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Attempts at the development of a recombinant African swine fever virus strain with abrogated *EP402R*, *9GL*, and *A238L* gene structure using the CRISPR/Cas9 system

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Abstract

Introduction: African swine fever (ASF) is a pressing economic problem in a number of Eastern European countries. It has also depleted the Chinese sow population by 50%. Managing the disease relies on culling infected pigs or hunting wild boars as sanitary zone creation. The constraints on the development of an efficient vaccine are mainly the virus' mechanisms of host immune response evasion. The study aimed to adapt a field ASFV strain to established cell lines and to construct recombinant African swine fever virus (ASFV) strain. **Material and Methods:** The host immune response modulation genes *A238L*, *EP402R*, and *9GL* were deleted using the clustered regularly interspaced short palindromic repeats/caspase 9 (CRISPR/Cas9) mutagenesis system. A representative virus isolate (Pol18/28298/Out111) from Poland was isolated in porcine primary pulmonary alveolar macrophage (PPAM) cells. Adaptation of the virus to a few established cell lines was attempted. The plasmids encoding CRISPR/Cas9 genes along with gRNA complementary to the target sequences were designed, synthesised, and transfected into ASFV-infected PPAM cells. **Results:** The reconstituted virus showed similar kinetics of replication in comparison to the parent virus isolate. **Conclusion:** Taking into account the usefulness of the developed CRISPR/Cas9 system it has been shown that modification of the *A238L*, *EP402R*, and *9GL* genes might occur with low frequency, resulting in difficulties in separation of various virus populations.

Keywords: African swine fever, CRISPR/Cas9, *EP402R*, *9GL*, *A238L*.

Introduction

African swine fever (ASF) was introduced to Poland in February 2014 most probably *via* a dead wild boar (27, 32, 34). In spite of efforts made to manage the population of wild boars and thus reduce the potential rate of African swine fever virus (ASFV) transmission between infected and susceptible animals, the disease is continuously spreading to new areas. Recently, numerous wild boar cases were reported for the first time in Lubuskie province in western Poland, about 300 km away from the nearest previously reported cases in Masovian province. This unexpected turn caused severe concern about further spread of the disease into Greater Poland province, where the pig population has high density, and also to neighbouring Germany. As a consequence of ASF prevalence among infected wild boars, the disease outbreaks in domestic pigs are closely aligned to the same areas where infected boars are found. Up to the end of February 2020 in Poland, the total number of ASF cases in wild boars had reached nearly 7,000, while in domestic pigs, 261 outbreaks had been notified. While ASF is not infectious to humans, it substantially impacts the national and international agriculture sector economically and socially (8). Because of the lack of an effective vaccine, the only control strategy stipulates two drastic measures: one, ruinous to pig producers, of

culling livestock and the other, of hunting campaigns in order to reduce wild boar populations to the minimum possible level of 0.1–0.5 animals per square kilometre (9, 14). The only reasonable means of ASF prevention is implementation of high level biosecurity practices in every kind of pig holding (15). Meanwhile, taking into account that currently the seventh wave of ASF is inflicting mortalities on domestic pigs in Poland, the potential risk of ASF occurrence is also real in commercial farrow-to-finish or fattening farms of up to 30,000 head.

ASF was originally described by Montgomery in 1921 in Kenya, but since then it has been present in many countries of Europe, South America, and recently also in Asia. Therefore, it has ceased to be considered an exotic disease (5, 9). ASFV is a large multi-enveloped DNA virus and the sole member of the *Asfivirus* genus in the *Asfarviridae* family (7). Its complex genome comprises a single, linear double-stranded DNA molecule, encoding genes essential in viral replication, virus assembly, and egress as well as responsible for immunological interactions with the host (7). The virus replicates predominantly in cells belonging to the mononuclear phagocyte system, specifically monocytes and macrophages, but nevertheless, other cell types may also be infected in the late stages of infection (6). ASFV possesses a unique feature to avoid recognition by and cytotoxic response of CD8+ cells (26, 33). Neutralising antibodies are not produced as they are in the in case of a number of other viral pathogens of pigs. Several viral genes which block or manipulate the host immune response have been identified (7). Among them, the *A238L* gene has an important role in the inhibition of expression of transcription factors involved in host immune response (29). Another gene, *EP402R* encoding CD2-like protein, is responsible for adsorption of erythrocytes around infected cells (haemadsorption) and facilitates virus spread in the host (7). Deletion or alteration of the *EP402R* gene in virulent ASFV strains leads to haemadsorption abrogation *in vitro*, furthermore in animal trials, it led to delayed viraemia and clinical signs, suggesting the crucial role of this gene in ASFV virulence (1–3, 11, 13, 21). Similarly, the studies conducted on ASFV strain lacking the *9GL* gene showed this gene's important role in maturation of fully infectious ASFV particles. Moreover, *in vivo* study demonstrated that the strain with deleted *9GL* was attenuated in pig model (19, 24).

