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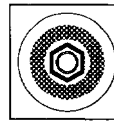
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Short Communication

Improvement of African swine fever virus neutralization assay using recombinant viruses expressing chromogenic marker genes

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Abstract

Antibody neutralization of African swine fever (ASF) virus measured by a plaque reduction assay presents frequent difficulties because of the absence or delay in plaque formation by many strains, especially low-passage viruses. To overcome this problem, a new ASF virus neutralization test has been developed. The new test consists of a conventional plaque reduction assay in which the viral plaques are detected by expression of marker genes. For the development of this neutralization assay 4 mutant viruses were generated by homologous recombination, containing β-galactosidase or β-glucuronidase reporter genes inserted into the thymidine kinase locus of the viral genome. These recombinant viruses have the following advantages with respect to parental viruses: (1) the neutralization assay takes less than a third of the time needed using non-recombinant viruses; (2) the small plaques can be detected more accurately by color contrast; and (3) the neutralization-resistant virus clones can be recovered easily post-plaque counting. Additionally, these recombinant viruses permit differentiation by chromogenic staining of individual infected pig macrophages, the natural host cell for ASF virus, facilitating neutralization assays in these primary cultures as described in cell lines.

Keywords: African swine fever virus; Neutralization test; β-Galactosidase recombinant; β;-Glucuronidase recombinant

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Plaque reduction assay is currently the most accurate method to determine in vitro African swine fever (ASF) virus neutralization capability of sera from infected or immunized pigs (Carrascosa et al., 1991; Angulo et al., 1993; Zsack et al., 1993). This is because the neutralization of this virus is often incomplete (80–98% of infectivity reduction) which enables virus neutralization analysis by inhibition of cytopathic effect. Only Vero cell-adapted virus strains, but not wild-type viruses, that only grow in pig macrophages, can be used in these studies, leading to marked differences between in vitro and in vivo studies. Additionally, the propagation of ASF virus in cell culture generates non-neutralizable variants (Zsack et al., 1993; Gómez-Puertas and Escribano, unpublished results) and modifications in the antigenic properties of the virus (García-Barreno et al., 1986) that can affect the neutralization results obtained using high-passage viruses.

An alternative to the use of these high-passage strains in neutralization assays could be the use of low-passage viruses. In these viruses, antigenic changes are not detected (García-Barreno et al., 1986) and they infect both Vero cells and pig macrophages. Nevertheless, these viruses show a very slow growing curve and cytopathic effect in Vero cells, making it difficult to detecting plaque formation, often taking up to 15 days.

An optimized neutralization assay is described using low-passage mutant ASF viruses expressing marker genes, that implement the technique by easy and early detection of plaques by color contrast. These new generated viruses significantly increase the sensitivity of virus plaque visualization, so that the detection of single infected cells, including pig macrophages, is possible. In addition, neutralization-resistant clones can be isolated, allowing the purification of putative antibody escape mutants for epitope mapping studies.

Three strains of ASF virus were selected for study: the high-passage virus Ba71V and the low-passage 608VR and 1207VR virus strains. The strain Ba71V (Enjuanes et al., 1976) has been passaged more than 100 times in Vero cells and is not infective for pigs. This virus was used as control of homologous recombination and plaque formation in Vero cell monolayers. The virulent strains 608VR and 1207VR, with low number of passages in Vero cells (13 and 15 times, respectively) form lysis plaques in Vero cell monolayers of smaller size than Ba71V, only detectable, with difficulty, 2 weeks after infection.

Recombinant variants from these 3 strains were obtained using the insertion/expression vectors pINS72 β-gal (Rodríguez et al., 1992) and pINS72 β-gus (R. García and J.F. Rodriguez, unpublished results), that introduce into the thymidine kinase (TK) locus of the viral genome a copy of the β-galactosidase or β-glucuronidase marker genes under the control of the ASF virus late promoter p72 (Fig. 1). All procedures used for the homologous recombination were as described (Rodríguez et al., 1992), and the virus mutants obtained were named: Ba71VΔTKβ-gal, 608VRΔTKβ-gus, 1207VRΔTKβ-gal and 1207VRΔTKβ-gus.

