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Safety and genetic stability of African swine fever virus vaccine candidate “ASFV-G-∆MGF” in an in vivo “reversion to virulence” study

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Article

Keywords: African swine fever, vaccine, reversion to virulence, genetic adaptation

Posted Date: August 17th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1922286/v1

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Abstract

African swine fever (ASF) has gained panzootic dimensions and commercial vaccines are still unavailable. Recently, a series of live attenuated vaccines has raised hope for an efficacious and safe vaccine, among them “ASFV-G-ΔMGF”. We tested the latter in a in vivo reversion to virulence study in accordance with International Cooperation on Harmonisation of Technical Requirements for Registration of Veterinary Medicinal Products guidelines. Upon forced animal passaging, a virus variant emerged that was associated with transient fever and an increased replication and shedding. However, all animals were healthy upon completion of the study and reversion to significant virulence was not observed. The genomic changes involved deletions and reorganizations in the terminal regions of the genome. While our study underscores that in-depth safety characterization is needed for live ASF vaccines, one should still conduct a thorough benefit risk analysis considering all safety and efficacy aspects when assessing their use in disease control.

1. Introduction

African swine fever (ASF) has recently spread in panzootic dimensions, exerting an immense pressure on the global pig industry and at the same time endangering entire populations of rare wild pig species (Luskin, Meijaard et al. 2021). The disease is caused by ASF virus (ASFV), a member of the genus *Asfivirus* within the family *Asfarviridae* (Alonso, Borca et al. 2018). Outside its sylvatic cycle in sub-Saharan Africa, the disease is characterized by a haemorrhagic fever with high lethality in domestic and wild suids, which represent the only susceptible mammals (Bosch, Barasona et al. 2020). Following an introduction into Georgia in 2007, ASF spread successively through eastern and central Europe, most of Asia, and recently to the Caribbean (Dixon, Stahl et al. 2020, Gonzales, Moreno et al. 2021, Sauter-Louis, Conraths et al. 2021). Without vaccines, the available control measures have failed to eliminate the disease in most countries affected by ASF (Dixon, Stahl et al. 2020). Thus, the call for a safe and efficacious vaccine is louder than ever, and research efforts to find solutions have recently intensified. Still, only a few attempts have produced successful vaccine candidates that have gone beyond proof-of-concept studies. To date, live attenuated vaccines (LAV) are the most promising concept, since complete protection against lethal field strains have only been shown with this group of vaccines (Muñoz-Pérez, Jurado et al. 2021). While reports in peer-reviewed publications of the first efficacious LAV prototypes raise hope for a licensable product on the horizon, there are still significant concerns with their safety (Bosch-Camós, Lopez et al. 2020). In particular, the inevitable ability of live vaccines viruses to replicate may result in in genetic mutations and adaption in target tissues.

In the present study, we evaluated vaccine candidate “ASFV-G-ΔMGF” (O'Donnell, Holinka et al. 2015), from here on called “ΔMGF”), a genetically modified LAV that has shown a most promising efficacy profile, in a standard *in vivo* reversion to virulence study in naïve weaner pigs. In short, the vaccine virus was passaged five times in domestic pigs in accordance with *VICH (International Cooperation on Harmonisation of Technical Requirements for Registration of Veterinary Medicinal Products) guideline 41*

for the examination of live veterinary vaccines in target animals for absence of reversion to virulence (reference number EMA/CVMP/VICH/1052/2004).

2. Methods

2.1 Experimental design

The VICH guideline 41 states that the study is to be carried out using the master seed at the maximum release titer expected in the recommended dose for five serial passages in target animals. The time interval between inoculation of the animals and harvest for each passage must be justified based upon the characteristics of the test organism. Moreover, the most sensitive class, age, sex and serological status of animals should be used. At least two animals are to be used for the first four groups and a minimum of eight for the fifth group. The initial administration and subsequent passages shall be carried out using a recommended route of administration or natural route of infection that is the most likely to lead to reversion to or increase in virulence and result in recovery of the organism following replication in the animal. Passage inocula should be collected and prepared from the most likely source of spread of the organism.

