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Article

Inactivation protocols for African Swine Fever Virus in serum and saliva samples

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Abstract:
African swine fever (ASF) is a notifiable viral disease of domestic and wild suids. Despite intensive research efforts, the pathogenesis of the disease is still far from being understood. Analysis of biomarkers in different body fluids may supplement traditional pathogenesis studies. As reliable protocols are often established in laboratories with lower biosafety, reliable inactivation of samples is crucial. The objective of this study was to find a procedure that inactivates the virus while preserving the biomarkers for downstream analyses. To this means, three different inactivation protocols were employed, namely Tergitol-type NP-40 (NP-40) and polyoxyethylene-p-t-octylphenol (Triton X-100), respectively, and one with 95 °C heating. It could be demonstrated that all samples treated with 0.5% (v/v) concentration of both detergents showed absence of virus infectivity. The same was true for heated samples. However, heated serum was not suitable for analyses. Next, the treatment impact on biomarker readouts was assessed. While all protocols had an impact on the detection of biomarkers, correlation was retained. Especially NP-40 could be the desired detergent for more accurate measurements while achieving efficient virus inactivation. Based on these studies, samples can be reliably inactivated for most biomarker analyses and thus broader interdisciplinary cooperation is possible.

Keywords: African swine fever; pathogenesis; biomarkers; serum; saliva; virus inactivation; detergent treatment; heat treatment; impact of treatment on biomarkers

1. Introduction

African swine fever virus (ASFV), an enveloped, double-stranded DNA virus which belongs to the *Asfarviridae* family, is the causative agent of African swine fever (ASF), a highly lethal disease in domestic and Eurasian wild suids [1]. For many decades, ASF has been endemic in many Sub-Saharan African countries and in Sardinia. Nonetheless, this scenario has radically changed after the introduction of the disease into Georgia in 2007 which was followed by the rapid spread of the virus to numerous eastern European countries, reaching the European Union in 2014 [1, 2]. From that year onwards, recordings of infected wild boar and domestic pigs have been reported in the Baltic States, Poland, Germany, Romania, and Bulgaria, amongst others [3]. Moreover, the virus spread to Asia and more recently to the Americas (OIE WAHIS visited online February 13th 2022). With that, the extent of ASF has now reached true pandemic dimensions [4].

Animals infected with ASFV show a panel of clinical signs that includes high fever, depression, and respiratory distress. In its worst disease course, ASF resembles a viral hemorrhagic fever with severe hemorrhages, lung edema, and neurological signs [5]. Pathomorphological changes include enlarged, hemorrhagic lymph nodes; reddening of tonsils; congestion of spleen or splenomegaly; petechiae in different organs such as the kidney, colon, or urinary bladder; and lung and gall bladder wall edema [2].

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Unfortunately, the pathogenesis of the disease has not been fully elucidated and the gaps in knowledge hamper the development of safe and effective vaccines, which are still lacking [6]. To further the understanding of the disease, various biomarkers of the immune system, inflammation, muscle damage, stress, oxidative status, or anaerobic metabolism, among others, can be studied directly from biofluids of affected animals [7]. Biochemical alterations have been described in ASF, such as increase in serum acute-phase proteins [8, 9]. Similarly, saliva has proven potential as biofluid for ASF diagnostics [10], biomarkers of stress, inflammation, immune system or redox homeostasis, as described in Cerón et al., [11] actually being employed to evaluate porcine health and welfare non-invasively. However, these research activities are confronted with the problem that sample material from ASFV infected animals can only be used under high containment conditions or must be subjected to reliable inactivation procedures prior to shipment.

Different virus inactivation protocols compatible with good performance in clinical chemistry and hematological analyses, have been described for enveloped viruses [12], including the inactivation through chemical non-ionic agents such as Tergitol-type NP-40 (NP-40) [13] or polyoxyethylene-p-t-octylphenol (Triton X-100) [10] that can also be combined with additional heat treatments [13].

