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Ineffective oral immunization of wild boar with the attenuated African swine fever virus NH/P68 grown in MA104 cell line

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ABSTRACT

African swine fever (ASF) is a highly contagious and lethal hemorrhagic disease affecting domestic pigs and wild boar, with serious consequences for animal health, the swine industry, and socio-economic stability. Given the lack of effective treatments and the limited success of conventional control measures, live attenuated vaccines (LAVs) have emerged as the most promising option, particularly for oral administration in free-ranging wild boar. The naturally attenuated genotype I isolate NH/P68 has shown protective potential in domestic pigs but retains residual virulence and persistence risks. To increase attenuation and facilitate large-scale production, the isolate was adapted to the MA104 continuous cell line. In this study, we assessed the safety and efficacy of NH/P68-MA104 in wild boar using an oral prime-boost immunization protocol (a primary dose of 10^4 $TCID_{50}$/mL followed by two booster doses of 10^5 $TCID_{50}$/mL), followed by a challenge with 10 HAD_{50}/mL of the virulent genotype II Armenia07 (Arm07) isolate. Oral administration of NH/P68-MA104 was safe, with no adverse effects observed throughout the vaccination period. Only one animal developed transient viremia, which was accompanied by clinical signs and a detectable antibody response. Two additional animals showed mild viremia, while the remaining animals displayed no evidence of infection or seroconversion before the challenge. After the challenge, vaccinated wild boar were not protected: all animals developed fever, viremia, and clinical signs consistent with acute ASF and succumbed at 14 ± 3 days post-challenge (dpc), comparable to in-contact and intramuscularly (IM) infected controls. These results demonstrate that oral immunization of wild boar with NH/P68-MA104 is safe but ineffective under the tested conditions. Continued research is needed to refine vaccine candidates and delivery strategies to achieve effective immunization of wild boar.

Introduction

African swine fever (ASF) is a highly contagious and lethal hemorrhagic disease affecting domestic pigs and wild boar, with severe consequences for animal health, pig production, and global trade (WOAH, 2025). The rapid and transcontinental spread of ASF in recent years has underscored the urgent need for effective control strategies to mitigate its devastating impact (Halasa et al., 2016; Brown et al., 2021; Ceruti et al., 2025). The disease is caused by a large, complex, double-stranded DNA virus, the only member of the *Asfarviridae* family, classified within the genus *Asfivirus* (Carrascosa et al., 1984; Alonso et al., 2018).

Since its introduction into the European Union (EU) in 2014, ASF has continued to expand steadily, affecting wild boar populations in Eastern Europe, including Poland, Latvia, Lithuania, and Estonia. Wild boar (*Sus scrofa*) have emerged as a major reservoir of ASF virus (ASFV), contributing significantly to the persistence and spread of the disease

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across Europe. Unlike African wild suids (*Phacochoerus*, *Potamochoerus*, *Hylochoerus*), which typically develop subclinical infections, Eurasian wild boar are highly susceptible and often experience severe disease (Blome et al., 2013; Pietschmann et al., 2015; Sánchez-Cordón et al., 2024). Between 2016 and 2023, ASF spread to several EU and non-EU countries, including Romania, Bulgaria, Germany, and the Western Balkans; with Romania and Croatia together accounting for 96 % of all reported cases by 2023 (EFSA, 2024). The situation worsened in 2024, with new outbreaks reported in Germany, Sweden, Croatia, and Italy, underscoring the urgent need for improved control measures, particularly in wildlife populations (Chenais et al., 2024).

ASF was first reported in Asia in 2018, with an initial outbreak detected in northeastern China, leading to devastating economic losses and large-scale culling campaigns (Wang et al., 2018; Ito et al., 2022; 2023; FAO, 2025). The virus rapidly spread across Southeast and East Asia, affecting Cambodia (2019), Laos (2019), Philippines (2019), South Korea (2019), Vietnam (2019), India (2020), Papua New Guinea (2020), Malaysia (2021), Bhutan (2021), Thailand (2022), Nepal (2022), and Singapore (2023), causing severe disruptions to pork production. Overall, 17 countries in the Asia-Pacific region have reported 3.566 ASF outbreaks in domestic and wild pigs to WOAH through the World Animal Health Information System (WAHIS) over the past five years. Only in Vietnam, nearly six million pigs, approximately 20 % of the national herd, were lost, reflecting the devastating impact of the ASF in the region (Nguyen-Thi et al., 2021).

In 2021, ASF re-emerged in the Western Hemisphere after four decades, registering outbreaks in the Dominican Republic and Haiti (WOAH WAHIS, 2025). These detections raised concerns about potential virus introduction into the Americas, where intensive pig production systems could face catastrophic losses estimated between $15 and $50 billion (Mikesell, 2020). This continued global spread, combined with limited veterinary infrastructure in some affected regions, emphasizes the need for coordinated surveillance and effective prevention strategies.

Among proposed control measures, live attenuated vaccines (LAVs) represent one of the most promising tools, offering the potential for long protection in both domestic pigs and wild boar (Bosch-Camós et al., 2020; Urbano & Ferereira, 2022; Ruedas-Torres et al., 2024). Efficient virus replication in the host organism is crucial for generating immunity and has been confirmed by recent trials, which have shown that both inactivated and subunit vaccines lack protection (Blome et al., 2014; Cadenas-Fernández et al., 2020; 2021), while attenuated vaccines, either naturally (Barasona et al., 2019; 2021) or obtained by genetic manipulation (Monteagudo et al., 2017; Borca et al., 2020), have shown promising results in terms of protection, in some cases approaching 100 % (Bosch-Camós et al., 2020; Urbano & Ferreira et al., 2022; Zhang et al., 2023). A significant milestone was reached in 2023, when the Vietnamese government approved the first two genetically engineered LAVs for domestic pigs: ASFV-G-ΔI177L (NAVET-ASFVAC®) and ASFV-G-ΔMGF (AVAC ASF LIVE®). Both vaccines, derived from the virulent ASFV Georgia 2007/1 isolate, were attenuated through targeted deletions of virulence-associated genes, primarily from multigene families (MGFs), and have shown encouraging efficacy in experimental trials (Borca et al., 2020; Swine Health Information Center, 2023; Fan et al., 2024; Chandana et al., 2024). However, these vaccines are currently licensed only for intramuscular (IM) use in domestic pigs, and their applicability for oral delivery or use in wild boar remains under evaluation within the framework of the European ASFaVIP project (HORIZON-CL6–2023-FARM2FORK-01).

