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DESCRIPTION

BACKGROUND OF THE INVENTION

Field of the Invention

[0001] This invention relates to a recombinant African Swine Fever Virus (ASFV) live attenuated candidate strain vaccine for the highly virulent Georgia 2007 isolate ASFV-G. The vaccine comprises the ASFV-G Δ9GLΔUK modified virus, a recombinant ASFV-G modified by deleting a large portion of the *9GL (8 119L)* gene and the *UK (OP96R)* gene.

Description of the Relevant Art

[0002] African Swine Fever (ASF) is a contagious viral disease of swine. The causative agent, ASF virus (ASFV), is a large enveloped virus containing a double-stranded DNA genome of approximately 190 kilobase pairs. ASFV shares aspects of genome structure and replication strategy with other large double-stranded DNA viruses, including the *Poxviridae, Iridoviridae* and *Phycodnaviridae* (Costard et al. 2009. Phil. Trans. Royal Soc. B 364:2683-2696). ASFV infections in domestic pigs are often fatal and are characterized by fever, hemorrhages, ataxia and severe depression. However, the course of infection varies, ranging from highly lethal to sub-clinical, depending on host characteristics and the particular virus strain (Tulman et al. 2009. Curr. Top. Microbiol. Immunol. 328:43-87).

[0003] Currently, the disease is endemic in more than twenty sub-Saharan African countries. In Europe, ASF is still endemic on the island of Sardinia (Italy) and new outbreaks have been declared in the Caucasus region since 2007, affecting Georgia, Armenia, Azerbaijan and Russia. Isolated outbreaks have been recently reported in Ukraine, Belarus, Lithuania, Latvia and Poland, posing the risk of further dissemination into neighbouring countries. The epidemic virus, ASFV Georgia 2007/1, is a highly virulent isolate belonging to the genotype II (Chapman et al. 2011. Emerging Infect. Ois. 17:599-605).

[0004] Currently, there is no vaccine available for ASF and disease outbreaks are controlled by animal quarantine and slaughter. Attempts to vaccinate animals using infected cell extracts, supernatants of infected pig peripheral blood leukocytes, purified and inactivated virions, infected glutaraldehyde-fixed macrophages, or detergent-treated infected alveolar macrophages failed to induce protective immunity (Coggins, L. 1974. Prog. Med. Virol. 18:48-63; Forman et al. 1982. Arch. Virol. 74:91-100; Kihm et al. 1987. In: African Swine Fever, Becker, Y. (ed), Martinus Nijhoff, Boston, pp 127-144; Mebus, C. A. 1988. Adv. Virus Res. 35:251-269). Homologous protective immunity does develop in pigs surviving viral infection. Pigs surviving acute infection with moderately virulent or attenuated variants of ASFV develop

long-term resistance to homologous, but rarely to heterologous, virus challenge (Hamdy and Dardiri. 1984. Am. J. Vet. Res. 45:711-714; Ruiz-Gonzalvo et al. 1981. In: FAOICEC Expert Consultation in ASF Research, Wilkinson, P. J. (ed), Rome, pp 206-216). Pigs immunized with live attenuated ASF viruses containing engineered deletions of specific ASFV virulence-associated genes were protected when challenged with homologous parental virus. Specifically, individual deletion of *UK (OP69R), 23-NL (DP71L), TK* (*A240L*) or *9GL* (*B119L*) genes from the genomes of pathogenic ASF viruses (Malawi Lil-20/1, Pretoriuskop/96/4, E70 and Georgia 2007) markedly attenuated the virus in swine and the animals immunized with these attenuated viruses were protected against challenge with homologous virus (Moore et al. 1998. J. Virol. 72:10310-10315; Lewis et al. 2000. J. Virol. 74: 1275-1285; Zsak et al. 1996. J. Virol. 70:8865-8871; Zsak et al. 1998. J. Virol. 72:1028-1035). These observations constitute the only experimental evidence describing the rational development of an effective live attenuated virus against ASFV. US 2016/082099 A1 and O'Donnell et al. 2015, J. Virol 89:8556-8566 relates to the construction of a recombinant Δ9GL virus derived from the highly virulent ASFV Georgia 2007 (ASFV-G) isolate in which additional ΔUK deletions have been engineered. Zsak et al 2015, J. Virol 72:1028-1035 relates to the use of ASFV-G ΔUK as potential vaccine. O'Donnel et al. 2016, Virus Research 221:8-14 discloses that ASFV-G ΔGL with additional mutations leads to an attenuated virus with suboptimal protection.

[0005] In particular, deletion of *9GL (B119L)* in highly virulent ASFV isolates Malawi Lil-20/1, Pretoriuskop/96/4, and Georgia2007 (Lewis *et al., supra;* Neilan et al. 2004. Virol. 319:337-342; O'Donnell et al. 2015. J. Virol. 89: 8556-8566) resulted in complete attenuation of these viruses in swine. Administration of Malawi Lil-20/1Δ9GL or Pretoriuskop/96/4 Δ9GL or the E70 ΔUK mutants to pigs via IM injection at a relatively high virus dose did not induce clinical signs, with all animals surviving the infection. Furthermore, IM inoculation of pigs with these viruses induced protection against challenge with virulent parental viruses (Zsak *et al.* 1998, *supra;* Lewis *et al., supra;* O'Donnell *et al., supra).* These observations constitute the only experimental evidence describing the rational development of an effective live attenuated virus against ASFV.

[0006] Since there are not ASFV vaccines currently available, the development of any experimental vaccine that may induce any type of protection against the lethal presentation of the disease is of great interest.

