Virus Research 213 (2016) 165–171

Contents lists available at ScienceDirect

Virus Research

journal homepage: www.elsevier.com/locate/virusres

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

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ARTICLE INFO

Article history:
Received 8 October 2015
Received in revised form
30 November 2015
Accepted 2 December 2015
Available online 4 December 2015

Keywords:
ASFV
African swine fever virus
Thymidine kinase

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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http://dx.doi.org/10.1016/j.virusres.2015.12.002
0168-1702/Published by Elsevier B.V.

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

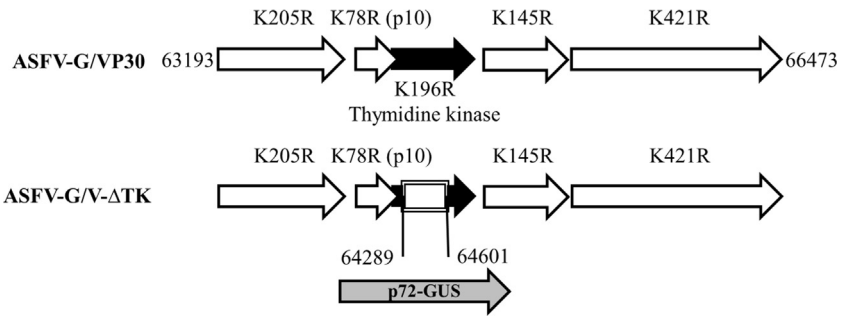

Fig. 1. Schematic representation of the thymidine kinase (TK) gene (*K196R*) region in ASFV-G/VP30 showing adjacent open reading frames. A 312 bp nucleotide deletion was introduced between positions 64289 to 64601 within the ORF of the *K196R* gene by inserting a p72-GUS reporter cassette using homologous recombination. This insertion resulted in the production of a TK-recombinant virus named ASFV-G/V-ΔTK.

expressed as the $\log_2$ of the inverse of the highest serum dilution showing reaction with the infected cells.

3. Results

3.1. Development of the ASFV-G/P-ΔTK deletion mutant

ASFV-G/P-ΔTK was constructed using ASFV-G/VP30 as the parental strain, a derivative of ASFV Georgia 2010 (Krug et al., 2015). ASFV-G/VP30, although adapted to replicate in Vero cells after 30 successive passages, is still virulent when intramuscularly (IM) inoculated into swine at a dose of 10^4 $TCID_{50}$ (Krug et al., 2015). From the 591-bp TK gene, a 312-bp region was deleted (between nucleotide positions 64,289 and 64,601) from ASFV-G/VP30 and replaced with a cassette containing the p72GUS reporter gene (p72GUSΔTK) by homologous recombination (see Section 2 and Fig. 1). The recombinant virus was obtained after 12 successive plaque purification events on monolayers of Vero cell cultures. The virus population obtained from the last round of plaque purification was amplified in Vero cell cultures to obtain a virus stock. To ensure the absence of parental ASFV-G, virus DNA was extracted from the virus stock and analyzed by PCR using primers targeting the MGF505 3R, β-Gus and TK genes. Only amplicons for the MGF- and β-Gus genes were detected in DNA extracted from the virus stock, whereas no amplicons were generated with primers targeting the TK gene (Fig. 2), indicating the lack of contamination of the ASFV-G/VP-ΔTK stock with ASFV-G/VP30.

3.2. Analysis of the ASFV-G/P-ΔTK genome sequence relative to parental ASFV-G/VP30 genome sequence

To evaluate the accuracy of the genetic modification and the integrity of the genome of the recombinant virus, the full genome sequence of ASFV-G/VP-ΔTK was obtained using NGS on the Ion Torrent PGM™ and compared to that of the parental ASFV-G/VP30 (Krug et al., 2015). A full-length genome comparison between parental ASFV-G/VP-ΔTK and ASFV-G/VP30 demonstrated no significant differences with the exception of the 312-bp deletion (between nucleotide positions 64,289 and 64,601) of the central area of the TK gene (K196R), which has been replaced by the p72GUSΔTK cassette. In addition, the location (nucleotide positions 178,631 to 183,475) and length (4,845 bp) of the gap present in the genome of the ASFV-G/VP30 (when compared with its parental virus ASFV-G) was also present in the genome of ASFV-G/VP30 (Krug et al., 2015). Besides the insertion of the cassette, no additional differences were observed between ASFV-G/VP-ΔTK and ASFV-G/VP30 genomes. Therefore, ASFV-G/VP-ΔTK virus did not accumulate any significant mutations during the process of homologous recombination and plaque purification.