In search for a protocol that ensures reliable ASFV inactivation in biofluids while preserving the informative value of downstream analyses of biomarkers, i.e. biomarkers of inflammation, immune system parameters, stress, and redox homeostasis, the present study was conducted using inactivation protocols with Triton X-100 or NP-40 and 95 °C heating.

2. Materials and Methods

2.1 Study Design

The study was conducted in three consecutive steps. Step one was carried out to show general inactivation of ASFV with the chosen detergent or heating protocol. To ease initial validation, the cell-culture adapted ASFV "ArmeniaΔ285LGFPhuCD4" variant was used. This virus isolate expresses the green-fluorescent protein (GFP) as fluorescent marker [14, 15]. Employing this virus, viral replication could be assessed through fluorescent readout on a robust cell culture and guaranteed sufficient high viral input for the different inactivation studies. Step two was done to transfer the inactivation protocols to biological samples, i.e. serum and saliva. These samples were derived from animals experimentally infected with the wild-type ASFV "Armenia 2008". Samples were taken at the humane endpoint at 7 days post inoculation. The third part aimed at the impact of the chosen protocols on relevant biomarkers. To this means, up to eleven biomarkers were measured in detergent-treated samples. A heat inactivation protocol using 95°C for 10 min was added for comparison.

2.1.1 Animal Trial Samples

Sera and saliva originated from three domestic pigs that had been oro-nasally inoculated with 10^4 hemadsorbing units 50% (HAD_{50}) of the highly virulent genotype II ASFV strain "Armenia 2008". The animal trial was approved by the competent authority (LALLF Rostock, Germany) under reference number 7221.3-2-011/19. All samples were stored at -80 C until use.

2.2 Detergent and heat treatments

In order to find the most suitable detergent concentration, a dilution-dependent inactivation experiment was first performed. Each sample was divided into six aliquots and subsequently treated. To this end, Triton X-100 (2% *v*/*v*; Sigma-Aldrich) and NP-40 (10% *v*/*v*; Thermo Fisher Scientific) were further diluted to the following final concentrations: 0.5%, 0.1% and 0.01% (*v*/*v*) and the chosen matrices were then exposed to the different detergent treatments. The residual virus infectivity was measured by a standard limiting dilution

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assay expressed as HAD_{50}/ml or Tissue Culture Infective Dose 50% ($TCID_{50}$/ml). Three independent experiments were carried out to evaluate the exposure effect to both detergents on the infectivity of ASFV. Additionally, heat inactivation experiment as comparison was also performed. To this end, sera and saliva samples were inactivated at 95 °C for 10 min in a water bath.

Following, the best performing concentration was chosen and samples were divided into four aliquots and treated as follows: (*i*) No inactivation treatment; (*ii*) Inactivation with a final concentration of 0.5% (*v*/*v*) NP-40 incubated 60 min at room temperature (RT); (*iii*) Inactivation with a final concentration of 0.5% (*v*/*v*) Triton X-100 incubated 60 min at RT; (*iv*) Heat inactivation at 95 °C for 10 min. Saliva and serum from negative donors and cell culture medium were included as negative controls and passaged in the same run.

2.4 Virus isolation and titration

Treated samples were next analysed by virus isolation. Hence, each sample was subjected to one blind passage in either primary porcine macrophages or in permanent WSL cells, each followed by two subsequent passages. Experiments using serum and saliva used hemadsorption tests on primary macrophages to quantify potential infectivity, and experiments conducted with the "ArmeniaΔ285LGFPhuCD4" used the GFP expression in WSL cells as read-out system.

WSL cells were cultivated in Iscove's Modified Dulbecco's Medium with Ham's F-12 Nutrient Mix (Thermo Fisher Scientific), 10% FBS and 1% penicillin and streptomycin (10,000 U/mL; Gibco). Virus isolation and titration on porcine macrophages were carried out with macrophages derived from peripheral blood mononuclear cells (PBMCs) as previously described [16].