In parallel, naturally attenuated isolates such as the genotype II Lv17/WB/Rie1 isolate, derived from wild boar, have demonstrated promising protection in both domestic pigs and wild boar. However, safety concerns remain, particularly regarding viral shedding and development of unacceptable post-vaccination reactions (Gallardo et al., 2018; 2021; Barasona et al., 2019; 2021; Kosowska et al., 2020; 2023). To address these challenges, research continues on gene-deleted derivatives of this isolate, aiming to improve safety while maintaining protective efficacy and DIVA capacity (Gallardo et al., 2024; González-García et al., 2025).

Another well-characterized LAV candidates from genotype I were the NH/P68 and OURT88/3 isolates, naturally attenuated, non-hemadsorbing viruses originally obtained from a chronically infected pig and soft ticks, respectively, during ASF outbreaks in the Iberian Peninsula (Leitão et al., 2001; Gallardo et al., 2015; 2018; Sánchez-Cordón et al., 2017). Experimental studies have shown that NH/P68 induces a mild, chronic form of ASF in domestic pigs, characterized by typical signs including necrotic skin lesions, joint swelling, intermittent fever, and viremia in a high proportion of infected animals (Gallardo et al., 2015). The virus exhibits prolonged persistence, with ASFV DNA detectable up to 99 days post-infection, and transmission to naïve in-contact pigs introduced 72 days later has been documented, raising concerns about potential silent dissemination (Gallardo et al., 2015).

To overcome these safety limitations, Gallardo and collaborators (2018) engineered a panel of recombinant NH/P68-derived mutants by deleting specific immunomodulatory and virulence-associated genes (A238L, A224L, EP153R, A276R). These recombinants were designed to improve safety while retaining immunogenicity. However, their protective efficacy varied markedly. For instance, the candidate NH/P68-ΔA276R, produced in a primary cell line, failed to protect pigs against the heterologous Arm07 challenge, despite evidence of viral replication *in vivo*. Similarly, NH/P68-ΔA238L produced in COS7 cells reduced adverse clinical signs compared with the parental virus but did not induce effective protection.

Considering the central role of wild boar in ASF epidemiology, oral vaccination remains one of the most practical and necessary strategies for controlling the disease in free-ranging populations (Brauer et al., 2006; Ballesteros et al., 2009). Among the available approaches, attenuation through serial cell passages has long been explored in ASF vaccine development (Masujin et al., 2021; Borca et al., 2021; Zhang et al., 2023). Earlier studies showed that repeated passage of virulent genotype I strains in porcine bone marrow and kidney cells could reduce virulence and elicit protection in immunized pigs (Zhang et al., 2021). However, field trials revealed major safety concerns, as many vaccinated animals developed chronic ASF, limiting the practical application of this method. Given these constraints, attention has shifted to the use of continuous cell lines for ASFV propagation, to enable standardized, scalable, and ethically acceptable vaccine production. Most current LAV candidates are still grown in primary porcine macrophages or monocytes, cells that are difficult to obtain, variable in quality, and unsuitable for large-scale manufacturing (Meloni et al., 2022; Thaweerattanasinp et al., 2024).

In this context, the adaptation of naturally attenuated isolates to continuous cell lines offers a potential dual benefit: increased biosafety through further attenuation and improved feasibility for industrial-scale production. MA104, a continuous cell line, has been proposed for this aim and supports ASFV replication with relative genomic stability (Meloni et al., 2022). To evaluate this strategy, we tested the safety and protective efficacy of the NH/P68 isolate adapted to the MA104 cell line (NH/P68-MA104), administered orally in a prime-boost regimen to wild boar. This approach aimed to assess whether the cell-adapted virus could serve as a viable oral vaccine candidate for ASF control in wildlife reservoirs, balancing safety, immunogenicity, and production feasibility.

Materials and methods

Animals

The experiment was conducted in the Biosafety Level 3 (BSL-3) animal facilities of the VISAVET Health Surveillance Centre at the Complutense University of Madrid, Spain. A total of 18 wild boar (15 females and 3 males), aged 3–4 months, were sourced from a commercial wild

boar farm in Andalusia, Spain. Before the study, all animals had been vaccinated against *Mycoplasma hyopneumoniae*, Aujeszky's disease virus, and porcine circovirus type 2. They also tested negative for *Mycobacterium bovis*, ASFV, and porcine reproductive and respiratory syndrome virus. Each animal was individually identified with an ear tag and acclimated to the facility conditions for one week before the start of the trial. Water and feed were provided *ad libitum* throughout the study.

All animal care and experimental procedures were conducted in accordance with good laboratory and animal welfare practices, following European, national, and regional regulations. The study was reviewed and approved by the Ethics Committee of the Complutense University of Madrid and the Comunidad de Madrid (PROEX reference 113.3/21).

Experimental design

The study involved a total of 18 wild boar, divided into two main experimental groups. The first group ($n = 12$) was orally immunized with 1 mL containing 10^4 $TCID_{50}$/mL of the attenuated ASFV NH/P68 isolate, grown in MA104 cells. A homologous booster dose of the same concentration was administered at 16 days post-immunization (dpi), followed by a second booster of 10^5 $TCID_{50}$ at 29 dpi. This vaccination phase extended over 33 days, allowing sufficient time for the development of a specific immune response prior to challenge. The immunization schedule was designed based on previous studies in wild boar using attenuated ASFV isolates (Barasona et al., 2019; 2021), with additional boosters included to enhance immune stimulation and compensate for the lower efficiency typically associated with oral delivery.

After this period, a second group of six animals was used for the challenge. Four of these animals were IM-inoculated with 10 HAD_{50}/mL of the highly virulent ASFV Arm07 isolate. The remaining two animals were not directly inoculated but were housed in direct contact with the infected wild boar to evaluate virus transmission through natural exposure (in-contact animals), following a shedder-pig challenge-exposure model.

