Intranuclear detection of African swine fever virus DNA in several cell types from formalin-fixed and paraffin-embedded tissues using a new in situ hybridisation protocol

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ABSTRACT

In this study, a new in situ hybridisation (ISH) protocol has been developed to identify African swine fever virus (ASFV) genome in formalin-fixed, paraffin-embedded tissues. Different digoxigenin labelled ASFV-probes were tested, including single ASFV-specific oligonucleotides, an 18.5 kb restriction fragment from the viral genome and the entire ASFV genome. The latter showed the highest sensitivity in all tissues tested, independently of the virus used for challenge: E75L or Ba71L. Although a similar ASFV genome distribution was observed, the number of ISH-positive cells was higher for Ba71L compared to E75L infected tissues. As expected, the monocyte-macrophage cell lineage was the main target cell for ASFV infection. Corresponding with the last stages of infection, ISH-positive signals were also found in other cell types, including endothelial cells, hepatocytes and neutrophils. Furthermore, two unexpected findings were also noticed: the detection of a specific ISH-signal in lymphocytes and a tendency to find the signal in the nucleus of infected cells. In summary, the present findings demonstrate the utility of this new ISH protocol to study ASFV pathogenesis and its potential use as a diagnostic tool.

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1. Introduction

African swine fever (ASF) is a highly contagious disease of domestic and wild pigs, which is currently included in the list of notifiable diseases to the Office International des Epizooties (OIE). Its etiological agent, the ASF virus (ASFV), is the only member of the Asfarviridae family (Dixon et al., 2005). ASFV is a large icosahedral enveloped virus that contains a linear double-stranded DNA genome ranging between 170 and 193 kb (Blasco et al., 1989; Chapman et al., 2008). The virus is well adapted to its natural hosts, warthogs (*Phacochoerus aethiopicus*), bushpigs (*Potamochoerus porcus*) and soft ticks (*Ornithodoros moubata*), in which ASFV is maintained in a sylvatic cycle causing unapparent infections (Anderson et al., 1998; Plowright et al., 1969; Thomson et al., 1980). Once ASFV infects domestic pigs, a range of clinical signs and lesions varying from peracute to chronic presentations can be found, depending on the virus isolate.

Since its first description in Kenya in the 1920s (Montgomery, 1921), the disease has been reported in several countries around the world, remaining endemic in Sardinia and in East and Southern Africa, where it represents a major threat for the development of pig industries (Dixon et al., 2004). Although transcontinental spread of ASF is considered a relatively rare event, a recent outbreak in the former Soviet republic of Georgia was confirmed by the OIE ASF reference laboratory in 2007. The disease spread to neighbouring countries and more than 80,000 pigs died or were slaughtered, causing a major economical impact (Rowlands et al., 2008). In recent reports, the Food and Agriculture Organization (FAO) authorities have reported the expansion of the disease as far as 2000 km East of its origin, in the Russian City of Saint Petersburg (www.fao.org) and menacing Eastern Asian countries, including China, the main world swine consumer and producer. The dramatic economical consequences that ASFV cause in the traditionally affected sub-Saharan countries, together with this new expansion wave, demonstrate the importance of control and eradication of this disease. As there is no vaccine available, the control of ASF is currently based on a rapid and accurate diagnosis and slaughter of infected animals. Current OIE diagnostic procedures for ASF

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are based on two differentiated groups. On one hand, the serological tests to detect ASFV antibodies by ELISA, immunoblotting or indirect fluorescent antibody (IFA), which are essential to follow the dynamics of moderately virulent ASF virus circulation. On the other hand, the techniques employed to directly detect the virus (haemadsorption), the ASFV nucleic acids (PCR and RT-qPCR), and the viral antigens (fluorescent antibody test or FAT) (Oura and Arias, 2008). To perform FAT (Bool et al., 1969), the only technique that allows the direct detection of the viral antigens in infected tissues, the maintenance of the cold chain is needed since fluorescence is carried out on cryostat sections and biosafety level 3 facilities are required due to the handling of highly infectious material. These facts, together with the traditional low sensitivity of the FAT technique, complicate its implementation under field conditions.

The aim of this work was to develop an optimised ISH protocol to detect ASFV on formalin-fixed paraffin-embedded tissues of experimentally infected pigs, aiming to provide a useful tool to study ASFV pathogenesis and for diagnostic purposes.