For the blind passages, 5 $x10^6$ cells of either WSL or PBMCs per well were seeded into 24-well Primaria plates (Corning, Durham, NC, USA) one or two days before inoculation, respectively. Then, 100 µl of 5-fold serially diluted samples (the dilution factors ranged from 1:5 to 1:1,600) were inoculated onto naïve WSL or macrophages. After 2 h incubation at 37°C to allow virus adsorption, cells were washed once with PBS and fresh medium was added. Cultures were examined daily for cytotoxicity with a light microscope and were cultivated for 5 days at 37°C. After a freeze-thaw cycle, virus titrations were carried out.

For proof of infectious virus, either 7.5 x 10^4 PBMCs or 3 x 10^5 WSL cells per well were seeded in a 96-well Primaria tissue culture plate (Corning), respectively. The following day, the attached cells were inoculated with 100 µl of supernatant from the blind or second passage in ten-fold dilutions from 10^{-1} to 10^{-8}. One day after inoculation, 20 µl of a 1% solution of red blood cells were added to each well containing primary macrophages. Plates were examined after 72 h. Each well with at least one hemadsorbing macrophage or green fluorescence, indicative of virus replication, read under light or epifluorescence microscope, respectively, was considered positive. Titers were calculated by the Spearman–Kärber method and expressed as log_{10} HAD_{50}, in serum; or $TCID_{50}$, in cell culture medium; with a limit of detection of $10^{1.75}$ per ml.

2.4 Effects of ASFV inactivation in different biomarkers

After determination of the effective concentration to inactivate ASFV, the possible effects of ASFV inactivation protocols in a battery of relevant biomarkers were evaluated. For this, a total of ten paired porcine saliva and serum samples were divided into 4 aliquots and treated as previously described in section 2.2.

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Cortisol and haptoglobin in saliva were measured using an AlphaLISA assay validated for its use in porcine samples [7], and were expressed in ng/ml. All other analytes (amylase in serum (IU/L) and alpha-amylase in saliva (IU/mL), adenosine deaminase (total: tADA, and isoenzyme ADA2, UI/L), total protein (TP, g/dl in serum and mg/dl in saliva), cupric reducing antioxidant capacity (CUPRAC, mmol trolox equivalents/L), and ferric reducing ability of saliva (FRAS, which has the same basis than ferric reducing ability of plasma or FRAP, mmol/L), trolox equivalent antioxidant capacity using the horseradish peroxidase (TEACH, mmol trolox equivalents/L), gamma-glutamyl transferase (GGT, IU/L), lactate dehydrogenase (LDH, IU/L) and haptoglobin in serum (g/L) were measured in an automated biochemical analyzer (Olympus AU400, Olympus Diagnostica GmbH, Ennis, Ireland) using commercial assays which were previously validated for their use in porcine samples [17, 18].

Normality in the distributions was evaluated using the D'Agostino and Pearson omnibus normality test, and data did not follow a normal distribution. To evaluate the effects of the different inactivation protocols on biomarkers, data were assessed using the One-Way ANOVA (Friedman multiple comparisons test). To determine the correlation between the different methods in both serum and saliva, the Spearman's correlation test was performed.

3. Results

3.1. Triton X-100 and NP-40 produce dilution-dependent inactivation

To establish the optimal NP-40 and Triton X-100 concentration, the WSL adapted ASFV strain, ArmeniaΔ285LGFPhuCD4, was exposed for 60 min to dilutions ranging from 0.5% (*v*/*v*) to 0.01% (*v*/*v*) (Fig. 1). A mean titer reduction of 5 Log $TCID_{50}$/ml was observed for both detergents when compared to the untreated sample. Absence of virus infectivity was observed in 0.5% NP-40 (Fig. 1 – A) and Triton X-100 (Fig. 1 – B) treated samples (showing also 99.99% infectivity reduction). Following the above described dilutions, no cytotoxic effects were observed. However, cell culture was incubated and tested for toxicity over 2 h. If cell toxicity (necrosis/detachment of cells) was observed during this time in one or more dilution levels, the respective dilution level was added in addition to a 24-well plate containing cell culture. Again, incubation and observation of toxicity was performed for 2 h.