SUMMARY OF THE INVENTION

[0007] The invention is set out in the appended set of claims. The embodiments and/or examples of the following description which are not covered by the appended claims are considered as not being part of the present invention. The references to methods of treatment in the subsequent paragraphs of this description are to be interpreted as references to the compounds, pharmaceutical compositions and medicaments of the present invention for use in a method for treatment of the human (or animal) body by therapy (or for diagnosis). $

[0008] We have developed the novel recombinant mutant ASFV-G Δ9GL/ΔUK virus, a modification of the ASFV-G (African Swine Fever Virus- Georgia 2007 isolate).

[0009] In accordance with this discovery, it is an object of the invention to provide the novel mutant ASFV-G Δ9GL/ΔUK virus, resulting from the deletion of a large portion of both the *9GL (B119L)* gene and the *UK (OP96R)* gene of the parental ASFV-G. The nucleotide sequence of ASFV-G Δ9GL/ΔUK (SEQ ID NO: 3) differs from the nucleotide sequence encoding the wild-type ASFV-G (SEQ ID NO: 1). The ASFV-G (wild-type) 9GL- encoded protein of 119 amino acids (SEQ ID NO: 2) and the ASFV-G (wild-type) UK - encoded protein of 95 amino acids (SEQ ID NO:25) differ from the mutant 9GL and UK proteins encoded by the mutant nucleotide sequence of ASFV-G Δ9GL/ΔUK (SEQ ID NO: 3). A mutant 9GL polypeptide of 61 amino acids (SEQ ID NO: 4) results from the deletion of amino acid # 11 through amino acid #68 of the wild-type 9GL polypeptide (SEQ ID NO: 2), and a mutant UK polypeptide of 10 amino acids (SEQ ID NO: 26) results from the deletion of amino acid #1 through amino acid #85 of the wild-type UK polypeptide (SEQ ID NO:25).

[0010] An added object of the invention is to provide immunogenic compositions comprising a viable ASFV-G Δ9GL/ΔUK virus.

[0011] An additional object of the invention is to provide a rationally designed live attenuated ASFV-G Δ9GL/ΔUK vaccine effective to protect an animal from clinical ASF disease when challenged with pathogenic ASFV-G.

[0012] A further object of the invention is to provide a genetic marker vaccine which can potentially distinguish between vaccinated animals and animals infected with ASFV-G.

[0013] Another object of the invention is to provide a live attenuated ASFV-G Δ9GL/ΔUK vaccine for use in the protection of swine against ASFV-G, wherein the vaccine comprises a mutant ASFV-G Δ9GL/ΔUK virus.

[0014] An additional object of the invention is to provide a method for distinguishing animals infected with ASFV-G from animals vaccinated with said rationally designed live attenuated ASFV-G Δ9GL/ΔUK vaccine, comprising a genetic DIVA strategy for differentiating vaccinated animals from wild-type infected animals.

BRIEF DESCRIPTION OF THE DRAWINGS

[0015] Figures 1A and 1B show the sequence alignment of ASFV-G *9GL (B119L)* gene-encoded polypeptides (Fig. 1A), and the ASFV-G UK (*DP96R*) gene-encoded polypeptides (Fig. 1B). Isolates of various temporal and geographic origins, including those from obtained from ticks and pig sources, were compared. The partial deletion introduced into ASFV-G that yielded ASFV-G Δ9GL/ΔUK virus is shown between brackets.

DETAILED DESCRIPTION OF THE INVENTION

[0016] We have developed an attenuated virus that can be used as a vaccine candidate through the approach of targeting both ASFV-G *9GL (B119L)* and ASFV-G *UK (OP96R)* genes for genetic modifications. Here we report the construction of a recombinant Δ9GL/ΔUK virus of the highly virulent ASFV Georgia 2007 isolate (ASFV-G). ASFV-G Δ9GL/ΔUK administered intramuscularly (IM) to swine at relatively high doses (10^4 or 10^6 HAD_{50}) does not induce disease. Animals infected with 10^4 or 10^6 HAD_{50} are protected against the presentation of clinical disease when challenged at 28 days post infection with the virulent parental strain Georgia 2007.

[0017] Although independent deletion of four different genes from ASFV has been shown to attenuate virulent viruses and although independent deletions of the NL (DP71L) (Zsak *et al.* 1996, *supra)* or the UK (DP69R) (Zsak *et al.* 1998, *supra)* genes from ASFV E75, deletion of the TK (A240L) gene (Moore *et al., supra)* from ASFV adapted to Vero cells, Malawi Lil-20/1 and Haiti, and deletion of the 9GL (B119L) gene also from Malawi Lil-20/1 (Lewis *et al., supra)* and Pretoriuskop/96/4 (Neilan *et al., supra)* isolates rendered recombinant deletion mutant viruses with significantly reduced virulence in swine, in all these cases, animals inoculated with each of these genetically modified viruses survived the infection and became protected against ASFV when challenged with the corresponding virulent parental virus, i.e., homologous challenge (Lewis *et al., supra;* Moore *et al., supra;* Neilan *et al., supra;* Zsak *et al.* 1996, *supra;* Zsak *et al.* 1998, *supra).* Those findings suggest that development of attenuated ASFV recombinant viruses by genetic manipulations of target genes is an effective approach for vaccine development.

[0018] However, their level of effectiveness in other ASFV isolates is not predictable. For example, the NL (DP71L) gene product exits in two different forms, a long (184 amino acids as in 23-NL) or a short form (70 to 72 amino acids) depending on the ASFV isolate (Zsak *et al.* 1996, *supra).* Although deletion of this gene in ASFV E70 isolate (short form) rendered an attenuated virus, the deletion of the NL (DP71L) gene from ASFV Malawi Lil-20/1 (long form) or Pretoriuskop/96/4 (short form) did not result in attenuation of the virus (Afonso et al. 1998. J. Gen. Virol. 79 (Pt. 10):2543-2547). Deletion of the TK (A240L) gene, a highly conserved gene among all ASFV isolates involved in DNA synthesis, has been introduced into pathogenic Vero cell-adapted Malawi Lil-20/1 and Haiti H811 viruses. The Malawi Lil-20/1 mutant virus was less virulent *in vivo* than the revertant virus (wild-type-like virus), but it was not completely attenuated (Moore *et al., supra).* The UK (DP69R) gene is located in the right variable region of certain ASFV isolates. Deletion of this gene from ASFV E70 isolates rendered a virus exhibiting reduced virulence (Zsak *et al.* 1998, *supra).* Although the UK (DP69R) gene is conserved, it is not present in every ASFV isolate (e.g. Malawi Lil-20/1), limiting its use as a candidate target gene for producing attenuated viruses.

