Sensitivity of African swine fever virus (ASFV) to heat, alkalinity and peroxide treatment in presence or absence of porcine plasma

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ABSTRACT

African swine fever virus (ASFV) is a highly resistant viraemic virus with devastating socio-economic impact. Its present epidemiology in Eastern Europe and Russia warrants increased biosecurity measures in Western Europe. This includes proactive precautions on traffic of pork products within and between areas that are officially free from ASF. Namely, delayed notification of clinical signs or introduction of a low-virulent strain in ASF-free areas could result in presence of ASFV in veterinary inspected pork and pork by-products. The present study evaluated sensitivity of ASFV to physical and chemical processing conditions that can be applied on abattoir collected blood for production of spray dried porcine plasma (SDPP). Standard endpoint dilution assays were used to determine the sensitivity of Vero-cell adapted Lisbon/60 strain ASFV to heat treatment (H) at alkaline conditions (A) with or without peroxide (P). Time (T) dependent inactivation was evaluated in presence or absence of porcine plasma. HAPT-treatment at H = 48 °C, A = pH 10.2 and P = 20.6 or 102.9 mM H_2O_2 during 10 min (T) inactivated (95LCL) 3.35, respectively, 4.17 $\log_{10}$ $TCID_{50}$ ASFV/ml plasma. In absence of plasma, 6.99 log-inactivation was reached within 5 min. Implementation of HAPT-treatment on plasma from ASFV-free areas provides an additional safety hurdle for derived blood products in the unlikely event that blood from few undetected infected pigs would contaminate pooled veterinary inspected blood. Such an additional processing step in the production of SDPP is thus a valuable precautionary measure to overcome a potential biosecurity-break that may arise during the high-risk phase between transboundary introduction of ASFV and first notification of the disease.

1. Introduction

Blood products are manufactured from abattoir collected blood of veterinary inspected swine. An anti-coagulant, usually trisodium citrate or tricalcium phosphate, is added during collection to enable fractionation by centrifugation. Optionally, plasma is ultrafiltrated to increase protein content and is dried by spray drying to obtain a highly digestible animal protein source. Main applications of resulting spray dried porcine plasma (SDPP) in animal feed include its use as a gelling agent in wet pet food, and to improve performance and gut health in young piglets (Polo et al., 2005a; Torrallardona, 2010). In short, SDPP is used in milk replacers, creep feed and weaning diets as an alternative source of lactogenic immunoglobins and other bioactive glycoproteins that are present in sow's milk. In-feed immunoglobins from SDPP offer passive mucosal immunity to the gut and plasmaborne growth factors promote maturation and integrity of the intestinal lining (Torrallardona, 2010; Peace et al., 2011; Perez-Bosque et al., 2016). Within the European Union, porcine blood products are approved as a feed ingredient for use in pet food and feed for non-ruminant food-producing animals, including fish and crustaceans, poultry and pigs (European Commission, 2009). Adequate sourcing and processing of the raw material is, however, essential to secure the safety of SDPP. Viral safety concerning emerging viraemic diseases, such as African swine fever (ASF) are to this respect of particular interest.

ASF is a highly lethal viral hemorrhagic fever of domestic pigs and European wild boar of all ages. Historical introductions of ASF from sub Saharan Africa to overseas territories were initiated by catering waste from aircrafts or ships fed to domestic pigs or improperly disposed and scavenged by wild boar. Untimely diagnosis of the index case and uncontrolled movement of infected domestic swine were the driving forces of rapid spread and spill-over to adjacent countries (Mur et al., 2011). Most recently, ASF was introduced into Georgia in 2007 from East Africa through catering waste from ships in the docks of Poti (Sánchez-Vizcaíno et al., 2012). Subsequently, the virus spread throughout the Caucasus into the Russian Federation, Ukraine, Belarus and an increasing number of bordering European Union (EU) member

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states. As of April 2018, ASF was diagnosed in EU member states Lithuania, Poland, Latvia, Estonia, Moldavia, Czech Republic and Romania (OIE, 2018). Current epidemiology in Eastern Europe intensifies the awareness of a real ASF threat to neighboring countries.

