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Laboratory methods to study African swine fever virus

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ABSTRACT

We summarize findings of comparative studies in different cells cultures susceptible to ASFV infection, through the analysis of virus components and infectious virus particles production, as alternative means to grow field and laboratory ASFV strains. We also provide different methods to assay the infectivity of ASFV samples and to purify the infective virus particles. Finally we describe the general strategy to construct virus deletion mutants that can be engineered to obtain attenuated ASFV strains suitable for vaccine approaches.

1. Introduction

African swine fever virus (ASFV) was discovered in 1921 (Montgomery, 1921) and is the causative agent of a highly contagious disease of swine prevalent in more than 20 sub-Saharan countries of Africa, but emerging lately in Caucasus region and Russian Federation in the Euro-Asian continent (Rahimi et al., 2010; Rowlands et al., 2008). These areas represent an enormous reservoir and a risk for the re-introduction of ASF into other countries. The course of the disease range from the acutely fatal hemorrhagic fever reported in the first descriptions to more attenuated, chronic or unapparent persistent infections (Vinuela, 1985) that have been shown to be increased in the course of the adaptation of ASFV to the domestic pig. In this particular host, the ASFV infects tissue macrophages, blood monocytes and, to a lesser extent, specific lineages of reticular, polymorphs, and megakaryocytic cells (Casal et al., 1984; Wilkinson, 1989), while some virus replication has been also described in endothelial cells (Wilkinson and Wardley, 1978), hepatocytes (Sierra et al., 1987), renal cells (Gomez-Villamandos et al., 1995) and neutrophils (Carrasco et al., 1996). Until recently, the wild type ASFV isolates obtained from the field could not be propagated in established cell lines in the laboratory, and porcine monocytes and macrophages were the only *in vitro* system available to mimic the natural ASFV infection, in which all the virus isolates readily grow. The description of the successful infection of COS.1 cells with many ASFV strains (Hurtado et al., 2010) unlocked many possibilities not only for the detection and diagnosis of ASFV field isolates, but also for titration and bulk production and purification of the virus. In this review we describe new data on the use of porcine established cell lines (IPAM and WSL) in the study of ASFV *in vitro* infections, as a possible alternative method to analyze the host cell response in the context of the natural target host (swine) for the virus.

The description of new cell lines susceptible to ASFV has provided the possibility of novel or re-adapted methods to quantify the virus infectivity, which will be comparatively discussed in this paper, besides the expected consequences of these observations in the improvement of the virus purification techniques.

Lastly, we address the current strategies based on ASFV-susceptible cell cultures, to construct recombinant viruses with deleted specific genes, and discuss the selection of ASFV genes in view of the development of an attenuated virus strain to be used as a safe and efficient vaccine against African swine fever.

2. Propagation of ASFV in cultured cells

2.1. Primary porcine cells

For many years since the beginning of the studies on ASFV, the analysis of the effects of the virus infection could only be performed on the natural target cells, namely the porcine monocytes–macrophages obtained either from blood or from specific organs/tissues. The use of these cells to propagate the virus and for ASF diagnosis by hemadsorption in infected monocytes

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was the subject of several historic publications (Enjuanes et al., 1976a; Hess, 1960; Malmquist, 1962; Malmquist and Hay, 1960), representing the only means to study ASFV samples until the first descriptions of some ASFV strains adapted to grow in established cell lines (Enjuanes et al., 1976a; Parker and Plowright, 1968).

The simplest way to prepare purified swine leukocytes from anticoagulated-blood samples (heparin, EDTA, citrate) requires its free sedimentation at an angle of about 45° (Tubiash, 1963) where most of the erythrocytes sediment in less than 1 h in the lower half of the glass container, before the PBMC (peripheral blood mononuclear cells) in the upper phase are washed and concentrated by successive low-speed centrifugations. This type of preparations further require the specific lysis of contaminant erythrocytes, which can be performed in NH_4Cl solutions, or even in distilled water, under highly-controlled conditions to avoid losses in PBMC viability. Several modifications have been found to help to increase the yield, specificity and/or speed of the purification method, for example, the use of some red blood cell aggregator like metrizamide or dextran-500 in the free sedimentation step, or more frequently, the centrifugation through a Ficoll-Paque (GE Healthcare) density gradient (Bach and Brashler, 1970), rendering purified PBMC preparations essentially free of erythrocytes and platelets. Standardized protocols for sample processing ensuring reliable results from downstream cell separation and functional analysis are now commercially available (for example, from STEMCELL Technologies or Miltenyi Biotec, among many others). Once the PBMCs are suspended in the suitable culture medium (usually DMEM or RPMI supplemented with fetal calf serum or swine serum), the cells are seeded on plastic plates, incubated for 2 or 3 days and then cleaned from non-attached cells by extensive washing, rendering a basically-pure culture of monocyte–macrophages.

An alternative source of macrophages can be obtained from pulmonary lavage of swine, a technique first described for rabbits (Myrvik et al., 1961) and then adapted to porcine species (Carrascosa et al., 1982). About 1×10^9 alveolar macrophages can be obtained from the broncho-alveolar lavage of a small pig (20–40 kg), and used immediately or conveniently stored frozen to be used for many years/assays. Porcine alveolar macrophages, can be kept frozen under liquid nitrogen, rendering, after thawing, monolayer cultures of alveolar macrophages suitable for virus titration or for biochemical and immunological studies, with the same results than those obtained with purified PBMCs. The advantage in this case is that successive experiments can be performed with the same cell stock (as an established cell line), ensuring the generation of more reproducible results than in a conventional primary culture.

Both porcine PBMCs and alveolar macrophages have been used to grow, diagnose and titrate the ASFV isolates obtained from field outbreaks. Most of these techniques have been developed following conventional methods in monolayer cell cultures, and even suspension cell culture procedures can be performed for mass production of ASFV stocks in swine alveolar macrophages (Carrascosa et al., 1982).

2.2. Monkey-derived established cell lines

The use of primary cells (PBMCs, tissue macrophages, etc.) to study ASFV infections is very convenient since they are the natural host cell for the virus, where most of the ASFV strains readily grow, but involves some drawbacks like (i) their lot-to-lot variations, hampering the reproducibility of the results achieved in these cultures, and (ii) their inability to grow in culture, which emphasizes the problem of producing them in sufficient amount to perform biochemical or structural studies. These issues were partially overcome by the adaptation of some ASFV isolates to grow in different established monkey cell lines like Vero, MS and CV (Carvalho et al., 1988; Enjuanes et al., 1976a; Hess et al., 1965; Parker and Plowright, 1968), facilitating many biological studies of the ASFV, but then restricting the analysis to those cell-adapted virus strains. The description of the susceptibility of COS.1 cells to many different ASFV isolates (Hurtado et al., 2010) has noticeably changed the scenery, allowing the direct infection of these cells with any field or laboratory-engineered virus strain without previous selection or adaptation. The sensitivity of COS cells to some ASFV isolates, and their use to construct deletion mutants in ASFV was already previously described, as well as their regular use for the transient and stable expression of ASFV genes in cell culture (Carrascosa et al., 1999; Galindo et al., 2000; Granja et al., 2006; Hurtado et al., 2004). The extension of the studies of COS.1 cells sensitivity to many ASFV isolates resulted in several improvements in both diagnostic and titration assays of ASFV, as for example, (i) when the low concentration of virus components or the poor quality of samples from the field outbreaks demand a previous amplification of the virus in cell culture, (ii) for the preparation of ASFV cytoplasmic soluble antigens for indirect ELISA and IPT tests to detect ASFV-specific responses in swine serum samples (Gallardo et al., 2012), or (iii) for the optimization of plaque assays (Bustos et al., 2002; Enjuanes et al., 1976a; Parker and Plowright, 1968), as inexpensive, easy, reproducible and quantitative methods for the evaluation of the infectivity titer of any ASFV sample (Hurtado et al., 2010).

