Suitability of faeces and tissue samples as a basis for non-invasive sampling for African swine fever in wild boar

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A B S T R A C T

A challenging aspect of ASFV control in wild boar populations is the design and implementation of effective surveillance and monitoring programmes, both for early warning, and to determine the ongoing epidemiological situation in an infected population. Testing blood samples requires invasive sampling strategies like hunting or capture of wild boar. Besides being biased towards healthy animals, such strategies are also linked to further spread of the virus. Non-invasive sampling strategies would increase the reliability of surveillance of ASFV in wild boar populations, without the negative side effects. This study evaluates the potential of faeces and tissue samples as a basis for non-invasive sampling strategies for ASFV in wild boar. In the acute phase (0–21 days after infection), in comparison with virus detection in blood, virus can be detected in faeces 50–80% of the time. This percentage decreases to below 10% for the subacute/chronic phase. ASFV DNA is quite stable in faeces. Half-lives range from more than 2 years at temperature up to 12 °C, to roughly 15 days at temperatures of 30 °C. In tissue samples, stored at 20 °C, half-lives mostly range from 1.7 to 7.4 days. The sample of preference is the spleen, where the highest titre and highest half-life of ASFV DNA are observed. The level and duration of excretion of ASFV in the faeces, combined with the stability of the DNA, suggest that sampling of faeces could be the basis for a non-invasive sampling strategy to monitor ASFV in wild boar.

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1. Introduction

African swine fever virus (ASFV), an enveloped double-stranded DNA virus, is the causing agent of African swine fever (ASF), a highly lethal, haemorrhagic disease of swine. ASF is present in several African countries and Sardinia (OIE, 2014). In 2007, after first being reported in Georgia, it spread to several neighbouring countries, including Armenia, Azerbaijan and the Russian Federation, where it is still circulating (Gogin et al., 2013). Recently, ASFV has also been reported in Ukraine, Belarus, Lithuania and Poland (OIE, 2014). The endemic presence of the virus in Russia, coupled with ongoing spread to neighbouring countries is being seen as a serious threat for pig populations in Europe and Asia. The reported presence of ASFV in wild boar populations has been of particular concern, due to their possible role as disease reservoirs (Blome et al., 2013; Jori and Bastos, 2009). In relation to their free-roaming behaviour, this may further complicate control efforts across national borders. One particularly
challenging aspect of ASFV control in wild boar populations is the design and implementation of effective surveillance programmes (Sanchez-Vizcaino, 2006).

To obtain the necessary epidemiological information from a potentially infected population, samples can usually be obtained from two sources: from hunted/captured animals, or from animals found dead/animal debris. Testing hunted and/or captured wild boar is heavily biased towards healthy animals (Artois et al., 2002), which will not result in correct information on current spread of ASFV in the population. Additionally, hunting and capturing are quite invasive practices, which may be linked to further disease spread (Choisy and Rohani, 2006; Ladomada, 2000). Among the non-invasive, alternative methods to hunting and capture methods in wildlife ecology, is sampling of faeces (Engeman et al., 2013). The design of a surveillance strategy employing faeces found in the field would require information on the duration and titres of ASFV shed in faeces, and stability of ASFV DNA in faeces. Especially on the latter, there is very little quantitative information available.

For an early detection of ASFV, after its first introduction into the population, wild boar found dead constitute the best sampling target. Although decomposed samples may not be suitable for virus isolation and immunofluorescence tests, testing by polymerase chain reaction (PCR) is considered valid (OIE, 2012). However, there is no information on decay rates of ASFV DNA in badly preserved tissues from infected pigs.

This study evaluates the diagnostic potential of faeces and tissue samples, as a basis for non-invasive sampling strategies for ASFV in wild boar, using domestic pigs as a model. The potential of faeces is evaluated by taking into consideration ASFV titres in faeces and duration of shedding, as well as the effect of time and temperature on the detection of ASFV DNA in faeces. Stability of ASFV DNA in tissues over time was investigated to determine the suitability of the PCR on testing wild boar in various stages of decomposition.

