Transboundary and Emerging Diseases

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RAPID COMMUNICATION

Experimental Transmission of African Swine Fever (ASF) Low Virulent Isolate NH/P68 by Surviving Pigs

C. Gallardo[1], A. Soler[1], R. Nieto[1], M. A. Sánchez[1], C. Martins[2], V. Pelayo[1], A. Carrascosa[3], Y. Revilla[3], A. Simón[1], V. Briones[1], J. M. Sánchez-Vizcaíno[4] and M. Arias[1]

[1] European Union Reference Laboratory for African Swine Fever, Centro de Investigación en Sanidad Animal, INIA-CISA, Madrid, Spain
[2] Faculdade Medicina Veterinária, Universidade Técnica Lisboa (FMV-UTL), Lisbon, Portugal
[3] Centro de Biología Molecular Severo Ochoa, Agencia Estatal Consejo Superior Investigaciones Científicas, CSIC-UAM, Madrid, Spain
[4] VISAVET, Dpto. Sanidad Animal, Facultad Veterinaria, Universidad Complutense de Madrid (UCM), Madrid, Spain

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Correspondence:
C. Gallardo. Centro de Investigación en Sanidad Animal (CISA-INIA), Ctra Algete el Casar s/n. 28130 Valdeolmos, Madrid, Spain.
Tel.: +34 916202300; Fax: +34 916202247;
E-mail: gallardo@inia.es

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Summary

African swine fever (ASF) has persisted in Eastern Europe since 2007, and two endemic zones have been identified in the central and southern parts of the Russian Federation. Moderate- to low-virulent ASF virus isolates are known to circulate in endemic ASF-affected regions. To improve our knowledge of virus transmission in animals recovered from ASF virus infection, an experimental *in vivo* study was carried out. Four domestic pigs were inoculated with the NH/P68 ASF virus, previously characterized to develop a chronic form of ASF. Two additional in-contact pigs were introduced at 72 days post-inoculation (dpi) in the same box for virus exposure. The inoculated pigs developed a mild form of the disease, and the virus was isolated from tissues in the inoculated pigs up to 99 dpi (pigs were euthanized at 36, 65, 99 and 134 dpi). In-contact pigs showed mild or no clinical signs, but did become seropositive, and a transient viraemia was detected at 28 days post-exposure (dpe), thereby confirming late virus transmission from the inoculated pigs. Virus transmission to in-contact pigs occurred at four weeks post-exposure, over three months after the primary infection. These results highlight the potential role of survivor pigs in disease maintenance and dissemination in areas where moderate- to low-virulent viruses may be circulating undetected. This study will help design better and more effective control programmes to fight against this disease.

Introduction

African swine fever (ASF) is a highly significant haemorrhagic viral disease that has serious sanitary and economic consequences due to associated high mortality rates and the international trade restrictions imposed after outbreaks. ASF affects only porcine species (both wild and domestic) of all breeds and ages, giving rise to a variety of clinical signs and lesions (Hess, 1981; Penrith and Vosloo, 2009a; Penrith, 2009b; Gómez-Villamandos et al., 2013; Sánchez-Vizcaíno et al., 2015). It is caused by a large complex DNA virus (ASFV) belonging to the *Asfarviridae* family for which no treatment or vaccine is currently available. Therefore, early detection is crucial in control and eradication strategies.

ASF epidemiology is very complex due to virus and host characteristics, as well as to the presence of reservoirs. The disease was first described in 1921 in Kenya and was confined to sub-Saharan Africa until 1957, when it was detected for the first time outside Africa, in Europe (Lisbon, Portugal). It soon spread to other European and American countries, from where the disease was successfully eradicated in most cases. Today, ASF is known to be endemic in sub-Saharan Africa and in Sardinia (Italy) (Costard et al., 2009; Arias and Sánchez-Vizcaíno, 2012; Sánchez-Vizcaíno et al., 2012; Penrith et al., 2013). In

2007, ASF was introduced into Georgia from East Africa and spread rapidly to Armenia, Azerbaijan and the Russian Federation (Rowlands et al., 2008; Food and Agriculture Organization, United Nations, 2013). The ongoing spread of ASF into the Ukraine (2012, 2014) and Belarus (2013), as well as into Russia affecting both wild boar and domestic pigs, placed neighbouring areas at risk. In early 2014, four European Union (EU) countries (Lithuania, Poland, Latvia and Estonia) were also affected, probably as a result of virus introductions by infected animals from neighbouring countries to the east along ecological wild boar corridors (Gallardo et al., 2014). Currently, the disease is threatening other regions in Europe and Asia due to the potential persistent spillover of the virus into adjacent areas.

