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Stability of African swine fever virus on spiked spray-dried porcine plasma

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Abstract

African swine fever (ASF) is a viral disease that affects members of the *Suidae* family. The notifiable disease is considered a major threat to the pig industry, animal health, and food security worldwide. According to the European Food Safety Authority, ASF virus (ASFV) survival and transmission in feed and feed materials is a major research gap. Against this background, the objective of this study was to determine the survival of ASFV on spiked spray-dried porcine plasma (SDPP) when stored at two different temperatures. To this means, commercial SDPP granules were contaminated with high titers of ASFV in a worst-case external contamination scenario. Three samples per time point and temperature condition were subjected to blind passaging on macrophage cultures and subsequent haemadsorption test to determine residual infectivity. In addition, viral genome was detected by real-time PCR. The results indicate that heavily contaminated SDPP stored at 4°C remains infectious for at least 5 weeks. In contrast, spiked SDPP stored at room temperature displayed a distinct ASFV titer reduction after 1 week (>2.8 log levels) and complete inactivation after 2 weeks (>5.7 log levels). In conclusion, the residual risk of ASFV transmission through externally contaminated SDPP is low if SDPP is stored at room temperature (21 ± 2°C) for a period of at least 2 weeks before feeding.

KEYWORDS
African swine fever virus, contamination of SDPP, disease introduction, risk factor, stability

1 | INTRODUCTION

African swine fever (ASF) is considered a major threat to the pig industry, animal health, and food security worldwide (Guinat et al., 2016). The causative agent, ASF virus (ASFV) is a complex, enveloped DNA virus of the *Asfarviridae* family with high tenacity (Alonso et al., 2018). Given the stability of ASFV under a wide range of ambient conditions, farmers and other stakeholders are concerned about ASFV transmission via feed and feed ingredients, but unfortunately the data basis is very limited. Against this background, the European Food Safety Authority (EFSA) defined research gaps regarding the potential for ASFV transmission through contaminated feed and feed materials (Alvarez et al., 2019). Of special interest could be feed with compounds of porcine origin, for example, spray dried porcine plasma (SDPP). SDPP is extensively used in pig starter diets and consistently improves growth performance and survival, especially under stressful conditions like pathogen challenge (Perez-Bosque et al., 2016). So far, there are very few studies on ASFV inactivation in contaminated porcine plasma. Kalmar et al. (2018) investigated a combination of physical and chemical processing conditions of liquid plasma, that is, heat

treatment (48°C), alkaline conditions (pH 10.2), and addition of peroxide (102.9 mM H_2O_2). This treatment led to an ASFV titer reduction of 4.17 $\log_{10}$ $TCID_{50}$/ml after 10 min. Blázquez et al. (2019) reported a titer reduction of 4.62 $\log_{10}$ $TCID_{50}$/ml after UV-C irradiation of liquid porcine plasma at a dose of 3000 J/L. The spray drying process itself with an inlet temperature of 200°C and a temperature of 80°C throughout its substance led to an ASFV titer reduction of 4.11 $\log_{10}$ $TCID_{50}$/ml (Blázquez et al., 2018). The above-mentioned studies addressed mainly contaminations that affected the raw materials, that is, plasma originating from infected pigs. However, concerns were also raised with regard to possible contamination scenarios after production, that is, during the filling process at the factory or upon transport. At the farm sites, SDPP granules are usually stored in closed containers at ambient temperature and are added to the main feed of weaned pigs.

The objective of the presented study was therefore to determine the stability of ASFV on contaminated SDPP after production when stored for different time periods (0, 7, 14, 21, 28, and 35 days) at two different temperatures (4°C and room temperature). For this purpose, commercial SDPP granules were spiked with high titer ASFV (10^6 50% hemadsorbing doses per ml [HAD_{50}/ml]). Successful contamination was demonstrated via real-time PCR analysis. Three samples per time point and temperature condition were subjected to a blind passage on peripheral blood mononuclear cell (PBMC)-derived macrophages and to subsequent haemadsorption tests (HAT) to determine residual infectivity.