The recent ASFV pandemic has highlighted the urgent need for vaccine development. Inactivated or subunit vaccines showed no protective immunity (12, 22), while some naturally attenuated strains isolated from the field are able to provide complete protection against virulent strains (2, 35). Partial or full protection was induced by live attenuated vaccines (LAVs) obtained by serial passages in cell cultures or targeted deletion of selected genes (16, 23–25). LAVs present the most promising candidates; nevertheless, further studies are required to guarantee their safety, long-term efficacy, and capacity to differentiate between infected and vaccinated animals. Investigations regarding ASFV vaccine are continuously impeded by deficient knowledge about virus interactions with host immunity, its genetic complexity, and technical difficulties such as the lack of established ASFV-permissive cell lines or low transfection efficiency in porcine macrophages (8).

During the last few years, CRISPR/Cas9 (clustered regularly interspaced short palindromic repeat/caspase 9) technology has become an exceptionally efficient method of genetic manipulation of distinct eukaryotic and prokaryotic organisms; moreover, this powerful tool facilitated targeted viral gene modifications (18). Originally, CRISPR/Cas9 was described as a component of the *S. pyogenes* immune system, which allows bacteria to combat phages in a simple but precise manner engaging endonuclease and RNA (17). In gene engineering applications, binding of Cas9 endonuclease to the target site is mediated by about 20-nt-long guide RNA (gRNA) complementary to the target sequence and a 3-nt-long protospacer adjacent motif (PAM) located immediately downstream of a given locus. Cas9 protein induces a double-strand break (DSB) within the target, about 3 or 4 nt upstream of the PAM sequence. Subsequently, the DSB is repaired by the non-homologous end joining (NHEJ) pathway, resulting in minor nucleotide insertions or deletions and therefore in disruption of the encoded gene open reading frame (ORF) (17).

ASFV replication was successfully inhibited by this RNA-directed DNA modification technique targeting the *p30* gene (13). This technology seems to be a promising approach to generation of a live attenuated ASFV vaccine strain. The study presented in this paper aimed to adapt an ASFV field isolate to established cell lines and to perform ASFV genomic manipulation in order to develop a recombinant strain lacking three immune response inhibition-related genes (*9GL*, *A238L*, and *EP402R*).

Material and Methods

Cell cultures and viruses. Porcine primary pulmonary alveolar macrophages (PPAMs) were purchased from the Technical University of Denmark (DTU, Lindholm, Denmark), primary cultures of pig bone marrow (PBM) cells were obtained by washing out the red bone marrow derived from the long bones of 20 kg Polish Large White pigs. Immortalised porcine alveolar macrophage (IPAM) and MARC-145, COS-1, and Vero cell lines were purchased from ATCC. PPAM, PBM, and IPAM cells were subcultured in RPMI 1640, 10% FBS, 1% antibiotic-antimycotic solution, 1 mM sodium pyruvate, and 1× non-essential amino acids. MARC-145 and COS-1 were maintained

in Dulbecco's modified Eagle medium (DMEM) and Vero in MEM, all supplemented with 10% FBS and 1% antibiotic-antimycotic solution. All cultures were grown at 37°C in a humidified atmosphere containing 5% CO_2.

The ASFV Pol18/28298/Out111 genotype II isolate was derived from the spleen of a pig which died during outbreak 111, (Chełm district, Poland, May 2018). Virus was isolated from clinical material as previously described (20). The titre was calculated using the Reed–Muench method (28).

The growth kinetics of isolated virus were evaluated in PPAM cells, which were infected with ASFV Pol18/28298/Out111 at a multiplicity of infection (MOI) of 0.1. Subsequently medium samples collected 0, 1, 2, 3, and 4 days post infection (dpi) were titrated.

Assessment of susceptibility of selected established cell lines to ASFV infection. Since field isolates of ASFV belonging to genotype II are not capable of infecting established cell lines, we attempted to adapt Polish field strain of ASFV to continuous cell lines, which were previously shown to facilitate replication of other ASFV genotypes. Nevertheless, up to date, only one paper reported successful adaptation of ASFV genotype II strain Georgia 2007/1 to such a culture (16).