[0019] The 9GL (*B119L)* gene is highly conserved among ASFV isolated and sequenced so

far, including those from both tick and pig sources. The fact that deletion of the gene from virulent Malawi Lil-20/1 (Lewis *et al., supra*) and Pretoriuskop/96/4 (Neilan *et al., supra)* effectively reduced virulence in swine and induced protection made 9GL (B119L) a strong candidate target gene for modification and production of attenuated virus that can confer effective protection against ASFV. Indeed, we found that the deletion of 9GL (B119L) from the ASFV-G isolate did not have the same effect in terms of attenuation and protection as reported for Malawi Lil-20/1 and Pretoriuskop/96/4. Only when ASFV-G Δ9GL was administrated at a low dose to swine was it possible to observe a decrease in virus virulence (O'Donnell *et al., supra*). We had also shown that a sub-lethal dose of ASFV-G Δ9GL was able to induce effective protection against the presentation of clinical disease after the challenge with homologous parental virus. The NL proteins encoded by E70 (short form) and Malawi Lil-20/1 (long form) differ significantly and that may explain the phenotypic differences observed in swine inoculated with the respective deletion mutant viruses. However, protein identity matrixes indicate that the 9GL protein is highly similar among ASFV isolates where ASFV-G, Malawi Lil-20/1, and Pretoriuskop/96/4 share over 93% amino acid identity, making it unlikely that ASFV attenuation relies solely on protein divergence. Since the well observed phenotypes are most likely mediated by the effect of multiple genes (Lewis *et al., supra;* Moore *et al., supra;* Neilan *et al., supra;* Zsak *et* al. 1996, *supra;* Zsak *et al.* 1998, *supra*), the evidence accumulated so far makes it difficult to speculate what is indeed the spectrum of genes mediating virulence in the ASFV Georgia 2007 isolate.

[0020] In summary, here we present evidence that deletion of both the 9GL (B*119L*) gene and the UK (*DP69R*) gene results in the attenuated recombinant ASFV-G Δ9GL/ΔUK virus. Intramuscular administration of ASFV-G Δ9GL/ΔUK to swine at relatively high doses (10^4 or 10^6 HAD_{50}) does not induce disease. Animals infected with 10^4 or 10^6 HAD_{50} are protected against the presentation of clinical disease when challenged at 28 days post infection with the virulent parental strain Georgia 2007.

[0021] A vaccine is defined herein as a biological agent which is capable of providing a protective response in an animal to which the vaccine has been delivered and is incapable of causing severe disease. Administration of the vaccine results in immunity from a disease; the vaccine stimulates antibody production or cellular immunity against the pathogen causing the disease. Immunity is defined herein as the induction of a significant higher level of protection in a population of swine against mortality and clinical symptoms after vaccination compared to an unvaccinated group. In particular, the vaccine according to the invention protects a large proportion of vaccinated animals against the occurrence of clinical symptoms of the disease and mortality. The vaccine of the invention herein is a genetically engineered mutant virus vaccine. A genetic marker vaccine is defined as a vaccine that, in conjunction with a diagnostic test, enables genetic differentiation of vaccinated animals from infected animals. A deletion mutation can be used to differentiate infected from vaccinated animals. A mutation is understood to be a change in the genetic information of a "wild-type" or unmodified *9GL* (*B119L*) and *UK (OP96R)* genes of a parent ASFV-G strain which is able to express native 9GL and UK proteins. Thus, the 9GL and UK polypeptides expressed by the ASFV-G Δ9GL/ΔUK

mutant virus is changed: the 9GL protein from ASFV-G Δ9GL/ΔUK has fewer amino acids than both the wild-type 9GL and the wild-type UK, as amino acids #11 through #68 are deleted in the 9GL polypeptide of ASFV-G Δ9GL and amino acids #1 to #85 are deleted in the UK polypeptide. The ASFV-G Δ9GL/ΔUK recombinant ASFV-G mutant comprises nucleotides encoding mutations in the ASFV-G 9GL and UK polypeptides. The mutation comprises a deletion of 58 amino acids of the 9GL protein and a deletion of 85 amino acids of the UK protein. The recombinant ASFV-G mutant ASFV-G Δ9GL/ΔUK is a live attenuated ASFV-G vaccine when used at IM inoculation doses of 10^4 HAD_{50} to 10^6 HAD_{50}.

[0022] A vaccine against ASFV-G is provided that comprises a ASFV-G Δ9GL/ΔUK mutant as defined above in a live form, and a pharmaceutically acceptable carrier or diluent. The vaccine according to the invention containing the live virus can be prepared and marketed in the form of a suspension or in a lyophilized form and additionally contains a pharmaceutically acceptable carrier or diluent customary used for such compositions. Carriers include stabilizers, preservatives and buffers. Suitable stabilizers are, for example SPGA (sucrose, phosphate, glutamate, and human. albumin), carbohydrates (such as sorbitol, mannitol, starch, sucrose, dextran, glutamate or glucose), proteins (such as dried milk serum, albumin or casein) or degradation products thereof. Suitable buffers are for example alkali metal phosphates. Suitable preservatives are thimerosal, merthiolate and gentamicin. Diluents include water, aqueous buffer (such as buffered saline), alcohols and polyols (such as glycerol).