ASFV strains are classified as highly, moderately or low virulent (Pan and Hess, 1984; Blome et al., 2013; Gómez-Villamandos et al., 2013; Sánchez-Vizcaíno et al., 2015). Highly virulent strains are usually responsible for the peracute and acute forms that give rise to high mortality rates that may reach 100% within 4–9 days post-infection. In peracute ASF, affected animals can die suddenly 1–4 days after the onset of clinical signs with no evident lesions in organs. Pigs showing the acute forms of the disease display mainly a febrile syndrome with erythema and cyanosis of the skin. Internal lesions are mainly characterized by hyperaemic splenomegaly and haemorrhages in organs, particularly in the visceral lymph nodes, with fluids in body cavities and fibrin strands on organ surfaces. The distribution and frequency of these lesions are variable, and most are seen in other swine diseases such as classical swine fever (CSF). Moderately virulent viruses lead to the appearance of acute and subacute forms. Pigs with subacute infection may have persistent or fluctuating temperature responses for up to 20 days, during which time some pigs stay in good condition, while others display the symptoms described above for the acute process form (but less severely) with mortality rates in the range 30–70%, usually after 20 dpi. In the chronic form of ASF, clinical signs and lesions are not specific and may persist for several months, giving rise to a range of illnesses, with symptoms such as skin ulcers and arthritis, stunted delayed growth, emaciation, lameness, pneumonia and abortion, but with low mortality rates. This form was described in the Iberian Peninsula and in the Dominican Republic in pigs affected by low-virulent viruses. It has been hypothesized that the chronic form may be the result of the natural attenuation of ASFV in the field or may have originated from the experimental vaccine virus released in Iberian Peninsula in the 1960s (Manso Ribeiro et al., 1963; Moulton and Coggins, 1968; Mebus et al., 1978; Mebus and Dardiri, 1979, 1980; Sánchez-Botija, 1982; Pan and Hess, 1984; Leitao et al., 2001; Sánchez-Vizcaíno et al., 2015; Portugal et al., 2015).

The *in vivo* experimental studies using ASFV isolates affecting Eastern European countries reveal the existence of virulent strains that induce acute forms of ASF provoking high mortality in both domestic and wild animals (Gabriel et al., 2011; Guinat et al., 2014; Gallardo et al., 2015; Pietschmann et al., 2015). However, since the introduction of the virus into the Russian Federation in 2007, ASF has become a large-scale epidemic involving both domestic pig and wild boar populations, with two recognized endemic zones in central and southern parts of the Russian Federation (Gogin et al., 2013). Once the disease is established as endemic in an area, a broad range of clinical symptoms and clinical onsets can be expected, although animals that survive for over a month are able to recover from the infection and even remain subclinically infected (Mebus et al., 1978; Mebus and Dardiri, 1979, 1980; Sánchez-Botija, 1982; Bech-Nielsen et al., 1995; Pérez et al., 1998; Arias and Sanchez-Vizcaino, 2002; Arias and Sánchez-Vizcaíno, 2012).

Although in past decades several *in vivo* experiments were performed to evaluate the role of survivor animals in the maintenance of the disease, to date this fact is still unclear (Wardley et al., 1983; Ordas et al., 1983; Penrith et al., 2013). The current ASF situation in the Russian Federation and the series of reported cases within the Eastern Europe highlight the need to study survivor pigs and their role in the transmission and maintenance of the virus in pig populations. Therefore, an experimental *in vivo* transmission study was performed using the ASFV NH/P68 strain, a low-virulent and non-haemadsorbing isolate that induces a chronic form of ASF (Leitao et al., 2001). Low-virulent ASF viruses provide useful models with high survival rates and thus are appropriate for assessing virus shedding, release and transmission. The results obtained demonstrated that the animals that recovered were still able to transmit the virus to a naive population three months after being infected and so may play a role in the spreading of the disease.

Methods

Cells and viruses

The NH/P68 isolate (NHV) is a non-haemadsorbing and low-virulent ASFV isolated from a pig chronically infected during the ASF epidemic in Portugal in 1968 that took place eight years after the disease's first appearance in that country (Leitao et al., 2001). The virus was propagated and titrated in primary swine macrophage cultures (Carrascosa et al., 2011). The ASFV BA71V, isolated in Spain in 1971 and adapted to Vero cells (ATCC CCL 81), was used for the production of the infected cell cultures for the microplates needed to perform the indirect immunoperoxidase test (IPT) (Gallardo et al., 2012; European Union Reference Laboratory (EURL) for African swine fever, 2014).

The MS stable monkey kidney cell line (ECACC, 91070510) was used for conventional soluble cytoplasmic antigen production after the infection with the ASFV MS-adapted E70 isolate (E70 MS 48), as described in the OIE Manual of diagnosis (World Organisation for Animal Health (OIE), 2014b).