2.3. Porcine established cell lines

There are many studies, however, in which the use of a cell system from the same species than the natural host is mandatory, as for example, in the analysis of the immune response of the host cell (cytokine induction, antigen presentation, etc.). In these cases it should be critical to maintain a more natural environment in the system thus retaining those components which can elicit and modulate the cellular immune response in swine (major histocompatibility complex antigens, immune receptors, etc.), with the added difficulty in the case of ASFV of being the antigen-presenting cells (monocytes and macrophages) the natural target cells for virus infection. This has encouraged the searching of a porcine cell line, ideally derived from a monocyte–macrophage lineage, which could be infected by ASFV while also being able to multiply in cell culture. A publication of Wardley et al. (1980) described the establishment of continuous macrophage cell lines from peripheral blood monocytes by a simple method of repeated medium changes, developing cell lines from seven different animal species (included swine) and sharing many characteristics (phagocytic ability, esterase-positivity and presence of Fc receptors) with the primary cells from which they were derived. It was also described in that publication the productive infection of the corresponding pig cell line with one attenuated ASFV strain, but no further information on the possible use of these methods to develop a porcine line suitable for ASFV infection has ever been reported, even though the experiment has been repeatedly attempted in many ASFV laboratories. The only exception, to our knowledge, is a porcine alveolar macrophage cell line named PM2, obtained in the University of James Cook in Australia (Nunn and Johnson, 1979), with no successful result in ASFV susceptibility already reported. Primary cultures of porcine aortic endothelial cells and bushpig endothelial cells have been also used in ASFV studies, mainly focused to the analysis of their role in the hemorrhagic pathogenesis of the ASF disease (Vallee et al., 2001), but with no practical advantages when compared to other porcine primary cell cultures, unless they might be established as immortalized cell lines (Carrillo et al., 2002).

Probably the most popular continuous porcine cell lines circulating among ASFV laboratories are those developed by Weingartl et al. (2002) from swine alveolar macrophages, establishing

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monomyeloid cell lines after transfection of primary porcine alveolar macrophages with plasmid pSV3neo carrying the SV40 large T antigen. Several clones were obtained from the original parental 3D4 clone 3D4 and their myeloid character together with the positivity to fagocytic and esterase activities was confirmed. However, the expression of the SV40 large T antigen gene was not confirmed in those clones, which were, nevertheless, still resistant to the selective antibiotic G418, suggesting that an alternative mechanism should account for the immortalization of the IPAM cells, as it has been named the cell line currently shared by different ASFV laboratories (immortalized porcine alveolar macrophages).

A new cell line (WSL) recently developed in Dr. Günther Keil laboratory (Friedrich-Loeffler Institut, Greifswald, Germany) has been used in studies of ASFV entry into susceptible cells (Hernaez and Alonso, 2010) and in limited experiments of infection of sensitive cells to different ASFV isolates (Carrascosa et al., 2011; Portugal et al., 2012). The WSL line is derived from wild boar lung cells and it has been claimed to be a macrophage cell line, but some information obtained from the original laboratory has indicated the possibility of being instead pulmonary fibroblasts.

2.4. Characterization of porcine cell lines

Aiming at clarifying the above mentioned question and to further characterize the available porcine cell lines, we have performed a set of experiments to detect in IPAM and WSL cells the presence of immune cell markers, which were stained by a panel of monoclonal antibodies (MAbs) and analyzed by flow cytometry. The origin and specificity of the MAbs used in these experiments are listed in Table 1.

Cell lines were grown in their corresponding culture medium, specifically DMEM supplemented with serum (5% of fetal calf serum (FCS) for Vero and COS.1 cells, 10% of porcine serum for swine macrophages), RPMI-1640 plus 5% FCS for IPAM cells, and RPMI:DMEM (1:1) plus 5% FCS for WSL cells, in a CO_2 incubator with humid atmosphere at 37 °C. For fluorescence activated cell scanning (FACS), cells were washed with PBS-staining buffer (1% BSA, 0.01% NaN_3, 1% FCS and 5 mM EDTA in PBS) before being incubated with different MAbs (Table 1) at a concentration of 50 μl/10^6 cells, for 1 h at 4 °C. Cells were washed again with PBS-staining buffer and incubated with the secondary antibody (specific against mouse IgG, Alexa 488 from Molecular Probes, diluted 1/400) for 20 min at 4 °C in the dark. Samples were then washed and resuspended in the dark in PBS-staining buffer with 1% p-formaldehyde. Analysis of the labeled populations was performed in a FACSalibur flow cytometer.

Table 1
Monoclonal antibodies used to characterize porcine cell lines.

MAb[a]	Specificity	Porcine marker
24BB7	P12 (ASFV attachment protein)	Negative
BL6H4	SLA-I	Nucleated cells
74/22/15	CD172a/SWC3	Panmyeloid marker
BL1H7	CD172a/SWC3	Panmyeloid marker
BL1H8	CD11a = LFA-1	Leucocyte marker
2F4/11	CD11R3	Myeloid marker
1F1	Sialoadhesin (CD169)	Macrophage-restricted marker
2A10	CD163	Macrophage-restricted marker

[a] All of the MAbs, with the exception of 24BB7 and 74/22/15, were a generous gift from Dr. Javier Dominguez (INIA, Spain).

FACS analysis was used for the characterization of both IPAM and WSL cells, while swine alveolar macrophages (Sw Mac) and Vero cells were also examined as positive and negative controls, respectively. As it was expected, FACS (Fig. 1) demonstrated the absence of staining of Vero cells with the panel of MAbs listed in Table 1, as well as the positive staining of swine macrophages with all of them, with the exception of the irrelevant 24BB7. Besides, a similar staining profile was found for both IPAM and WSL cells, with positive results for SLA-I (stained by BL6H4) and for SWC3a myeloid marker (stained by MAbs 74/22/15 and BL1H7). However, negative staining was found for CD11a, CD11R3 and CD169 (by MAbs BL1H8, 2F4/11 and 1F1, respectively). The staining by MAb 2A10 (specific for CD163) was contradictory and it has not been included in the figure. Essentially the same results were obtained in a parallel experiment in Dr. Javier Dominguez laboratory (INIA, Spain), determining that some specific porcine macrophage markers were present both in IPAM and WSL cells, but other myeloid markers were not. Overall, our conclusion is that both cell lines (IPAM and WSL) seem to be quite similar, and both of them probably share a macrophage lineage origin with the loss of some specific myeloid markers.

