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Molecular contamination of an animal facility during and after African swine fever virus infection

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Abstract

Introduction: The molecular contamination of an animal facility was investigated during and after an infection with highly pathogenic African swine fever virus (ASFV) among domestic pigs. The investigation evaluated the risk of indirect transmission of the disease and indicated points that may facilitate cleaning and disinfection processes. **Material and Methods:** Six domestic pigs were infected oronasally with the highly pathogenic Georgia 2007 strain. Environmental samples from the floors, walls, rubber floor mats, feeders, drinkers, high-efficiency particulate-absorbing filter covers and doors were collected 7 days post infection (dpi), 7 days later and 24 h after disinfection of the facility. The samples were investigated by real-time PCR and *in vitro* assays to find genetic traces of ASFV and infectious virus. **Results:** Typical clinical outcomes for ASF (*i.e.* fever, apathy, recumbency and bloody diarrhoea) were observed, and all animals died or required euthanasia before or at 9 dpi. No infectious virus was found in environmental samples at the sampling time points. Genetic traces of ASFV were found in all locations except the doors. The initial virus load was calculated using real-time PCR threshold cycle values and was the highest at the drain. A statistically significant decrease of virus load over time was found on non-porous surfaces mechanically cleaned by water (the floor and drain). **Conclusion:** The gathered data confirmed different routes of virus excretion (oral and nasal, faeces and urine, and aerosol) and showed virus locations and different initial concentrations in the animal facility. Maintaining the facility with mechanical cleaning and using personal protection (gloves) and hand disinfection may efficiently minimise the risk of further virus spread. Together with the results of previously published studies, the present investigations' failure to isolate infectious virus may suggest that if stable environmental conditions are assured, the time needed before the introduction of new herds into previously ASF-affected farm facilities could be shortened and in this way the economic losses caused by the disease outbreak mitigated.

Keywords: African swine fever, pigsty, indirect transmission, persistence, risk.

Introduction

African swine fever (ASF) is one of the most dangerous and devastating diseases of domestic pigs and wild boar. The disease poses a serious risk to the worldwide trade in pork meat, causing enormous economic losses. Since 2005, 74 countries have confirmed cases of ASF on their territory (29). The causative agent of ASF, African swine fever virus (ASFV), belongs to the *Asfarviridae* family. Its large genome (170 to 193 kilobase pairs), a double-stranded DNA, encodes more than 150 proteins (21). The virus has an ability to evade the host immune system, and causes massive, devastating inflammation processes leading to up to 100% mortality among affected animals (5, 26, 28). The complex structure of the virus and wide

range of its genes prevent the development of an effective vaccine against ASF. Despite the promising results shown by several studies of vaccine candidates, currently there is no vaccine against the disease accepted as safe in the EU market (24, 31). Managing the disease relies on prevention by using strict biosecurity measures, early detection and culling of infected pigs. Such an approach has serious socio-economic impact (6, 13). Early restoring of pig production on farms affected by ASF could mitigate the economic losses; however, maximum safety should be observed in early restarting to prevent further disease spread.

According to the literature, ASFV is among the persistent pathogens in the environment, but the virus can be easily eradicated with most common disinfectants (10, 11, 14). Direct contact plays an important role in the transmission of the disease. Infected animals excrete the virus with oral and nasal fluids, faeces and urine. African swine fever virus can also be transmitted over short distances *via* aerosol. Contact with the blood of infected animals and their carcasses poses the highest risk of infection, as in those matrices the virus load is particularly large (27). The presence of blood (*e.g.* in bloody diarrhoea or after veterinary procedures) or any excreta in animal houses may result in contamination of the environment, and a consequent possibility of indirect transmission of ASF has been reported by several authors (18). On the one hand, field studies suggest that low farm biosecurity and contaminated fomites (*e.g.* clothes and vehicles) are the most probable facilitators of infection (17, 30). On the other hand, experimental infection of sentinel animals that had contact with contaminated environments could not be demonstrated at all (7) or was possible only very soon (*i.e.* one day) after removing infected pigs from the pigsty (19). A contaminated environment may pose a risk of ASF transmission because the virus persists in it. Davies *et al.* (4) proved that infectious ASFV could be found in faeces and urine for maximums of 15.33 and 3.71 days, respectively, depending on the temperature. Nuanualsuwan *et al.* (16) showed that at 25°C, infectious ASFV could be recovered from different fomites for no longer than 17 days (from non-porous materials) or 22 days (from porous materials). Therefore, the virus' persistence as well as the characteristics of the individual farming systems affected by ASFV should be taken into consideration when biosecurity measures are implemented.