To characterize the growth rate and plaque size phenotype of the recombinant and parental viruses, Vero cell monolayers were infected with 100 PFUs of each virus and the plaque formation was studied. Plaque detection was carried out using chromogenic dyes (X-gal or X-gluc) with recombinant viruses or crystal violet when staining the parental viruses. The first observation was that the mutation in TK does not affect the

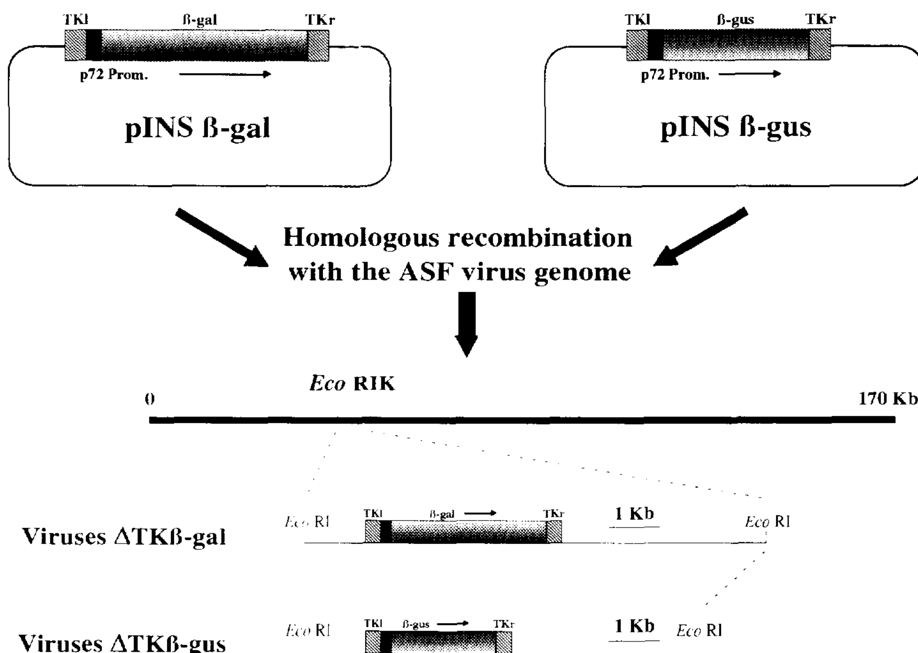


Fig. 1. Predicted genomic structure of the recombinant ASF viruses ΔTKβ-gal and ΔTKβ-gus. Homologous recombination between the virus genome and the plasmid vectors pINS β-gal and pINS β-gus should lead the insertion of the β-gal and β-gus genes, fused to the virus promoter p72 into *Eco*RI K genomic fragment.

plaque size comparing parental and recombinant variants analyzed by the Crystal violet method (data not shown). Then, the time in which it was possible to detect virus-infected cells by Crystal violet plaque staining or by chromogenic dye (X-gal or X-gluc) in recombinant viruses was compared. It was found that virus plaques produced by the high-passage virus Ba71V were detected at least 4 days earlier by the chromogenic staining than by the conventional Crystal violet method (Fig. 2A).

The same comparison made with the low-passage strains 1207VR and 608VR and their recombinant derivatives 1207VRΔTKβ-gal, 1207VRΔTKβ-gus and 608VR ΔTKβ-gus (Fig. 2B, C) revealed greater differences among both virus plaque detection methods than in the high-passage virus Ba71V. In these viruses, 100% detection of the viral plaques took less than 6 days by chromogenic dye, in contrast to the 15–20 days required by conventional Crystal violet staining. The development of virus plaques visualized by chromogenic dye is shown in Fig. 3.

Comparative neutralization assays of parental and recombinant low-passage viruses using a pool of 3 sera from ASF virus-vaccinated pigs with the attenuated virus strain E75CV1-4 (Ruiz Gonzalvo et al., 1986; Alcaraz et al., 1992) showed identical results to the neutralization titer of the tested sera with both types of viruses (Fig. 4A). However, neutralization tests carried out with recombinant ASF viruses took 4 days, while parental

