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Short communication

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

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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, 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

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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

att: attenuated; vir: virulent; mod vir: moderately virulent. #: numbered as in Fig. 1.
[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).

amino acids. Cells were cultured at 37 °C in medium supplemented with 5% heat-inactivated fetal calf serum.

The 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 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 1–3 pfu per cell. After 2 h 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 determine 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, MA 01821, 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

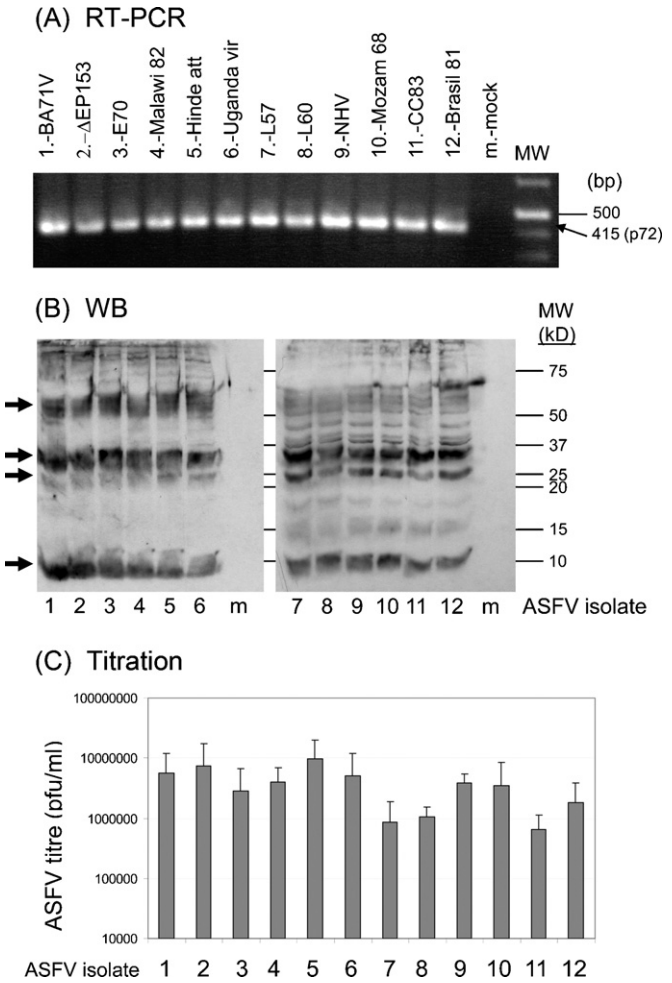

Fig. 1. Infection of COS-1 cells by different ASFV stocks. COS-1 cells infected with the indicated virus sample at an m.o.i. of 3 pfu per cell, were incubated at 37 °C and collected at 70 h after infection to determine the presence of p72-specific mRNA by RT-PCR (panel A), and for the detection of virus-specific 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.