2. Materials and methods

2.1. Animal experiment design

Animal experiments were performed with three different ASFV isolates: Brazil’78, Malta’78 and Netherlands’86 (de Carvalho Ferreira et al., 2012). Brazil’78 behaved as highly virulent in these experiments, while Malta’78 and Netherlands’86 behaved as moderately virulent. Further details regarding animal experiment design (e.g. number of inoculated and contact pigs, age, inoculation dose) can be found in de Carvalho Ferreira et al. (2012). Although the experiments were carried out in domestic pigs, wild boars are expected to have comparable pathogenesis and excretion patterns (Blome et al., 2013; Jori and Bastos, 2009). In these experiments, all pigs became infected with ASFV either by inoculation or contact with infected pigs. The experiments were approved by the Ethical Committee for Animal Experiments of the Animal Sciences Group.

2.2. Collection and processing of faeces

To estimate the amounts of excreted virus and duration of virus excretion, 20 pigs infected with Malta’78 and 10 pigs with Netherlands’86 were sampled daily from dpi 0 to 13, three times per week from dpi 14 to 27, and twice per week from dpi 28 until dpi 70. Ten pigs infected with Brazil’78 were sampled three times per week, up until the end of the experiment, on 9 dpi (de Carvalho Ferreira et al., 2012). Pigs infected with Malta’78 and Netherlands’86 were sampled for a total of 33 times and the pigs infected with Brazil’78 for a total of 5 times.

To estimate the stability of ASFV DNA, 24 additional faeces samples were collected in larger amounts (on average 27 g, ranging from 1.5 to 78 g) from pigs showing acute clinical signs: 8 originating from pigs infected with the Brazil’78 isolate, 10 from pigs infected with Malta’78 and 6 from pigs infected with Netherlands’86. Samples were taken 8–9 dpi in the Brazil’78 group, and between 7 and 15 dpi in the Malta’78 and Netherlands’86 groups. Each faecal sample was homogenized and divided in 4 equal amounts, placed in 50 ml tubes and kept at different temperatures: 5 °C, 12 °C, 20 °C, or 30 ºC, for up to 35 days. Depending on the amount of faeces, 4–10 time points were sampled from each tube.

All faecal suspensions were made by adding 1 g of faeces to 9 ml Eagle’s minimum essential medium (EMEM Gibco, Invitrogen, Breda, The Netherlands) with 10% heat-inactivated pig serum and 10% antibiotics solution ABII (1000 U/ml penicillin, 1 mg/ml streptomycin, 20 µg/ml fungizone, 500 µg/ml polymyxin-B and 10 mg/ml kanamycin), and vortexed with glass beads. After centrifugation (2500 × g for 15 min) the supernatants were stored at −70 ºC until they were analysed by quantitative real-time polymerase chain reaction (qPCR).

2.3. Collection and processing of tissue samples

In total, tissue samples from 22 pigs were collected, of which 10 had been infected with the Brazil’78 isolate, 6 with the Malta’78 isolate and 6 with the Netherlands’86 isolate. Tissue samples were collected from pigs that died as a result of the ASFV infection, with necropsies taking place between 7 and 9 days post-infection (dpi) in the Brazil’78 group, between 9 and 18 dpi in the Malta’78 group, and between 18 and 25 dpi in the Netherlands’86 group.

