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ORIGINAL ARTICLE

Virulent strain of African swine fever virus eclipses its attenuated derivative after challenge

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Abstract African swine fever (ASF) is one of the most devastating diseases affecting the swine industry worldwide. No effective vaccine is currently available for disease prevention and control. Although live attenuated vaccines (LAV) have demonstrated great potential for immunizing against homologous strains of African swine fever virus (ASFV), adverse reactions from LAV remain a concern. Here, by using a homologous ASFV Congo strain system, we show passage-attenuated Congo LAV to induce an efficient protective immune response against challenge with the virulent parental Congo strain. Notably, only the parental challenge Congo strain was identified in blood and organs of recovered pigs through B602L gene PCR, long-range PCR, nucleotide sequencing and virus isolation. Thus, despite the great protective potential of homologous attenuated ASFV strain, the challenge Congo strain can persist for weeks in recovered pigs and a recrudescence of virulent virus at late time post-challenge may occur.

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Introduction

African swine fever (ASF) is a highly contagious haemorrhagic disease that causes rates of death approaching 100% among domestic pigs and wild boars [9, 15, 42]. The disease is caused by the ASF virus (ASFV), which is the sole member of the *Asfarviridae* family [3, 7, 10].

Devastating ASF outbreaks and the continuing disease epidemic in the Caucasus and western Russia (2007 – to date) and, more recently, in Eastern Europe (2014-to date) highlight ASF as arguably the greatest emerging disease threat facing the swine industry worldwide [13, 25, 29, 38]. No effective and safe ASF vaccine is currently available, and progress is hindered by a lack of knowledge as to the extent of ASFV strain diversity and the viral antigens responsible for protection in pigs [14, 21]. Available data from vaccination/challenge experiments in pigs indicate that homologous, avirulent strains induce protective immunity against virulent ASFV challenge [19, 41, 43]. Several reports have presented encouraging data indicating that avirulent ASFV strains provide effective live-attenuated vaccine (LAV) tools for studying both disease pathogenesis and protective immune responses [2, 20, 33]. However, there is still great concern over the safety of live-attenuated ASFV vaccines, and thus their implementation is highly limited.

ASFV homology, in terms of cross protection, is a phenomenon primarily dependent on virus origin or in vitro adaptation. Intriguingly, some genetically distinct ASFV strains show high cross protection potential [32]. One of the ways to assign ASFV homology is a serologic typing based on a hemadsorbtion inhibition assay (HAI). HAI suggests that several antigenic types of ASFV exist and that each type induces protection against all ASFV strains that belong to the same type [23, 35–37]. In the referred

studies, several ASFV immunotypes (serogroups) were available that made it possible to conduct a comparative analysis of live-attenuated and virulent ASFV strains in a vaccination/challenge experiment.

Several studies have shown efficient cross-protective immunity against virulent ASFV using recombinant [22, 28, 44] ASFV strains. Most of this work focuses on a degree of induced protection and identification of ASFV virulence factors, but only a few have gone further to understand the interaction between the vaccine and challenge virus. Thus, Carlson and colleagues [5] revealed that recombinant and challenge viruses might persist simultaneously in recovered animals, but the dynamics of this interaction was not studied. The tissue localization, time of persistence and comparative quantitative analysis of viruses' distribution remained unsolved.

Considering these questions about the potential for convergent persistence of LAV and virulent ASFV strains and the possible interference between live-attenuated and virulent ASFV strains, we sought to determine the kinetics of virulent and attenuated ASFV strains in recovered pigs and how to differentiate highly homologous ASFV strains in vivo.

Methods

Viruses and pigs

Here, we used the attenuated ASFV strain KK262 (Genotype I, Serogroup 2) to induce immunity against ASF as well as the virulent parental strain K49 (Genotype I, Serogroup 2) [4, 24]. The ASFV K49 was initially isolated from the Congo in 1949 and was designated as a highly virulent virus because it caused high mortality in domestic pigs; they died 6-9 days after infection and had no detectable antibodies. KK262 is an attenuated derivative of K49 with 50 serial passages in porcine kidney cell lines (SPEV) and 262 passages in porcine bone marrow cell culture [35].

