Deletion of E184L, a putative DIVA target from the pandemic strain of African swine fever virus, produces a reduction in virulence and protection against virulent challenge

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Abstract

African swine fever (ASF) is currently causing a major pandemic affecting the swine industry and protein availability from Central Europe to East and South Asia. No commercial vaccines are available, making disease control dependent on the elimination of affected animals. Here, we show that the deletion of the ASFV E184L gene from the highly virulent ASFV-Georgia2010 (ASFV-G) isolate produces a reduction in virus virulence during the infection in swine. Forty percent (40%) of domestic pigs intramuscularly inoculated with a recombinant virus lacking the E184L gene (ASFV-G-ΔE184L) experienced a significantly (5 days) delayed presentation of clinical disease and, overall, had a 60% rate of survival when compared to animals inoculated with the virulent parental ASFV-G. Importantly, all animals surviving ASFV-G-ΔE184L infection developed a strong antibody response and were protected when challenged with ASFV-G. As expected, a pool of sera from ASFV-G-ΔE184L-inoculated animals lacked any detectable antibody response to peptides partially representing the E184L protein, while sera from animals inoculated with an efficacious vaccine candidate, ASFV-G-ΔMGF, strongly recognize the same set of peptides. These results support the potential use of the E184L deletion for the development of vaccines able to differentiate infected from vaccinated animals (DIVA). Therefore, it is shown here that the E184L gene is a novel ASFV determinant of virulence that can potentially be used to increase safety in pre-existing vaccine candidates as well as to provide them with DIVA capabilities. To our knowledge, E184L is the first ASFV gene product experimentally shown to be a functional DIVA antigenic marker.

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Importance:

No commercial vaccines are available to prevent African swine fever. The ASF pandemic caused by the Georgia (ASFV-G) strain is seriously affecting pork production in a contiguous geographical area

from Central Europe to East Asia. The only effective experimental vaccines are viruses attenuated by deleting ASFV genes associated with virus virulence. Therefore, identification of such genes is of critical importance for vaccine development. Here we report the discovery of a novel determinant of ASFV virulence, the E184L gene. Deletion of the E184L gene from the ASFV-G genome (ASFV-G-ΔE184L) produced a reduction in virus virulence and, importantly, animals surviving infection with ASFV-G-ΔE184L were protected from developing ASF after challenge with the virulent parental virus ASFV-G. Importantly, the virus protein encoded by E184L is highly immunogenic, making a virus lacking this gene a DIVA vaccine candidate that allows the differentiation of infected from vaccinated animals. Here we show that unlike what is observed in animals inoculated with the vaccine candidate ASFV-G-ΔMGF, ASFV-G-ΔE184L-inoculated animals do not mount a E184L-specific antibody response, indicating the feasibility of using the E184L deletion as the antigenic marker for the development of a DIVA vaccine in ASFV.

Introduction

African swine fever virus (ASFV) is a large and structurally complex virus, which is currently causing a disease pandemic affecting swine production in several countries from Eastern to Central Europe and Southeast Asia. As a result, the disease has caused devastating economic losses in swine production as well as a shortage in worldwide protein availability. The ASFV strain causing this pandemic is a highly virulent isolate identified during the initial 2007 outbreak in the Republic of Georgia, ASFV Georgia 2007/1 (ASFV-G). This initial 2007 outbreak outside of Africa was the initial event that caused the current pandemic, with all isolated strains having sequence similarity to this initial strain.

ASFV is an enveloped virus with a double-stranded DNA genome of approximately 180-190 kilobase pairs encoding for approximately 150-160 ORFs (1). The functions of most ASFV proteins encoded within these ORFs are unknown or have only been predicted using functional genomics (1, 2), and very few have had an experimental function described.

Currently, there is no vaccine to prevent ASF, consequently, the control of the disease relies on the quarantine and elimination of affected animals. Several experimental live attenuated vaccines have been shown to induce protection against infection with historical virulent virus strains (3, 4) and against the current pandemic strain (5-10). Generally, animals inoculated with attenuated viruses containing genetically engineered deletions of virus genes involved in the process of virulence are protected against infection with the homologous virulent parental virus (3-10). Therefore, the identification and genetic manipulation of virus genes associated with virulence is necessary for the rational design of genetically modified virus strains to be used as live attenuated ASFV vaccine candidates.

Here we report the identification of a novel determinant of ASFV virulence, the E184L gene. An ASFV-G recombinant virus without the E184L gene, ASFV-G-ΔE184L, has a reduced virulence when

inoculated in swine and animals surviving the infection are protected against challenge with the virulent parental virus. We also demonstrate that E184L is a highly immunogenic protein, as evidenced during inoculation with the vaccine candidate ASFV-G-ΔMGF; this immune response is completely absent in ASFV-G-ΔE184L-infected animals. Therefore, deletion of the E184L gene can act as an antigenic marker to develop DIVA vaccines that allow the differentiation of infected and vaccinated animals.

Materials and Methods

Cell culture and viruses

Culture of primary swine macrophages was performed as described elsewhere (11). Briefly, blood mononuclear leukocytes were separated over a Ficoll-Paque density gradient (Pharmacia, Piscataway, N.J.). Monocyte/macrophage cells were cultured in plastic Primaria tissue culture flasks (Falcon; Becton Dickinson Labware, Franklin Lakes, N.J.) in macrophage media: RPMI 1640 Medium (Life Technologies, Grand Island, NY) with 30% L929 supernatant and 20% fetal bovine serum (HI-FBS, Thermo Scientific, Waltham, MA) at 37°C under 5% CO_2. After 48 hours of incubation, adherent cells were detached from the tissue culture with a solution containing 10 mM EDTA in phosphate buffered saline (PBS) and detached cells were then reseeded into Primaria T25, 6- or 96-well dishes at a density of $5x10^6$ cells per ml for use in assays 24 hours later.

Comparative growth curves to study growth kinetics between parental ASFV-G and recombinant viruses were performed in primary swine macrophage cell cultures. Macrophage monolayers were infected (MOI = 0.01) for 1 hour and then the inoculum was removed, the cells rinsed twice with PBS, once with macrophage media and incubated at 37°C under 5% CO_2. At 2, 24, 48, 72 and 96 hours post-infection (hpi) cell cultures were frozen at <−70°C and the thawed lysates were clarified by centrifugation to eliminate cell debris and used to determine virus titers in primary swine macrophage cell cultures. All samples were run simultaneously to avoid inter-assay variability. Presence of infectious virus was detected by hemadsorption (HA) and virus titers calculated using the Reed and Muench method (12).

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.

Detection of E184L transcription

Real-time PCR analysis was used to assess the expression profile of the gene E184L during the infection of ASFV-G in cultures of porcine macrophages. For this purpose, six well plates containing cell cultures of porcine macrophages ($1x10^7$ cells per well) were infected in triplicate with a stock of ASFV-G using a MOI of 1. Plates were incubated at 37°C and RNA extractions were conducted at 4, 6, 8 and 24 hours post-infection.

RNA extraction was carried out using the RNeasy Kit (QIAGEN) following the manufacturer's instructions. Afterwards, RNA was treated with 2 units of DNase I (BioLabs) following the manufacturer's protocol. Final reactions were purified using the Monarch® RNA Cleanup Kit (New England BioLabs, Inc.). RNA was quantified and 1µg was used to produce cDNA using qScript cDNA SuperMix (Quanta bio) following the manufacturer instructions.

Using the sequence of the ASFV Georgia 2007/1 strain (GenBank data base LR743116) as a reference, primers and probes were designed using the RealTime qPCR Assay Entry tool from Integrated DNA Technologies (IDT) (https://www.idtdna.com/scitools/Applications/RealTimePCR/). For E184L primers, forward: 5'-AAAATCACACCCGAAAACCAAG-3', reverse: 5'-GTGAGAATACATAAG GGTTTGCG-3', and probe: 5'-FAM/AAAACACCTTGCAAAGCCGACTCATC/MGBNFQ-3'. The CP204L (p30) gene was used as a control for the quantification of an early expression gene of ASFV: forward: 5'-GACGGAATCCTCAGCATCTTC-3', reverse: 5'- CAGCTTGGAGTCTTTAGGTACC-3', and probe 5'-FAM/TGTTTGAGCAAGAGCCCTCATCGG/MGBNFQ3'. Additionally, as a control for a late expression gene of ASFV, we use the gene B646L (p72) using a qRT-PCR previously published (13). Also, the housekeeping gene β-actin was used as an endogenous control to validate the quality of the extraction and the RNA concentration from different infections performed in this experiment.

All qRT-PCR assays were conducted on a 7500 Real-time PCR system (Applied Biosystems), using the TaqMan Universal PCR Master Mix (Applied Biosystems catalog # 4304437) following this protocol for master mix preparation (1x): Universal mix 12.5 µl, water 7.05 µl, forward primer (50µM) 0.1 ul, reverse primer (50µM) 0.1 ul, probe (10µM), and DNA 5µl. Conditions of amplification were as follows: One step at 55°C for 2 min, followed by one denaturation step at 95°C for 10 min, then 40 cycles of denaturation at 95°C for 15 s and annealing/extension at 65°C for 1 min.

Construction of the recombinant ASFV-G-ΔE184L

Recombinant viruses were generated by homologous recombination between the corresponding parental genome (either ASFV-G or ASFV-G-ΔMGF) and recombination transfer vector p30mCherryΔE184L by infection and transfection procedures using swine macrophage cell cultures as previously described in detail (14) . Development of the recombinant vaccine candidate ASFV-G-ΔMGF was previously described (7). The recombinant transfer vector p30mCherryΔE184L contains flanking genomic regions to the amino acid residues 1 and 157 of the E184L gene, mapping approximately 1kbp to the left and right of these amino acids, along with the reporter gene cassette containing the mCherry gene with the ASFV p30 early gene promoter, p30mCherry. This construction created a 471bp deletion in the E184L ORF (Fig. 1). The coding region of the C-terminus of E184 was left intact but believed not to be expressed due to the lack of a promoter or start codon, as this portion of E184L overlaps with another ASFV protein C-terminus QP383R, leaving this section allows for QP383R to be properly expressed. The recombinant transfer vector p30mCherryΔE184L was obtained by DNA synthesis (Epoch Life Sciences Missouri City, TX, USA).