3.3. Comparative replication of ASFV-G/VP-ΔTK and ASFV-G/VP30

In vitro growth characteristics of ASFV-G/VP-ΔTK was first evaluated in Vero cell cultures and compared to parental ASFV-G/VP30 in a multistep growth curve. Cell cultures were infected with either virus at a MOI of 0.01 and samples were collected at 2, 24, 48, 72 and 96 h post-infection (hpi). ASFV-G/VP-ΔTK displayed a decreased growth kinetic when compared with that of the parental ASFV-G/VP30 (Fig. 3A). ASFV-G/VP-ΔTK yields were 10–100 times lower than those of ASFV-G/VP30 regarding the time point considered.

In addition, the ability of ASFV-G/VP-ΔTK to replicate in primary swine macrophage cell cultures, the primary cell targeted by ASFV during infection in swine, was compared relative to the parental ASFV-G/VP30. Growth analysis was performed under similar conditions as described when Vero were used (MOI = 0.01, with sampling times at 2, 24, 48, 72 and 96 hpi). Results demonstrated (Fig. 3B) that ASFV-G/VP-ΔTK displayed a decreased growth kinetic when compared with that of the parental ASFV-G/VP30 (Fig. 3B), showing yields 100–1000 times lower than those of ASFV-G/VP30 regarding the time point considered. Therefore, deletion of the TK gene causes a clear disadvantage in virus replication regardless of the cell type considered.

3.4. Assessment of ASFV-G/VP-ΔTK virulence in swine

In order to evaluate the effect of the deletion of the TK gene in ASFV-G/VP-ΔTK virulence, 80–90 pound pigs (n = 5) were IM inoculated with 10^4 $TCID_{50}$ of either ASFV-G/VP-ΔTK or the parental ASFV-G/VP30. As previously described (Krug et al., 2015), animals infected with 10^4 $TCID_{50}$ of ASFV-G/VP30 exhibited increased body temperature (>104 °F) by 6–8 days post-infection followed by the appearance of clinical signs associated with the disease including anorexia, depression, purple skin discoloration, staggering gait and diarrhea. Signs of the disease aggravated progressively over time and animals either died or were euthanized *in extremis* by days 8–9 post-infection (Table 1). Conversely, animals infected with ASFV-G/VP-ΔTK did not present any signs of clinical disease during the entire observation period (21 days). An additional experiment was then performed where animals with the same characteristics ($n = 5$) were IM inoculated with a higher dose (10^6 $TCID_{50}$) of ASFV-G/VP-ΔTK. This group of animals also remained clinically normal throughout the 21-day observation period (Table 1). Therefore, partial deletion of the TK gene completely attenuated the virulent ASFV-G/VP30.

Viremia in experimentally inoculated animals was quantified at different days post-infection in swine macrophage cell cultures. As expected, animals inoculated with 10^4 $TCID_{50}$ of virulent parental ASFV-G/VP30 had very high virus titers in blood with viremia titers reaching values as high as 10^7 to 10^8 $TCID_{50}$/ml by the time of death