Experimental design and sampling collection

In vivo experiments were conducted in the BSL3 animal facilities at CISA-INIA in accordance with Spanish and European regulations and EC Directive 86/609/EEC, and following the recommendation 2007/526/EC for the accommodation and care of animals used for experimental and other scientific purposes. Four hybrid pigs (named C1 to C4) were inoculated intramuscularly with 10^5 50% tissue culture infectious doses per ml (TCID50/ml) of the NHV. At 72 days post-inoculation (dpi), two additional pigs (CC13 and CC14) were introduced into the same box as in-contact (exposed) pigs. The severity of the disease was expressed using a clinical score, obtained by summing the score of seven clinical signs recorded on a daily basis (Table 1). For antibody kinetics and viraemia studies, paired EDTA blood and sera samples were collected from pigs at 0, 4, 8, 11, 14, 18, 21, 30, 37, 44, 52, 58, 65, 72, 79, 86, 93, 100, 107, 114, 121, 128 and 134 dpi. The virus was found to be present in 19 tissue samples (liver, spleen, tonsil, heart, lung, kidney, intra-articular tissues of joints and submandibular, retropharyngeal, inguinal, popliteal, mesenteric, mediastinal, gastro-hepatic, splenic and renal lymph nodes) obtained from the inoculated animals, which were euthanized, respectively, at 35 (C1), 65 (C4), 99 (C3) and 134 (C2) dpi, and from the in-contact pigs, euthanized at 42 (CC14) and 62 (CC13) days post-exposure (dpe) (114 and 134 dpi, respectively).

Table 1. List of clinical signs used to create the ASF clinical score

1. Anorexia	0. no abnormality
	1. Mild (reduced eating)
	2. Moderate (only picking at food)
	3. Severe (not eating)
2. Recumbence	0. no abnormality
	1. Mild (stillness)
	2. Moderate (get up only when touched)
	3. Severe (remain recumbent when touched)
3. Skin Haemorrhage/ Cyanosis	0.no abnormality
	1. Mild
	2. Moderate
	3. Severe
4. Swelling	0. no abnormality
	1. Mild (joint swelling)
	2. Moderate (lameness, necrotic foci)
	3. Severe (severe lameness, impaired walking)
5. Laboured breathing and/or coughing	0. no abnormality
	1. Mild
	2. Moderate
	3. Severe
6. Ocular discharge	0. no abnormality
	1. Mild
	2. Moderate
	3. Severe
7. Digestive findings	0. no abnormality
	1. Mild (normal diarrhoea<24 h)
	2. Moderate (diarrhoea, vomiting>24 h)
	3. Severe (bloody diarrhoea)

Sample analysis

DNA was extracted from all organ homogenates and blood samples using the High Pure PCR Template Preparation kit (Roche) and amplified using OIE conventional (Agüero et al., 2003; World Organisation for Animal Health (OIE), 2014b) and real-time PCR (King et al., 2003; World Organisation for Animal Health (OIE), 2014b). Samples with recorded threshold cycle number (Ct) < 40.0 were considered positive and samples with no recorded Ct value were considered negative (World Organisation for Animal Health (OIE), 2014b). Virus isolation and titration were performed using porcine peripheral blood macrophages (PBM) (Carrascosa et al., 2011). Detection of ASFV-specific antibodies was performed in serum using the commercial ELISA (®INGENASA-INGEZIM PPA COMPAC K3 INGENASA, Madrid, Spain), the OIE-Indirect ELISA (World Organisation for Animal Health (OIE), 2014b), and the IPT validated by the EURL (Gallardo et al., 2012; European Union Reference Laboratory (EURL) for African swine fever, 2014).

Results

Inoculated animals

Clinical findings and gross lesions

The inoculated animals developed variable clinical manifestations ranging from subclinical infection (C2) to clinical signs of different intensity – more intense in C1 than in C3 followed by C4 – associated with a chronic form of ASF initiated at 14 ± 3 dpi (Fig. 1a). The predominant clinical symptoms were (i) recurrent transient fever, mainly in pigs C1 and C4 (Fig. 1b), (ii) joint swelling (moderate to severe) with reddened areas of skin that became raised and necrotic (Fig. 2a), (iii) gradual weight loss and (iv) respiratory distress with spasmodic coughing over the period of a month, most evident in C3. Animals were slaughtered at 35 (C1), 65 (C4), 99 (C3) and 134 (C2) dpi to determine the presence of ASFV in tissues. Macroscopic lesions found during necropsies performed at 35 and 65 dpi included fibrinous