Next Generation Sequencing (NGS) of ASFV genomes

ASFV DNA was extracted from infected cells and quantified as described earlier. Full-length sequence of the virus genome was performed as described previously (15) using an Illumina NextSeq500 sequencer.

Animal experiments

Animal experiments were performed under biosafety level 3AG conditions in the Plum Island Animal Disease Center (PIADC) animal facility following protocols approved by the PIADC Institutional Animal Care and Use Committee of the US Departments of Agriculture and Homeland Security (protocol number 225.04-16-R, 09-07-16).

ASFV-G-ΔE184L virulence was evaluated by comparing it to parental ASFV-G using 80-90 pound commercial breed swine. Groups of pigs (n=5) were intramuscularly (IM) inoculated with 10^2 HAD_{50} of either ASFV-G-ΔE184L or ASFV-G. Presence of clinical signs (anorexia, depression, fever, purple skin discoloration, staggering gait, diarrhea and cough) and changes in rectal temperature were recorded daily throughout the experiment. In protection experiments, animals inoculated with ASFV-G-ΔE184L, ASFV-G-ΔMGF or ASFV-G-ΔMGF/ΔE184L were IM challenged 28 days later with 10^2 HAD_{50} of parental ASFV-G. Presence of clinical signs associated with the disease was recorded as described earlier (7).

Detection of anti-ASFV antibodies

ASFV antibody detection used an in-house ELISA performed as described previously (16). Briefly, ELISA antigen was prepared from ASFV-infected Vero cells. Maxisorb ELISA plates (Nunc, St Louis, MO, USA) were coated with 1 µg per well of infected or uninfected cell extract. The plates were blocked with phosphate-buffered saline containing 10% skim milk (Merck, Kenilworth, NJ, USA) and 5% normal goat serum (Sigma, Saint Louis, MO). Each swine serum was tested at multiple dilutions against both infected and uninfected cell antigen. ASFV-specific antibodies in the swine sera were

detected using an anti-swine IgG-horseradish peroxidase conjugate (KPL, Gaithersburg, MD, USA) and SureBlue Reserve peroxidase substrate (KPL). Plates were read at OD630 nm in an ELx808 plate reader (BioTek, Shoreline, WA, USA). Sera titers were expressed as the log10 of the highest dilution where the OD630 reading of the tested sera at least duplicates the reading of the mock infected sera.

Specific peptide slides representing partial sequences of ASFV proteins p72 (residues 34-53), p54 (residues 138-160) and the carboxy end of E184L (residues 163-177, 164-178, 165-179, 166-180, 167-181, 168-182, 163-177, 169-183, 170-184) were manufactured by PEPperPRINT (Heidelberg, Germany). Microarray analysis was conducted based on PEPperCHIP Immunoassay Protocol provided by the array manufacturer. In brief, the peptide microarray slides were incubated with 1.5 ml of standard buffer (PBS, 0.05% Tween20, pH 7.4) (Sigma-Aldrich) at room temperature for 15 minutes and blocking buffer (TRIS buffered saline at pH 7.6 with 1% BSA) for an additional 30 minutes. After removal of blocking buffer, the arrays were incubated with staining buffer (standard buffer with 10% of the blocking buffer) containing Cy5-labelled mouse anti-HA (positive control, PEPperPRINT) and Cy3-labelled goat anti-swine IgG (Jackson ImmunoResearch, West Grove, PA) for 45 minutes. The microarrays were washed three times with standard buffer and scanned with a GenePix 4000B scanner (Molecular Devices, Downington, PA). After scanning, the microarrays were incubated again with staining buffer for 15 minutes followed by incubation with staining buffer containing the serum sample at 1:1000 dilution overnight at 4^{o}C, followed with incubation of the staining buffer containing Cy3-labelled goat anti-swine IgG for 45 minutes. After washing three times, the microarrays were scanned again at the same setting as the first scan. Fold changes were calculated by dividing the signal intensity of positives with the negatives.

Results

Conservation of E184L gene across different ASFV isolates

ASFV E184L gene encodes for a 184 amino acid protein and is positioned on the negative strand between nucleotide positions 163174 and 162620 of the ASFV-G genome (Fig. 1). The translated product of the ASFV E184L gene is a 22KD protein of unknown function (17) expressed during the virus replication cycle in pigs (18), inducing a strong antibody response (19).

To assess the nucleotide and amino acid homology among different isolates of ASFV representing the genetic diversity of gene E184L, multiple pairwise comparisons were performed using the algorithm clustalW. In general, the average homology at nucleotide and amino acid levels were calculated to be 95.65% and 92.67%, respectively. However, we found that there is a disparate range of homology at nucleotide (90.42 - 99.80%) and amino acid (83.60 - 99.45%) levels, indicating varying conservation of the E184L protein among some ASFV isolates (Fig. 2). In this context, examples of low levels of amino acid homology (83.60%) can be appreciated between the isolate RSA 2 2008 and isolates Kenya 1950, Malawi, Tengani 62, Ken.rie 1, R35, Liv 13/33, Uvira B53, Ken05/Tk1, and Ken 06. Bus. On the other hand, an example of high level of conservation (99.45%) was found between the isolate Georgia 2007 and isolates Mkuzi 1979, Warthog, Tengani 62, Malawi, Benin 97/1 and the low virulent isolate OURT 88/3. Interestingly, no differences were found at nucleotide and amino acid levels within the Eurasian lineage isolates, indicating conservation of the E184L gene within this lineage. Also, it strongly suggests stasis of the E184L gene during the evolution of this lineage.

E184L is a late transcribed gene

To determine whether the E184L gene is transcribed during the infectious cycle, a time course experiment was performed to analyze the kinetics of RNA transcription in primary swine macrophages infected with ASFV strain Georgia. Swine macrophage cultures were infected with an MOI = 1 with

ASFV-G and cell lysate samples were taken at 4, 6, 8, and 24 hpi. The presence of E184L RNA was detected by RT-PCR as described in the Material and Methods section. Transcription of E184L was detected starting at 6 hpi and remained stable until 24 hpi (Fig. 3). The pattern of expression of the well-characterized ASFV early protein p30 (CP204L) and the late protein p72 (B646L) has been previously described and is used here as a representation of early and late transcription profiles. Expression of E184L practically overlaps with that of the p72 gene. Therefore, the ASFV E184L gene encodes for a protein that is expressed late in the virus replication cycle.

Development of the E184L gene deletion mutant in the ASFV-Georgia isolate

Although it is known that the E184L gene is expressed during infection in pigs and induces a strong antibody response, the biological function of the gene remains completely unknown. To investigate the function of the E184L gene during virus infection in cell cultures and its impact on disease phenotype, a recombinant virus harboring a deletion of the E184L gene was developed (ASFV-G-ΔE184L) from the parental highly virulent ASFV Georgia 2010 (ASFV-G). The E184L gene was replaced by a cassette containing the fluorescent reporter gene, mCherry, under the ASFV p30 promoter (Fig. 1). The recombinant ASFV-G-ΔE184L was purified by limiting dilution based on the presence of fluorescent activity.

To evaluate the accuracy of the genetic modification introduced in ASFV-G-ΔE184L as well as the integrity of the virus genome, the full genomic sequence of the recombinant virus was obtained using Next Generation Sequencing (NGS). Comparison of ASFV-G-ΔE184L and ASFV-G genomic sequences showed a deletion of 471 nucleotides (covering nucleotide positions 162704 and 163174) corresponding with the deletion of the E184L gene (Fig. 1). Additionally, the ASFV-G-ΔE184L genome possesses an insertion of 3,944 nucleotides, consistent with the introduction of the p30mCherry cassette substituting the E184L gene. No unwanted additional genomic modifications were found in the rest of

the ASFV-G-ΔE184L genome. No E184L gene sequences were detected by NGS indicating the purity of the recombinant virus stock.

Replication of ASFV-G-ΔE184L in primary swine macrophages

The impact of the removal of the E184L gene from the genome of ASFV-G was assessed by a growth kinetics study using primary swine macrophage cultures, the main cell type targeted by ASFV during infection in swine. The kinetics of replication of ASFV-G-ΔE184L were compared with that of the parental ASFV-G in multistep growth curves (Fig. 4). Primary cultures of swine macrophages were infected (MOI of 0.01) and samples were collected at 2, 24, 48, 72 and 96-hours post-infection (hpi). The analysis of the results indicate that ASFV-G-ΔE184L exhibited a replication kinetic significantly diminished compared to that of the parental ASFV-G. ASFV-G-ΔE184L titers are between 10- and 100-fold lower than those of ASFV-G, depending on the time point considered. Therefore, deletion of the E184L gene moderately diminished the virus' capability to replicate in swine macrophage cultures.