A group of six cross-bred pigs (Large White and Landrace, 30 to 35 kg, animal numbers DP 5.1; DP 5.2; DP 5.3; DP 5.4; DP 4.5; DP 4.6) were intramuscularly immunized with ASFV KK262 ($2x10^6$ hemadsorbing unit [HAU]) to induce an immune response. The inoculum was an African green monkey kidney tissue cell line (COS-1) lysate infected with ASFV KK262. The pigs were boosted at day 21 with the same dose of virus and then challenged intramuscularly at day 42 with the highly virulent homologous strain K49 (10^2 HAU). The immunization and challenge doses for this experiment were chosen based on previous work [4]. Pigs were observed daily according to a welfare schedule to monitor their health status and record the clinical signs after infection [30].

The control group (n = 3; animal numbers DP 2.4, DP 4.3, and DP 4.4) was challenged with 10^2 HAU ASFV K49, and clinical signs were recorded daily. The inoculum was lyophilized swine blood infected with ASFV K49 strain diluted in phosphate-buffered saline.

Experiments with domestic pigs were performed with the approval (reference number 33-11-0132/16.06.2016) of the Federal Agency of Scientific Organization and the Institute's Research Ethics Committee and were conducted in animal biosecurity facilities (77.99.03.001.J.000702.0405) at the National Research Institute for Veterinary Virology and Microbiology (VNIIVViM, Pokrov).

All methods were performed in accordance with Russian legislation and did not require further approval by the authorities.

PCR

Peripheral blood samples were collected at day 0 and at different days post vaccination (7, 14, 21, 28, 35, and 42 dpv) and challenge (3, 5, 7, 14, 21, and 28 dpc) for viremia detection using an OIE-prescribed real-time PCR assay [18]. Viral DNA was extracted from the tissue and blood samples of the ASF infected pigs by using a QiaAmp DNA mini kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. The genome copies of the viral DNA were calculated using recombinant plasmid pGEM-T-easy (Promega, Fithcburg, USA) with an insertion of the B646L (p72) gene from the ASFV strain, Stavropol 01-08 (Genotype II, Serogroup VIII). The genome copies of ASFV DNA per gram were also measured in tissue samples (spleen, kidney, liver, submandibular lymph nodes, and gastrohepatic lymph nodes). Viral load with multiple data points per group were assessed by one-way ANOVA or Kruskal–Wallis one-way ANOVA with ranked data, as implemented in GraphPad Prism (GraphPad Software, USA). Statistically significant results are noted in the figures and text.

For the differentiation of virulent/attenuated ASFV viruses, the following oligomers were used to amplify the central variable region (CVR) within the B602L gene: forward 5'-AAT GAA GGC AAA CTC TAG G-3' and reverse 5'- GGG AGC AGT ATA TTC GAC-3'. The ASFV B602L gene PCR was carried out in a Maxygene thermal cycler (Axygene, New York, USA) with the following conditions: 95 °C for 4 minutes, 40 cycles of 95 °C for 30 seconds, 56 °C for 30 seconds, and 72 °C for 30 seconds. The reaction mixture was set up with 12.5 µl of 2 x Phusion® Hot Start Flex Master Mix (New England Biolabs, Ipswitch, USA) with 1 µl (10 mM) of each primer,

5 µl of cDNA, and PCR grade RNase-free water up to 25 µl.

Long range PCR for the ASFV genome

A primer-walking method from Portugal et al. [31] and Goller et al. [12] was adapted to generate 10-15 kb overlapping fragments for the ASFV strains from different serogroups (Supplementary materials). Briefly, ASFV long range PCR was carried out with Long Amp® DNA Polymerase (New England Biolabs, Ipswitch, USA). The reaction mixture and PCR conditions were set up according to the manufacturer's recommendations.

Nucleotide sequencing

Amplified fragments of the B602L gene were sequenced after purification from a 2% agarose gel with a commercial kit, Qiaquick DNA gel purification kit (Qiagen GmbH, Hilden, Germany), by using an ABI Prism Big dye terminator ready reaction cycle sequencing kit with a 3130 DNA sequencer (Applied Biosystems, Waltham, USA), according to the manufacturer's recommendations. Products of the sequencing reaction were purified with a BigDye Xterminator kit (Applied Biosystems, Waltham, USA). Chromatograms were manually edited and assembled using CAP [16]. The nucleotide sequences were aligned using the MUSCLE algorithm implemented in MEGA 5.0 [40].