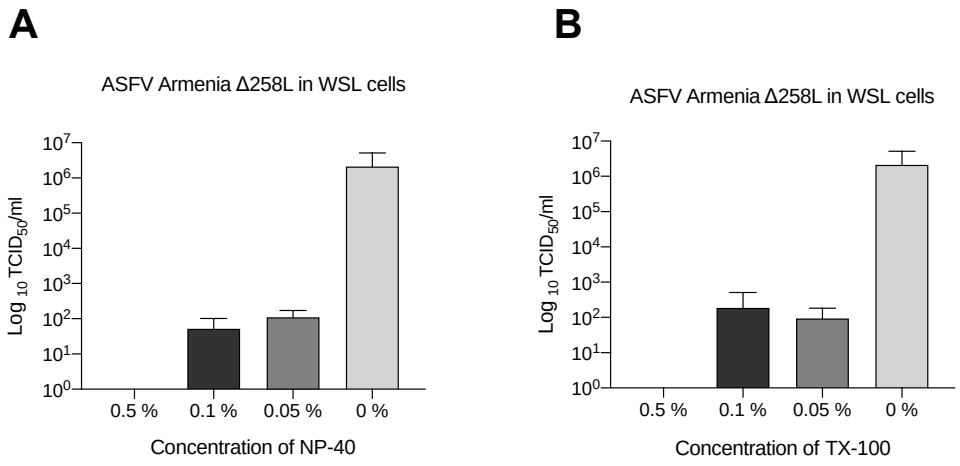

Figure 1. Inactivation of ASFV in cell culture supernatant by Triton X-100 and NP-40. ASFV containing cell culture supernatant was treated with three detergent dilutions ranging from 0.5% (v/v) to 0.0% (v/v) NP-40 (**A**) and Triton X-100 (**B**) for 60 min at RT. After the incubation period, both treated and untreated samples were back-titrated by limiting dilution on WSL cells. Infectivity is expressed as Log $TCID_{50}$/ml (limit of detection of $10^{1.75}$ Log $TCID_{50}$/ml).

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Subsequently, serum and saliva were used in order to test the impact of the matrix on the virus inactivation protocols. As shown in Fig. 2 A–B, 0.1% and 0.05% detergent concentrations revealed a similar efficacy in serum samples, with an average reduction of the virus titer of 2 Logs HAD_{50}/ml compared to the untreated sample. This scenario differed when samples were treated with 0.5% (*v*/*v*) concentration (Fig. 2 A–B), where absence of virus infectivity was notable in treated sera (≥6 $\log_{10}$ infectivity reduction). All saliva samples obtained for this study, including non-treated specimens, were negative for infectious virus.