During the necropsy, 5 g of spleen and liver, one retropharyngeal lymph node, one parotid lymph node and the entire tonsil were collected. These tissues were placed at room temperature (approximately 20 ºC). The tissues were sampled after 0, 1, 2, 3, 5, 7, 14, 21 days. On every sampling day, 0.1 g of tissue was excised, and placed in a “MagNA Lyser Green Beads” tube (Roche Applied Science, Mannheim, Germany) filled with 600 µl of EMEM with 5% heat-inactivated pig serum and 10% antibiotics solution ABII. The sample was then homogenized in a MagNA Lyser instrument (Roche Applied Science, Mannheim, Germany) for 30 s at 3500 × g, and then centrifuged for 1 min at 9500 × g. A volume of 100 µl of tissue suspension was suspended in 200 µl of medium. Samples were stored at −70 ºC until analysis by qPCR.
2.4. Quantitative real-time polymerase chain reaction

Samples were analysed by qPCR to determine the concentration of viral DNA, according to the procedure described in de Carvalho Ferreira et al. (2012). The viral DNA concentration of each individual sample was calculated using a standard curve. Results were expressed as TCID₅₀ eq./g. TCID₅₀ equivalents do not necessarily represent infectious virus, but represent a relative measure of the amount of viral DNA present in a sample.

2.5. Statistical analysis

The ASFV titres in faeces were compared between samples originating from pigs infected with different ASFV isolates, the Brazil, Malta and Netherlands groups; and between different disease phases (acute and subacute/chronic). The acute phase of disease was assumed to start at the moment of infection (coinciding with inoculation time in inoculated pigs, or estimated in contact exposed pigs (de Carvalho Ferreira et al., 2013)), and end 21 days post infection, when clinical signs were no longer apparent. After this period, the subacute/chronic period ensued, a phase marked by few clinical signs and low excretion titres (de Carvalho Ferreira et al., 2012). Since data were not normally distributed, the non-parametric Mann–Whitney–Wilcoxon test was performed between groups, within disease phase and between disease phases, within groups.

For each individual pig, and for each disease phase, the number of virus excretion days (VED) was determined by adding up all days with a positive qPCR result in the faeces. Although shedding in faeces can be sporadic or intermittent, particularly in subacute disease (de Carvalho Ferreira et al., 2012), for simplicity, missing days in-between samplings were assumed to show the same results as non-missing days, being then interpolated as follows: between two positive or negative results, the intermediate days were respectively positive or negative, and between a positive and negative result the intermediate days were considered 50% positive. The duration of ASFV excretion in faeces for each virus strain was expressed as the ratio between the sum of VED and the sum of pig days (PD, being the number of days each pig was still part of the study).

The fraction of days in which ASFV could be detected in faeces was also compared to the fraction of days ASFV could be detected in blood (EDTA stabilized), the latter being the standard diagnostic sample for live animals (OIE, 2012). Corresponding blood data from the same experiment (de Carvalho Ferreira et al., 2012) was used, with samples being collected three times per week from dpi 0 to 27 and twice per week from dpi 28 to 70.

The percentage of days with detectable levels of ASFV in faeces, relative to days with detectable viraemia (ASFV in faeces relative to ASFV in blood (FrB%)), was calculated by applying the formula:

\[
\text{ASFV in faeces relative to ASFV in blood (FrB%)} = \frac{\text{of days with positive faeces samples}}{\text{of days with viraemia}} \times 100
\]

In order to estimate the decay rate of ASFV in faeces samples originating from acutely infected pigs, the log₁₀ ASFV titres measured by qPCR were analysed using a linear mixed effects model, with sample as random effect, time as continuous variable and ASFV isolate and temperature as categorical variables. Possible interactions between variables time, isolate and temperature were taken into account during model building.

The log₁₀ ASFV titres in tissue samples measured by qPCR were analysed by a linear mixed effect model, with pig as random effect, time as continuous variable, and both ASFV isolate and tissue type as categorical variables. Possible interactions between time, isolate and tissue were taken into account during model building.

The ASFV titres at the start (D0) were compared between tissues, within ASFV isolate; and between ASFV isolate within tissue by applying the non-parametric Mann–Whitney–Wilcoxon test.