Transboundary introduction of ASF virus (ASFV) in ASF-free countries results in massive compulsory culling of swine and suspension of international trade of pork and live animals. As spread into new regions is likely to occur in the near future, increased ASFV-specific biosafety measures are urged at all levels of the pig production and distribution chain. This includes prevention of unknowingly spread of ASFV between ASF-free countries during the high-risk phase, which is the period from initial introduction until official diagnosis and notification. Spread by veterinary inspected pork and pork by-products from ASF-free countries would require an unlikely coincidence of circumstances within the index-case. In particular, delayed notification or introduction of a low virulent strain have to take place in conjunction with hampered traceability in order to fail timely withdrawal of products manufactured from abattoir inspected pork and pork by-products.

A worrisome limited survey in pig farmers showed that only 91% (20/22) of German and 88% (150/171) of Bulgarian respondents intended to comply to the mandatory immediate notification of suspicion of ASF. Motivations for delayed notification included concerns about adverse effects on their reputation, fear of penalties and the belief of being able to handle the outbreak themselves. Some respondents declared they would try to sell their pigs as soon as possible (Vergne et al., 2016). As ASF is characterized by prominent clinical disease and severe internal lesions, veterinary inspection at slaughter will intercept acutely and subacutely infected animals. Preclinical viraemic animals would likely pass inspection, which emphasizes the importance of traceability. Emergence of an attenuated low-virulent strain causing chronic infections and apparently healthy carriers may more importantly impede prompt ante and post mortem recognition of ASF (Penrith and Vosloo, 2009; Sánchez-Vizcaíno et al., 2014). Such attenuated strains are reputed to emerge in endemic regions, as is the current situation in Transcaucasia and Russia. ASFV, however, does not readily undergo genetic modifications when passaged in pigs or ticks (Boinas et al., 2004). Notably, it is hypotized that the low-virulent strains isolated during the previous European outbreak where derived from the laboratory-attenuated experimental vaccine that was massively released in Portugal and Spain in the 1960's (Boinas et al., 2004; Penrith and Vosloo, 2009; Sánchez-Vizcaíno et al., 2012). Experimental infections with high and low doses of Russian, Caucasian and Lithuanian field isolates indicate that the strain introduced in Georgia in 2007 has remained virulent so far (Pietschman et al., 2015; Vlasova et al. 2015; Gallardo et al., 2017). Even though, emergence of carrier animals cannot be excluded (Vlasova et al., 2015).

Besides epidemiologic considerations in viral risk assessment, virus-inactivation studies should include highly resistant model viruses when evaluating the efficacy of processing methods for raw materials in the production of food or feed ingredients. Heat treatment of animal plasma in industrial settings is primarily limited by technical feasibility and aspired preservation of bioactive properties of end products. Namely, heat-induced denaturation and gelling of plasma proteins impairs further processing, reduces gel strength when applied in meat products or wet pet food, and obliterates beneficial effects on gut health when used in animal feed. HAT-treatment, defined as moderate heating (H, 48 °C) of plasma at alkaline conditions (A, pH 10.2) for a short period of time (T, 10 min), has been shown to efficiently inactivate porcine epidemic diarrhea virus (PEDV) (Quist-Rybachuk et al., 2014). As ASFV is reputed to be a highly resistant virus, we added hydrogen peroxide (P, H_2O_2) as supplemental treatment condition to evaluate the feasibility to inactivate ASFV in absence and presence of porcine plasma. Therefore, the present study evaluated the efficacy HAT and HAPT-treatment of porcine plasma in the production of spray dried porcine plasma (SDPP) to inactivate ASFV as a model virus of highly resistant envelope viruses that are adapted to survival in animal plasma.

Table 1

Treatment conditions of media spiked with ASFV lab strain Lisbon/60. All combinations of treatment conditions were tested in separate test tubes containing 9 ml medium (MEM or porcine plasma) and 1 ml virus stock[d]. Virus was added after temperature and pH adjustment of media.

Treatment Conditions	Heat[a] (H)	Alkalinity[b] (A)	Peroxide[c] (P)	Time (T)
	H: °C	A: pH	P: mM H_2O_2	T: min
Exp. 1 (n = 1)	21	7.5	0	0 (starting titer)
	4	7.5 or 10.2	0 or 92.6	90
	21	7.5 or 10.2	0 or 92.6	1 or 60
	48	7.5 or 10.2	0 or 92.6	30
Exp. 2 (n = 4)	48	7.5	0	0 (starting titer)
	48	10.2	0	1, 5, 10, 15, 20, 25, 30, 45 or 60
Exp. 3 (n = 3)	48	7.5	0	0 (starting titer)
	48	10.2	20.6 or 102.9	1, 5, 10 or 20
Termination	Cooling	Neutralization	Inactivation	
	37	7.5	0–90 μl catalase	

[a] 4 °C, ice water; 21 °C, room temperature; 48 °C, water bath, 37 °C, cooling in ice water (± 50 s).