Assessment of ASFV-G-ΔE184L virulence in swine

Evaluating the impact of the removal of the A137R gene from the ASFV-G genome on virus virulence in swine was assessed by experimentally infecting domestic pigs with ASFV-G-ΔE184L, for comparison with animals infected with parental virulent ASFV-G. Groups of five 80-90 pound pigs were IM inoculated with 10^2 HAD_{50} of either ASFV-G-ΔE184L or ASFV-G and observed for 28 days. All five animals infected with ASFV-G had increased body temperature (>104 $^{\circ}$F) by day 4-5 post-infection followed by development and rapid progression of clinical ASF signs, with all animals euthanized *in extremis* by 7 days post-infection (pi) due to the severity of the disease (Table 1 and Figs. 5 and 6). The five animals infected with ASFV-G-ΔE184L presented with a heterogenous response. All animals had increased body temperature (>104 $^{\circ}$F) by day 10-11 pi. Two of those animals started showing clinical signs of the disease (anorexia, depression, skin lesions and, later, incoordination) which

evolved during 2-3 days to a more severe form of the disease with the animals being euthanized by day 13-14 pi. The remaining three animals in the group did not show any clinical sign of the disease besides the initial rise in body temperature remaining clinically normal until day 28 pi (Fig. 5 and 6). Therefore, deletion of the E184L gene resulted in an attenuation of the ASFV-G strain, with 40% of infected animals experiencing a significantly delayed disease onset before developing lethal ASF disease and 60% of animals exhibiting a late and transient rise of body temperature with no additional clinical signs.

The level of virus replication, as represented by the viremia values, was analyzed in both groups of animals. As expected, the animals infected with ASFV-G had high titers ($10^{5.5}$ - 10^{8} HAD_{50}/ml) by day 4 pi, which rapidly increased (around $10^{8.5}$ HAD_{50}/ml) by day 7 pi, when all animals were euthanized (Fig. 7). Conversely, ASFV-G-ΔE184L-infected animals had viremia kinetics that paralleled the development of clinical signs. Animals had viremia titers ranging between $10^{2.5}$ - $10^{6.5}$ HAD_{50}/ml by day 4 pi, increasing to titer values of $10^{5.5}$ - $10^{7.5}$ HAD_{50}/ml by day 7 pi. Viremia titers progressively decreased until day 28 pi reaching titer values between $10^{4.5}$ - 10^{7} HAD_{50}/ml. The two euthanized animals had final titers 10^{7} HAD_{50}/ml. Therefore, disappearance of ASFV virulence caused by deletion of the E184L gene is accompanied by a reduced but stable virus replication presenting long viremias with relatively moderate disease.

Protective efficacy of ASFV-G-ΔE184L against challenge with ASFV-G

Infection with attenuated strains of ASFV consistently protect animals against the disease caused by the virulent parental virus. The ability of ASFV-G-ΔE184L to induce protection against disease caused by parental ASFV-G was assessed in the animals surviving the ASFV-G-ΔE184L infection. The three animals were challenged IM 28 days after ASFV-G-ΔE184L infection with 10^{2} HAD_{50} of ASFV-G. An additional group of five naïve animals were challenged under the same conditions as a control group.

Animals in the control group started displaying ASF-related clinical signs 4-5 days post-challenge (dpc), with quick progression to severe disease; all animals were euthanized by day 7 dpc (Table 2, Figs. 5 and 6). Conversely, all three animals infected with ASFV-G-ΔE184L did not display any clinical signs of ASF during the 21-day observational period. Therefore, ASFV-G-ΔE184L infection induced protection against development of ASF when challenged with the highly virulent parental strain ASFV-G.

After challenge, virus titers in the control animals were high (ranging between $10^{6.5}$ - 10^{7} HAD_{50}/ml) by day 4 pi, increasing (ranging $10^{7.5}$ - 10^{8} HAD_{50}/ml) by day 7 pi, when all animals were euthanized (Fig. 7). After challenge with ASFV-G, none of the three ASFV-G-ΔE184L-infected animals developed viremia values higher than titers present at the time of challenge. The titers in these animals progressively decreased until the end of the observational period (21 days after challenge) when no detectable titers were found in the animals, while the remaining one animal had a viremia titer of $10^{4.5}$ HAD_{50}/ml at the end of the experiment.

Antibody response in animals infected with ASFV-G-ΔE184L

Although the immune mechanisms producing protection in animals infected with attenuated strains of virus is still under controversy, our experience working with several vaccine candidates is that the only parameter consistently associated with protection is the presence of ASFV-specific circulating antibodies (6, 7, 9, 10, 16, 20). Therefore, we tried to associate the presence of anti-ASFV circulating antibodies in ASFV-G-ΔE184L-infected animals with protection against challenge. A robust virus-specific antibody response, detected using an in-house developed direct ELISA, was detected in the sera of all three animals (Fig. 8). Antibody response, mediated by IgG isotype, was detected in all the animals by day 11 pi remaining high until the day of the challenge. Therefore, in agreement with

315 previous reports a close association exists between presence of anti-ASFV antibodies at the moment of
316 challenge and protection of the animals.

317 The E184L protein has been shown to elicit a strong immune response when used as an
318 individual protein (19) and, more importantly, during viral infection (21). Therefore, the E184L gene
319 product is potentially a target for the development of a DIVA test to discriminate between animals
320 immunized with a recombinant vaccine lacking the E184L gene from those infected with wild-type
321 ASFV. In this study, we assessed the potential of E184L as a DIVA marker. The response of a pool of
322 sera from animals immunized with a vaccine candidate, ASFV-G-ΔMGF (7), to a set of 15mer partially
323 overlapping peptides representing the carboxyl end of the translated sequence of the E184L gene, was
324 evaluated in a peptide array assay and compared to that raised in the ASFV-G-ΔE184L-infected animals.
325 Synthetic peptides representing partial amino acid sequences of highly immunogenic ASFV proteins p72
326 and p54 were included as positive controls. Results demonstrated that pooled sera from both ASFV-G-
327 ΔMGF and ASFV-G-ΔE184L-infected animals strongly recognized peptides representing amino acid
328 sequences of the control antigens p72 and p54 (Fig. 9). In addition, pooled sera from ASFV-G-ΔMGF-
329 infected animals also strongly recognizes the E184L peptides. Conversely, and as expected, the pooled
330 sera from ASFV-G-ΔE184L-infected animals failed to recognize any of the peptides representing the
331 E184L amino acid sequence. These results together with the positive response found after vaccination
332 with ASFV-G-ΔMGF (Figure 9), affirm the potential use of the E184L gene as the molecular basis of a
333 DIVA marker in the ASFV genome.

334 **Development of an ASFV vaccine candidate harboring E184L deletion as potential DIVA marker**

335 Deletion of E184L does not lead to a complete attenuation of ASFV-G; therefore, to be used as a
336 potential DIVA marker, removal of the gene should be performed in a ASFV vaccine candidate with a
337 minimal safety profile. To test the DIVA functionality of E184L we deleted the gene from the vaccine

candidate ASFV-G-ΔMGF (7).The ΔMGF deletion was constructed using a p72Gus reporter gene cassette as described previously (7), allowing for the same strategy and methodology to develop the resulting recombinant virus, ASFV-G-ΔMGF/ΔE184Lthat was already described for ASFV-G-ΔE184L. The E184L gene was replaced by the p30mCherry cassette provoking the same modifications in the ASFV-G-ΔMGF/ΔE184L genome as those described in ASFV-G-ΔE184L (Figure 1).

The effect of the E184L gene deletion on the replication ability of ASFV-G-ΔMGF was assessed by a growth kinetics study in primary swine macrophages of the ASFV-G-ΔMGF/ΔE184L compared with that of ASFV-G, and the parentals ASFV-G-ΔMGF and ASFV-G-ΔE184L in multistep growth curves (Fig. 4). Interestingly, ASFV-G-ΔMGF/ΔE184L exhibited a replication kinetic almost indistinguishable from that of ASFV-G-ΔE184L and approximately 10-fold lower kinetics than those of ASFV-G and ASFV-G-ΔMGF. Therefore, deletion of the E184L gene produced a moderate decrease in the ability of the resulting virus (ASFV-G-ΔE184L and ASFV-G-ΔMGF/ΔE184L) to replicate when compared with the corresponding parental virus.

The potential use of ASFV-G-ΔMGF/ΔE184L as a DIVA marker experimental vaccine was tested in domestic pigs in comparison with the efficacy of the parental ASFV-G-ΔMGF vaccine candidate. Two groups of five 80-90 pounds pigs were IM inoculated with either 10^4 HAD_{50} or 10^6 HAD_{50} of ASFV-G-ΔMGF/ΔE184L while a third group was inoculated with 10^4 HAD_{50} of ASFV-G-ΔMGF. All animals were observed for 28 days with no development of clinical disease.

Analysis of the viremia titers in all groups showed that, as already reported (12), animals infected with ASFV-G-ΔMGF displayed a heterogenous pattern with three animals presenting low to medium ($10^{2.5}$ - $10^{5.5}$ HAD_{50}) titers between day 4 to 28 pi and the other two not producing detectable titers at all during the pre-challenge period (Fig. 10A). Interestingly, in both groups of animals

inoculated with ASFV-G-ΔMGF/ΔE184L no viremia titers were detected in any of the animals at any time point tested.

According to the viremia kinetics, antibody titers developed in ASFV-G-ΔMGF-infected animals starting at day 11 pi, increasing through days 14 to 21 pi, reaching highest titers by the day of challenge (Fig. 10B). Conversely, no detectable antibody titers were found at any time point in animals inoculated with ASFV-G-ΔMGF/ΔE184L with the exception of very late low titers in two animals receiving 10^6 HAD_{50}.