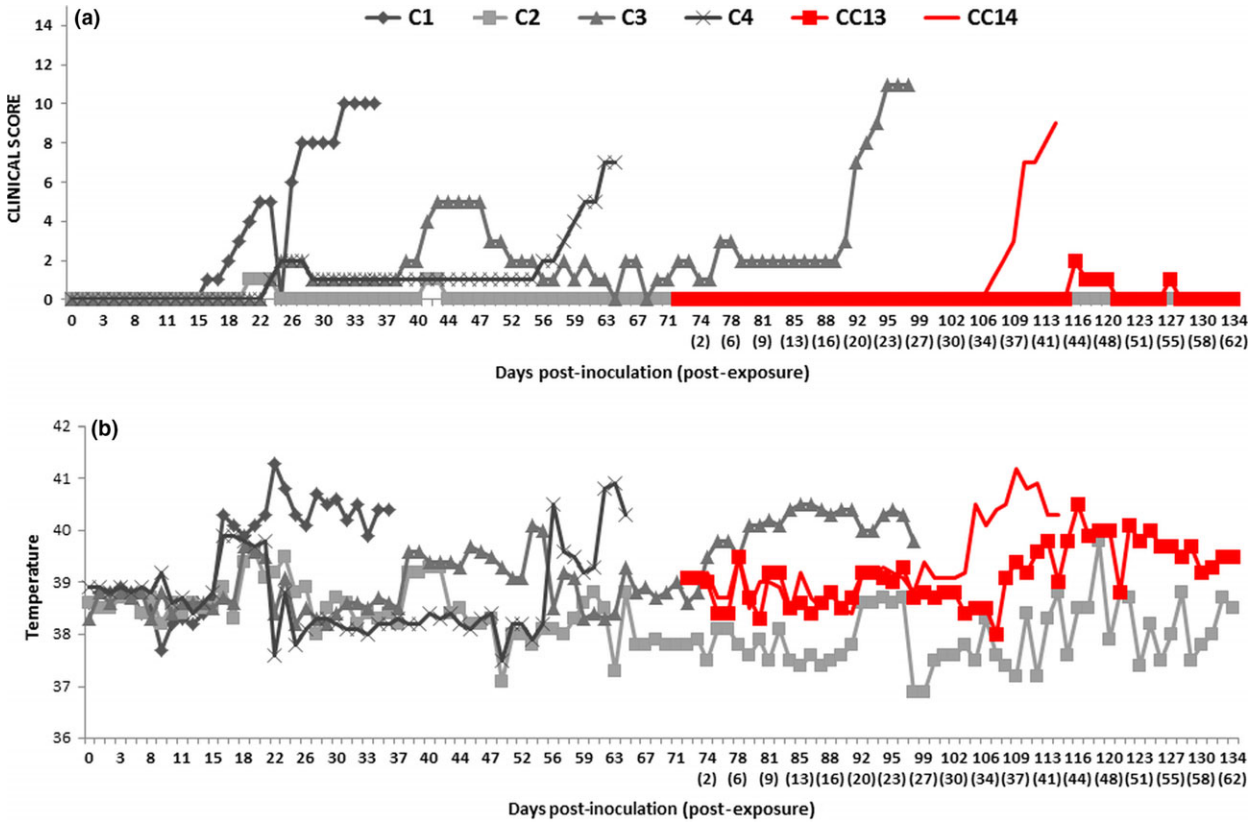

Fig. 1. Clinical score (a) and temperature (b) in the NHV ASFV-inoculated (in grey) and in-contact (in red) groups.

pericarditis and oedematous and partially haemorrhagic lymph nodes, mainly gastro-hepatic, renal and mediastinal. The pig slaughtered at 99 dpi (C3) had severe pneumonia with caseous lobular consolidation and calcification of areas of the lungs (Fig. 2b) and focal lesions in the thoracic lymph nodes. No significant lesions were found in the asymptomatic animal (C2) slaughtered at 134 dpi.

Viremia, humoral response and ASFV distribution in tissues

Viremia was detected to a variable extent in the inoculated pigs (Fig. 3a). In pig C1, which developed a more intense chronic form of infection, viremia was detected at 18 dpi and remained positive until slaughtered at 35 dpi. Infectious viruses were recovered from all positive blood samples including the PCR-positive blood samples obtained from pig C4 at 21, 58, 62 and 65 dpi. Interestingly, despite having similar Ct values, no infectious virus was recovered from pig C3, which developed a viremia that lasted for over two months (14–99 dpi). The subclinical infected pig C2 was aviraemic on all analysis days. Anti-ASFV-specific antibodies were detected at 8 dpi in all pigs using IPT and at 8–14 dpi (depending on the animal) by the ELISA tests. The anti-ASFV antibody titre was quite low in the asymptomatic animal, whereas symptomatic animals yielded very high values ($>10^5$) until the end of the experiment (Fig. 3b).

The ASFV genome was detected by PCR in all tissue samples obtained from the two inoculated animals slaughtered at 35 and 65 dpi. After three passages in PBM cells, the ASFV was recovered from 16 (84.21%) and 12 (63.15%) different tissue samples. In the animal euthanized at 99 dpi, the virus genome was detected in 12 of the 19 (63.1%) collected tissues, although virus could only be recovered from the lung and mediastinal lymph node. However, these samples yielded high titre values ($>10^7$TCDI50/ml). No positive ASFV result was obtained from samples collected from the inoculated animal slaughtered at 134 dpi (C2). The results are summarized in Table 2.

In-contact animals

Clinical findings and gross lesions

At 72 dpi, two in-contact pigs (CC13 and CC14) were introduced into the same box as the inoculated animals C2 and C3. At the time of exposure, both inoculated animals had high antibody titre levels and one (C3) had a weak viremia (Fig. 3a,b). In one of the in-contact pigs (CC14), the clinical signs relating to a chronic form of ASF began at 30–