2 | MATERIALS AND METHODS

2.1 | Sample preparation

SDPP from a commercial producer (APC, Villarrobledo, Spain) was used in this study. Seventy grams of SDPP (humidity max. 9.0%) was contaminated with 10.5 ml of an ASFV suspension with a titer of 10^6 HAD_{50}/ml (ASFV strain "Armenia08"). The contamination procedure was performed in a zipper bag and divided into two steps: first step was blending 70 g of the SDPP granules with 5 ml virus suspension, and the second step was blending the previous mixture with additional 5.5 ml virus inoculum, separated by a 15 min drying period with thorough shaking. Blending was performed using an intranasal mucosal atomization device (MAD Nasal; Wolfe Tory Medical, Salt Lake City, USA) to nebulize the virus suspension over the SDPP granules. After additional 15 min drying and shaking at room temperature, contaminated SDPP was dispensed as 2 g aliquots in 50 ml tubes (Sarstedt, Nümbrecht, Germany) and stored at 4°C or at room temperature (21 ± 2°C). To achieve a basic statement in this limited laboratory study, SDPP samples were taken as biological triplicates for each sampling point on days 0, 7, 14, 21, 28, 35, and stored at −80°C until further analysis. As negative control (NC), triplicate samples of uncontaminated SDPP were stored immediately at −80°C together with an aliquot of the original virus suspension (VS T_0). Furthermore, 900 μl of the virus suspension was also incubated at 4°C or at room temperature (21 ± 2°C) for 7 days or 35 days and subsequently stored at −80°C.

Prior to analysis, the −80°C stored SDPP samples (2 g) were resuspended thoroughly with 10 ml sterile distilled water with 1% antibiotic-antimycotic mix (Gibco Antibiotic-Antimycotic 100x; Thermo Fisher Scientific, Schwerte, Germany) by shaking and vortexing. Two milliliter of the reconstituted plasma was stored at −80°C for real-time PCR analysis to prove successful contamination. The remaining plasma was used as inoculum for a blind passage on PBMC-derived macrophages to determine whether any residual infectious ASFV could be detected.

2.2 | Isolation of macrophages, blind passage, and HAT

For blind passage and HAT, isolation of PBMC derived macrophages was performed from ethylenediaminetetraacetic acid (EDTA)-anticoagulated blood from healthy domestic pigs as previously described (Fischer et al., 2020). PBMCs were seeded into six-well tissue culture plates (Primeria; Corning, Durham, USA) with a density of 1×10^7 cells/ml in Iscove's Modified Dulbecco's Medium with Ham's F-12 Nutrient Mix (Thermo Fisher Scientific, Schwerte, Germany) supplemented with 10% fetal calf serum (FCS) and 1% antibiotic-antimycotic mix (Thermo Fisher Scientific, Schwerte, Germany). Cells were cultured at 37°C in a humidified atmosphere containing 5% CO_2 for 24 h. Subsequently, medium was changed to remove nonadherent cells, adherent macrophages were rinsed with 1 ml phosphate-buffered saline (PBS) and afterward wells were replenished with medium supplied with 2 ng/ml GM-CSF (granulocyte macrophage colony-stimulating factor; Biomol, Hamburg, Germany) to facilitate maturation of macrophages. PBMCs were incubated for 24 h as described above. For blind passaging, medium was removed and replaced by 1 ml of reconstituted plasma (inoculum). After a 2 h adsorption period at 37°C, the inoculum was discarded and cultures were rinsed with 1 ml PBS. Afterward, fresh medium without granulocyte-macrophage colony-stimulating factor (GM-CSF) was added. Plates were incubated for 72 h and then stored at −80°C until real-time PCR analysis and processing in HAT.

For proof of infectious virus, PBMCs were seeded into 96-well microplates (Primeria; Corning, Durham, USA) with a density of 5×10^6 cells/ml (100 μl per well) and cultured as described above. Subsequently, 100 μl of each blind passage sample was used to inoculate four wells of a 96-well plate, respectively (quadruplicate values). Furthermore, virus suspension samples were prepared for endpoint titration. To this means, 10-fold dilution series of these samples were inoculated in quadruplicate. After a 24 h incubation at 37°C, 20 μl of a 1% homologous erythrocyte suspension in PBS was added to each well. For read-out, cultures were analyzed for haemadsorption reactions (formation of rosettes) over a period of 4 days. In a second-round HAT, virus positive blind passage samples and virus suspension samples were titrated in quadruplicate to obtain endpoint titers of biological replicates. Because of virus dilution and the number of replicate wells, the limit of detection is 1.75 $\log_{10}$ 50% hemadsorbing doses per ml (HAD_{50}/ml). Titers were calculated using the method of Reed and Muench (1938) and were expressed as $\log_{10}$ HAD_{50}/ml. Graphical representation, calculations, and linear