R statistical software (R Development Core Team) package “nlme” was used for this analysis (Pinheiro et al., 2013). All models were selected based on the Akaike Information Criterion (AIC). Statistical differences were considered when \( p < 0.05 \). The linear regression slopes calculated for the tissues and faeces were used to determine the half-life of ASFV DNA according to the formula:

\[
\text{Half-life} = \frac{-\log_{10}(2)}{\text{slope}}
\]

3. Results

3.1. Excretion of ASFV DNA through faeces

Virus DNA could be detected in faeces from 4 dpi. After 30–35 dpi faeces samples were rarely qPCR-positive (de Carvalho Ferreira et al., 2012). The percentage of days that ASFV DNA could be detected in faeces (VED/PD) ranged from 39% to 57% in the acute phase, and from 5.9% to 7.6% in the subacute/chronic phase (Table 1). The FrB% in the acute phase was higher in the Netherlands group (82%) than in the Brazil and Malta groups (50–53%). In the subacute/chronic phase the FrB% was much lower: 7.6–8.1%.

Median titres in faeces, in the acute phase, were highest for the Brazil’78 isolate: 2.55 log₁₀ TCID₅₀ eq./g (0.40–4.44). Although median titres in the Malta and Netherlands groups were lower than in the Brazil group during the acute phase, the differences were not statistically significant (Table 1). During the subacute/chronic phase, titres were significantly lower than in the acute phase, with no significant difference between pigs infected with Malta’78 and Netherlands’86.

3.2. Stability of ASFV DNA in faeces

The half-lives of ASFV in faeces did not differ significantly (\( p > 0.05 \)) across virus isolates. The stability of the ASFV DNA in faeces is highly dependent on temperature (Table 2). At 5 °C and 12 °C hardly any decay of virus DNA was observed, with half-lives of close to 4 and 2 years respectively. At higher temperatures the half-life of ASFV DNA in faeces ranged from 22 days (at 20 °C) to 15 days (at 30 °C).
Table 1
Duration of ASFV excretion and titres in faeces. Duration of virus excretion is expressed as the number of VED/PD (absolute and in percentage), and as a percentage relative to virus detection in blood (FrB%). ASFV titres are expressed as log10 TCID50 eq/μg, both median and range. Results are shown separately for the acute phase (0–21 days after infection) and the subacute/chronic phase (22–70 days after infection). Significant differences between medians between the acute and the subacute/chronic disease phases are shown by different letters; significant differences between ASFV isolates (within the same disease phase) are shown by different digits.

| Group | Acute phase (0–21 dpi) | | Subacute/chronic phase (22–70 dpi) |
|-------|------------------------|-------|----------------------------------|------------------|
|       | Duration of excretion  | ASFV | Duration of excretion            | ASFV |
|       | in faeces              | in faeces | % FrB | in faeces | in faeces | % FrB | Median | Range [min; max] | % FrB | Median | Range [min; max] | % FrB |
|       | VED/PD in days (%)     |       |       |       |       |       |       |       |       |       |       |       |
| Brazil’78 (10 pigs) | 34/87 (39%) | 53% | 2.551 | [0.40; 4.41] | ncp | ncp | ncp | ncp | ncp | ncp | ncp | ncp | ncp |
| Malta’78 (20 pigs)  | 148/384 (39%) | 50% | 1.064 | [-0.67; 5.36] | 49/649 (7.6%) | 7.6% | 0.77a,1 | [-0.54; 2.40] | ncp | ncp | ncp | ncp | ncp |
| Netherlands’86 (10 pigs) | 98/172 (57%) | 82% | 0.164 | [-0.99; 3.07] | 11/185 (5.9%) | 8.1% | -0.32b,1 | [-0.56; 0.18] | ncp | ncp | ncp | ncp | ncp |

VED, virus excretion days; PD, pig days; FrB%, faeces relative to blood; min, minimum titre observed; max, maximum titre observed; ncp, no chronic phase.

Table 2
Half-life values of the ASFV DNA (measured by qPCR) in faeces originating from infected pigs, at different temperatures: 5°C, 12°C, 20°C and 30°C. No significant differences between strains were observed. Significant differences between temperatures are shown by different digits.