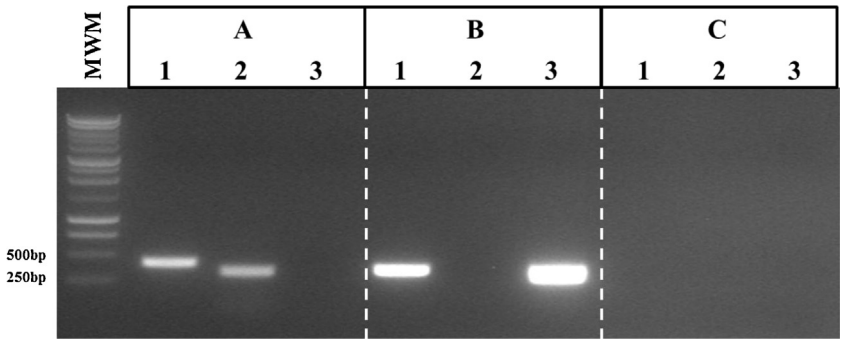

Fig. 2. PCR analysis of ASFV-G/VP-ΔTK virus stock DNA using specific primers targeting MGF505 3R (lane 1), βGus (lane 2), or TK (lane 3) genes. DNA from ASFV-G/VP-ΔTK (A) or parental ASFV-G/VP30 (B) was assessed along with non-DNA template control (C) samples. MWM: molecular weight marker.

Table 1
Swine survival and fever response following infection with ASFV-G/VP-ΔTK or parental ASFV-G/VP30.

Virus	No. of survivors/total	Mean time to death (days ± SD)	Fever		
			No. of days to onset (days ± SD)	Duration No. of days (days ± SD)	Maximum daily temp (°F ± SD)
ASFV-G/VP30 10^4 TCID	0/4	8.75 (0.96)	7.2 (0.84)	2 (0. 71)	106.4 (0.67)
ASFV-G/VP-ΔTK 10^4 TCID	4/4	–	–	–	102.7 (0.19)
ASFV-G/VP-ΔTK 10^6 TCID	4/4	–	–	–	103.7 (0.4)

(–) Animals did not die or they did not present fever.

(Fig. 4). Conversely, animals inoculated with 10^4 or 10^6 $TCID_{50}$ of mutant ASFV-G/VP-ΔTK had almost undetectable virus titers in blood throughout the experimental period. Therefore, although ASFV-G/VP-ΔTK is able to replicate in swine macrophage cell cultures, it appears that it is not efficient in replicating in animals since no viremia titers could be detected.

3.5. Animals inoculated with ASFV-G/VP-ΔTK are not protected against challenge with virulent parental virus

In order to assess the effect of inoculation with ASFV-G/VP-ΔTK on the induction of protection against challenge with the virulent parental virus, those animals surviving ASFV-G/VP-ΔTK infection were IM challenged with 10^3 HAD_{50} of ASFV-G. Both groups, animals inoculated with either 10^4 or 10^6 $TCID_{50}$ of ASFV-G/VP-ΔTK, were challenged at 28 dpi with 10^3 HAD_{50} of virulent parental ASFV-G. Animals were monitored daily for clinical signs and body temperature.

Two naive animals that were challenged using the same route and dose served as the control group. These animals started displaying ASF-related signs by 5 days post-challenge (dpc), evolving to a more severe disease in the following days and all animals dying or being euthanized around 8 dpc. Similarly, all animals receiving either 10^4 or 10^6 $TCID_{50}$ of ASFV-G/VP-ΔTK developed a disease indistinguishable in severity and kinetics to that observed in the mock vaccinated control animals (data not shown). Therefore, infection with ASFV-G/VP-ΔTK does not induce protective immunity against challenge with the virulent parental ASFV-G. In accordance with the lack of protection all animals infected with either 10^4 or 10^6 $TCID_{50}$ of ASFV-G/VP-ΔTK lack any detectable ASFV-specific antibodies at the time of challenge (28 dpi) detected using an in-house immunohistochemistry assay or ELISA (data not shown).

4. Discussion

There are no commercial vaccines to prevent ASF. Experimental protection against challenge with virulent ASFV isolates has been partially achieved by immunizing swine with experimental subunit vaccines, as expressed proteins or DNA vaccines (Lacasta et al., 2014; Argilaguet et al., 2012,2013; Barderas et al., 2001), and in a more effective way by pre-infecting pigs with homologous live attenuated virus strains. Attenuated ASFVs have been developed by either adaptation to grow in different cell substrates (Enjuanes et al., 1976; Pires et al., 1997; Ruiz Gonzalvo et al., 1986; Tabares et al., 1987; Krug et al., 2015) or by genetic manipulation, deleting specific genes by homologous recombination (Zsak et al., 1996; Zsak et al., 1998; Moore et al., 1998; Lewis et al., 2000; Neilan et al., 2004; O'Donnell et al., 2015a,2015b). These recombinant deletion mutant viruses have significantly reduced virulence in swine and animals inoculated with each of these genetically modified viruses survived the infection when challenged with the virulent parental virus (Lewis et al., 2000; Moore et al., 1998; Neilan et al., 2004; Zsak et al., 1996,1998; O'Donnell et al., 2015a,2015b). Therefore, development of attenuated ASFV recombinant viruses by genetic manipulation of specific genes appears to be an effective approach for vaccine development.

