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The use of COS-1 cells for studies of field and laboratory African swine fever virus samples.

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ABSTRACT

Different naturally occurring, cell adapted or genetically manipulated stocks of African swine fever virus were able to infect directly cultures of COS-1 cells, producing extensive cytopathic effects and amounts from 10^6 to 10^7 of infective progeny virus per ml. The induction of late virus-specific proteins, demonstrated by RT-PCR and immunoblotting, and the development of lysis plaques by all the virus samples tested so far, allowed the optimization of both titration and diagnostic assays, as well as the proposal of a method for selection of virus clones during the generation of virus mutants with specific gene deletions.

African swine fever (ASF) virus is the causative agent of a highly contagious disease of swine prevalent in more than 20 sub-Saharan countries of Africa, with devastating effects in the areas where the disease is endemic, these areas representing an enormous reservoir and a risk for the re-introduction of ASF into other countries. ASF virus (ASFV) is a complex enveloped virus which contains a double-stranded DNA molecule surrounded by several layers of protein and lipid with an isometric shape of about 200 nm in diameter (Breese and DeBoer, 1966; Carrascosa et al., 1984). The virus has been classified as the only member of the *Asfarviridae* family (Dixon et al., 2004) and it infects domestic and wild pigs of the *Suidae* family, as well as ticks of the Ornithodoros genus, leading to a range of conditions from acutely fatal hemorrhagic fever to chronic or inapparent persistent infection (Vinuela, 1985). In domestic pigs the virus infects tissue macrophages, blood monocytes and, to a lesser extent, specific lineages of reticular, polymorphs, and megakaryocytic cells (Casal et al., 1984; Wilkinson, 1989).

Wild type ASFV isolates do not replicate in conventional cell cultures. Porcine monocytes and macrophages are the *in vitro* systems selected to mimic natural ASF virus infection, in which most of the virus stocks readily grow. These cells have been used for many years for the diagnosis and titration of both hemadsorbing (Enjuanes et al., 1976) and non-hemadsorbing (Carrascosa et al., 1982) ASFV isolates, but, being primary cells, they often reveal divergences and they are difficult to obtain in sufficient amounts as those required for biochemical studies. Adaptation of some ASFV isolates for growth in different cell lines allowed many experimental approaches and the development of suitable plaque formation assays to evaluate the infectivity titers using more simple, reproducible and quantitative methods (Bustos et al., 2002; Enjuanes et al., 1976; Parker and Plowright, 1968). Nevertheless, these assays were confined to cell culture-adapted virus strains.

On the other hand, as there is no available vaccine against ASF, the rapid and accurate laboratory diagnosis of ASFV-positive and carrier animals is critical for the control of virus outbreaks; The procedures developed so far include the detection in clinical samples of infectious virus (titration), viral antigens/antibodies (ELISA and immunoblotting) or genomic DNA (PCR) (Aguero et al., 2003; Barderas et al., 2000; Oura et al., 1998; Pastor et al., 1992; Pastor et al., 1989; Zsak et al., 2005). In many cases, the low concentration of virus components, the poor quality of samples, or the requirement to confirm an ambiguous result, demand the previous amplification of the virus in cell culture.

The sensitivity of COS cells to some ASFV strains, and its use to construct a deletion mutant in ASFV, have been previously described (Carrascosa et al., 1999; Galindo et al., 2000; Granja et al., 2006; Hurtado et al., 2004). These cells have been also routinely used for the transient and stable expression of ASFV genes in cell culture, but the analysis of its sensitivity to different ASFV field isolates had not been already performed. The COS-1 cell line used in this study was originally obtained from the American Type Culture Collection (CRL-1650**,** Manassas, VA 20108 USA) and it was grown in Dulbecco’s modified Eagle Medium (DMEM) supplemented with 2 mM L-glutamine, 100 U of gentamicin per ml, and non essential amino acids. Cells were cultured at 37ºC in medium supplemented with 5% heat-inactivated fetal calf serum.