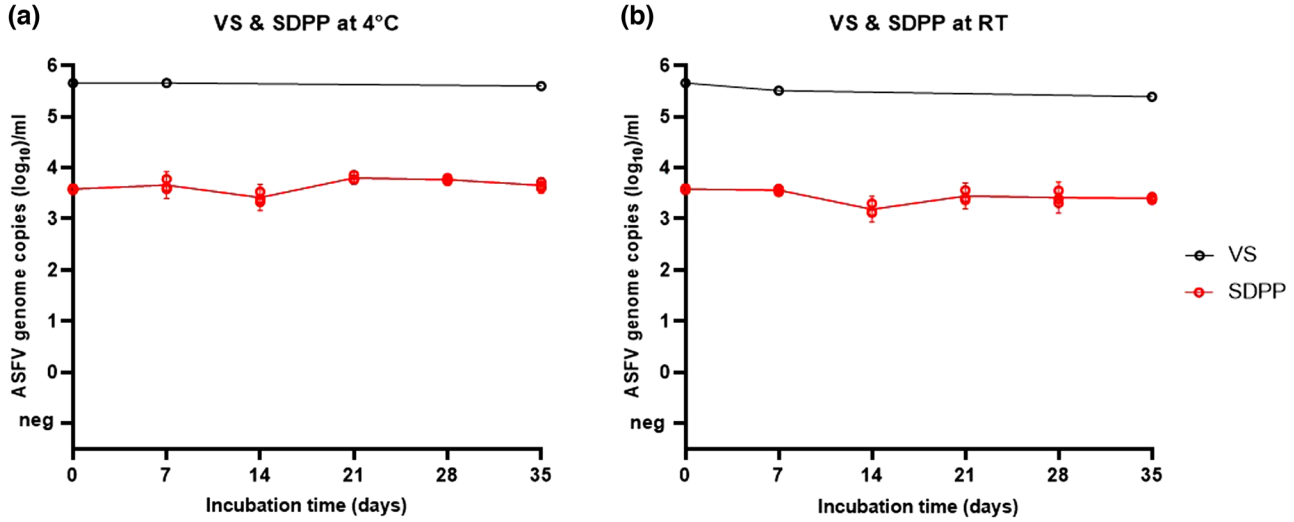

FIGURE 1 Copy numbers of virus suspension (VS) and inoculum (SDPP) at 4°C (a) and room temperature 21 ± 2°C (b) over time. Open circles indicate individual replicate values, solid lines and error bars represent the mean and standard deviation. Neg: negative result

regression analysis were performed using GraphPad Prism 8 (GraphPad Software Inc, San Diego, USA) and Excel version 2013 (Microsoft GmbH, Unterschleißheim, Germany), respectively. Decimal reductions (*D*-values) of virus titers over time were calculated as the negative reciprocal of the slope of the linear regression model (Jelsma et al., 2019).

2.3 | Nucleic acid extraction and real-time PCR

Prior to real-time PCR analysis, viral nucleic acids from all samples (inoculum, blind passage, and virus suspension) were extracted using the NucleoMag VET kit (Macherey-Nagel, Düren, Germany) on the automated KingFisher 96 flex platform (Thermo Fisher Scientific, Schwerte, Germany) according to the manufacturer's recommendations. Subsequently, nucleic acids were analyzed using a published real-time PCR (King et al., 2003) in combination with an internal control based on an enhanced green fluorescent protein (EGFP) detection system (Hoffmann et al., 2006) on a Biorad CFX real-time cycler (Bio-Rad Laboratories, Hercules, USA). For each real-time PCR, a quantification cycle (Cq) value was determined according to the PCR cycle number at which the fluorescence of the reaction crosses a value that is statistically higher than the background, which is determined by the Biorad CFX software. Using a dilution series of an in-house ASFV DNA standard, the genome copies in the respective samples were determined. For generation of the ASFV standard, DNA from an ASFV "Armenia08" PBMC culture supernatant was extracted using the QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations. Subsequently, the DNA concentration was determined by spectrophotometry using a Nanodrop 2000c (Thermo Fisher Scientific, Schwerte, Germany), and the exact number of DNA molecules was calculated using an online tool (http://www.molbiol.edu.ru/eng/scripts/0107.html).