Virus isolation

Primary swine monocyte/macrophage cultures from peripheral swine blood were prepared for ASFV isolation. The inoculum was the blood and tissue homogenates from immunized/challenged animals. The swine blood samples were frozen and thawed 3 times and were then diluted 10 times in PBS before inoculation. The 10% homogenized tissue suspension treated with gentamycin (50 µg/ml) was used to inoculate swine monocytes/macrophages. The sample was declared negative after three serial passages in cell culture. Attempts at virus isolation were performed according to the accredited protocol of the EU and OIE Reference Laboratory for ASF.

Enzyme-linked immune assay for ASFV antibodies detection

Serum samples were harvested at day 0 and at different days post-immunization (7, 14, 21, 28, 35, and 42 dpv) and through 28 dpc and stored at −80 °C until use. An ELISA kit (Ingezim PPA COMPPAC, INGENASA, Madrid, Spain) was used to detect specific ASFV antibodies.

Results

Animal experiments

A classic vaccination/challenge experiment was designed to test the kinetics of both viruses and the possibility of differentiation of parental and derivative ASFV strains in vivo.

Clinical findings and virus detection

Transient fever was detected in all pigs immunized with KK262 at days 3 and 24 post vaccination (dpv) (Figure 1A). Reduced feed intake and slight depression were observed in pigs after several days of elevated body temperature. The ASFV KK262 strain was detected by qPCR from 3 to 42 dpv (Figure 2). Recovering pigs from the immunized group, regardless of their gender and body weight, all showed no clinical signs associated with the disease. All animals presented with good health, appetite and activity. In contrast the pigs from the control group succumbing to the challenge virus presented typical symptoms of acute disease. All pigs showed high febrile temperatures at 3 dpc (40.8 - 41.7 °C) and demonstrated severe ASFV clinical signs: redness of the skin of the ears, abdomen, and legs, a strong depression, laboured breathing, anorexia and ataxia. All animals from the control group demonstrated a loss of appetite and recumbency. Nevertheless bleeding from the nose or rectum was not observed. The pigs in the control group were found in a moribund state at 5-7 dpc and were euthanized. All 6 surviving pigs from the vaccinated group did not show detectable viraemia through 28 dpc. The results of the animal experiment are summarized in Table 1.

An antibody response appeared by 12-14 dpv in all pigs that had been immunized with ASFV KK262 and lasted until the end of experiment (70 dpv). The levels and kinetics of specific anti-ASFV antibodies were not defined in this experiment.

The recovered pigs were euthanized at 35 dpc, at which point they were in a clinically healthy state. The pigs inoculated with ASFV KK262 and challenged with ASFV K49 did not show any pathological signs of ASFV. Only the spleens were slightly enlarged, but the spleen sizes were still within expected biological variation. Gross lesions in the pigs from the control group were similar to those described for classical ASF (i.e., enlarged haemorrhagic gastrohepatic lymph nodes, enlarged spleens, lung oedema, and petechial haemorrhages in the kidneys of some pigs).

Viral load and virus isolation

Blood obtained at different time points post infection as well as tissue samples (spleen, kidney, liver, submandibular lymph node, gastrohepatic) from euthanized pigs were