[0023] If desired, the live vaccines according to the invention may contain an adjuvant. Examples of suitable compounds and compositions with adjuvant activity are well known in the art. Furthermore, nucleic acid sequences encoding polypeptides for pharmaceutical or diagnostic applications, in particular immunomodulators such as lymphokines, interferons or cytokines, may be incorporated into the vaccine.

[0024] A vaccine according to the invention can be prepared by conventional methods such as those commonly used for the commercially available live attenuated ASFV vaccines. Briefly, a susceptible substrate is inoculated with the ASFV-G Δ9GL/ΔUK mutant and propagated until the virus has replicated to a desired titer after which ASFV-G Δ9GL/ΔUK -containing material is harvested. Subsequently, the harvested material is formulated into a pharmaceutical preparation with immunizing properties.

[0025] Every substrate which is able to support the replication of ASFV-G Δ9GL/ΔUK viruses can be used in the present invention, including primary cultures of swine peripheral blood macrophages.

[0026] The vaccine may be administered by intramuscular, subcutaneous or intranasal inoculation or injection in an amount which is effective to protect the animal against challenge by a virulent strain of ASFV-G. This amount may vary according to the animal being inoculated, taking into consideration the size and weight of the animal. The vaccine according to the invention comprises an effective dosage of the ASFV-G Δ9GL/ΔUK mutant as the active

component, i.e. an amount of immunizing ASFV-G Δ9GL/ΔUK material that will induce immunity in the vaccinated animals, swine, against challenge by a virulent ASFV-G. Immunity is defined herein as the induction of a significant higher level of protection in a population of swine against mortality and clinical symptoms after vaccination compared to an unvaccinated group. In particular, the vaccine according to the invention prevents a large proportion of vaccinated animals against the occurrence of clinical symptoms of the disease and mortality. Typically, the live vaccine can be administered in a dose of 10^4 HAD_{50} to 10^6 HAD_{50}. Effective amounts may be experimentally determined as necessary by those of skill in the art by following the guidance provided, for example, by Example 6.

[0027] In addition to the ASFV-G Δ9GL/ΔUK mutant, the invention can also include combination vaccines comprising a vaccine strain capable of inducing protection against another porcine pathogen.

[0028] The ASFV-G Δ9GL/ΔUK vaccine described above, in conjunction with a diagnostic method, has the potential of distinguishing between animals that are vaccinated with it and animals that are infected with naturally occurring ASFV-G strains or vaccinated with conventional ASFV-G vaccines.

[0029] The present invention also provides an invaluable tool to monitor ASFV-G control measures that may lead to eradication of ASFV-G if applied in large scale stamping out programs. This tool concerns a method for determining ASFV-G infection in swine comprising the step of examining a sample of the animal for the presence of nucleotides encoding the wild-type ASFV-G 9GL and UK proteins versus the polynucleotide encoding the shorter ASFV-G Δ9GL and ΔUK polypeptides due to deletions in the *9GL (B119L)* gene and the *UK (DP96R)* genes of ASFV-G Δ9GL/ΔUK. The sample of the animal used in this method may be any sample in which ASFV-G versus ASFV-G Δ9GL/ΔUK genetic differences allowing for differentiating of natural infection versus vaccination can be detected by genetic DIVA.

EXAMPLES

[0030] Having now generally described this invention, the same will be better understood by reference to certain specific examples, which are included herein only to further illustrate the invention and are not intended to limit the scope of the invention as defined by the claims.

EXAMPLE 1

Cell Cultures and Viruses

[0031] Primary swine macrophage cell cultures were prepared from defibrinated swine blood

as previously described by Zsak *et al.* (1996, *supra*). Briefly, heparin-treated swine blood was incubated at 37°C for 1 hour to allow sedimentation of the erythrocyte fraction. Mononuclear leukocytes were separated by flotation over a Ficoll-Paque (Pharmacia, Piscataway, N.J.) density gradient (specific gravity, 1.079). The monocyte/macrophage cell fraction was cultured in plastic Primaria (Falcon; Becton Dickinson Labware, Franklin Lakes, N.J.) tissue culture flasks containing macrophage media, composed of RPMI 1640 Medium (Life Technologies, Grand Island, NY) with 30% L929 supernatant and 20% fetal bovine serum (HI-FBS, Thermo Scientific, Waltham, MA) for 48 hours at 37°C under 5% CO_2. Adherent cells were detached from the plastic by using 10 mM EDTA in phosphate buffered saline (PBS) and were then reseeded into Primaria T25, 6- or 96-well dishes at a density of 5×10^6 cells per ml for use in assays 24 hours later.

[0032] Virus titration was performed on primary swine macrophage cell cultures in 96-well plates. Virus dilutions and cultures were performed using macrophage medium. Presence of virus was assessed by hemadsorption (HA) and virus titers were calculated by the Reed and Muench method (1938. Amer. J. Hygiene 27:493-497).

[0033] 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.