[b] Alkalinisation of media with 2.5 M NaOH to reach the final pH upon addition of virus stock, neutralization with 0.22 M H_2SO_4.

[c] Via addition of 35% peroxide stock solution (10.3 M H_2O_2).

[d] 4.71, 4.62 and 8.35 $\log_{10}$ $TCID_{50}$ ASFV/ml virus stock in experiment 1, 2 and 3, respectively.

2. Materials and methods

2.1. Experimental design

Sensitivity of ASFV was evaluated for combinations of physical and chemical treatment conditions of heat (H), alkaline pH (A) and hydrogen peroxide (P) during different time intervals (T) (Table 1). Spike-inactivation assays with ASFV were performed in two test media: minimum essential medium (MEM, Gibco Life technologies, Paisley, UK) and porcine plasma (Veos NV, Zwevezele, BE). Initial and residual virus infectivity was determined via standard endpoint dilution assays performed in duplicate. Vero cell adapted Lisbon/60 ASFV strain was used for all assays (kindly provided by Professor C. Martins, Faculdade de Medicina Veterinária Universidade Técnica de Lisboa, Portugal). Effects of HAPT-treatment on nutritional content of liquid plasma was evaluated by analysis of dry matter (ISO 6496), crude protein (ISO 16634-1), crude ash (ISO 5984) and sodium content (ICP-OES) of three samples before and after industrial processing.

2.2. Test media and treatment conditions

The plasma used was of porcine origin and was commercially collected at Belgian slaughterhouses. The anticoagulant was trisodium citrate. Plasma was kept refrigerated until sterile filtration through a 450 nm cellulose acetate and a 220 nm PES membrane bottle top filter (VWR, Radnor, PA, USA) and stored at −20 °C until use. Prior to the assays, the plasma was thawed at room temperature and heat inactivated at 56 °C for 30 min in order to avoid gelation when incubated in cell culture media. Table 1 presents the treatment conditions tested in experiment 1, 2 and 3. All combinations of treatment conditions, including the different incubation times, were tested in separate 50 ml polypropylene tubes (Falcon®, VWR, Leuven, Belgium) containing 9 ml medium (MEM or plasma) to which 1 ml virus stock was added. Plasma and MEM were equilibrated to the required temperature and pH prior to addition of virus stock. Correct temperatures were reached by submerging the test tubes in ice water (4 °C), water at room temperature (21 °C) or a water bath (48 °C). The pH of test media was adjusted using

2.5 M NaOH and was set to reach the desired final pH upon addition of virus stock. Hydrogen peroxide (Persynt® 350 Spray, Evonik, Hanau, DE) was added immediately after addition of virus. Starting titer at time 0 min was determined in temperature equilibrated media at neutral pH. Treatments where terminated after preset incubation times by briefly cooling each sample in ice water, neutralization of pH with diluted sulfuric acid (0.22 M H_2SO_4) to pH 7.5 and inactivation of peroxide with catalase (Multifect® CA1000L, DuPont Industrial Biosciences, Rochester, NY, USA). Peroxide in test samples at 0, 20.6, 92.6 and 102.9 mM H_2O_2 was inactivated with 0, 20, 90 and 100 μl catalase, respectively. Complete inactivation of peroxide was verified with test strips (Quantofix® Peroxide 100, Macherey-Nagel GmbH & Co KG, Düren, DE).

2.3. Analyses

Absence of ASFV in native plasma was verified by real-time polymerase chain reaction (RT-PCR) as described in Tignon et al. (2011). No CPE was observed when plasma was cultured on Vero cells. Initial and residual virus titers were determined via standard endpoint dilution assays on Vero cells. In short, test samples were 10-fold serially diluted in MEM without addition of antibiotics or fetal calf serum and 100 μl of each of eight dilutions per test sample inoculated in duplo on 96-well plates with a one-day old monolayer of Vero cells in replacement of the culture medium. After three days of cultivation at 37 °C in presence of 5% CO_2, the cytopathogenic effects where read under a phase-contrast microscope and 50% tissue culture infective dose ($TCID_{50}$) calculated using the Reed-Muench method. The theoretical lower detection limit (LDL) was $0.5 \log_{10} TCID_{50}$/ml.