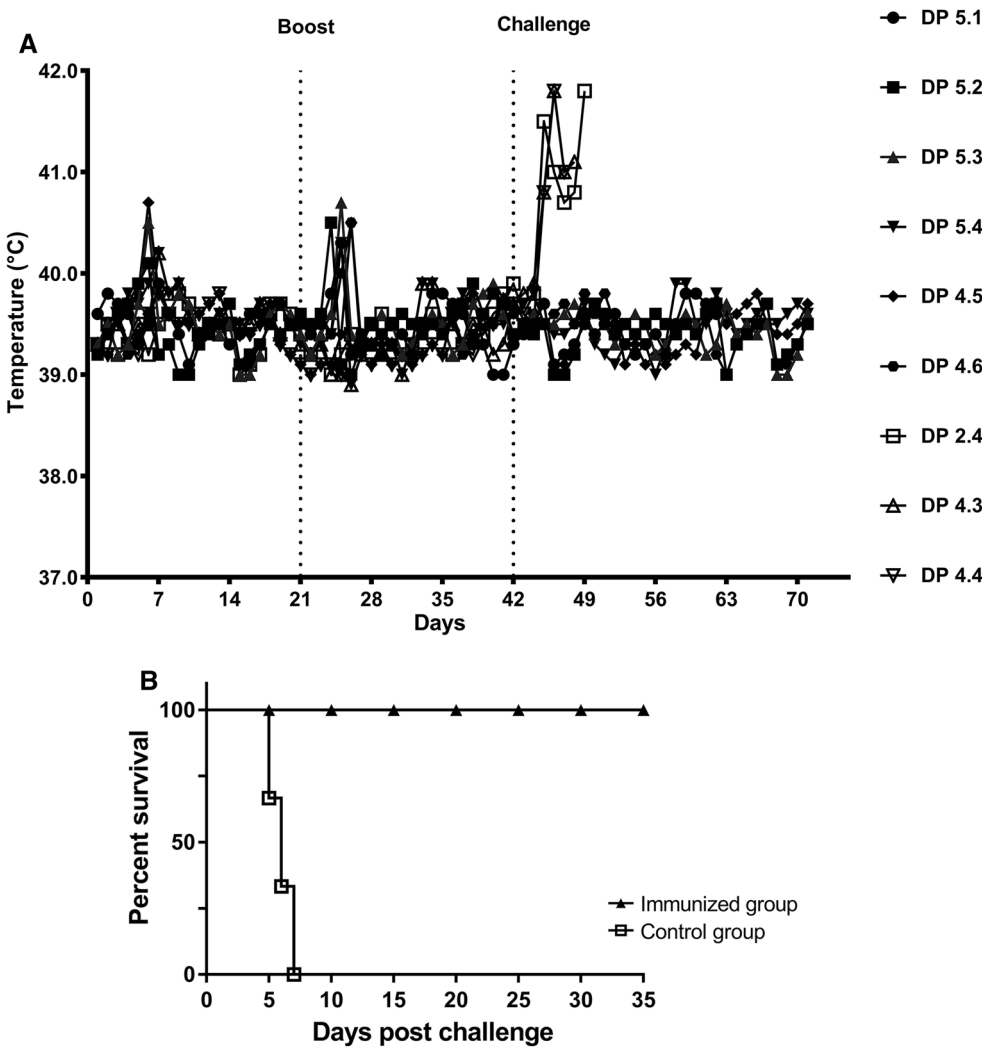

Fig. 1 The results for the immunization/challenge experiment using ASFV KK262 and ASFV K49. 6 pigs were immunized with ASFV KK262, boosted with the same dose of virus at day 21 and then challenged with 100 HAU of ASFV K49 at day 42. The control group (n = 3) were non-immunized and challenged with 100 HAU of ASFV K49. (A) ASFV fever kinetics for the immunized and control groups. (B) Survival analysis for all inoculated groups

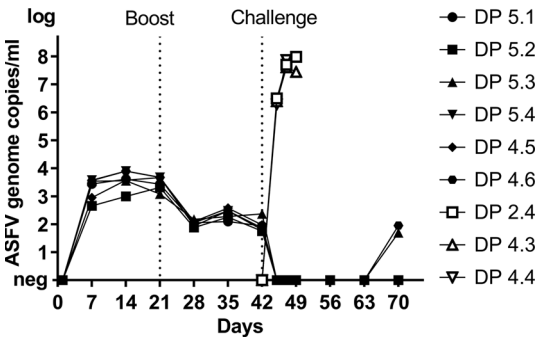

Fig. 2 The results of qRT-PCR analysis for ASFV DNA in EDTA-blood samples. The ASFV genome copies in the EDTA-blood samples were calculated using a real-time PCR assay as described elsewhere [18] with a pGEM-p72 recombinant plasmid being used as a standard. The mean values, standard deviations, minimal and maximum values for all inoculated groups are depicted in the graph

used for ASFV DNA detection using qPCR and virus isolation. ASFV DNA was detected in blood samples from 3 dpv but unexpectedly could not be detected from the blood of all animals after the challenge until 28 dpc. Low levels of ASFV DNA were detected in the blood of two pigs (DP 4.6 and DP 5.3) at 28 dpc. Those pigs did not show any clinical signs of ASF and remained healthy until the end of the experiment (Figure 2).