ASFV isolates

For immunization purposes, the attenuated, non-hemadsorbing genotype I ASFV isolate NH/P68-MA104 was used. The attenuated isolate was obtained from the European Union Reference Laboratory (EURL) for ASF, Centro de Investigación en Sanidad Animal, Instituto Nacional de Tecnología Agraria y Alimentaria, Consejo Superior de Investigaciones Científicas (CISA-INIA-CSIC), Valdeolmos, Spain. The virus was initially isolated in porcine blood monocytes (PBM) and subsequently passaged five times in MA104 cells for propagation (Thaweerattanasinp et al., 2024). Viral titer was defined as the amount of virus infecting 50 % of the tissue culture ($TCID_{50}$/mL).

For the challenge, the virulent hemadsorbing genotype II ASFV isolate Arm07 was used. The virus was propagated in PBM (Gallardo et al., 2015). The lethal effect of this isolate has been widely described in pigs and wild boar (Gallardo et al., 2018; Sunwoo et al., 2019; Rodriguez-Bertos et al., 2020). Viral titer was defined as the amount of virus causing hemadsorption in 50 % of infected cultures (HAD_{50} /mL).

Laboratory procedures

Throughout the study period, blood samples were collected from each animal twice a week. Both EDTA-anticoagulated whole blood and coagulated blood (for serum preparation) were obtained. Viral DNA was extracted from 200 μL of EDTA blood using the High Pure Template Preparation Mix Kit (Roche Diagnostics GmbH, Mannheim, Germany), following the manufacturer's protocol. Detection of ASFV DNA was performed by quantitative real-time PCR (qPCR) using the Universal Probe Library (UPL) method recommended by the WOAH and described by Fernández-Pinero et al. (2013). A sample was considered positive when the quantification cycle (Cq) value exceeded background fluorescence within 40 amplification cycles. Positive and negative controls were included in both DNA extraction and qPCR runs to ensure validity.

Serum samples were tested for ASFV-specific antibodies using a commercial ELISA kit targeting the VP72 antigen (Ingezim PPA Compac K3, Ingenasa, Madrid, Spain), according to the manufacturer's instructions. The assay reports a sensitivity of 98 % and a specificity of 100 %.

Clinical signs and euthanasia

Animals were monitored daily throughout the study to assess health status, using a video surveillance system (Hikvision iVMS-4200, Hikvision®, Hangzhou, China) complemented by routine veterinary inspections. Clinical progression was evaluated using a quantitative clinical scoring (CS) system specific to ASFV infection in wild boar, as previously described by Cadenas-Fernández et al. (2020). The scoring system encompassed a range of clinical parameters, including fever, behavior, body condition, skin alterations, ocular or nasal discharge, joint swelling, as well as respiratory, digestive, and neurological signs. Each parameter was scored on a scale from 0 to 4, with 4 indicating the most severe manifestation. Fever was defined as a rectal temperature exceeding 40 °C.

Animals that reached a pre-established humane endpoint (CS > 18) or exhibited any severe clinical sign (score of 4) for more than two consecutive days were humanely euthanized. Euthanasia was carried out via intravenous injection of T61® (Intervet, Spain) following sedation with an IM combination of tiletamine-zolazepam (Zoletil 100 mg/mL, Virbac, France) and medetomidine (Medetor, Virbac, France), as described by Barasona et al. (2013).

Post-mortem examination and tissue sampling

A thorough *post-mortem* examination was conducted to evaluate the presence of macroscopic lesions consistent with ASF (Galindo-Cardiel et al., 2013; Rodríguez-Bertos et al., 2020). A total of 20 tissue samples were collected from each necropsied animal, including major lymph nodes (mandibular, renal, mediastinal, retropharyngeal, mesenteric, prescapular, gastrohepatic, and inguinal), along with key visceral organs such as the spleen, liver, lung, heart, kidney, brain, urinary bladder, intestine, diaphragm, bone marrow, and synovial membranes from both forelimb and hindlimb joints. The presence of ASFV DNA in these tissues was assessed using a real-time PCR assay based on the UPL method, as previously described by Fernández-Pinero et al. (2013).

Full genome sequencing

The NH/P68-MA104 strain, obtained after five passages of parental NH/P68 in the MA104 cell line, was subjected to full genome sequencing to assess the *in vitro* genetic stability of the vaccine candidate. In detail, DNA libraries were prepared as described previously (Gallardo et al., 2024). Afterwards, 1 μg of the library was hybridized to a custom-designed probe library, encompassing the entire ASFV genome (ASFSeqCapv1.0 SeqCap EZ developer library, NimbleGen, Roche, Madison, USA), and following the SeqCap EZ Library SR User's Guide for Illumina. Then, the captured DNA library was purified and finally sequenced on Illumina MiSeq system (MiSeqv2 500-cycle, 2 ×250 bp).

CLC Genomics Workbench (version 20.0.4) was used for reads analysis. For variant calling, reads were mapped to the reference NHV genome sequence available in GenBank (NC_044943) through VarScan2 (minimum read depth 20x; min. reads with variant 5x; min. variant freq 0.05). DNA reads have been deposited in GenBank under the BioProject ref. PRJNA1366406.

Statistical analysis

All statistical analyses were performed using SPSS version 28 (IBM Corp., Armonk, NY, USA). Data distribution was assessed using the Kolmogorov–Smirnov test to determine normality. Since most continuous variables did not follow a normal distribution, non-parametric tests were applied.

Survival analysis was conducted using the Kaplan–Meier method, and differences between groups were evaluated with the Mantel–Cox log-rank test. Comparisons of continuous variables, such as the onset of viremia and Cq values, were performed using the Mann–Whitney *U* test.

A p-value < 0.05 was considered statistically significant in all analyses.

Results

Genetic analysis of NH/P68-MA104 isolate

Whole-genome sequencing of the NH/P68-MA104 isolate yielded high-quality data, with DNA reads mapping to the parental NH/P68 reference genome (GenBank NC_044943) at an average depth exceeding 1000x. Variant analysis revealed 20 major mutations (10 SNPs and 10 indels; variant frequency ≥ 0.5) and 35 minor variants (frequency > 0.05 and < 0.5) distributed across the genome (**Supplementary Material**), indicating that the virus maintained a high degree of genetic stability after five passages in the MA104 cell line.