2. Materials and methods

2.1. ASFV-infected pig tissues

Four Large White × Landrace pigs were challenged intramuscularly with 10^4 haemadsorbing dose 50 (HAD_{50}) of the ASFV virulent isolates Ba71L or E75L (2 pigs with each one of the viruses) grown in pig leucocytes (L) and isolated from different Spanish locations in 1971 and 1975, respectively. Two extra-pigs remained uninfected and were used as controls.

Pigs were euthanized for humanitarian reasons at 7 days post-inoculation (dpi), showing typical acute ASF clinical signs such as high fever, depression, weakness and anorexia. Tissue samples including liver, spleen, kidney, lung, and gastro-hepatic lymph node were collected, fixed by immersion in 10% neutral buffered formalin and embedded in paraffin. Sections (3–4 μm thick) were cut and placed onto Capillary Gap slides (Dako, Barcelona, Spain).

2.2. DNA probes

Different probe approaches were tested: (i) a pool of three DIG-oligonucleotide probes (40 nt in size), complementary to three viral gene sequences encoding the structural p30 (5′-DIG-ctt gag cct gat gtt cag tat acc ctt gcc ctg cat ata t-3′), p54 (5′-DIG-gag aag aag ctt ggt ggg gtg act ggt gac aaa cac tca c-3′) and p72 (5′-DIG-tct tcg att tga ctc aaa gtg ggt tcg ggg tcg ggt ttc c-3′) proteins; (ii) the 18.5 kb SalI fragment (SD probe) of the genomic Ba71V DNA (Almendral et al., 1984), labelled with DIG High Prime system (Roche, Barcelona, Spain); and (iii) the total E75L viral DNA (complete genome probe) isolated from purified virions. For the latter purpose, an animal was inoculated intramuscularly with 10^5 HAD_{50} of the pathogenic ASFV isolate E75L. The pig was killed at 5 dpi and the virus was purified from the red blood cell fraction obtained from 500 ml of whole blood, essentially following the protocol described by Wesley and Tuthill (1984), except that virus was banded on 20%/60% rather than 25%/60% sucrose step gradients. In addition, virus preparations were treated with DNase (50 pg/ml) followed by 1% Tween-80 in order to remove contaminating cellular DNA before loading onto sucrose gradients. DNA was prepared from isolated virus after proteinase K treatment (250 μg/ml) followed by phenol extraction. Total E75L viral DNA was digested with MboI before labelling using the DIG High Prime system (Roche).

After DIG-labelling, probes were purified with the Qiagen Nucleotide removal kit (Qiagen, Madrid, Spain).

A DIG-oligonucleotide probe (41 nt in size), complementary to the porcine circovirus type 2 (PCV2)-Rep gene (5′-DIG-cct tcc tca tta ccc tcc tcg cca aca ata aaa taa tca aa-3′) (Kennedy et al., 2003) was used as negative control.

2.3. In situ hybridisation

Paraffin-embedded sections were dewaxed in xylene, rehydrated through an ethanol series (100%, 96%) and washed in Automation buffer (GeneTex, Inc., CA, USA). Tissue sections were either digested in 0.25 mg/ml proteinase K in 2× SSC for 10 min at 37 °C or with 0.3% (w/v) pepsin in 2× SSC for 10 min at 37 °C, followed by incubation at 105 °C for 8 min. After pre-treatment with proteinase K or pepsin, all sections were washed in Automation buffer. Three hundred nanograms of DNA probes labelled with DIG were precipitated with 3 μg of salmon sperm DNA and resuspended in 33 μl of a hybridisation mix (2× SSC, 10% dextran sulphate, 50% formamide, 1% Tween-20). The probes were then denatured at 75 °C for 10 min and preannealed at 37 °C for 90 min. DIG-oligo probes were denatured at 95 °C for 10 min and cooled rapidly on ice until hybridisation.

Tissue sections were denatured at 75 °C for 10 min in 70% formamide/2× SSC. Hybridisation was performed in a humidified box at 37 °C overnight under coverslips sealed with rubber cement.

