NPB FINAL RESEARCH GRANT REPORT

African Swine Fever Research in Vietnam, NPB Project #PR-005359

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Project Topics:

This project is comprised of 3 distinct research projects to address the following research topics:

Topic #1 – Verification of the inactivation of ASFv in compost using bioassay

Topic #2 – Assessing the viability of ASFv in the deep burial environment

Topic #3 – Assessing the viability of ASFv in manure slurry and the surface water environment

For clarity, each research topic is reported individually below.

Topic #1 – Verification of the inactivation of ASFv in compost using bioassay

Industry Summary:

African swine fever virus (ASFv) continues to be a major concern for swine producers in both the United States and Vietnam due to the lack of effective vaccines and the high risk of transmission. Safe and practical disposal of infected carcasses is critical to prevent further spread of the disease. Research conducted in Vietnam demonstrated that ASFv in infected swine carcasses can be effectively inactivated through composting. The study used a combination of PCR, cell culture, and bioassay methods to confirm virus inactivation. Results showed that while ASFv DNA could still be detected by PCR, the virus itself was no longer infectious after only 16 hours of composting in soft tissues such as the spleen and within 3 days in bone marrow. These findings provide strong evidence that composting can rapidly inactivate ASFv when piles are properly constructed and managed. This means swine producers can rely on composting as a safe, biosecure, and practical method for on-farm carcass disposal following an ASF outbreak. Further studies are needed to confirm the same level of inactivation under different environmental conditions and with different composting materials.

Key Findings:

- Temperatures of compost piles reached 69–70 °C within 2–3 days and remained above 60 °C for 10 days
- Composting can rapidly inactivate ASFv in pig carcasses

- Cell culture and bioassay confirmed the inactivation of ASFv in spleen samples after 16 h and in bone marrow samples after 3 days of composting
- Composting offers a practical, biosecure, and environmentally safe carcass disposal method for ASF outbreaks.

Keywords: African swine fever; Composting; Swine carcasses; Bioassay; Inactivation

Scientific Abstract:

Introduction:

African swine fever (ASF) is a highly fatal disease of pigs caused by a virus belonging to the family *Asfarviridae*, resulting in significant economic losses in global swine industries [1, 2]. ASFv was first discovered in the 1920s, and all genotypes are available in Africa. In Europe, the genotype I ASFv emerged in the 1950s, but it was eradicated in Europe, except in Sardinia, by the mid-1990s. Since August 2018, a highly virulent genotype II ASFv has spread to China and other Asian countries, including Mongolia, Vietnam, Cambodia, Laos, North Korea, the Philippines, Myanmar, South Korea, Indonesia, and other Asia-Pacific countries [3]. Vietnam has also been one of the most severly impacted countries, with the first outbreak occurring in February 2019 in the northern part of the country near China. Around 6 million pigs were culled in Vietnam between February 2019 and May 2020 [4, 5]. Previous attempts to develop a vaccine against ASFv have failed to produce protective immunity. Current methods of disease control include isolating affected areas and destroying infected or exposed animals [6]. Currently, many methods of animal carcass disposal are applied. In the event of a disease outbreak, mass destruction of infected and exposed animals is required. Biosafety agencies in the United States, Canada, Australia, and New Zealand have identified composting as the preferred method of disposing of animal carcasses, both in routine and emergencies. This method is effective and economical in the disposal of animal carcasses and the inactivation of pathogens [7].