2.5. Infection of porcine cell lines with different ASFV isolates

An important issue to be defined is the general susceptibility of porcine cell lines to ASFV infection. Published data on the

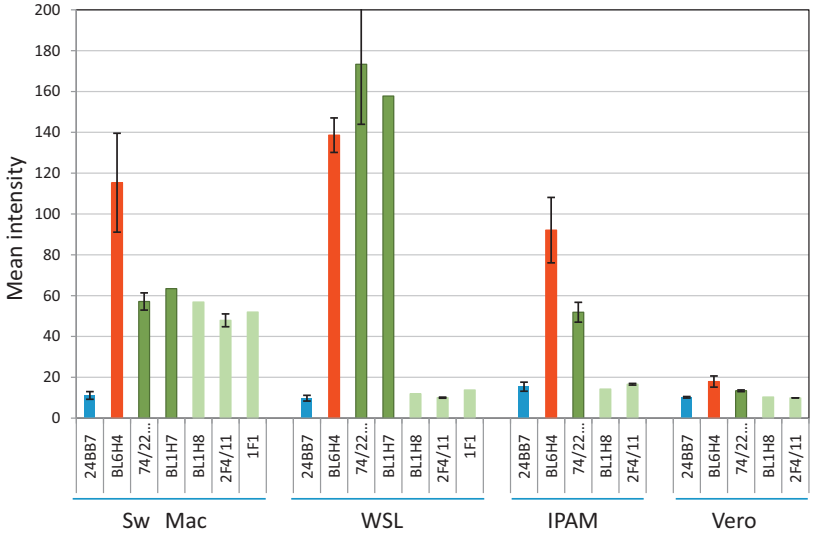

Fig. 1. Characterization of porcine cell lines. Cultures of Sw Mac, WSL, IPAM and Vero cells were incubated with MAbs specific for different porcine markers (listed in Table 1) and the mean intensity of staining was determined by FACS analysis. Results are representative of several experiments performed in two different laboratories.

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Table 2
ASFV strains selected to analyze the virus susceptibility of porcine cells.

ASFV strain	Virulence[c]	Hemadsorbing	Origin
BA71V (V9)[a]	att	+/−	European
ΔEP153R[b]	att	non-	European
E70 (Spain 70)	vir	+	European
Malawi 82	vir	+	African
Hindeatt	att	+	African
Uganda vir	vir	+	African
Lisbon 57	vir	+	European
NH/P68 (NHV)	att	non-	European
CC83	vir	+	European
Uganda att	att	+	African

[a] Adapted to Vero cells.
[b] Lab-engineered strain generated from BA71V.
[c] att, attenuated; vir, virulent.

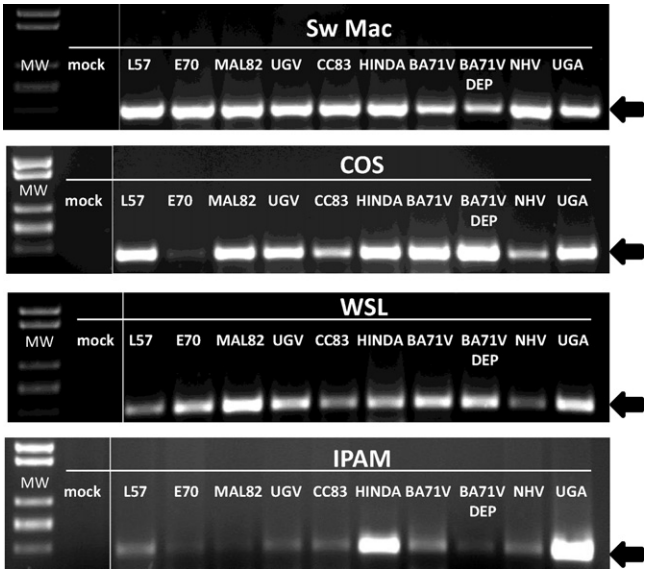

Fig. 2. Determination of ASFV-specific late transcription. Cultures of each cell line (Sw Mac, COS.1, WSL or IPAM) were infected at an m.o.i. of 3 pfu/cell with the ASFV isolates indicated in the figure (and listed in Table 2), washed away from the non-adsorbed virus and incubated in culture medium. ASFV mRNA specific for the late structural protein p72 was detected by RT-PCR in cell extracts collected at 24 h after infection. Molecular weight markers are shown (MW) in the left lane. The presence of a band of 422 bp (arrow) implies the effective transcription of the late virus gene.

production of infective virus for some ASFV isolates in these cells included: (i) Vero-adapted Lisbon 61 and Lillie SI 85, in IPAM cells (Weingartl et al., 2002), (ii) Vero-adapted Ba71V and 608VR13 strains, in WSL-R cells (Hernaez and Alonso, 2010), and (iii) Ba71V, E70, Lisbon 60 and NH/P68 in both IPAM and WSL cells (Carrascosa et al., 2011). In order to expand and improve the knowledge of the virus cycle into porcine cell cultures, a number of ASFV strains (up to 10, with different origins and degree of virulence, listed in Table 2) were selected to infect four cell cultures (Sw Mac, COS.1, IPAM and WSL). The infected cultures were used to determine the transcription of late virus genes, the synthesis of late virus proteins and the production of infective virus particles at the end of the virus cycle.

To perform these analysis preconfluent cultures of each one of the cell lines selected were infected, in their corresponding culture medium described above, with the ASFV isolates listed in Table 2, at an m.o.i. of about 3 pfu/cell. After 2 h of adsorption, the non-adsorbed virus was washed away, and cultures were incubated in fresh medium at 37 °C up to different times after infection: 24 h for RT-PCR analysis of ASFV gene transcription, 40 h for WB assays of late-induced virus proteins, and 48–72 h for the titration of the infective virus production.

The transcription of ASFV genes was determined by detection of mRNAs specific for the late structural virus protein p72, by RT-PCR with primers designed for the B646L open reading frame. Cultures were collected at 24 hpi and RNA was extracted and quantified after the addition of TRizol Reagent (Invitrogen Life Technologies). High Capacity RNA-to-cDNA kit (Applied Biosystems) was used for the reverse transcription of total RNA (2 μg) into cDNA. PCR amplification was performed with p72 specific primers, by 30 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min and extension at 72 °C for 1 min. Amplified cDNAs were analyzed by agarose gel electrophoresis. As it is shown in Fig. 2, all the cell lines were positive for p72 transcription after infection with the ASFV isolates tested, while no virus transcription was detected in mock-infected cultures. There were no major differences in the intensity of bands detected in Sw Mac, COS.1 or WSL infected cells (with the exception of a slight band in COS.1 cells infected by E70). However, in the case of IPAM cells, two virus isolates (Hinde att and Uganda att) exhibited a very intense band, while only slight bands could be detected in the infection with the rest of ASFV stocks, suggesting a more efficient infection of IPAM cells with these two virus isolates.

The synthesis of late virus proteins was determined in cultures collected at 40 hpi. Cells were washed with PBS and lysed in TNT buffer (20 mM Tris–HCl pH 7.2, 0.2 M NaCl, 1% Triton X-100) supplemented immediately before use with protease inhibitor cocktail tablets (Roche). BCA Protein Assay Reagent (Pierce, Cultek) was used to determine protein concentration. Samples of 20 μg of protein were analyzed by 12% SDS-PAGE and electroblotted onto an Immobilon PVDF transfer membrane (Amersham). Membranes were incubated with a primary antibody specific for ASFV-late induced proteins (del Val and Vinuela, 1987) and then exposed to horseradish peroxidase-conjugated secondary antibody followed by chemiluminiscense detection (ECL, Amersham). The synthesis of virus proteins induced late in the infection was demonstrated in COS.1 (Fig. 3B) and WSL (Fig. 3C) cultures infected with all of the ASFV isolates tested so far. The same result was obtained in Sw Mac (Fig. 3A), with the exception of Ba71V (V9) and ΔEP isolates, where the ASFV-specific protein bands were hardly detected. A limited infection was also reported in Sw Mac infected by Ba71V (Zsak et al., 2001), although virus production could be clearly detected (Bustos et al., 2002). Our results indicated a very low synthesis of virus proteins in Sw Mac infected with the Ba71V-derived isolates, at least at the time selected, which may be too late in the infection for WB assays, considering that the virus cycle in Sw Mac is shorter by one half from that observed in WSL or IPAM cells (unpublished observations). Regarding IPAM cells, positive detection of ASFV-specific bands were clearly observed (Fig. 3D) in Hinde att and Uganda att infections (confirming the RT-PCR results), and also in E70 and, in a minor extent, in CC83 and Malawi infections.

