

Duration of Immunity following oral and intramuscular Immunization with ASFV-G- Δ I177L

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Article

Keywords: Domestic Pig, African Swine Fever Virus, Vaccine, Live Attenuated Vaccine, Immunity, Duration of Immunity

Posted Date: September 16th, 2026

DOI: <https://doi.org/10.21203/rs.3.rs-10876664/v1>

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Additional Declarations: Competing interest reported. VF, KM, MBe, SB, and AS are currently involved in (or employed by) the EU-funded project ASFaVIP. This project investigates the potential of the live-attenuated vaccine candidate ASFV-G- Δ I177L for licensing on the European market. Members of FLI have no direct financial interest in the candidate ASFV-G- Δ I177L. Members of Zoetis, namely EV, and MBa produced and provided the experimental vaccines (passages of master seed virus) for this study. Zoetis holds a non-exclusive license to produce commercial vaccines based on ASFV-G- Δ I177L and is an associate partner in the abovementioned EU project. The remaining author(s) declared that this work was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Abstract

African swine fever virus (ASFV) remains a global threat to pig production and wildlife conservation. While selected Asian countries approved live attenuated vaccines like the deletion mutant *ASFV-G-ΔI177L*, a licensed vaccine for the European market is pending, requiring evaluation of efficacy and duration of immunity. Therefore, 15 domestic pigs each were immunized orally or intramuscularly with *ASFV-G-ΔI177L*. Vaccination was well tolerated and induced seroconversion in all intramuscularly and eight orally immunized animals. *ASFV-G-ΔI177L* was detectable for up to four months. ASFV-specific antibodies initially declined but remained detectable throughout the observation period. Six months after vaccination, all animals were challenged with highly virulent ASFV *Armenia08*. Control animals, oral non-responders, and two intramuscularly immunized pigs reached humane endpoints, whereas all orally immunized responders and the remaining intramuscularly immunized animals were protected. Orally immunized animals generally exhibited lower viral loads and shedding. Furthermore, surviving animals largely cleared challenge virus within 28 days, although signs of an unrelated concurrent infection were observed. These findings show that *ASFV-G-ΔI177L* confers protection against virulent ASFV challenge for six months, while indicating that protective immunity may begin to decline around this time.

1 Introduction

African swine fever (ASF), caused by the ASF virus (ASFV), poses a major threat to domestic pig production worldwide. The ongoing panzootic is caused by highly virulent genotype II ASFV strains, which typically induce an acute hemorrhagic disease with high case fatality in infected pigs [1]. Currently, control measures are largely limited to outbreak responses, e.g., by culling affected herds and implementation of movement and trade restrictions, but effective preventative measures beyond biosecurity are still missing [2]. The most promising prevention strategy would be the use of safe and protective vaccines, preferably also administrable orally to immunize wild boar and contain viral spread in the field [3, 4]. While many different vaccine approaches have been assessed, the only efficacious and protective candidates against ASFV so far were live attenuated vaccines (LAVs) [4, 5]. These usually rely on deletion of single or multiple viral genes, but development is hindered by imperfect understanding of both ASFV gene function and genome organization, as well as host responses against ASFV [6, 7]. Still, several protective LAV candidates have been proposed in the past, and two, *ASFV-G-ΔI177L* and *ASFV-G-ΔMGF*, are already commercially licensed in Vietnam, and undergo licensing procedures in several other countries, including India, Malaysia, and Myanmar [4, 5, 8].

Among the available LAV candidate vaccines, *ASFV-G-ΔI177L* has been studied most extensively. Originally generated by Borca *et al.* in 2020, it has shown its robust protection in numerous subsequent studies [9–14]. However, while *ASFV-G-ΔI177L* demonstrated an overall beneficial safety profile in young animals, later studies revealed negative impacts of immunization in older pigs [15, 16]. In addition, LAVs carry inherent risks, like reversion to virulence or recombination with wildtype viruses. Such events were recently reported in studies with adult boars, pregnant sows, and Vietnamese field isolates that carried LAV-derived genes despite no history of vaccination in the respective region [15–18]. These results highlight the need for thorough safety assessment of every LAV candidate prior to widespread use. However, given the severity of

ongoing ASFV outbreaks, and the risk of further spread and introduction into new pig populations, the benefit-risk assessment of ASFV-G- Δ I177L might overall still be favorable [19].

To secure market authorization within the European Union, regulatory authorities require comprehensive safety and efficacy evaluations through a series of mandated studies. European Union Regulation 2019/6, the European Pharmacopoeia, the World Organization for Animal Health (WOAH) Terrestrial Manual, and related guidelines stipulate that ASFV vaccine safety assessments must be conducted in the target species under controlled experimental conditions. These evaluations should include dose-ranging studies and repeated administrations, with endpoints encompassing the observation of clinical signs, assessment of transmission potential, quantification of viremia, analysis of tissue dissemination, evaluation of genetic stability following serial passages, and duration of immunity in immunized animals.

In this study, the duration of immunity after oral and intramuscular immunization with ASFV-G- Δ I177L was assessed six months after vaccination by challenge infection with the highly virulent ASFV *Armenia08*. This trial was conducted in accordance with VICH (International Cooperation on Harmonization of Technical Requirements for Registration of Veterinary Medicinal Products) guideline 44 (Guideline on duration of immunity achieved by veterinary vaccines, EMA/CVMP/IWP/224985/2025). We investigated clinical signs, viremia, viral shedding and dissemination, and humoral responses after immunization and challenge infection, following standard regulatory guidelines for veterinary vaccine safety studies. By characterizing the duration of immunity of the promising LAV candidate ASFV-G- Δ I177L, this study provides essential data for a future licensing process and vaccine research.