Composting is the process of promoting natural biological decomposition under aerobic conditions [7]. Pathogen inactivation is facilitated by the heat produced by aerobic microorganisms during the decomposition of animal carcasses. Temperatures in the compost pile of around 55-60˚C for several days are sufficient to eliminate most pathogenic viruses, bacteria, fungi, and protozoa [8]. However, some spore-forming bacteria and prions may survive composting [9]. Composting is aerobic in nature, requiring adequate oxygen to sustain biodegradation. The availability of oxygen and the moisture content of the compost pile are critical for successful composting [10]. The oxygen concentration of the compost pile should not be less than 5% (by volume). For optimal composting, a C: N ratio of 25:1–30:1 (w/w), a moisture content of 50–60% (by weight), a porosity of 35 to 45%, and an oxygen level higher than 10% are recommended [7]. Covering materials are equally important to successful composting. Carbon materials help retain heat, absorb excess moisture, and create a barrier that helps deter insects and scavengers. Sawdust, rice hulls, straw, and wood chips are popular materials today because they are inexpensive, retain moisture, and heat well [11, 12]. Previous studies have demonstrated that composting is capable of rapidly destroying many infectious pathogens in pigs, such as foot and mouth disease virus (FMDv), porcine epidemic diarrhea virus (PEDv), and ASFv [11,13–15]. However,

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the inactivation of ASFv in compost has not been confirmed on animals yet. This study was conducted to verify the inactivation of ASFv in compost using a bioassay in addition to cell culture.

Objectives: This research aimed to determine the viability of ASFv in compost using Real-time PCR (qPCR), cell culture, and bioassays.

Materials & Methods:

Compost pile construction

Three market-size hogs with clinical signs typical of ASF were used for this study. Before constructing the compost piles, the carcasses were necropsied to confirm ASF infection, identified by pathological lesions including hemorrhagic spleen and lymph nodes, as well as an excess of yellow fluid in the heart. qPCR also confirmed the presence of ASFv in spleen samples collected from each of the pigs (Figure 1).

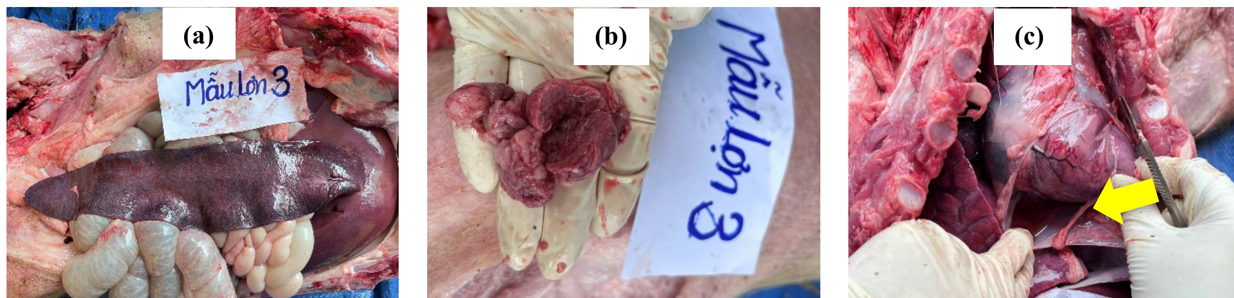

Figure 1. Pathological lesions of swine carcasses use for composting in this study. Enlarged and hemorrhagic spleen (a) and lymph nodes (b). Excess of yellow fluid in the heart (c).

After confirmation, 3 static aerated compost piles were constructed according to the USDA Large Animal Protocols with some modifications at the Veterinary Hospital of the Vietnam National University of Agriculture (VNUA). Approximately 7–8 cubic yards of rice hulls were used to compost an infected swine carcass of 60–80 kg. A layer of rice hulls (40-60 cm) was employed as the base of each compost pile. The swine carcass was then placed on the base. The spleen samples and femur bone samples were stored in the Dacron in situ bags (Ankom, NY, USA), placed in a tea strainer attached to a metal cord to facilitate retrieval, and then placed around the carcass. Finally, the carcass was covered with rice hulls (40-60 cm). The temperature of the compost piles was monitored daily at 45 cm (18 “) and 90 cm (36”) depths using a data logger and a handheld thermometer.