All three groups were challenged at 28 days pi with virulent ASFV-G along with 5 naïve pigs used as the control group. Control animals developed the expected clinical disease with a rise in body temperature by day 4 dpc with a rapid progression in severity until all animals were euthanized by day 7 pc (Fig. 10C). After the challenge, as described earlier (7), animals infected with ASFV-G-ΔMGF remained clinically normal throughout the observational period. On the other hand, all animals inoculated with ASFV-G-ΔMGF/ΔE184L developed a clinical disease undistinguishable from that experienced by the control animals, being euthanized by day 7 pc. Again, viremia profiles followed presentation of clinical signs (Fig. 10A). The control group developed high titers ($10^{7.05}$ - $10^{7.8}$ HAD_{50}) by day 4 pc reaching $10^{7.05}$ - $10^{8.55}$ HAD_{50} by time they were euthanized. After challenge, all animals infected with 10^4 HAD_{50} of ASFV-G-ΔMGF except one (which remined aviremic) developed viremia titers ranging from $10^{2.55}$ to $10^{6.55}$ HAD_{50} at different times pc. Animals receiving 10^4 HAD_{50} of ASFV-G-ΔMGF/ΔE184L ranged from $10^{4.55}$ to $10^{7.55}$ HAD_{50} by day 4 pc reaching high titers ($10^{7.3}$ to $10^{8.5}$ HAD_{50}). Those animals inoculated with 10^6 HAD_{50} of ASFV-G-ΔMGF/ΔE184L displayed a range of titers (from undetected to $10^{5.05}$ HAD_{50}) by day 4 pc, reaching high titers ($10^{5.8}$ - $10^{8.5}$ HAD_{50}) in all euthanized animals at day 7 pc.

Discussion

Experimental vaccines based on the use of live attenuated strains is a practical approach toward the development of an effective ASF vaccine. Attenuated virus strains can be developed using different approaches, from the use of natural attenuated field isolates (22), to attenuation by adapting virulent field isolates to growth in cell cultures (23, 24), or attenuation by genetic manipulation, deleting viral genes associated with virulence (3-10, 25-29). The latter appears effective and, perhaps, is a safer methodology when compared to the use of naturally attenuated isolates. In the referred examples genetic manipulation causing deletions of single genes or a group of genes produced attenuated virus strains that induce protection against the virulent parental virus. Here, we present the identification of a previously uncharacterized ASFV gene, E184L, as a novel viral genetic determinant of virulence. Deletion of E184L partially attenuates ASFV-G in swine, when used at doses of 10^2 HAD_{50}. Interestingly, only six other genetic modifications have been shown to decrease virulence in the highly virulent ASFV Georgia isolate or its derivative isolates. In our laboratory, we showed that deletion of the 9GL and UK genes; a deletion of a group of six genes from the MGF360 and 530 or the I177L gene induced a complete virus attenuation when inoculated IM in a dose range of 10^3 to 10^6 HAD (6, 7, 10). We also have shown that the deletion of I177L when inoculated IM was 100% effective at doses as low as 10^2 HAD (10). This low dose potency was maintained when an additional deletion in the Left variable region was introduced to adapt the ASFV-G-ΔI177L experimental vaccine to cell culture (27). More recently, results using virulent field isolates from China have shown that deletion of MGF110-9L or MGF505-7R gene induce complete or partial attenuation when IM tested at 10 HAD (25, 30), while deletion of a group of genes, L7L-L11L (28), in a recombinant virus IM inoculated at doses of 10^3 to 10^6 HAD induce partial attenuation. Therefore, deletion of the E184L gene constitutes the seventh genetic modification leading to a decrease in virulence of the Georgia 2007 virus or its derivatives. Importantly, along with the

surviving animals infected with ASFV-G-ΔE184L, only recombinant virus lacking I177L, 9GL, 9GL/UK, CD2/UK, MGF, L7L-L11L or MGF/CD2 genes, in the context of the Georgia 2007 or its derivative isolates, have been used as experimental vaccines to protect against the corresponding virulent parental virus (5-10, 28, 29).

Because residual virulence remained using the ASFV-G-ΔE184L recombinant virus, it is clear that the individual deletion of E184L cannot be used in the development of an attenuated virus strain but in combination with other gene deletions to achieve complete attenuation. The deletion of E184L in the context of a vaccine candidate genome would potentially serve to both increase vaccine safety and provide a negative antigenic marker in the vaccine candidate. The recognized immunogenicity of the E184L protein product (19) constitutes a critical characteristic for its potential use as an antigenic marker to develop a DIVA-compatible vaccine. Our results demonstrated that E184L deletion can be used as a negative antigenic marker in a potential live attenuated vaccine. Animals surviving inoculation with ASFV-G-ΔE184L mounted a vigorous ASFV-specific antibody response while failing to recognize the E184L protein product. To our knowledge, this constitutes the first report describing a specific ASFV protein functioning as an antigenic DIVA marker in pigs vaccinated with an experimental attenuated ASFV vaccine that confers protection against virulent challenge.

We attempted to present a proof of concept of E184L gene deletion in a genome of the experimental attenuated vaccine candidate ASFV-G-ΔMGF. Deletion of E184L clearly affects the ability of ASFV-G-ΔMGF/ΔE184L to replicate *in vivo*. Interestingly, this replication deficiency does not appears to be so drastic in primary macrophage cell cultures indicating the existence of other to-be-determined host factors involved in the decreased replication of ASFV-G-ΔMGF/ΔE184L in pigs. The reduced replication resulting from multiple gene deletions from the virus genome is not an uncommon event. Subsequent deletion of genes from a virus genome already containing deletion of genes

associated with virulence usually decreases the ability of the novel recombinant virus to replicate, particularly in inoculated swine. Some example of this phenomenon are the deletion of six genes of the MGF360/505 family, or the CD2-like gene, from the genome of the ASFV-G-Δ9GL virus (20, 31), the deletion of the NL or UK genes in naturally attenuated OURT T88/3 (32), the deletion of CD2-like gene in HLJ/18-6GD (29), the deletion of UK in CD2-deleted virus (8), the deletion of UK from ASFV-G-Δ9GL (6) , or the deletion of NL and UK genes from ASFV-G-Δ9GL (31). The outcome of these combined deletions is unpredictable, in some cases producing a desirable increase of virus attenuation (6, 8, 29) and in others, as in the case of ASFV-G-ΔMGF/ΔE184L, a decreased efficacy in inducing protection by the modified vaccine candidate harboring the novel deletion (20, 31-33). Therefore, deleting the E184L gene from the genome of a vaccine candidate to gain DIVA functionality will need to be clinically evaluated to ensure the resulting virus still efficaciously protects animals.

We believe the results presented here demonstrate that deletion of the E184L gene should be considered as a valid approach to produce a vaccine candidate with DIVA capability and to potentially increase vaccine safety. To our knowledge, E184L is the first ASFV gene candidate functionally tested as a negative antigenic marker for the development of live attenuated ASF vaccines with serological DIVA capability.

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SC0014664. All opinions expressed in this paper are the author's and do not necessarily reflect the policies and views of USDA, ARS, APHIS, DHS, DOE, or ORAU/ORISE.

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Conflict of Interest

The authors Douglas Gladue and Manuel Borca have a patent for ASFV-G-ΔMGF as a live-attenuated vaccine for African swine fever. US patent # US9528094B2.

Table 1. Swine survival and fever response following infection with 10^2 HAD_{50} doses of ASFV-G-ΔE184L or parental ASFV-G.

Virus (HAD_{50})	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	0/5	7 (0) [(1)]	4.6 (0.55)	2.4 (0.55)	105.52 (0.79)
ASFV-G-ΔE184L	3/5	13.5 (0.71) [(1)(2)]	10.5 (0.71) [(2)]	3 (0) [(2)]	105.45 (0.07)

(1) All animals were euthanized due to humanitarian reasons following the corresponding IACUC protocol.

(2) Data referred to the only two animals in the group developing disease. The other three animals remained clinically normal during the observational period.

Table 2. Swine survival and fever response in ASFV-G-ΔE184L-infected animals with 10^2 HAD_{50} ASFV-G virus 28 days later.

Virus (10^2 HAD_{50})	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)
Mock	0/5	7 (0) [(1)]	4.2 (0.45)	2.8 (0.45)	105.98 (0.94)
ASFV-G-ΔE184L	3/3	-	-	-	103.06 (0.29)

[(1)] All animals were euthanized due to humanitarian reasons following the corresponding IACUC protocol.

Figure Legends

Fig. 1. Diagram indicating the position of the E184L open reading frame in the ASFV-G genome. The donor plasmid with the homologous arms to ASFV-G and the mCherry under control of the p30 promoter in the orientation as indicated. The final genomic changes introduced to develop ASFV-G-ΔE184L where the sequence of the donor plasmid mCherry reporter is introduced to replace the ORF of E184L as indicated. The nucleotide positions refer to the positions of the ORF of E184L in ASFV-G or the residual portion of E184L that remains in ASFV-G- ΔE184L.

Fig. 2. Multiple sequence alignment of the indicated ASFV isolates of viral protein E184L. Twenty-two protein sequences representing the genetic diversity of gene E184L of ASFV at GenBank database were used to conduct this alignment. To assess the nature of the replacements at multiple residues, conservation scores based on the biological properties of each amino acid were included, being the lower scores associated with more divergent replacements. Symbols (*) indicate residue conservation or (+) replacement for an amino acid with similar properties. Analysis was conducted on the Jalview software version 2.11.1.3, using the ClustalW algorithm.

Fig. 3: Expression profile of E184L gene of ASFV during the infection in porcine macrophages. qRT-PCR analysis was performed to assess the profile expression of E184L at different hours post-infection. As controls for this analysis genes encoding p30 (early expression) and p72 (late expression) were used for this experiment. The housekeeping B-actin was use as endogenous control for the analysis.

Fig. 4: *In vitro* growth characteristics of parental ASFV-G, and recombinant ASFV-G-ΔE184, ASFV-G-ΔMGF and ASFV-G-ΔMGF/ΔE184L. Primary swine macrophage cell cultures were infected (MOI=0.01) with each of the viruses and virus yield titrated at the indicated times post-infection. Data represent means from three independent experiments. Sensitivity of virus detection: $\geq$ 1.8 $\log_{10}$ HAD_{50}/ml. Significant differences in viral yields between ASFV-G-ΔE184L vs ASFV-G (*) and

between ASFV-G-ΔMGF vs ASFV-G-ΔMGF/ΔE184L (*) are shown at specific times points. Statistical analysis was conducted by the unpaired T test using the two-stage step-up (Benjamini, Krieger and Yekutieli) method, assuming individual variance for each time point. p-values <0.05 were considered statistically significant.