The above-mentioned recommendations were implemented as follows: Dose and route of inoculation were chosen to maximize the chance of reversion to or increase in virulence, representing a worst-case scenario of vaccine virus transmission. Considering this, undiluted master seed virus (MSV) was used and day 7 was set as timepoint for organ and blood collection for passaging. At this point, recovery of vaccine virus had the highest chance based on our experience from previous studies. Along the same lines, an intramuscular transfer of material was chosen to maximize the chance of infection given the experience that the parenteral route is much more efficient than an oral or oro-nasal inoculation (Guinat, Gogin et al. 2016). Given the low detection rate in previous trials, the study was performed in groups of ten, 6-10-week-old weaned naïve pigs which were obtained from the breeding unit of the Friedrich-Loeffler-Institute (FLI) in Mariensee, Germany, and moved to the high containment facilities of the FLI on the Isle of Riems, Germany. Before inoculation, blood of each pig was collected in an EDTA tube for reference purposes. At the respective day of study completion, a full pathological examination was performed based on the modified protocol published by Galindo-Cardiel (Galindo-Cardiel, Ballester et al. 2013) as previously described (Sehl, Pikalo et al. 2020). Blood as well as tissue samples including spleen, lung, liver, tonsil, kidney, salivary gland and gastro-hepatic and mandibular lymph nodes were collected upon necropsy. Pigs of the first four passages were observed for seven days post inoculation (dpi). Passage five was observed for 21 dpi, and oral, nasal and rectal swabs were collected weekly to trace vaccine virus shedding during the last passage. The animal experiment was approved by the State Office for Agriculture, Food Safety and Fishery in Mecklenburg-Western Pomerania (LALFF M-V) under reference number 7221.3-1-020/21.

2.2 Cells and titrations

Titrations and virus isolations were performed on porcine peripheral blood mononuclear cell (PBMC) derived macrophages obtained as previously described (Fischer, Mohnke et al. 2020). For titrations, cells were seeded in 96-well culture plates (Primaria; Corning) with 100 µl / well at a density of 5x10[illegible] cells/ml and cultivated as described before (Fischer, Pikalo et al. 2021). 100 µl of the respective samples diluted in cell culture medium at factors 10^{-1} to 10^{-8} was added to each well for end point titration. 20 µl of a 1% suspension of erythrocytes from the same donor pig in PBS was added after 24 h. For determination of titers, infected wells were read 48 and 72 h post infection using the hemadsorption as read-out. The HAD_{50} was calculated according to the method by Kaerber (1931).

2.3 Passaging of the virus

A pure pre-master master seed virus grown on primary swine macrophages was prepared by USDA-ARS at the Plum Island Animal Disease Center and transferred to Zoetis. Master seed virus grown on a proprietary commercial permanent cell line was subsequently prepared by Zoetis and provided to the FLI ready to use.

Five groups of ten pigs each were inoculated intramuscularly with 1 mL of the respective virus suspension into the right side of the neck using 2 mL syringes with 21G cannulas.

Group one received the “ΔMGF” MSV at a dose of 1.75×10^{6} HAD_{50}/ml. Organs were sampled after each passage and screened by qPCR for the presence of ASFV genome. For subsequent passages, tissues with the highest genome loads were selected, pooled at equal proportions, and homogenized in PBS at 20 Hz for 30 seconds in a grinding jar set compatible with the TissueLyser II (QIAGEN) to receive a 1 % tissue suspension. A total of 1.5 g of tissue was weighed and suspended in 13.5 mL of PBS for preparation of each inoculate. After centrifugation at 3500 rpm for 5 minutes, supernatants were obtained and administered to the following passage group. Tissue homogenate from passage one was back titrated to $10^{4.25}$ HAD_{50}/mL. The subsequent inoculates contained $10^{2.25}$ (P2), 10^{4} (P3) and $10^{5.75}$ HAD_{50}/mL (P4). An overview of the study design and the organs chosen for passaging is provided in Fig. 1.

2.4 Laboratory investigations

2.4.1 Preparation of samples and qPCR

Tissue samples were homogenized in 1 mL phosphate buffered saline (PBS) with a metal bead on a TissueLyser II (QIAGEN) at 30 Hz for 3 minutes, then centrifuged at 14000 rpm for 5 minutes. Swab samples were soaked in medium for 1h, then thoroughly vortexed and aliquoted. All samples were stored at -80°C or immediately processed.