Legislation stipulates the procedure for introduction of a new pig herd onto a farm previously affected by ASF; in Poland it permits housing a new herd on a farm after 40 days, counting from the day when cleaning and disinfection processes are completed. Rehoused animals should serve as sentinels and be tested for the presence of specific anti-ASFV antibodies after 45 days. Shortly after the negative results are obtained, a new herd can be introduced safely. Alternatively, pigs may be brought onto the farm again 6 months from the completion of the cleaning and disinfection processes without retesting for antibodies (8). Such a long period ensures the safety of a newly introduced herd; however, in light of recently published studies in this area, from the economic point of view it may be open to discussion if this duration is necessary for the inactive phase (7, 19).

In the present study the molecular contamination of an animal facility was evaluated during and after highly pathogenic ASFV infection, to investigate the virus' location and its initial amount in the environment. Isolation of infectious virus from collected samples was also attempted, to indicate the risk to animal safety from a contaminated environment. Different routes of transmission were focused upon, *i.e.* direct contact with oral and nasal excretions (the feeder, drinker and walls), contact with faeces (the drain, the floor and rubber floor mats), the aerosol route (the high-efficiency particulate-absorbing (HEPA) filter cover) and *via* personnel contact (doors).

Material and Methods

Animals. The data presented in this study were gathered during an independent animal trial from a group of six 10-week-old Danbred Duroc domestic pigs. The animal experiment was approved by the Local Ethical Committee for Animal Experiments in Lublin (under approval number 82/2022). All procedures including euthanasia were performed in compliance with current legal regulations.

Virus. The virulent ASFV genotype II Georgia 2007 strain at a dose of 1×10^5 50% haemadsorbing doses (HAD_{50}) per animal was used for intranasal infection. The virus was isolated by Dr. Linda Dixon at the Pirbright Institute, Woking, UK, and kindly provided for the present study by the Institut de Recerca i Tecnologia Agroalimentàries – Centre de Recerca en Sanitat Animal, Barcelona, Spain.

Facility. Animals were kept in a biosafety level 3 animal facility at the National Veterinary Research Institute (Puławy, Poland) and provided with feed and water *ad libitum*. Before the experiment, the state of health of all the animals was evaluated through veterinary examination, and the six pigs were confirmed to be free of ASFV by testing with a Virotype Real-time PCR kit (Qiagen, Hilden, Germany). The facility maintains a stable temperature ranging from 20 to 24°C, humidity at 55% (±10%), 12–15 air exchange per hour and airflow not exceeding 0.3 m/s. Pigs were kept without other bedding than rubber mats ensuring their welfare.

Cleaning and disinfection. The facility floor was cleaned with tap water on a daily basis during the trial by the facility staff. Seven days from the end of the experiment, the facility was decontaminated with 35% hydrogen peroxide vaporisation (Bioquell Z, Andover, UK).

Shedding assessment. Oral, nasal and rectal swabs were collected at 0 and 7 days post infection (dpi).

Additional samples were taken during necropsy. Swabs were placed into tubes containing 1 mL of phosphate-buffered saline, then incubated at room temperature for 10 min and vortexed. An aliquot of 200 µL of each sample was reserved for DNA extraction and real-time PCR analysis.

Environmental sample collection. Environmental samples were collected when the infection was ongoing at 7 dpi, at 14 dpi (before decontamination) and at 15 dpi (24 h after decontamination). Two millilitres of Roswell Park Memorial Institute 1640 medium (PAN Biotech, Aidenbach, Germany) as a growth medium (GM) supplemented by 1% Antibiotic-Antimycotic Solution (Sigma-Aldrich, St. Louis, MO, USA) and 10% foetal bovine serum (GIBCO, Thermo Fisher Scientific, Waltham, MA, USA) was placed into a plastic tube. Designated spots were thoroughly scrubbed by swabs and immersed in the medium. Swabs from the floor (five spots), feeder (one spot), drinker (one spot), a rubber mat (one spot), a wall at a height of about 30 cm (one spot), the HEPA filter cover (one spot), and the doors at a height of about 120 cm (one spot) were collected in triplicate. Samples were aliquoted and stored at −80°C for real-time PCR and *in vitro* analyses.