2.4. Statistics

Linear regression analysis was used to construct first order kinetics survival curves with incubation time as independent variable and residual infectivity as dependent variable. Curve estimation with fixed intersection at incubation time 0 min was based on 3–4 independent replicate assays and included verification of compliance to normal distribution and homoscedasticity of data. The decimal reduction time (D value) or time required to inactivate 90% of the initial virus population ($1 \log_{10} TCID_{50}$ reduction) was calculated as the negative inverse of the slope of the semi-logaritmic survival curve. Half-life ($t_{1/2}$) was calculated as the time needed to inactivate 50% of the initial virus population. Inactivation parameters of HAT, HAPT-LP (low peroxide) and HAPT-HP (high peroxide) treatment were considered significantly different at $p \leq 0.050$ in case of non-overlapping 95% confidence intervals (95% CI). Where appropriate, 95% lower confidence limits (95LCL) or 95% upper confidence limits (95UCL) are provided. Effects of HAPT-treatment on nutrient content of liquid plasma were analyzed using the paired Student's *t*-test and data expressed on dry matter basis (%DM) are provided as mean ± se. Statistical analysis was done in Sigmaplot 13.0 (Systat Software Inc., San Jose, CA, USA).

3. Results

3.1. Preliminary data suggest synergism of heat, alkalinity and peroxide in inactivation of ASFV

Alkalinisation of test media to pH 10.2 required 0.042% NaOH in MEM and 0.1% NaOH in porcine plasma. A small reduction in ASFV titer was noticed in MEM within one min treatment with either 0.042% NaOH or 92.7 mM H_2O_2 at room temperature (21 °C). Data on combined treatment of alkalinisation to pH 10.2 and addition of peroxide suggested a synergistic effect on the inactivation of ASFV at room temperature. Either alkalinisation or peroxide treatment were sufficient to inactivate a starting titer of $4.12 \log_{10} TCID_{50}$ ASFV per ml MEM to below LDL when incubated for 90 min at 4 °C, 60 min at 21 °C or 30 min
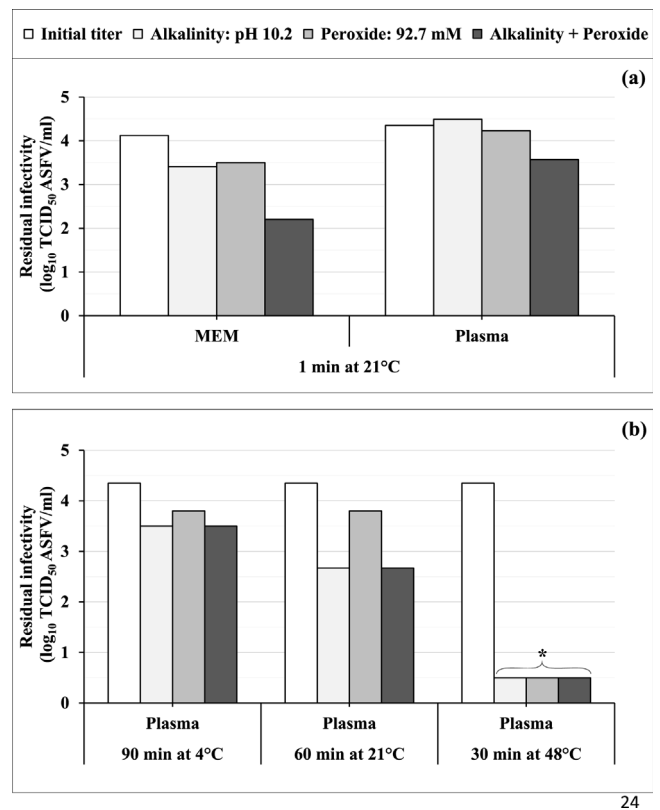

Fig. 1. Preliminary data (n = 1) on time and temperature dependent inactivation of ASFV lab strain Lisbon/60 by alkaline and peroxide treatment in presence or absence of porcine plasma. *Inactivation below the lower detection limit (LDL) of $0.5 \log_{10} TCID_{50}$ ASFV/ml. All treatments shown in panel (b) inactivated the viral spike in MEM to below the LDL.