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Figure 2. Construction of compost piles

Sample Preparation

Sample bags were withdrawn from compost piles at 1, 4, 8, 16, 24 h, day 3, day 5, day 7, day 14, and day 28. Each sample was thoroughly homogenized in Phosphate-Buffered Saline (PBS) using a Retsch MM400 (Retsch, Dusseldorf, Germany) and then centrifuged at 3000 rpm for 10 minutes. The supernatant was collected for viral DNA detection and virus isolation using qPCR and cell culture.

Detection of African Swine Fever Virus

The extraction of ASFv DNA was carried out using a MagMAX™ Viral/Pathogen Nucleic Acid Isolation Kit (Thermo Fisher, MA, USA) following the manufacturer's instructions. Next, qPCR was performed according to the previous method described by Tignon et al. (2011) [16] using a TaqMan probe (5′-FAM-TTCCATCAAAGTTCTGCAGCTCTT-TAMRA-3′) and primer pair (Forward, 5′-TGCTCATGGTATCAATCTTATCG-3′; Reverse, 5′-CCACTGGGTTGGTATTCCTC-3′). The PCR mixture (25 µL) consisted of 5 µL of nuclease-free water, 12.5 µL of PCR master mix 2X (Invitrogen Superscript III qRT-PCR, Thermo Fisher, USA), 2.5 µL of the primer-probe mix (forward primer (0.6 µM), reverse primer (0.6 µM), a TaqMan probe (0.3 µM)), and 5 µL of DNA template. A thermal cycling machine (CFX96, BioRad Laboratories, Hercules, CA, USA) was used to carry out a PCR amplification program consisting of 1 cycle at 95 °C for 2 min, 45 cycles at 95 °C for 15 s, and 60 °C for 45 s.

Isolation of African Swine Fever Virus

Porcine Alveolar Macrophages (PAMs) were thawed in a water bath at 37 °C before centrifuging at 2000 rpm for 10 min at room temperature. The pellet was collected and then washed with 5 mL of PBS buffer, mixed with 10 mL of culture medium (RPMI 1640, 10% Fetal bovine serum) to obtain a cell concentration of 5×10^6 cells/mL. An aliquot of cell suspension was transferred into a flat-bottom microplate and incubated for 16–24 h at 37 °C in 5% CO_2. Next, PAMs were infected with 100 µL of 10-fold serial dilutions of the prepared sample and incubated for 30 min under the same conditions as mentioned above. Wells

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uninoculated with the sample were used as negative controls, while wells spiked with haemadsorbing ASFv were used as a positive control. Following the incubation, the suspension was discarded. The wells were added with 200 μL of fresh RPMI supplemented with 10% fetal bovine serum, 1% antibiotics and antifungus, and 10% swine erythrocytes and incubated at 37 °C in 5% CO_2. The PAM cells in the plate were observed under a microscope daily for 7 days for the presence of cytopathic effect (CPE) or haemadsorption (HAD). Virus concentration results are reported as HAD_{50}.

Bioassay

A bioassay was performed to confirm the inactivation of ASFv in compost piles. The spleen samples collected at 16 h were pooled with bone marrow samples at day 3 as the first set of samples used for bioassay, as they were the first date samples positive for viral DNA detection but negative for virus isolation. Similarly, the spleen samples collected at 24 h were pooled as the second set used for bioassay.

For each bioassay, a total of 12 healthy pigs of 20 kg (8-week-old) purchased from an ASF-free farm were divided into an experiment group (4 pigs), a positive control group (4 pigs), and a negative control group (4 pigs). The pigs in the experiment group received 1 mL of intramuscular injection of the supernatant produced from the pool sample of bone marrow and spleen, while the positive control group received supernatant from the samples at day 0. For the negative control, pigs will be intramuscularly injected with 1 mL of Ringer's lactate. The clinical signs of ASF were monitored daily. On days 3, 4, 5, 6, 10, and 25 after injection, blood samples of each pig were collected for the detection of ASFv using qPCR and cell culture as mentioned above.