DNA extraction and real-time PCR. Extraction of DNA was carried out on the Qiagen DNA Mini Kit protocol (Qiagen), with further use of the Virotype ASFV PCR Kit (Qiagen) for a subsequent real-time PCR reaction in a Rotor-Gene Q thermocycler (Qiagen) according to the manufacturer's instructions.

Infectious virus isolation. Porcine primary pulmonary alveolar macrophages were collected by lung lavage from uninfected donor pigs and seeded at 1×10^5 cells per well in GM supplemented by erythrocytes (1:300 v/v) in a 96-well plate. One hundred microlitres of each filtered (0.45 µm) environmental sample was added to the respective wells in triplicate. In parallel, the negative control (medium) and positive control (Georgia 2007, 1×10^3 HAD_{50}/mL) were prepared in the same way. The plates were incubated at 37°C in 5% CO_2 and inspected for the presence of haemadsorption and cytopathic effect on the 0, 4th and 7th days of incubation. In addition, cells and medium were collected on days 0 and 7 of incubation for real-time PCR analyses after three freeze-thaw cycles.

Initial virus load estimation. The initial virus load was estimated for non-infectious samples based on the threshold cycle (Ct) values, as previously described (27).

Statistical analysis. Statistical analysis of initial virus load in samples from different time points was performed using the Kruskal–Wallis test in GraphPad Prism 8.4.2 (GraphPad Software, San Diego, CA, USA). The significance for statistical difference was defined as P-value < 0.05.

Results

Infection outcomes and virus shedding. Fever followed by apathy in infected animals could be seen beginning from 4 dpi. Infection developed and resulted in the deaths of 100% of the animals by 9 dpi. During the disease, three out of the six animals reached the humane endpoint and were euthanised at 8 dpi because they were suffering from bloody diarrhoea, severe dyspnoea and recumbency. The evolution of fever and the survival rate in this group are presented in Fig. 1.

Up to the 7th dpi (the first environmental sampling time point), four out of the six animals remained in the facility, and at this time, the presence of ASFV genetic material was confirmed in the rectal swabs of two out of the four animals and the oral and nasal swabs of all four animals. All swab samples collected from all six animals during necropsy were PCR positive, with noticeably higher initial virus loads. The mean virus loads of swab samples collected at 7 dpi and during necropsy based on the Ct values is presented in Fig. 2.

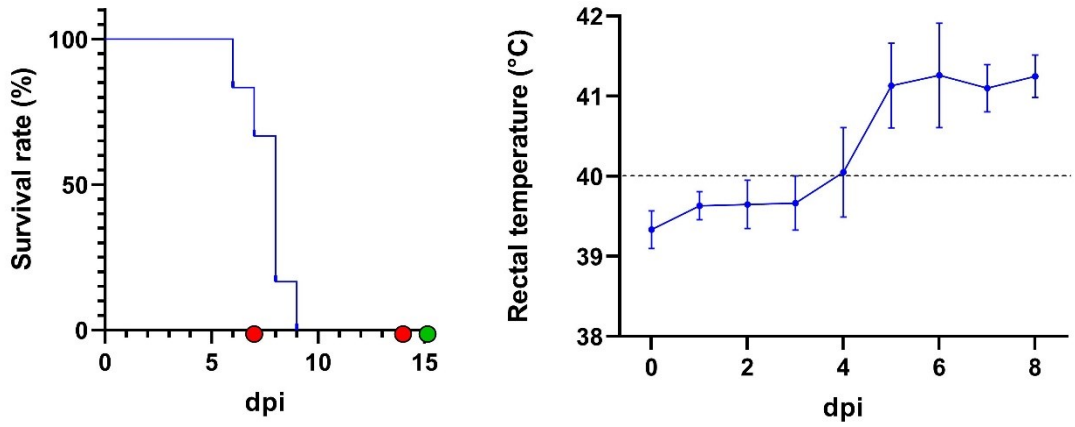

Fig. 1. Survival rate and fever evolution during experimentally induced African swine fever in 10-week-old Danbred Duroc pigs. Dots indicate environmental sampling timepoints. Red dots – before disinfection; green dot – after disinfection; dashed black line – fever threshold; dpi – days post infection

Table 1. Numbers of positive qPCR-samples in tested animal facility locations and their mean threshold cycle (Ct) values