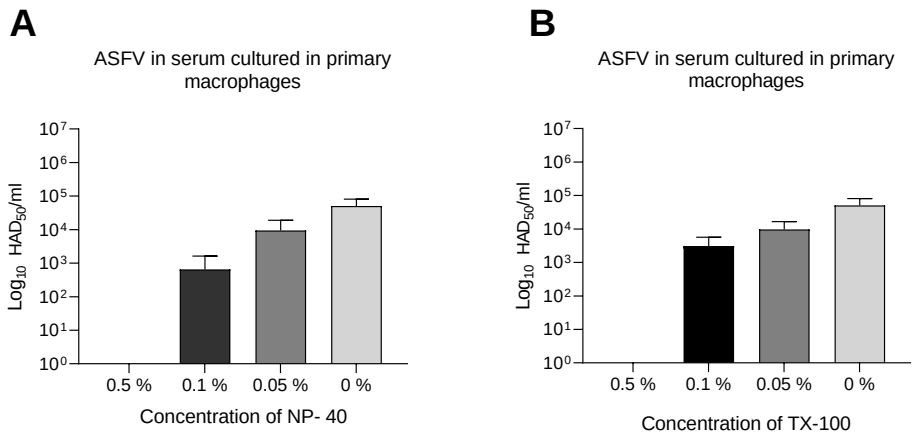

Figure 2. Inactivation of ASFV in serum by Triton X-100 and NP-40. ASFV containing sera were treated with three detergent dilutions ranging from 0.5% (*v*/*v*) to 0.1% (*v*/*v*) NP-40 (**A**) and Triton X-100 (**B**) for 60 min at RT. After the incubation period, both treated and untreated samples were back-titrated by limiting dilution on primary macrophages. Infectivity is expressed as Log HAD_{50}/ml (limit of detection of $10^{1.75}$ Log HAD_{50}/ml).

3.2. Virus infectivity

To determine whether 0.5% (*v*/*v*) Triton X-100 and NP-40 completely abolished infectivity after treatment, up to three passages were carried out for each of the treated samples. As summarized in Table 1, all detergent-treated samples were negative in the virus isolation assay and demonstrated no residual infectivity upon three consecutive passages of the inoculated cells. In contrast, control samples not treated with detergents showed no obvious loss in virus titers. The effect of heat combined with detergents was also tested along the detergents. Similarly, no infectivity was detected in any of these heat-treated samples in three consecutive passages of inoculated cells (Table 1), while the virus control retained the virus titer (Table 1). Saliva samples, including non-treated samples, showed lack of infectivity during the experiment.

Table 1. Effects of detergent and heat treatments on ASFV infectivity.

Treatment	Infectious titers before treatment	Titers after treatment	Titers after virus isolation at the 3rd cell passage
0.5% (*v*/*v*) Triton X-100			

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ASFV in porcine serum (HAD_{50})	5.07×10^4	negative	negative
ASFV in porcine saliva (HAD_{50})	negative	negative	negative
ASFV in culture medium ($TCID_{50}$)	2.12×10^6	negative	negative
	0.5% (*v*/*v*) NP-40		
ASFV in porcine serum (HAD_{50})	5.07×10^4	negative	negative
ASFV in porcine saliva (HAD_{50})	negative	negative	negative
ASFV in culture medium ($TCID_{50}$)	2.12×10^6	negative	negative

3.3. Effects of ASFV inactivation in different biomarkers

The impact of ASFV inactivation protocols on each biomarker concentration is shown in Table 2. All sera heated at 95 °C were coagulated and therefore not suitable for any measurements. In serum, TX-100 did not affect significantly ADA2, FRAP nor LDH, while amylase, ADA, total protein, CUPRAC, TEACH, GGT and haptoglobin were affected. ASFV NP-40-based inactivation protocol did not affect cortisol, amylase, Hp, CUPRAC, TEACH, GGT nor TP, but ADA, FRAP and LDH showed differences in comparison to untreated aliquots.

In saliva, TX-100-based inactivation not affected the levels of cortisol, alpha-amylase, Hp, ADA, CUPRAC, TEACH and PT; while altered FRAS, GGT and LDH; Cortisol, alpha-amylase, Hp, ADA, CUPRAC, TEACH, GGT and LDH were not affected by NP-40 , opposing protein content and FRAS. 95ºC heating changed significantly the levels of all measured biomarkers except for CUPRAC, TEACH and TP. Correlation between untreated aliquots and TX-100, NP-40 and 95 °C heating in saliva and serum are shown in Table 3. With the exception of amylase, tADA, ADA2, GGT and LDH at 95 °C heating, all evaluated correlations were positive with coefficients > 0.73 and statistically relevant.

Table 2. Biomarkers after different ASFV inactivation treatments. For easier interpretation, results were normalized according to NT measurements, which was considered 100%, and expressed as median (25–75 percentile). Asterisk indicates differences of statistical relevance vs. NT measurements (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$). NT: no inactivation treatment (control); NP-40: 0.5% NP-40; TX100: 0.5% Triton X-100; 95 °C: 95 °C heating for 10 min.