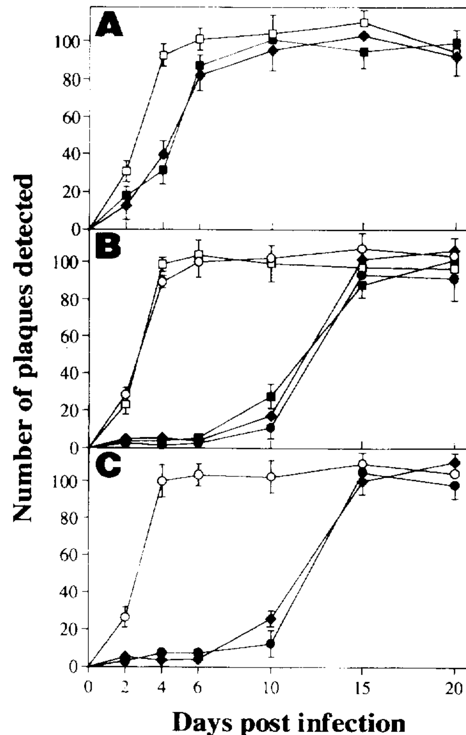
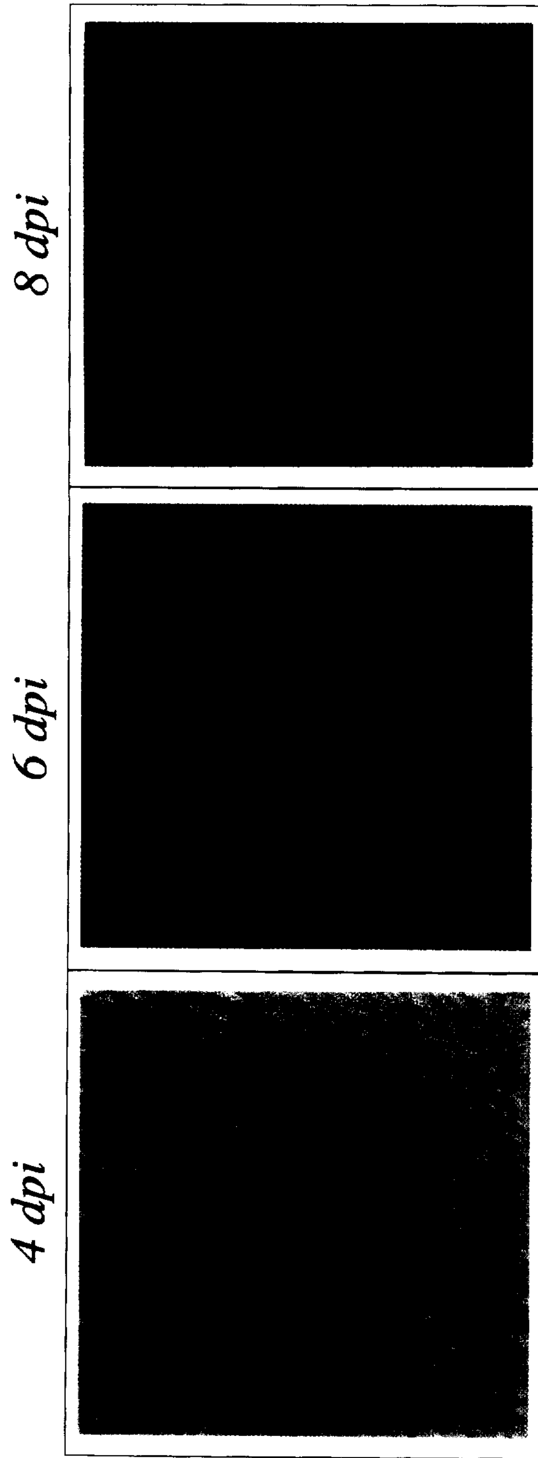


Fig. 2. Kinetic of plaque appearance in confluent monolayer Vero cell cultures infected with 100 plaque forming units (PFU) of parental and recombinant derivative viruses. At the indicated days postinfection, viral plaques were visualized using Crystal violet staining or X-gal or X-gluc chromogenic dye. A: comparison of virus plaque detection by Crystal violet staining of Ba71V (■) and Ba71VΔTKβ-gal (♦) infected cell monolayers or chromogenic staining of Ba71VΔTKβ-gal-infected cells with X-gal (□). B: comparison of virus plaque detection by Crystal violet staining of 1207VR (■), 1207VRΔTKβ-gal (♦) and 1207VRΔTKβ-gus (●) virus-infected cell monolayers or by chromogenic staining with X-gal or X-gluc of infected cells with 1207VRΔTKβ-gal (○) and 1207VRΔTKβ-gus (□) viruses, respectively. C: comparison of plaque detection by Crystal violet staining of 608VR (♦) and 608VRΔTKβ-gus (●) virus-infected cells and virus plaque detection by chromogenic staining with X-gluc of 608VRΔTKβ-gus (○) infected cells. Results show mean ± S.E.M. of 3 independent experiments.

viruses took up to 15 days for the final reading of the test. Fig. 4B and C show chromogenic dye with X-gal and X-gluc, respectively, of virus plaques formed after virus infection prior to incubation with a non-immune serum or anti-ASFV serum.