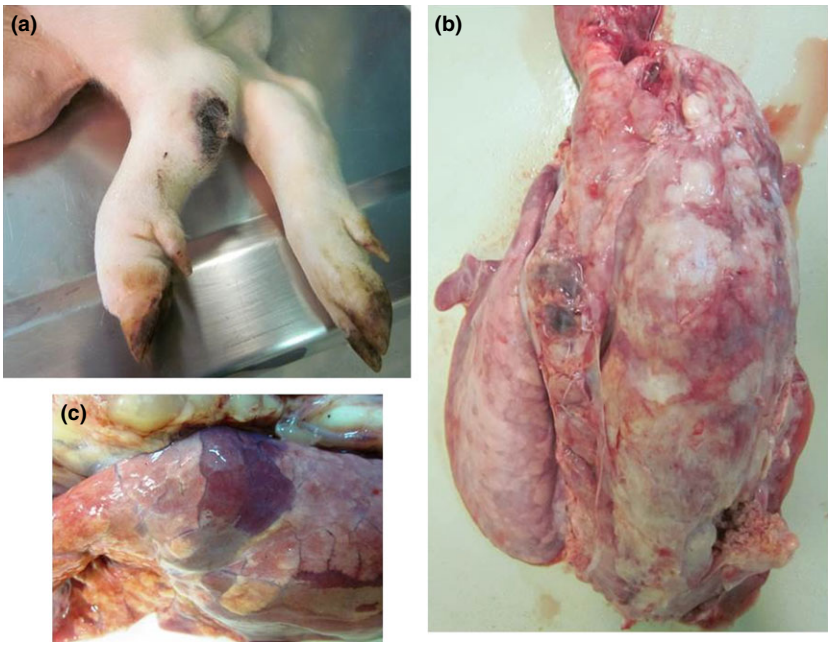

Fig. 2. (a) Necrotic area and joint swelling of left metatarsus; C1, slaughtered at 35 dpi. (b) Severe bilateral caseous pneumonia; mediastinal lymph nodes appear enlarged; C3, slaughtered at 99 dpi. (c) Consolidation areas (darker) and caseous lobular pneumonia; a slight opacity of pleura can also be noted; CC14, slaughtered at 42 dpe.

32 dpe and resembled those observed in the inoculated animals, which is, mainly joint swelling, temperature above 40°C, and respiratory disorders (Fig. 1a,b). The second in-contact pig (CC13) did not develop any detectable clinical signs other than a weak peak of fever over 40°C at 45 dpe (Fig. 1b). The in-contact pigs were euthanized at 42 dpe (CC14) and 62 dpe (CC13) to assess lesions and virus presence in tissues. The post-mortem examination revealed macroscopic lesions including caseous necrosis and focal mineralization of the lungs, fibrin deposits in tracheal lumen, and oedematous and partially hyperaemic bronchial lymph nodes. Theses lesions were more intense in pig CC14 slaughtered at 42 dpe than in pig CC13 (Fig. 2c).

Viremia, humoral response and ASFV distribution in tissues

The first viraemia peak was identified in pig CC14 at 28 dpe, prior to the appearance of clinical signs, whereas in pig CC13 two peaks of viraemia were detected at 49 and 56 dpe (Fig. 3a). Antibodies were found in both animals from 35 dpe to the end of the experiment (Fig. 3b). The presence of ASFV was demonstrated by PCR in all tested tissues in the animal slaughtered at 42 dpe, with titres in the range $10^{6.3}$–$10^{8.3}$ TCID50/ml. In the exposed pig CC13 (62 dpe), a positive PCR result was obtained in 10 of 19 (52.5%) tissues samples, although no virus could be recovered after three passages in primary cultures (Table 2).

Discussion

When introduced into a region or a domestic pig population, ASF is typically associated with high mortality rates and a rapid spread of outbreaks (Costard et al., 2009; Sánchez-Vizcaíno et al., 2012). However, several studies have demonstrated that in areas where ASF becomes endemic, increased numbers of subacute, chronic and subclinical infections also occur, and that mortality rates decline over time. In such situations, the clinical manifestations of this disease are more variable and difficult to recognize in the field. The infection can persist for several months with no particular obvious symptoms in the infected animals, other than stunting or emaciation, or may even mimic certain other illnesses (Mebus et al., 1978; Mebus and Dardiri, 1979, 1980; Thomson et al., 1979; Hess, 1981; Wilkinson et al., 1981, 1983; Wilkinson, 1984; Sánchez-Botija, 1982; Nsalambi, 1993; Boinas et al., 2004; Penrith et al., 2013; Sánchez-Vizcaíno et al., 2015).

Although no long-term carrier state has yet been experimentally demonstrated, subclinically infected, chronically infected or survivor pigs are likely to play an important role in the epidemiology of the disease, that is in disease persistence in endemic areas or in sporadic outbreaks or ASF introduction into free zones (Allaway et al., 1995; Arias and Sanchez-Vizcaino, 2002; Arias and Sánchez-Vizcaíno, 2012; Penrith and Vosloo, 2009a; Penrith, 2009b; Costard et al., 2009). Field studies in affected regions such as Brazil and the Iberian Peninsula (1979–1981) have revealed that 3.5% and 0.6% of new outbreaks, respectively, are thought to be caused by seropositive domestic pigs that had recovered from an initial infection (Sánchez-Botija, 1982; Vigário et al., 1983; Ordas et al., 1983; Bech-Nielsen et al., 1995). The presence of subclinical and chronic forms in endemic regions could be related to the acquired immunity