Among the major variants, 10 SNPs and 2 single-nucleotide insertions were located within coding regions, all positioned in the right half side of the viral genome. The two insertions affected CP204L and MGF-360–16R genes and were also identified in the parental NH/P68 available at CISA-INIA-CSIC, which was the original stock passaged in MA104 cell line. These insertions would produce a frameshift resulting in shorter proteins compared to the reference NH/P68 sequence published in GenBank (Table 1).

In contrast, the 10 SNPs identified exclusively in NH/P68-MA104 (all C→T transitions) resulted in non-synonymous changes affecting proteins MGF-100–3L, I7L, I8L, and the regulation of I9R gene (Table 1).

Vaccination period

During the vaccination period, only one wild boar orally inoculated with the NH/P68-MA104 isolate developed a detectable antibody response against the ASFV VP72 antigen. This animal had been involved in a fight within the pen, which likely contributed to the onset of the viremia, detected at 9 dpi and persisting for approximately one week. Clinical signs included decreased activity, anorexia, locomotor difficulties, and moderate dyspnea, although fever was not observed. To prevent unnecessary suffering, the animal was euthanized at 16 dpi with a CS of 11 and viremia with a mean of Cq values of 33.20.

Two additional animals developed viremia at 6 and 19 dpi, with an average of Cq values of 39.97 ± 2.16. These animals showed mild fever (40.3 ± 0.09°C) and a CS of 2. Before the challenge, no other animal from either group displayed clinical signs indicative of ASFV infection or immunization. Apart from the single seropositive case, no ASFV-specific antibodies were detected in the serum of any other wild boar.

Challenge period

After 33 days of the vaccination period, the virulent ASFV was introduced by four IM-challenged animals. These animals developed clinical signs consistent with acute ASFV infection within 4 ± 2 days post-challenge (dpc). One animal died spontaneously, while the remaining three were euthanized between 6 and 8 dpc due to reaching the pre-established humane endpoint. The two in-contact control animals, co-housed with the IM-inoculated group, became infected through direct transmission and died at 12 and 15 dpc, respectively (Fig. 1). All six animals exhibited fever (IM-inoculated animals 40.32 ± 0.27°C; in-contact 40.07 ± 0.60°C), and clinical signs including lethargy, anorexia, slight erythema, locomotor difficulties, and dyspnea. High loads of ASFV DNA were detected in their blood, starting at 4 ± 1 dpc in IM-inoculated animals (mean Cq: 17.96 ± 3.03) and at 7 dpc in in-contact animals (mean Cq: 28.15 ± 7.94) (Fig. 2). No ASFV-specific antibodies were detected in any of these animals during the study period.

Following the virulent ASFV challenge, no ASFV-specific antibody response was detected in the vaccinated animals at any point during the study. These animals were not protected against the virulent challenge with the ASFV Arm07 isolate and succumbed to the disease at 14 ± 3 dpc, similar to the IM-inoculated and in-contact control groups (Fig. 1). Survival analysis revealed statistically significant differences in survival time among the vaccinated group, IM-challenged animals, and in-contact controls, as determined by the Mantel-Cox test ($\chi 2 = 5.026$, 10.698, and 4.060, respectively; $p = 0.025$, 0.001, and 0.044).

Wild boar orally inoculated with the NH/P68-MA104 isolate began to show clinical signs consistent with ASF infection at 9 ± 2 dpc. Observed symptoms included fever (39.21 ± 0.66°C), lethargy, anorexia, erythema, ocular discharge, mild dyspnea, and coughing, reaching the human endpoint at 19 dpc. Viremia was detected starting at 11 ± 4 dpc, with the mean of Cq values of 19.60 ± 3.47 recorded at the end of the study (Fig. 2). No statistically significant differences in the

Table 1
Major variations identified in gene regions of the NH/P68-MA104 genome compared to the *GenBank* reference NH/P68 sequence (acc. no. NC_044943).

Nt position in NC_044943	Gene Affected	Function	Reference NHV strain (NC_044943)	NH/P68-MA104-P5	Protein variation
110742	**CP204L**	Encode the immunodominant protein p30, necessary for virus internalization into host cells	(-)	T	Frameshift, CP204L termination (10 aa shorter). Possible error in large homopolymer region
163379	**MGF 360–16 R**	Host immune response by suppressing induction of type I IFN. Not essential for the virus virulence	(-)	A	Frameshift, MGF 360–16 R termination (2aa shorter). Possible error in large homopolymer region.
166351	**MGF**	Unknown	C	**R (C/T)**	No change in the aa sequence
166392	**100–3 L**		C	**R (C/T)**	D (ASP)→ N (ASN) or no change in the aa sequence
166603	**I7L**	Unknown	C	**T**	D (ASP)→N (ASN)
166606			C	**T**	D (ASP)→N (ASN)
166712			C	**T**	M (MET)→I (ILE)
166813			C	**T**	D (ASP)→N (ASN)
167122	**I8L**	Unknown (Not essential for the virus virulence)	C	**T**	R (ARG)→K (LYS)
167132			C	**T**	D (ASP)→N (ASN)
167135			C	**T**	D (ASP)→N (ASN)
167576	**Upstream I9R**	Gene regulation	C	**T**	

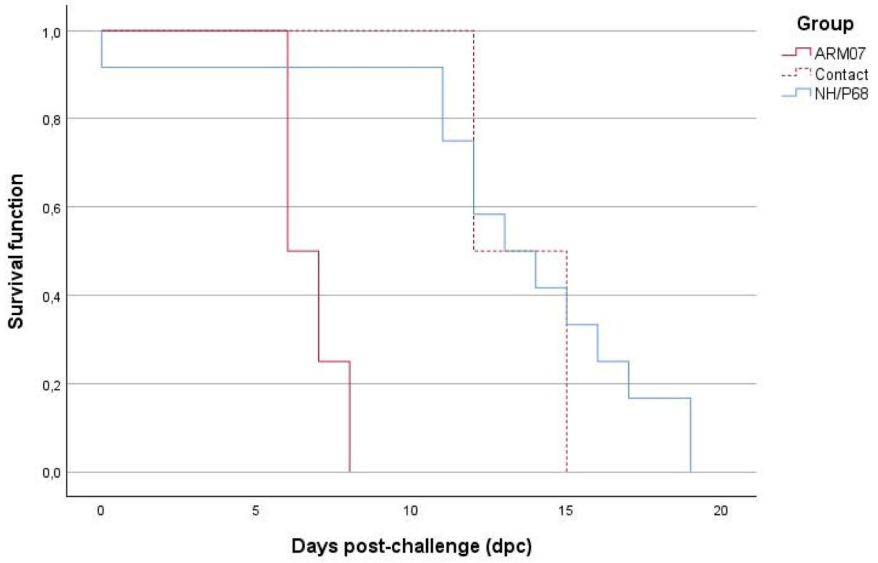

Fig. 1. Kaplan–Meier survival curves showing the survival of wild boar following different treatments: oral administration with the attenuated ASFV NH/P68-MA104 isolate ($n = 12$; blue line), negative control group ($n = 2$; dashed red line), and IM challenge with the highly virulent ASFV Arm07 isolate ($n = 4$; solid red line).