	Serum			**Saliva**			
	NT	TX-100	NP-40	NT	TX-100	NP-40	95ºC
Cortisol	100	112 (105 - 127)*	102 (98.7 - 112)	100	117 (88.7 - 155)	136 (104-235)	0 (0 – 0)*
Amilase/Alpha-amilase	100	91.6 (89.7 - 92.6)***	99.2 (97.1 - 100)	100	90.8 (88.4 - 102)	95.3 (91.6 - 113)	0 (0 - 0)***
Haptoglobin	100	83.5 (71.8 - 100)*	96.5 (88.7 - 113)	100	91.5 (81.7 – 119)	128 (114 – 182)	0 (0 – 0)**
tADA	100	91 (88.5 - 95.2)***	93.7 (86.9 - 97.1)*	100	95.8 (91.5 - 98.5)	100 (93.9 - 104)	0 (0 - 0.1)***
ADA2	100	108 (105 - 110)	112 (111 - 114)***	100	97.4 (88.8 - 114)	103 (91.4 - 120)	0 (0 - 19.3)**
CUPRAC	100	118 (112 - 124)*	112 (110 - 118)	100	101 (95.5 - 113)	110 (103 - 112)	98.4 (94.7 - 101)
FRAP/FRAS	100	98.2 (96.9 - 99.8)	98 (93.8 - 98.8)**	100	90.1 (87.3 -93.3)*	91.4 (88.5 - 94.2)*	98. (88.3 - 105)

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TEACH	100	93.4 (90.3 - 95.8)***	97.7 (94 - 100)	100	99.4 (94.9 - 101)	104 (94.9 - 108)	81.7 (78.3 - 90)**
GGT	100	97.8 (96 - 99.2)*	99.8 (97.9 - 101)	100	193 (131 - 288)*	123 (111-167)	0 (0 - 25)*
LDH	100	100 (97.8 - 103)	102 (102 - 104)*	100	56.2 (46.2 - 71.4)*	98 (88.3 - 101)	0.1 (0 - 2.4)***
TP	100	93.5 (91-7 - 95.5)*	102 (100 - 103)	100	91.5 (86.7 - 94.5)	124 (118 - 134)*	120 (115 - 123)

Table 3. Correlation coefficients between different inactivation treatments and untreated samples, for each biomarker. NT: no inactivation treatment (control); NP-40: 0.5% NP-40; TX100: 0.5% Triton X-100; 95ºC: 95ºC heating for 10 min. For an easier interpretation, P < 0.05 are highlighted in bold.

	Serum		**Saliva**		
	TX-100	NP-40	TX-100	NP-40	95ºC
Cortisol	**r = 0.879; P = 0.001**	**r = 0.867; P = 0.002**	**r = 0.4895; P = 0.11**	**r = 0.6294; P = 0.032**	r = 0.0843; P = 0.895
Amylase/Alpha-amilase	**r = 0.9879; *P* = 0.001**	**r = 0.9879; *P* = 0.001**	**r = 1; *P* = 0.001**	**r = 1; *P* = 0.001**	r = 0.1741; *P* = 0.8
Haptoglobin	**r = 0.9758; *P* = 0.001**	**r = 0.9879; *P* = 0.001**	**r = 0.9429; P = 0.017**	**r = 1 ; P = 0.003**	r = 0.1243; P = 0.79
tADA	**r = 0.9726; *P* = 0.001**	**r = 0.9605; *P* = 0.001**	**r = 0.9879; *P* = 0.001**	**r = 0.9515; *P* = 0.001**	r = -0.2593; *P* = 0.4656
ADA2	**r = 0.8831; *P* = 0.001**	**r = 0.9319; *P* = 0.001**	**r = 0.9205; *P* = 0.001**	**r = 0.9146; *P* = 0.001**	r = -0.02055; *P* = 0.9558
CUPRAC	**r = 0.7333; *P* = 0.02**	**r = 0.9879; *P* = 0.001**	**r = 0.9273; *P* = 0.001**	**r = 0.9515; *P* = 0.001**	**r = 0.9636; *P* = 0.001**
FRAP	**r = 0.8061; *P* = 0.007**	**r = 0.9515; *P* = 0.001**	**r = 1; *P* = 0.001**	**r = 0.9758; *P* = 0.001**	**r = 0.9785; *P* = 0.001**
TEACH	**r = 0.7939; *P* = 0.009**	**r = 0.903; *P* = 0.009**	**r = 0.9758; *P* = 0.001**	**r = 0.9636; *P* = 0.001**	**r = 0.9758; *P* = 0.001**
GGT	**r = 0.9273; *P* = 0.001**	**r = 0.9879; *P* = 0.001**	**r = 0.924; *P* = 0.001**	**r = 0.9024; *P* = 0.001**	r = -0.1524; *P* = 0.716
LDH	**r = 0.8061; *P* = 0.007**	**r = 0.9273; *P* = 0.001**	**r = 0.8788; *P* = 0.001**	**r = 0.9273; *P* = 0.001**	r = 0.4282; *P* = 0.219
TP	**r = 0.7815; *P* = 0.016**	**r = 0.8452; *P* = 0.006**	**r = 0.9636; *P* = 0.001**	**r = 0.9879; *P* = 0.001**	**r = 0.9879; *P* = 0.001**