EXAMPLE 2

Construction of the recombinant ASFV-G Δ9GL/ΔUK

[0034] Recombinant ASFVs were generated by sequential homologous recombination between the parental ASFV genome and recombination transfer vectors in infection and transfection procedures using swine macrophage cell cultures (Neilan *et al., supra;* Zsak *et al.* 1996, *supra*). First, recombinant transfer vector (p72GUSΔ9GL) containing flanking genomic regions including portions of 9GL mapping to the left (1.2 kbp) and right (1.15 kbp) of the gene and a reporter gene cassette containing the β-glucuronidase (GUS) gene with the ASFV p72 late gene promoter, p72GUS was used. This construction created a 173-nucleotide deletion in the 9GL ORF (amino acid residues 11 to 68) (see Fig. 1). Recombinant transfer vector p72GUSΔ9GL was obtained by DNA synthesis (GenScript, Piscataway, NJ, USA). Macrophage cell cultures were infected with ASFV-G and transfected with p72GUSΔ9GL. Recombinant viruses representing independent primary plaques were purified to homogeneity by successive rounds of plaque assay purification. The recombinant virus was obtained after 11 successive plaque purification events on monolayers of primary swine macrophage cell cultures. The produced intermediate recombinant virus, ASFV-G Δ9GL, was then used as parental virus in infectious/transfection procedures using a recombinant transfer vector that would produce deletion of the UK gene from the virus genome. Recombinant transfer vector (p72mCherryΔUK) containing flanking genomic regions of UK mapping to the left (1.156 kbp)

and right (1.190 kbp) of the gene and a reporter gene cassette containing the mCherry gene with the ASFV p72 late gene promoter, p72mCherry was used. Recombinant transfer vector p72mCheryΔUK was obtained by DNA synthesis (GenScript, Piscataway, NJ, USA). This construction created a 255-nucleotide deletion in the UK ORF (amino acid residues 1 to 85) (see Fig. 1). The second recombination event replaced the UK gene by the cassette containing the fluorescent gene mCherry under the ASFV p72 promoter. Recombinant virus was selected after 10 rounds of limiting dilution purification based in the fluorescent activity. The virus population obtained from the last round of purification was amplified in primary swine macrophage cell cultures to obtain a virus stock.

EXAMPLE 3

Full Genome Sequence Analysis: ASFV-G Δ9GL/ΔUK Relative to Parental ASFV-G

[0035] To evaluate the accuracy of the genetic modification and the integrity of the genome of the recombinant virus, full genome sequences of ASFV-G Δ9GL/ΔUK and parental ASFV-G were obtained using Next Generation Sequencing (NGS) and compared (Table 1). First, a full-length genome comparison between parental ASFV-G and ASFV Georgia 2007/1 (Chapman *et al., supra*) was performed. ASFV DNA was obtained from the cytoplasm of infected cells using the Trizol method (Life Technologies, Grand Island, NY, USA). DNA concentration was determined using the Qubit® dsDNA HS assay kit (Life Technologies) and read on a Qubit® 2 Flourometer (Life Technologies). One microgram of virus DNA was enzymatically fragmented to obtain blunt end fragments in a length range of 200-300 bp using the Ion Shear™ Plus reagent kit (Life Technologies) and incubated at 37°C in a Peltier Thermal Cycler DNA Engine Tetrad 2. After shearing, the fragmented DNA library was loaded onto a DNA chip (Agilent, Santa Clara, CA, USA) and analyzed using a 2100 Bioanalyzer (Agilent) to assess DNA size distribution and size range. Fragmented DNA was ligated to Ion-compatible adapters and library barcodes, followed by nick-repair to complete the linkage between adapters and DNA inserts using the Ion Plus Fragment Library kit (Life Technologies). The adapter-ligated library was size-selected for optimum length on 2% Agarose Gel Cassettes (Sage Science, Beverly, MA, USA) using the Pippin Prep™ instrument (Sage Science). Library concentration was normalized using the Ion Library Equalizer™ Kit (Life Technologies). Next, the DNA library was clonally amplified onto Ion Sphere™ Particles (IPS) generating template-positive ISPs using the Ion PGM™ Template OneTouch™ 2 200 Kit (Life Technologies) with the Ion OneTouch™ 2 Instrument (Life Technologies). Before proceeding to enrichment, quality assessment of non-enriched template-positive ISPs was performed using the Ion Sphere™ Quality Control assay kit (Life Technologies) and a Qubit® 2 Flourometer instrument. The template-positive ISPs were then enriched using the Ion PGM™ Template OneTouch™ 2 200 Kit (Life Technologies) and Ion OneTouch™ ES instrument (Life Technologies) to eliminate template-negative ISPs

and to denature DNA on template-positive ISPs. Using the Ion PGM™ 200 Sequencing v2 Kit (Life Technologies), enriched template ISPs were prepared for sequencing and loaded onto either Ion 314™ or Ion 316™ Chip v2 (Life Technologies) and run on the Ion PGM™ Sequencer (Life Technologies). Obtained sequences were then trimmed using Galaxy (Retrieved from the Internet: usegalaxy.org/) NGS QC and Manipulation tools. Sequences were aligned and analyzed using Sequencher 5.2.2 (Genecodes) and CLC Genomics Workbench (CLCBio) software.

[0036] The following differences were observed between these two viruses (nucleotide positions are provided based on ASFV Georgia 2007/1, GenBank accession FR682468): (i) two nucleotide insertions, T at position 433 and A at position 441 in a non-coding segment of the genome; (ii) two nucleotide deletions, T at position 1602 and T at position 1603 in the MGF 360-1L gene ORF resulting in a frameshift; (iii) a nucleotide deletion, T at position 1620 in the MGF 360-1L gene ORF resulting in a frameshift; (iv) a nucleotide mutation, A to G at position 97391 resulting in a silent mutation in ORF B438L; (v) a nucleotide mutation, C to G at position 166192 resulting in a residue substitution (Ala to Pro) at residue position 85 in ORF E199L; and (vi) a nucleotide insertion, T at position 183303, a non-coding segment of the genome (Table 1). Second, a full-length genome comparison between ASFV Δ9GL/ΔUK and parental ASFV-G was performed. The DNA sequence assemblies of ASFV Δ9GL/ΔUK and ASFV-G revealed a deletion of 173 nucleotides in 9GL gene corresponding with the introduced modification. The consensus sequence of the ASFV Δ9GL/ΔUK genome showed an insertion of 2324 nucleotides in 9GL gene corresponding to the p72-βGUS cassette sequence introduced to generate a 173 nucleotide deletion in the targeted gene. In addition, the DNA sequence assemblies of ASFV-G Δ9GL/ΔUK and ASFV-G revealed a deletion of 255 nucleotides in UK gene corresponding with the introduced modification. The consensus sequence of the ASFV-G Δ9GL/ΔUK genome showed an insertion of 937 nucleotides in UK gene corresponding to the p72-mCherry cassette sequence introduced to generate a 255 nucleotide deletion in the targeted gene. Besides the insertion of the cassette, only one additional difference was observed between ASFV-G Δ9GL/ΔUK and ASFV-G genomes, a G to C nucleotide mutation at position 36,465 resulting in a residue substitution (Glu to Gln) at residue position 224 in ORF MGF 505-4R. In summary, ASFV-G Δ9GL/ΔUK virus did not accumulate any significant mutations during the process of homologous recombination and plaque purification (Table 1).