Time	Number of positive samples (Mean Ct (±SD))							
	Floor	Wall	Door	HEPA filter cover	Drain	Feeder	Drinker	Mat
T1	14/15 (35.9 (±1.6))	3/3 (33.3 (±1.9))	0/3	2/3 (37.3 (±0.4))	2/3 (28.7 (±1.4))	3/3 (34.3 (±1.9))	3/3 (33.0 (±0.8))	2/3 (38.3 (±0.6))
T2	4/15 (38.2 (±0.6))	2/3 (36.7 (±2.2))	0/3	1/3 (36.7 (±0.0))	1/3 (32.4 (±0.2))	3/3 (35.9 (±1.2))	3/3 (36.7 (±1.6)	3/3 (37.8 (±0.9))
T3	5/15 (39.0 (±0.8))	2/3 (35.6 (±2.9))	0/3	1/3 (39.1 (±0.0))	1/3 (32.9 (±0.6))	3/3 (36.2 (±1.6))	3/3 (34.6 (±1.6))	3/3 (37.4 (±0.7))

SD – standard deviation; T1 – ongoing infection (7 days post infection (dpi)); T2 – after infection (T1 + 7, *i.e.* 14 dpi), T3 – 24 h after disinfection (15 dpi); HEPA – high-efficiency particulate-absorbing

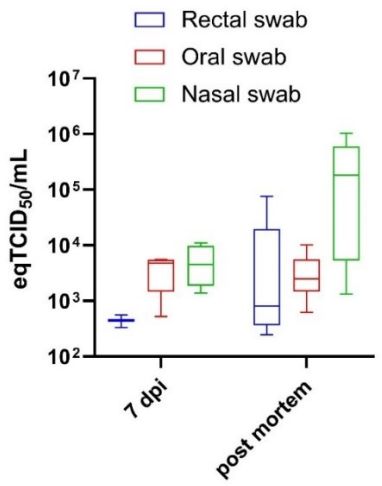

Fig. 2. Estimated initial virus load in swab samples collected from 10-week-old Danbred Duroc pigs during experimentally induced African swine fever at 7 days post infection (dpi) and necropsy. The boxes represent the 50% between the 25 and 75% quartiles. The line inside the box indicates the median. The whiskers denote maximum and minimum values. $eqTCID_{50}$ – 50% tissue culture infectious dose equivalent

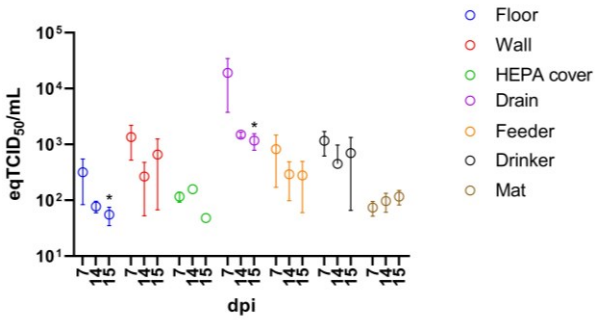

Fig. 3. Initial load of African swine fever virus in different pigsty locations after experimental infection and its change over time. Statistically significant decreases of estimated initial virus load were observed at 15 dpi at the floor and drain spots – locations mechanically cleaned by water. Error bars indicate standard deviation. $eqTCID_{50}$ – 50% tissue culture infectious dose equivalent; HEPA – high-efficiency particulate-absorbing; * – statistically significant (P-value 0.002 (floor), 0.0393 (drain))

Environmental samples. The presence of ASFV DNA could be confirmed in all tested locations except the doors. However, infectious virus could not be recovered in any of the samples collected at any time point. The numbers of qPCR-positive samples at the three sampling time points are presented in Table 1.

The highest estimated initial virus load was found while the infection was ongoing (7 dpi) at the drain (reaching 1.92×10^4 $eqTCID_{50}$/mL). A noticeably decreasing tendency in estimated virus load over time was found at the floor and drain spots at 15 dpi, when the mean virus load had decreased to 5.5×10^1 50% tissue culture infectious dose equivalents ($eqTCID_{50}$)/mL and 1.2×10^3 $eqTCID_{50}$/mL, respectively. These decreases were statistically significant changes, which was shown by a P-value of 0.002 for the floor and one of 0.0393 for the drain. It is noteworthy that the mean initial virus load at feeder and drinker spots was slightly higher than at faeces and urine or aerosol contact spots (*i.e.* the mats, the floor and the HEPA filter cover). The estimated initial virus loads in different locations and their change over time is presented in Fig. 3.

