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Research Article

Inefficient Transmission of African Swine Fever Virus to Sentinel Pigs from an Environment Contaminated by ASFV-Infected Pigs under Experimental Conditions

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Knowledge about African swine fever virus (ASFV) transmission and its survival in the environment is mandatory to develop rational control strategies and combat this serious disease in pigs. In this study, the risk that environmental contamination poses for infection of naïve pigs was investigated. Naïve pigs were introduced as sentinels into contaminated pens kept at ambient temperature (about 18–22°C) either on the same day or up to 3 days after ASFV-infected pigs were removed. Three experiments were carried out in which four to six pigs per pen were inoculated with virulent ASFV isolates OURT88/1 (genotype I), Georgia 2007/1, or POL/2015/Podlaskie (genotype II), respectively. The majority of the inoculated pigs developed acute disease but with no evident haemorrhagic lesions or haemorrhagic diarrhoea and were culled at the predefined humane endpoint. The levels of ASFV DNA detected in the blood of the infected animals reached 10^{7-9} genome copies/ml before euthanasia. Environmental swabs were taken from different surfaces in the animal rooms, as well as from faeces and urine, close to the time of introduction of the naïve animals. Relatively low quantities of virus DNA were detected in the environmental samples, in the range of 10^{3-7} genome copies per swab or per gram and ml of faeces and urine. No infectious virus was recovered from these environmental samples. Neither clinical signs nor virus genomes were detected in the blood of any of the sentinel pigs over a period of 2 to 3 weeks after exposure, indicating that transmission from the ASFV-contaminated environment did not occur. Interestingly, viral DNA was detected in nasal and oral swabs from some of the sentinel animals at early days of exposure (ranging between $10^{3.7-5.8}$ genome copies per swab), though none of them developed ASF. The results indicate a relatively low risk of ASFV transmission from a contaminated environment under the conditions provided in these experimental studies and in the absence of bloodshed from infected animals.

1. Introduction

African swine fever (ASF) is a severe haemorrhagic disease with a high case fatality rate in domestic pigs and wild boar. It is caused by the African swine fever virus (ASFV), a large, cytoplasmic, double-stranded DNA virus that is the only member of the *Asfarviridae* family. Safe and efficient commercial vaccines are not yet available to aid disease control.

A long-established wildlife reservoir of ASFV is present in East Africa in warthogs and soft ticks from the genus *Ornithodoros* that inhabit their burrows. These and other wild suids in Africa, including bush pigs, show no disease and develop a low transient viremia but animals can remain persistently infected for long periods [1, 2]. In contrast, infected domestic pigs and wild boar develop high titres of virus in blood and direct transmission occurs readily between them [3]. The large numbers of wild boar in many European countries provide a wildlife reservoir for infection of domestic pigs.

Indirect transmission by various mechanisms is also recognized as an important transmission route. The virus can be transmitted indirectly via pork products and virus-contaminated fomites [4, 5]. Long-distance jumps of the virus usually involve transmission via pork meat containing the virus [6]. Contaminated feed or water supplies as well as wild boar carcasses can also provide sources of infection for spread of the virus among wild boar and spillover into domestic pigs [7]. Interestingly, different feed materials with a low moisture content (hay, straw, and grain) have been shown to promote ASFV stability and survival when compared to other matrices with a higher moisture content (soil, water, and leaf litter) [8].

Mechanical transmission by biting flies has been suggested to play a role in virus transmission but has been little studied. Two studies showed that ASFV survived for 48 hr in stable flies fed on infected blood with high viremia (10^8 HAD_{50}/ml) [9] or 12 hr after feeding on blood with lower viremia (5×10^5 $TCID_{50}$/ml) [10]. In the study by Mellor et al. [9], ASFV was transmitted to pigs by the biting flies that had been blood fed 1 and 24 hr before feeding on the animals. It has also been shown that ingestion of stable flies fed on ASFV infected blood also resulted in infection of pigs [11]. ASFV DNA has been detected in hematophagous insects on ASF outbreak farms, where ASFV-infected pigs were still present or had been culled [12]. Furthermore, in an ASF outbreak area, hematophagous insects carrying blood meals including ASFV DNA were captured on the windows of a high biosecurity pig farm that was free of ASF, hence indicating a potential risk for introduction of ASFV [13, 14].

Aerosol transmission of ASFV has been detected over short distances within buildings but wider dispersion by aerosol is not thought to occur [15, 16]. As a large DNA virus, ASFV is physically very stable over a wide range of pH values and temperatures [17, 18] and can survive for extended periods in contaminated materials posing additional problems for control. A recent EFSA scientific opinion reviewed literature on the survival of ASFV in different matrices and estimated the risk these posed for virus transmission in different scenarios [5]. Very high levels of virus are present in the blood of pigs showing clinical signs of acute ASF (up to 10^{8-9} $TCID_{50}$ or HAD_{50}/ml). A very early study showed that blood collected after death with ASF and stored at room temperature in the dark for 140 days was still infectious as shown by inoculation of another pig [18]. ASFV was also observed to survive in chilled blood for an extended period of time, some 525 days [17]. Thus, materials contaminated with infected blood pose a high risk for spread of virus. Although much lower levels of virus are found in excretions, ASFV can also survive in these for several days. In faeces, with an initial titre of $10^{4.83}$ $TCID_{50}$/g, collected from animals showing acute disease, ASFV survived for up to 8.5 days when the faeces were stored at 4°C, for 5 days at 21°C and 3 days at 37°C [19]. In an early study, virulent virus was still present in faeces kept at room temperature in the dark for up to 11 days, with these faeces inducing ASF and death in 7 days after being fed to a susceptible pig [18]. This study also found that virus in urine collected after death survived at room temperature for at least 2 days and caused ASF after being fed to a susceptible pig. However, after storage for longer than 2 days, the urine was less likely to cause infection. More recently, it was observed that ASFV in the urine from infected animals, with initial titres of $10^{2.2-3.8}$ $TCID_{50}$/ml, could survive, on average, for 15 days when chilled, for 5 days at room temperature or for 3 days when incubated at 37°C [19]. In agreement with these results, a recent study did not detect infectious virus in faeces and urine from ASFV-infected pigs and wild boar after 1 week of storage at room temperature [20]. Water troughs are shared by the animals in a pig pen and hence if water becomes contaminated, it may also spread infection, especially since ASFV seems to be highly stable in water. Experimentally contaminated water, with an initial titre of $10^{6.5}$ HAD_{50}/ml stored frozen (−16 to −20°C) or chilled (4–6°C), contained viable ASFV for at least 60 days, and when stored at room temperature (22–25°C) was infectious for 50 days [21].

No data were found estimating ASFV survival on straw, a commonly used bedding material in pig farms [5]. However, it would be expected that straw in housing with infected pigs may be contaminated with faeces and urine as well as blood and hence constitute an important source for virus transmission.

The infectious dose of ASFV varies according to the route of infection. Using the intranasal route, it has recently been shown that the minimum infectious dose for a highly virulent genotype II ASFV was very low, since five hemadsorbing units (HAU) resulted in infection [22]. For oral infection, the minimum infectious dose of virulent genotype II ASFV in liquid was estimated to be 10^0 $TCID_{50}$, while it was 10^4 $TCID_{50}$ in feed (median infectious dose was $10^{1.0}$ $TCID_{50}$ for liquid and $10^{6.8}$ $TCID_{50}$ for feed) [23]. This is a large difference, for which the basis is not known. It has been suggested that liquid provides a suitable substrate for contact between the virus and the tonsils. In an earlier study, the intranasal/oral infectious $dose_{50}$ (ID_{50}) and the intravenous/intramuscular (IV/IM) ID_{50} of a moderately virulent isolate of ASFV were determined to be 18,500 and 0.13 HAD_{50}, respectively, and a highly virulent isolate required approximately 10-fold more virus to cause infection by the intranasal/oral route [24]. Although these studies vary in their estimates of infectious dose, they confirm that ASFV can readily be transmitted by the oral–nasal route. Since infectious virus can survive for several days, at the range of temperatures where pigs are reared, blood and secretions may pose a risk for virus transmission.

Although progress has been made in understanding mechanisms and risks posed by different indirect routes of ASFV transmission, gaps in knowledge remain. In early studies, transmission to pigs from an environment contaminated

with ASFV was observed when a contaminated pen had been left empty for 3 days, but not for 5 days [18]. In more recent experiments [25, 26], it was shown that pigs that were introduced into the contaminated environment (average temperature 21°C) at 1 day after removal of ASFV-infected animals, developed clinical disease. However, pigs introduced into the contaminated pens after 3, 5, or 7 days did not develop signs of ASF. The results from the studies suggested a relatively narrow window of time for transmission, but further studies were needed to confirm this.