2 Materials and Methods

2.1 Animal Husbandry

Prior to obtaining animals, the present study was approved by the competent regional authority, the State Office for Agriculture, Food Safety and Fishery in Mecklenburg-Western Pomerania (LALLF MV), filed under reference number VI-722-00000-2025/105-001. This research was conducted in accordance with European and German animal welfare regulations, including EU Directive 2010/63/EC and institutional guidelines. The animals were purchased from a commercial vendor (Gut Schweinezucht Alt Gaarz/Blücherhof GmbH & Co. Landwirtschafts-KG) and kept in the high containment facility at Friedrich-Loeffler-Institut (FLI) Riems, Germany. Prior to the study, all animals tested negative for ASFV genome and ASFV-specific antibodies. The animals underwent an acclimatization phase of one week prior to immunization. Initially, 30 6-week-old Large White domestic pigs of both sexes were kept in two stables (each with 15 animals and 24 m²). At the age of approximately 6 months, the animals were divided into a total of four stables, each containing groups of seven or eight animals. Five additional age-matched Large White domestic pigs of both sexes from the same vendor were added to the study prior to challenge infection. They were kept together with the other animals in separate compartments within two stables together with the previously immunized animals.

2.2 Experimental Design and Sample Preparation

The animals were divided into two groups with 15 domestic pigs each and immunized orally or intramuscularly with 1 ml of 10^6 HAD₅₀/ml or 1 ml of 10^2 HAD₅₀/ml ASFV-G- Δ I177L, respectively (Fig. 1A), and observed for six months. The vaccine virus was prepared to industry-grade standards by Zoetis and shipped to the study facility ready-to-use. Back titration confirmed vaccine virus titers. Due to a low response rate in the oral group, all animals in the oral group received an oral booster immunization with the same dose one month after the initial immunization. In accordance with international guidelines, and reflecting the lifetime of a fattening pig, the duration of immunity was assessed by highly virulent challenge infection six months after the initial immunization. For this, five additional age-matched animals were added as infection controls, and all animals underwent oro-nasal challenge infection with 10 ml of 10^5 HAD₅₀/ml ASFV *Armenia08*.

After immunization and challenge infection, animal well-being, rectal temperature, and potential clinical signs were individually assessed daily, and evaluated by a clinical scoring system involving various parameters of animal behavior and potential disease progression that was previously established at our institute for immunization trials involving this and other vaccine candidates [15]. Animals were euthanized upon reaching a moderate endpoint, defined as reaching an overall sum of 10 score points or more, having fever (rectal temperature > 40.0°C) lasting over three days with or five days without additional clinical signs, or when clinical signs persisted without improvement and likely caused intolerable suffering.

Samples (nasal and oral swabs, blood) were initially taken at weekly intervals (7, 14, 28 days post vaccination, dpv) for the first month and then monthly until challenge infection six months (168 dpv) after immunization. After challenge, samples were taken 4, 7, 14, and 28 days post challenge (dpc). For sampling, the animals were restrained and blood was drawn from the jugular vein (*Vena jugularis externa*) using aspiration tubes (KABE Labortechnik), and swabs were taken from the nasal and oral cavity. Blood was further prepared for virus detection (EDTA blood) or serology (serum).

Upon reaching the moderate endpoint or at the end of the observation period 28 dpc, all animals were subjected to necropsy. Animals were sedated using a combination of 1 mg/kg azaperone (Stresnil, Elanco) and 2.2 mg/kg ketamine (cp-pharma). Euthanasia was conducted by intracardial injection of pentobarbital sodium (Release, WDT). Several tissues were sampled and subjected to laboratory analyses (tonsil, salivary gland, lung, liver, spleen, kidney, bone marrow) as well as several lymph nodes (LN): *cervicalis superficialis* (LN cs) mandibular (LN mand), *retropharyngeales mediales* (LN medret) gastrohepatic (LN) gh, and inguinal (LN ing). To obtain homogenates, an organ piece (~ 200 mg) was placed in a 2 mL centrifugation tube with 1 mL PBS and a 5 mm metal bead, then subjected to homogenization at 30 Hz for 3 min using a tissue lyzer (TissueLyzer II, Qiagen).

2.3 DNA Extraction and PCR Analysis

100 μ l EDTA-blood or organ homogenates were used for nucleic acid extraction using the NucleoMag VET Kit (Macherey-Nagel) on a KingFisher Flex 96 system (Thermo Fisher Scientific) according to the manufacturer's instructions. ASFV-negative serum was used as a negative extraction control. Presence of ASFV genomes was assessed by real-time quantitative PCR (qPCR) using the virotype ASFV 2.0 PCR kit

(Indical Bioscience) on a CFX Opus real-time PCR cycler (Bio-Rad Laboratories) according to the manufacturer's instructions. ASFV genome copy numbers were calculated by utilizing a PCR standard containing a defined amount of extracted ASFV DNA (8×10^7 genome copies/ml) in RSB50 buffer (50 ng/ μ L carrier RNA, 0.05% Tween 20, 0.05% sodium azide in RNase-free water).

To differentiate vaccine and challenge virus genomes in samples collected after challenge infection, a quadruplex PCR assay targeting *B646L* (p72), *I177L*, *mCherry*, and porcine β -actin (*ACTB*) as internal control was established. The assay enabled discrimination between of ASFV-G- Δ I177L (*B646L*+, *I177L*-, *mCherry*+), ASFV *Armenia08* (*B646L*+, *I177L*+, *mCherry*-), while simultaneous detection of *I177L* and *mCherry* indicated the presence of both viruses in the same sample. Reactions were performed in a total volume of 25 μ L with 5 μ L DNA template, and primer and probe sequences together with their final concentrations are listed in Table 1. Cycling conditions consisted of an initial Taq polymerase activation at 95°C for 15 min, followed by 45 cycles of denaturation at 95°C for 60 s and annealing/extension at 60°C for 60 s, with fluorescence signals (FAM, HEX, TEX, and Cy5) recorded during the annealing/extension step.