Discussion

After infection and replication in the host's lymphatic organs, ASFV spreads through the pig's body with blood and can soon be found in almost all tissues (1). The virus can be secreted with oral fluid, faeces and urine (4, 27). Shedding of the virus and the presence of dead animals in the pigsty worsen environmental contamination, pose a risk for indirect transmission of ASF and consequently block the sale of animals (2, 23). Presently, the only action to control the disease is the imposition of biosecurity measures (6). Knowledge of ASFV locations and virus load may facilitate cleaning and disinfection processes in the pigsty after culling, leading to effective eradication of the pathogen and fast reintroduction of animals onto a previously affected farm.

In this study, well-established shedding could be seen at 7 dpi. The presence of the virus' DNA in all tested matrices is in accordance with previously published findings (9, 22, 25). The environmental samples collected in the present study reflected the ongoing infection situation and consequent facility contamination. Genetic traces of ASFV were found on surfaces that may have had contact with faeces and urine (the floor, walls and drain) and with oral and nasal excretions (the feeder and drinker). The latter outcome may suggest that the virus could be transmitted not only *via* surfaces but also *via* feed and water, which partially confirms other authors' findings (3, 15, 18). The aerosol

route was confirmed by finding traces of ASFV genetic material on the HEPA filter cover, which is in line with the observation of Olesen *et al.* (20) of proven infection of animals by this route over short distances.

In this study we were not able to isolate infectious virus from environmental samples. Attempts at infectious virus isolation directly from oral fluid were not successful either in the studies of Guinat *et al.* (9) or those of Davies *et al.* (4); however, the latter confirmed that infectious virus could be found in faeces and urine for up to approximately 5 days of the period of its shedding in similar environmental conditions (21°C). The results of our study may suggest that excretion of the virus to the environment renders it inactive, even when the initial virus load was high (as it was at the drain). These observations partially support those of the studies of Eblé *et al.* (7) and Olesen *et al.* (18), where infection of sentinel pigs was not possible at all or was possible no more than one day after removing ASF-affected animals from the facility. In light of this and previously published studies, the time needed for the introduction of a new pig herd onto a previously ASF-affected pig farm could be probably revised and shortened, on condition that similar environmental conditions are maintained.

The absence of genetic material of ASFV on doors indicates that using personal protective equipment (gloves) and disinfecting hands during standard veterinary procedures prevents further spreading of the virus. It was also noticeable that the estimated initial virus load decreased significantly on surfaces which had been cleaned mechanically with tap water. This showed that even simple maintenance of a pig-farming facility could minimise the risk of the pathogen's transmission, but only on non-porous surfaces. Porous materials (*i.e.* in the present case, the rubber mats) were resistant to water washing and their estimated initial virus load did not decrease. This finding concurred with that of studies performed by Krug *et al.* (12) and Nuanualsuwan *et al.* (16), which indicated difficulties in effectively disinfecting porous materials.

Besides its use in proving the restoration of satisfactory hygiene standards, examination of environmental samples could be useful in epidemiological analysis, *e.g.* when ASF-affected pigs are transported illegally off a pig farm. Based on the results from this study, the presence of ASFV could be confirmed in the facility, especially in the places that had contact with sewage, such as the drain, but also on porous material and in the ventilation system.

To the best of our knowledge, this is the first report on contamination of a facility housing pigs during and after ASF infection. The gathered data provide useful practical knowledge, facilitating processes of cleaning and disinfection, and bring additional insight into the mechanism of indirect transmission of ASF and the virus presence in pig husbandry facilities.

Conflicts of Interests Statement: The authors declare that there is no conflict of interests regarding the publication of this article.

Financial Disclosure Statement: This study received no external funding.

Animal Rights Statement: The experiment was approved by the Local Ethical Committee for Animal Experiments in Lublin (approval number 82/2022). All procedures were carried out according to the "Ustawa z dnia 15 stycznia 2015 r. o ochronie zwierząt wykorzystywanych do celów naukowych lub edukacyjnych (Dz. U. z 2015, poz 266)" (Polish Act of 15 January 2015 on the protection of animals used for scientific or educational purposes (Official Journal of Laws 2015, item 266) based on Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes.

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