4. Discussion

African swine fever has become a pandemic threat to sustainable pig production and still, safe and efficacious vaccines are lacking and the knowledge of beneficial and detrimental host responses remains sketchy. To join forces and to make use of interdisciplinary possibilities in sample analyses, virus inactivation procedures that do not have a critical impact on the parameters in question are needed.

Along these lines, our study set out to find inactivation protocols for biological samples intended for biomarker analyses. In detail, the effectiveness of heating, Triton X-100, and NP-40 for the inactivation of ASFV in porcine saliva and serum samples was evaluated. For this, samples were first subjected to different detergent concentrations and heating in order to evaluate which protocols successfully inactivate ASFV. Taken together, these results suggest that Triton X-100 or NP-40 at 0.5% (*v*/*v*) concentration are highly effective in eliminating the infectivity of both extracellular and intracellular ASFV particles. Then, these detergent concentrations and 95 °C heating were tested in a battery of biomarkers that are applied in porcine medicine, including markers of stress, inflammation, immune system and oxidative status. The final inactivation protocols presented here agree with previously related data on detergent-treatment [13, 19-21] to inactivate various viruses.

These protocols have the additional advantage that they employ economical reagents and do not need expensive infrastructure.

Detergent treatment of potentially infectious matrices was introduced into the manufacturing processes of medical products more than 20 years ago [22] and has been considered to have contributed to the overall safety of laboratory procedures. Additionally, when compared with heat inactivation procedures, detergents seems to imply less significant impact on the clinical chemistry and the analysis of hematology parameters [23]. Although NP-40 has less extensively been used for virus inactivation, Triton X-100 is a well-established method because of its lack of interference in the analysis of biofluids, such as human plasma and blood [24]. Hersberger, Nusbaumer [25] additionally showed that the latter does not seem to interfere with the performance of most chemistry and hematological assays, although Rubio, Franco-Martínez [23] reported that its presence at 0.5% concentration might interfere with the colorimetric lamp. However, the possible influence of the detergents used in this study on the analysis of porcine serum biomarkers and/or analytical tests remains to be studied.

The evaluated biomarkers represent analytes used to evaluate the general health status in pigs. In concrete, biomarkers to evaluate stress (cortisol, salivary alpha-amylase), inflammation (haptoglobin), immune system parameters (adenosine deaminase), and the oxidative status (TEAC, CUPRAC and FRAP/FRAS) were included. In addition, other biomarkers commonly employed namely total protein, GGT and LDH were evaluated [11].