In the current study, we investigated the potential role of environmental contamination in pig housing for transmission of ASFV. Three experiments were carried out in which naïve pigs were introduced into pens that had recently housed pigs showing acute disease after inoculation with virulent isolates of ASFV: OURT88/1 (genotype I), Georgia 2007/1 or POL/2015/Podlaskie (genotype II). The naïve pigs were introduced on the same day, or 1–3 days after the infected pigs were removed and the levels of viral DNA in different surface swabs and excretions were evaluated at different days before and during exposure. None of the introduced pigs became infected suggesting that the risk of transmission from environmental contamination is low, although we detected relatively low levels of virus genome in environmental samples collected from rooms that had housed the infected pigs and also in some oral/nasal swabs from the introduced animals.

2. Materials and Methods

2.1. Virus Isolates and Cell Culture. The OURT88/1 (genotype I) and Georgia 2007/1 (genotype II) virulent isolates of ASFV have been described previously [27, 28]. Virus stocks were prepared by infection of primary porcine bone marrow cells and titrated by limiting dilution in porcine bone marrow cells seeded in 96 well plates using a hemadsorption (HAD) assay and are expressed as HAD_{50} /ml, as described previously [29]. The ASFV POL/2015/Podlaskie (genotype II) was isolated, as previously described [16]. The virus was prepared by infection of porcine pulmonary alveolar macrophages (PPAM) and titrated in PPAM seeded in 96-well plates using an immunoperoxidase monolayer assay (IPMA) as described previously [16, 30] with titres presented as $TCID_{50}$/ml [31].

2.2. Animal Housing and Ethical Approval. Animal experiments 1 and 2 were carried out at The Pirbright Institute under license 7088520 issued by the UK Home Office under the Animals (Scientific Procedures) Act (1986) (ASPA) and were approved by the Animal Welfare and Ethical Review Board. The animals were housed in the SAPO4 high containment large animal unit at The Pirbright Institute in accordance with the Code of Practice for the Housing and Care of Animals Bred, Supplied, or Used for Scientific Purposes. Bedding and species-specific enrichment were provided throughout the study to ensure high standards of welfare. Clinical scoring was carried out daily [32] and pigs that reached the scientific or moderate severity humane endpoint, as defined in the project license, were euthanized by an overdose of anaesthetic.

Animal experiment three was performed in BSL3 facilities at the Centre de Recerca en Sanitat Animal (IRTA-CReSA, Barcelona, Spain). The experiment was conducted in accordance with EU legislation on animal experimentation (EU Directive 2010/63/EU). A commercial diet for weaned pigs and water were provided *ad libitum*. Rectal temperatures and clinical signs were recorded for each pig on a daily basis. A total clinical score was calculated per day based using a previously described system [33]. Pigs were euthanized, after reaching the humane endpoints set in the study, by intravascular injection of Pentobarbital following deep anesthesia.

2.3. Animal Experiments Design. For experiments 1 and 2, female Landrace × large white × Hampshire pigs about 23–26 weeks of age were obtained from a high-health status farm in the UK and after a 7-day settling-in period were challenged intramuscularly with 10,000 HAD_{50} infectious units of virulent ASFV. In experiment 1, five animals (numbered 41–45) were inoculated with OURT88/1 isolate. In experiment 2, six animals (numbered 1–6) were inoculated, three with OURT88/1 and the other three with Georgia 2007/1, all kept in the same pen. The infected animals were euthanized 5 days after infection when reaching the humane endpoint. The premises were minimally cleaned between days 3 and 5 of infection (removal of gross faeces contamination and any blood postsampling, as well as ensuring the pigs had a clean area to eat) and after that were left completely uncleaned until two sentinel pigs were introduced into the room. The sentinel pigs were 6–8 weeks of age and had also had a 7-day settling in period. In Experiment 1, sentinels (numbered 46 and 47) were introduced to the premises 1 day after removal of the infected animals and, in Experiment 2, two sentinels (numbered 7 and 8) were introduced on the same day. Minimal cleaning was then restarted the following day and the sentinel animals were monitored for clinical signs over a period of 14 days. Figure 1 shows the design of experiment 1 including days on which samples were collected. During the experiments, temperature and relative humidity of the premises housing the animals were recorded daily and were 18.4–19.1°C and 45%–53.2%, respectively. Air exchange rates in the animal housing rooms were approximately 13 per hour.

For experiment 3, 24 male Landrace × large white pigs about 8 weeks of age were obtained from a conventional Spanish swine herd. After an acclimatization period of 1 week, 12 pigs (numbered 1–12) were challenged intranasally with 10,000 $TCID_{50}$, as already described [33]. The infected animals were euthanized 6 days after inoculation when the humane endpoint was reached. The 12 inoculated pigs were housed in three separate high containment units (termed boxes 4, 5, and 6): pigs 1–4 in box 4, pigs 5–8 in box 5, and pigs 9–12 in box 6 (Figure 2). The three pens were identically designed with slatted (2/3) and solid (1/3) flooring. The three boxes had a room volume of 70 m^3, an average temperature of 22°C (±0.19°C), and 11–16 air renewals per hour. No cleaning was performed during the course of the

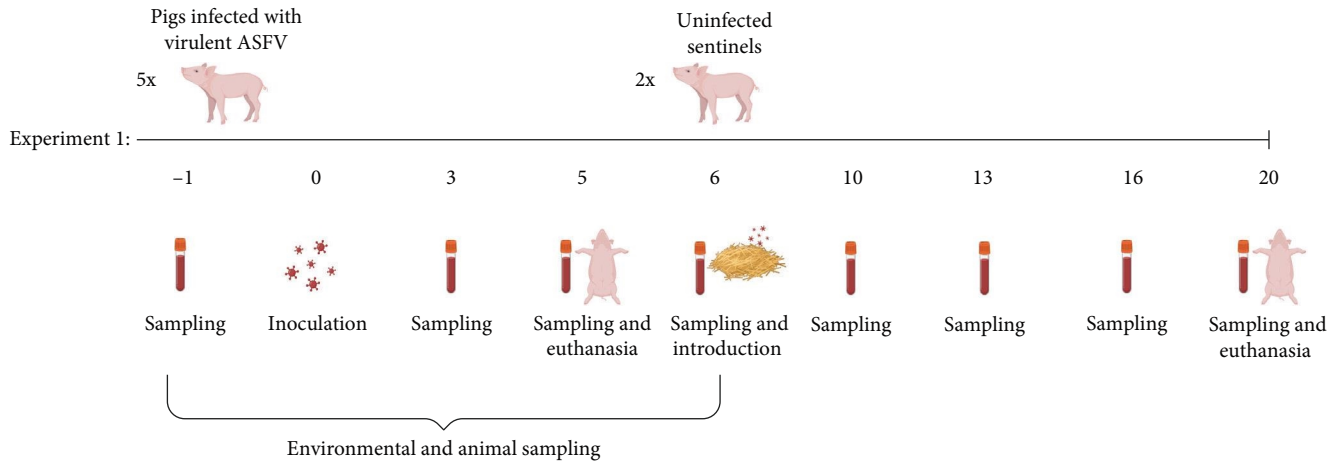


FIGURE 1: Plan of animal experiment 1 involving animal and environmental sampling and sentinel monitoring for ASF transmission. Two sentinel pigs were introduced into the premises 1 day after euthanasia of infected controls. Numbers below the line represent days of the experiment with reference to the inoculation day (0). Created with https://BioRender.com.

experiment since the slatted floors at the eating area did not generate this need for minimal cleaning and no visual blood contamination was seen in these pens before introduction of the sentinel pigs. Enrichment materials (rope, toys) were available within all pens. Following euthanasia of the 12 inoculated pigs, all material, including feed, faeces and toys, remained in the pens. No visual blood contamination was present in the pens following euthanasia of pigs 1–12. In order to avoid excessive drying, the pen floors were covered with plastic (approximately 52 cm above the floor). Under these conditions, the three pens within boxes 4, 5, and 6 were left empty for 1-, 2- or 3-days following removal of the ASFV-infected pigs. Subsequently, 12 sentinel pigs (numbered 13–24) were introduced into the contaminated pen environments in the following order: pigs 13–16 were introduced into box 6 at 1 day, pigs 17–20 into box 5 at 2 days, and pigs 21–24 into box 4 at 3 days post-euthanasia of the ASFV-infected pigs, respectively (Figure 2).

2.4. Animal Sampling. In experiments 1 and 2, blood samples were collected from the infected animals at 1, 3, and 5 days post-inoculation (dpi) and from the sentinel animals at 0, 4, 7, 10, and 14 days post-exposure (dpe) (Experiment 1, Figure 1). Nasal swabs were also collected during experiment 2 from the infected animals on 2, 3, and 5 dpi and from the sentinels at 9 and 14 dpe and placed in 1 ml DMEM with 2% FCS. All samples were stored at −80°C until analysis.