Fig. 5: Kinetics of body temperature values in pigs IM inoculated with 10^2 HAD_{50} of either ASFV-G-ΔE184L, or ASFV-G (ASFV-G 1) before and after the challenge with 10^2 HAD_{50} of ASFV-G (ASFV-G 2)**.** Each curve represents individual animal's values in each of the group.

Fig. 6: Kinetics of mortality in pigs IM inoculated with 10^2 HAD_{50} of either ASFV-G-ΔE184L or ASFV-G (ASFV-G 1) before and after the challenge with 10^2 HAD_{50} of ASFV-G (ASFV-G 2).

Fig. 7: Viremia titers detected in pigs IM inoculated with 10^2 HAD_{50} of either ASFV-G-ΔE184L or ASFV-G (ASFV-G 1) before and after the challenge with 10^2 HAD_{50} of ASFV-G (ASFV-G 2). Each curve represents values from individual animals in each group. Sensitivity of virus detection: $\geq$ 1.8 $\log_{10}$ HAD_{50}/ml.

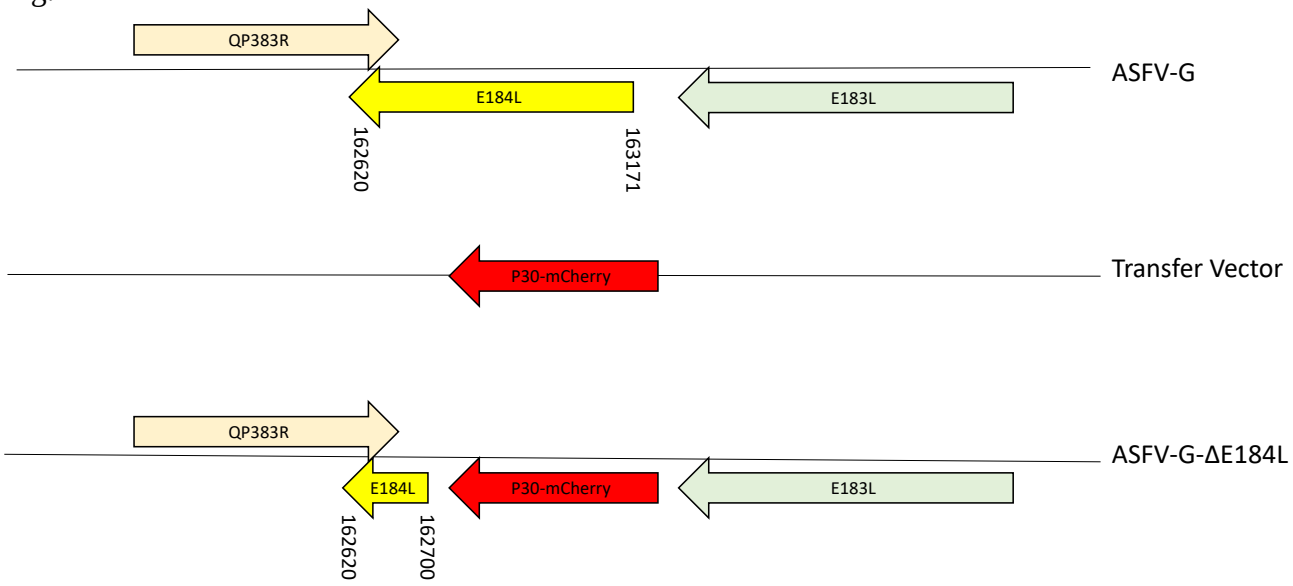
Fig. 8: Anti-ASFV antibody titers detected by ELISA in pigs IM inoculated with 10^2 HAD_{50} of ASFV-G-ΔE184L. Each point represents values from individual animals.

Fig. 9: Antibody response to ASFV E184L protein, p54 and p72 in pool of sera of animals inoculated with either ASFV-G-ΔE184L or ASFV-G-ΔMGF detected by peptidearray. Results are presented as Signal/Noise OD values (and their SD) of each pool sera related to OD of a pool of pre-immune sera. E184L peptides 1, 2 and 3 represent amino acid residues at positions 163-177, 167-181 and 170-184 of the E184L protein, respectively.

Fig. 10: (A) Viremia titers detected in pigs IM inoculated with either 10^4 or 10^6 HAD_{50} of ASFV-G-ΔMGF/ΔE184L or 10^4 HAD_{50} of ASFV-G-ΔMGF before and after the challenge with 10^2 HAD_{50} of ASFV-G (ASFV-G 2). Each curve represents average values and corresponding SD from each animal

group. Sensitivity of virus detection: $\geq$ 1.8 $\log_{10}$ HAD_{50}/ml. **(B)** Anti-ASFV antibody titers detected by ELISA in pigs IM inoculated with either 10^4 or 10^6 HAD_{50} of ASFV-G-ΔMGF/ΔE184L or 10^4 HAD_{50} of ASFV-G-ΔMGF. Each point represents average values and corresponding SD from each animal group. **(C)** Kinetics of mortality in pigs IM inoculated with either 10^4 or 10^6 HAD_{50} of ASFV-G-ΔMGF/ΔE184L or 10^4 HAD_{50} of ASFV-G-ΔMGF before and after the challenge with 10^2 HAD_{50} of ASFV-G.