Table 1. Summary of differences between the full-length genome sequence of ASFV-G Δ9GL/ ΔUK and the parental ASFV-G compared with ASFV Georgia07/1*

		Virus	
NPN*	**Type of Modification**	**ASFV-G**	**ASFV-G Δ9GL/ ΔUK**
433	T insertion	+	+
411	A insertion	+	+
1602	MGF 360-1L TT deletion FS@	+	+

		Virus	
NPN*	**Type of Modification**	**ASFV-G**	**ASFV-G Δ9GL/ ΔUK**
1620	MGF 360-1L T insertion FS	+	+
36465	MGF 505-4R G to C Glu224Gln	-	+
97391	B438L A to G SM#	+	+
166192	E199L C to G Ala85Pro	+	+
183303	T insertion in a NCR+	+	+

* Nucleotide Position Number (based on the sequence of ASFV Georgia 2007/1 isolate published by Chapman *et al.* 2011)
@ Nucleotide modification causes frameshift in the corresponding ORF
# Nucleotide modification causes silent mutation
+ Non-Coding Region

EXAMPLE 4

Assessment of ASFV-G Δ9GL/ΔUK Virulence in Swine

[0037] 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.

[0038] ASFV-G Δ9GL/ΔUK was assessed for its virulence phenotype relative to the virulent parental ASFV-G virus using 80-90 pound commercial breed swine. Five pigs were inoculated intramuscularly (IM) either with 10^4 or 10^6 HAD_{50} of either ASFV-G Δ9GL/ΔUK or with 10^4 HAD_{50} of ASFV-G virus. Clinical signs (anorexia, depression, fever, purple skin discoloration, staggering gait, diarrhea and cough) and changes in body temperature were recorded daily throughout the experiment. In protection experiments animals were IM inoculated with 10^4 HAD_{50} or 10^6 HAD_{50} and 28 days later IM challenged with 10^3 HAD_{50} of parental virulent ASFV Georgia2007 strain. Presence of clinical signs associated with the disease was performed as described earlier.

[0039] All 80-90 pounds pigs inoculated via IM with 10^4 HAD_{50} of ASFV-G exhibited increased body temperature (>104 °F) by 3 to 4 days post-infection. Pigs presented clinical signs associated with the disease including anorexia, depression, purple skin discoloration,

staggering gait and diarrhea (Table 2). Signs of the disease aggravated progressively over time and animals either died or were euthanized *in extremis* by days 7 or 9 post-infection. Conversely, animals inoculated via IM with either 10^4 or 10^6 HAD_{50} of ASFV-G Δ9GL/ΔUK did not present any signs of clinical disease during the entire observation period (21 days). Therefore, deletion of 9GL and UK genes produced a complete attenuation of the parental virulent ASFV-G. Deletion of UK gene enlarges attenuation of the ASFV-G Δ9GL to the extent that 10^6 HAD_{50} of ASFV-G Δ9GL/ΔUK is completely attenuated while animals inoculated with 10^4 HAD_{50} of ASFV-G Δ9GL presented a variable level of disease (O'Donnell *et al., supra*).

Table 2. Swine survival and fever response following infection with ASFV-G Δ9GL/ΔUK and parental ASFV-G viruses.

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 10^2 HAD_{50}	0/5	7.6 (0.55)	3.6 (0.55)	4 (0.5)	105.6 (0.7)
ASFV-G Δ9GL/ΔUK 10^4 HAD_{50}	5/5	-	-	-	103.2 (0.32)
ASFV-G Δ9GL/ΔUK 10^6 HAD_{50}	5/5	-	-	-	103.4 (0.59)

EXAMPLE 6

Protective Efficacy of ASFV-G Δ9GL/ΔUK Against Challenge with Parental ASFV-G

[0040] Since pigs inoculated via IM with 10^4 HAD_{50} -10^6 HAD_{50} of ASFV-G Δ9GL/ΔUK survived the infection without signs of the disease, groups of animals (n=5) infected with either 10^4 or 10^6 HAD_{50} of ASFV-G Δ9GL/ΔUK were challenged via IM with 10^3 HAD_{50} of parental ASFV-G at day 28 post-inoculation (homologous challenge). Five naive animals that were challenged using same route and dose served as non-inoculated/ challenged control group. The five ASFV-G Δ9GL/ΔUK-inoculated and challenged animals remained completely asymptomatic during all the observational period (21 days) with the exception of two animals immunized with 10^6 HAD_{50} of ASFV-G Δ9GL/ΔUK showing a slight and transient rise in body temperature (Table 3). All the animals in the mock inoculated/challenged control group developed disease

with a clinical course similar to that observed in animals inoculated with 10^4 HAD_{50} of ASFV-G (see above). Therefore, ASFV-G Δ9GL/ΔUK is able to induce protection against the presentation of clinical disease when challenged with the highly virulent parental virus.