In order to perform adaptation, subconfluent monolayers of the IPAM, MARC-145, COS-1, and Vero cell lines were infected either with spleen homogenate or virus stock previously isolated in PPAM cells. The infected cultures were then incubated for five days, and subsequently, 10 passages were performed. A real-time PCR according to Fernandez-Pinero (10) was used to control virus replication. At 5 dpi of the tenth passage, cells were fixed, and an immunoperoxidase test (IPT) was carried out in order to confirm or rule out the presence of infected cells, since neither haemadsorption nor cytopathic effect was observed.

Construction of CRISPR/Cas9 expression plasmids. In total, six DNA fragments encoding gRNA which aligns to six selected regions within the *9GL*, *A238L*, and *EP402R* ASFV genes (two target sites per gene) were designed (Table 1). Target sequences were unique in comparison to the rest of genome and were located adjacent to a 3-nt-long PAM sequence serving as a binding signal for Cas9 nuclease. The pGK1.1 vector was obtained by cloning the designed gRNA encoding oligonucleotides into a vector backbone (Creative Biogene, Shirley, NY, USA). Target sequences including PAM and their positions are presented in Table 1.

The plasmid encodes an endonuclease Cas9 (Csn1) and two antibiotic resistance genes (kanamycin and puromycin) to facilitate selection of positive clones. The sequence encoding gRNA and CRISPR RNA is encoded under the control of a U6 promoter.

E. coli G10 cells were transformed with the obtained construct and provided by Creative Biogene as bacterial glycerol stocks. *E.coli* were cultivated overnight in LB medium supplemented with kanamycin, then sedimented by centrifugation, and subjected to plasmid DNA extraction with a NucleoSpin Plasmid Miniprep Kit (Machery Nagel, Hoerdt, Germany) according to the manufacturer's protocol. The correctness of the obtained plasmids was verified by a conventional PCR utilising a forward primer aligning to the right end of the U6 promoter (CATATGCTTACCGTAACTTGAAAG) and reverse complement target sequences as the reverse primer. The annealing temperature was set at 50°C. The plasmids were used for further transfection of selected cell lines.

Transfection. Six plasmids containing the CRISPR/Cas9 encoding cassette, specific for six target sequences (two vectors per gene, each of which targeted two distinct sequences), were designed for transfection. During this study, two transfection kits were tested, namely the Xfect (TaKaRa Bio, Mountain View, CA, USA) and the GeneJect transfection reagents (A&A Biotechnology, Gdynia, Poland). The former is a biodegradable transfection polymer stated by the producer to have a very low cytotoxicity profile and high transfection efficiency (up to 97%). The latter has reduced cell toxicity compared to lipofectin; however, its toxicity may increase at low confluence. A comparison of the protocols for the transfection kits used during this study is shown in Table 2. The Xfect and GeneJect transfection reagents were used for transfection of primary cells (PPAM) and a continuous cell line (Vero), and the outcomes informed the selection of the appropriate transfection kit and target cells, which is schematised in Fig. 1. The transfected cells were intended for further virus infection.

Infection. Infection was carried out in 24-well plates with 1×10^6 cells/mL and an MOI of 0.1. After 48–72 h, the haemadsorption phenomenon was noticeable, and at 7 dpi, 100 µL of thrice freeze-thawed medium was transferred to fresh PPAM cell cultures to observe replication of the virus.

Transfected cells (PPAMs and primary PBM cells) were subjected to 24- or 48-h puromycin selection, and subsequently infected with ASFV Pol18/28298/Out111 in the presence of pig erythrocytes in order to observe haemadsorption. At 5 dpi, the plates were freeze-thawed three times, the total DNA was extracted, and ASFV presence was confirmed by real-time PCR (10). The targeted *9GL*, *A238L*, and *EP402R* genes of the ASFV genome were amplified by conventional PCR and 1.4-, 0.759-, and 1,139-kbp regions covering the whole genes of interest were sequenced. Moreover, amplification of the *A224L* gene was performed as a reaction control. The primer characteristics are given in Table 3.