All organ samples from the pigs of the control group were found to be strongly positive for ASFV by qPCR (mean values range 2.71E+06 - 1.12E+07 genomes per gram). In contrast, a very low level (mean values range 9.29E+02 – 7.88+01 genomes per gram) of the ASFV genome was detected in some tissue samples (spleens, submandibular lymph nodes, gastrohepatic lymph nodes) in 2 pigs (DP 4.6 and DP 5.3) from the immunized group. ASFV DNA was not detected in liver and kidney samples from any of the pigs.

The qPCR positive blood and tissue samples from pigs DP 4.6 and DP 5.3 were used for virus isolation. Primary swine macrophage cultures were infected with blood or a 10% organ suspension. Based on the results of the haemadsorbtion test (HAT), ASFV was successfully

Table 1 The results of a homologous ASFV Congo immunization/challenge experiment

Groups	n	Average viremia prechallenge (42 dpv)	Percentage of animals with viremia prechallenge	Percentage of animals with anti-ASFV antibodies Ω	Challenge with ASFV K49 102 HAU							
					TTF #	TTD Ψ	Viraemic load at days post challenge τ					%*
							3	5	7	14	21	28	
Immunized with ASF KK262	6	0.9E+01 (0.4)	100	100	3.6 (0.2)	-	-	-	-	-	-	0.7E+0.1 (0.4)	33
Control (Mock)	3	0	0	0	3.0 (0.0)	6.0 (0.3)	7.9E+06 (0.3)	8.2 E+07 (0.4)	9.4 E+07 (0.5)	-	-	-	-

DP domestic pigs with individual numbers

TTF, Mean time-to-fever in days post challenge, with SE in parenthesis

Ψ TTD, Mean time-to-death in days post challenge, with SE in parenthesis

τ Mean viral load in genome copies per ml in blood, with SE in parenthesis

Ω Percentage of animals in group developing positive antibody titers using INGENASA ELISA

***** Percentage of animals in group demonstrating viral load in blood at 28 dpc. In 2 animals out of 6 ASFV was successfully isolated from blood and tissue samples in swine macrophages

isolated from blood (28 dpc) and tissue samples (spleen, gastrohepatic and submandibular lymph nodes) from pigs DP 4.6 and DP 5.3 (Table).

ASFV long-range PCR

Examining the genetic basis for attenuation might help to identify the most variable parts of the ASFV genome, reveal new genetic markers for virus evolution and find possible recombinant variants in the case of co-infection. The genomes of ASFV virulent strains and their attenuated derivatives from 7 different serogroups (SG) were amplified using long-range PCR and a primer-walking method. ASFV virulent and attenuated strains, totalling 14 ASFV strains, were analysed in pairs. The central parts of the genomes (conserved between ASFV and homologous viruses) remained unchanged in all of the studied isolates. The most significant changes in size for the amplified fragments were detected in the left variable region (LVR) and the right variable region (RVR) of the ASFV genomes. The sizes of these differences approached 10 kb for several ASFV strains (in the LVR). The attenuated ASFV strains demonstrated large deletions of varying size in the LVR genomic regions containing genes from the MGF505/360 multi-gene family. In the RVR region in the attenuated ASFV strains, smaller deletions of up to 4 kb were detected in regions containing MGF100 members.

Additionally, the long range PCR was performed to gain an understanding of which strain/strains was detected in the blood and tissue samples from recovered pigs.

The results of the long range PCR of the ASFV LVR and RVR with a DNA template extracted from blood (28 dpc) and tissue samples (spleens, submandibular lymph nodes, gastrohepatic lymph nodes) from qPCR-positive recovered pigs (DP 4.6 and DP 5.3) showed the presence of only ASFV K49 genome fragments.