In experiment 3, unstabilized blood (to obtain serum), EDTA-stabilized blood (EDTA blood) and oral, nasal, and rectal swabs were collected prior to inoculation at 0 dpi and at 3, 5, and 6 dpi (euthanasia) (Figure 2). Urine samples were obtained on an occasional basis, i.e., if the pigs urinated while the personnel were in the pens, urine was collected with a tube during urination. Prior to their introduction into the three contaminated pens in boxes 4–6, unstabilized blood, EDTA blood and oral, nasal, and rectal swabs were collected from the sentinel pigs. After introduction into the contaminated pens, blood and swabs samples were collected from the 12 pigs, as shown in Figure 2. All swabs were collected into 1 ml 1x PBS.

2.5. Environmental Sampling

2.5.1. Pilot Studies to Test Detection of ASFV DNA on Spiked Surfaces. The efficacy of recovering virus from a smeared surface and from straw was tested in pilot experiments. Samples (50 μl) of ASFV (strain BA71V) with titres of 10^4, 10^5, and 10^6 $TCID_{50}$/ml were spiked directly onto the surface of Petri dishes and smeared over the surface or dropped onto small clumps of straw of approximately 1 cm^3. The surfaces were briefly allowed to dry and then swabbed either using pieces of normal household electrostatic dust swabs (Minky Homecare, Rochdale, UK) with an approximate size of 4 × 4 cm, or round-tip traditional cotton swabs. Both types of swabs were placed into 1.5 ml of either culture medium (DMEM with 10% FBS and penicillin/streptomycin) or PBS and kept for 2 hr at 4°C to elute the virus. The spiked straw clumps were also placed directly into the same volumes of either PBS or culture medium. Nucleic acids were extracted from 140 μl of each sample and also from the original virus dilutions with the "QIamp viral RNA extraction kit" (Qiagen) and eluted in 60 μl elution buffer according to manufacturer's instructions. Viral DNA was detected by PCR from 2 μl of each sample using primers for the ASFV B646L (VP72) gene (CTGCTCATGGTATCAATCTTATCGA, GATACCACAA GATCRGCCGT, 200 nM), Platinum Blue PCR SuperMix (Invitrogen) in total volumes of 20 μl. The PCR program 3 min 94°C; 35 cycles of 20 s 94°C, 20 s 58°C, 20 s 72°C was used. Amplification products were visualized using 1.5% agarose gel electrophoresis.

2.5.2. Sampling of Animal Premises. In premises with infected animals in experiments 1 and 2, dust swabs were used to sample different surfaces, i.e., walls, floor, bedding (especially showing evident presence of animal excretions), and the rim of water bowls. The blood collection points of some of the infected animals were also sampled as positive controls of the swabbing technique and recovery of viral DNA. In animal experiment 1, dust swab areas were approximately 50 cm^2 (5 × 10 cm) and after collection the samples were kept

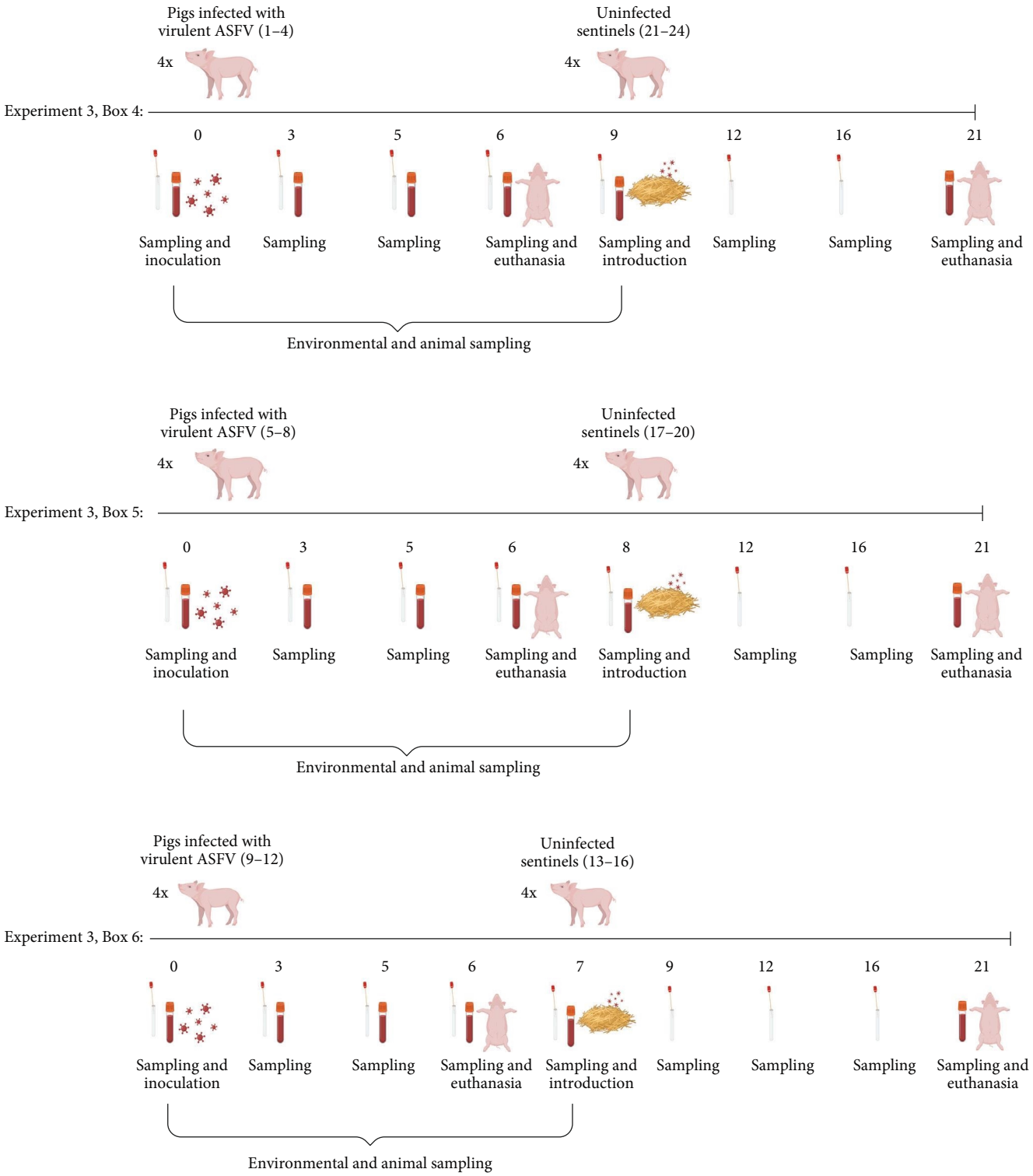


FIGURE 2: Overview of study design in boxes 4–6 in Experiment 3. Numbers below the line represent days of the experiment relative to the inoculation day (0). Created with https://BioRender.com.

refrigerated at 4°C until transport to the lab and then immersed in 4 ml PBS at 4°C for 2 hr. DNA was extracted as described above using the "QIamp viral RNA extraction kit" (Qiagen) in duplicate from each swab. In animal experiment 2, a similar sampling method was followed but dust swab sizes were reduced to approximately 20 cm^2 (4 × 5 cm) and immersed in 2 ml PBS at 4°C for 2 hr. DNA was extracted from 100 μl of each sample in duplicate using the automated extraction "Kingfisher Flex Extraction System" and Magvet Universal Isolation kit (Thermo Fisher Scientific, LSI MV384).

In the pens housing the infected pigs in experiment 3, floor swabs and faeces were collected from 0 dpi (before inoculation) and until the day of euthanasia (6 dpi). In addition, these samples were obtained on the day of introduction of the sentinel pigs (0 dpe) in each of the three pens. Floor swab samples were collected in 1 ml 1x phosphate buffered saline (1x PBS) (Thermo Fisher Scientific). Faecal homogenate suspensions (10%, w/v) were prepared in 1x PBS with 5% foetal bovine serum (FBS), streptomycin (Sigma–Aldrich), neomycin (Sigma–Aldrich), amphotericin (Sigma–Aldrich), and benzylpenicillin (Sigma–Aldrich). They were homogenized by rigorous vortexing with glass beads (Merck Millipore) and centrifuged at 950x *g* for 10 min. Floor swabs in 1x PBS were vortexed and centrifuged briefly. Recovered supernatants were used for DNA extraction (see Section 2.6).