The African swine fever virus (ASFV) stocks selected in this study (Table 1) were obtained from the laboratory collection (Garcia-Barreno et al., 1986) and most of them were available from the Community Reference Laboratory for ASF (Department of Exotic Diseases, Centro de Investigación en Sanidad Animal, Valdeolmos, Madrid, Spain). They represent a broad perspective of the many field or laboratory-manipulated virus stocks presently available, including virulent and non-virulent, hemadsorbing or not, as well as African, European and American samples. The field ASFV isolates were propagated (from 2 to 6 passages) from frozen stocks on swine macrophages and titrated by hemadsorption and plaque assay as previously described (Carrascosa et al., 1982; Enjuanes et al., 1976). The Vero-adapted African swine fever virus (ASFV) strain BA71V and the deletion mutant ΔEP153R were grown and titrated on Vero cells as described (Galindo et al., 2000).

As a first screening to determine the ability of the different virus stocks to infect COS-1 cells, we analyzed the presence of mRNA specific for the p72 ASFV gene, which is transcribed late in the virus infection cycle, and it codes for the major capsid viral protein. Indeed, many routine diagnosis tests for ASFV developed so far are based on the detection of p72-related components (DNA, protein or antibody). To perform this analysis, pre-confluent cultures of COS-1 cells were infected with the indicated ASFV isolate at an m.o.i. of 1 to 3 pfu per cell. After 2h of adsorption the remaining virus was washed away, and cultures were incubated in fresh medium until extensive cytopathic effect was evident (about 70 hpi). An aliquot of cells collected from the culture medium was reserved in each case to evaluate the total virus production (see below). The samples were divided in two tubes and centrifuged to analyze in each pelleted fraction the presence of p72-specific mRNA (by RT-PCR) and ASFV-specific virus proteins induced late in the infection (by immunoblotting), respectively. For RT-PCR analysis, total RNA was isolated using the Trizol reagent (Invitrogen, Carlsbad CA 92008, USA). RNA (1 μg) was reverse transcribed to single-stranded cDNA with Revertaid H Minus First Strand cDNA synthesis kit (Fermentas, Burlington, Canada), following the manufacturer's recommendations and the DNA was PCR-amplified with Amplitaq DNA polymerase (Roche, Basel, Switzerland) and the following primers:

p72 forward: 5´-CGCGGATCCATGGCATCAGGAGGAG-3´

p72 reverse: 5´-CGCGAGATCTAGCTGACCATGGGCC-3´

and then analyzed by electrophoresis in 0.7% agarose gels containing ethidium bromide. For Western blot analysis, cells were lysed in TNT buffer (20 mM Tris–HCl, pH 7.5, 0.2 M NaCl, 1% Triton X100) supplemented with protease inhibitor cocktail tablets (Roche, Basel, Switzerland). Proteins (30 μg) were subjected to sodium dodecyl sulfate –12% polyacrylamide gel electrophoresis, and then electroblotted onto a PVDF-Immobilon (Millipore, Billerica, MA01821, USA) membrane. After reacting with primary antibody specific for late ASFV-induced virus proteins (del Val and Vinuela, 1987), membranes were exposed to horseradish peroxidase-conjugated secondary anti-rabbit antibody (Amersham, GE Healthcare, Chalfont St. Giles, United Kingdom), followed by chemiluminescence (ECL, Amersham, GE Healthcare, Chalfont St. Giles, United Kingdom) detection by autoradiography. As it is shown in Fig. 1, all of the tested ASFV stocks were able to induce the synthesis of p72-specific mRNA (Fig. 1A). Moreover, the synthesis of many ASFV proteins induced late in the infection was also detected in COS cells infected with everyone of the virus stocks, revealing a number of viral specific bands that were absent in the mock-infected samples (Fig. 1B).