The production of infective virus was assayed by plaque titration on COS.1 cell monolayers, assuming that all the viruses produced in the different cell lines used here maintained the ability to infect COS.1 cells as their original virus stocks (Hurtado et al., 2010). The results shown in Fig. 4 demonstrated that all the ASFV isolates tested so far were able to induce a productive infection both in COS.1 and in WSL cells, with titres ranging from 10^5 to 10^7 pfu/ml. Sw Mac yielded virus productions ranging from 10^5 to 10^6 pfu/ml, except in the case of Ba71V and ΔEP isolates, with very low productions (in the range of 10^3 pfu/ml), as expected from the WB results above described. In the case of IPAM cells, the highest titres (10^6–10^7) were found in Hinde att and Uganda att, confirming that these isolates were the best virus producers in IPAM cultures, while the rest of the viruses yielded lower amounts of infective progeny, in the range of 10^4–10^5 pfu/ml.

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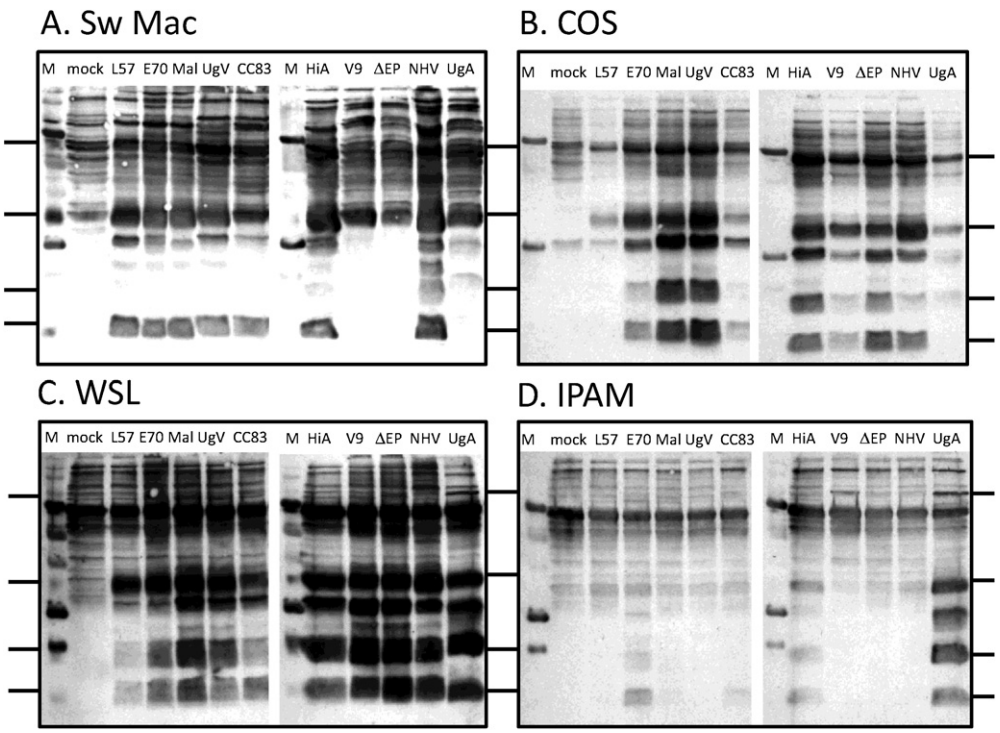

Fig. 3. Analysis of ASFV-specific late translation. The synthesis of virus proteins induced late in the infection was determined in cell extracts collected at 40 h after ASFV infection (see Fig. 2), by Western blotting with an anti-late specific ASFV proteins rabbit antiserum. Results shown for Sw Mac (A), COS.1 (B), WSL (C) and IPAM (D) cells infected with the ASFV isolates indicated in the figure (and listed in Table 2), or non-infected (mock). Molecular weight markers are shown in the left lane (M). The positions of major ASFV-induced proteins are indicated by black lines at the left and right side of each panel.

From the results obtained in the infection of sensitive cells with a panel of 10 ASFV isolates, we propose the following advices:

- COS.1 cells can be used to grow and titrate any ASFV isolate;
- WSL cells can be infected with any ASFV isolate, and they are an ideal system to study virus infection in a porcine context. However they cannot be used for titration since they do not develop a clear cytopathic effect;
- Sw Mac, susceptible to any ASFV isolate (with a limited infection in the case of Ba71V), are suitable for analysis of the virus infection in the natural target cell, and to perform the hemadsorption assay for virus titration;
- IPAM, sensitive to only some of the ASFV isolates (Hinde att, Uganda att, E70, CC83) tested so far, are a good alternative for viruses with a higher response, as for example, the Uganda att isolate.

3. Growth and purification of ASFV

3.1. Growth on roller bottles and purification by Percoll gradients: alternatives

About 30 years ago, several methods for the purification of ASFV particles were published, which included fluorocarbon extraction (Larenaudie et al., 1965), isoelectric (Stone and Hess, 1965) and polyethylene glycol (Enjuanes et al., 1976b) precipitation, equilibrium centrifugation in sucrose and sucrose-cesium chloride mixtures (Polatnick et al., 1974), hydroxyapatite treatment and

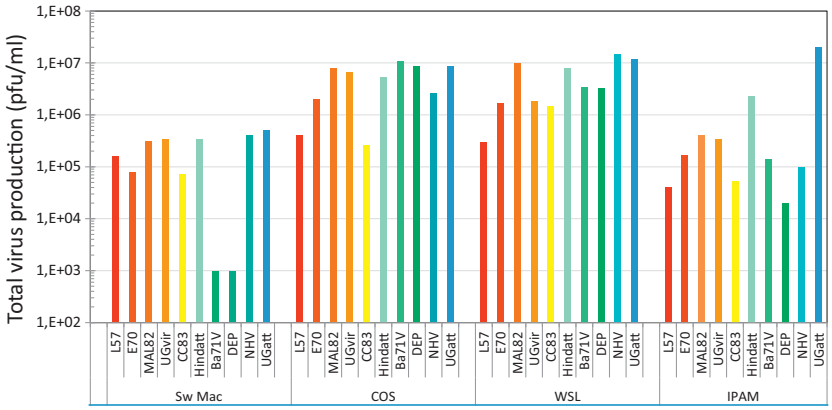

Fig. 4. Production of infective virus. Cultures of each cell line (Sw Mac, COS.1, WSL or IPAM) infected as indicated in Fig. 2 were collected at 48–72 hpi for titration of total virus production: infected cells suspended in their own culture medium were disrupted by sonication and the infectivity evaluated by plaque assay on COS.1 cell monolayers. Results are representative of 2 independent experiments, and correspond to the same samples analyzed in Figs. 2 and 3.