Table 1
Sequences of primers and probes for the quadruplex PCR for ASFV strain identification.

Target	Primer/ Probe	Sequence (5'--> 3')	Fluorophore/Quencher	Final concentration [nM]
<i>B646L</i>	Forward	TGCTCATGGTATCAATCTTATCG	FAM/BHQ1	800
	Reverse	CCACTGGGTTGGTATTCCTC		800
	Probe	TTCCATCAAAGTTCTGCAGCTCTT		100
<i>I177L</i>	Forward	GCATAATTATCAAATGCGAAGG	HEX/BHQ1	400
	Reverse	TGTGGAAAGTTAATGATCAGGG		400
	Probe	TCCGTATAAAATCCTAGCTTGCCGGT		100
<i>mCherry</i>	Forward	GCTTCTTGGCCTTGTAGGTG	Texas red/BHQ2	400
	Reverse	CAGAGGCTGAAGCTGAAGGA		400
	Probe	CGGCGGCCACTACGACGCTG		100
β -actin	Forward	AGCGCAAGTACTCCGTGTG	Cy5/BHQ2	200
	Reverse	CGGACTCATCGTACTCCTGCTT		200
	Probe	TCGCTGTCCACCTTCCAGCAGATGT		100

2.4 Hemadsorption Test and Virus Isolation

Hemadsorption Tests (HAT) assessed both the viral titer of the ASFV vaccine and the presence of infectious ASFV in tissue samples. PBMCs from EDTA blood of a healthy donor pig were isolated using a

1:10 mix with 10% Hanks dextran (Sigma-Aldrich), incubated 90 min at room temperature, washed twice with PBS containing 2.5 mM EDTA, and resuspended in cell culture media (Dulbecco's Modified Eagle's Medium, supplemented with 10% fetal calf serum and 0.01% Penicillin/Streptomycin, Gibco). Animal-specific erythrocytes were diluted 1:10 and stored at 4°C. PBMCs were seeded at 5×10^5 cells/well in 96-well plates with and incubated at 37°C with 5% CO₂ for 24 h. Recombinant porcine colony-stimulating factor 2 (CSF2, 2 ng/ml, Biomol) was added for maturation, and after another 24 h, samples for virus titration were added. Post-inoculation, donor-specific erythrocytes were added at 1:40 ratio to detect rosette formation on infected monocytes/macrophages, evaluated after 24 and 48 h.

Detection of infectious virus was performed in tonsil, salivary gland, spleen, and mandibular lymph nodes. To enhance detection sensitivity in samples with low viral quantities, a blind propagation step of all samples tested was implemented prior to viral load quantification. For this process, PBMCs were seeded into a 24-well culture plate at a density of 2.5×10^6 cells per well, in the presence of 2 ng/ml CSF2. After 24 h, 100 µl of the respective organ homogenate were added in duplicate and incubated for 72 h. Post-incubation, plates were frozen at - 80°C for a minimum of 24 h to induce cell lysis and ASFV release. Subsequently, supernatants containing infectious virus were subjected to HAT. Each technical replicate ($n = 2$) of the blind passage was independently tested in four replicates ($n = 4$) via HAT.

2.5 Serology

Antibody kinetics were assessed with two commercial ELISAs, detecting antibodies against ASFV-p32 (ID Screen African Swine Fever Competition; ID.vet) or ASFV-p72 (Ingezim PPA COMPAC; Gold Standard Diagnostics) according to the manufacturers' instructions.

Serum samples were also evaluated by immunoperoxidase test (IPT), which served as the serological reference assay due to its superior sensitivity. IPT was done following the European Union Reference Laboratory for ASF (EURL-ASF) protocol for the detection of antibodies against ASFV by IPT (SOP/CISA/ASF/IPT/1, 2018) with slight modifications regarding the strain and cell type. Samples positive in IPT were titrated in twofold dilutions, starting at 1:40, to determine semi-quantitative antibody titers of each positive serum sample.

2.6 Next-Generation Sequencing

Unbiased metagenomic sequencing

Lung tissue homogenates from two pigs (samples #86 and #91) were subjected to unbiased metagenomic analysis at the FLI. For each sample, 250 µL of mechanically homogenized lung tissue lysate was mixed with 750 µL TRIzol LS (Thermo Fisher). RNA was extracted using the RNAdvance kit (Thermo Fisher), quantified using a NanoPhotometer (Thermo Fisher), and concentrated using Agencourt RNAClean XP beads (Beckman Coulter). Double-stranded cDNA was generated using SuperScript IV (NEB). The resulting nucleic acids were fragmented using a M220 focused ultrasonicator (Covaris), purified and concentrated using Agencourt AMPure XP beads, and used for library preparation with the Next Fast DNA Library Prep Set (NEB) for Ion Torrent (Thermo Fisher). Libraries were purified, size selected using AMPure XP beads, quality

assessed using the Agilent High Sensitivity DNA kit, and quantified using the QIAseq Library Quant Assay Kit for Ion Torrent.