The virus-induced proteins in COS cells were assembled into infectious viral particles, yielding titers from 10^6 to 10^7 pfu per ml when assayed by plaque titration in COS cell monolayers, as shown in Fig. 1C, where it is represented the mean values and standard deviations obtained from 2 to 5 independent determinations. The expected yield for pathogenic ASFV in pig macrophage cultures ranges from 10^7 to 10^8 HADU_{50} per ml (data not shown) when assayed by hemadsorption (Carrascosa et al., 1982;

Enjuanes et al., 1976), but the difference in productivity found among COS and macrophage cultures should be most probably assigned to the higher sensitivity of the hemadsorption test as compared to the plaque titration assay. The plaque test developed in COS cells was similar to that described for ASFV titration in Vero cells (Enjuanes et al., 1976). Briefly, subconfluent cultures of COS-1 cells grown on multiwell dishes were infected with 10-fold dilutions of virus stocks in culture medium, shacked every 10 min for 2 h, and carefully overlaid, without removing the inoculum, with agar-medium made up by mixing one volume of DMEM (2X) medium with 4% fetal calf serum and 160 μg/ml of DEAE-dextran, and one volume of freshly prepared 1.4% agar noble (Difco, Lawrence Kansas 66044 USA) in distilled water, equilibrated at 45ºC. Plates were incubated at 37ºC for 5 to 7 days and stained with 2% crystal violet in 5% formaldehyde. An illustration of the plaques developed by each ASFV isolate in COS cell monolayers is presented in Fig. 2A, where plaques were stained with 2% crystal violet at day 5 after infection. Several virulent ASFV isolates (Malawi 82, Lisbon 57, Mozam 69 and Brazil 81) developed large plaques in COS cell monolayers, but some others, like E70, Uganda vir., Lisbon 60 and CC83 yielded standard small-sized lysis plaques, as did the attenuated stocks (Ba71V, ΔEP153R, Hinde att., and NHV). Therefore, it was not possible to establish any correlation between plaque size and virulence, hemadsorbing properties or origin of the virus stocks. However, all of the four ASFV isolates yielding large-sized plaques in COS-1 cells developed an aggressive and rapid cytopathic effect, and they were also able to produce large plaques in Vero cell monolayers (data not shown), where the standard-sized plaques produced by cell-adapted ASFV strains (like Ba71V and ΔEP153R) required the standard period (5 to 6 days after infection) to be detected. Information regarding the sensitivity of different cells to ASFV stocks is of the major importance, not only to determine their capacity to detect, grow and titrate these viruses, but also to define possible candidates with increased ability to cross host-specific barriers, which should be manipulated and controlled even more carefully than the rest of field ASFV isolates. In any case, and taking into account the results presented in this study, we propose the use of COS-1 cells to improve the detection, growth and titration of field and laboratory-engineered ASFV stocks.

The ability of the E70 virus isolate to grow in COS cell monolayers has been previously exploited to generate mutant viruses defective in the viral A238L gene (Granja et al., 2006) after disrupting the open reading frame by insertion of the *Escherichia coli β-glucuronidase* gene. Considering the results presented above on the sensitivity of COS-1 cells to different ASFV stocks, we can anticipate that the construction of defective mutants in specific viral genes might be boarded in all the ASFV stocks able to grow in this cell line, as it was made before in the Vero cell-adapted BA71V ASFV strain (Galindo et al., 2000; Garcia et al., 1995; Granja et al., 2004; Nogal et al., 2001; Rodriguez et al., 1993). To illustrate the type of result expected in the selection of a virus recombinant expressing the marker gene LacZ under the control of the ASFV promoter p72, a suitable dilution of the deletion mutant ΔEP153R generated from the BA71V ASFV strain (Galindo et al., 2000) was subjected to plaque assay in COS-1 cell monolayers in 6-well tissue culture plates, as above indicated. Samples at day 4 after infection were incubated with the substrate X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, 300 μg/ml in fresh semi-solid medium, as indicated elsewhere (Garcia et al., 1995)), and then stained at day 5 after infection with crystal violet as above indicated. A perfect match of the plaques developed by both staining is shown in Fig.2B, corroborating that all the plaque-forming units present in the virus stock were expressing the marker gene, supporting the use of the plaque assay for the selection and purification of virus recombinants engineered with selectable marker genes.

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FIGURE LEGENDS

Fig.1. Infection of COS-1 cells by different ASFV stocks. COS-1 cells infected with the indicated virus sample at an m.o.i. of 3 pfu per cell, were incubated at 37ºC and collected at 70 h after infection to determine the presence of p72-specific m-RNA by RT-PCR (panel A), and for the detection of virus-specific late induced proteins by immunoblotting (panel B). The molecular weight markers (MW) are shown at right and the position of the major ASFV-induced proteins at left by arrows. The production of infective progeny virus was also determined by titration on COS-1 cell monolayers (panel C): mean and standard deviation from 2 to 5 independent experiments are represented. ASFV stocks are numbered as described in the top of the figure and in Table 1.