535 Fig.1



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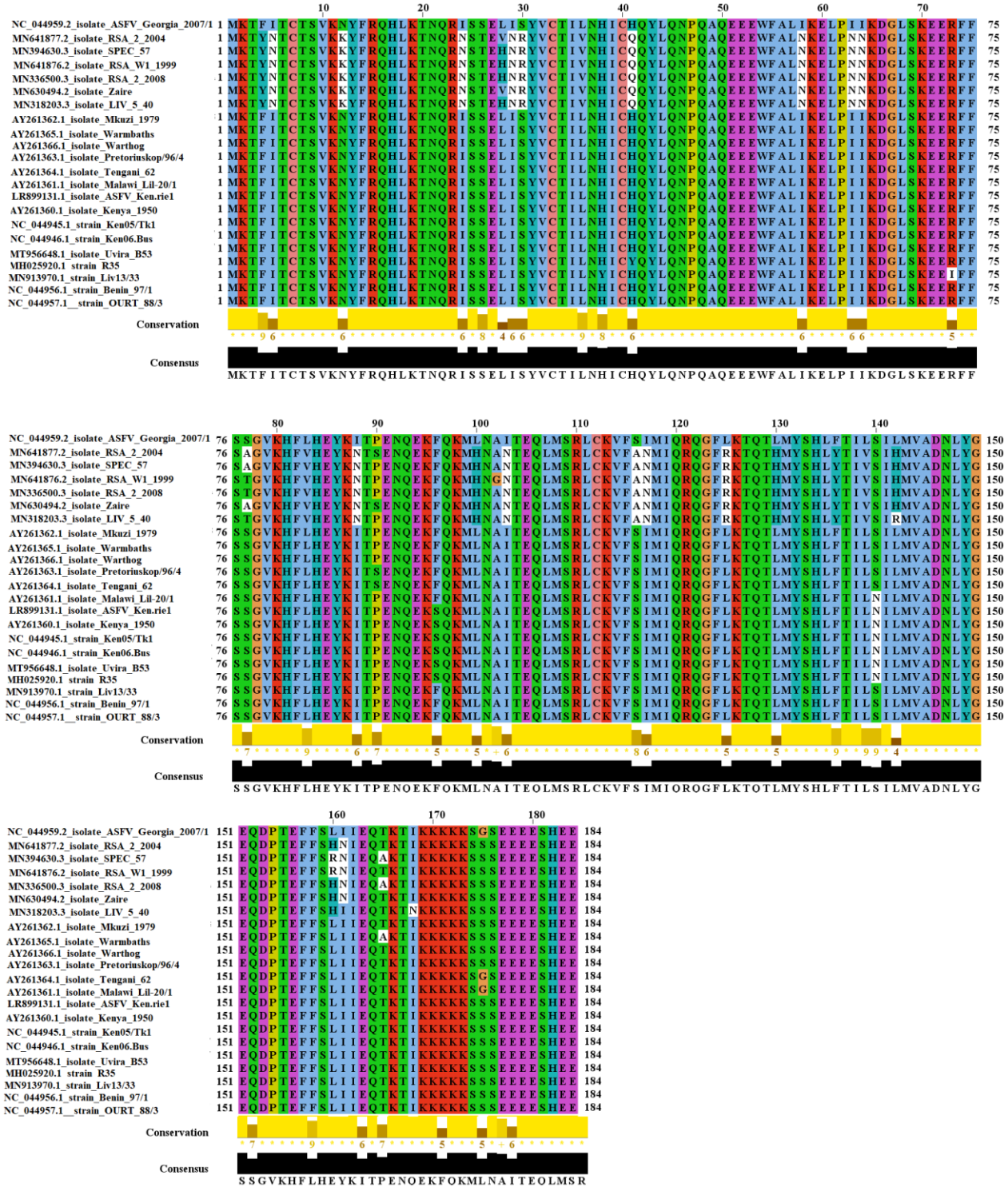
538 Fig. 2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NC_044956.1_strain_Benin_97/1
NC_044957.1__strain_OURT_88/3
Conservation
Consensus
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AY261362.1_isolate_Mkuzi_1979
AY261365.1_isolate_Warmbaths
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Conservation
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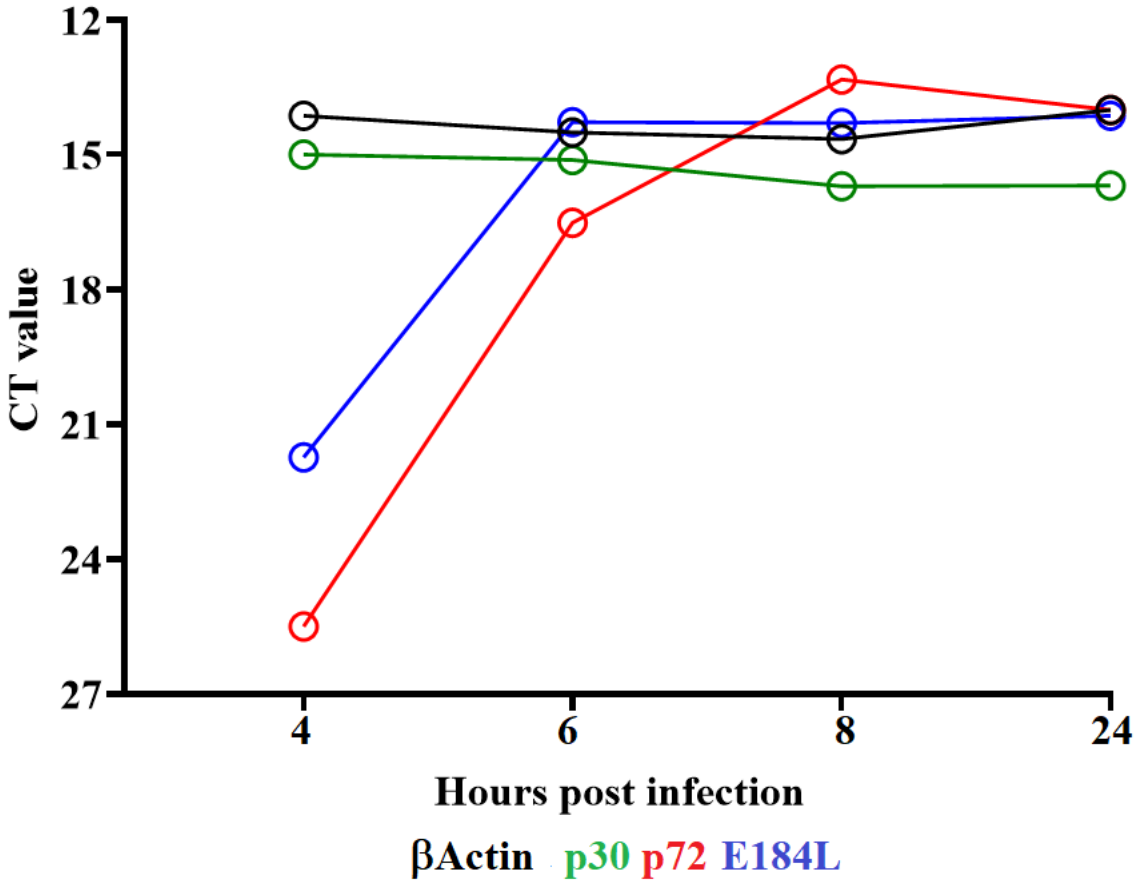
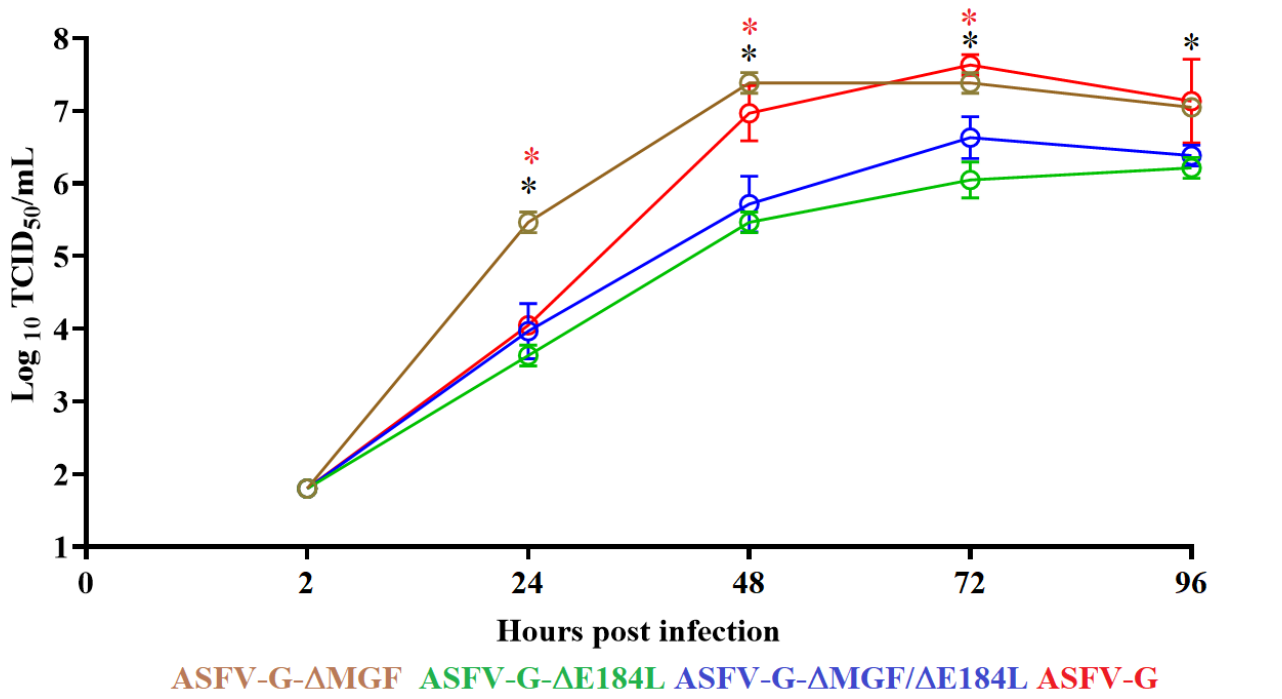
539

Fig. 3


12
15
18
21
24
27
CT value
4
6
8
24
Hours post infection
βActin
p30
p72
E184L

544 Fig. 4
545



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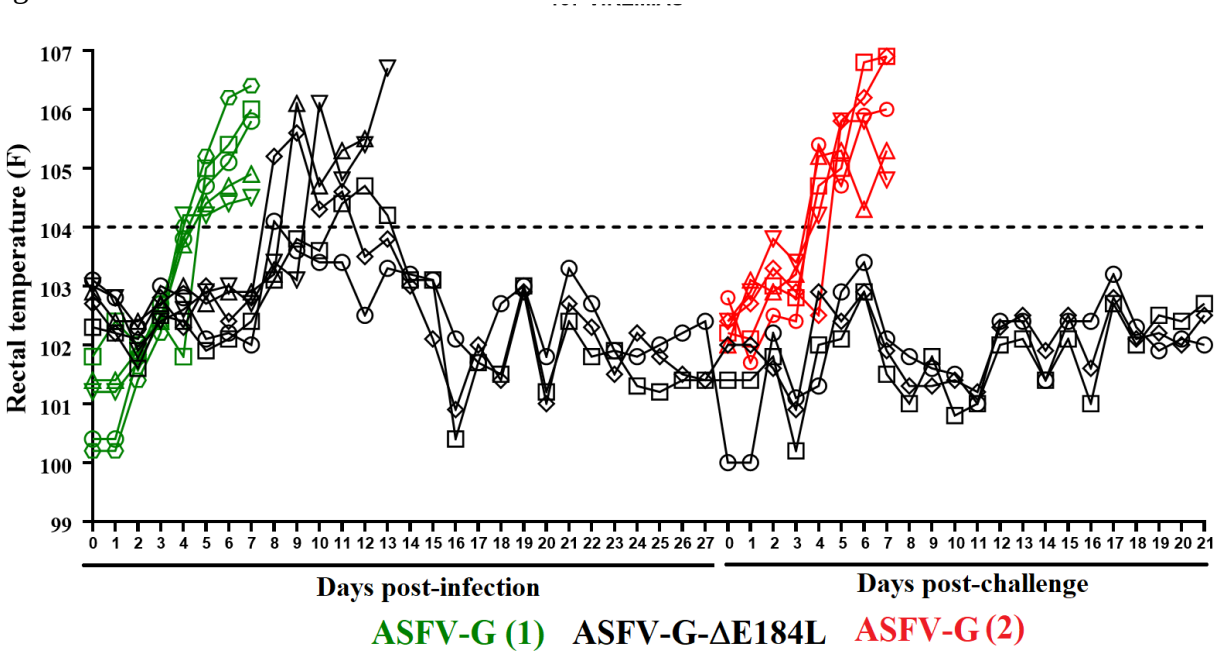
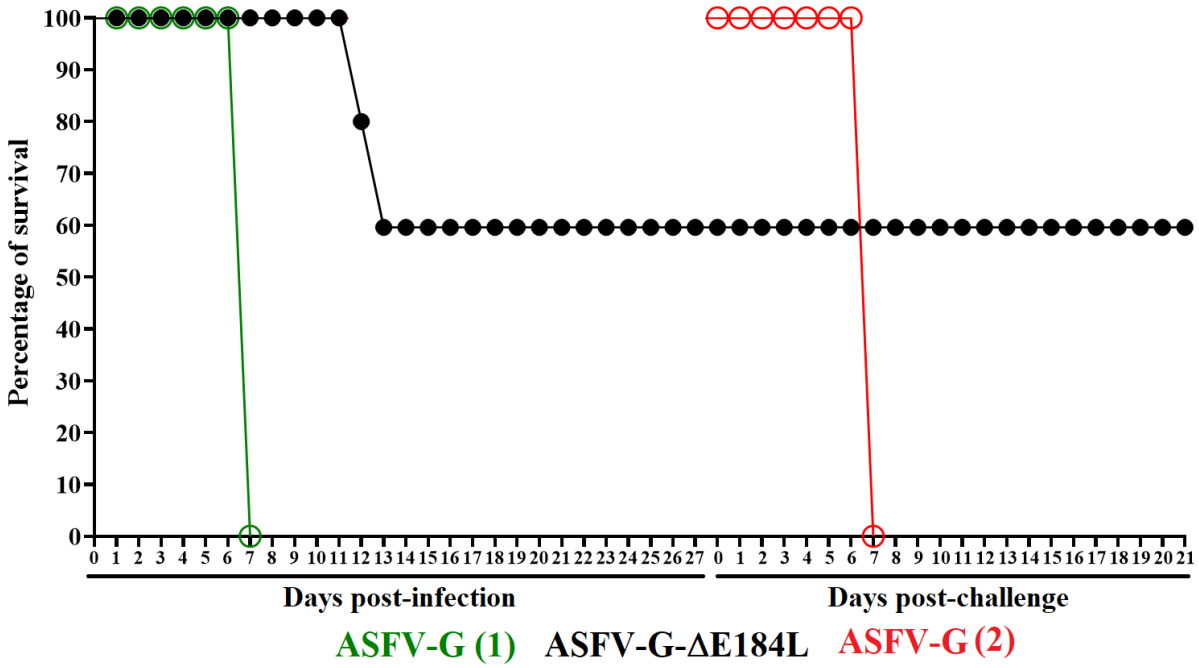
547 Fig. 5