Preparations were immersed in 2× SSC buffer to allow the coverslips to drop off. Slides were immersed twice in a wash solution composed of 0.5× SSC, 0.4% Tween-20, 0.25% Brij-35 for 5 min, followed by another 5-min wash in 0.25× SSC, 0.4% Tween-20, 0.25% Brij-35 at 37 °C. Then, tissue slides were blocked twice in 1% (v/v) sheep serum (Sigma, Madrid, Spain)/1× TBS (400 mM Trizma base, 3 M NaCl, pH7)/0.3% Triton X-100 (blocking solution) at room temperature (RT) for 5 min. The detection system consisted of an anti-digoxigenin antibody conjugated to alkaline phosphatase, diluted 1/500 in blocking solution for 1 h at 37 °C, and the substrate NBT/BCIP (nitroblue tetrazolium/5-bromo-4-chloro-3-indolphosphate). Tissue sections were finally counterstained with fast green, covered with coverslips and examined by light microscopy.

To validate the specificity of the newly developed ISH protocols, several controls were included. On one hand, the ASF-specific probes were tested on tissues from naïve pigs, non-infected with ASFV, and on the other, the ISH procedure was developed in ASFV-infected tissues using a PCV2-probe (Kennedy et al., 2003) as negative control for the assay.

The amount of ASFV-specific labelling was semi-quantitatively assessed by comparing the number of positive cells using five microscopic fields per tissue (screened at 200× magnification). Low, moderate and high amount of ASFV-stained cells were defined as averages of <10, 10–20 and >20 positive cells per microscopic field, respectively.

2.4. Double staining: in situ hybridisation and immunohistochemistry

In order to confirm the presence of ASFV nucleic acid in T-lymphocytes, a double ISH–immunohistochemistry (IHC) was performed. ISH was performed as described above and using the complete genome probe and the proteinase K treatment. IHC to identify T-lymphocytes was performed firstly using a polyclonal anti-human CD3 antibody (Dako) following a previously described protocol (Chianini et al., 2003). Briefly, endogenous peroxidase activity was blocked by immersing the tissue sections in a 3% H_2O_2 in methanol for 30 min. Then, proteolytic enzyme digestion was performed using 0.1% (w/v) protease XIV in 1× TBS for 8 min at 37 °C. Blocking was carried out with 2% BSA in 1× TBS

Table 1
In situ hybridisation results using the 18.5 kb SalI fragment of the genomic Ba71V DNA (SD) and the total E75L viral DNA (CG) probes for the detection of African swine fever virus genome in different tissues of experimentally infected pigs with isolates Ba71L and E75L and their corresponding negative control animals. Comparison of results using proteinase K and pepsin as enzymatic treatments is given.

Treatment	Probe	Virus	Animal	Liver	Spleen	Lung	LN	Kidney
Proteinase K	SD	Ba71L	a	+	−	+	+	+
			b	+++	++	+++	+++	+++
		E75L	c	−	−	−	+	−
			d	−	−	−	−	−
		Neg	e	−	−	−	−	−
			f	−	−	−	−	−
	CG	Ba71L	a	+++	+	++	++	+++
			b	+++	+++	+++	++	+++
		E75L	c	+	+/−	++	++	+
			d	−	−	++	++	+
		Neg	e	−	−	−	−	−
			f	−	−	−	−	−
Pepsin	SD	Ba71L	a	−	−	+	+	−
			b	+++	+	+++	++	+++
		E75L	c	−	−	−	−	−
			d	−	−	−	−	−
		Neg	e	−	−	−	−	−
			f	−	−	−	−	−
	CG	Ba71L	a	++	++	++	++	+
			b	+++	++	+++	++	+++
		E75L	c	−	−	+	++	−
			d	−	+	+	+	+
		Neg	e	−	−	−	−	−
			f	−	−	−	−	−

CG (E75L complete genome derived probe); LN (lymph node); SD (18.5-kb fragment Ba71V DNA derived probe); Neg (non-ASFV-infected tissue); ISH score (number of positive cells found in a given tissue); − (negative); + (low amount, average of <10 labelled cells/microscopic field); ++ (medium amount, average of 10–20 labelled cells/microscopic field); +++ (high amount, average of >20 labelled cells/microscopic field).

at RT for 1 h. The primary polyclonal anti-human CD3 antibody, diluted 1/100 in blocking solution, was then incubated overnight at 4 °C. Detection was performed with biotinylated goat anti-rabbit (Dako) diluted 1/200 in blocking solution and the ABC complex system (Thermo scientific, Rockford, USA), diluted 1/100 in 1× TBS. Both were applied for 1 h at RT. Sections were finally incubated in diaminobenzidine (DAB)–hydrogen peroxide solution for 10 min, counterstained with Harris' haematoxylin, dehydrated, covered with coverslips and examined by light microscopy. Negative control procedures included the lack of primary antibody for CD3 marker for the IHC and ASFV-probe for the ISH procedures.