onset of viremia or Cq values were observed between vaccinated and in-contact controls (Mann-Whitney U test: $p > 0.05$).

Post-mortem examination

During the *post-mortem* examination, all the vaccinated and control animals presented pathological lesions consistent with ASF infection. The main findings included moderate to severe accumulation of yellowish to reddish fluid in the abdominal cavity (ascites), thoracic cavity (hydrothorax), and pericardial sac (hydropericardium). Pulmonary changes were characterized by a lack of collapse, diffuse congestion, mild to moderate distension of interlobular septa (interstitial edema), and alveolar edema with foamy material in the trachea. Multifocal subpleural ecchymoses and petechiae were also present on the lung surface. Reddish areas of consolidation, located in the cranial and middle lobes and compatible with bronchopneumonia, were occasionally noted. Congestion and enlargement of the spleen (splenomegaly), liver (hepatomegaly), and multiple lymph nodes (lymphadenomegaly) were consistently observed. Haemorrhages of varying severity were particularly evident in the renal, gastrohepatic, mesenteric, ileocecal, and submandibular lymph nodes. Additional findings included hyperemia and multifocal petechial haemorrhages in the kidneys, epicardium and endocardium, gastric mucosa, and intestinal serosa and mucosa, most prominently in the colon. Less frequent haemorrhages were observed on the surface of the pancreas and adrenal glands, and in the serosa and mucosa of the urinary bladder. The brain exhibited marked congestion of submeningeal blood vessels (Fig. 3).

In general, no effect of immunization was observed in terms of ASFV DNA load across tissues. All animals, regardless of treatment group, showed high levels of viral DNA in the majority of sampled tissues and organs (Fig. 4). Cq values were consistently low in key target tissues such as lymph nodes, spleen, liver, and bone marrow. No statistically significant differences in ASFV DNA levels (Cq values) were detected between groups for any tissue analyzed (Kruskal-Wallis: $p > 0.05$).

Discussion

Despite decades of research, the development of a safe, effective, and field-deployable vaccine against ASF remains one of the greatest challenges in animal health. This need has grown increasingly urgent given the continued global spread of ASF and the critical role that wild boar play in the virus's maintenance and dissemination (Schulz et al., 2021). Among the various approaches, LAVs have shown the greatest potential, as evidenced by recent experimental successes; however, none have yet been successfully implemented in wild populations, where oral administration remains the only practical route (Brauer et al., 2006; Ballesteros et al., 2009; Barasona et al., 2019; Pachauri et al., 2024).

Previous studies have demonstrated that attenuated genotype I strains can confer solid protection against homologous virulent genotype I isolates in domestic pigs (Leitão et al., 2001; King et al., 2011; Dixon et al., 2017; Gallardo et al., 2018). To our knowledge, this is the first study to evaluate the efficacy of an attenuated genotype I isolate against a virulent genotype II isolate (Arm07) in wild boar.

The NH/P68 isolate used in this study is a naturally attenuated, non-hemadsorbing, and non-lethal virus. It has shown protective efficacy against homologous genotype I strains, such as the L60 isolate (Leitão et al., 2001; Gil et al., 2008; Gallardo et al., 2018). However, as observed with other genotype I attenuated viruses, including OURT88/3, this protection does not generally extend to heterologous genotype II isolates (Mulumba-Mfumu et al., 2016; Gallardo et al., 2018). An exception was reported by Monteagudo et al. (2017) where recombinant genotype I isolate BA71ΔCD2 was able to confer complete protection against the heterologous genotype II Georgia 2007/1 isolate, with cross-protection correlating with the induction of specific $CD8^+$ T cells recognizing antigens from both genotypes. These studies, therefore, dig deeply into the breadth of immunity conferred by a genotype I vaccine when faced with a genotype II challenge.

In this study, we evaluated the protective efficacy of the NH/P68-MA104 isolate, a naturally attenuated genotype I ASFV isolate adapted to the commercial cell line, using an oral prime-boost protocol in wild boar. According to Gallardo et al. (2018), ASFV NH/P68 produced in primary porcine alveolar macrophages (PAMs) successfully protected domestic pigs against challenge with Arm07, preventing death from acute infection. In contrast, recombinant viruses derived from NH/P68 produced in immortalized cell lines failed to provide the same level of protection. This discrepancy highlights that the protective potential of NH/P68 may depend on how the vaccine is produced and administered. In Gallardo et al.'s study (2018), the virus was grown in porcine cells and was likely delivered parenterally, conditions that differ markedly from the oral prime–boost approach used in wild boar here. Thus, factors such as the production cell line and vaccination route could be crucial in determining vaccine efficacy.