DNA was extracted using the NucleoMag Vet Kit (Machery-Nagel) on the KingFisher® extraction platform (Thermo Scientific). qPCR for the detection of ASFV genome was either conducted according to the protocol published by King, Reid et al. (2003) or with commercial virotype 2.0 ASFV (Indical Bioscience)

on C1000™ thermal cyclers with the CFX96™ Real-Time System (Biorad). An in-house ASFV full genome standard was employed for calculation of genome copies and harmonization between runs.

2.4.2 Whole-genome sequencing

For whole-genome sequencing, a minimum of 100 ng of DNA was sent to and sequenced by Eurofins Genomics. This service included preparation of a 450 bp DNA sequencing library using a modified version of the NEBNext Ultra™ II FS DNA Library Prep Kit for Illumina and sequencing on an Illumina NovaSeq 6000 with S4 flowcell, XP workflow and in PE150 mode (Illumina).

2.4.3 Data analysis

The sequence data received from Eurofins Genomics was quality trimmed and mapped against a previously produced MSV whole-genome sequence as reference using Newbler 3.0 (Roche) with default parameters. Mapped reads were extracted and assembled using SPAdes 3.13 in the mode of error correction prior to assembly and standard parameters. The resulting contigs were mapped against the ASFV MSV reference sequence and the contigs was curated and assembled manually in Geneious Prime. For validation of the assembly and mean coverage determination, all reads were mapped against the final contig again using Newbler 3.0 with default parameters.

2.4.4 Tailored qPCR

For the identification of the novel ASFV variant, two qPCRs were designed using Geneious Prime (Biomatters) spanning the reorganization site at the 5’ end (probes directly positioned at the reorganization site) with one specifically recognizing the ΔMGF MSV (FAM-labeled) and one recognizing the ΔMGFnV (HEX-labeled) (see Table 1 and supplementary table 3).

Table 1
List of modified genes found in the ΔMGFnV genome

Gene	Type	Minimum	Maximum	Function
MGF 360-1La CDS	Deletion	1,63	2,463	Unknown
MGF 360-1Lb CDS	Deletion	2,391	2,711	Unknown
MGF 360-2L CDS	Deletion	2,797	3,885	Unknown
KP177R CDS	Deletion	4,026	4,559	P22, structural protein, transmembrane domain
L83L CDS	Deletion	4,696	4,941	Early gene, located in cytoplasm, interacts with IL1B, non-essential
L60L CDS	Deletion	5,042	5,2	Unknown
MGF 360-3L CDS	Deletion	5,359	6,429	Unknown
MGF 110-1L CDS	Deletion	6,822	7,466	Early gene. Membrane protein. Might play a role in virus cell tropism, and may be required for efficient virus replication in macrophages (inferred from homology).
ASFV G ACD 00090 CDS	Deletion	7,465	7,578	Unknown
MGF 110-2L CDS	Deletion	7,646	7,96	Early gene. Might play a role in virus cell tropism, and may be required for efficient virus replication in macrophages (inferred from homology).
MGF 110-3L CDS	Deletion	8,057	8,431	Unknown
ASFV G ACD 00120 CDS	Deletion	8,542	8,766	Unknown
MGF 110-4L CDS	Deletion	8,745	9,119	Early gene, causes the redistribution of lumenal ER protein to an enlarged ERGIC compartment. Glycosylated.
MGF 110-5L-6L CDS	Deletion	9,308	9,925	Early gene, might play a role in virus cell tropism, and may be required for efficient virus replication in macrophages (inferred from homology).

Gene	Type	Minimum	Maximum	Function
MGF 110-7L CDS	Deletion	10,132	10,545	Glycosylated, might plays a role in virus cell tropism, and may be required for efficient virus replication in macrophages (inferred from homology).
285L CDS	Deletion	10,86	11,144	Transmembrane domain.
DP60R CDS	Deletion	232	396	Transmembrane domain, glycosylated.
ASFV G ACD 01990 CDS	Deletion	517	663	Unknown
ASFV G ACD 01980 CDS	Duplication	1,354	1,548	Unknwon
MGF 360-21R CDS	Duplication	1,537	2,607	Might play a role in virus cell tropism, and may be required for efficient virus replication in macrophages (inferred from homology).
ASFV G ACD 01960 CDS	Duplication	3,053	3,184	Unknown
MGF 360-19Rb CDS	Duplication	3,253	3,522	Might play a role in virus cell tropism, and may be required for efficient virus replication in macrophages (inferred from homology).
MGF 360-19Ra CDS	Duplication	3,536	4,345	
ASFV G ACD 01940 CDS	Duplication	4,498	4,656	Unknown
DP96R CDS	Duplication	4,956	5,246	Unknown
DP71L CDS	Duplication	5,345	5,557	Interacts with the host phosphatase PP1 catalytic subunit (PPP1CB) and recruits it to dephosphorylate EIF2S1/eIF2alpha and therefore restores the host translation that has been shut-down by the host. Also inhibits the EIF2S1/eIF2alpha-ATF4-DDIT3/CHOP pathway.
MGF 360-18R CDS	Duplication	5,54	6,253	Might play a role in virus cell tropism, and may be required for efficient virus replication in macrophages (inferred from homology).
L11L CDS	Duplication	6,483	6,764	Membrane protein.