Fig.2. Plaques developed in COS-1 cell monolayers by different ASFV stocks. Sub-confluent cultures of COS-1 cells in multiwell plates were infected with suitable dilutions of the indicated ASFV stock (panel A) or with ΔEP153R (panel B), and overlaid with solid culture medium. Plaques were stained with crystal violet (CV) at day 5 after infection (panel A), or pre-stained with X-Gal at day 4 and then with CV at day 5 after infection (panel B).

Figure 1

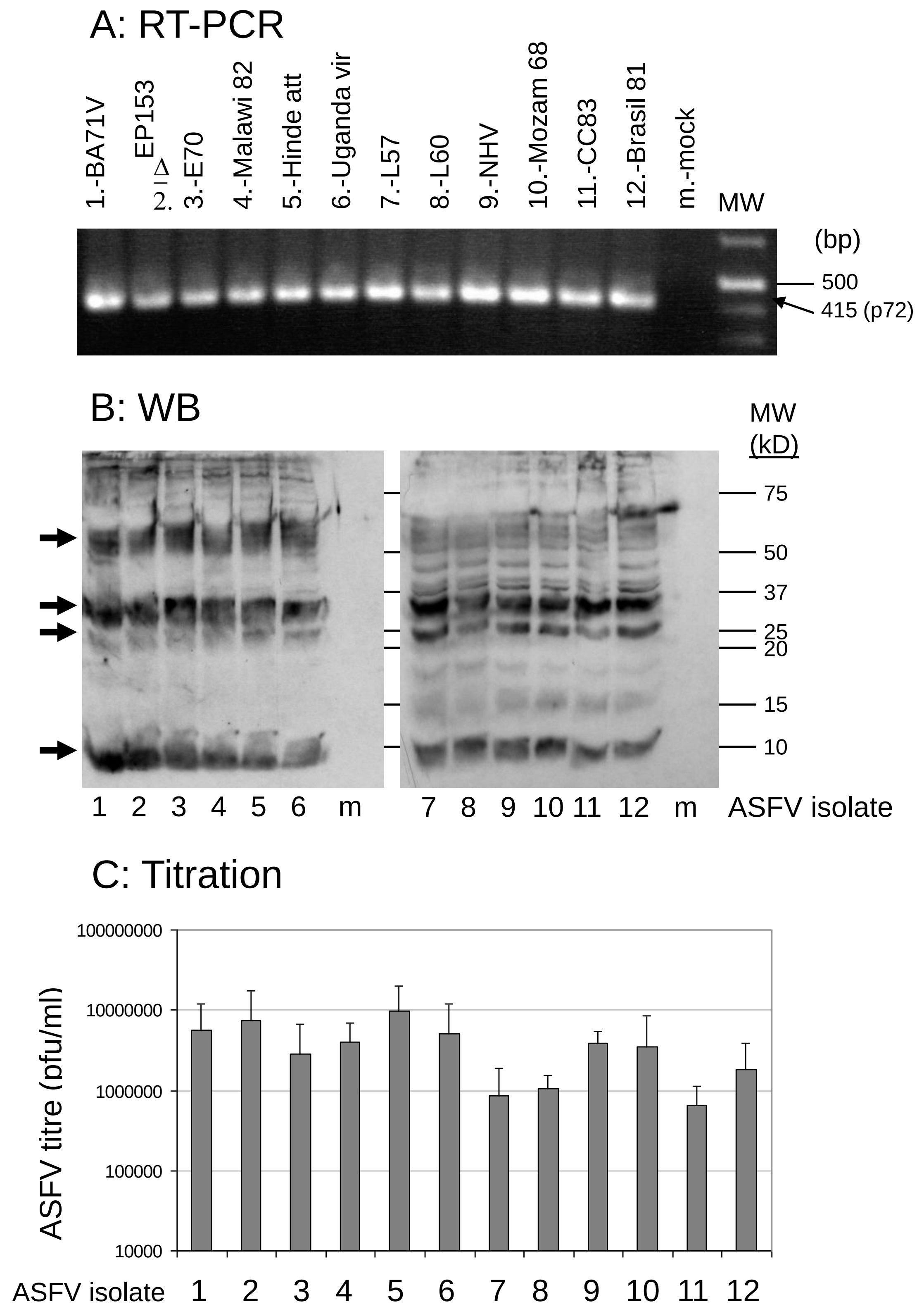
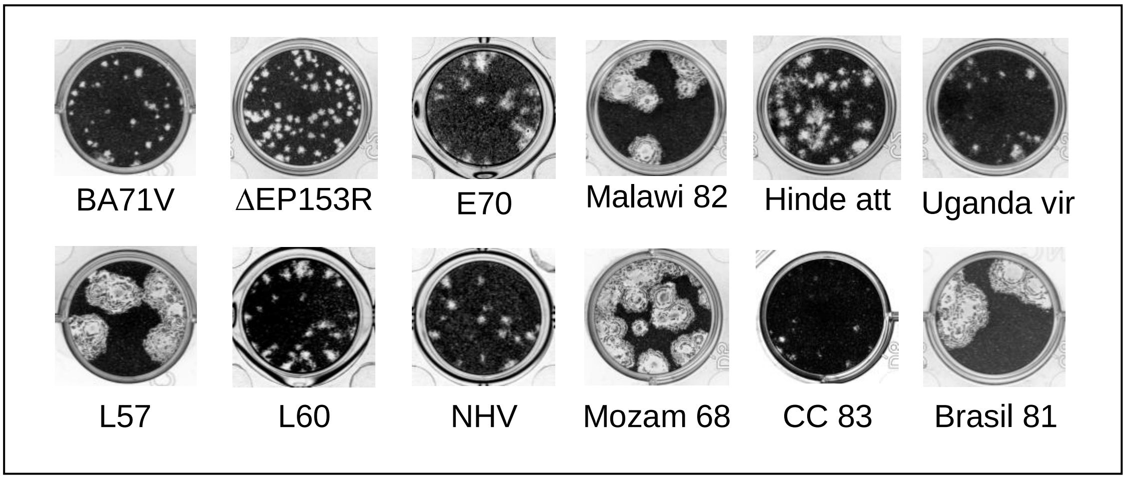