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Half-life values (days) of ASFV [95% CI]</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>1568 [29.9; inf]</td>
</tr>
<tr>
<td>12</td>
<td>660 [29.5; inf]</td>
</tr>
<tr>
<td>20</td>
<td>21.7 [12.7; 75.4]</td>
</tr>
<tr>
<td>30</td>
<td>15.3 [10.1; 31.8]</td>
</tr>
</tbody>
</table>

inf, infinite, slope > 0; CI, confidence interval.

3.3. Detection of ASFV DNA in tissues

Median DNA titres in tissue samples ranged from 3.17 to 5.38 log10 TCID50 eq/μg (Table 3). Overall, titres were significantly higher for the Brazil’78 isolate, compared to the other two isolates. Titres were also significantly higher in the spleen, compared to the retropharyngeal lymph node, the tonsil and the liver.

Virus DNA was most stable in spleen tissue, in which the highest half-life values were observed (Table 4). An exceptionally high half-life value was observed in spleen samples from pigs infected with the Malta’78 isolate, but this coincided with a very wide confidence interval.

4. Discussion

A non-invasive sampling strategy for ASF in wild boar to monitor an infected population, based on sampling of faeces offers good perspectives. Especially during the acute phase (up to 21 dpi), virus titres in faeces are high enough for detection by PCR. Although virus is less often present in faeces than in blood, this could be easily compensated for...
by a higher number of samples. In addition, virus DNA in faeces is sufficiently stable to allow for detection over a long time, with half-life values of more than 4 years at temperatures of 4°C and around 15 days at temperatures of 30°C. Sampling carcasses from wild boar found dead is the best method for early detection of ASFV in the wild boar population; virus titres in several tissue samples are high enough for reliable detection. Half-life values for ASFV DNA in these tissues vary from approximately 2–5 days at room temperature (approximately 20°C). This study therefore shows that decomposing tissue samples can remain suitable for diagnostic testing for a considerable period of time.

Virus DNA can be detected in faeces from 4 dpi, and most of the virus excretion through faeces will take place in the first 30–35 dpi. However, not all pigs shed ASFV in the faeces during the whole of this period. During the acute phase, ASFV can be detected in faeces in 40–60% of the days. This translates to 50–80% of the days compared to viraemia. This lower sensitivity could be compensated for by collecting a higher number of samples. Testing faeces during the acute phase could, therefore, be a good alternative for testing blood samples. After the acute phase, ASFV excretion levels quickly drop, and compared to testing blood samples for the presence of virus, faeces become a relatively poor diagnostic sample, as faeces will not allow for a reliable monitoring of chronically infected pigs. However, especially for highly virulent strains, faeces samples seem to be a promising alternative. The highly virulent strain circulating in Russia results in 100% lethality after 10 days of infection (Gogin et al., 2013), and should therefore compare quite well to the Brazil’78 isolate used in this study.

Several studies have been published on sampling wild boar faeces for different purposes (Acevedo et al., 2007; Ebert et al., 2012), namely for estimating population size (Ebert et al., 2012; Fickel and Hohmann, 2006). Although faeces sampling still involves a considerable effort, the lower bias and large number of samples (hundreds of faeces pellets can be found in this way) (Ebert et al., 2012), make faeces sampling a very interesting non-invasive sampling option. Field persistence of faeces pellets depends on many factors, but studies have shown that 74% of the faeces pellets will last for at least 1 month and 64% for at least 2 months (Hone and Martin, 1998). Faeces samples were exposed to laboratory conditions that may not accurately reflect the range of conditions faeces are exposed to in the field. Daily and inter-daily temperature variation, seasonal variation and hydric stress, for instance, may all affect the stability of ASFV in faeces found in the field. Given the remarkable stability of ASFV DNA in faeces, even at temperatures of 30°C, virus can be expected to remain detectable for a long period of time, particularly in comparison with the relatively shorter period during which faeces pellets can be found in the field.