Template preparation was performed using the Ion Chef system, followed by sequencing on an Ion Torrent S5 XL platform. Sequence data were processed using an extended version of the RIEMS pipeline. Putative viral reads were manually validated by BLASTn searches against the NCBI nucleotide database. In addition, reads without initial taxonomic assignment, together with reads classified as viral, were assembled into longer contiguous sequences and analyzed by BLASTn against the NCBI nucleotide database. Where appropriate, translated amino-acid sequences were queried against the NCBI non-redundant protein database using BLASTp. The analytical workflow followed previously described principles for diagnostic metagenomic analysis and RIEMS-based taxonomic classification.

2.7 Data Visualization

The differences between the groups are presented for descriptive purposes but were not examined further. Visualization was performed using GraphPad Prism 10.6 (GraphPad Software Inc., Dotmatics, USA). Unless otherwise indicated, data are shown as mean (SD).

3 Results

3.1 Clinical course of oral or intramuscular immunization with *ASFV-G-ΔI177L*

Two groups of 6-week-old domestic pigs ($n = 15$ each) were orally or intramuscularly immunized once with 10^6 HAD₅₀/ml or 10^2 HAD₅₀/ml *ASFV-G-ΔI177L*, respectively (Fig. 1A). Viremia in orally immunized animals was generally short (up to one month post immunization) and to only moderate levels (up to 10^7 genome copies/ml, Fig. 1B). Since only four animals were viremic at least once after immunization, it was decided to administer a booster immunization to this group 28 dpv, identical to the initial immunization (10^6 HAD₅₀/ml *ASFV-G-ΔI177L*, orally). This increased the number of animals with detectable ASFV presence to six, which were both viremic at the next sampling point (56 days after initial immunization, 28 days after boost). This booster immunization was also not associated with any clinical signs (Supplementary Fig. 1A). All intramuscularly immunized animals were viremic 14 dpv at the latest and at least once during the sampling period and were therefore not boosted (Fig. 1B). Some animals were only transiently viremic and cleared the vaccine virus within a few weeks (56 dpv). However, some intramuscularly immunized animals with viral loads up to $\sim 10^{10}$ genome copies/ml blood early after immunization remained viremic for months (up to 112 dpv).

Both oral and intramuscular immunization were well tolerated (Fig. 1C). No clinical signs or fever were observed after oral immunization. Intramuscular immunization resulted in transient fever up to 41°C 8–12 days post vaccination (dpv) in some animals. However, no or only minor additional clinical signs, e.g. mild fatigue, were observed in these animals. Low and transient levels of ASFV genomes were detected in nasal and oral swabs (Supplementary Fig. 1B), mainly after intramuscular immunization 7 dpv. All orally immunized animals, except one, were ASFV-positive 28 dpv, but this was because the animals were not separated after sampling and booster application to increase potential contacts to the vaccine virus.

3.2 Clinical course of challenge infection with highly virulent ASFV *Armenia08* six months after oral or intramuscular immunization with ASFV-G- Δ I177L

Six months after the initial immunization, all immunized animals and five age-matched naïve control animals from the same breeding facility were oro-nasally infected with highly virulent ASFV *Armenia08* (10 ml of 10^5 HAD₅₀/ml). At the time of infection, all animals were free of vaccine virus in blood and swabs. All control animals as well as eight of the orally immunized animals developed fever up to 42°C and moderate clinical signs within a few days, and had to be euthanized at the moderate endpoint within a week after challenge infection (Fig. 2A). Of note, two intramuscularly immunized animals developed fever and clinical signs as well and had to be euthanized 7 and 21 dpc. One of them, euthanized 7 dpc, was unable to fight off the challenge infection and was viremic concurrently with the unprotected non-responders. The other animal, euthanized 21 dpc, was the one viremic for the longest time after immunization.

Many of the remaining intramuscularly immunized animals displayed intermittent fever and unspecific clinical signs (mainly lethargy and coughing) between 7 dpc and the end of the study 28 dpc (Fig. 2B). During necropsy, the lungs displayed lesions but were overall inconclusive. Samples of lung lesions from two animals were investigated by metagenomic analysis in next-generation sequencing (NGS) and revealed a viral pathogen (Teschovirus, *Picornaviridae*), which was found false positive by specific PCR. Bacterial reads were mostly qualitative, not quantitative (e.g. *Burkholderiaceae*, *Enterobacteriaceae*, *Phyllobacteriaceae*) and were found to be unlikely to have caused the pneumonia lesions and clinical signs observed. *Mycoplasmataceae* rendered 7.000 and 23.000 reads and together with the pathological findings of patchy discoloration in the lung, it is most likely that these pigs had mycoplasma-associated pneumonia.

All animals that were ultimately euthanized at the humane endpoint after challenge infection displayed high viral loads in the blood (up to $\sim 10^{10}$ genome copies/ml). Animals that survived the challenge infection were only transiently viremic and cleared the virus within 14 days (Fig. 2C). However, there were high viral loads detected in nasal and oral swabs after challenge in virtually all animals, predominately 7 dpc (Fig. 2D). Viral loads in naïve control pigs were similar to oral non-responders (Supplementary Fig. 2A)

3.3 Viral dissemination after challenge infection with ASFV *Armenia08*

During necropsy at the humane endpoint or at the end of the study, various samples of lymphatic and non-lymphatic tissues were collected and investigated for presence of genome or infectious ASFV (Fig. 3). Viral loads were generally comparable in animals that were euthanized within the same timeframe, irrespective of immunization route (Fig. 3A). High levels of ASFV were detected in control animals as well as in oral non-responders. Of note, the intramuscularly immunized animal that was euthanized 7 dpc displayed comparable ASFV loads in its tissue. Animals that were sampled at the end of the study were largely negative for ASFV (Fig. 3A), with only few lymph nodes or other tissue still showing low ASFV loads. Infectious ASFV was detected in all animals euthanized at the humane endpoint (Fig. 3B). Viral loads in animals sampled at the end of the study were too low for detection of infectious virus. To discriminate whether the virus found after challenge was ASFV *Armenia08* or reactivated ASFV-G- Δ I177L, all blood samples after challenge were subjected to a qPCR detecting B646L (p72), I177L, and mCherry. All samples

tested positive for *B646L* and *I177L* (present in both *ASFV-G-ΔI177L* and *ASFV Armenia08*) but negative for *mCherry* (present only in *ASFV-G-ΔI177L*), confirming presence of challenge virus (Supplementary Table 1).