2.5.3. Air Sampling of Animal Premises. Two systems were used to collect air samples during animal experiment 2. One was a handheld AirPort MD8 (Sartorius, Epsom, UK) that was used to collect samples from directly above the pigs for 5 min at a flow rate of 50 l/min. Aerosol particles are retained on a gelatin filter (nominal pore size 3 μm) attached to the front of the device, through which air is drawn. The gelatin filter was dissolved in 10 ml RPMI with 10% FCS and penicillin and streptomycin after sampling. The second was a wet-walled cyclone Coriolis microair sampler (Bertin Technologies, Aix-en-Provence, France) that was used to collect air samples from the room during husbandry and sampling. The Coriolis sampler was placed at a height of 1.1 m close to the extraction vent for the room, run for 30 min at a flow rate of 300 l/min, and aerosolized material was collected into 10 ml of RPMI medium with penicillin and streptomycin and 10% FCS. Air samples were stored at −80°C until nucleic acids extraction from 100 μl of each, in duplicate, using the automated extraction "Kingfisher Flex Extraction System" and Magvet Universal Isolation kit (Thermo Fisher Scientific, LSI MV384).

2.6. ASFV Genome Detection by qPCR. For animal experiments 1 and 2, nucleic acids extracted from environmental samples as described above (2.5.2) were analyzed for the presence of ASFV DNA by qPCR, as described previously [34]. Extracted nucleic acids (5 μl) were tested per sample in duplicate. ASFV DNA quantification in whole EDTA blood collected from the animals was performed similarly after extraction in duplicate from each blood sample using the extraction system Magvet Universal Isolation kit (Thermo Fisher Scientific, LSI MV384) and automated extraction with a Kingfisher Flex Extraction System, as described above.

In experiment 3, DNA was purified from EDTA blood, nasal, oral, rectal, and floor swab samples, urine, and faecal supernatants using a MagNA Pure 96 system (Roche) and analyzed for the presence of ASFV DNA by qPCR essentially as described previously [16, 35] but using the Bio-Rad CFX Opus Real-Time PCR System (as previously described [14]). Results are presented as viral genome copy numbers per milliliter (EDTA blood, swab supernatant, urine), or per gram (faecal suspension supernatants) using a standard curve based on a 10-fold dilution series of the pVP72 plasmid [33]. A positive result in the qPCR was determined to be a threshold cycle value (Cq) at which FAM (6-carboxy fluorescein) dye emission increased above background within 42 cycles (as previously described [14]).

2.7. Detection of Infectious ASFV Using Virus Isolation in Cells. Aliquots of nasal swabs and air samples collected during animal experiment 2 were inoculated onto primary porcine bone marrow cell cultures. A sample (0.8 ml) of each nasal swab (out of 1 ml total) and 1 ml of each air filter sample (out of 10 ml total volume) were added to the cells cultivated in six-well plates with RPMI medium containing 10% FBS and penicillin/streptomycin in 3 ml culture volume. The cells were then incubated and observed for development of hemadsorption, as above described [29], for a period of 5 days after which the plates were frozen at −80°C. After thawing and centrifuging at 600x *g* for 5 min, aliquots of 1 ml of supernatant from each of the first inoculation wells were used to inoculate new primary cultures. These were again incubated and observed for 5 days for development of hemadsorption.

In experiment 3, swab samples, urine, and faecal suspension supernatants were analyzed for the presence of infectious ASFV by virus isolation in PPAM [25, 16]. The cells were maintained in minimum essential medium (MEM, Gibco) with 5% FBS in NUNC 24-well plates (Thermo Fisher Scientific). Prior to inoculation of cells, PBS from the swab samples, faecal suspensions, and urine samples were filtered, using 0.45 μm syringe filters (Merck Millipore) and the clarified samples (100 μl) were added to MEM (100 μl) containing antibiotics and 10% FBS prior to addition to 1 ml PPAM (2×10^6 cells/ml). In one trial, the inoculum was removed from the cells after incubation at 37°C for 1 hr, and the cells were then washed twice with PBS. MEM, containing 5% FBS, streptomycin, neomycin, amphotericin, and benzylpenicillin, was added to the cells and incubated at 37°C (5% CO_2) for 3 days. In another trial, PPAM (2×10^6 cells/ml with 5% FBS) was incubated with the inoculum for 3 days, i.e., without its removal, at 37°C (5% CO_2). In both trials, following the 3 days, the cells were harvested by freezing and 100 μl of the harvested first passage was inoculated onto 1 ml fresh PPAM (2×10^6 cells/ml with 5% FBS) in NUNC 24-well plates. Following 3 days of incubation (37°C, 5% CO_2), virus-infected cells were identified using an immunoperoxidase monolayer assay (IPMA) essentially as described previously [16, 30]. Red-stained (virus infected) cells were identified under a light microscope.

2.8. Antibody Detection. Blood from the sentinel animals at the termination of experiment 2 was tested for the presence of anti-ASFV antibodies using lateral flow test devices (INGEZIM PPA CROM, R.11.PPA.K41, Ingenasa). Serum samples obtained at euthanasia from the inoculated and the sentinel pigs in experiment 3 were tested for the presence of anti-ASFV antibodies using the INgezim PPA Compac kit (Ingenasa), according to the manufacturer's instructions.

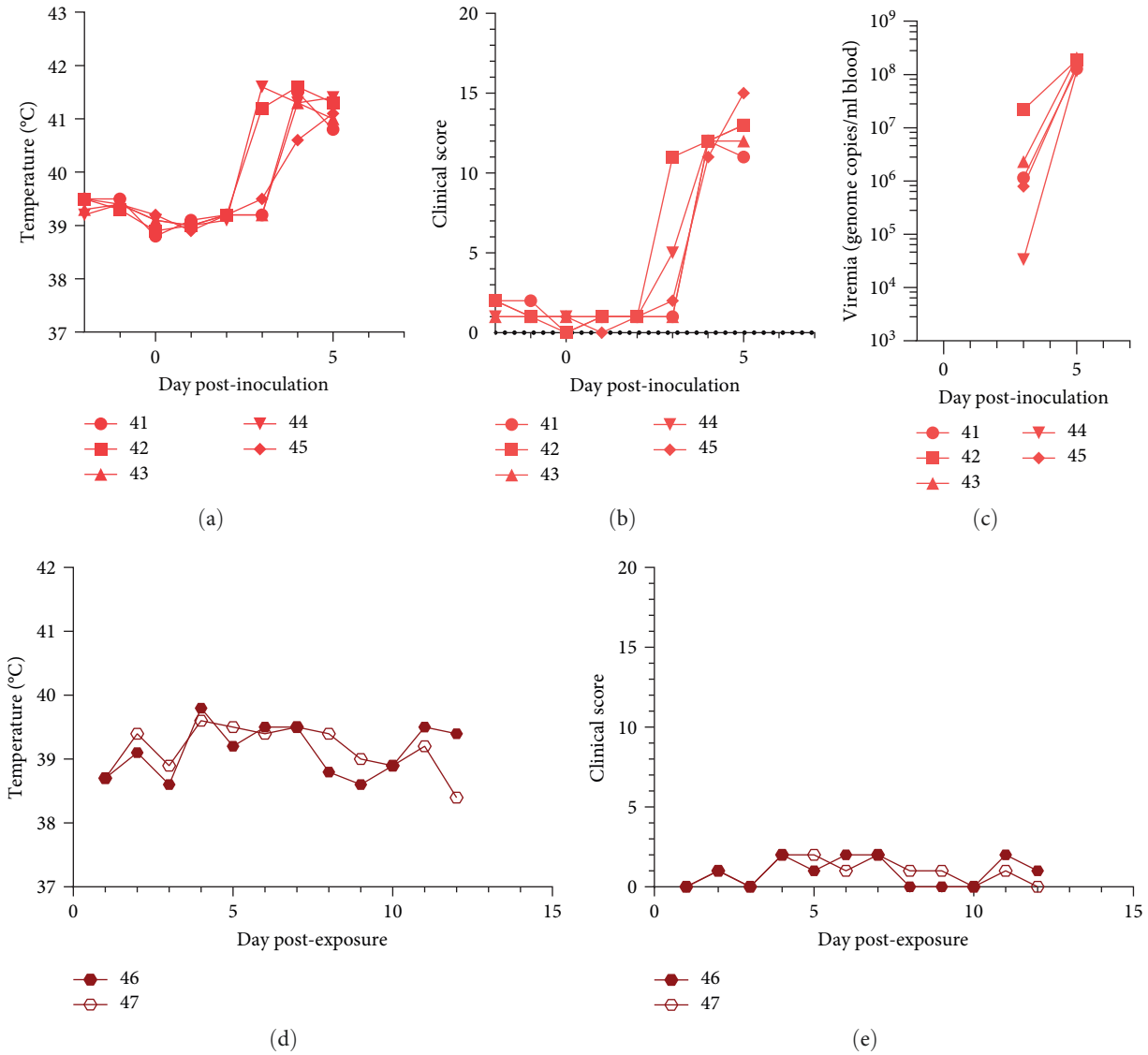


FIGURE 3: Clinical parameters for inoculated animals (a, b) and sentinels (d, e) and viremias of the inoculated animals (c) during Experiment 1.