Gene	Type	Minimum	Maximum	Function
I10L CDS	Duplication	7,003	7,515	Structural protein, in virion, Transmembrane protein. In viral envelope.
I9R CDS	Duplication	7,591	7,881	Transmembrane protein
ASFV G ACD 01870 CDS	Duplication	7,844	7,981	Unknown
I8L CDS	Duplication	8,076	8,387	Non-essential. Unknown.
hypthetical CDS	Duplication	8,434	8,541	Unknown
I7L CDS	Duplication	8,601	8,909	Transmembrane protein
MGF 100-3L CDS	Duplication	9,008	9,316	Might play a role in virus cell tropism, and may be required for efficient virus replication in macrophages (inferred from homology).
MGF 100-1L CDS	Duplication	9,681	10,106	Unknown
MGF 505-11L CDS	Duplication	10,225	11,853	Might play a role in virus cell tropism, and may be required for efficient virus replication in macrophages (inferred from homology).
MGF 360-16R CDS	Duplication	11,933	12,991	Might play a role in virus cell tropism, and may be required for efficient virus replication in macrophages (inferred from homology).
DP238L CDS	Duplication	13,168	13,884	Unknown
I196L CDS	Duplication	13,978	14,586	Late gene. Unknown.
I177L CDS	Duplication	14,579	15,112	Late gene. Single pass membrane protein. Gylcosylated.
I215L CDS	Duplication	15,153	15,791	Early and late gene. Accepts ubiquitin from the E1 complex and catalyzes its covalent attachment to other proteins. Performs the second step in the ubiquitination reaction that targets specifically a protein for degradation via the proteasome. By controlling the ubiquitination status of specific host proteins, the virus may target them to degradation and thereby optimize the viral replication. Knockdown impairs viral infection, with lower number of synthesized viral genomes and lower viral progeny.
I329L CDS	Duplication	16,08	17,069	Late gene. Single-pass type I membrane protein. Highly glycosylated.

Gene	Type	Minimum	Maximum	Function
I73R CDS	Duplication	17,279	17,497	Unknown
I243L CDS	Duplication	17,581	18,312	Late gene. Transcription factor S-II-related protein
DP60R CDS	Duplication	416	580	Transmembrane domain, glycosylated
ASFV G ACD 01990 CDS	Duplication	701	847	Unknown

2.4.5 Growth kinetics

The novel virus variant ΔMGFnV was isolated from the blood of pig #22 of the fifth passage and cultivated on macrophages in T25 cell culture flasks (Primaria; Corning) obtained as described above. Absence of the MSV virus was assured by tailored qPCR. Growth kinetics were conducted on macrophages. To this end, T25 flasks were infected at a multiplicity of infection of 0.1 with either the ΔMGF MSV, the ΔMGFnV or both viruses simultaneously. After two hours of incubation, medium supernatants were removed, cells were rinsed once with PBS- and flasks were resuspended with cell culture medium. 300 µl of supernatant were removed at -2 hours post infection (hpi, before adding the virus solution), 0 hpi (after incubation), and 4, 8, 12, 24, 48 and 72 hpi and immediately stored at -80°C. Samples were analyzed by the commercial ASFV real-time PCR system virotype 2.0 (Indical Bioscience GmbH) and tailored qPCR for differentiation between ΔMGF MSV and ΔMGFnV replication, and by titration on macrophages using the methods described above.