3.4 Antibody kinetics after oral or intramuscular immunization with *ASFV-G-ΔI177L* and challenge infection with highly virulent *ASFV Armenia08*

The serological responses after oral or intramuscular immunization and highly virulent challenge infection were investigated employing two commercially available ELISAs (against ASFV-p32 and ASFV-p72), as well as an indirect immunoperoxidase test (IPT, Fig. 4). All animals responded to intramuscular immunization within 14 days at the latest and were clearly positive in all assays. In contrast, oral immunization was less efficient, with only four responders after the initial immunization until 28 dpv. After booster immunization, four additional animals seroconverted, two of them without prior detection of vaccine virus. Antibody levels of two animals fell below the assay thresholds in both commercial ELISAs around 140 dpv and were negative at the time of challenge infection. A similar trajectory was not observed in the IPT. However, antibody levels in some animals decreased up to 10,000-fold over the course of the study and did not recover after challenge infection. Moreover, there was considerable variance within each group, with antibody titers varying 100- to 1,000-fold between the animals.

There was a minor boost detectable after challenge infection in all serological assays (Fig. 4A-D). The two orally immunized animals that were negative in both commercial ELISAs were positive again 14 dpc at the latest. Naïve control pigs did not seroconvert after infection with *ASFV Armenia08* (Supplementary Fig. 2B). Overall, the antibody levels in responding animals were similar in both groups, and orally immunized animals that responded to the vaccine did not differ considerably from intramuscularly immunized animals.

4 Discussion

African swine fever remains one of the most devastating transboundary diseases of pigs, with prevention still relying largely on containment measures in the absence of an approved vaccine in most regions. Vaccine candidates, particularly LAVs like *ASFV-G-ΔI177L*, currently represent the most advanced approach, yet key knowledge gaps remain regarding long-term immunity and safety under field-relevant conditions. Here, we assessed the duration of immunity induced by the LAV candidate *ASFV-G-ΔI177L*. In accordance with international regulations and guidelines, two groups of domestic pigs were orally or intramuscularly immunized with *ASFV-G-ΔI177L* and challenged with highly virulent *ASFV Armenia08* six months later to cover the usual lifespan of a domestic fattening pig. While most responders were protected from fatal disease, some intramuscularly immunized animals had to be euthanized despite having comparable results in laboratory assessments as survivors. Interestingly, we found considerable viral loads in nasal and oral swabs early after challenge in non-responders of both groups, and intramuscularly immunized but not orally immunized animals. Moreover, surviving animals displayed mild to moderate clinical signs unrelated to ASF until the end of the study.

Oral immunization was overall less efficient in inducing responses. However, all orally immunized animals that eventually responded (*i.e.*, seroconverted) were protected from highly virulent challenge infection, while some intramuscularly immunized animals succumbed despite seroconversion. Why a proportion of orally

immunized animals failed to mount a protective immune response remains unclear. Unsuccessful establishment of the vaccine virus infection cannot be excluded and may represent the most plausible explanation. The underlying causes could include administration-related factors as well as individual differences in the oral environment or early virus-host interactions, all of which warrant further investigation. Relatively high ASFV loads in all swabs of orally immunized animals during the immunization itself at least exclude immediate viral clearance as a cause. Interestingly, immunization by the oral route was initially better tolerated and protection against oro-nasal challenge infection was ultimately more robust, *i.e.*, all orally immunized responders survived and were able to prevent viremia in most cases. Nevertheless, all but one animal were positive for ASFV in nasal and oral swabs post-challenge. This reflects the superior efficacy of mucosal immunity induced by oral immunization, largely restricting ASFV to the oro-nasal cavity while preventing systemic spread, despite overall lower viral loads after immunization and decreasing antibody levels. The key advantage of oral immunization is its multi-layered protection conferred by secretory antibodies and tissue-resident memory lymphocytes: first, in the mucosal surface, effectively inhibiting or blocking viral entry and spread, and second, systemically, providing a failsafe in case mucosal defenses are breached [20]. This has been shown to induce well-tolerable but still more robust protection by various vaccines, arguably most prominently and successfully in the human polio vaccine, one of the major successes of vaccinology to date [21]. Given that natural ASFV infection is thought to occur predominantly via the oro-nasal route, these findings further support oral immunization as a promising strategy for controlling ASF in free-ranging wild boar populations. Although oral vaccination is inherently more variable than intramuscular administration and several aspects of early vaccine-host interactions remain poorly understood, these limitations are not unique to ASFV and have not precluded the successful implementation of oral vaccination strategies against other animal diseases, *e.g.*, rabies or classical swine fever. Achieving sufficiently high vaccine titers remains an important consideration for bait development and large-scale production [22, 23].