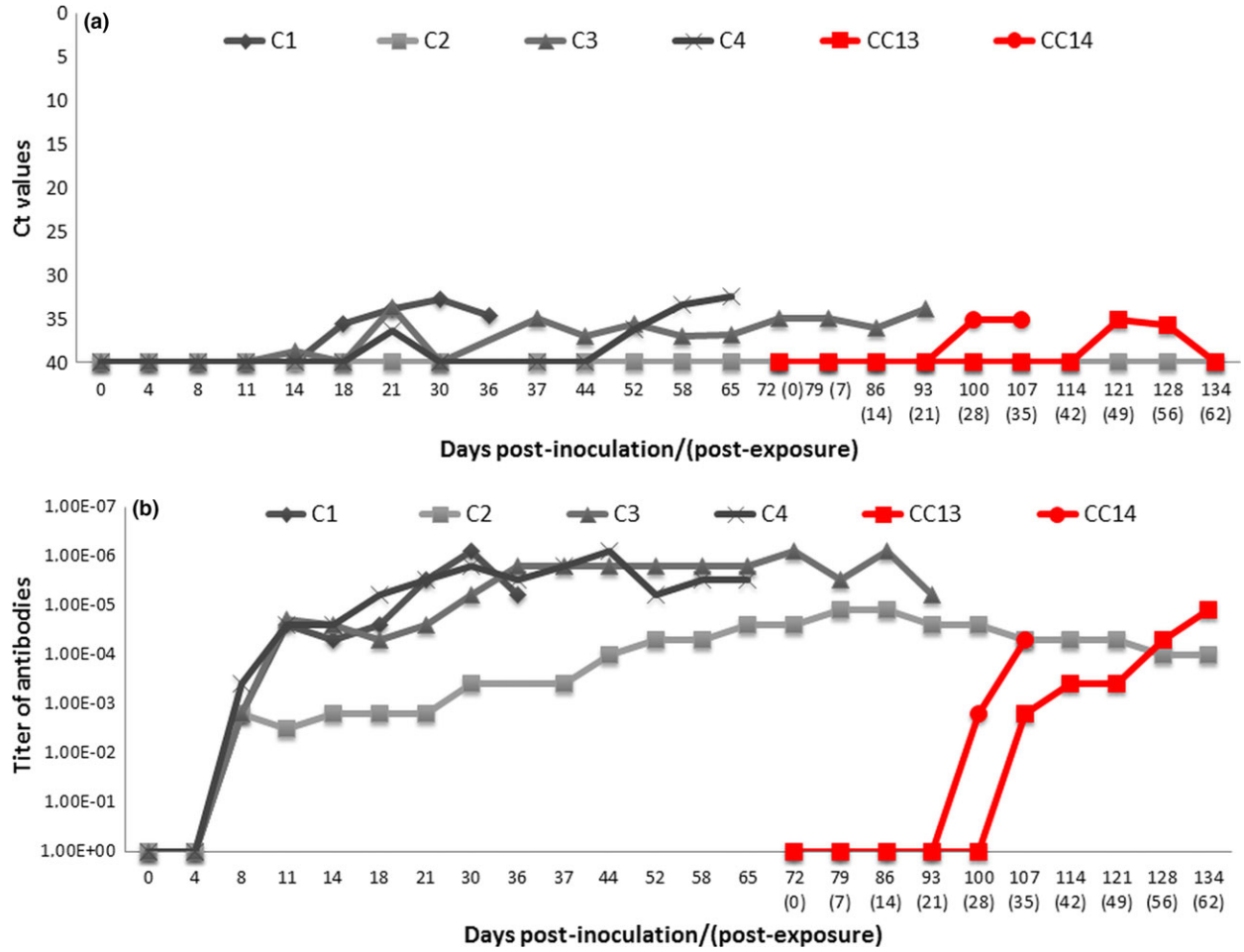

Fig. 3. Viremia (a) and anti-ASFV antibody titre measured using the OIE real-time PCR and the IPT, respectively, in the group of the inoculated (grey lines) and *in*-contact pigs (red lines) introduced at 72 dpi. The days post-exposure are indicated in brackets.

from previous exposure to lower doses of virus and/or the presence of related viruses of reduced virulent that may have emerged after many years of circulation in domestic pig populations. In the Iberian Peninsula, the release in the field of more than 400 000 doses of vaccine based on attenuated ASFV resulted in viruses of lower pathogenicity which gave rise to atypical form of the disease (Penrith et al., 2004; Penrith and Vosloo, 2009a; Penrith, 2009b; Sánchez-Vizcaíno et al., 2015; Portugal et al., 2015).