3. Results

3.1. Animal Experiments and Result of Naïve Pig Exposures. The aim of the experiments was to assess the risk that environmental contamination poses for infection of naïve pigs introduced to contaminated pens on different days after infected pigs were removed. In experiment 1, a group of five pigs (numbered 41–45) were inoculated with 10,000 HAD_{50} infectious units of virulent ASFV isolate by the intramuscular route (0 dpi). Clinical signs typical of acute ASF including increased temperature rising above 41°C, anorexia and increasing lethargy were detected from 3 dpi (Figures 3(a) and 3(b)) but no clear haemorrhagic faeces or other haemorrhagic lesions or excretions were observed. All pigs reached the predefined humane endpoint at 5 dpi and were culled. Measurement of viremia by qPCR confirmed the expected high levels (above 10^8 genome copies per milliliter of blood) by 5 dpi (Figure 3(c)). The room was then left completely uncleaned for 1 day and two sentinel pigs (numbered 46 and 47) were introduced into the room. Over the period of 14 days, until the end of the experiment, no clinical signs of ASF were observed in the sentinel pigs (Figures 3(d) and 3(e)). Blood samples collected along this period also showed no detectable ASFV genomes in either of the animals. Thus, neither of the sentinels became infected and environmental transmission did not occur. In experiment 2, we shortened the time between removal of infected animals and introduction of sentinels. Two sentinel animals were introduced on the same day as a group of six infected animals with virulent ASFV were removed due to reaching the predefined humane endpoint. All six of the directly inoculated pigs developed clinical signs from 3 dpi and were culled at 5 dpi, as in experiment 1 (results not shown). All animals had very high blood viremias as detected by qPCR on day 5 with titres ranging from $10^{7.69}$

Table 1: ASFV genome copy numbers in blood and in nasal swabs of inoculated animals during animal Experiment 2 and presence of infectious virus in nasal swabs.

	3 dpi			5 dpi		
	Genome per milliliter of blood (log_{10})	Genome per nasal swab (log_{10})	Infectious virus in nasal swabs	Genome per milliliter of blood (log_{10})	Genome per nasal swab (log_{10})	Infectious virus in nasal swabs
Pig 1	7.79	n.d.	No	9.11	6.13	No
Pig 2	6.55	n.d.	No	8.80	6.16	Yes
Pig 3	7.99	n.d.	Yes	8.75	n.a.	n.a.
Pig 4	4.61	n.d.	No	8.37	5.70	No
Pig 5	5.45	n.d.	No	8.39	5.86	No
Pig 6	4.09	n.d.	No	7.69	5.39	No

Genome copy numbers were determined by qPCR and represent the average for each sample tested in duplicate. Virus isolation was performed in primary macrophage cell cultures. n.d., not detected; n.a., not available (nasal swab was not collected).

to $10^{9.11}$ (Table 1). We could clearly detect viral DNA in nasal swabs at this timepoint, with $10^{5.39}$–$10^{6.16}$ copies per swab, but not 2 days earlier, i.e., on day 3. Infectious virus was isolated from the nasal swabs of two animals, one at day 3 and another at day 5 post-infection (Table 1 and Figure S2). However, in experiment 2, again no clinical signs or viremia were detected in the sentinel pigs during the 15-day period of exposure, and no ASFV-specific antibodies were detected in the blood of the animals either. Nasal swabs collected at 9 and 14 dpe were also negative for viral DNA (not shown). Thus, in experiment 2, transmission from environmental contamination to naïve pigs also failed when these were exposed to the contaminated environment on the same day as the acutely infected animals were removed from the premises.

In experiment 3, pigs 1–12 in boxes 4–6 (see Figure 2) were inoculated intranasally with a virus suspension containing 10,000 $TCID_{50}$/2 ml. At 4 dpi, three out of four inoculated pigs in boxes 4 (pigs 1, 2, and 4) and 6 (pigs 10, 11, and 12) presented with high fever (rectal temperature above 41°C). In box 5, two out of four inoculated pigs presented with high fever at 5 dpi (pigs 7 and 8). Clinical signs became apparent from 4 dpi (boxes 4 and 6) or 5 dpi (box 5) and included depression, anorexia, mildly labored breathing, hyperemia of the skin and cyanosis on the ears and distal limbs, blood in faeces (pig 10, box 6 at 6 dpi, 1 day before introduction of sentinel pigs into this pen), and vomiting (pig 8, box 5). At 6 dpi, pig 2 was found dead upon entering box 4 (so no clinical score was registered on this day for this pig). Foam was observed from the nostrils of this pig. The remaining 11 inoculated pigs were euthanized on this day. Pigs 1, 4 (box 4), 7, 8 (box 5), 10, 11, and 12 (box 6) had reached the predetermined humane endpoints. The remaining four, pigs 3 (box 4), 5, 6 (box 5), and 9 (box 6) were euthanized for animal welfare reasons. Rectal temperatures and clinical scores for the inoculated pigs are shown in Figures 4(a) and 4(b). All but pigs 3 (box 4) and 5 (box 5) had shown clinical signs of ASF. Measurement of viremia by qPCR confirmed the expected high levels (above $10^{8.7}$ genome copies per milliliter of blood) by 6 dpi in all animals except pig 3 in box 4 that had approximately 10^5/ml and pig 5 in box 5 did not show viremia (Figure 4(c)). Levels of viral DNA detected in nasal, oral, and rectal swabs obtained from the inoculated pigs at 6 dpi are shown in Table 2. Most pigs in box 4 (pigs 1, 2, and 4) had high levels of viral genome in nasal swabs with at least 10^8 copies/ml (pig 3 had $10^{4.4}$), oral swabs with $10^{4.6–7}$, and rectal swabs with $10^{6.2–7.2}$ (except again for pig 3). Pigs in box 5 (pigs 5–8) had lower ASFV DNA levels in nasal swabs than the previous group, with no viral DNA in one of the animals and $10^{4.8–7.8}$ copies/ml in the other three; only one animal had viral DNA in its oral swab with $10^{5.1}$ copies and three of the animals had rectal swabs with $10^{3.7–6.3}$ copies/ml. All pigs in box 6 (pigs 9–12) had quite high levels of ASFV DNA in nasal swabs, with $10^{6.8–8.6}$ copies/ml, oral swabs with $10^{6.2–7.1}$/ml, and rectal swabs with $10^{6.2–7.5}$/ml.

Following exposure to the contaminated environments after 1 day (box 6), 2 days (box 5), or 3 days (box 4) following removal of the infected pigs, none of the sentinels (pigs 13–24, four per box), developed clinical signs that would indicate an ASFV infection. Rectal temperatures and clinical scores obtained from these pigs are shown in Figures 4(d) and 4(e).

No ASFV DNA was detected in EDTA blood obtained from the sentinel pigs (data not shown). After their introduction to the contaminated environment, ASFV DNA was detected in several nose and mouth swabs (Table 2). The highest prevalence and most viral DNA in swabs were observed in the sentinel pigs that were introduced into box 6 1 day after euthanasia of inoculated animals. At 2 dpe, all oral swabs from pigs in this group were positive for ASFV DNA ($10^{4.5–5.8}$ genome copies) and viral DNA was also detected in the nasal swabs ($10^{3.9–4.9}$) from two of the animals. At 5 dpe, however, there was a reduction in the number of positive swabs and by 9 dpe only one of the animals had a positive oral swab with $10^{4.7}$ genome copies (Table 2). Following introduction to box 5, at day 2 after the infected animals were removed, only one sentinel had a positive oral swab at 4 dpe with $10^{4.4}$ genome copies and no swabs were positive at 8 dpe (Table 2). In box 4, where sentinels were introduced at day 3, ASFV DNA was only detected in oral swabs of two of the animals at both 3 and 7 dpe with $10^{3.9–4.6}$ genome copies (Table 2). Despite the apparent uptake of ASFV by the sentinel animals from the contaminated environment, as evidenced by the presence of viral DNA in nasal and oral swabs at least at early days post-exposure, all sentinel pigs were euthanized after 3 weeks exposure to the contaminated environment without evidence of infection by