Table 1. Target DNA sequences of *A238L*, *EP402R*, and *9GL* genes of African swine fever strains. Nucleotide positions refer to the ASFV Georgia 2007/1 genome sequence (GenBank accession number FR682468.1). PAM sequences are marked in bold

Name	Sequence	Position	Length
A238L-1	**CCG**AAATAGCCCAACACCCCTTC	50.832–50.851	20
A238L-2	TCGCATCTATTGACAATCCA**CGG**	51.027–51.046	20
EP402R-1	**CCT**CATAATGATGTATTTGATAC	73.627–73.646	20
EP402R-2	**CCT**GCTACTCCCCCAAATATCAC	73.705–73.724	20
9GL-1	**CCA**GTACTGAAAGTCCTCCGAG	95.099–95.117	19
9GL-2	**CCA**GTATTTAGGTCCCCAATGCA	95.285–95.304	20

Table 2. Transfection protocol. The main conditions for two applied kits are presented

Parameter	Xfect	GeneJect
Plasmid DNA	5 µg	0.4 µg
Total growth medium volume	1,000 µL	1,000 µL
Transfection reagent	1.5 µL	2 µL
Incubation time for nanocomplexes creation	10 min/RT	30 min/RT
Incubation time with target cells	4 h/37°C	24–72 h/37°C
Additional steps	Disposal of medium Replacement with fresh growth medium	-
Control of transfection effect	48 h	Within 24–72 h incubation time

RT – room temperature

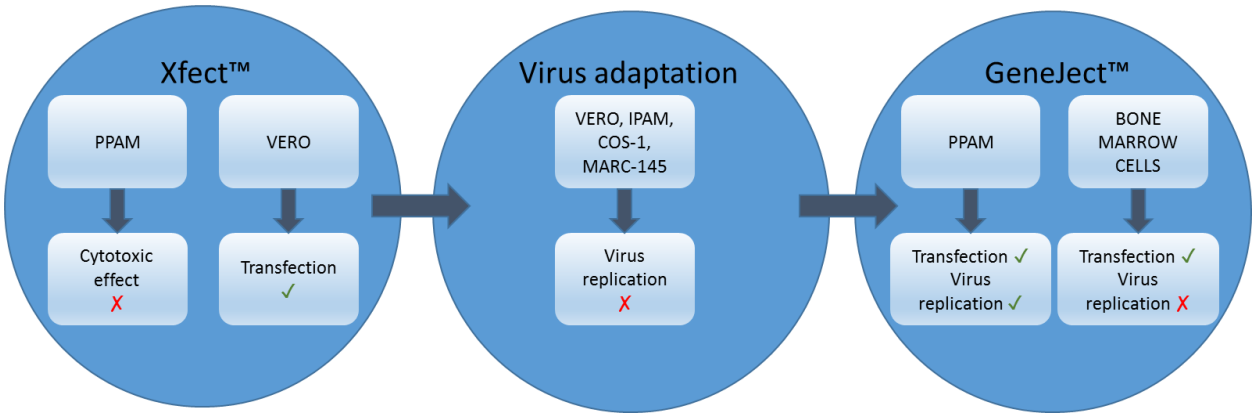

Fig. 1. Schematic representation of the selection of the suitable transfection kit and target cells

Table 3. Primers used for amplification of the region of interest covering target genes. Nucleotide positions refer to the ASFV Georgia 2007/1 genome sequence (GenBank accession number: FR682468.1)

Name	Sequence	Position	Tm (Primer melting temperature)
A238L-F	TTGGACACAGGAAACGATCT	50.370–50.389	49.7°C
A238L-R	ATATGGGAAAAGGGCCTGGC	51.302–51.283	53.8°C
EP402R-F	ACTATATTATAAAACATATG	73.341–73.360	37.4°C°
EP402R-R	TGCATGTGATGGAAATCGGT	74.594–74.575	49.7°C
9GL-F	GCCTCACTATCGATCGGCAA	94.046–94.065	53.8°C
9GL-R	ACTGGCTGGAATTACGCCAA	95.450–95.431	51.8°C
A224L-F	AAAAGCTATTTGTTTATCCCCA	46.266–46.287	47.4°C
A224L-R	CCTTCAATTGAGGATGATCATT	47.057–47.036	49.2°C

Results

Field ASFV strain. ASFV Pol18/28298/Out111 isolated in PPAM cells reached a titre of 6.06 $\log_{10}HAD_{50}$/mL. The haemadsorption phenomenon was easily observable from 1 dpi and was characteristic for the ASFV genotype II, currently circulating in Europe and Asia. The replication kinetics assessed in PPAM were comparable to previously reported growth kinetics of the reference Georgia 2007/1 strain (23). Sanger sequencing of the three target genes showed 100% identity with this strain.