3. Results

3.1 Clinical Observations

No clinical abnormalities or fever were observed during the first animal passage (see Fig. 1). In the second animal passage, however, three pigs displayed a transient rise in body temperatures to up to 40.4°C. Beginning from passage three and in both subsequent passages, high fever to a maximum of 42.1°C (pig #31, P4, 6 dpv, supplementary Fig. 1) was observed in numerous animals with a peak at around five and six dpi. In passage five, a body temperature of 41° C or above was recorded in nine out of ten animals for at least one day. Elevated body temperatures were clinically mirrored by mild to moderate signs of anorexia and apathy, which were scored to a maximum of three cumulative clinical score points in a single animal in passage four (animal #31, 6 dpi). All other clinical observations between passages three and five were very mild and resulted in only one cumulative clinical score point. In passage five, with an extended observation period of 21 dpi, normalization of body temperatures and the subsequent disappearance of clinical abnormalities were observed after the fever peak. Animals were clinically healthy at the end of the 21-day observation period of passage five, with the exception of animal #1110,

which showed transient lameness (not related to ASFV) on 14 and 15 dpi, recovering thereafter. One animal (#21) displayed a complete loss of sensory and motoric function of the front right leg immediately after inoculation. Treatment of the clinically diagnosed nerval lesion with dexamethasone did not result in any improvement of the lameness, so the animal was euthanized for ethical reasons on 7 dpi.

3.2 Pathological findings

All pigs were subjected to detailed pathological examination. Overall, very few lesions were detectable in all passages, i.e. slightly enlarged lymph nodes and pulmonary consolidation. No correlation or significant difference was observed when comparing lesions with passage level or time point after inoculation, i.e. 7 versus 21 days (data not further shown).

3.3 Laboratory investigations

3.3.1 Detection of ASFV genomes

qPCR screening of the samples taken from the first passage yielded less than $7.2x10^1$ ASFV genome copies (gc) per 5 µl template from the entire sample set (shown in Fig. 3). In two pigs, vaccine virus could not be detected. In passage two, no more than $5x10^1$ gc were detected in a single sample and in five pigs, vaccine virus was not detected at all. In the third passage, however, all pigs were positive in at least one sample and up to $1.8x10^3$ gc were quantified. All pigs were positive for ASFV genome on passage four with a maximum $2.6x10^3$ gc in a sample. In passage five, again, all animals were positive for ASFV genome, however with slightly lower genome loads in the different tissues but comparable loads in blood to the previous passages (prolonged monitoring phase of 21 dpi, see Fig. 4). In pig #21, up to $2.6x10^3$ gc were detected (euthanized on 7 dpi).

Investigation of different swab samples showed low viral genome loads in seven out of ten animals in at least one type of swab on seven dpi (maximum of 4 gc in a sample). On 14 dpi, six out of nine animals were positive and three out of nine were positive 21 dpi. Interestingly, higher genome loads were detected from swabs at 21 dpi than in the weeks before (see supplementary table 1).

3.3.2 Whole-genome sequencing

In two samples of passage four, an ASFV variant was detected characterized by a large deletion at the 5'-end of the genome. This deletion of 11197 bp leads to the loss of 18 previously annotated ASFV genes (see Fig. 2 and Table 1 for detailed and functional findings). Interestingly, the deletion is accompanied by a duplication of 18592 bp from the 3'-end of the genome which are bound to the 5'-end in reverse complementary orientation leading to the duplication of 29 genes.

The new virus variant that evolved during *in vivo* passaging was named "ΔMGFnV".

3.3.3 Screening for the novel virus variant ΔMGFnV

Using tailored qPCR, the emergence of the novel virus variant ΔMGFnV was tracked back to pig #1081 of passage one, the spleen of which was part of the organ pool for subsequent passaging (see supplementary table 3). In this animal, a mixed infection of the MSV and ΔMGFnV was observed, while in six other pigs only the wild-type MSV was detected. In the second passage, the ΔMGFnV was detected in two pigs (#1080 monoinfection, #1073 mixed infection with MSV), while three pigs were positive for just the MSV. In passage three, all positive pigs harbored the ΔMGFnV, one individual (#1082) as a monoinfection and nine other animals as mixed infection with the MSV. The picture remained similar after the fourth passage: All pigs were positive for ΔMGFnV, one of which by monoinfection and nine out of ten were also infected with the MSV. In passage five, ΔMGFnV was detected as a monoinfection in four pigs, while the other six animals were coinfected with both the MSV and the variant.