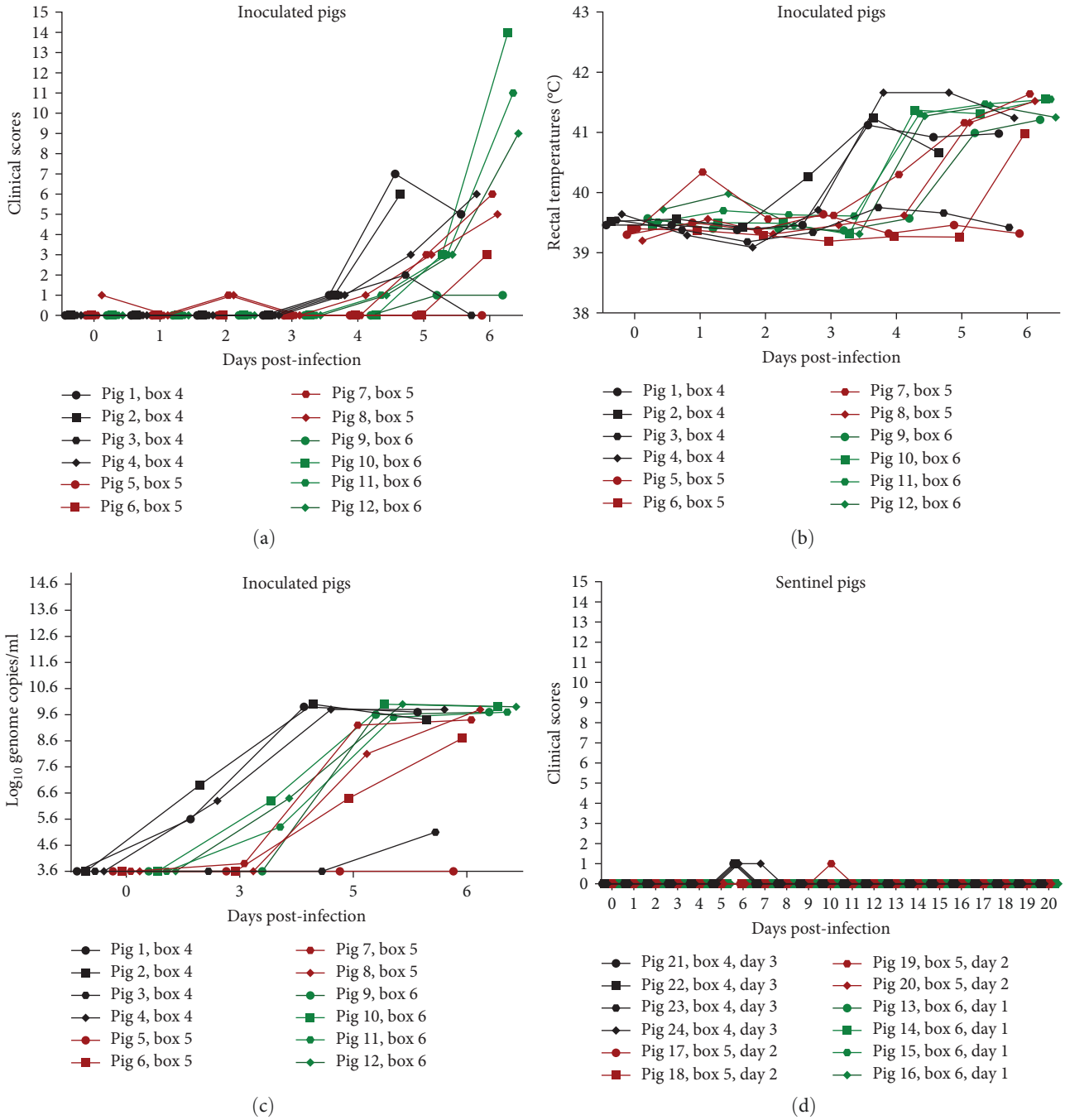
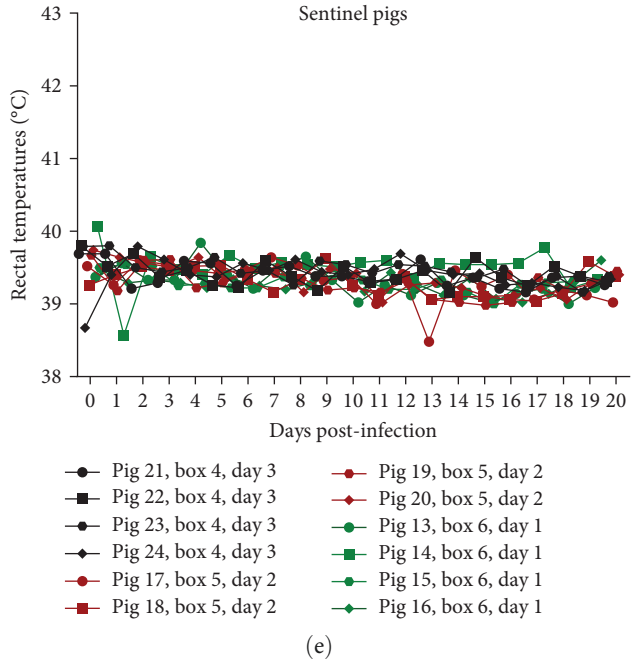


Figure 4: Continued.



(e)

Figure 4: Clinical parameters from the inoculated and sentinel pigs in Experiment 3. Inoculated pigs clinical scores (a), rectal temperatures (b), detection of ASFV DNA in EDTA-blood (c), sentinel's clinical scores (d), and rectal temperatures (e). In panel (c), the detection threshold for ASFV DNA is $10^{3.6}$ genome copies/ml. The data on clinical scores and rectal temperatures from pigs 9–12 have been published previously for a different study [33] and are shown here for completeness.

Table 2: qPCR results for ASFVDNA detection in faecal and urine samples and in floor, nasal, oral, and rectal swabs obtained in the different boxes during Experiment 3.

	Panel A					
Box 4	Log_{10} genome copy numbers/ml or g obtained at different days post infection/exposure					
	4	5	6 (Euthanasia)	9 (Introduction)	12	16
Faeces	No Ct	5.4	No Ct	5.2	—	—
Floor swabs	No Ct	4.3	No Ct	—	—	—
Urine	4.0	5.6	—	—	—	—
Nasal swab, pig 1	—	—	8.0	—	—	—
Nasal swab, pig 2	—	—	8.4	—	—	—
Nasal swab, pig 3	—	—	4.4	—	—	—
Nasal swab, pig 4	—	—	8.0	—	—	—
Oral swab, pig 1	—	—	7.0	—	—	—
Oral swab, pig 2	—	—	6.3	—	—	—
Oral swab, pig 3	—	—	4.6	—	—	—
Oral swab, pig 4	—	—	5.6	—	—	—
Rectal swab, pig 1	—	—	6.2	—	—	—
Rectal swab, pig 2	—	—	7.2	—	—	—
Rectal swab, pig 3	—	—	No Ct	—	—	—
Rectal swab, pig 4	—	—	6.3	—	—	—
Nasal swab, pig 21	—	—	—	No Ct	No Ct	No Ct
Nasal swab, pig 22	—	—	—	No Ct	No Ct	No Ct
Nasal swab, pig 23	—	—	—	No Ct	No Ct	No Ct
Nasal swab, pig 24	—	—	—	No Ct	No Ct	No Ct
Oral swab, pig 21	—	—	—	No Ct	No Ct	No Ct
Oral swab, pig 22	—	—	—	No Ct	4.2	4.5
Oral swab, pig 23	—	—	—	No Ct	No Ct	No Ct
Oral swab, pig 24	—	—	—	No Ct	4.6	3.9

Table 2: Continued.

Box 5	Panel B Log_{10} genome copy numbers/ml or g obtained at different days post infection/exposure					
	4	5	6 (Euthanasia)	8 (Introduction)	4	16
Faeces	No Ct	No Ct	No Ct	No Ct	—	—
Floor swabs	No Ct	No Ct	No Ct	—	—	—
Urine	—	—	4.7	—	—	—
Nasal swab, pig 5	—	—	No Ct	—	—	—
Nasal swab, pig 6	—	—	4.8	—	—	—
Nasal swab, pig 7	—	—	7.5	—	—	—
Nasal swab, pig 8	—	—	7.8	—	—	—
Oral swab, pig 5	—	—	No Ct	—	—	—
Oral swab, pig 6	—	—	No Ct	—	—	—
Oral swab, pig 7	—	—	No Ct	—	—	—
Oral swab, pig 8	—	—	5.1	—	—	—
Rectal swab, pig 5	—	—	No Ct	—	—	—
Rectal swab, pig 6	—	—	3.7	—	—	—
Rectal swab, pig 7	—	—	4.7	—	—	—
Rectal swab, pig 8	—	—	6.3	—	—	—
Nasal swab, pig 17	—	—	—	No Ct	No Ct	No Ct
Nasal swab, pig 18	—	—	—	No Ct	No Ct	No Ct
Nasal swab, pig 19	—	—	—	No Ct	No Ct	No Ct
Nasal swab, pig 20	—	—	—	No Ct	No Ct	No Ct
Oral swab, pig 17	—	—	—	No Ct	4.4	No Ct
Oral swab, pig 18	—	—	—	No Ct	No Ct	No Ct
Oral swab, pig 19	—	—	—	No Ct	No Ct	No Ct
Oral swab, pig 20	—	—	—	No Ct	No Ct	No Ct