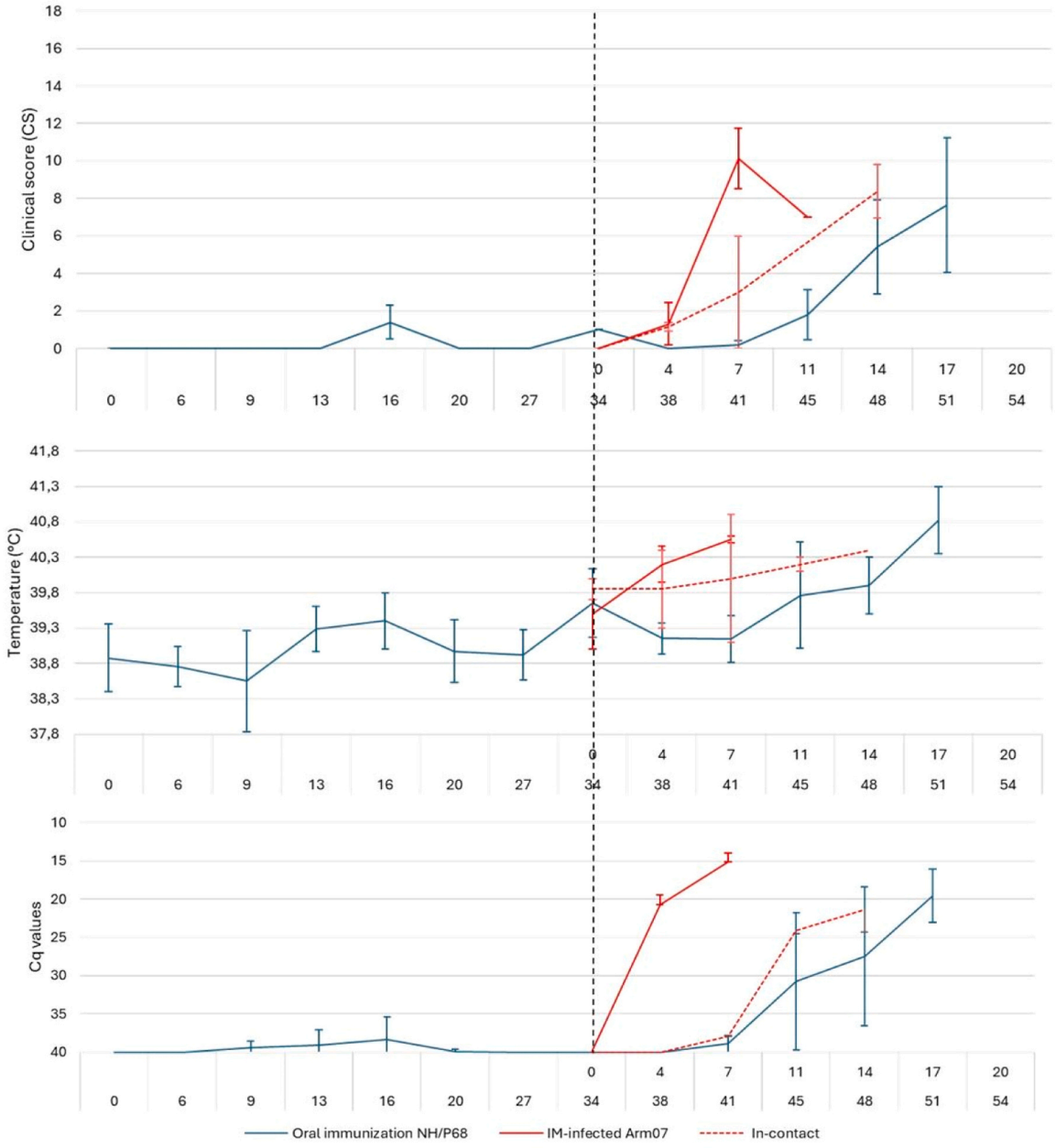

Fig. 2. Average of clinical scores, body temperatures, and Cq values from real-time PCR of wild boar following different treatments: oral inoculation with the attenuated ASFV NH/P68-MA104 isolate ($n = 12$; blue line), IM challenge with the virulent ASFV Arm07 isolate ($n = 4$; red line), and in-contact animals ($n = 2$; dashed red line). Error bars represent standard deviation (SD). The timeline includes days post-immunization (dpi) covering the prime, boost, and challenge phases, followed by days post-challenge (dpc).

The vaccine candidate appeared safe during the immunization period, as most animals showed no adverse effects or abnormal clinical signs prior to exposure. Only three animals developed clinical manifestations: two had mild fever, while the third showed more pronounced signs due to causes unrelated to the experiment and was therefore excluded from further analysis. However, despite this acceptable safety profile, the immunization failed to confer protection, as ASFV-specific antibody responses were minimal – only one animal seroconverted. Consequently, upon exposure to the virulent Arm07 isolate, all vaccinated animals developed acute clinical ASF indistinguishable from non-immunized controls, with high viremias and typical lesions, and ultimately succumbed to the disease.

It is generally recognized that oral ASF vaccination requires substantially higher doses than IM administration to achieve effective systemic replication and stimulate a robust immune response (Deutschmann et al., 2022). However, despite delivering two booster doses at 16 and 29 dpi to enhance viral exposure and immune priming, no improvement in immune activation or protection was observed. These findings suggest that, under the tested protocol, the administered dose and delivery strategy were insufficient to induce effective immunity in wild boar.

Given that protection against ASFV relies on a complex interplay between humoral and cellular immune responses (Franzoni et al., 2023; Xu et al., 2023; Pedrera et al., 2024), and although protection is mainly mediated by cellular immunity, particularly by CD8 + T lymphocytes (Pedrera et al., 2024), previous studies have shown that the magnitude of the humoral response at the time of challenge correlated positively with protection (Orosco, 2023). Likewise, animals that failed to seroconvert after vaccination generally succumbed to the disease. We hypothesized that the NH/P68-MA104 vaccine candidate might still