at 48 °C. Presence of plasma seemed to protect ASFV from inactivation by alkalinisation or peroxide treatment. In addition, these preliminary data suggest a temperature dependency for speed and magnitude of inactivation of ASFV. In plasma, only combined treatment of NaOH and peroxide resulted in a small but noticeable drop in ASFV titer within one minute at 21 °C. Sensitivity of ASFV to alkaline conditions strongly increased with increasing temperature. Peroxide did, however, not have a potentiating effect of alkalinisation of plasma when incubated for prolonged times at 4–21 °C. Moreover, ASFV was in presence of plasma hardly sensitive to peroxide treatment at these temperature conditions. Still, ASFV showed sensitivity to peroxide in neutral plasma when incubated at 48 °C (Fig. 1). Protein (CP), ash (CA) and sodium (Na) content of industrial plasma samples (n = 3) was 76.5 ± 0.3%DM CP, 15.4 ± 0.2%DM CA, and 6.0 ± 0.2%DM Na, expressed on dry matter (DM) basis. HAPT-treatment did not significantly affect CP or CA content, but tended to increase sodium content with 0.4 ± 0.1%DM (p = 0.060).

3.2. ASFV showed low sensitivity to HAT treatment in presence of plasma

The titer of ASFV Lisbon/60 strain in virus stock was $4.62 \pm 0.05 \log_{10} TCID_{50}$/ml. In four independent assays, heat-alkalinity-time treatment at 48 °C and pH 10.2 consistently inactivated a starting titer of $3.51 \pm 0.10 \log_{10} TCID_{50}$ ASFV per ml MEM within 25 min to below LDL. Sensitivity of ASFV to HAT-treatment in presence of plasma was lower, more variable and occurred according to a broken stick model. Here, the initial titer ($3.53 \pm 0.10 \log_{10} TCID_{50}$/ml) was inactivated to below LDL within 30 min in only two of four assays. Overall, ASFV showed high resistance to HAT-treatment. The D value in absence of plasma (8.7 min, 95% CI 8.1–9.4 min) was similar to the D
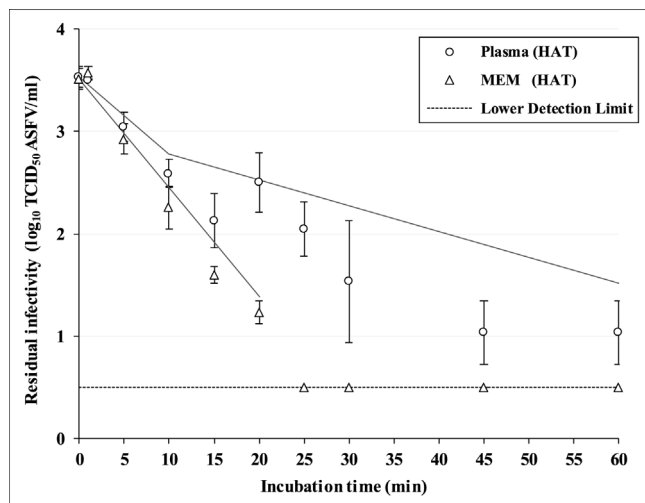
Fig. 2. Inactivation of ASFV lab strain Lisbon/60 during heat (48 °C) treatment in alkaline conditions (pH 10.2) in absence or presence of porcine plasma (n = 4, mean ± se). Inactivation by HAT-treatment in presence of plasma occurred according to a first order broken stick model. The 95% upper confidence limit survival curves are represented by full lines for HAT-treatment of plasma (0–10 min and 10–60 min) and MEM (0–20 min).

Fig. 3. Inactivation of ASFV lab strain Lisbon/60 during heat (48 °C), alkalinity (pH 10.2) and peroxide (LP: 20.6 mM H_2O_2 or HP: 102.6 mM H_2O_2) treatment in absence or presence of porcine plasma (n = 3, mean ± se). Short-term 95% upper confidence limit survival curves are represented by dashed (LP) and full lines (HP) for HAPT-treatment of plasma (0–10 min) and MEM (0–1 min).

value during the initial inactivation phase in presence of plasma ($D_{0-10\ min}$ = 10.6 min, 95% CI 8.9–13.3 min). In the second phase, the $D_{10-60\ min}$ value in presence of plasma was significantly increased to 30.8 min (25.1–39.7 min) (Fig. 2, Table 2).