The addition of 0.5% (*v*/*v*) TX-100 to serum produced changes in 8 of the evaluated biomarkers, namely amylase, tADA, protein content, CUPRAC, TEACH, GGT, cortisol and haptoglobin. However, a strong positive correlation with NT ($r > 0.95$, $P < 0.05$) were found in amylase, tADA, GGT and Hp, while cortisol, protein content, CUPRAC and TEACH showed coefficients of correlation > 0.73 ($P < 0.05$). In saliva, TX-100 did not alter the levels of analytes significantly, with the exception of a reduction in FRAS and LDH and an increase in GGT. In these cases, the correlation coefficients were $r = 1$, $P < 0.001$; $r = 0.88$, $P < 0.01$ and $r = 0.92$, $P < 0.01$, respectively. Therefore, although TX-100 caused changes in the majority of biomarkers evaluated –especially in serum-, the high correlation coefficients observed in relation to NT aliquots suggest it is an adequate alternative to inactivate ASFV or other enveloped viruses in porcine samples.

NP-40 was the inactivation treatment that caused less alteration in biomarkers' levels. In serum, it altered the levels of tADA, ADA2, FRAP and LDH; while in saliva total proteins and FRAS were the only biomarker that changed significantly. However, the correlations observed in biomarkers between untreated and NP-40 inactivated samples were in all cases strong with a correlation coefficient $r > 0.9$ (with the exception of total protein in serum that was 0.85). Therefore, the addition of NP-40 to porcine and saliva samples would be the election method for the selected biomarkers.

The use of 95ºC heating for 10 minutes is a rapid protocol that only requires a heating bath, and no additional reagents need to be added to samples. In the case of serum, 95ºC heating caused all samples to coagulate, preventing their analyses. Therefore, this method is not recommended for serum samples unless the solid state of the sample will not interfere with the measurement technique. In the case of saliva samples, 95ºC heating causes a marked decrease in cortisol, haptoglobin, alpha-amylase, tADA, ADA2, TEACH and LDH, reaching zero concentrations in most cases. For TEACH, heating causes an 18.3% median reduction, although data was strongly correlated with NT ($r = 0.9636$, $P > 0.001$). GGT concentration also decreased to 0 UI/L, although in this case the difference was considered no significant. Although it was not statistically relevant, 95ºC heating produced a 20% median increase in total protein ($r = 0.9636$, $P < 0.001$), which can be related to protein denaturalization caused by heat. Overall, 95ºC could be employed for the measurement of CUPRAC and FRAP with confidence; the strong correlation observed between no

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treated aliquots and 95ºC in protein content and TEACH also allow their measurement and posterior correction; while it is not recommended for the measurement of other biomarkers such as cortisol, haptoglobin, alpha-amylase tADA and ADA2.

Based on our results, NP-40 is the inactivation protocol that causes fewer alterations in biomarker concentrations in comparison to untreated aliquots, and thus, could be evaluated in first time for other biomarkers. However, Triton X-100 could also be a suitable inactivation treatment since correlations coefficients with untreated aliquots are in most cases close to 1. Last, inactivation with 95ºC for 10 minutes coagulates serum samples preventing their measurements, and in saliva it was the treatment that causes most alterations in biomarkers levels, although some of them are not affected.

The use of serum and saliva samples from ASFV-infected domestic pigs allowed us to test the inactivation of the ASFV in complex biological matrices. Therefore, suggesting that these treatments could as well be use in other biological matrices, such as blood and other biological fluids. During the present study we have been able to report that, when used alone and/or combined with heat, Triton X-100 and NP-40 are able to rapidly inactive the infectivity of a large-enveloped DNA virus, such as ASFV, at a concentration of 0.5% without interfering with the measurement of important porcine biomarkers. Because of the high effectiveness of the currently described methodology, it can be considered sufficient to render samples non-infectious, hence contributing to mitigate the risk for the animal health.

5. Conclusions

While we did observe an impact of the virus inactivation procedures on the read-out of biomarker detection techniques, correlation still warrants analyses. The best combination from our study was NP-40 at a concentration of 0.5%. Based on our data, future animal experiments will be supplemented with biomarker analyses.

Supplementary Materials: The following supporting information can be downloaded at: www.mdpi.com/xxx/s1, Figure S1: title; Table S1: title; Video S1: title.

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