Prolonged non-ASF disease after challenge mainly presented as respiratory disease with mild lethargy, reduced feed uptake, and recurring mild fever. This might indicate a compromised immune response against pathogens newly introduced by comingled control animals after challenge infection, manifesting in a reduced ability to clear pathogens. These observations are reminiscent of post-acute infection syndrome (PAIS), which describes persistent unspecific symptoms after complete or partial clearance of the initial pathogen and was most recently discussed in the context of SARS-CoV-2 but has also been described for numerous other (human) pathogens [24]. Major PAIS symptoms include fatigue and flu-like symptoms, which in pigs might manifest as lethargy, fever, respiratory disease like coughing, or reduced feed uptake, which were the main clinical signs observed in most animals after challenge in this study. While the underlying mechanisms remain unclear, the main hypotheses include pathogen remnants (undetectable in standard assays), autoimmune responses, and tissue damage [24]. While immunopathology has been hypothesized earlier, induction of autoimmunity during ASF in pigs has never been explored. The more favorable tolerability and apparently more robust protection observed in orally immunized responders may be related to the induction of immune defenses at the portal of entry. Because ASFV infection is thought to occur predominantly via the oro-nasal route, local mucosal immunity—including secretory antibodies and tissue-resident memory lymphocytes—could promote rapid containment of challenge virus at the mucosal surface and thereby limit systemic dissemination, while systemic immunity provides an additional layer of

protection. Dysregulated immunity during infection, often observed and discussed during ASF [7, 25–28], have been shown to induce autoimmune responses, *i.e.*, autoantibodies targeting tissue or cytokines, autoreactive mononuclear cells, or autoreactive T cells, either by molecular mimicry, bystander activation, or release of self-antigens in inflammatory environments (epitope spreading) [29, 30]. These processes might explain at least some of the observed immunopathology during ASF but require further studies.

Few other long-term studies investigating ASF have been done so far. In one, the naturally attenuated ASFV *OURT88/3* was evaluated as a potential LAV candidate. However, intramuscular immunization did not induce robust protection against the highly virulent ASFV *Benin 97/1* five months (130 dpv) after inoculation [31]. In a study more closely related to this, we recently examined the protective potential of another naturally attenuated ASFV strain, ASFV *Estonia14*, in a long-term challenge study with the same highly virulent challenge virus as in this study (ASFV *Armenia08*) [32]. Oro-nasal inoculation with ASFV *Estonia14* induced robust immunity in all animals for at least six months, as evidenced by complete clinical protection. However, despite being clinically inapparent, some animals shed infectious ASFV *Armenia08* after challenge [32]. Interestingly, another study with a similar setup (inoculation with ASFV *Estonia14* and challenge infection with ASFV *Armenia08*) but using conventionally-reared and specific-pathogen-free (SPF) domestic pigs found somewhat contrasting results: while SPF pigs remained healthy after challenge infection, all conventionally-held pigs displayed significant clinical disease and a 60% lethality rate [33]. Despite the apparent similar setup, there were considerable differences between both studies, as we discussed previously [32]: besides husbandry conditions and microbiome composition, there were differences in the age at immunization, the interval between immunization and challenge, and doses used for immunization and challenge infection [32, 33]. In the present study, while there was a 10,000-fold difference in the immunization titers (2×10^6 HAD₅₀/ml orally vs. 10^2 HAD₅₀/ml intramuscularly), the number of infectious units that successfully established infection remains unknown, especially after oral immunization. Likewise, although intramuscular vaccination resulted in high levels of vaccine virus replication (up to $\sim 10^{10}$ genome copies/ml), this alone was not predictive of long-term protection, as the animal with the most prolonged viremia post-vaccination ultimately succumbed to challenge infection. In line with this, previous studies indicated that the amount of available antigen in the host, either administered directly or induced by LAV or mRNA, needs to be neither too low nor too high to induce protective immunity with robust recall and effector functions [34–36]. This is difficult to realize and assess for LAVs.

The present work assessed humoral responses only. Future studies investigating the cellular immune response might show additional differences between orally and intramuscularly immunized animals. Although antiviral responses against ASFV are not fully understood, it is undisputed that T cells play a major role [7, 37]. Given the overall superior results of oral immunization in responders, a stronger focus on mucosal immune responses and tissue-resident T cells might lead to valuable insights. Induction of long-lasting cellular immunity at the site of viral entry would confer better protection

Overall, ASFV-G- Δ I177L conferred substantial protection against an otherwise lethal ASFV challenge for around six months after immunization, with all orally responding animals and the majority of intramuscularly immunized animals remaining protected. The loss of protection in two intramuscularly vaccinated pigs suggests that protective immunity may begin to decline in some individuals around six

months post vaccination, as previous studies report full protection after earlier challenge infection [12, 13]. Alternatively, intramuscular immunization might not confer full protection in all vaccinated pigs in this age cohort and with the dose used in this study. These findings further emphasize that correlates of durable protective immunity against ASFV remain incompletely understood. Defining these correlates will be essential for the rational optimization of vaccination strategies and will support the further development, evaluation and long-term implementation of ASF vaccines.

Declarations

Conflict of Interest

VF, KM, MBe, SB, and AS are currently involved in (or employed by) the EU-funded project ASFaVIP. This project investigates the potential of the live-attenuated vaccine candidate *ASFV-G-ΔI177L* for licensing on the European market. Members of FLI have no direct financial interest in the candidate *ASFV-G-ΔI177L*. Members of Zoetis, namely EV, and MBa produced and provided the experimental vaccines (passages of master seed virus) for this study. Zoetis holds a non-exclusive license to produce commercial vaccines based on *ASFV-G-ΔI177L* and is an associate partner in the abovementioned EU project.

The remaining author(s) declared that this work was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author Contributions

AS: Conceptualization, Methodology, Investigation, Visualization, Formal analysis, Writing – review & editing.

KM: Methodology, Investigation, Formal analysis, Writing – review & editing.

PD: Investigation, Writing – review & editing.

SW: Investigation, Writing – review & editing.