Box 6	Panel C Log_{10} genome copy numbers/ml or g obtained at different days post infection/exposure						
	4	5	6 (Euthanasia)	7 (Introduction)	9	12	16
Faeces	No Ct	4.3	5.5	5.5	—	—	—
Floor swabs	No Ct	No Ct	6.6	—	—	—	—
Urine	—	4.9	5.8 – 7.0	—	—	—	—
Nasal swab, pig 9	—	—	7.4	—	—	—	—
Nasal swab, pig 10	—	—	8.1	—	—	—	—
Nasal swab, pig 11	—	—	6.8	—	—	—	—
Nasal swab, pig 12	—	—	8.6	—	—	—	—
Oral swab, pig 9	—	—	6.4	—	—	—	—
Oral swab, pig 10	—	—	6.6	—	—	—	—
Oral swab, pig 11	—	—	6.2	—	—	—	—
Oral swab, pig 12	—	—	7.1	—	—	—	—
Rectal swab, pig 9	—	—	6.2	—	—	—	—
Rectal swab, pig 10	—	—	7.5	—	—	—	—
Rectal swab, pig 11	—	—	6.4	—	—	—	—
Rectal swab, pig 12	—	—	7.5	—	—	—	—
Nasal swab, pig 13	—	—	—	No Ct	No Ct	4.2	No Ct
Nasal swab, pig 14	—	—	—	No Ct	No Ct	No Ct	No Ct
Nasal swab, pig 15	—	—	—	No Ct	4.9	No Ct	No Ct
Nasal swab, pig 16	—	—	—	No Ct	3.9	3.7	No Ct
Oral swab, pig 13	—	—	—	No Ct	5.3	4.9	No Ct
Oral swab, pig 14	—	—	—	No Ct	4.5	No Ct	No Ct
Oral swab, pig 15	—	—	—	No Ct	5.2	4.9	No Ct
Oral swab, pig 16	—	—	—	No Ct	5.8	5.6	4.7

Sentinel pigs were introduced to the contaminated environment on different days following euthanasia of the infected pigs: 3 days in box 4 (panel A), 2 days in box 5 (panel B), and 1 day in box 6 (panel C). Swab samples at 0 dpe were collected prior to the introduction of the pigs into the contaminated environment in the three boxes. Numbers are log_{10} genome copy numbers/ml (swab samples and urine) or log_{10} genome copy numbers/g (faeces).

Table 3: ASFV genome copy numbers in the environment of premises with infected animals and in the blood of the animals during Experiments 1 and 2.

Day post-inoculation (dpi)	Experiment 1			Experiment 2		
	Swab surface	Genome copies per swab (log_{10})	Genome copies per milliliter blood (log_{10}) ($n = 5$)	Swab surface	Genome copies per swab (log_{10})	Genome copies per milliliter blood (log_{10}) ($n = 6$)
3 dpi	Bleed 1	4.11	4.52–7.34	Bleed 1	n.a.	4.09–7.99
	Bleed 2	5.10		Bleed 2	n.a.	
5 dpi	Bleed 1	7.58	8.08–8.32	Bleed 1	8.52	7.69–9.11
	Bleed 2	7.92		Bleed 2	9.25	
	Floor 1	2.86		Floor 1	n.d.	
	Floor 2	n.d.		Floor 2	4.09	
	Wall 1	2.70		Wall 1	n.d.	
	Wall 2	n.d.		Wall 2	n.d.	
	Bedding 1	n.d.		Bedding 1	4.60	
	Bedding 2	n.d.		Bedding 2	n.d.	
	Water bowl 1	n.d.		Water bowl 1	5.06	
	Water bowl 2	n.d.		Water bowl 2	n.d.	
6 dpi	Bedding 1	2.74	n.a.	Bedding 1	3.79	n.a.
	Bedding 2	2.87	n.a.	Bedding 2	n.d.	n.a.

Swabs were collected from different surfaces of the premises or from areas around the bleeding of two of the infected pigs (Bleeds 1 and 2). Total genome copy numbers of each swab were determined by qPCR and represent the average for each sample tested in duplicate. For comparison, the levels of ASFV genome in the blood of the animals occupying the premises are also shown. Genome copies per milliliter of blood represent the range of values determined in the infected group of pigs. n.a., not available; n.d., not detected.

ASFV. No ASFV-specific antibodies were detected in the blood of the animals after the 21 days exposure.

3.2. Estimation of Levels of Virus Contamination in Environmental Samples in Rooms Housing Infected Pigs. Pilot experiments were carried out to compare swabbing methods to recover virus from surfaces. In these experiments, 50 μl of ASFV containing 5×10^4, 5×10^3, and 5×10^2 $TCID_{50}$ was spiked directly onto Petri dishes or onto small clumps of straw. The surfaces were then swabbed, and ASFV DNA was detected by PCR. PCR fragments could be weakly detected only from spiking with 5×10^4 $TCID_{50}$ collected with either dust or cotton swabs or directly from straw. PBS was the most effective for elution. Control samples of ASFV DNA gave clear positive results (see Figure S1). This showed that the sample swabbing method using dust swabs and PBS for viral elution was sufficiently sensitive to detect 5×10^4 $TCID_{50}$ of virus spiked on straw and on surfaces by conventional PCR.

During animal experiments 1 and 2, we assessed the recovery of ASFV DNA from roughly 20 cm^2 areas sampled with dust swabs in the premises housing ASFV infected animals. A similar sampling regime was followed as used during previous experiments with foot-and-mouth disease virus (FMDV) in which FMDV nucleic acid (RNA) was readily detected in most samples [36]. During animal experiment 1, the room housed five ASFV infected pigs. Two swabs each were collected from the floor, wall, straw bedding, and water bowl at day 1 before infection and days 3 and 5 post-infection with virulent ASFV OURT88/1, on the same days that blood samples were collected from each of the inoculated animals. The animals were euthanised on day 5 at the humane endpoint. As control for the recovery of ASFV DNA by the swabbing technique, the skin area surrounding the site of blood collection from two of the animals at days 3 and 5 post-infection was also swabbed. Swabs were also collected from the environment following removal of the ASFV infected pigs and introduction of the sentinel pigs, on days 6 and 7 post-inoculation (or 0 and 1 dpe). Using the swabbing method, a low amount of ASFV DNA (500–750 genome copies) was detected by qPCR in eluates from the swabs of a few of the environmental surfaces at days 5 and 6 post-infection: on day 5 in one swab from the floor and one from a wall, and on day 6 in both swabs from straw bedding (Table 3, Experiment 1). None of the swabs from day 7 or from the day before infection showed detectable ASFV DNA (not shown). ASFV DNA was always detected in positive control samples (swabs around the site of blood collection from pigs): approximately 10^4 and 10^5 genomes on day 3 rising to 4 and 8×10^7 on day 5. The level of genome copy numbers per milliliter determined directly from the blood of the infected animals by qPCR ranged between 10^4 and 10^7 at day 3 and was approximately 10^8 at day 5 (Table 3, Experiment 1 and Figure 2(c)). The difference was less than 1 $\log_{10}$ in genome copies detected between the swabs from areas around the sites of pig bleeding and the blood samples on day 5 but this is likely to be due to lower recovery of DNA from the swabs. Detecting ASFV DNA in environmental samples with lower viral loads may be difficult and require a larger sampling area.

In experiment 2, swab sizes and elution volumes were reduced to approximately half to concentrate the potential ASFV DNA from contaminated surfaces and increase sensitivity of detection. Swabs were collected from the premise's surfaces on days 3, 5 (0 dpe), and 6 (1 dpe) and on day 5 also from the blood collection points on the animals. This time, viral DNA levels detected on the premise's surfaces were higher than in experiment 1, in the range of $10^{3.8}$–10^5 copies on days 5 and 6, detected on the floor, bedding, and a water bowl (Table 3, Experiment 2). Increased genome copy levels were also present in the blood of some of the infected animals in comparison to experiment 1 at day 5 dpi, which may have led to more virus being released to the environment. The more concentrated elution of the swabs may also have contributed to the higher levels of detection of ASFV DNA during experiment 2. Air samples were also collected from the premises during the second experiment, on 0, 3, 5, and 6 dpi using two different devices (MD8 and Coriolis). No viral DNA or infectious virus was detected on any of the sampled days, suggesting that ASFV was not aerosolized to a high level during the experiment and may have been efficiently removed by the ventilation system.