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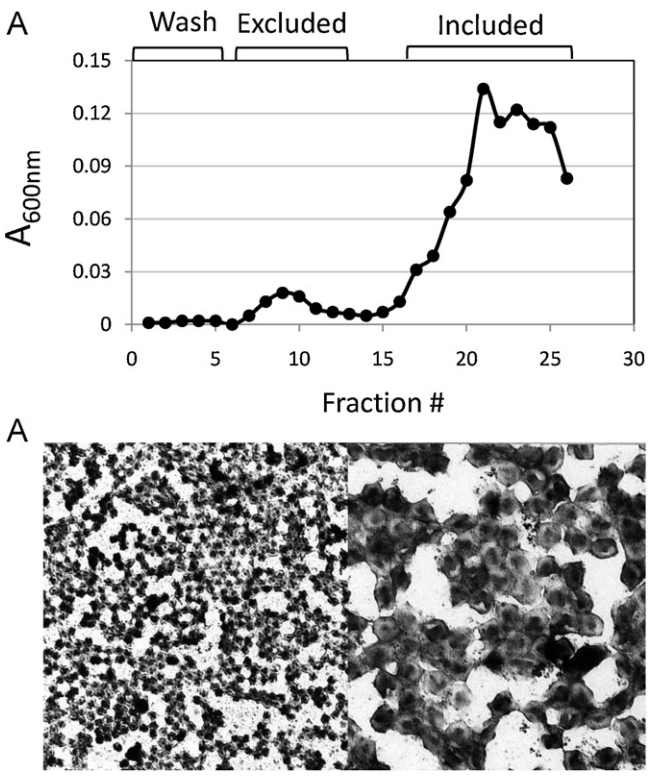

Fig. 5. Purification of ASFV particles after Percoll sedimentation. (A) Gel filtration on Sephacryl S1000. Extracellular ASFV purified by Percoll sedimentation and subjected to chromatography on Sephacryl S1000 column. Percoll determined by precipitation with 10% TCA. Virus proteins were detected in the excluded fractions while Percoll particles appeared in the included volume. (B) Electron micrographs at different magnification of Percoll-purified virus particles. Negative staining of virus samples from the excluded volume (panel A) concentrated in an Airfuge.

potassium tartrate centrifugation (Kuznar et al., 1980), in virus samples treated or not with non-ionic detergents and high-salt buffers (Black and Brown, 1976). None of these reports were successful in terms of yielding final preparations with good recoveries of virus infectivity and reasonably purified from cellular contaminants. The description in 1985 of a new method of purification based on equilibrium centrifugation in Percoll density gradients of extracellular virions produced in infected Vero cells (Carrascosa et al., 1985), revealed the first protocol for the efficient removal of contaminant cell membranes and the best recovery of infectious ASFV particles reported so far. Briefly, the extracellular virus produced in Vero cells grown and infected in roller bottles with the Vero-adapted BA71V strain, was pooled from the culture medium, clarified to remove cell debris and concentrated by high-speed sedimentation. The concentrated virus pellet was then subjected to centrifugation in a Percoll (45%) density gradient, where the virus particles could be found at a density of 1.095 g/ml released from the cell contaminants, a heterogeneous fraction banding at densities from 1.02 to 1.08 g/ml and mainly consisting on membrane fragments and vesicles (Carrascosa et al., 1985). The virus preparations could be removed from most of the Percoll contamination by chromatography on Sephacryl-S1000 columns: a typical result is shown in Fig. 5, with the virus particles arising as a small peak in the excluded volume (panel A) far from the main peak found in the included fractions containing the Percoll granules. Electron micrographs (Fig. 5B) revealed a purified virus preparation only composed of homogeneous and complete virus particles, and free from cell debris, vesicles, membrane fragments and Percoll granules. The Percoll-purified ASFV particles sedimented as a single component in isokinetic sucrose gradients and exhibited the highest ratios in all of the biological parameters analyzed (Carrascosa et al., 1985), as the specific infectivity (2.7×10^7 pfu/μg of protein), the DNA–protein ratio (0.18 ± 0.02) or the overall recovery of infectivity throughout the entire process ($15.2 \pm 8.8\%$), in comparison to any previous or subsequent method ever published for ASFV purification.

The Percoll method has been routinely used in many ASFV laboratories as the best alternative whenever a pure preparation of undamaged infective virions was needed, but some other methods have also been used elsewhere to prepare faster and/or easier concentrated and partially purified ASFV stocks. There are reports, for example, using centrifugation through a sucrose cushion of the virus pellet obtained after high speed centrifugation: this step should render a partial release of cellular membranes, but the virus particles are still associated to cell contaminants, while a significant reduction of virus infectivity is often obtained as a concomitant effect whenever other density solutions (sucrose, tartrate, dextran, etc.) are used instead of Percoll/PBS in the purification protocol (data not shown).

Another question regarding ASFV purification derives from unpublished observations in different laboratories, in which the application of the Percoll method to virus samples produced in porcine primary cultures (monocytes or macrophages) did not produce the expected result in terms of purified virus preparations: in fact, it seems that the ASFV band, released from the cell contaminants during the centrifugation in Percoll gradients, was not produced in those cases, or exhibited a density different to 1.095 g/ml and closer to that of the cellular membranes, probably because the type of association between ASFV particles and cell contaminants is different depending on the type of cell used to produce the virus. The main consequence of this problem was that the ASFV field isolates, which were routinely grown only on porcine primary cell cultures, could not be purified by the Percoll method. This situation has been notably improved after the description of different established cell lines (COS, IPAM, WSL) available for the infection and production of ASFV field isolates (Carrascosa et al., 2011; Hurtado et al., 2010; Portugal et al., 2012), as described above in this report. The use of these cells to grow any ASFV isolate is expected to facilitate the purification of the corresponding virus particles by the Percoll method, which must render purified virus preparations like those obtained from infected Vero cells.

4. Infectivity assays for ASFV

4.1. HAD (or CPE) on swine monocyte–macrophages

The detection and quantification of ASFV samples can be performed by evaluation of a structural component of the virion or a virus product induced during the infection, with techniques like ELISA, PCR, WB or fluorescent focus assay (Aguero et al., 2003; Barderas et al., 2000; Oura et al., 1998; Pastor et al., 1989, 1992; Zsak et al., 2005). It is more relevant, however, to evaluate the samples for their ability to produce infective virus, and the exploitation of a characteristic feature of the swine monocytes infected with ASFV, which developed a rosette of erythrocytes around the infected cell, has been the basis of a classical assay by "hemadsorption," widely used both for diagnostic purposes and for virus titrations (Enjuanes et al., 1976a). Detailed protocols of this assay, as well as those evaluating the cytopathic effect (suitable for non-hemadsorbing ASFV isolates) produced in macrophage cultures, both assays based on semi-quantitative estimation by limit dilution, have been described in a recent review (Carrascosa et al., 2011). These methods can be used for titration of any ASFV isolate, since the indicator cell is the natural target cell for virus infection, but they involve the use of primary cells which can be more difficult to manage than established cell lines, as discussed above.

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4.2. Plaque assays (Vero, COS.1 or swine macrophages)

The adaptation of some ASFV isolates to grow in established cell lines like PK or Vero, allowed the use of plaque formation assays (Enjuanes et al., 1976a; Hess, 1971; Parker and Plowright, 1968) to evaluate the infectivity in virus samples with a simpler, inexpensive and quantitative method than those based on the hemadsorption or cytopathic effect of infected cells. The plaque assays demonstrated a linear dose–response curve, and higher sensitivity and reproducibility than the former methods for ASFV titration, but they were obviously restricted to the cell culture-adapted ASFV strains.

The development of an efficient method to generate plaques on cultures of swine monocyte/macrophages (Bustos et al., 2002) was aimed as an alternative for quantitative titration and for plaque isolation of virus clones. The assay is suitable for any ASFV isolate both from the field or lab-engineered (see an example in Fig. 6A), exhibiting the advantages associated to plaque assays in terms of linearity, sensitivity, regularity, effortless and economy, but with the same disadvantages derived from the use of primary cells, which may pose in this case some additional problems, for example, in the generation of a suitable pseudo-monolayer of macrophages (to visualize the lysis plaques) due to the differences from lot-to-lot stocks of primary cells.