MBa: Resources, Investigation, Writing – review & editing.

EV: Conceptualization, Resources, Investigation, Writing – review & editing.

DH: Resources, Investigation, Writing – review & editing.

MBe: Conceptualization, Writing – review & editing, Supervision.

SB: Conceptualization, Investigation, Funding acquisition, Writing – review & editing, Supervision.

VF: Conceptualization, Methodology, Investigation, Formal analysis, Visualization, Writing – original draft, Writing – review & editing, Supervision.

Funding

This research was funded by the EU Horizon Europe grant ASFaVIP (African Swine Fever attenuated live Vaccines In Pigs) via the HORIZON-CL6-2023_FARM2FORK-01 call under ID 101136676.

Acknowledgments

The authors thank Matthias Jahn, Dominique Lux, Patrice Mary, and Ramona Klüsener for outstanding animal caretaking, Ulrike Kleinert for excellent technical assistance, and Andreas Bath and Marco Beerbohm for exceptional support during necropsies. We also thank Theresa Holzum, Isabella Hrabal, Thaddäus Ebinger, and Frank Fischer for their help during sample collection and preparation. We also thank the members of the ASFaVIP consortium for their contributions to discussions prior to and during this trial.

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Figures

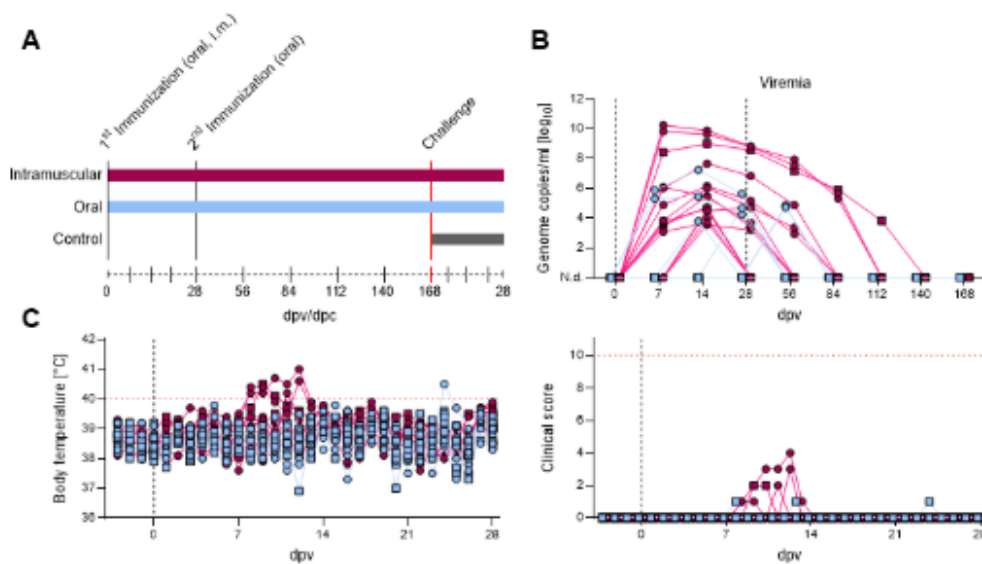


Figure 1

Study design and clinical course after oral or intramuscular immunization with ASFV-G-ΔI177L. (A)

Illustration showing the overall experimental setup. Two groups of domestic pigs were immunized either intramuscularly (red) or orally (blue) with ASFV-G-ΔI177L and observed for six months (168 days) before highly virulent challenge infection. Control animals (grey) were added prior to challenge infection. **(B)** Viremia levels assessed by qPCR and **(C)** Clinical signs, assessed by body temperature (left) and clinical score (right), after oral or intramuscular immunization with ASFV-G-ΔI177L. Animals that ultimately succumbed after challenge infection are shown as squares, surviving animals are shown as circles. dpv, days post vaccination; dpc, days post challenge.