ASFV B602L gene PCR

ASFV B602L gene PCR was used to differentiate ASFV virulent strains from attenuated derivatives. A preliminary study of ASFV strains from different SGs revealed a small-scale insertion (up to 50 bp) in several attenuated ASFV strains. However, not all of the attenuated ASFV strain B602L gene variations were found. The most gradual variations in B602L gene size were detected in the ASFV attenuated strains that were obtained via extensive adaptation in heterologous cell lines (e.g., Vero, CV-1, COS-1, A4C2/9K). These data confirm the results of previous work [17, 34, 39] showing that the progressive adaptation of ASFV in vitro may lead to significant changes in the B602L gene.

Additional nucleotide sequencing confirmed an increased number of repeats (amino acid tetramers) in the B602L genes in several ASFV strains. Thus, the ASFV KK262 strain, compared with the ASFV K49 strain, had a 36 bp insertion in the B602L gene.

The nucleotide sequences of the B602L gene from ASFV K49 and ASFV KK262 have been submitted to GenBank under accession numbers KX375344 and KX375345, respectively.

In the current work, the B602L gene was used to differentiate attenuated and virulent ASFV strains in the clinical samples. The B602L gene was amplified from the blood (28 dpc) and tissue samples (spleens, submandibular lymph nodes, gastrohepatic lymph nodes) from pigs DP 4.6 and DP 5.3. According to the lengths of the B602L PCR

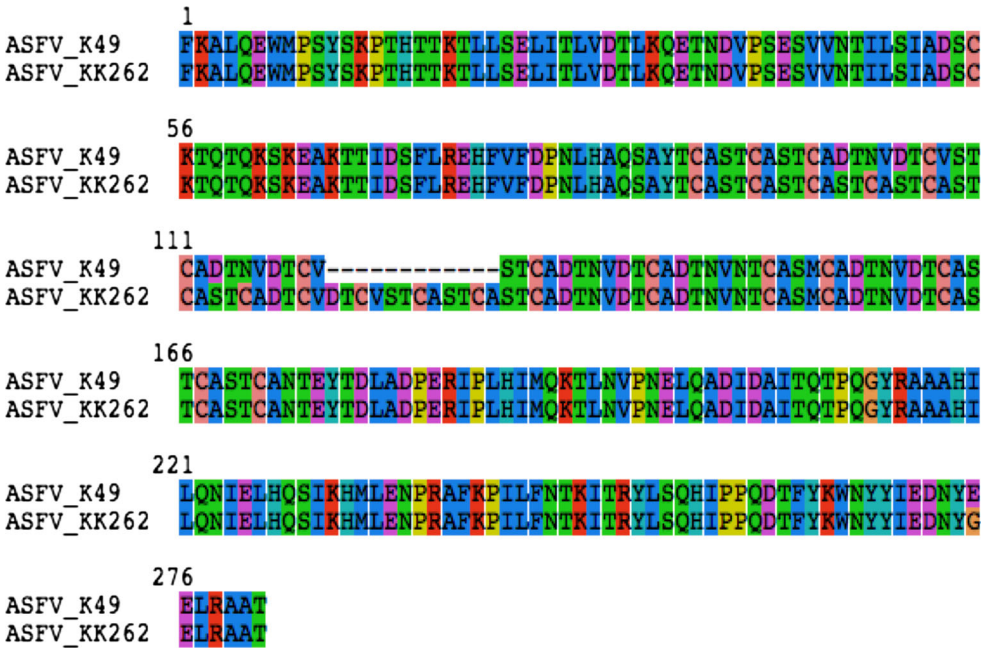

Fig. 3 Partial amino acid sequence alignment of the tetrameric tandem repeats identified within the central variable region (CVR) of B602L gene from ASFV K49 (virulent) and ASFV KK262 (attenuated). ASFV KK262 contains a 12 amino acid insertion in the CVR of the B602L gene

products, only the ASFV K49 B602L specific sequence was detected. The difference of 36 bp allowed us to differentiate virulent ASFV K49 from attenuated ASFV KK-262 strains. All analysed samples were additionally sequenced and confirmed to be ASFV K49 specific sequences (Figure 3).

Therefore, based on the results of B602L gene PCR and nucleotide sequencing, exceptionally virulent ASFV K49 was found at 28 dpc in the blood and tissue samples of vaccinated/challenged domestic pigs.