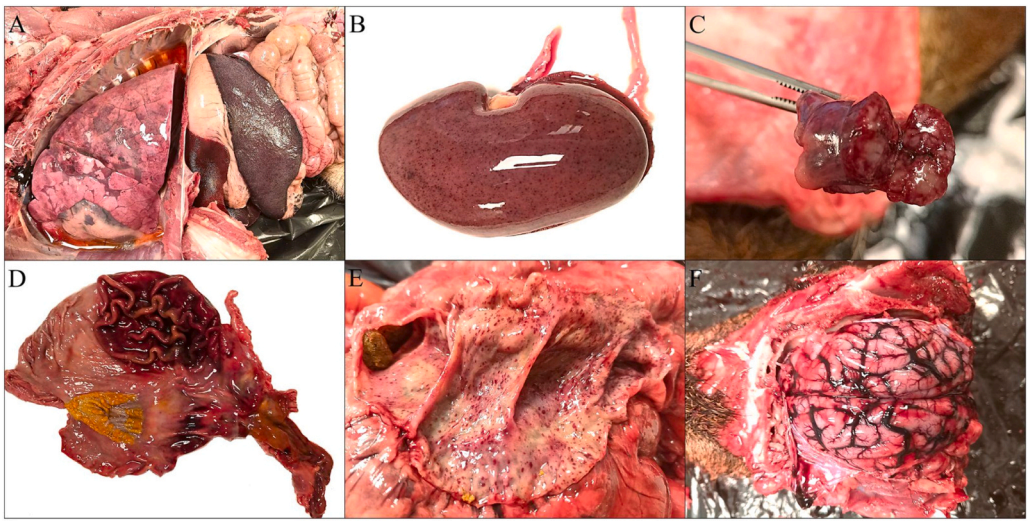

Fig. 3. Representative macroscopic lesions observed in wild boar during the *post-mortem* examination. **(A)** Hydrothorax, pulmonary edema, congestion, reddish areas of consolidation, multifocal subpleural ecchymoses and petechiae, and splenomegaly. **(B)** Multifocal cortical petechiae in the kidney. **(C)** Subcortical and medullary hyperemia and hemorrhage in the lymph node. **(D)** Hyperaemia and inflamed mucosal surfaces in the gastric body. **(E)** Hyperemia and multifocal petechiae in the colon. **(F)** Marked congestion of submeningeal blood vessels in the brain.

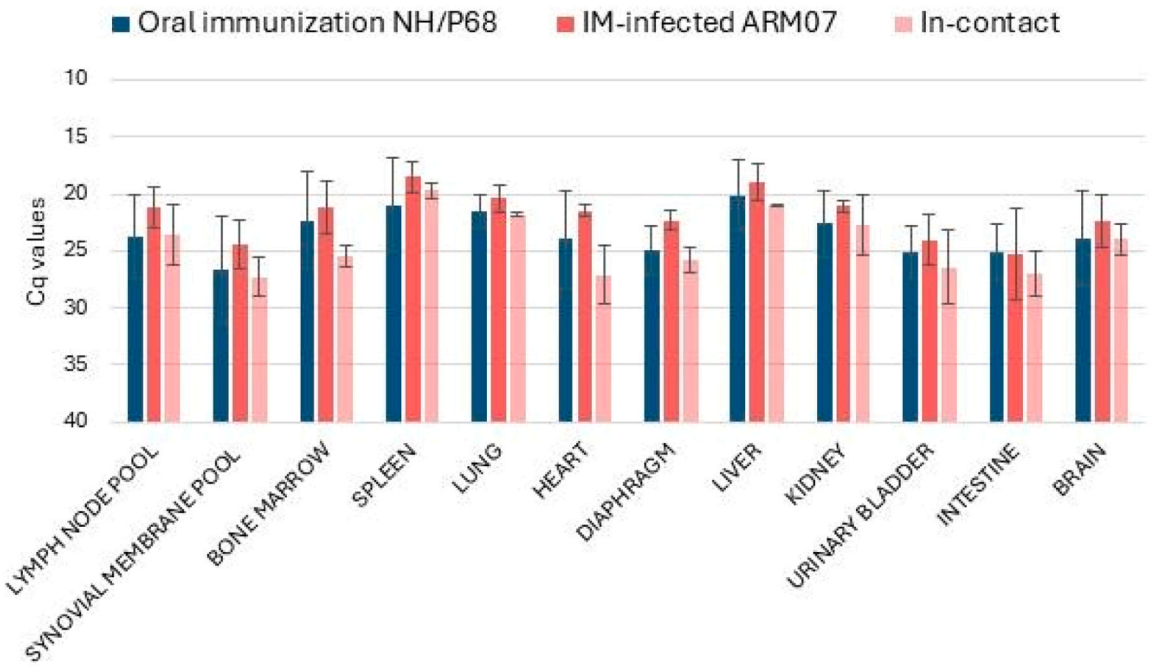

Fig. 4. ASFV DNA levels (Cq values by qPCR) in tissue and organ samples from wild boar orally inoculated with the attenuated NH/P68-MA104 isolate (blue), in-contact exposed animals (light red), and IM-challenged controls inoculated with the virulent Arm07 isolate (red). No statistically significant differences in viral DNA levels (Cq values) were detected among the groups (Kruskal-Wallis: $p > 0.05$). Error bars represent standard deviation (SD). Synovial membrane pool includes samples from both forelimb and hindlimb joints. Lymph node pool includes the prescapular, mandibular, medial retropharyngeal, superficial inguinal, gastrohepatic, renal, mediastinal, and mesenteric lymph nodes.

provide some level of cellular immunity, even in the absence of detectable antibodies (Schäfer et al., 2022). However, we did not directly measure cell-mediated immune responses in these animals. The uniformly poor outcomes suggest that if any ASFV-specific T-cell immunity was induced, it was insufficient to influence disease progression. To investigate this possibility, we employed a shedder-pig challenge model designed to better mimic natural transmission compared to direct inoculation. In this model, viral exposure occurs progressively rather than all at once. Under these conditions, vaccinated animals and in-contact controls showed slightly longer survival times than IM-infected wild boar, most likely due to the more gradual infection dynamics and extended incubation periods characteristic of this approach (de CarvalhoFerreira et al., 2013; Guinat et al., 2016), which underscores that a robust immune activation did not occur in our orally vaccinated wild boar.

A key factor potentially influencing the outcome of this study is the adaptation of the NH/P68 isolate to the MA104 cell line. Whole-genome sequencing of NH/P68-MA104 indicated that the viral genome remained largely stable after five passages; however, a small set of high-frequency mutations was detected, particularly C→T transitions and

potential indels, affecting genes such as MGF-100–3L, I7L, I8L, and the regulatory region of I9R. Several of these mutations clustered in genomic regions previously associated with cell-line adaptation in ASFV (Krug et al., 2015; Rodríguez et al., 2015; Wang et al., 2021), consistent with early steps of adaptation to MA104 cells. Nevertheless, no direct association could be established between these genomic changes and the reduced immunogenicity observed *in vivo*.

Insertions were also detected in CP204L and MGF-360–16R. These insertions would introduce frameshifts, resulting in shorter predicted proteins compared with the reference NH/P68 sequence deposited in GenBank. However, because these insertions are located within homopolymeric regions, they may reflect sequencing artifacts—an acknowledged limitation of next-generation sequencing technologies in these contexts. Furthermore, their presence in both the parental NH/P68 stock and the MA104-adapted virus suggests that not all of the observed differences arose during the adaptation process.