Rectal temperature (F)
107
106
105
104
103
102
101
100
99
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
Days post-infection
Days post-challenge
ASFV-G (1)
ASFV-G-ΔE184L
ASFV-G (2)

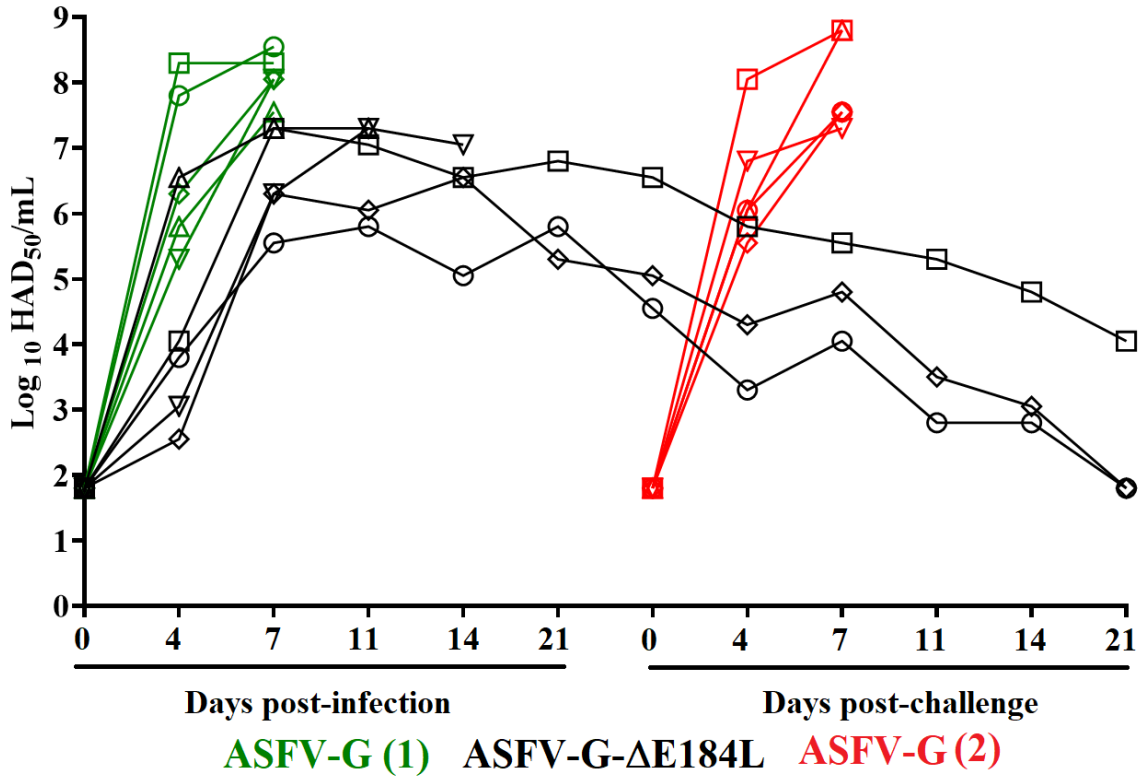
548

549 Fig. 6


Percentage of survival
100
90
80
70
60
50
40
30
20
10
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
Days post-infection
Days post-challenge
ASFV-G (1)
ASFV-G-ΔE184L
ASFV-G (2)

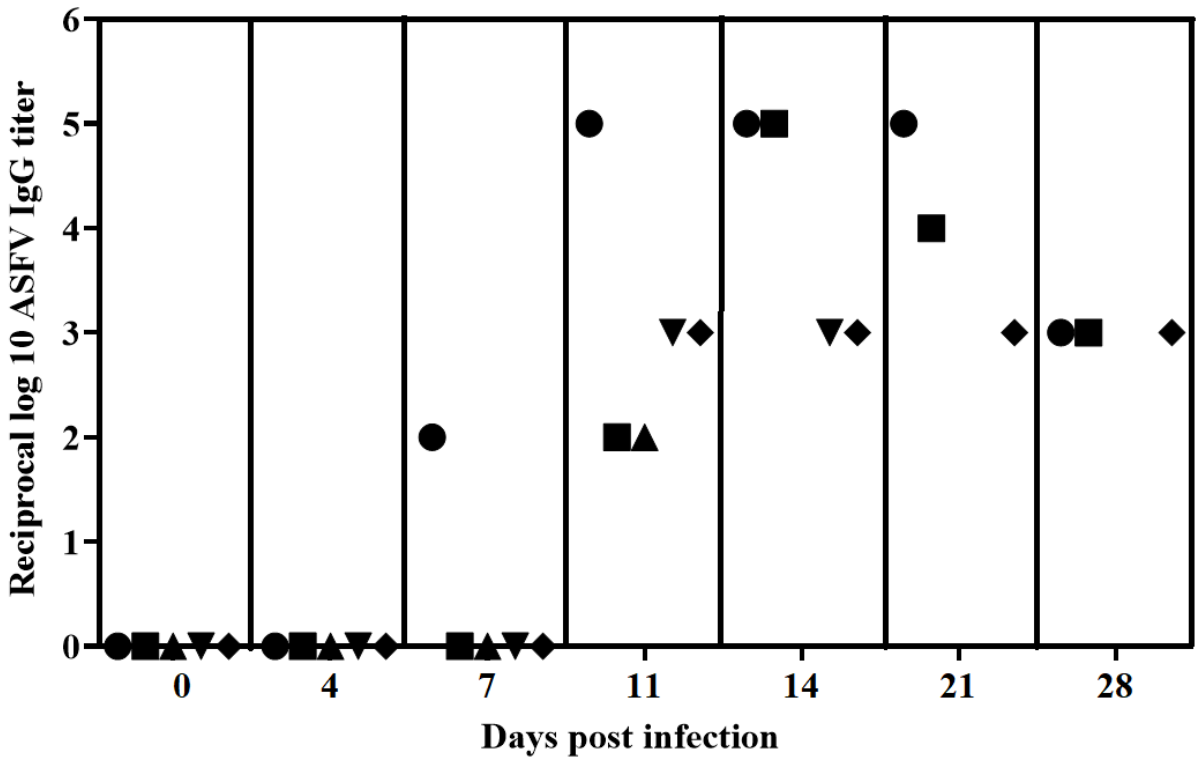
550

551 Fig. 7


Log 10 HAD50/mL
9
8
7
6
5
4
3
2
1
0
0
4
7
11
14
21
0
4
7
11
14
21
Days post-infection
Days post-challenge
ASFV-G (1)
ASFV-G-ΔE184L
ASFV-G (2)

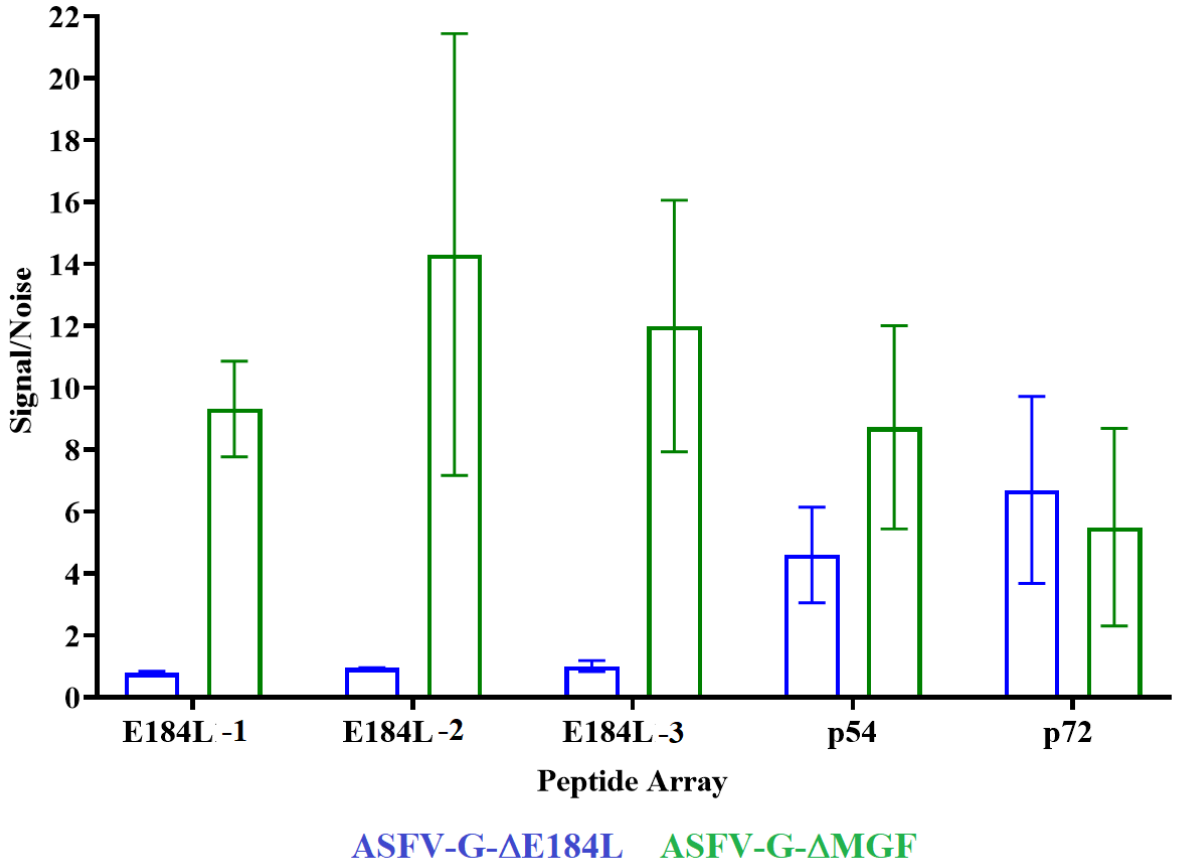
552

553 Fig. 8


Reciprocal log 10 ASFV IgG titer
6
5
4
3
2
1
0
0
4
7
11
14
21
28
Days post infection