To date, the best choice for ASFV plaque titration seems to be the assay described in COS.1 cells (Hurtado et al., 2010), which allows the quantification of infective virus in different naturally occurring, cell adapted or genetically manipulated ASFV samples, by a simple and reliable plaque assay. The sensitivity of COS cells to some ASFV strains, and its use in the construction of ASFV deletion mutants or in the expression of ASFV genes in cell culture, had been previously published (Carrascosa et al., 1999; Galindo et al., 2000; Granja et al., 2006; Hurtado et al., 2004; Salguero et al., 2008), while the extension of the analysis of susceptibility and diagnosis to a broad spectrum of ASFV isolates, representing virulent, attenuated, hemadsorbing or not, field or lab-engineered, African and European strains, has been more recently reported (Gallardo et al., 2012; Hurtado et al., 2010). A detailed description of the basic protocols for plaque assay of ASFV samples both in Vero and COS.1 cells is available (Carrascosa et al., 2011): briefly, subconfluent cultures of Vero or COS.1 cells grown on multiwell dishes were infected with 10-fold dilutions of virus stocks in culture medium, and carefully overlaid, without removing the inoculum, with agar-medium made up by mixing one volume of DMEM (2×) medium with 4% fetal calf serum and 160 μg/ml of DEAE-dextran, and one volume of freshly prepared 1.4% agar noble in distilled water, equilibrated at 45 °C. Plates were incubated at 37 °C for 5–7 days and stained with 2% crystal violet in 5% formaldehyde. An illustration of the plaques developed by several ASFV isolates in COS.1 cell monolayers is shown in Fig. 6B, where plaques were stained at day 5 after infection.

5. Use of cell cultures to construct ASFV deletion mutants

5.1. Deletion of ASFV genes

A common approach to study the function of a particular protein is the generation of virus mutants in which the gene encoding that polypeptide is partially or totally deleted. This approach has been found to be decisive for the successful determination of the role of several ASFV genes in the infective virus cycle, that could be demonstrated after the deletion of the viral gene from different parental viruses by homologous recombination in susceptible cells (see method below) subjected to transfection with a specific deletion plasmid and infection with the parental ASFV strain (Galindo et al., 2000; Granja et al., 2004; Moore et al., 1998; Neilan et al., 1999; Nogal et al., 2001; Oliveros et al., 1999; Redrejo-Rodriguez et al., 2006; Rodriguez et al., 1993; Salguero et al., 2008; Zsak et al., 1996, 1998, 2001). As above mentioned, the possibility to grow many ASFV isolates in the established cell line COS.1 (Hurtado et al., 2010) has allowed the generation of recombinant viruses with specific genes deleted from practically any ASFV isolate, by using techniques based on lysis plaque identification and selection. A number of laboratories have been, or they are now, involved in the process of construction of ASFV deletion mutants, and several of them are described in Table 3. The deletion of A224L and A238L virus genes has been also produced in the parental L60 and NHV isolates by homologous recombination in COS.1 cells (Dr. Yolanda Revilla, CBMSO, Spain, unpublished data).

5.2. General strategies to construct virus deletion mutants

We describe below a number of techniques developed to assist the construction of ASFV deletion mutants, all of them based on the use of virus-susceptible cell cultures to facilitate the homologous recombination process between parental virus DNA and specifically-designed deletion plasmid vectors.

5.2.1. Chromogenic plaque assay

The genetic manipulation of the ASFV genome by homologous recombination between the viral DNA and plasmid vectors transfected into the infected cell, was first described in 1992 (Rodriguez et al., 1992), but its practical application was only opened in 1995 by the generation of plasmid vectors designed to facilitate the deletion of ASFV genes (Garcia et al., 1995). These vectors, named p72GUS10T and p72GAL10T, respectively, were based on pUC119 plasmids, and contained the coding sequence of the corresponding β-glucuronidase (GUS) or β-galactosidase (GAL) reporter gene from *Escherichia coli* fused to the ASFV p72 (major viral structural protein) promoter, and followed by a signal for the 3' end formation of ASFV mRNAs (10T). The presence of selectable marker genes like GUS or GAL, encoding proteins whose enzymatic activities on specific substrates (5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (X-gluc) for GUS, or 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) for GAL) develop an intense blue precipitate, facilitated the identification and isolation of recombinant viruses in which the deletion cassette was inserted. These deletion plasmids must be also engineered by incorporating into the polylinker regions the left and right flanking sequences found up and downstream of the ASFV gene to be deleted. These flanking sequences may recombine with the corresponding regions in the genome of a wild type ("parental") virus, replacing the viral gene by a reporter bacterial gene under the p72 promoter, thus providing to the recombinant virus the selectable property of the marker gene.

As an example, we have summarized in Fig. 7 the process of construction of a virus recombinant with the EP153R gene deleted from the NHV isolate of ASFV. A deletion plasmid was generated (Galindo et al., 2000) with the GAL (LacZ) reporting gene inserted between the flanking sequences of the viral gene to be deleted, and under the p72 promoter, designed to facilitate the replacement of a genomic DNA fragment of 333 bp (covering the majority of EP153R) with the marker gene. The plasmid was transfected into COS.1 cell monolayers using LipofectAMINE Plus reagent and then infected with the parental virus (NHV isolate). Cultures were incubated until extensive cytopathic effect was developed (usually 2–3 days) to allow the process of homologous recombination to proceed, harvested and then serially diluted to infect fresh cultures of COS.1 cells. After virus adsorption the inoculum was removed and solid medium was layered on the cultures, as indicated to perform the plaque assay. The presence of lysis plaques was monitored from the third day of infection onward, to select the best moment in which the plaques

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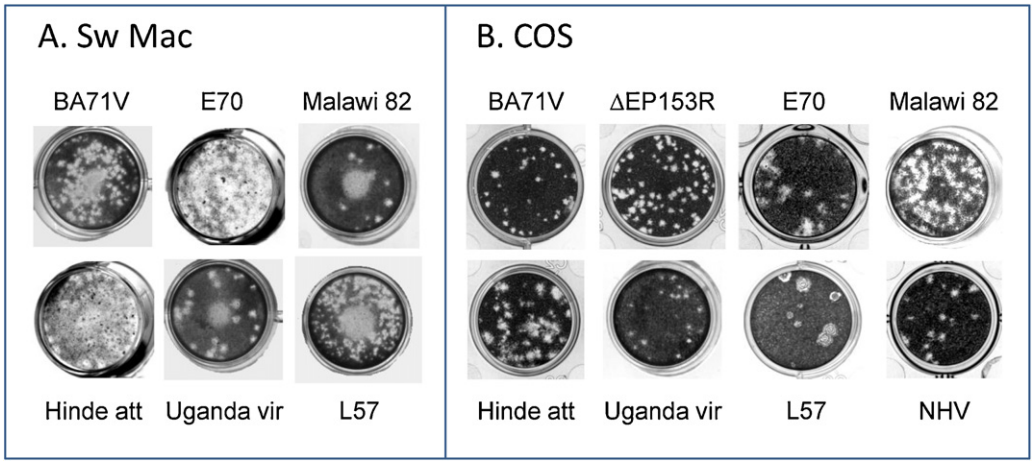

Fig. 6. Lysis plaques produced by different ASFV isolates in susceptible cell monolayers. (A) Alveolar swine macrophages were seeded to generate a quasi-monolayer culture in multiwell plates for the plaque assay of ASFV. Wells infected with a suitable dilution (to produce isolated lysis plaques) and stained with crystal violet at day 5 after infection are shown. (B) COS.1 cells were seeded to produce preconfluent monolayers, which were infected with serial dilutions of the ASFV isolates indicated. Lysis plaques were stained with crystal violet at days 5–7 after virus infection.

were just visible but small enough to avoid their contamination with adjacent plaques (4th to 5th day post inoculation). Cultures were then incubated with the specific substrate (X-gal) to stain the recombinant plaques (immersed into a broth of unstained wild-type virus plaques), and the best candidates (intense-blue plaques with the smaller contamination of parental virus and in the highest dilution as possible) were selected to be picked up in order to begin a new round of infection-plaque selection-purification. After the selected recombinant plaques were collected, the plates were stained with crystal violet (CV) to reveal all virus plaques present in the monolayer. As it can be seen in Fig. 7, as the successive rounds of purification proceeded, differences between the total number of plaques (CV-stained) and the number of recombinant plaques (X-gal-stained) were decreasing, obtaining in the last rounds (VII–VIII in this case) a perfect coincidence between the images for total and recombinant lysis plaques.