3.3.4 Comparative growth kinetics

The purity of the respective ΔMGF and ΔMGFnV isolates as well as the presence of both isolates in the coinfected cell culture was confirmed by tailored qPCR. After incubation of the virus suspensions (0 hpi), titers between $10^{2.75}$ (MSV + nV, nv) and 10^3 HAD_{50}/ml (MSV) were detected from supernatants in HAT (see supplementary table 4). Increase of titers developed uniformly with a maximum logarithmic deviation of 0.5 at a single point in time. Viruses grew up to $10^{6.75}$ HAD_{50}/ml (MSV and nV monoinfecton) and 10^7 HAD_{50}/ml (coinfection).

When testing samples from the kinetics in the tailored qPCR, for the FAM channel (detection of ΔMGFnV, a mean deviation of 2.9 % was recorded from ASFV genome detection in virotype 2.0. For the HEX channel (nV detection), mean deviation from virotype 2.0 was 8.55% (shown in supplementary table 4). Variant ΔMGFnV reached cq values of roughly 17 in mono- and coinfection at 48 and 72 hpi, while growth of the nV variant yielded cq values of roughly 19 (monoinfection) or 21 (coinfection), at the respective times. Considering the beforementioned deviations in the tailored qPCR, growth kinetics of both isolates developed quite uniformly.

4. Discussion

As the global spread of ASF continues, the situation for pig holders and nature conservationists has never been as tense as it is now. Millions of pigs are at risk, representing the livelihoods of farmers, and entire species of certain wild suids, e.g. bearded pigs, that are now threatened with extinction (Ewers, Nathan et al. 2021). Against this background, a vaccine is urgently needed to complement available control measures. In this context, we may not be in the position to wait for a perfect vaccine candidate and should rather stress the application of a practicable solution as fast as reasonably possible. On the other hand, we cannot afford to compromise on vaccine safety, or as Gavier-Widen, Stahl et al. (2020) put it, allow hasty solutions. Experiences in Spain and Portugal from the last century using attenuated field isolates (Petisca 1965), and possibly very recently in China (https://www.reuters.com/article/us-china-swinefever-vaccines-insight-idUSKBN29R00X, visited May 15th 2022), show us that premature field testing of live vaccines can cause prolonged forms of ASF with extended shedding and delayed clinical

characteristics, often below the detection limit. The use of vaccine viruses with unacceptable residual virulence or that revert to virulence can lead to an iatrogenic, self-sustaining infection cycle with increasing virulence. This would further complicate eradication efforts and these scenarios must be avoided under all circumstances.

Consequently, we took one of the few fully efficacious and possibly licensable vaccine candidates, “ΔMGF”, and examined its safety profile in terms of genetic stability and reversion to virulence under a worst-case scenario.

In our traditional reversion to virulence study over five animal passages, we observed the occurrence of a virus variant in one animal of passage one that subsequently overgrew the wild-type MSV. This variant was genetically characterized by a large deletion at the 5’-end of the genome and an accompanied duplication at the 3’-end. Clinically, the variant was associated with a slightly increased virulence, e.g. induction of a short episode of fever in most animals in the later passages. However, all animals even in the final 5th passage were clinically inapparent at the end of the experiment, showed no evidence of incipient, chronic infection, and showed little or no vaccine virus in the tissues tested.

For further characterization of the “ΔMGFnV”, we conducted comparative *in vitro* growth kinetics of both viruses in primary macrophages, revealing no indications for a significant advantage in *in vitro* replication of the variant virus in this setup. Clear limits in explanatory power should be considered, however, since modifications in the MGF regions are known to have effects on interferon expression (Wang, Kang et al. 2021), and the full consequences of such changes are probably only observable *in vivo*. The underlying factors causing the *in vivo* replication advantages of ΔMGFnV remain therefore unanswered, stressing that many of these questions can only be addressed by further animal experiments due to the highly complex virus-host interactions of ASFV.

Whether our findings showing genetic changes and a slight rise in virulence after *in vivo* passaging disqualify the vaccine candidate is a matter of critical debate, since the mode of transmission is highly artificial (selecting particularly positive samples for further passaging and intramuscular injection of tissue homogenates), and there is no evidence of reversion to the original levels of high virulence of ASFV “Georgia07”. In this context, it could be discussed whether a brief period of fever can be tolerable for an efficacious and therefore otherwise practicable first generation ASFV vaccine. A prerequisite for this assumption would be that the novel variant is genetically stable and does not mark the beginning of a maintained process of further genetic adaption, only mirrored by this first mutation.