Sampling faeces may be useful in monitoring ASFV infections with highly virulent strains (such as the strain that is currently circulating in Russia), especially when combined with testing wild boar found dead. When faeces pellets are collected with regular time intervals, in previously defined plots, it is also possible to easily include time as a factor when describing the epidemiological situation. In regions where moderately virulent ASFV strains circulate among wild boar, the number of PCR-positive faeces pellets to be found will be even higher. Pigs infected with moderately virulent strains survive longer, and since at least a fraction will continue shedding ASFV for some time, these pigs will produce more PCR-positive faeces pellets. So, for a correct interpretation of the local situation, when designing monitoring programmes, the virulence of the circulating strain will have to be considered. Furthermore, it should be taken into consideration that positive faeces samples will mainly represent wild boar in the acute phase of the infection. Chronic carriers, mainly occurring after infection with moderately virulent strains, will not be detected by testing faeces. This should not be a problem, as information on acutely infected wild boar should be sufficient for a good insight in the local epidemiological situation.

Early detection of ASF in wild boar populations will mainly depend on diagnostic tests carried out on animals found dead. Given the high lethality of many ASFV strains, death occurs relatively quickly after the infection. When a dead wild boar is then found in the field, the carcass has often started decomposing. Tissue samples obtained from such a carcass are unfit for testing by virus isolation and immunofluorescence tests, but are acceptable for testing by PCR (OIE, 2012). With half-lives of roughly 2–5 days in several different tissues, ASFV DNA is more stable than other relevant viruses in wild boar. The RNA of classical swine fever virus (CSFV) for instance, has half-life values of 1–2.5 days (Weesendorp et al., 2010). For ASFV detection, all tissues investigated are suitable for diagnostic purposes but the preferred sample would be spleen, where initial titres at the moment of death are highest. However, the final sample taken in the field may also depend on the condition of the carcass to be sampled.

Several factors will affect the time frame in which a carcass remains present in the field (Carter et al., 2007; DeVault et al., 2003). Without scavenger intervention, pig carcasses have been shown to fully decompose due to insect activity between 3 (Grassberger and Frank, 2004) and 5 weeks (Amendt et al., 2004). Still, soft tissues are expected to decompose comparatively faster. Given the initial titres at the time of death, and the decay values for ASFV DNA, it is likely that ASFV can be detected by PCR, at least as long as tissue samples can be obtained.

Decay rates of ASFV DNA in tissues were investigated at one temperature only. Although at higher temperatures half-life values are expected to be shorter, in faeces a temperature increase from 20°C to 30°C did not lead to dramatic decrease in half-life values. Therefore, considering the timeframe in which a carcass can be sampled before it is fully decomposed, higher temperatures (for temperate climates) are not likely to be a limiting factor.

The current study was carried out with infected domestic pigs as a model for wild boar. Experimental infections in wild boar are difficult to carry out, especially when they need to be sampled often. Moreover, both types of pigs belong to the same species (Sus scrofa), and can be considered as non-adapted to infections with ASFV, unlike
wart hogs (Phacochoerus africanus) (Jori and Bastos, 2009). Experimental infections with an isolate representative for the strains circulating in Russia have shown a similar virulence and pathogenesis in wild boar as in domestic pigs (Gabriel et al., 2011). Furthermore, wild boars seem to excrete virus in similar quantities as domestic pigs (Jori and Bastos, 2009). For the purpose in this study, the domestic pig was therefore considered as a good model for wild boar.

In conclusion, it is shown here that ASFV DNA is relatively stable in both tissues and faeces. During the time period that these materials can be expected to remain present in the field, ASFV can still be detected with a high confidence. The presence of virus in faeces of infected wild boar allows for non-invasive surveillance strategies that can be carried out independently of hunting or capture practices. Field tests in regions where ASF occurs in wild boar would be helpful to further develop these surveillance strategies, through which ultimately a better and much needed insight can be gained in the role of wild boar in the ASF epidemiology.

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