Table 3. Swine survival and fever response in ASFV-G Δ9GL/ΔUK- infected animals challenged with parental ASFV-G viruses.

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)
Mock	0/5	7.6 (0.55)	3.6 (0.55)	5 (0.50)	105.6 (0.7)
ASFV-G Δ9GL/ΔUK 10^4 HAD_{50}	5/5	-	-	-	102.7 (0.63)
ASFV-G Δ9GL/ΔUK 10^6 HAD_{50}	5/5	-	10.5 (9.45)*	4 (2.84)*	103.6 (1.21)
* Data are based in 2 out 5 animals presenting transient rise of body temperature. #The animals IM infected with 10^4 or 10^6 HAD_{50} of ASFV-G Δ9GL/ΔUK were IM challenged 28 days later with 10^3 HAD_{50} of ASFV-G virus.					

[0041] In summary, here we present evidence that deletion of the *9GL* and UK genes drastically alter virulence of ASFV-G producing a completely attenuated virus named ASFV-G Δ9GL/ΔUK. Animals immunized with ASFV-G Δ9GL/ΔUK were protected against challenge with the virulent parental ASFV-G.

[0042] The foregoing description and certain representative embodiments and details of the invention have been presented for purposes of illustration and description of the invention.

REFERENCES CITED IN THE DESCRIPTION

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KRAV

1. En rekombinant mutant afrikansk svinepestvirus - Georgien-2007-isolat (ASFV-G) Δ9GL/ΔUK-virus.

2. En rekombinant mutant ASFV-G Δ9GL/ΔUK-virus med SEQ ID NO: 3.

3. Vaccinesammensætning omfattende den rekombinante mutante ASFV-G Δ9GL/ΔUK-virus ifølge krav 1 eller krav 2.

4. En levende, svækket ASFV-G Δ9GL/ΔUK-vaccine til anvendelse i beskyttelse af svin mod ASFV-G, hvor vaccinen omfatter den rekombinante mutante ASFV-G Δ9GL/ΔUK-virus ifølge krav 1 eller krav 2 i en mængde, der er effektiv til at beskytte svinet mod klinisk ASF-G.

5. Vaccine til anvendelse ifølge krav 4, hvor mængden, der er effektiv til at beskytte svinene mod klinisk ASF-G, er 10^4 HAD_{50} til 10^6 HAD_{50} ASFV-G Δ9GL/ΔUK-virus.

6. Fremgangsmåde til at differentiere et pattedyr vaccineret med en levende, svækket ASFV-G Δ9GL/ΔUK-vaccine omfattende den rekombinante mutante ASFV-G Δ9GL/ΔUK-virus ifølge krav 1 eller krav 2 fra et ikke-vaccineret pattedyr inficeret med ASFV-G, hvor fremgangsmåden omfatter: at analysere en prøve fra et testpattedyr, der har behov for at blive evalueret, for tilstedeværelsen af et gen, der normalt er til stede i vildtype ASFV-G men ikke i ASFV-G Δ9GL/ΔUK-virusset, der anvendes til at vaccinere testpattedyret.