The mutant detection qPCR described here was also used retrospectively for representative samples from several efficacy tests with the ΔMGF vaccine candidate, with clearly negative results (data not further shown).

It should be noted, however, that in this case a viral variant with altered geno- and phenotypic properties has already emerged in the first passage, i.e. after application of the MSV. This phenomenon did not occur in any of the previous studies, but it is relevant because it could also happen in the field during

intensive use. It is also remarkable that the mechanism of a large deletion complemented by reorganization of genomic regions has been observed for an ASFV strain under natural circumstances before (Zani, Forth et al. 2018). We may have unraveled a common mechanism of ASFV for genetic adaption when a certain selection pressure is applied.

For a fact-based benefit-risk assessment, further studies with the evolved virus variant are needed, which should address excretion, long-term effects, and transmission to naive contact animals.

In general, our study confirms that even the most promising ASF live vaccine candidates require very comprehensive safety testing (Gavier-Widen et al., 2020). However, it also provides a first indication of what an attenuated ASF vaccine virus would need to do to increase its replication efficacy in the animal or to compensate for deletions in the MGF region. This knowledge can be deepened and used to devise strategies to make these changes even more difficult for the virus.

If field application is considered after benefit-risk-assessment, one should apply genetic tools to differentiate infected from vaccinated animals (DIVA). The PCR described here could aid such approaches. Moreover, conditional licensing under controlled conditions could be a solution to obtain field data for final decisions on the use of the vaccine to complement national control measures.

Declarations

Acknowledgements

The authors would like to thank Ulrike Kleinert, Robin Brandt and Christian Loth for their excellent technical support and further the animal caretakers Matthias Jahn, Steffen Brenz and Domenique Lux for their help in the stable.

Author Contributions

Conceptualization, P.D., E.V., J.C.M., A.U., M.B., and S.B.; methodology, P.D., T.C., J.S.-E., M.B. and S.B..; formal analysis, P.D., T.C., J.S.-E., J.-H.F., and S.B.; investigation, P.D., T.C., J.S.-E. and J.-H.F.; resources, M.B. and S.B..; data curation, P.D. and S.B.; writing—original draft preparation, P.D. and S.B.; writing—review and editing, S.B., E.V., J.C.M., A.U. and M.B.; visualization, P.D., J.-H.F and S.B.; supervision, S.B. and M.B.; project administration, S.B., E.V., J.C.M., A.U. and M.B. funding acquisition, S.B. and M.B. All authors have read and agreed to the published version of the manuscript.

Competing Interests statement

Elisenda Viaplana, Jose Carlos Mancera and Alicia Urniza are employed by Zoetis. Zoetis has a commercial license for the vaccine candidate described in the manuscript. The overall project, coordinated by Sandra Blome at the FLI, received funding by Zoetis, including the salary of Paul Deutschmann. All other authors declare no competing interests.

Ethical Statement

All applicable animal welfare regulations including EU Directive 2010/63/EC complied with for the animal experiment. The animal experiment was approved by the competent authority (Landesamt für Landwirtschaft, Lebensmittelsicherheit und Fischerei (LALLF) Mecklenburg-Vorpommern) under reference number 7221.3-2-020/20.

Data Availability Statement

All relevant data to support the findings described in the text are included in the main text or in the supplementary materials. Additional data is available from the corresponding author upon reasonable request.

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Figures

Figure 1

Overview of the passaging groups and the study design. Tissues used from the respective animals for further passaging are depicted as organs in the center. Clinical and virological results throughout the study are visualized on the right side. Created with BioRender.com