Adaptation to established cell lines. Unfortunately, despite strenuous attempts at the development of a strain adapted to established cell lines, each of the tested cells (IPAM, MARC-145, COS-1, and Vero) was incapable of facilitating and maintaining ASFV replication during ten passages. Neither cytopathic effect nor haemadsorption phenomenon were visible; moreover, real-time PCR results clearly confirmed that the ASFV Pol18/28298/Out111 strain (both directly from clinical samples and previously isolated in PPAMs) is not able to grow in the selected continuous cell lines because viral DNA content fell successively, disappearing completely between the second and eighth passages. The IPT assay performed during the tenth passage showed the absence of replicating virus. This result is in line with previous reports that ASFV genotype II strains replicate in primary cells belonging to the mononuclear phagocyte system (6) rather than in continuous cells.

Vector design. In order to confirm the correctness of the extracted plasmids, a conventional PCR amplifying the region between the 3′ end of the U6 promoter and the target sequence was performed. The obtained products were about 100 bp in size, which was consistent with expectations and evidenced the presence of the gRNA encoding sequence within the vector backbone.

Transfection. As regards transfection, the X-fect reagent yielded successful results exclusively in an established cell line (Vero), which was able to grow stably in medium supplemented with 1 μg/mL of puromycin (Fig. 2).

Nevertheless, these transfection reagents transpired to be cytotoxic to PPAM cells, and when used according to the manufacturer's protocol, caused cell death almost immediately. For this reason, the GeneJect transfection kit dedicated to more susceptible and hard-to-transfect cells was used with PPAM and PBM cells and exerted no cytotoxic effect. It is apposite to confirm that transfection of each of the six plasmids was conducted separately in order to determine whether any of the induced mutations directly affected virus replication.

Infection. Puromycin-resistant PPAMs and PBM cells were infected with ASFV Pol18/28298/Out111, 24 or 48 h post transfection, and the virus was allowed to propagate for seven days, during which haemadsorption was observed. Subsequently, a real-time PCR was performed. The obtained results are included in Table 4 and clearly show that virus replication is present in PPAM cells.

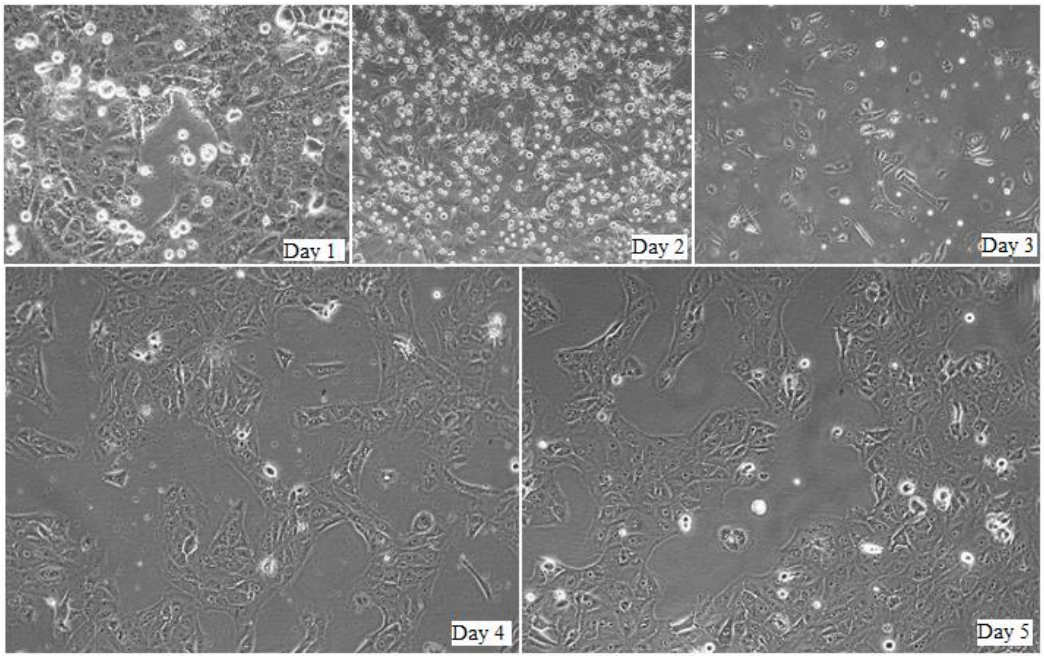

Fig. 2. Vero cells transfected with constructed CRISPR/Cas9 plasmid. In order to confirm successful transfection, a puromycin selection was applied, which led to an increased rate of cell death during the first three days post transfection, and later the cell culture started to grow in antibiotic supplemented medium (magnification 200 ×)