3 | RESULTS AND DISCUSSION

3.1 | Real-time PCR analysis of virus suspensions, spiked SDPP, and blind passages

The virus suspension was used to contaminate SDPP contained high genome copy numbers (5.66 $\log_{10}$/ml, Figure 1). As a control, the original virus suspension was stored up to 35 days under the same conditions as the SDPP samples. Irrespective of the storage temperature (4°C or room temperature, respectively), ASFV genome copy numbers were constant over the entire observation period in the virus suspension (copy numbers between 5.66 and 5.39 $\log_{10}$/ml). However, copy numbers do not allow any conclusions on the amount of infectious virus.

In comparison, the contaminated SDPP (3.58 $\log_{10}$/ml), used as inoculum for the blind passage, contained lower genome copy numbers than the initial virus suspension (5.66 $\log_{10}$/ml) due to dispersion on SDPP. Such a dispersion effect was also seen after contamination of field crops (Fischer et al., 2020). Overall ASFV genome was detectable in all samples, indicating a successful contamination of SDPP (Figure 1). Furthermore, genome copy numbers of SDPP did not display any distinct decline over time, irrespective of the storage temperature. The remarkable stability of ASFV DNA after heat treatment or drying is already described under laboratory conditions (Fischer et al., 2020) and under field conditions (Zani et al., 2020). In the untreated plasma NC, no ASFV genome was detected (Table S1).

PCR analyses after blind passages of contaminated SDPP stored at 4°C revealed that these samples were highly genome positive during the entire observation period (copy numbers between 7.32 and 6.52 $\log_{10}$/ml, Figure 2a). These values exceed the viral load of the initial virus suspension, indicating a considerable amplification of ASFV on macrophages during the blind passages. In contrast, only a single blind passage replicate of SDPP stored at room temperature for

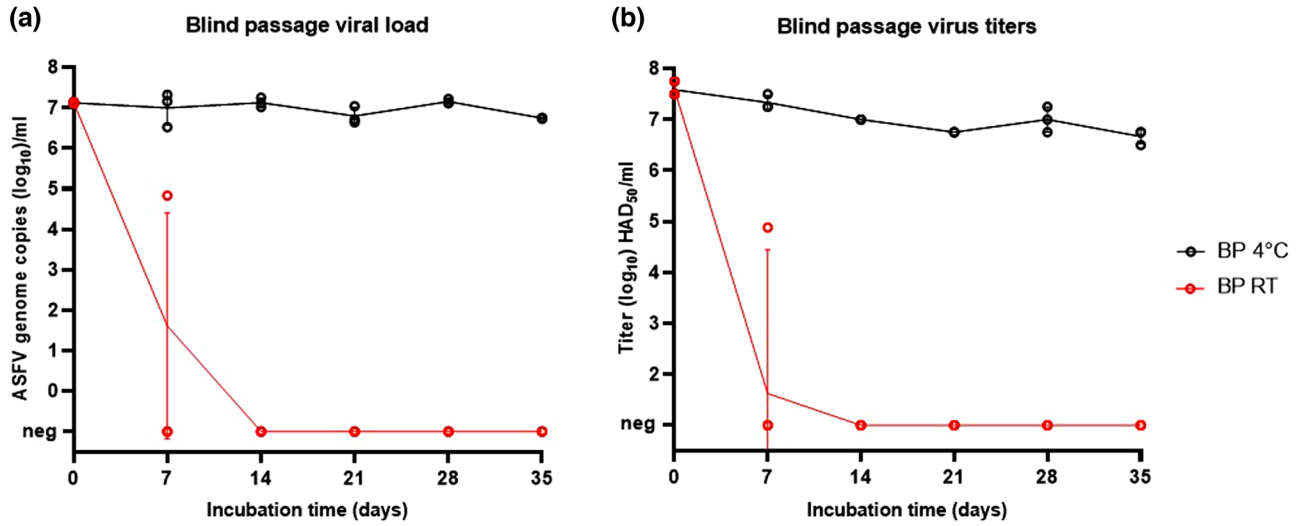

FIGURE 2 Representation of the viral load as copy numbers (a) and of virus titers (b) after blind passage (BP). Each open circle represents an individual replicate value, solid lines and error bars represent the mean and standard deviation. Neg: negative result

1 week was PCR positive (4.88 $\log_{10}$ genome copies/ml, Figure 2a and Table S2).