3.3. HAPT treatment efficiently inactivated ASFV in presence or absence of plasma

A high titer virus stock of 8.35 ± 0.51 $\log_{10}$ TCID$_{50}$ ASFV Lisbon/60 strain per ml was obtained by ultracentrifugation of cultured virus for 16 h at 15,000*g*. In three independent spike-inactivation assays, addition of peroxide strongly increased sensitivity of ASFV to HAT-treatment at 48 °C and pH 10.2 in presence or absence of porcine plasma. In absence of plasma, over 4 $\log_{10}$ TCID$_{50}$/ml reduction (95LCL) of Lisbon/60 ASFV was reached within 1 min HAPT treatment, with similar D-values for HAPT-LP and HAPT-HP (p > 0.050). In each of six assays, inactivation of the initial titer of 6.99 ± 0.37 $\log_{10}$ TCID$_{50}$ ASFV/ml MEM to below LDL was reached within 5 min. In presence of plasma, the initial titer was inactivated to below LDL in only one of three assays of both HAPT conditions. Required incubation
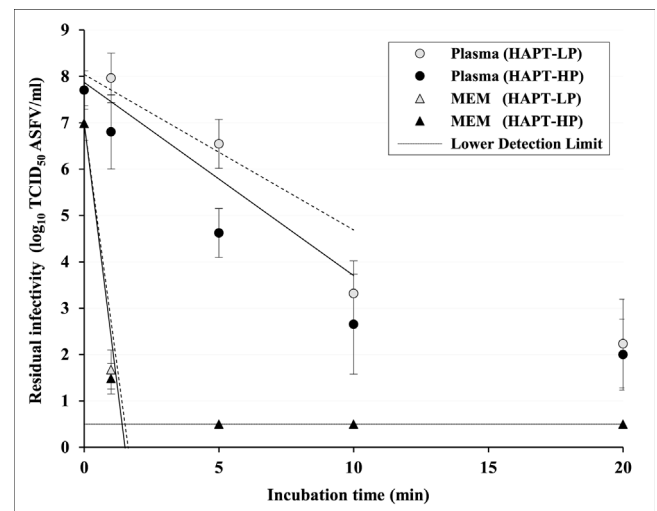
times to inactivate 7.12 $\log_{10}$ TCID$_{50}$/ml plasma were 10 min and 20 min by HAPT-HP and HAPT-LP, respectively. Initial titers in the other assays in plasma were higher (8–8.5 $\log_{10}$ TCID$_{50}$ ASFV/ml plasma). The potentiating effect of peroxide on inactivation of ASFV by heat and alkaline treatment of virus-spiked plasma was largely temporary, with maximal effect in the first ten min of incubation. Survival curves showed similar D-values at low (D_{0-10} = 2.3 min, 95% CI 1.8–3.0 min) and high (D_{0-10} = 1.8 min, 95% CI 1.5–2.4 min) peroxide dose (p > 0.050). Prolonged treatment resulted in numerically higher D_{0-20}-values (p > 0.050) (Fig. 3, Table 2).

4. Discussion

ASFV is a large enveloped DNA-virus with a capsid of several concentric lipid and protein layers below the external lipid membrane (Cobbold and Wileman, 1998). Due to its complex structure, ASFV generally shows much higher tolerance to inactivation compared to many other enveloped viruses. Its reputed high resistance to inactivation is illustrated by its long persistence during dry curing methods of pork. Complete loss of infectivity in Spanish and Italian dry-cured hams

Table 2
Sensitivity of ASFV lab strain Lisbon/60 to heat treatment in alkaline conditions without or with peroxide (HAT or HAPT) in absence or presence of porcine plasma. (Heat, H: 48 °C; alkalinity, A: pH 10.2; Peroxide, LP: 20.6 mM H_2O_2 or HP: 102.9 mM H_2O_2).

Matrix	Treatment	Replicates	Time (min)	Mean survival curve[a]	D-value (s)		$t_{1/2}$ (s)	Inactivation[b]	
					mean	95% CI	95UCL	mean	95LCL
Plasma	HAT	n = 4	0–10	y = 3.53–0.0941x	638	531–800	241	0.94	0.75
			10–60	y = 2.78–0.0325x	1846	1504–2381	717	1.63	1.26
	HAPT-LP	n = 3	0–10	y = 8.04–0.4441x	135	108–179	54	4.44	3.35
			0–20	y = 8.04–0.3252x	182	152–234	70	6.49	5.13
	HAPT-HP	n = 3	0–10	y = 7.87–0.5515x	109	88–144	43	5.50	4.17
			0–20	y = 7.87–0.3668x	164	131–219	66	7.32	5.48
MEM	HAT	n = 4	0–20	y = 3.51–0.1152x	521	483–566	170	2.30	2.12
	HAPT-LP	n = 3	0–1	y = 6.99–5.3133x	11	10–14	4	5.31	4.40
			0–5	y = 6.99–1.4524x[c]	(41)	(29–74)	(22)	6.99 (± 0.37) to < LDL	
	HAPT-HP	n = 3	0–1	y = 6.99–5.5067x	11	9–13	4	5.51	4.69
			0–5	y = 6.99–1.4599x[c]	(41)	(28–76)	(23)	6.99 (± 0.37) to < LDL	