Target gene analysis. The viruses obtained in the second passage after 48 h incubation with puromycin were selected for further analysis. A conventional PCR was performed in order to amplify viral target genes as well as the *A224L* gene which was used as the PCR control for each of the obtained knock-out (KO) viruses (Fig. 3). Lack of *A224L* amplification in KO-9GL1 and KO-EP402R2 was probably a consequence of the low ASFV DNA concentration (35.97 and 36.25 Cq values in qPCR, respectively). Therefore, only KO-9GL2 and KO-EP402R1 were analysed further. The results showed no PCR product of the *9GL* and *EP402R* sequences for cells transfected with each of the plasmid variants (Table 5). However, the cells transfected with the A238L-1 and A238L-2 target sequence-encoding plasmids showed appropriately sized bands; therefore, the PCR product was subjected to further sequencing. The findings revealed the 100% nucleotide identity of the obtained recombinant viruses with the parent ASFV Pol18/28298/Out111.

Replication kinetics. The in vitro replication kinetics of each of the six generated deletion mutants and WT-ASFV/Pol18/28298/O111 were evaluated using a culture of primary swine macrophages. The cells were infected with the analysed viruses at an MOI of 0.1, and samples were collected at 0, 1, 2, 3, and 4 dpi. All evaluated strains showed similar growth kinetics to each other and their parent strain (Fig. 4).

Table 4. Results obtained in real-time PCR

Target site	PPAM Puromycin 24 h		PPAM-Puromycin 48 h		PBM Puromycin 24 h		PBM Puromycin 48 h	
	I	II	I	II	I	II	I	II
9GL-1	33.89[h]	33.85[h]	34.14[h]	35.97	31.95[h]	-	31.48[h]	38.62
9GL-2	34.49[h]	31.05[h]	34.38[h]	31.99[h]	31.65[h]	33.55[h]	32.37[h]	34.08
A238L-1	32.26[h]	31.3[h]	32.75[h]	31.26[h]	32.34[h]	28.5[h]	32.88[h]	-
A238L-2	32.92[h]	31.93[h]	32.92[h]	29.88[h]	31.93[h]	30.52[h]	33.61[h]	35.54
EP402R-1	31.92[h]	38.71	33[h]	29.76[h]	32.74[h]	34.21	32.9[h]	33.79[h]
EP402R-2	31.79[h]	28.82[h]	32.8[h]	36.25	32.89[h]	38.69	32.59[h]	34.81

[h] – haemadsorption; I (II) – first (second) passage after 24 (48) h of incubation with puromycin

Table 5. Results of conventional PCR

Material	PCR target			
	9GL	*A238L*	*EP402R*	*A224L*
KO-9GL1	(2)−	n/a	n/a	(5)−
KO-9GL2	(3)−	n/a	n/a	(6)+
KO-A238L1	n/a	(8)+	n/a	(11)+
KO-A238L2	n/a	(9)+	n/a	(12)+
KO-EP402R1	n/a	n/a	(14)−	(17)+
KO-EP402R2	n/a	n/a	(15)−	(18)−
WT	(4)+	(10)+	(16)+	(7, 13, 19)+

Numbers in brackets represent sample numbers in Fig. 3. KO – knock-out targets; WT – wild type; n/a – not applicable