3. Results

3.1. ISH for the detection of ASFV DNA in infected tissues

Independently of the tissue tested, no ISH-specific signal was detected when using the pool of the three DIG-oligonucleotide probes. In clear contrast, both the SD and the complete genome (CG) derived probes gave positive signals in most ASFV-infected tissues; the latter probe was the most sensitive (Table 1). Thus, the SD probe was able to detect the presence of the ASFV genome mainly in the tissues infected by Ba71L and only to a low extent in the E75L infected lymph node. On the other hand, the CG probe hybridised specifically with both E75L and Ba71L infected tissues; the number of ISH-positive cells was higher for the latter (Table 1).

Proteinase K treatment was more efficient than pepsin digestion, yielding a higher and more intense ISH-signal (Table 1), better tissue integrity and lower background. Negative controls were performed on tissues obtained from infected or non-infected animals hybridised either with the PCV2-probe or the ASFV-CG probe, respectively. As expected, these ISH controls yielded negative results on all tested tissues.

3.2. Cell tropism and organ distribution of ASFV (Ba71L and E75L) in infected pig tissues

ASFV genome distribution in tissues and the intracellular location of the signal were studied using the combination of the probe (CG) and the enzymatic treatment (proteinase K) that yielded the best results. ISH staining distribution (mainly in perinuclear cytoplasmic location, but also into the nucleus) and intensity (mild, moderate or strong) were recorded for each examined tissue. Spleen showed a high number of moderate to strong intracytoplasmic and intranuclear positive staining in macrophages of the white and red pulp. The amount of ASFV-positive macrophages was higher in red pulp than in white pulp. Labelled cells in spleen also included circulating macrophages, tingible body macrophages, and lymphocyte-like cells. Lymph nodes showed strong intranuclear and intracytoplasmic staining in cortical and medullary macrophages (most of them in the perifollicular areas), and moderate to strong intranuclear positive staining in small to middle-sized intravascular mononuclear cells, possibly lymphocytes or immature lymphoblasts. The liver showed moderate to strong positive intracytoplasmic labelling in hepatocytes (Fig. 1A and B), as well as strong intranuclear and intracytoplasmic positive staining in Kupffer cells, stellate cells and endothelial cells. The kidney had moderate to strong intranuclear positive staining in circulating intravascular macrophages and mild to moderate staining in glomerular endothelial cells (Fig. 1C and D). Finally, lungs showed mild intracytoplasmic positive staining in endothelial cells, moderate to strong intranuclear positive staining in pulmonary alveolar (Fig. 1E and F) and intravascular macrophages, neutrophils and small to middle-sized intravascular mononuclear cells, possibly lymphocytes or lymphoblastic cells.