3.2 | Endpoint titers of virus suspension and blind passage

In general, endpoint titers of the virus suspensions remained stable over a 5-week storage period at 4°C (*D*-value: 416 days). At room temperature, virus titers declined from 6.0 to 4.13 $\log_{10}$ HAD_{50}/ml over time (Table S3, *D*-value: 18 days). Dee et al. (2018) also observed a comparable titer reduction (from 5.0 to 3.0 $\log_{10}$ $TCID_{50}$) in a virus stock during 30 days of storage mimicking transatlantic shipment conditions. Overall, the virus suspension remained infectious over the entire observation period, irrespective of the storage temperature.

Regarding blind passage samples of contaminated SDPP stored at 4°C, virus titers remained constant over the entire study period (between 7.75 and 6.50 $\log_{10}$ HAD_{50}/ml, Figure 2b, *D*-value: 42 days). Thus, contaminated SDPP stored at 4°C was infectious for at least 5 weeks. In contrast, blind passage samples of contaminated SDPP stored at room temperature displayed only residual ASFV infectivity (one out of three replicates HAT positive) and a distinct titer reduction after 1 week (Figure 2b, *D*-value: 2 days). After 2 weeks, complete ASFV inactivation was observed. This finding is in accordance with the corresponding real-time PCR results (Figure 2a). The higher ASFV tenacity at low temperatures is already described in literature (EFSA, 2014) and can be partially explained by the kinetics of general degradation processes. Ubiquitous proteases and nucleases are more active at room temperature than at 4°C and thus virus particles are degraded more rapidly at ambient temperature.

In comparison to porcine epidemic diarrhea virus (PEDV), which is fully inactivated on spray dried bovine plasma after 3 weeks at 4°C or after 1 week at room temperature (Pujols & Segalés, 2014), ASFV displayed a higher stability on SDPP. Analogous to Pujols and Segalés (2014), in this study also cell culture methods and blind passaging were used for virus isolation. Pujols and Segalés (2014) used the cytopathic effect for the detection of PEDV infectivity, whereas in this study, the HAT was used for the detection of infectious ASFV. In our opinion, these methods are comparable. The higher tenacity of ASFV may be due to the high complexity of the ASFV virus particle and the stability of the genomic DNA in contrast to the small PEDV virus particle containing a less stable RNA genome.

When judging these results, the sensitivity of the HAT detection system should be taken into consideration. Compared with a bioassay, that is, the inoculation of pigs, the HAT is less sensitive (Heuschele, 1967). However, this discrepancy in sensitivity should not be a concern in this case since Blázquez et al. (2020) demonstrated that the minimum infectious dose in feed mixed with ASFV contaminated liquid porcine plasma was higher than 5.0 $\log_{10}$ $TCID_{50}$/pig (50% tissue culture infectious dose per pig).

3.3 | SDPP as a risk factor for ASFV transmission

It has been shown that the spray drying process itself has an inactivating effect on various viruses affecting pigs, like swine vesicular disease virus (SVD), pseudorabies virus (PRV), porcine reproductive and respiratory syndrome virus (PRRSV), or ASFV (Blázquez et al., 2018; Polo et al., 2005; Pujols & Segalés, 2014; Pujols et al., 2007). Hence, we investigated the contamination of SDPP after processing as well as the possibility of residual ASFV infectivity after an initial titer reduction during production. Therefore, this study provides valuable data for evaluating the risk of ASFV transmission via externally contaminated SDPP. Based on the obtained results, we conclude that the residual risk

of re-contamination of SDPP that is produced under European standards can be mitigated by room temperature storage at 21 ± 2°C for at least 14 days.

AUTHOR CONTRIBUTIONS

Conceptualization, Melina Fischer and Sandra Blome; Data curation, Melina Fischer; Funding acquisition, Sandra Blome and Martin Beer; Investigation, Melina Fischer and JP; Methodology, Melina Fischer, and Jutta Pikalo; Visualization, Melina Fischer; Writing – original draft, Melina Fischer and Sandra Blome; Writing – review & editing, Jutta Pikalo and Martin Beer. All the authors have read and agreed to the published version of the manuscript.

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ETHICAL STATEMENT

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to. No ethical approval was required as this study did not involve animal experimentation.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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