To assess the complete inactivation of the EP153R gene, COS.1 cells were infected with the parental NHV virus or one of the purified clones of the recombinant virus (named D1 and D2), and analyzed at 24 hpi both for the presence of DNA corresponding to p72 or EP153R viral genes (PCR, Fig. 8A) and for the lack of expression of these genes by RT-PCR (Fig. 8B). As it is shown in the figure, both ASFV genes were detected in cells infected by NHV, and they were not present in mock-infected samples, as expected. The presence of the p72 gene and of mRNA specific for this gene was also identified in all of the virus-infected samples, while specific products from EP153R gene were absent in the cultures infected by both (D1 and D2) deletion mutants, confirming the effective inactivation of the EP153R gene in these recombinant viruses.

5.2.2. *EGFP-fluorescent technique*

It has been recently published an alternative method for the generation of recombinant ASFV, also suitable for some field isolates. This method is based on GFP expression and 5-bromo-2'-deoxyuridine selection (BrdU) (Portugal et al., 2012) and it takes advantage of the WSL cell line described in this report, which they found to be suitable for the propagation of the NHV isolate of ASFV, as it can be also noticed in Fig. 4 of this report. For the generation of recombinant viruses they constructed a transfer plasmid with the EGFP open reading frame (under the transcriptional control of the viral p72 promoter) flanked by segments of the viral TK gene, to provide sequences for homologous recombination. After transfection with the plasmid into WSL cell monolayers, the cultures were infected by parental NHV isolate, and further incubated to allow homologous recombination and productive replication of recombinant viruses (detected as autofluorescence foci of rounded cells). Cells from these foci were aspirated and used to inoculate BrdU-resistant Vero (VeroTK$^-$) cells in the presence of the drug for positive selection of recombinants. This step is not particularly successful since, as stated by the authors, NHV replicates only inefficiently in Vero TK$^-$ cells. After one or two rounds of positive selection (the authors claimed that theoretically only one round is needed), the infected cell extracts can be used to infect WSL cells for the production of high titre stocks of recombinant viruses.

Table 3
Virus genes inactivated from different parental ASFV isolates by deletion after homologous recombination.

ASFV gene	Homolog	Parental ASFV	Cell	Reporter gene	Reference (first description)
EP402R	CD2	Ba71V	Vero	GAL	Rodriguez et al. (1993)
DP71L	NLS	Several	Several	GUS	Zsak et al. (1996)
K169R	TK	Malawi LiL-20	Vero	GUS	Moore et al. (1998)
DP96R	UK	E70	Sw Mac	GAL	Zsak et al. (1998)
E165R	dUTPase	Ba71V	Vero	GAL	Oliveros et al. (1999)
EP153R	C-type lectin	Malawi LiL-20	SwMac	GUS	Neilan et al. (1999)
EP153R	C-type lectin	Ba71V	Vero	GAL	Galindo et al. (2000)
EP153R	C-type lectin	NHV/E70	COS	GAL	Unpublished
MGF360/530	Multigene-Families	Ba71V	Vero	GUS	Zsak et al. (2001)
A224L	IAP	Ba71V	Vero	GUS	Nogal et al. (2001)
A238L	IkB	Ba71V	Vero	GUS	Granja et al. (2004)
E296R	AP endo-nuclease	Ba71V	Vero	GUS	Redrejo-Rodriguez et al. (2006)
A238L	IkB	E70	COS	GUS	Salguero et al. (2008)

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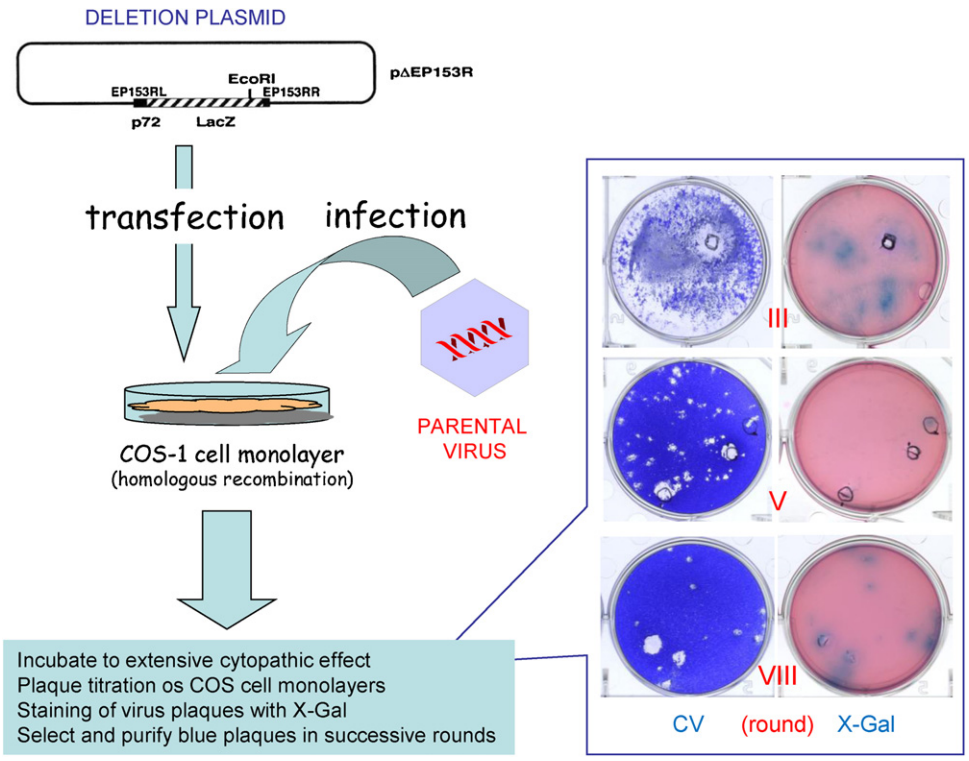

Fig. 7. Generation of an ASFV deletion mutant. A deletion plasmid, based on the p72GAL10T (obtained from pUC119) with the GAL (LacZ) reporter gene under the viral p72 promoter, was engineered to replace a large fragment of the EP153R gene of ASFV by inserting the flanking sequences up and down-stream of the virus gene into the polylinker regions. The deletion plasmid was used to transfect monolayers of COS.1 cells, which were subsequently infected with the parental virus, in this case the NHV isolate, at an m.o.i. of 2 pfu/cell. Cultures were then incubated, to allow the homologous recombination process, and plaque titrated as indicated in the figure. The presence of lysis plaques of recombinant or total (wild type + recombinant) viruses were detected by specific staining with X-gal or CV, respectively, along the successive rounds of purification (results on rounds III, V and VIII, are shown in the figure).