Due to the fact lymphocyte-like cells were labelled, a double ISH–IHC technique was performed in different infected tissues (liver, spleen, lung, and gastro-hepatic lymph node) using a CD3 (pan T-marker) antibody. Double positive cells showing an intranu-
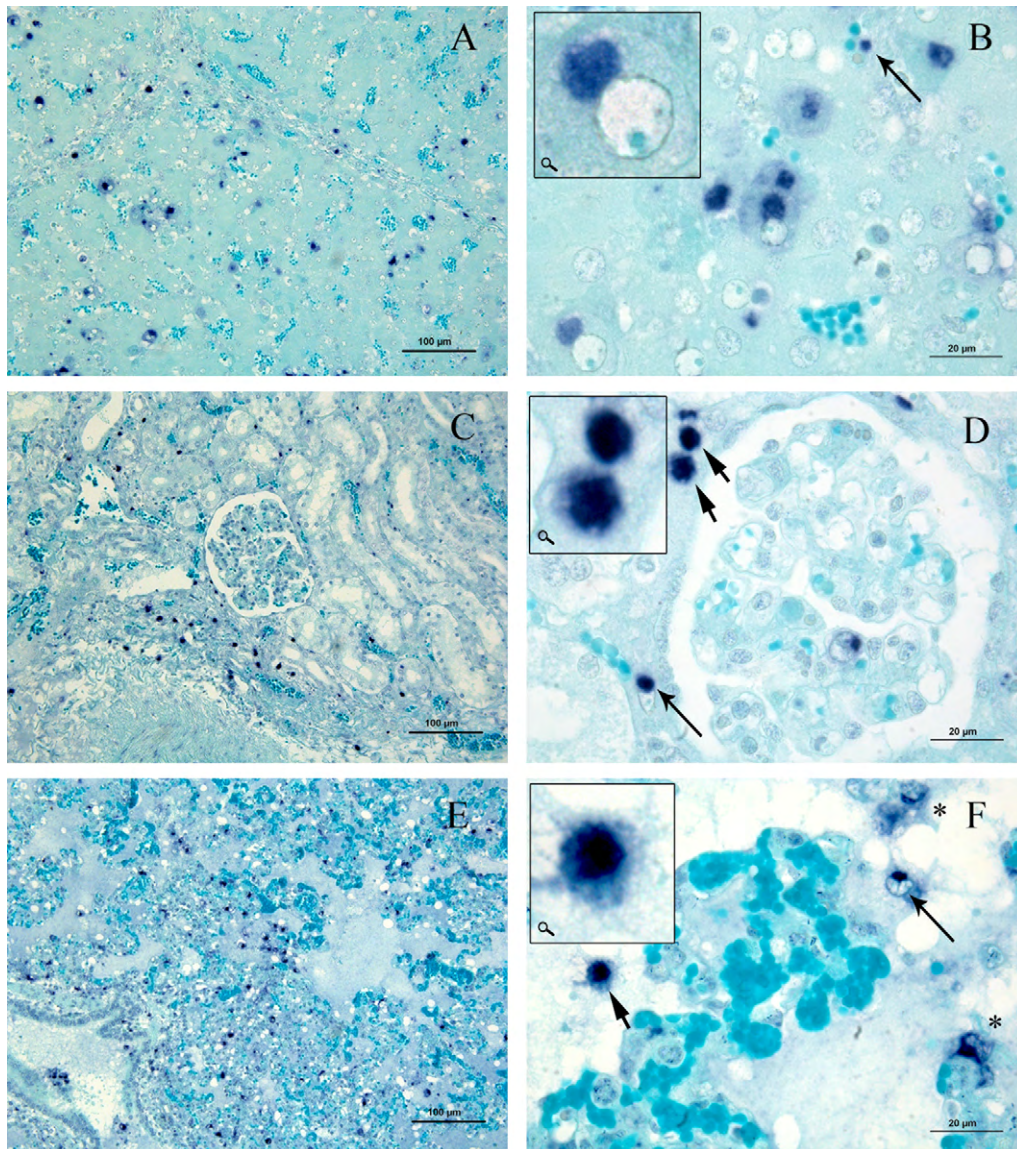
Fig. 1. Localisation of ASFV DNA by in situ hybridisation; fast green counterstain. *Liver*: (A) high numbers of labelled hepatocytes disseminated across the hepatic parenchyma. (B) Strong positive intracytoplasmic (perinuclear) labelling in hepatocytes (detail in the inset) with a strong positive intranuclear lymphocyte-like cell (arrow). *Kidney*: (C) high numbers of positive cells in the interstitium of the kidney. (D) Strong positive intranuclear (short arrows; detail in the inset) and perinuclear (long arrow) labelling in macrophage-like cells in periglomerular location. Also, a positive perinuclear labelling in the mesangium is seen. *Lung*: (E) high numbers of labelled interstitial and alveolar macrophages throughout the lung parenchyma. (F) Strong intranuclear (short arrow and detail in the inset) and intracytoplasmic (long arrow) specific signal in pulmonary alveolar macrophages. Intracytoplasmic labelling in cells resembling type 2 pneumocytes (asterisks) is also seen.

clear ASFV positive staining were observed in the lung (Fig. 2A), spleen (Fig. 2B), and gastro-hepatic lymph node (Fig. 2C) confirming the detection of ASFV genome in T-cells.

