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Deletion of the thymidine kinase gene induces complete attenuation of the Georgia isolate of African swine fever virus

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ABSTRACT

African swine fever virus (ASFV) is the etiological agent of a contagious and often lethal viral disease of domestic pigs. There are no vaccines to control Africa swine fever (ASF). Experimental vaccines have been developed using genetically modified live attenuated ASFVs obtained by specifically deleting virus genes involved in virulence, including the thymidine kinase (TK) gene. TK has been shown to be involved in the virulence of several viruses, including ASFV. Here we report the construction of a recombinant virus (ASFV-G/V-ΔTK) obtained by deleting the TK gene in a virulent strain of ASFV Georgia adapted to replicate in Vero cells (ASFV-G/VP30). ASFV-G/P-ΔTK demonstrated decreased replication both in primary swine macrophage cell cultures and in Vero cells compared with ASFV-G/VP30. *In vivo*, intramuscular administration of up to 10^6 $TCID_{50}$ of ASFV-G/V-ΔTK does not result in ASF disease. However, these animals are not protected when challenged with the virulent parental Georgia strain.

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1. Introduction

African Swine Fever Virus (ASFV), a large enveloped virus with a double-stranded (ds) DNA genome of approximately 180–190 kilobase pairs (Costard et al., 2008), causes a spectrum of disease (from highly lethal to sub-clinical) depending on the virus strain (Tulman et al., 2009). Virulent ASFV infections in domestic pigs are fatal and characterized by fever, hemorrhages, ataxia and severe depression.

The current epidemiological situation of ASF (endemic in several sub-Saharan African countries and in Sardinia) is particularly important for outbreaks recorded in the Caucasus region since 2007 (affecting Georgia, Armenia, Azerbaijan and Russia and more recently in Ukraine, Belarus, Lithuania, Latvia and Poland), threatening to disseminate into neighboring Western European countries (Chapman et al., 2011).

There is no vaccine available for ASF and the disease is controlled by quarantine and elimination of affected animals. Protective immunity does develop in pigs surviving viral infection against reinfection with homologous viruses (Hamdy and Dardiri, 1984; Ruiz-Gonzalvo et al., 1981). Pigs immunized with live attenuated ASF viruses developed by deleting specific ASFV virulence-associated genes (*UK*, *23-NL*, *TK*, *9GL* or *MGFs*) were protected when challenged with homologous virulent parental virus (Lewis et al., 2000; Moore et al., 1998; Zsak et al., 1996,1998; O'Donnell et al., 2015a,b). These reports are the only experimental evidence describing the rational development of an effective live attenuated virus against ASFV.

ASFV thymidine kinase (TK), a viral enzyme involved in synthesis of deoxynucleoside triphosphates (Martin Hernandez and Tabares, 1991) has been shown to not be essential for virus replication in cell cultures (Martin Hernandez et al., 1995; Rodriguez et al., 1992) but recombinant ASFV having the TK gene removed could not replicate in swine macrophages (Moore et al., 1998). Deletion of the TK gene both in herpesviruses and poxviruses showed the gene is not essential for replication in cultured cells (Dubbs and Kit, 1964; Panicali and Paoletti, 1982), but was associated with a reduction in virus virulence (Buller et al., 1985; Field and Wildy, 1978; Kochneva et al., 1994). Similarly, deletion of the TK gene from the ASFV Malawi isolate results in significant virus attenuation (Moore et al., 1998).

Here we report the development of a recombinant virus (ASFV-G/V-ΔTK) engineered by deleting the TK gene from the genome of a virulent ASFV Georgia isolate that has been adapted to replicate in Vero cells (ASFV-G/VP30) while still causing disease in domestic pigs inoculated with the virus. Compared with the parental virus,

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ASFV-G/P-ΔTK demonstrated decreased replication efficiency both in primary swine macrophage cell cultures and in Vero cells. ASFV-G/V-ΔTK is completely attenuated when administered IM to swine at a dose as high as 10^6 $TCID_{50}$ although, interestingly, these, animals are not protected when challenged with the virulent parental Georgia strain.

2. Materials and methods

2.1. Cell cultures and viruses

ASFV Georgia (ASFV-G) was a field isolate kindly provided by Dr. Nino Vepkhvadze, from the Laboratory of the Ministry of Agriculture (LMA) in Tbilisi, Republic of Georgia. ASFV-G/VP30 was developed by serial passages in Vero cells as described by Krug et al. (2015).

Growth kinetics was assessed either in Vero cells or in primary swine macrophage cell cultures. Vero cells were obtained from ATCC and sub-cultured in DMEM with 10% FC while primary swine macrophage cell cultures were, prepared as described by Zsak et al. (1996). In either case, preformed monolayers were prepared in 24-well plates and infected at a MOI of 0.1. After 1 hour of adsorption at 37 °C under 5% CO_2 the inoculum was removed and the cells were rinsed two times with PBS. The monolayers were then rinsed with media and incubated for 2, 24, 48, 72 and 96 h at 37 °C under 5% CO_2. At appropriate times post-infection, the cells were frozen at ≤−70 °C and the thawed lysates were used to determine titers by $HAID_{50}$/ml in primary swine macrophage cell cultures or $TCID_{50}$/ml in Vero cell cultures. All samples were run simultaneously to avoid inter-assay variability. Virus titration was performed on primary swine macrophage or Vero cell cultures in 96-well plates. Presence of virus was assessed by hemadsorption (HA) or immunocytochemistry (using an anti-ASFV p30 monoclonal antibody produced in APHIS, PIADC); virus titers were calculated by the Reed and Muench method (1938).