Among these viral genes is the one encoding for the enzyme thymidine kinase (TK), involved in synthesis of deoxynucleoside triphosphates (Martin Hernandez and Tabares, 1991). The ASFV TK gene has been shown to not be essential for virus growth 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).

As described, ASFV-G/VP-ΔTK possesses a reduced ability to replicate when compared with its parental virus. This disadvantage was evidenced when evaluated either in Vero cells or primary cultures of swine macrophages. Similarly, recombinant ASFV-ΔTK produced in strains Malawi and Haiti (which were also partially adapted to replicate in Vero cells) also exhibited yields 100 to 1000 time lower than the parental viruses in swine primary macrophages (Moore et al., 1998).

In swine, these ΔTK strains, as it is reported here for ASFV-G/VP-ΔTK, presented an attenuated phenotype producing a very mild form of the disease (Moore et al., 1998). In fact, based on the reported data, ASFV-G/VP-ΔTK appears to present a more attenu-

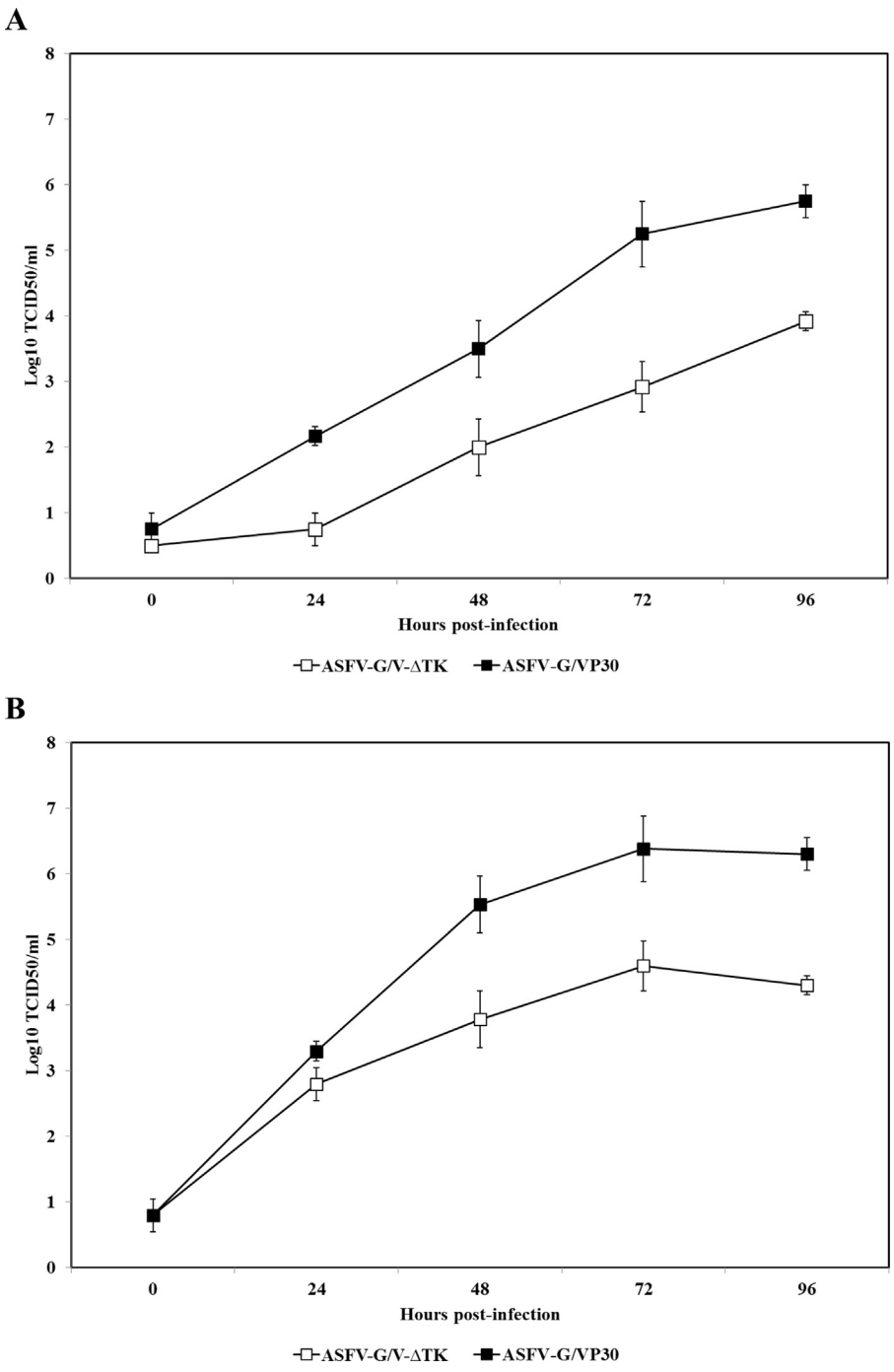

Fig. 3. *In vitro* growth kinetics of ASFV-G/VP-ΔTK or parental ASFV-G/VP30. Vero cell line (A) or primary swine macrophage cell (B) cultures were infected (MOI = 0.01) with either ASFV-G/VP-ΔTK or ASFV-G/VP30 viruses. Virus yields obtained at the indicated times post-infection were titrated in Vero cell cultures. Data represent means and standard deviations from two independent experiments. Sensitivity of virus detection: ≥1.8 $TCID_{50}$/ml.

ated phenotype than Malawi or Haiti ΔTK strains since no presence of clinical signs were detected in ASFV-G/VP-ΔTK-infected animals even when they were inoculated with a dose of 10^6 $TCID_{50}$, while some of the animals infected with the Malawi-ΔTK virus did not survive the infection or presented a transient fever (Moore et al., 1998). Accordingly, levels of viremia in animals infected with ASFV-G/VP-ΔTK were almost undetectable while levels above 10^4 $TCID_{50}$ were achieved in animals infected with Malawi-ΔTK virus (Moore et al., 1998). Importantly, while animals surviving the Malawi-ΔTK infection were protected against challenge with parental virulent virus (Moore et al., 1998), none of those infected with ASFV-G/VP-ΔTK survived challenge with ASFV-G.

Possible differences between the pathogenesis and protective effect of infection with ASFV-G/VP-ΔTK and Malawi-ΔTK viruses may be due to the different genetic background of the strains used as parental virus in each of the cases. Comparing the full length genomic sequences of the Vero-adapted parental viruses and corresponding non-Vero-adapted field isolates showed interesting differences. While no notable differences were found between the field isolate Malawi LiL-20/1 and its Vero-adapted version, Malawi LiL-20/1 V (data not shown), significant differences