The NH/P68 isolate was adapted to MA104 cells to enable efficient propagation, since many ASFV isolates (including unadapted NH/P68) replicate poorly in available porcine cell lines (Lee et al., 2025). MA104 cells, derived from green monkey kidney epithelium, are widely used in virology for their scalability and consistency in vaccine production (Meloni et al., 2022). However, these cells are evolutionarily distant from ASFVs natural target cells, porcine monocytes and alveolar macrophages. Effective oral vaccination depends not only on the virus's ability to cross mucosal barriers (Lavelle & Ward, 2022) but also on its capacity to replicate in these porcine immune cells to initiate a strong immune response.

Although MA104 adaptation may improve attenuation and biosafety, it can also drive genomic changes that reduce viral tropism for porcine macrophages. While some genotype II isolates maintain stable sequences for key structural proteins (p72, p54, p30) after passage in MA104 (Kwon et al., 2022), other studies report the emergence of deletions, such as those affecting MGF110–9L, that impair replication in porcine target cells and diminish immunogenicity (Gallardo et al., 2018; Li et al., 2023; Thaweerattanasinp et al., 2024), particularly when delivered orally, where efficient local replication is essential. Similar progressive reductions in macrophage tropism have been documented in ASFV strains adapted to Vero or HEK293T cells, where cumulative mutations and deletions accompany attenuation and loss of virulence (Krug et al., 2015; Wang et al., 2021).

Although several studies have shown that extensive adaptation of ASFV to non-porcine continuous cell lines can lead to progressive loss of macrophage tropism, reduced infectivity, and over-attenuation, the genomic changes identified in NH/P68-MA104 do not fully mirror these previously reported patterns. For example, Wang et al. (2021) demonstrated that continuous passaging of the HLJ/18 strain in HEK293T cells resulted in major deletions in MGF300 and MGF360, accompanied by markedly reduced replication in primary porcine macrophages. Similarly, Krug et al. (2015) reported that prolonged adaptation of the Georgia 2007 isolate to Vero cells produced large deletions and numerous point mutations, particularly at the variable genome ends, leading to complete loss of replication in porcine macrophages after 110 passages.

These studies illustrate how deep adaptation in non-porcine cell lines can cause cumulative genomic erosion and profound loss of virulence and immunogenicity. However, the NH/P68-MA104 virus was only passaged five times, showed no large deletions, and retained an overall stable genome, with only a limited set of point mutations and potential indels. Thus, while cell-line adaptation remains a plausible contributing factor to the reduced immunogenicity observed *in vivo*, the magnitude and nature of the mutations detected in NH/P68-MA104 do not fully resemble the dramatic genomic remodeling described in these prior studies. This indicates that other factors, such as oral delivery or insufficient replication in target tissues, likely played a more important role in the lack of vaccine efficacy observed here.

Environmental monitoring and sample collection were performed at various time points throughout the study to evaluate potential ASFV circulation among animals (data not shown) (Kosowska et al., 2021; Barroso-Arévalo et al., 2023; Kwon et al., 2024). No ASFV DNA was detected in any of these samples prior to challenge, indicating that the orally administered NH/P68-MA104 isolate did not spread between animals, suggesting an absence of virus shedding during immunization. Following the challenge with the virulent Arm07 isolate, however, ASFV DNA became detectable in several environmental samples. This coincided with the onset of acute ASF in all animals, confirming that viral replication and shedding occurred after exposure to the virulent isolate rather than during the immunization phase.

These findings were further supported by the *post-mortem* examinations. Oral immunization with NH/P68-MA104 failed to reduce ASFV replication, tissue viral load, or the severity of lesions after challenge with the virulent Arm07 isolate. Gross pathological findings in vaccinated animals were indistinguishable from those observed in in-contact and IM-infected controls. Marked splenomegaly, lymphadenomegaly with hemorrhages, pulmonary edema, and multifocal hemorrhages in several organs were consistently recorded across all groups (Galindo-Cardiel et al., 2013; Rodríguez-Bertos et al., 2020; Sánchez-Cordón et al., 2024). These results indicate a complete absence of vaccine-derived protective mechanisms.

In summary, our results highlight the complex interplay between vaccine characteristics, delivery route, and host factors in ASFV immunity. While adaptation of NH/P68 to the MA104 cell line improves manufacturability and regulatory compliance, it may also compromise viral fitness and immunogenicity in the oral vaccination context. Taken together, the evidence indicates that NH/P68-MA104 is not a suitable candidate for oral vaccination in wild boar under the conditions tested. Further optimization is imperative to develop LAVs that are both safe and effective for this key reservoir host. Future efforts should consider using genotype II–based attenuated isolates to better match the circulating ASFV isolates and ensure that vaccine viruses achieve sufficient replication to induce immunity without shedding or reversion to virulence. Achieving this balance will be crucial before any oral ASF vaccine can be responsibly deployed in wild boar populations.

Declaration of generative AI and AI-assisted technologies in the manuscript preparation process

During the preparation of this work, the authors used ChatGPT (developed by OpenAI) in order to improve and correct the English language of the manuscript, and Perplexity.ai to cross-check and verify information. After using these tools, the authors reviewed and edited the content as needed and take full responsibility for the content of the published article.

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CRediT authorship contribution statement

Aleksandra Kosowska: Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Methodology, Investigation, Formal analysis, Data curation. **Mónica Sánchez-Segovia:** Writing – review & editing, Writing – original draft, Visualization, Validation, Formal analysis, Data curation. **Sandra Barroso-Arévalo:** Writing – review & editing, Methodology, Investigation. **Jovita Fernández-Pinero:** Writing – review & editing, Methodology,

Investigation, Formal analysis, Data curation. **Néstor Porras:** Writing – review & editing, Visualization, Methodology, Investigation, Formal analysis, Data curation. **José A. Barasona:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization. **Lidia Sánchez-Morales:** Writing – review & editing, Investigation. **Marta Díaz-Frutos:** Writing – review & editing, Investigation.

Conflict of Interest

The authors declare no conflict of interest.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.tvjl.2026.106554.

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