Fig.1

Figure 2

A. ASFV isolates



B. ΔEP153R

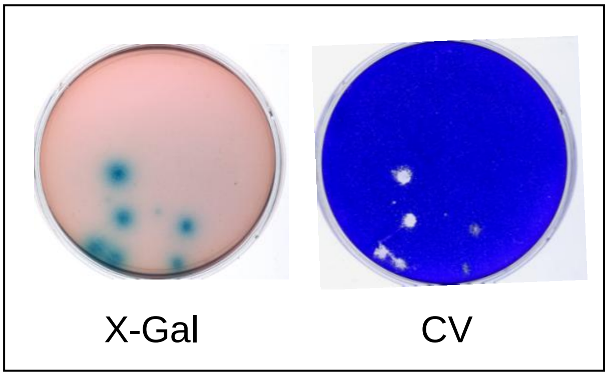


Fig.2

Table 1. ASFV stocks selected to analyze COS-1 cell sensitivity

ASFV stock	Virulence	Hemadsorbing	Origin	#
BA71V[a]	att	+/-	European	1
ΔEP153R[b]	att	non	European	2
E70 (Spain 70)	vir	+	European	3
Malawi 82	vir	+	African	4
Hinde att.	att	+	African	5
Uganda vir.	vir	+	African	6
Lisbon 57	vir	+	European	7
Lisbon 60	vir	+	European	8
NHV[c]	att	non	European	9
Mozam 68	vir	+	African	10
CC83	vir	+	European	11
Brazil 81	mod vir	+	American	12

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att: attenuated; vir: virulent; mod vir: moderately virulent

[a] Adapted to Vero cells (Enjuanes et al., 1976)

[b] Lab-engineered strain (Galindo et al., 2000)

[c] Non-hemadsorbing virus (Gil et al., 2003)

#: numbered as in Fig. 1