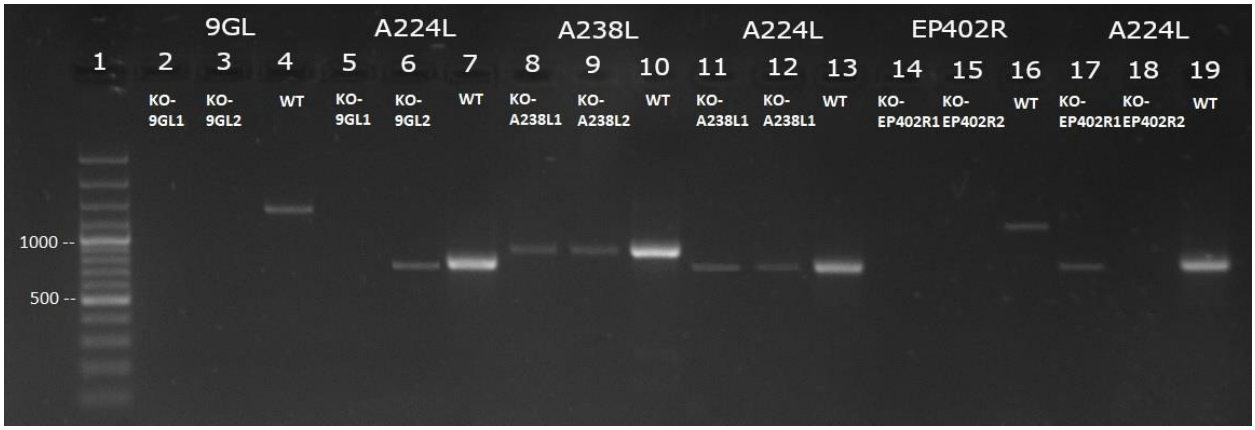

Fig. 3. Agarose gel (2%) showing the results of the conventional PCR to amplify whole sequences of targeted genes. Gene names at the top represent the amplified region. Numbers below gene names correspond to Table 5 numbers in brackets, 1000/500 – band size markers