In experiment 3, viral DNA was also detected in urine and faeces excreted into the pens housing the infected pigs and in floor swabs (see Table 2). The level of contamination of the three boxes seemed to differ. Viral DNA was detected in floor swabs and in urine and faecal samples obtained prior to or on the day of introduction of sentinel pigs into boxes 4 (3-day group) and 6 (1-day group), but in box 5 (2-day group) only one urine sample was positive 2 days before introduction. The swab samples from the inoculated animals in box 5 also showed lower prevalence and levels of ASFV DNA than in the other two boxes and one of the animals was not viremic (see Figure 4), which may explain the lower environmental contamination detected in this box. The level of ASFV DNA in the faecal samples and floor swabs in boxes 4 and 6 varied from $10^{4.3}$ to $10^{5.5}$ genome copies/g and $10^{4.3}$ to $10^{6.6}$ genome copies/ml, respectively. The level in urine obtained from the inoculated pigs in the three boxes varied from 10^4 to 10^7 genome copies/ml (Table 2). However, no infectious virus was detected in the urine samples, faeces supernatants, or floor swabs following two passages in PPAM (not shown).

4. Discussion

In three separate transmission experiments, we failed to detect transmission of ASFV to naïve pigs introduced into rooms which recently had housed pigs with acute ASFV infection. From previous studies, we knew that the amount of virus in urine, faeces, and oral and nasal secretions is much lower than in blood from infected animals. Infected blood may be present in the environment from excretions or scratches on pigs making transmission more likely. At later stages of infection, bloody diarrhoea may be observed but it was rarely observed in our experiments probably because in experimental settings, pigs are usually culled for humane reasons before reaching these late (terminal) stages of the infection.

Overall, in the current study, in experiments 1 and 2, we detected only low levels of ASFV genome in environmental samples from surfaces including walls, floor, water bowls, and straw bedding ($10^{2.7}$–10^5 genomes in swabs) on days 1 and 0 before exposure of sentinels, introduced to the premises 1 day after, or on the same day as, removal of acutely infected animals. This suggests very low level of virus contamination in the environment consistent with the lack of transmission from the environment to sentinel pigs. In experiment 3, susceptible pigs were introduced to ASFV contaminated pens either 1, 2, or 3 days after removal of ASFV infected animals (boxes 6, 5, and 4, respectively). Environmental sampling in the pens before or on the day of introduction showed viral DNA present in faeces and floor swabs only in boxes 4 and 6 ($10^{4.3}$–$10^{5.5}$ genome copies/g and $10^{4.3}$–$10^{6.6}$ genome copies/ml, respectively) but not in box 5. ASFV DNA was detected in urine samples from all pens prior to introduction of sentinel pigs, with 10^4–10^7/ml genome copies. The level of contamination within the three pens seemed to depend on the course of infection in the nasally inoculated pigs housed within them. Hence, in box 5, in which only half of the inoculated pigs had a severe course of infection, contamination levels of the pen seemed to be lower, when compared to boxes 4 and 6, where more inoculated pigs showed severe symptoms of the disease. In addition, the level of contamination of the pens also correlated with the degree of detection of ASFV DNA in nasal and oral swab samples obtained from the sentinel pigs, especially at earlier days of exposure, denoting uptake of virus from the environment. Thus, in box 5, which seemed to be only mildly contaminated, viral DNA was only detected in one oral swab from one sentinel pig out of four, whereas in box 4, oral swabs from two sentinels were positive and in box 6 oral swabs from all four sentinels and additionally three nasal swabs were positive. Further, in box 6, one of the infected pigs was observed to have blood in faeces (at euthanasia, 1 day prior to the introduction of sentinel pigs) which probably contributed to the increased environmental contamination in this box. Even with the highest contamination level and earliest introduction of the sentinels, no ASFV transmission occurred in box 6.

In an earlier study, transmission to pigs via an environment contaminated with ASFV was observed when a contaminated pen had been left empty for 3 days but not for 5 days [18]. Other previous experiments [25, 26] showed that pigs were introduced into the contaminated environment at 1 day after removal of infected animals with acute disease, developed clinical disease within 1 week, and both ASFV DNA and infectious virus were detected in their blood. However, pigs introduced into the contaminated pens after 3, 5, or 7 days did not develop signs of ASF and no viral DNA was detected in blood samples within the following 3 weeks. The results suggested a relatively narrow window of time for transmission, but further repetitions were needed to confirm this. This short window could also be related to the limited half-life of infectious virus in the environment. The authors reported clinical signs including mild rectal bleeding in one of the directly infected pigs occupying the pen before exposure of the healthy pigs when environmental transmission was observed, as well as presence of viral DNA in faeces in the environment 1 day before exposure. Especially the contamination of the environment with blood from an acutely diseased animal, which typically contains a high titre of infectious virus, was probably a main factor for transmission. This and other environmental conditions, including humidity and rate of air changes, may explain differences between the earlier experiments and the current study. For example, we observed that drying of ASFV samples resulted in a 10-fold loss of virus titre (unpublished results). In the studies demonstrating transmission of ASFV to pigs via a contaminated pen environment, hay [18] or large amounts of straw [25] were left within the contaminated pens. One study did report that matrices with a low moisture content (hay, straw, and grain) can provide a suitable environment to ensure ASFV viability when compared to matrices with a higher moisture content (soil water and leaf litter) when stored at cooled temperatures [8]. Infectious ASFV was detected for up to 56 days in spleen tissue from ASFV-infected pigs incubated with straw and hay, and for up to 28 days in spleen tissue incubated with grain, when the samples were kept at 4°C. At room temperature, a rapid decay of the virus within all matrices was observed and no infectious virus was detected after incubation for 7 days in hay, straw, or grain [8]. Perhaps ASFV transmission via contaminated pens after 1 day [25] or 3 days [18] was also observed as a result of a stabilizing effect of the bedding material on the virus. In experiments 1 and 2 in the current study, we also detected viral DNA in straw used as bedding material on the days of introduction of susceptible animals, although the levels were not high ($10^{2.7}$–$10^{4.6}$ genome copies). The environment into which the pigs were introduced in experiment 3 had no straw and had become very dry when compared to the environment that the pigs were introduced in Olesen et al.'s [25] study, where a thick layer of straw prevented the environment from drying out. Furthermore, pigs introduced into an environment containing straw on the floors could be more eager to investigate this environment, via eating and moving of the straw, when compared to pigs introduced into a pen with no bedding material.

The premises housing ASFV-infected animals are typically contaminated via the different animal excretions and sometimes blood. Animals experimentally infected (by the intramuscular route) with highly virulent isolates of ASFV develop clinical signs of acute disease between days 3 and 5 after infection concomitant with excretion of infectious virus [19, 37]. Previously, we detected infectious virus and viral DNA in urine and faeces collected from infected animals at the onset of pyrexia (≥40°C) [19]. In urine, we could detect infectious virus at approximately 10^3 $TCID_{50}$/ml and viral genome at 2.5×10^4 copies/ml. In faeces, virus was intermittently detected but was present in some samples at up to 6.8×10^4 $TCID_{50}$/g or 10^7 genomes/g. In rectal swabs taken from animals after the onset of clinical signs, between days 3–6 after infection, up to 10^2 HAD_{50}/ml and 10^{3-4} genome copies/ml could be detected and in nasal swabs, up to 10^4 HAD_{50}/ml and 10^5 genome copies/ml [37].

Infectious virus was not detected in oral swabs although low levels of genome could be detected. In blood, much higher levels of virus and genome were detected, up to 10^{6-8} $TCID_{50}$/ml and 10^{6-8} genome copies/ml [19, 37]. Therefore, the swabbing technique and qPCR detection of ASFV genome should be sufficiently sensitive to detect contamination with these secretions and excretions and especially with blood, if present in the swabbed areas. The results from pilot experiments showed that the method used in experiments 1 and 2 should be sufficiently sensitive to detect levels of virus in environmental samples that are similar to levels excreted in urine, faecal, or oral samples and in any blood present. However, excretions and secretions from pigs may be spread unevenly in the rooms and detection may require sampling over a larger area than we sampled. Alternative methods for environmental sampling which could be applied to wider areas are likely to improve detection of low levels of contamination by ASFV. One method that could also circumvent safety concerns about collecting infectious materials for analysis used a sponge impregnated with a solvent to inactivate virus and could detect similar amounts of DNA to traditional cotton swabs [38].

In conclusion, the results of this study demonstrate that, within our experimental setting, indirect transmission of ASFV via an environment contaminated with excretions from ASFV-infected pigs is very inefficient when viral DNA levels are similar to those detected in this study and no obvious contamination with blood is present.

Data Availability

The data (values for means and used to build graphs) used to support the findings of this study are available from the corresponding author upon request.

Disclosure

The manuscript was already published as a preprint [39] based on the link https://www.biorxiv.org/content/10.1101/2023.09.28.559902v1.

Conflicts of Interest

The authors declare no conflicts of interest.

Acknowledgments

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Supplementary Materials

Figure S1: detection of ASFV DNA on spiked surfaces using different swabs and elution media. Figure S2: detection of infectious ASFV in nasal swabs from inoculated animals in Experiment 2. (*Supplementary Materials*)

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