Despite field evidence, the exact role of seropositive survivor animals in the maintenance of ASFV among pig populations is still unclear. In this study, a formal procedure to assess the capacity of ASFV-infected survivor pigs to transmit the virus after 72 days of primary infection was carried out. In addition, the presence of the virus in tissue samples over time was evaluated by a regular follow-up of clinical infection, bleeding and euthanasia at different times post-infection. Different parameters such as viraemia and humoral response, as well as pathological findings, were analysed in experimentally infected and exposed pigs. In agreement with the previous findings (Leitao et al., 2001), three of the four experimentally NHV-inoculated pigs developed a chronic form of ASF to variable degrees of intensity with common features such as swelling above leg joints, necrotic foci in the skin and clinical respiratory symptoms. Animals had recurring cycles of pyrexia and viremia and the virus was isolated from the blood during periods of high temperature for at least 65 dpi (C4), even though the cycle threshold (Ct) values were >30. Lesions revealed at necropsy mainly consisted of chronic fibrinous pericarditis and caseous necrosis, and the calcification of the lungs, related to the presence of pneumonia. Such respiratory symptoms and lesions associated with chronic forms of ASF have been often recognized as a consequence of bacterial secondary infections (Coggins and Colgrove, 1968; Moulton and Coggins, 1968; Moulton et al., 1975; Pan et al., 1975). An analysis of virus replication in the tissues of pigs slaughtered between 35 and 134 dpi showed

Table 2. ASF virus detection in tissues collected from the NHV-inoculated and NHV-exposed animals

	Pig C1 d35pi			Pig C4 d65pi			Pig C3 d99pi			Pig C2 d134pi			Pig CC14 d34pe			Pig CC13 d62pe		
	PCR			PCR			PCR			PCR			PCR			PCR		
Tissue identification	Ct	Result	VIc	Ct	Result	VI	Ct	Result	VI	Ct	Result	VI	Ct	Result	VI	Ct	Result	VI
Submandibular LNa	21.68	POS	POS	22.1	POS	POS	31.87	POS	NEG	No Ct	NEG	NEG	29.87	POS	POS	No Ct	NEG	NEG
Retropharyngeal LN	30.6	POS	POS	21.84	POS	POS	33.97	POS	NEG	No Ct	NEG	NEG	37	POS	NEG	No Ct	NEG	NEG
Mediastinal LN	27.29	POS	POS	20.75	POS	POS	22.68	POS	POS	No Ct	NEG	NEG	29.64	POS	NEG	32,83	POS	NEG
Gastro-hepatic LN	29.45	POS	POS	26.06	POS	POS	34.45	POS	NEG	No Ct	NEG	NEG	34.71	POS	POS	37,5	NEG	NEG
Renal LN	31.99	POS	NEG	22.23	POS	POS	28.66	POS	NEG	No Ct	NEG	NEG	32.5	POS	POS	No Ct	POS	NEG
Splenic LN	29.56	POS	POS	25.74	POS	POS	No Ct	NEG	NEG	No Ct	NEG	NEG	33.43	POS	POS	No Ct	NEG	NEG
Mesenteric LN	35.94	POS	NEG	24.06	POS	POS	36.16	POS	NEG	No Ct	NEG	NEG	34.75	POS	POS	35,43	POS	NEG
Popliteal LN	24.5	POS	POS	23.73	POS	POS	30.68	POS	NEG	No Ct	NEG	NEG	30.39	POS	POS	No Ct	NEG	NEG
Inguinal LN	26.31	POS	POS	27.91	POS	POS	No Ct	NEG	NEG	No Ct	NEG	NEG	30.03	POS	POS	No Ct	NEG	NEG
Tonsil	30.07	POS	POS	23.95	POS	POS	27.6	POS	NEG	No Ct	NEG	NEG	32.59	POS	POS	No Ct	NEG	NEG
Heart	28.44	POS	POS	30.05	POS	NEG	No Ct	NEG	NEG	No Ct	NEG	NEG	32.46	POS	POS	32,65	POS	NEG
Lung	33.69	POS	NEG	20.9	POS	POS	23.97	POS	POS	No Ct	NEG	NEG	21.33	POS	POS	31,05	POS	NEG
Liver	34.07	POS	POS	28.25	POS	NEG	33.63	POS	NEG	No Ct	NEG	NEG	34.49	POS	NEG	No Ct	NEG	NEG
Spleen	29.83	POS	POS	24.27	POS	POS	30.07	POS	NEG	No Ct	NEG	NEG	28.82	POS	POS	36,65	POS	NEG
Kidney	32.46	POS	POS	30.67	POS	NEG	34.95	POS	NEG	No Ct	NEG	NEG	31.43	POS	POS	37.09	POS	NEG
Back right IAb	33.91	POS	POS	27.7	POS	NEG	No Ct	NEG	NEG	No Ct	NEG	NEG	27.1	POS	POS	No Ct	NEG	NEG
Back left IA	27.92	POS	POS	29.12	POS	NEG	No Ct	NEG	NEG	No Ct	NEG	NEG	25.15	POS	POS	No Ct	NEG	NEG
Front right IA	26.55	POS	POS	31.38	POS	NEG	No Ct	NEG	NEG	No Ct	NEG	NEG	18.56	POS	POS	34.75	POS	NEG
Front left IA	30.49	POS	POS	29.87	POS	NEG	No Ct	NEG	NEG	No Ct	NEG	NEG	23.41	POS	POS	38.98	POS	NEG

[a]Lymph node.

[b]Intra-articular tissues.