Discussion

Despite complete or near-complete suppression of ASFV replication in recovered pigs, ASFV may persist indefinitely under immune dysfunction conditions [11]. Long-term persistence of ASFV DNA was established in swine monocyte/macrophages up to 500 days post infection [6]. In several studies, the low level of ASFV DNA in blood and tissue in immunized/challenged pigs has been confirmed [8, 19]. It is clear that solid protective immunity is induced in pigs immunized with LAV or moderately virulent ASFV strains. Notably, pigs surviving ASF infection develop long-term resistance to homologous, but not heterologous, ASFV strains.

The immunization/challenge experiments with recombinant LAV demonstrated that both the vaccine and the challenge virus could be detected in the blood of survived pigs [26, 27]. In contrast, in short-term immunization/challenge experiments with E75CV1/E75 the challenge virus was absent in recovered pigs [20]. The persistence of attenuated or moderately virulent ASFV strains in infected animals might play an essential role in the process of protection against challenge with a virulent ASFV strain [5]. The exact mechanism for this type of competition between ASFV strains remains unknown and may strictly depend on the model being used.

Clearly, homologous protection induced by live-attenuated or moderately virulent ASFV strains is a unique model for understanding ASFV pathogenesis and vaccine development [20]. Nevertheless, in some cases, unacceptable post-vaccination reactions might lead to severe clinical signs and disease progression.

In this study, we demonstrate that ASFV Congo model is a relevant system to study protective immunity. Overall, 100% of the pigs were fully protected and did not show any clinical ASFV symptoms. Intriguingly, ASFV DNA was not detected in most of the pigs after the challenge through to 28 dpc. We found that ASFV localized to the local replication sites (gastrohepatic lymph nodes) and remained dormant.

A low level of viremia was detected in 2 of the 6 pigs at 28 dpc in the immunized/challenge group. The virulent ASFV K49 was identified in blood and in the spleen, gastrohepatic and submandibular lymph nodes of these 2 pigs. The four other pigs were in very good health until the end of the experiment.

The CVR region (B602L gene) is a well known genetic marker for ASFV variability, but in this study the B602L gene was used for the first time to differentiate attenuated and virulent ASFV strains in clinical samples. The CVR variants ASFV K49 and ASFV KK262 were also characterized based on the presence of amino acid tetramers,

using the one letter code lately proposed previously [1]. The results showed additional TRS in the B602L gene of ASFV KK262. The CVR of the B602L gene of attenuated ASFV KK262 consisted of 21 repeats (A A A A A A B B A A A B N B T D B N A A F), whereas the parental virulent ASFV K49 had only 18 (A A B N A B N A B N B T D B N A A F). This result should be considered further in a more detailed analysis of the molecular epidemiology of ASF outbreaks that use CVR as a genetic marker.

Our observation that virulent ASFV may eclipse attenuated homologous strains *in vivo* is important because it suggests potential hazards, e.g. disease occurrence, even in immune pig populations. It is clear that immune pigs remain susceptible to ASF infection and that virulent ASFV may rebound at a late stage post infection and become dominant in the host. This research stresses the need for further detailed study of the biosafety of LAV against ASFV, i.e. using different virus models, large groups of animals and separate routes of immunization. We cannot exclude the possibility that in our Congo model the attenuated ASFV strain might persist in recovered pigs, but this probably represents a minor virus population.

We have clearly demonstrated that a recrudescence of virulent virus may occur at least 28 days post challenge in recovered animals. Highly homologous ASFV strains are different in the LVR and the B602L gene and can be easily identified in clinical samples. Furthermore the B602L gene ontology should be considered in ASF evolution and ecology studies.

Additional information Accession codes: the obtained nucleotide sequences for the B602L gene from ASFV K49 and ASFV KK262 have been deposited in GenBank under accession numbers KX375344 and KX375345, respectively.

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Author contributions IT, AM and GB wrote the main manuscript text and designed the experiments. SM and AK worked with animals. IT sequenced ASFV strains and assembled the sequences. DK designed the experiments and analyzed the data. All authors reviewed the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare no competing financial interests.

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