4. Discussion

In situ hybridisation (ISH) on formalin-fixed, paraffin-embedded tissues has become an important method to detect different pathogen genomes in veterinary diagnosis (Furuta et al., 1990; Jung and Chae, 2004; Okjin, 2004; Rosell et al., 1999). Although different electron microscopy and immunohistochemistry methods have been performed for studying the ASFV pathogenesis (Carrasco et al., 1996b; Fernández et al., 1992a,b; Gómez-Villamandos et al., 1995, 1996; Hervás et al., 1996), only one ISH method has been described so far (Oura et al., 1998), with no further applications reported, most probably due to the cost, technical complexity and time required to complete the assay.

In this study, a new optimised protocol to detect the ASFV genome in formalin-fixed, paraffin-embedded tissues has been developed. Using DIG-probes, ASFV-positive signals were identified in different cell types from several infected tissues. However, sensitivity was different depending on the probe used. While the ASFV DNA signal was detected using SD and CG DIG-probes, no signal was obtained using DIG-oligonucleotide probes. DIG-oligonucleotide probes have often been used for visualizing transcription of specific genes (Tyagi, 2009) that usually display high numbers of mRNA copies. These kind of short probes have been successfully used in the detection of small viruses such as human parvovirus B19 (P-B19) (Cubie et al., 1995) and PCV2 (Rosell et al., 1999). However, some viral characteristics such as genome size, replication cycle and/or the presence of envelope might interfere in the capability of probes to efficiently reach their targets. While PCV2 and P-B19 viruses have a non-enveloped single stranded DNA genome, ranging from 1.8 to 5 kb in size, ASFV
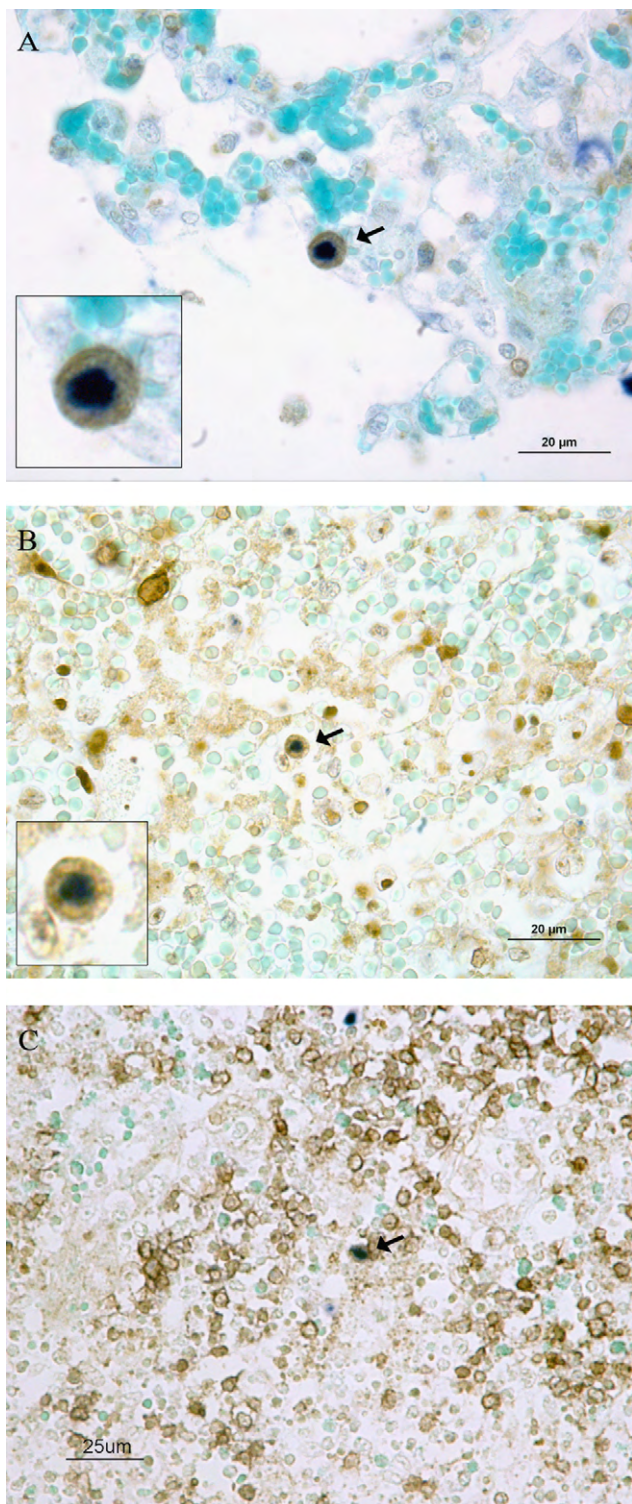
Fig. 2. Double positive staining (ASFV in situ hybridisation followed by CD3 immunohistochemistry) in different ASFV-infected tissues; Harris' haematoxylin counterstain. Strong intranuclear ASFV nucleic acid labelling in CD3-positive lymphocytes (arrows and insets) in (A) lung, (B) spleen, and (C) gastro-hepatic lymph node.