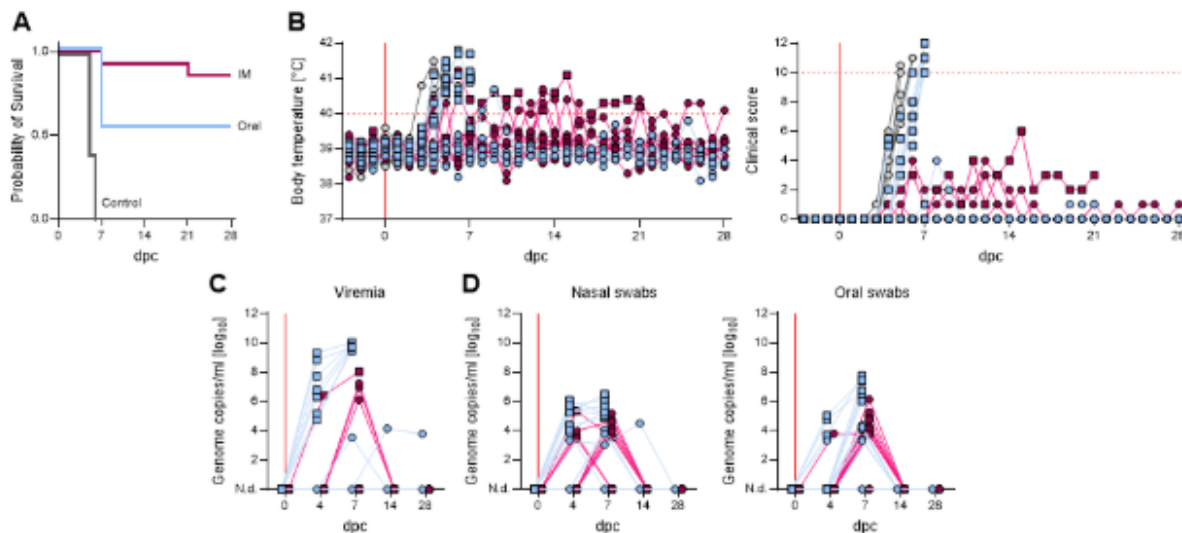


Figure 2

Clinical course of challenge infection with highly virulent ASFV *Armenia08* six months after oral or intramuscular immunization with ASFV-G- Δ I177L. (A) Kaplan-Meier survival curve of domestic pigs after highly virulent challenge infection with ASFV *Armenia08* six months after intramuscular (IM, red) or oral (blue) immunization with ASFV-G- Δ I177L and of non-immunized control animals (grey). (B) Clinical signs, assessed by body temperature (left) and clinical score (right), (C) viremia, and (D) viral shedding in nasal (left) and oral (right) swabs after highly virulent challenge infection. Immunized animals that ultimately succumbed after challenge infection are shown as squares, immunized animals that survived are shown as circles. dpc, days post challenge

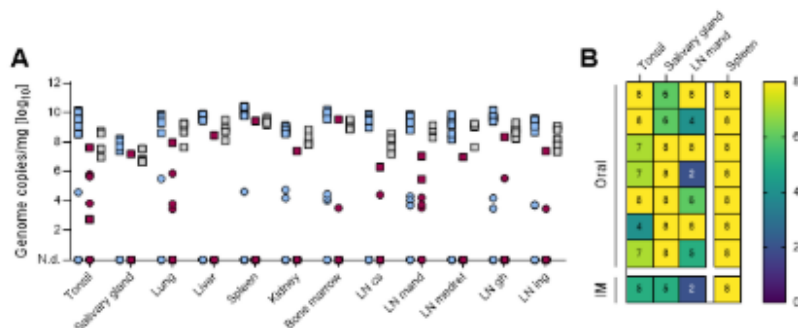


Figure 3

Viral dissemination after challenge infection with highly virulent ASFV *Armenia08* six months after oral or intramuscular immunization with ASFV-G- Δ I177L. (A) Detection of ASFV genomes in tissue samples of domestic pigs taken before (squares) or at the end of the study (28 dpc, circles) after highly virulent challenge infection with ASFV *Armenia08* six months after intramuscular (red) or oral (blue) immunization with ASFV-G- Δ I177L and of non-immunized control animals (grey). (B) Detection of infectious ASFV by hemadsorption test in the indicated tissues of animals that were euthanized at the humane endpoint.

Numbers indicate the number of virus-positive wells of a total of eight replicates. IM, intramuscular immunization; LN, lymph node; cs, *cervicalis superficialis*; mand, mandibular; medret, *retropharyngeales mediales*, gh, gastrohepatic; ing, inguinal.

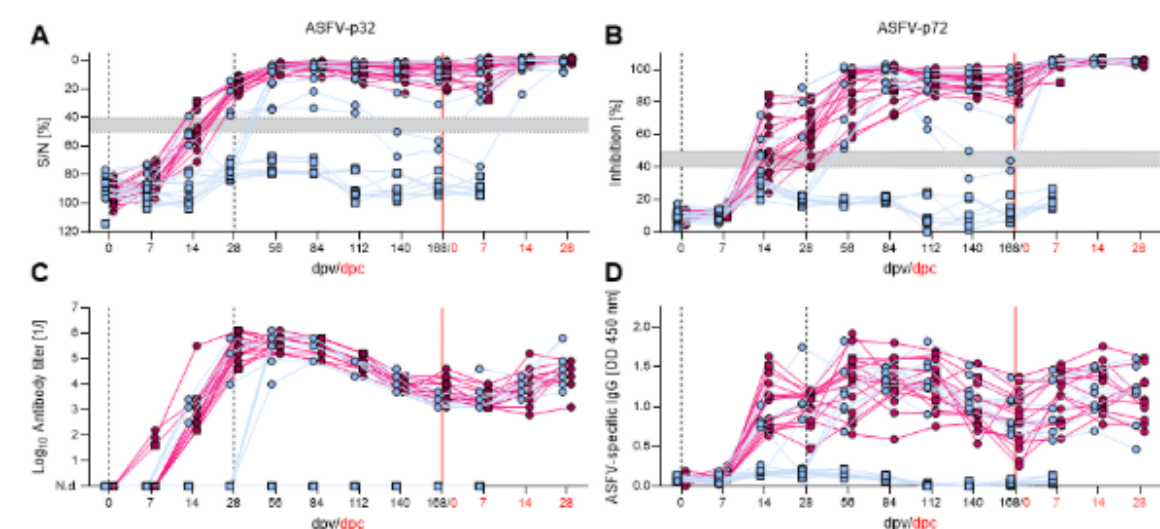


Figure 4

Humoral responses after oral or intramuscular immunization with ASFV-G-ΔI177L and challenge infection with highly virulent ASFV Armenia08 six months later. Serum samples were taken over the course of the study from animals immunized intramuscularly (red) or orally (blue) with ASFV-G-ΔI177L. Commercially available ELISAs were used to assess antibody levels against (A) ASFV-p32 and (B) ASFV-p72. (C) Semi-quantitative antibody titers assessed by indirect immunoperoxidase test. (D) Detection of ASFV-specific IgG. Vertical black dotted lines indicate times of immunizations, solid red lines indicate challenge infection. Animals that ultimately succumbed after challenge infection are shown as squares, surviving animals are shown as circles. Grey areas in A and B indicate assay thresholds, values above are considered positive. dpv, days post vaccination; dpc, days post challenge

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