[c]Virus isolation result after three passages in PBM.

evidence of virus at up to 99 dpi in lung and thoracic lymph nodes at levels that were sufficient to infect naive pigs (>10^7TCDI50). The persistence of the virus in organs such as the respiratory tract implies a risk factor for virus transmission to other animals, which thus could contribute to the spread and maintenance of the disease. This finding has been reported in other experimental studies that have identified tissues as a source of virus at up to 180 dpi during persistent infections with moderately virulent isolates (Mebus and Dardiri, 1980; Wilkinson et al., 1981; Wilkinson, 1984; Hamdy and Dardiri, 1984; Oura et al., 1998). More recently, certain studies have reported that in subacute infections using ASFV isolates of moderate virulent (Brazil 1978, Malta 1978 and Netherlands 1986), viraemic surviving pigs were able to shed virus from their oropharynx for at least 70 days (de Carvalho Ferreira et al., 2012, 2013a,b). In comparison with the animals that succumb to the disease, it seems clear that pigs that recover may shed virus for up to a month after the disappearance of clinical signs. Lower amounts and frequency of shedding were observed after 30 dpi, although transmission to susceptible pigs may still be possible, through either direct or indirect contact (de Carvalho Ferreira et al., 2012).

One inoculated animal (C2) remained asymptomatic throughout the experimental study, although its antibody response detected at 8 dpi and maintained until 134 dpi shows that it was infected with ASFV. The non-appearance of clinical signs might be linked to low transient levels of viremia that probably appeared before the collection of the first blood samples. The absence of ASFV in tissues samples (taken at 134 dpi) may reflect the existence of low transient levels of viremia in that particular pig. Lower anti-ASFV-specific antibody titres were detected in the asymptomatic animal than in pigs that developed chronic infection. This agrees with the previous observations (Leitao et al., 2001) that have identified two clinical groups in pigs infected with NHV ASFV: (i) pigs developing chronic-type ASF lesions with viraemia and fever after 14 dpi and high levels of anti-ASFV-specific antibodies and (ii) pigs remaining asymptomatic that are neither viraemic nor febrile at 14 dpi and have relatively low levels of anti-virus antibody titres.

By introducing two naïve pigs at 72 dpi, we aimed to determine the ability of chronically ASFV-infected animals to transmit ASFV to susceptible populations. Despite the small number of pigs used in the study, the results obtained show that persistently infected pigs can efficiently transmit the virus to naive pigs after a period of even four (CC14) or five (CC13) weeks after the initial exposure, that is over three months after the virus was first inoculated. In spite of the fact that at the time of the exposure only one of the previously inoculated animals had a weak viremia peak (Ct ~ 35), the virus was easily transmitted, probably due to the presence of infectious ASFV in the pigs' respiratory tracts. As reported above for the inoculated pigs, one of the in-contact animals developed a mild course of chronic ASF and one remained asymptomatic. These mild clinical signs could easily remain unnoticed under field conditions, and only laboratory tests, necessary for confirming transient viremia and the presence of anti-ASFV-specific antibodies in exposed animals, can make them detectable. These data support the idea that control–eradication programmes in areas with a clear endemic status should include parallel routine laboratory monitoring, together with the regular clinical inspection of animals by keepers. It is important to underline that an effective control programme will always require laboratory monitoring. However, in some endemic areas of sub-Saharan Africa, governments and farmers would not be able to afford such intense controls. Nevertheless, the reinforcement of preventive biosecurity measures to guarantee the safe production and marketing of pigs and pig products in these regions could be promoted as a means of optimizing control strategies based on risk reduction that would lessen the laboratory costs of contingency and control plans.

Summarizing, this work demonstrates that pigs infected with low-virulent strains of ASFV are able to transmit the virus within three months of infection and that infectious virus are retained in tissues for up to 99 dpi. These findings confirm previous experimental studies that report virus shedding for at least 70 days (de Carvalho Ferreira et al., 2012; de Carvalho Ferreira et al., 2013a; de Carvalho Ferreira et al., 2013b) and the persistence of virus in tissues for up to six months (Wilkinson, 1984; Oura et al., 2005). By contrast, other studies report that the survivor pigs experimentally infected with Brazilian and Dominican Republic isolates excrete the virus intermittently for up to a month and that transmission to contact animals could only occur during this period. Long-surviving seropositive animals were not able to transmit the virus beyond 135 and 110 days to susceptible pigs after a contact period of 14 days (Wilkinson et al., 1981; Mebus and Dardiri, 1980). The failure of in-contact pigs to become infected during different experiments when exposed might be due to a short exposure period as this disease is not highly contagious (Wilkinson et al., 1981; Penrith et al., 2004).

Existent data undoubtedly provide evidence that survivor animals that have recovered from ASFV infection can still play a role in virus transmission and dissemination. We cannot exclude the possibility that this virus may establish a latent infection in some tissues and, under certain natural or induced conditions (transport, underfeeding, immunosuppression, etc.), may be reactivated, thereby facilitating its transmission. These possibilities are clearly important and need to be investigated, along with the fate of the virus in pigs that recover from the disease.

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