is an enveloped virus that contains a linear double-stranded DNA genome ranging between 170 and 193 kb. Furthermore, PCV2 and P-B19 virus replication is strictly nuclear, in contrast with ASFV that experiences two different replication stages (Rojo et al., 1999) that might complicate its full detection by the small oligonucleotide probes (40 nt in length each one). This hypothesis seems to be con-

firmed since the SD probe showed lower sensitivity than full-length probes.

Using 10^4 HAD$_{50}$ of either Ba71L or E75L, no significant differences were observed for the time of death of the animals, the viremia kinetics and the disease time-course (data not shown). However, Ba71L tissues were more severely damaged and samples showed a larger number of ISH-positive cells than those form E75L infected pigs. The fact both viruses were isolated within the same country 4 years apart, allows us to speculate that Ba71L may be the ancestor of E75L, and the latter viral strain might be slightly attenuated after 4 years circulating in Spanish pig farms. The minimal lethal dose (LD$_{50}$) for each viral isolates and phylogenetic studies are currently being conducted in order to shed some light on this issue.

As expected, ASFV DNA was mainly found in cells of the monocyte-macrophage lineage, the main target cells for ASFV replication (Fernández et al., 1992a; Gómez-Villamandos et al., 1995; Oura et al., 1998; Rodríguez et al., 1996). The ASFV genome was also found in other cell types, including endothelial cells, neutrophils and hepatocytes, as was expected during the late stages of ASFV infections (Carrasco et al., 1996b; Fernández et al., 1992a,b; Gómez-Villamandos et al., 1995, 1996; Oura et al., 1998; Rodríguez et al., 1996). Discrepancies in ASFV cell distribution at terminal stages of infection have been reported before. Thus, while infection of alveolar macrophages was not detected in lungs from the virulent Spanish E70 or African Malawi isolates, either by IHC or ISH, respectively (Fernández et al., 1992b; Oura et al., 1998), viral particles were found in the latter using electron microscopy (Carrasco et al., 1996b). In the present study, infected alveolar macrophages were observed for both virus isolates, therefore pointing towards sensitivity of the technique as a key issue to explain the dichotomy of the already published results, not ruling out differences between each virus isolate tropism.

Together with the expected intracytoplasmic (mainly perinuclear) localisation of the ASFV genome in infected cells, a proportion of them showed intranuclear staining, which is a novel finding of the present study; intranuclear location of the ASFV genome in infected tissues has not been reported previously. Intranuclear labelling might be the in vivo reflection of an early stage of infection as has been previously reported in vitro, after infection of tissue culture cells (García-Beato et al., 1992; Rojo et al., 1999; Tabarés and Sánchez Botija, 1979). The strong signal found in cells with intranuclear labelling might reflect that the nuclear phase is a key event during viral infection in vivo and emphasizes the high sensitivity of the ISH protocol used in this study. Intriguingly, intranuclear positive staining was also observed in a low number of T-lymphocytes, most probably indicating there is an small proportion of this cellular subset that are susceptible to ASFV infection at later stages of the disease, confirming previous reports showing intracellular ASFV particles in lymphocytes (Carrasco et al., 1996a) and opening new concerns about their implication in ASFV pathogenesis.

The results here presented demonstrate the utility of this ISH protocol to study ASFV pathogenesis and open new avenues to be used in the future as a diagnostic tool.

Acknowledgements

which also financed the CONSOLIDER-"Porcivir" CDS2006-00007 and AGL2007-66441-C03-01/GAN research projects.

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