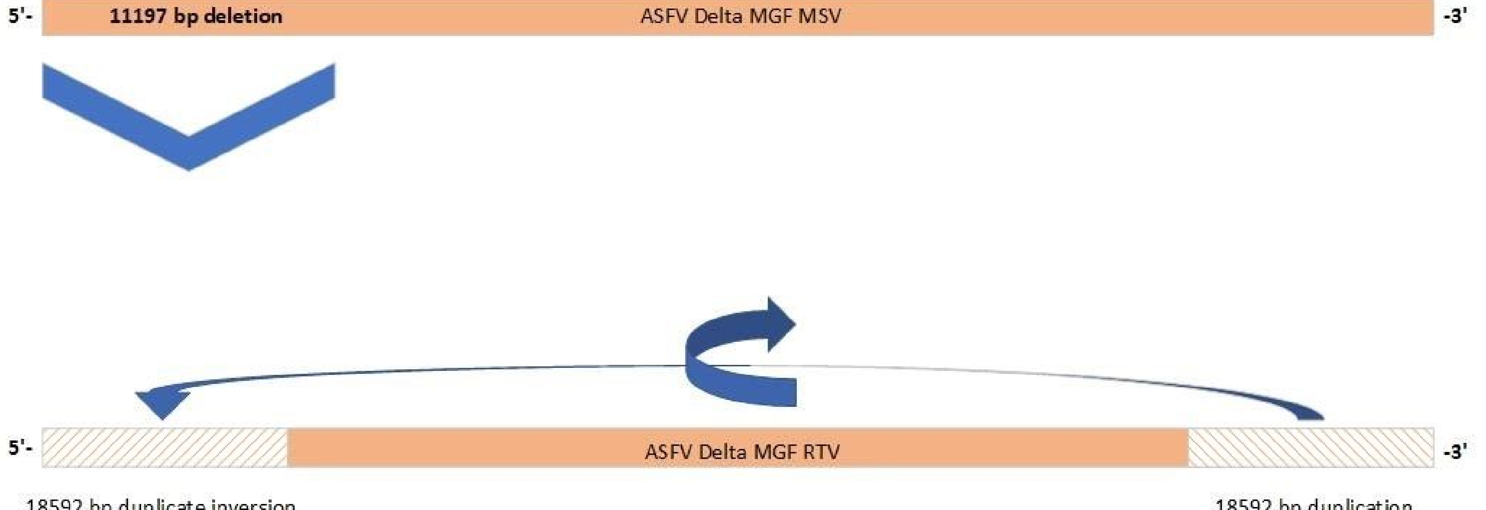

Figure 2

Schematic overview of the mutation characterized by duplicate inversion identified in the novel variant. The genome of "DMGF" with the deletion is depicted on top and the genome of the "DMGFnv" with the duplicate inversion mutation is represented at the bottom.

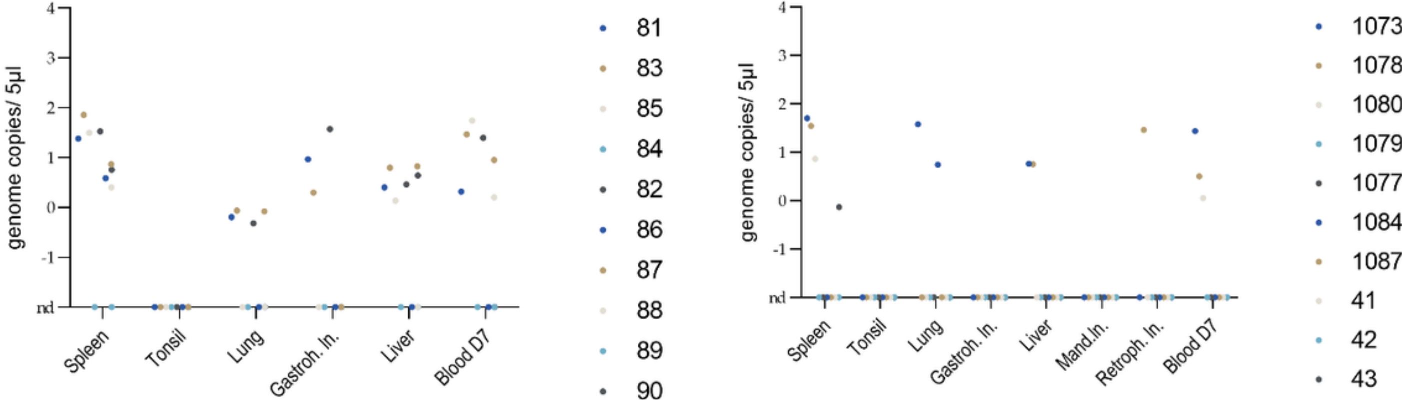

Figure 3

Genome load in the sample sets in passage 1 (top left), 2 (top right), 3 (bottom left) and 4 (bottom right) after 7 days of observation. Individual blots mark tissue samples from respective animals in each passage.

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