[a] y = y_0 + ax with y, y_0 = titer, initial titer ($\log_{10}$ TCID$_{50}$/ml) and x = time (min) (all p-values ≤ 0.001).

[b] $\log_{10}$ TCID$_{50}$/ml.

[c] Assumptions for linear regression are not met.

from acutely infected swine lasted up to 140 days in Iberian and Serrano ham, and between 300 and 399 days in Parma ham (McKercher et al., 1987; Mebus et al., 1993). High tolerance to long periods of dry conditions was also shown in contaminated lyophilized blood, where ASFV remained infective after 7 year storage at 4–8 °C (Kovalenko et al., 1695). Besides resistance to heat ($D_{56\,°C}$ = 12.9 min in Earl's saline) and desiccation, ASFV has shown tolerance to extreme pH values of 3.9–10.1 at 18 °C for over 20 h (Plowright and Parker, 1967). It should be noticed that differences in inactivation kinetics may occur between wild-type and laboratory-adapted virus strains and between wild-type strains in general. Plowright and Parker (1967), for instance, showed somewhat higher sensitivity of ASFV isolate F86 (France 1964) to pH treatment at both ends of the pH range compared to ASFV isolate Tengani (Malawi 1962), both cultured in primary pig bone marrow cells. The mechanisms involved in productive entry into susceptible host cells may depend on both the strain and the cell type used for virus culture (Sobhy, 2017). This may in turn influence *in vitro* results on residual infectivity following exposure to physical or chemical treatment conditions. Yet, in depth study of the predominant mechanism of entry (macropinocytosis) of ASFV isolate E70 in immortalized porcine alveolar macrophages (IPAM) revealed to occur highly similar compared to entry of Vero cell adapted ASFV Ba71V in IPAM or Vero cells (Sánchez et al., 2012).

Not only feasibility, but also efficacy of physical and chemical treatment of potentially contaminated material depends on the matrix involved. The considered virus furthermore determines magnitude as well as direction of matrix effects. Presence of non-specific anti-viral compounds in seronegative plasma, for instance, strongly increased sensitivity of the enteric PED virus to HAT treatment at 48 °C and pH 10.2 ($D_{HAT,\ MEM}$ = 114 s and $D_{HAT,\ 90\%\ plasma}$ = 35 s; Quist-Rybachuk et al., 2014). PEDV RNA may transiently be present in blood during acute infection, but the natural infection cycle of PEDV does not include a viraemic phase. Infectious virions could, moreover, not be demonstrated in acute phase serum (Gerber et al., 2014). Altogether, this supports a hypothesis of absence of evolutionary adaptation mechanisms in PEDV to resist the non-specific defense system of serum. Plasma being a hostile environment for PEDV thus may explain increased susceptibility of PEDV to HAT treatment in presence of seronegative plasma. The present study on ASFV showed low sensitivity to HAT treatment and presence of plasma resulted in a tenfold higher tolerance to HAPT treatment. Viraemic spread is, however, essential in the infection cycle of ASFV. Increased susceptibility of ASFV in presence of plasma is thus not expected. On the contrary, a protective effect of blood proteins against inactivation of ASFV is in line with results of Plowright and Parker (1967) who observed an almost threefold faster inactivation of ASFV in serum-free Earl's saline treated at 37 °C compared to treatment in presence of serum ($D_{37\,°C,\ Earl's\ saline}$ = 27.1 h and $D_{37\,°C,\ 25\%\ serum}$ = 79.4 h). At 56 °C, the protective effect of plasma was much smaller ($D_{56\,°C,\ Earl's\ saline}$ = 12.9 min; $D_{56\,°C,\ 25\%\ serum}$ = 18.6 min). Progeny of survivors demonstrated the same inactivation pattern, which indicates a non-genetic basis (Plowright and Parker, 1967). In contrast to increased tolerance of ASFV in presence of plasma, both thermal and chemical (NaOH) inactivation of ASFV occurred more rapidly in pig slurry compared to MEM. In pig slurry, sensitivity of ASFV increased with increasing protein and total solid levels (Turner and Williams, 1999).