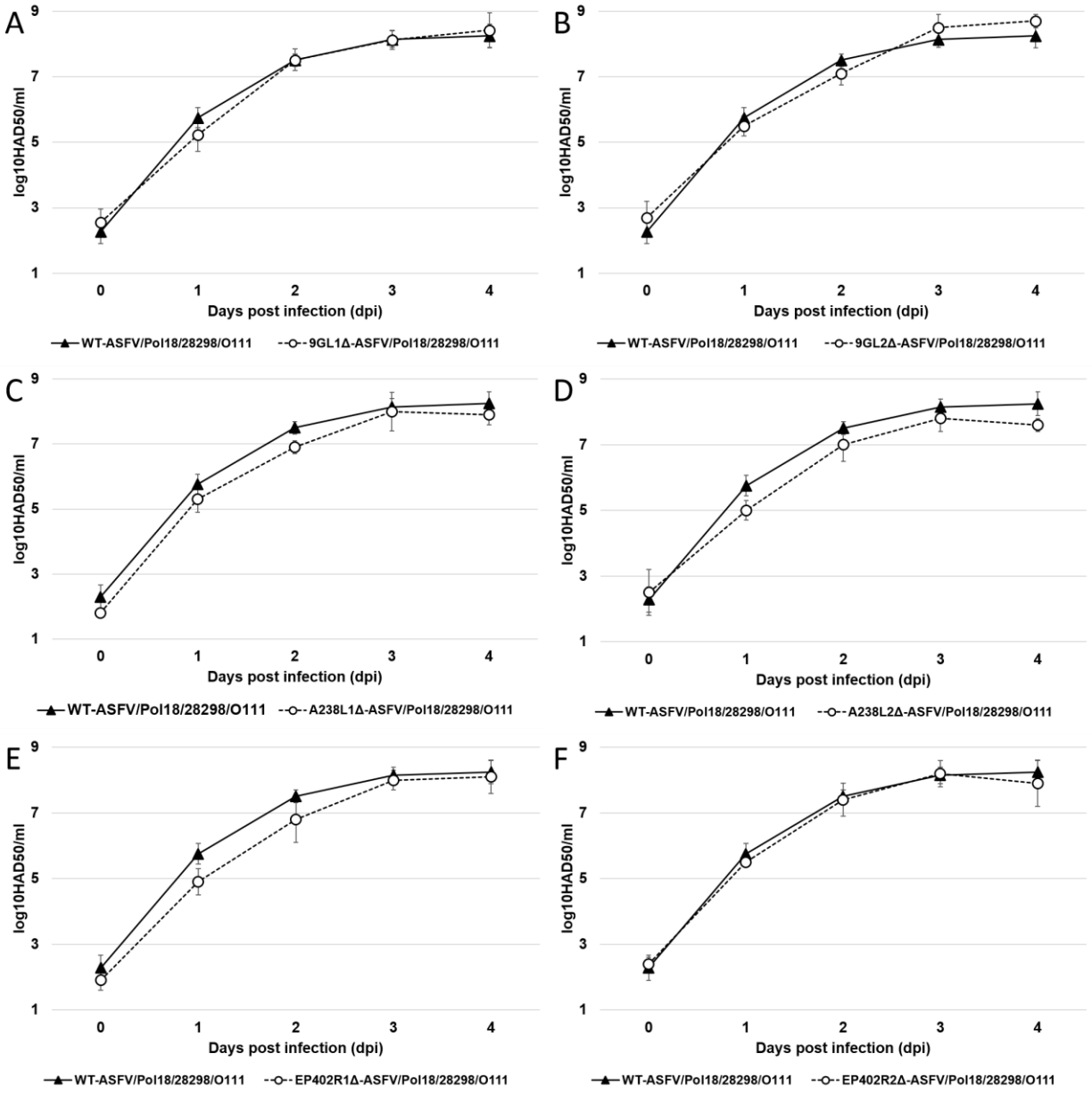

Fig. 4. *In vitro* replication kinetics of the six generated deletion mutant (A – 9GL1Δ; B – 9GL2Δ; C – A238L1Δ; D – A238L2Δ; E – EP402R1Δ; F – EP402R2Δ) and parent ASFV/Pol18/28298/O111 isolates. PPAM cell cultures were infected (MOI 0.1) with both strains, and subsequently virus titres were estimated daily over 96 h post infection. Data represent means and standard deviations from three independent experiments. The sensitivity of virus detection was 1.8 HAD_{50}/mL

Discussion

To date, numerous research efforts have been made to develop a vaccine against ASFV; nevertheless, the only currently available methods to control the disease are based on early detection and implementation of strict biosecurity measures. However, these have proved to be insufficient to limit worldwide ASF spread; therefore, the generation of a safe, effective vaccine is urgently required. So far, attempts at its development have included inactivated viruses, recombinant proteins, DNAs-, and LAVs-vaccines, but only the last approach seems to be a promising strategy to generate protective immunity in pigs and wild boars (30). LAVs which showed high levels of protection were obtained by serial passaging in cell cultures or by rational genetic modification of virulent strains. Moreover, naturally attenuated, low virulent ASFV isolates present a promising alternative to developing protection against lethal challenge with ASF virus homologues (2). The CRISPR/Cas9 technique has so far shown itself to be an effective approach for manipulation of the ASFV genome, efficiently inhibiting virus replication *in vitro* (13). Nevertheless, since highly virulent ASFV strains are only capable of infecting and efficiently replicating in primary cells (31), attempts to develop a genetically engineered virus are even more difficult.

Despite our best efforts to adapt Polish ASFV strains to numerous established cell lines, neither serial

passages nor modifications of the medium composition in order to sensitise cells facilitated efficient virus replication in them. Transfection of continuous cell lines (Vero) was easier to conduct, observe, and control than transfection of primary cells; therefore, such an approach might be much more suitable for virus genetic manipulation purposes due to the constant process of cell proliferation. However, since established cell lines are not permissive of ASFV genotype II replication, and numerous attempts to adapt the virus have failed, the possibility of using stable cell lines in further studies must be discounted. As primary cells were found to be highly sensitive to transfection reagents, genetic manipulation of ASFV genotype II using plasmid-delivered modification systems such as CRISPR/Cas9 in combination with PPAM cell culture is severely constricted. Moreover, the lack of a gene encoding a fluorescent protein such as green or red (GFP or RFP) within the vector backbone caused the selection of positive transfected clones to be based only on antibiotic resistance genes. Such selection is not an optimal approach for primary, non-dividing cells; therefore, determination of the successfully transfected cell proportion is difficult, and may lead to replication of WT virus. Lack of amplification of the *9GL* and *EP402R* genes in the conventional PCR precluded unarguable confirmation of changes in its nucleotide sequences. However, similar growth kinetics of the altered and parent strains and the observation of a haemadsorption phenomenon in a strain intended not to include the *EP402R* gene may respectively suggest that only a small proportion of viruses underwent successful modification of the *9GL* and *EP402R* genes. As regards the *A238L* gene, sequencing explicitly excluded any changes in its nucleotide sequence.

There are few possible explanations of the obtained results, two of which being that Cas9 nuclease has a low or limited affinity to the selected target sites and that deletions or insertions induced by the NHEJ pathway repair do not significantly affect protein function. Although CRISPR/Cas9 is considered a method which revolutionised genetic engineering, using this RNA-guided genome modification system with regard to ASFV is strictly limited. So far, only a few papers have reported successful CRISPR/Cas9 targeted ASFV genome modification. The authors were able to get less than 1% recombinant virus on the initial transfection, suggesting that the method is useful but needs further optimisation to improve its efficiency (4, 13). The other possibility is that CRISPR/Cas9 system performance is simply insufficient to edit all copies of virus genome before ASFV DNA is packaged in its protein envelope during the virus replication cycle in the cell.

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Animal Rights Statement: Not applicable.

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