Infection of pig macrophages obtained from peripheral blood or lung with the recombinant virulent viruses showed a similar infection cycle as their parental counterpart. By chromogenic dye, the visualization of single infected pig macrophages at 14 h postinfection was possible by staining with X-gal or X-gluc (300 μg/ml) directly on 96-well plaques (Fig. 5A) or after cytocentrifugation (5×10^6 cells/ml) and counter-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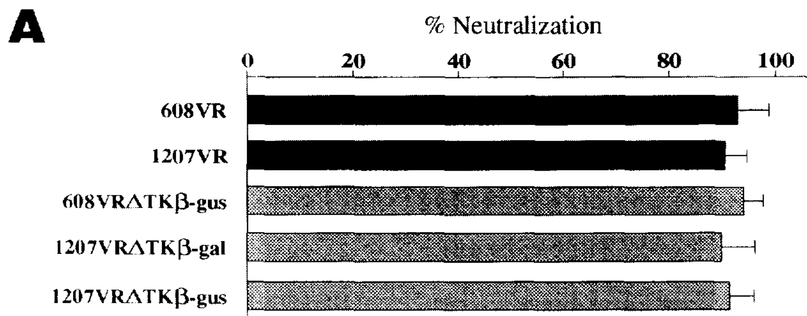

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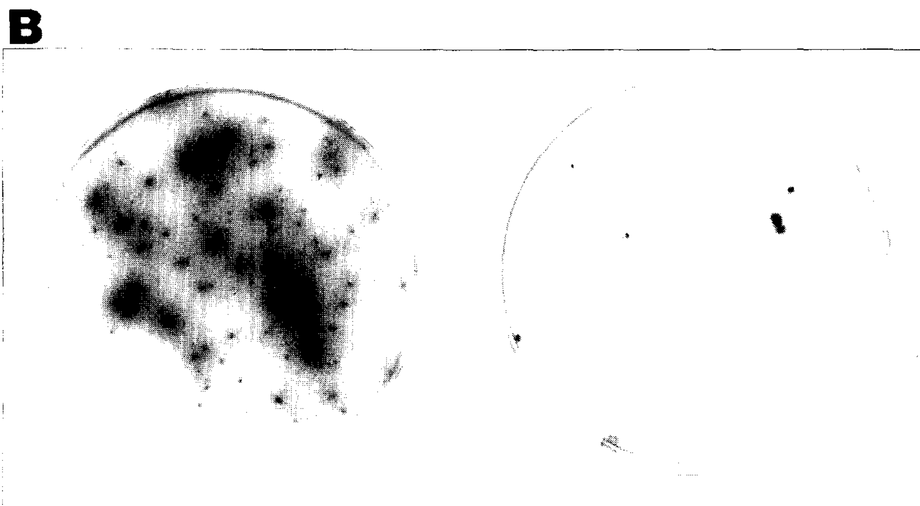

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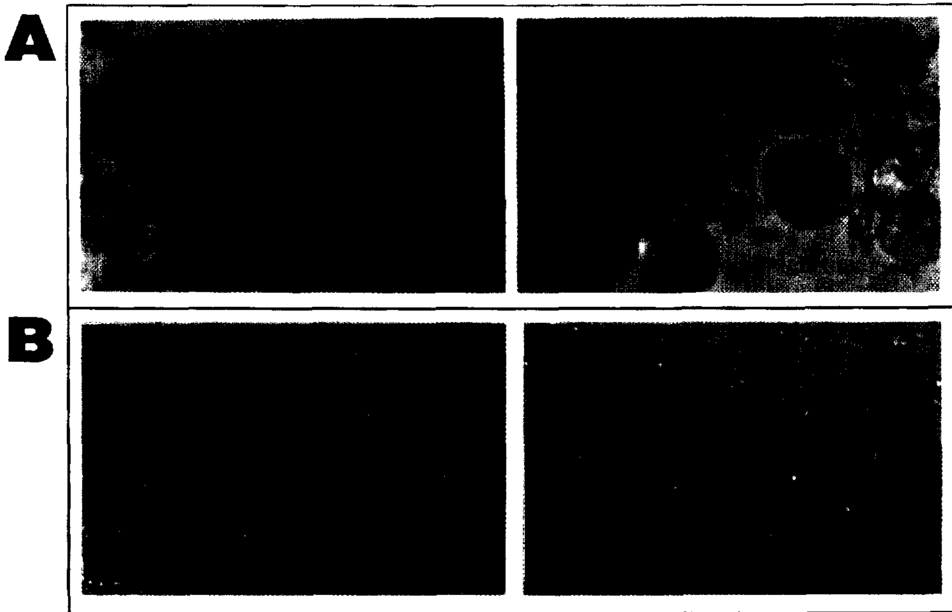