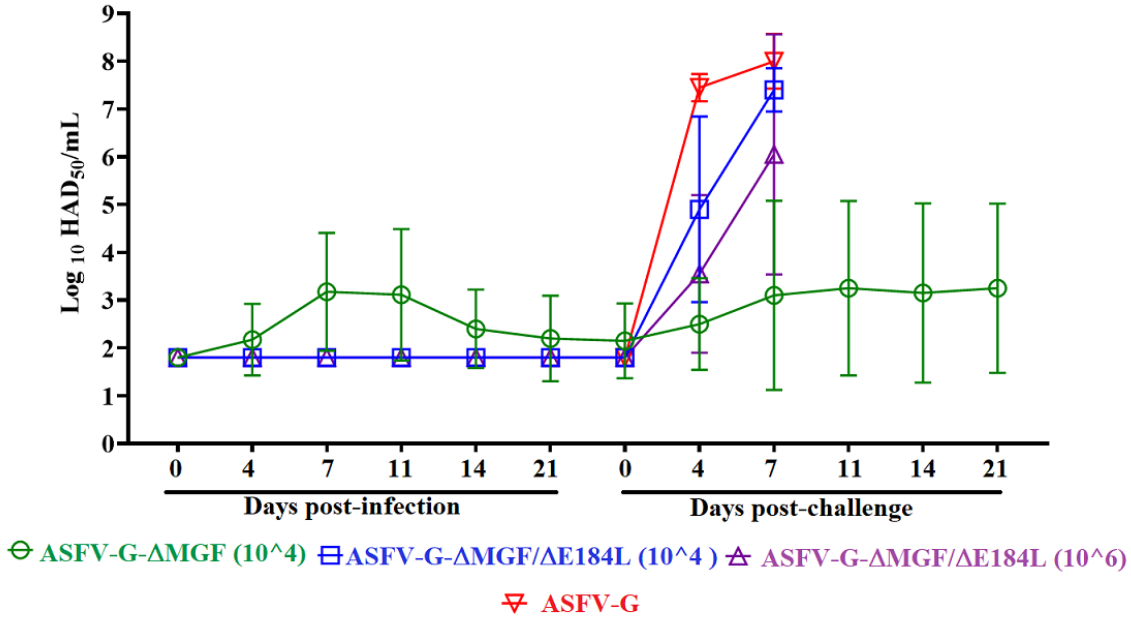
554

555 Fig. 9


Signal/Noise
22
20
18
16
14
12
10
8
6
4
2
0
E184L -1
E184L -2
E184L -3
p54
p72
Peptide Array
ASFV-G-ΔE184L
ASFV-G-ΔMGF

556

557 Fig. 10A



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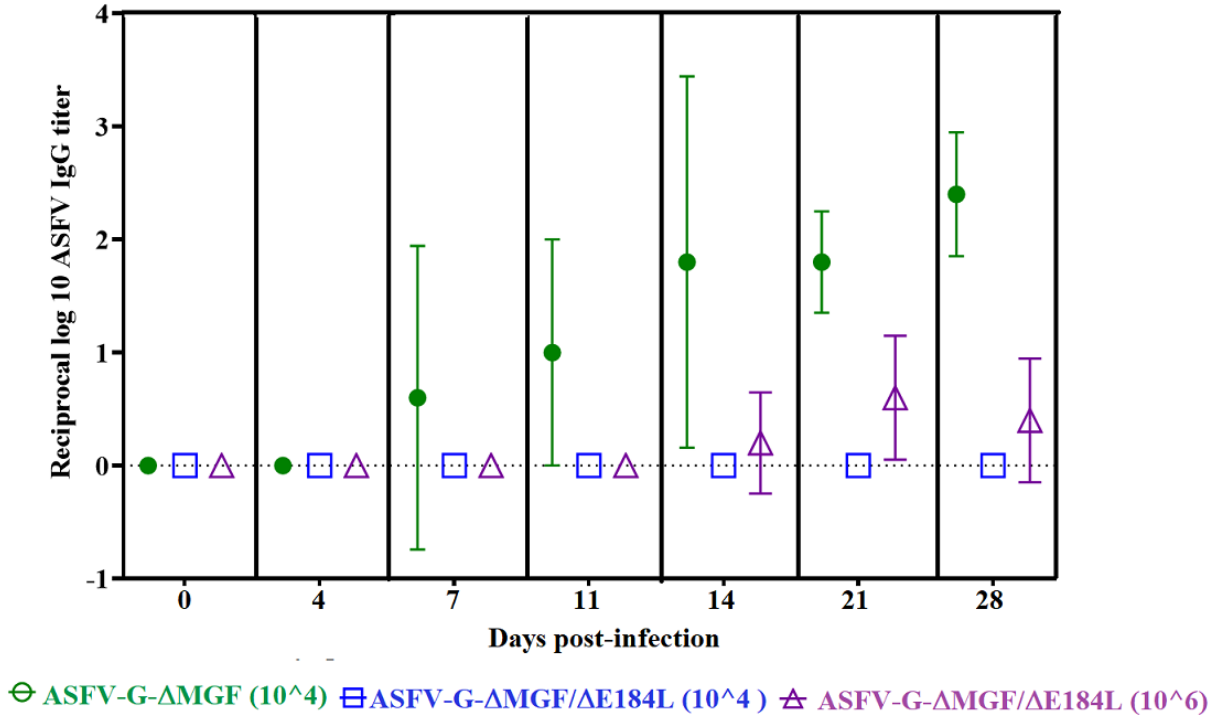
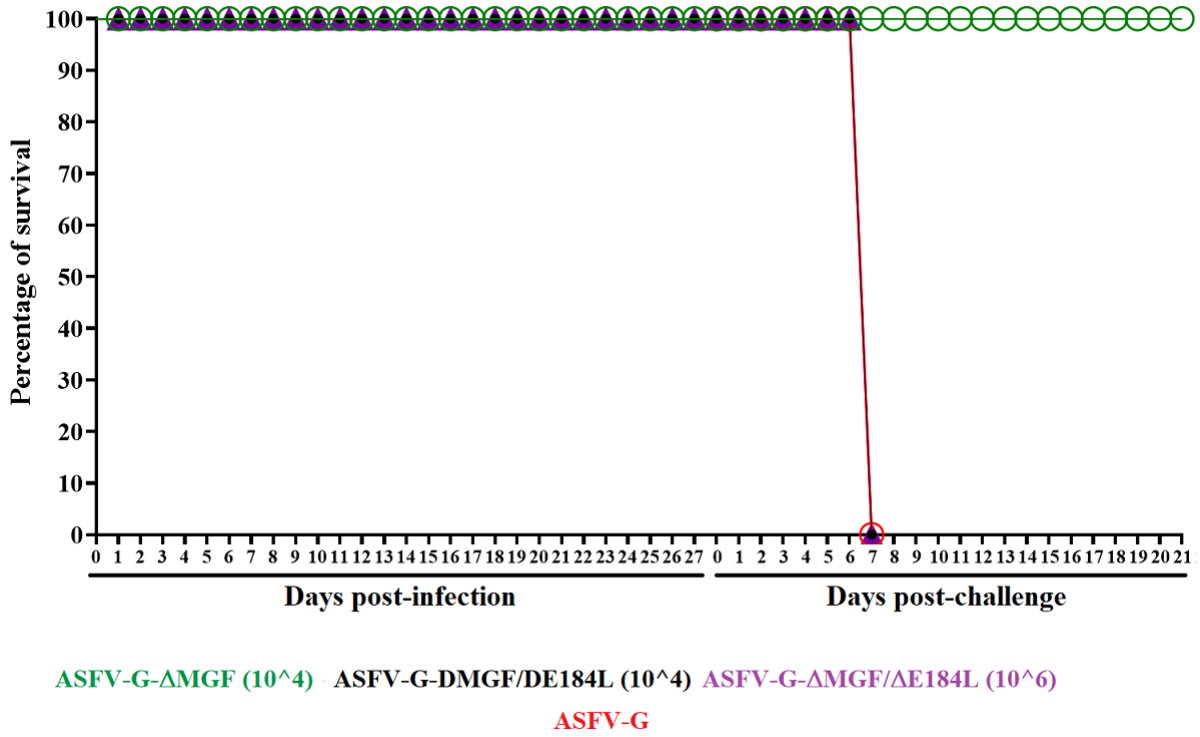
559 Fig. 10B



560

561 Fig. 10C



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564

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