Although it represents a novel and rapid approach to generate ASFV recombinants, the method requires the selection of recombinants in VeroTK$^-$ cells, limiting its application again to parental virus isolates able to replicate in this established cell line. The generation of a TK-deficient variant of the WSL cell line will help to increase the application of this technique to other ASFV field isolates, as shown in this report.

5.2.3. Sequential deletion of virus genes

With the chromogenic technique described in Section 5.2.1, the use of two marker genes like GUS and GAL allowed the generation of ASFV recombinants with up to 2 deletions, each of them screened by its corresponding substrate X-gluc or X-gal, respectively. Interestingly, the possibility to sequentially delete combinations of genes from the ASFV genome has also been very recently reported (Abrams and Dixon, 2012), and it is equally based on the use of susceptible cell cultures to perform the homologous recombination. In this publication, several virus genes were initially deleted from the Vero-adapted Ba71V strain, by the use of transfer plasmids engineered to recombine with the specific ASFV gene to be deleted and containing bacteriophage IoxP sequences flanking the GUS marker gene. After the selection and purification of the corresponding (1st generation) recombinant virus in cultures of Vero cells, the GUS gene was removed by site-specific recombination between the two IoxP sites involving expression of the bacteriophage Cre recombinase enzyme. Then, the 2nd generation recombinant viruses were screened for white plaques (developed after the removal of the GUS) gene, and further purified to be used in the construction of a new successful deletion at a second locus on the Ba71V genome (with the same homologous recombination process in Vero cell cultures) using again the GUS reporter gene as described above. The process should be applied successively

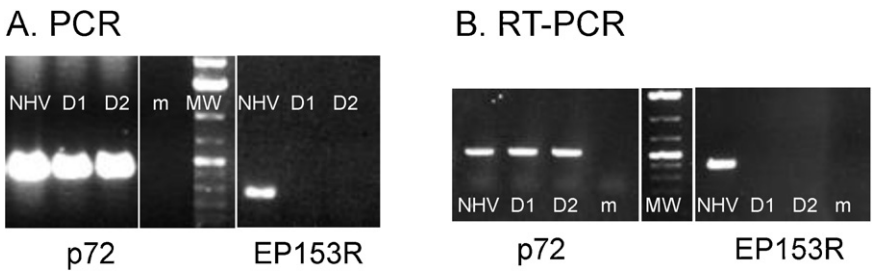

Fig. 8. Presence and expression of p72 and EP153R genes in COS.1 cells infected by ASFV recombinants with the EP153R gene deleted. Two virus clones (D1 and D2), with the EP153R gene interrupted as indicated in Fig. 7 from the NHV isolate, were assayed for the presence of p72 and EP153R genes (A, PCR) or specific transcripts (B, RT-PCR), in samples of purified DNA (1 μg) or RNA (2 μg, DNase-pretreated), respectively, extracted at 24 hpi. Control lanes with mock (m) or NHV-infected extracts, and molecular weight markers (MW) are also shown.

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for the sequential deletion of several virus genes, providing the removal of the GUS marker gene before each deletion step. Besides, and although initially refered for the tissue culture adapted strain Ba71V of ASFV, the cre/loxP technology should be applied to create multiple gene deletions on field virus strains by infection and transfection of susceptible COS.1 cells (Hurtado et al., 2010).

As an overall conclusion of the different techniques reported above, it is clear that all of them required the use of ASFV-susceptible cell cultures to generate virus recombinants with specific genes deleted. Although some authors still propose the use of isolated pig bone marrow macrophages for the infection/transfection step with field isolates, we encourage the use, if possible, of COS.1 cells as the most suitable cell system in chromogenic plaque-purification technologies, and with a broad spectrum of susceptibility to laboratory and field ASFV isolates. Whenever multiple gene deletions are demanded, the cre/loxP technology is the best recommendation, while the EGFP-fluorescent technique will be best applied whenever a TK-deficient variant of the WSL cell line is available to be used with many field virus isolates.

5.3. *ASFV virulence genes: inducible expression approach: perspectives on ASFV vaccines*

One of the main applications expected for the construction of ASFV recombinants with specific genes deleted, inactivated or switched of (in virus mutants which may express, in an inducible way, the viral gene), is the possibility to disable the expression of virus genes involved in virulence. This will lead to attenuate high- or moderately-virulent ASFV field isolates, which can then be used in vaccine strategies against ASF. The inducible method mentioned above was first described in the publication of Garcia-Escudero et al. (1998) in which a technique based on the *Escherichia coli* Lac repressor-operator system was applied to the isopropyl β-D-thiogalactoside (IPTG)-inducible expression of ASFV p72 gene, to analyze the function of virus genes. Since this publication, a number of ASFV genes has been studied with the support of this technique (p17, pE248R, pB602L, pB438L, p54, S273R, pp220, pp62 and pE120R, among others) (Alejo et al., 2003; Andres et al., 2001, 2002; Epifano et al., 2006a,b; Rodriguez et al., 2004, 2009; Suarez et al., 2010a,b), most of them involved in the assembly and maturation of the ASFV particles in the infected cell. The method is particularly remarkable for the study of essential viral genes absolutely required for productive virus infection, which cannot be deleted because its inactivation renders a non-viable virus particle.

Anyone of the techniques for the inactivation of ASFV genes described above can be used to disable the expression of virus genes involved in virulence. There are many publications reporting the role of several ASFV genes in the control of virulence (for a review see Dixon et al., 2004): for example, those involved in the control of the immune response and apoptosis in the infected cell, like A238L (IkB-homolog; Miskin et al., 2000; Revilla et al., 1998), A224L (Iap-homolog; Nogal et al., 2001), A179L (Bcl-2-homolog; Revilla et al., 1997), EP153R (C-type lectin-homolog; Hurtado et al., 2004, 2011) or EP402R (CD2-homolog; Rodriguez et al., 1993; Rowlands et al., 2009), involved in the modulation of processes like NFkB/NFAT transcription factors activities, programmed cell death induction, MHC-I-dependent antigen presentation, cytokine induction and cytolytic activities of CTL and NK cells. These activities may affect to the host antiviral response, and thus have an important impact in the virulence of ASFV infection. Besides, other ASFV genes have been described as directly involved in virulence, including NL-S (the short form of the NL gene similar to the neurovirulence ICP34.5 gene of herpes simplex virus; Zsak et al., 1996) and UK gene (Zsak et al., 1998), and some members of the multigene families 360 and 530 have been shown to have a role in virus cell tropism and efficient replication in macrophages (Neilan et al., 2002; Zsak et al., 2001). Although conflicting evidences (presence in avirulent strains, pathogenicity unaffected after their deletion from virulent isolates, etc.) have been reported in many of the ASFV genes above mentioned, all of them are currently considered as candidates for inactivation from less virulent strains of ASFV (King et al., 2011), in order to obtain a fully attenuated virus model for ASFV vaccination. The virus virulence is a property undoubtedly influenced by many determinants, probably including some or all of the above mentioned ASFV genes, together with other additional genes that might be found in the next future as involved in the control of host responses to the virus infection. A considerable input is currently focused, not solely in the generation of an attenuated virus model, but also in viral DNA or protein mimicking peptides (Ivanov et al., 2011) approaches, as candidates for protective vaccines against ASF, with very promising results. It is now conceivable, and realistically expected, that some ASFV-derived product able to induce a protective immunity in pigs will be delivered in a short time to efficiently combat against the ASF disease.

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