative terms or diagnoses, to add precision to histopathology databases concerning the relevant histological changes. As it has been seen in others pathological approaches, specialized pathological techniques such as in situ hybridization, immunohistochemistry, blind scoring or morphometry, may be valuable to clarify specific alterations (Maronpot, 2006). These techniques should be directed only to answer a specific scientific question and not be used as routine screening tools (Maronpot, 2006; Ballester et al., 2010).

In conclusion, the present guideline offers a basic approach to pathological evaluation of ASFV-inoculated pigs and suggests some tools to improve data collection and analysis. This information may be of special interests by researchers focused on ASFV vaccine development.

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