2.2. Construction of recombinant ASFV-G/VP-ΔTK

Recombinant ASFVs were generated by homologous recombination between the parental ASFV genome and a recombination transfer vector following infection and transfection of swine macrophage cell cultures (Zsak et al., 1996). The recombinant transfer vector (p72GUSΔTK) contained flanking genomic regions, which included the left arm, located between genomic positions 63,236 to 64,282, and the right arm, located between genomic positions 64,602 to 65,674 and a reporter gene cassette containing the β-glucuronidase (GUS) gene with the ASFV p72 late gene promoter, p72GUS (Zsak et al., 1998). This construction created a 312 nucleotide deletion within the TK gene (K196R) between nucleotide positions 64,289 to 64,601 (Fig. 1). Recombinant transfer vector p72GUSΔTK was obtained by DNA synthesis (Epoch Life Sciences, Sugar Land, TX, USA). Vero cell cultures were infected with ASFV-G/VP30 and transfected with p72GUSΔTK. Recombinant viruses representing independent primary plaques were purified to homogeneity by successive rounds of plaque assay purification.

2.3. Polymerase chain reaction (PCR)

Purity of ASFV-G/VP-ΔTK in the virus stock was assessed by PCR. Detection of the TK genes was performed using the following pair of primers: forward 5′ CACTCCGGTATACAGCTACG 3′; reverse 5′ GGCCAATATACTTAACCC AGC 3′. Detection of the β-Gus gene was performed using the following pair of primers: forward 5′ GCCGATTATCATCACCGAATAC3′; reverse 5′TGCGCCA GGAGAGTTGTTGATTC3′. Presence of ASFV DNA was detected using the following pair of primers: forward 5′CGTAGAGCTGTTGTTGTCAT 3′; reverse 5′CAGAGAGAATACAGCTG TAGGTCT3′, which detect the presence of the MGF505 3R gene.

2.4. Sequencing of PCR products

PCR products were sequenced using the dideoxynucleotide chain-termination method (Sanger et al., 1977). Sequencing reactions were prepared with the Dye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA). Reaction products were sequenced on a PRISM 3730xl automated DNA sequencer (Applied Biosystems). Sequence data were assembled with the Phrap software program (http://www.phrap.org), with confirmatory assemblies performed using CAP3 (Huan and Madan, 1999). The final DNA consensus sequence represented an average five-fold redundancy at each base position. Sequence comparisons were conducted using BioEdit software (Tom Hall, Ibis Biosciences, Carlsbad, CA, Copyright 1997–2013).

2.5. Next generation sequencing (NGS) of ASFV genomes

ASFV DNA was extracted from infected cells and quantified as described earlier (Krug et al., 2015). Full-length sequencing of the virus genome was performed as described elsewhere (Krug et al., 2015). Briefly, one microgram of virus DNA was enzymatically sheared and the resulting fragmented DNA size distribution was assessed. Adapters and library barcodes were ligated to the fragmented DNA. The appropriate size range of the adapter-ligated library was collected using the Pippin Prep™ system (Sage Science) followed by normalization of library concentration. The DNA library was then clonally amplified onto ISPs and enriched. Enriched template ISPs were prepared and loaded onto Ion chips for sequencing. Sequence analysis was performed using Galaxy (https://usegalaxy.org/) and CLC Genomics Workbench (CLCBio).

2.6. Animal experiments

Animal experiments were performed under biosafety level 3 conditions in the animal facilities at PIADC following a protocol approved by the Institutional Animal Care and Use Committee.

ASFV-G/VP-ΔTK was assessed for its virulence phenotype relative to the parental ASFV-G/VP30 virus using 80–90 pound commercial breed swine. Five pigs were inoculated intramuscularly (IM) either with 10^4 $TCID_{50}$ of ASFV-G/VP-ΔTK or ASFV-G/VP30 (additional experiments were performed using 10^6 $TCID_{50}$ of ASFV-G/P-ΔTK). Clinical signs (anorexia, depression, fever, purple skin discoloration, staggering gait, diarrhea and cough) and changes in body temperature were recorded daily throughout the experiment.

To assess the protective effect of ASFV-G/VP-ΔTK, ASFV-G/P-ΔTK-infected animals were IM challenged with 10^3 HAD_{50} of highly virulent parental ASFV-G at 28 days post-infection. Clinical signs (as described above) and changes in body temperature were recorded daily throughout the experiment.

2.7. Detection of anti-ASFV antibodies

Anti-ASFV antibodies in sera of infected animals were quantified using an in-house immunohistochemistry assay. Vero cells were infected (MOI = 0.1) with ASFV-G/VP30 (Krug et al., 2015) in 96 well plates. Two-fold dilutions of the sera were incubated for 1 hour at 37 °C in the 96-well ASFV-infected cell monolayer. After washing, the presence of anti-ASFV antibodies was detected by using a commercial anti-swine peroxidase labeled mouse immunoglobulin and a peroxidase substrate (Vector Laboratories, CA). Titers were