Fig. 5. Chromogenic staining of pig macrophages infected with the recombinant virus 608VRΔTKβ-gus. Visualization of single infected pig macrophages at 14 h postinfection by staining with X-gal (A) directly on 96-well plaques or (B) after cytocentrifugation and counterstaining with Nuclear red.

staining with nuclear red (Fig. 5B). In both cases, macrophages were fixed with 2% formaldehyde, 0.2% glutaraldehyde previous to chromogenic dye incubation. The stained pig macrophages corresponding to infected cells, some also presenting hemadsorbing properties characteristic of this virus, could easily be counted. Neutralization

Fig. 4. Influence of the use of parental and recombinant viruses on neutralization test results. Comparative neutralization percentages obtained with a pool of 3 anti-ASF virus sera using the parental and recombinant low-passage 608VR, 1207VR, 608VRΔTKβ-gus, 1207VRΔTKβ-gal and 1207VRΔTKβ-gus ASF viruses. One hundred PFU of virus were mixed with a neutralizing pool of sera diluted 1 : 5, using preimmune swine serum as negative control. Plaques produced by the non- neutralized virus particles of 608VR and 1207VR virus strains were visualized by Crystal violet staining at 15 days postinfection (dark bars). The non-neutralized virus particles by the pool of sera of 608VRΔTKβ-gus, 1207VRΔTKβ-gal and 1207VRΔTKβ-gus viruses were revealed by chromogenic staining with X-gluc or X-gal at 4 days postinfection (light bars). A: neutralization index of the pool of sera against the different viruses calculated as a percentage of plaque reduction with respect to the number of plaques obtained after incubation of the viruses with the preimmune serum. Results show mean ± S.E.M. of 3 independent experiments. B and C: viral plaques on Vero cell monolayers visualized by X-gal or X-gluc chromogenic dyes on the fourth day after infection with the viral strains 1207VRΔTKβ-gal (B) or 1207VRΔTKβ-gus (C) preincubated with swine preimmune serum (left) or immune pool of sera (right).

assays using pig macrophages and recombinant viruses were carried out detecting the infected cells by chromogenic dye at 14 h postinfection. Identical neutralization results as above were found in Vero cells, were obtained in macrophages with the same sera and viruses (data not shown). The detection by chromogenic dye of pig macrophages infected with recombinant viruses, opens the possibility of using genetically recombinated virulent viruses growing only in primary cultures of monocyte/macrophage cells.

Another advantage of the use of recombinant viruses in neutralization assays is the possibility of easy recovery of viable non-neutralizable virus clones. Virus from chromogenic stained plaques of different size, produced by non-neutralized viruses were recovered, diluted in cell culture medium and subsequently plaqued, showing unaltered cell infectivity (data not shown). This possibility will lead to the study of the generation of neutralizing resistant viruses to polyclonal or monoclonal antibodies, facilitating the mapping of neutralizing epitopes of the virus.

At present, only a non-pig-infective high-passage ASF virus, Ba71V has been genetically manipulated. The demonstration that viruses with a reduced number of passages in Vero cells, that remain infective for pigs, can be genetically manipulated offers the possibility of obtaining in vivo information about genes involved in virulence or pathogenesis. On the other hand, the expression of marker genes during ASF virus pig infection may become a tool for in vivo studies about tropism, pathogenesis or latency (Stoddart et al., 1994).

In conclusion, the use of recombinant derivatives of low-passage ASF virus strains in neutralization tests is a novel and advantageous modification of the conventional test, that permits more reliable and rapid information of the neutralization index of the tested sera.

Acknowledgements

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