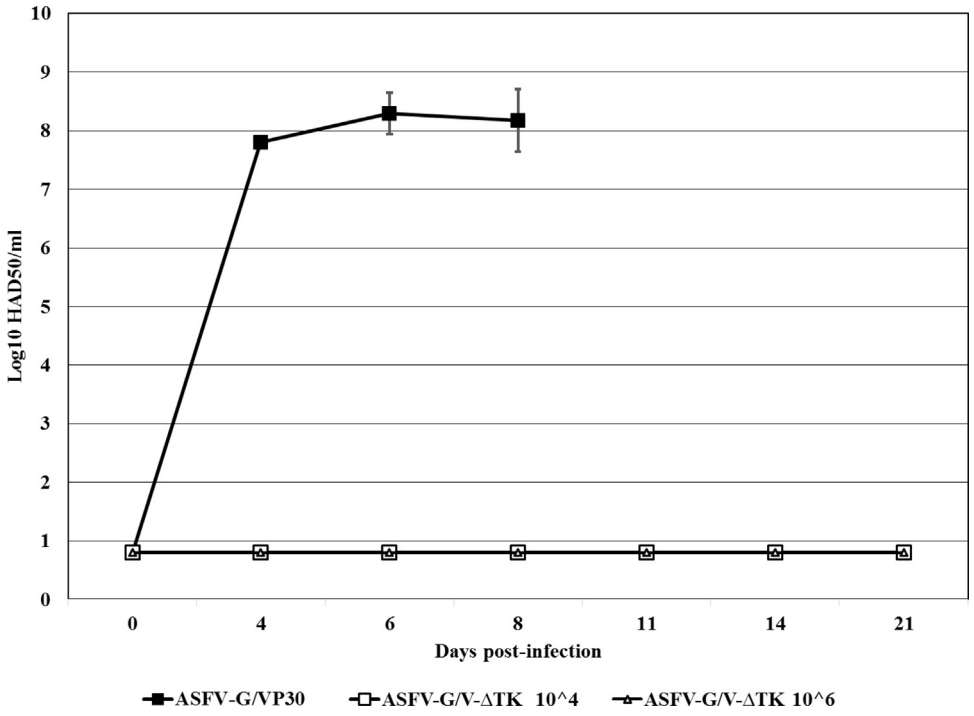

Fig. 4. Virus titers in clinical blood samples from pigs infected with either 10^4 (A) or 10^6 (B) $TCID_{50}$ of ASFV-G/VP-ΔTK compared with those from pigs infected with parental ASFV-G/VP30. Values are expressed as $\log_{10}$ HAD_{50}/ml. Sensitivity of virus detection: $\geq\log_{10}$ 0.8HAD_{50}/ml.

were observed between ASFV-G and its Vero adapted derivative ASFV-G/VP30 (Krug et al., 2015). These observations may indicate that Malawi LiL-20/1 V is less divergent from its parental virus than ASFV-G/VP30 is from ASFV-G, supporting the concept that Malawi LiL-20/1 V is less attenuated than ASFV-G/VP30. Therefore, it could be expected that their corresponding ΔTK derivatives will behave similarly to the corresponding parental viruses, with ASFV-G/VP-ΔTK being completely attenuated but also replicating less aggressively *in vivo*, failing to mount an effective immune response in the infected animal as the Malawi-ΔTK does (Moore et al., 1998).

The study of the role of ASFV genes using single gene deletion virus mutants has demonstrated certain heterogeneity in regards to the function of a similar gene in the context of different virus isolates. Thus, deletion of the NL gene has been shown to attenuate virulent isolate E75 (Zsak et al., 1996) but does not alter virulence of isolate Malawi (Afonso et al., 1998). In addition, deletion of 9GL is very effective in attenuating virulence of Malawi isolate, even at doses of 10^6 HAD_{50} (Lewis et al., 2000), while does not abolish virulence in isolate Gerogia2007 when administered at just 10^4 HA_{50} (O'Donnell et al., 2015b). In fact, most of the studies involving deletion of a particular gene have been carried out using only one virus isolate opening the question if a gene would play exactly the same role in different virus isolates. This is very relevant issue considering that different ASFV isolates may present a wide phenotypic variability particularly in terms of their virulence in swine. This issue is also important since it may affect the development of live attenuated vaccines, particularly when you consider the existing limitation in the inability to induce effective cross-protection between different ASFV isolates. This causes the need to develop a homologous attenuated strain to each of the virulent field isolates.

In summary, we report here the first evidence that deletion of the thymidine kinase gene is able to completely attenuate a virulent ASFV isolate although animals infected with the attenuated strain do not develop a protective immune response when challenged with the virulent parental ASFV.

Acknowledgements

We thank the Plum Island Animal Disease Center Animal Care Unit staff for excellent technical assistance. We wish to particularly thank Mrs. Melanie V. Prarat for editing the manuscript. This project was funded through an interagency agreement with the Science and Technology Directorate of the U.S. Department of Homeland Security under Award Numbers HSHQDC-11-X-00077 and HSHQPM-12-X-00005. We would like to thank ARS/USDA-University of Connecticut SCA# 58-1940-1-190 for partially supporting this work.

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