7. Fremgangsmåde ifølge krav 6, hvor trinnet med at analysere prøven udføres med et PCR-baseret assay.

8. Fremgangsmåde ifølge krav 6, hvor analysetrinnet udføres med et antistofdetektionsassay eller et ELISA.

DRAWINGS

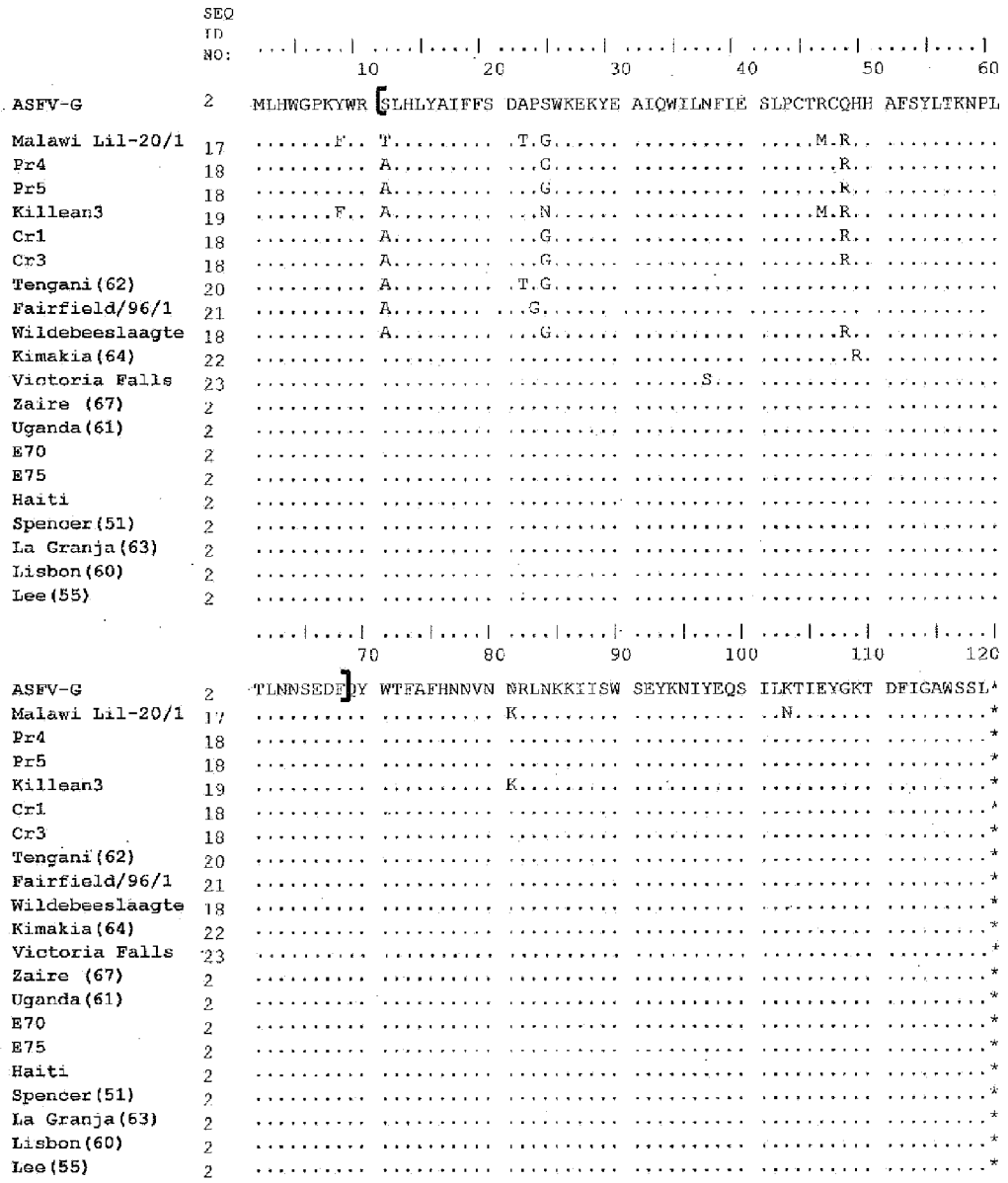
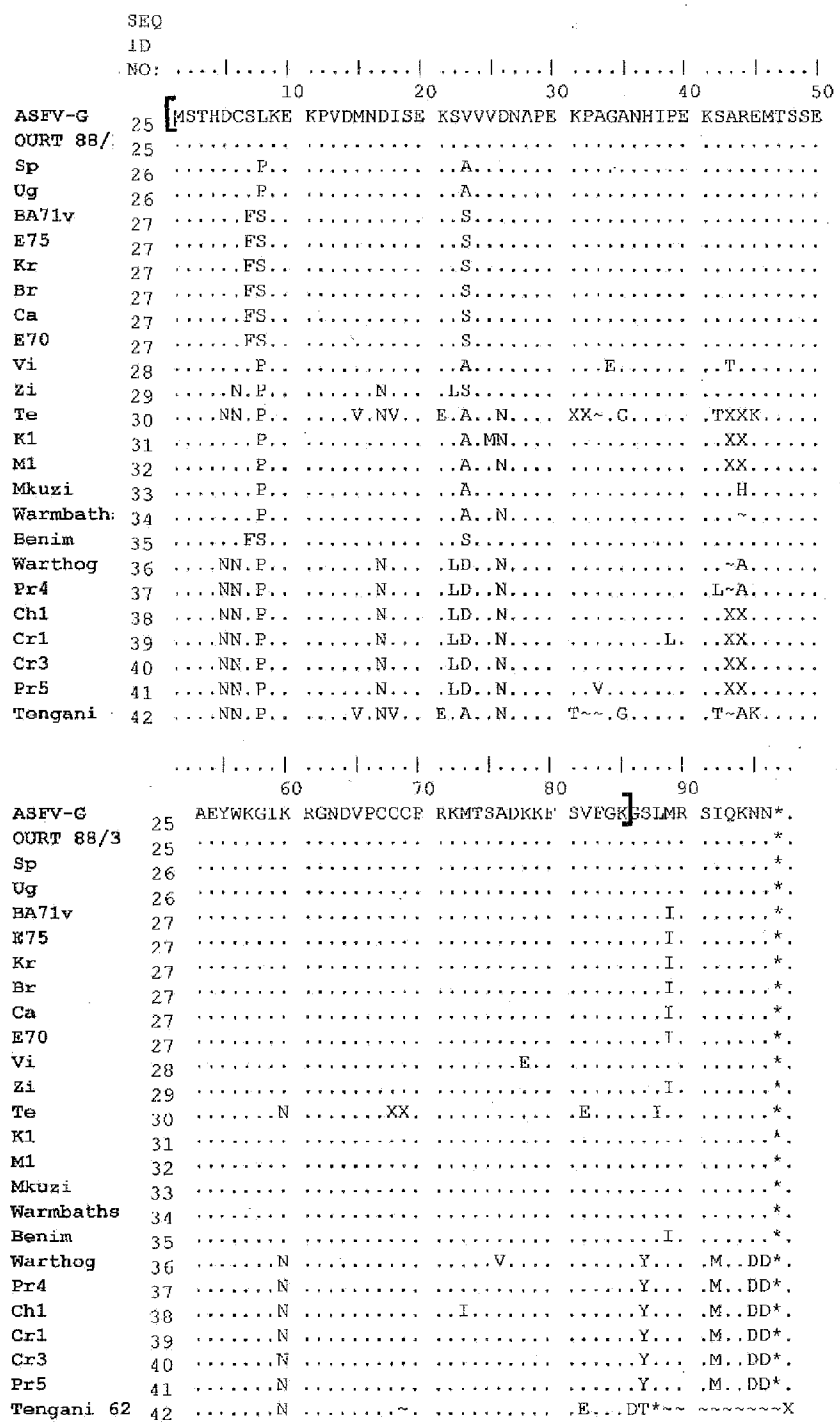


Fig. 1A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Ug 26
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Kr 27
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E70 27
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Zi 29
Te 30
K1 31
M1 32
Mkuzi 33
Warmbaths 34
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Pr4 37
Ch1 38
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Cr3 40
Pr5 41
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Kr 27
Br 27
Ca 27
E70 27
Vi 28
Zi 29
Te 30
K1 31
M1 32
Mkuzi 33
Warmbaths 34
Benim 35
Warthog 36
Pr4 37
Ch1 38
Cr1 39
Cr3 40
Pr5 41
Tengani 62 42

Fig. 1B

SEKVENSLISTE

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