Temperature-dependent sensitivity of ASFV to alkaline treatment, as observed in the present study, is in line with results of Turner and Williams (1999). Still, HAT-treatment at 48 °C and pH 10.2 did not result in substantial inactivation of ASFV within 10 min. Because production of functional SDPP limits severity of heat and pH conditions, we added hydrogen peroxide as an additional treatment condition instead of increasing temperature, alkalinity or incubation time. Both liquid and vaporized hydrogen peroxide are extensively used to inactivate microorganisms in medical, laboratory and food applications where decomposition products need to be non-toxic and environmentally

friendly (Linley et al., 2012). Even at a level as low as 20.6 mM H_2O_2, ASFV showed high sensitivity to HAPT-treatment with initial half-lives of only 4 s and 54 s in absence and presence of porcine plasma, respectively. The virucidal and bactericidal effects of hydrogen peroxide are attributed to oxidative damage to specific proteins, DNA or RNA and lipid membranes such as the viral envelope or bacterial cell wall (Linley et al., 2012). Spray drying of plasma may as well contribute to the ASFV safety of SDPP. Reported viral inactivation by spray drying of plasma amounts 4–5 $\log_{10}$ per ml for swine vesicular disease virus, pseudorabies virus, reproductive and respiratory syndrome virus and PEDV (Polo et al., 2005b; Pujols and Segales, 2007; Pujols et al., 2007; Gerber et al., 2014; Opriessnig et al., 2014).

In conclusion, presence of ASFV in abattoir collected blood of veterinary inspected swine from ASF-free areas is highly unlikely. Still, blood from few undetected infected animals could result in low amounts of ASFV in pooled veterinary inspected blood. This could result from pre-clinical viraemic pigs or chronically infected carriers, which are more likely to occur in case of delayed notification of clinical signs in swine premises or in the occasion of introduction of a low virulent strain. This risk is mitigated by mandatory traceability and timely intervention of product withdrawal following the first clinical outbreak. Virus inactivation by processing of raw material is an additional safety measure to assure absence of infectious ASFV in derived blood products that are eventually applied in meat products or used as feedstuff for swine. The 95LCL of inactivation of ASFV lab strain Lisbon/60 by HAPT-treatment at 48 °C, pH 10.2 and 20.6 (or 102.9) mM H_2O_2 for 10 min on porcine plasma is 3.35 (or 4.17) $\log_{10}$ $TCID_{50}$ ASFV/ml plasma. Presuming similar sensitivity of wild-type ASFV strains, such a wet processing step on plasma sourced from allegedly ASF-free areas would inactivate an equivalent of blood from about 66 (or 444) undetected infected pigs each with a viremia of 6 $\log_{10}$ $TCID_{50}$ ASFV/ml plasma in 96,000 L pooled blood for the production of a 4-t batch of SDPP. A potential load of 6 $\log_{10}$ $TCID_{50}$ ASFV/ml plasma from clinically unaffected animals is a wide overestimation. In general, viraemia and clinical signs overlap both in onset and peak of disease, with viraemia titers of 6 to over 8 $\log_{10}$ $TCID_{50}$/ml blood in moribund animals (Carvallho Ferreira et al., 2012; Gallardo et al., 2017). Reported occurrence of pre-clinical viraemia amounts 1.5–3.5 $\log_{10}$ $TCID_{50}$ ASFV/ml blood up to 48 h prior to appearance of clinical disease (Karalyan et al., 2012; Vlasova et al., 2015). Onisk et al. (1994) noted 2 $\log_{10}$ $TICD_{50}$/ml blood in apparently healthy carrier animals that where chronically infected with attenuated strain E75-CV1. HAPT-treatment of pooled plasma is nonetheless a valuable precautionary measure in the production of SDPP as dilution alone would only reduce infective titer without any inactivation of infectiousness. The present study demonstrates feasibility of inactivation of ASFV lab strain Lisbon/60 by processing of raw material in the production of SDPP. This model can be used to evaluate sensitivity of other viraemic and non-viraemic viruses to improve safety of blood products. Such a precautionary safety hurdle is complementary to veterinary control measures put in place by the national authorities.

Competing interests

Acknowledgements

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