Results

Temperature profile of compost piles

Fig. 3 shows the temperature profile of 3 static aerated compost piles. Generally, the temperature profiles of the three piles were relatively similar. The temperatures of pile 2 and pile 3 increased rapidly and reached the peak of approximately 70˚C after 2 days post-pile construction, while that of pile 1 achieved the highest temperature of nearly 70˚C at day 3 of composting. Afterwards, the temperatures of 3 piles decreased slightly but still maintained above 60 ˚C until day 10. The temperature gradually declined, reaching approximately 44.5˚C at day 30. During the experiment period, the air temperature varied from 24 ˚C to 39 ˚C.

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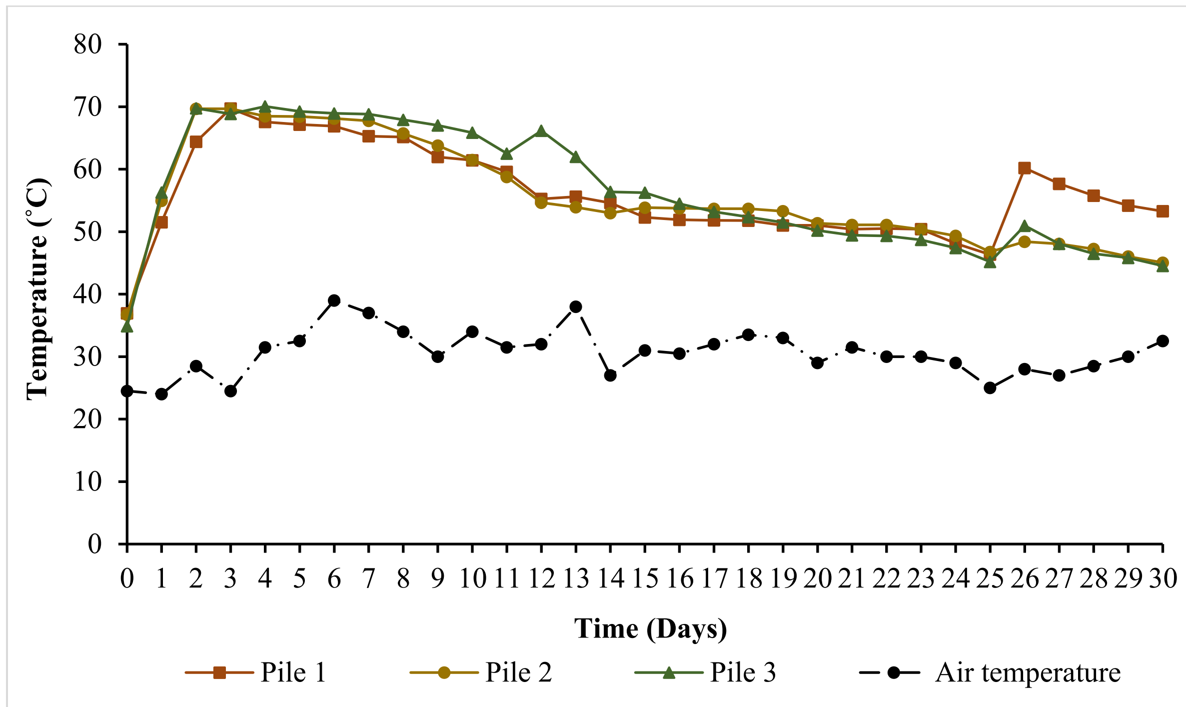

Figure 3. Temperature profiles of compost piles

Detection and Isolation of the African Swine Fever Virus

A qPCR test showed that 3 pigs used for the construction of compost piles were positive for ASFv (Fig. 4). The Ct value of spleen samples of 3 pigs ranged from 19.15 to 21.38 at day 0, while Ct values of bone marrow samples on day 0 varied from 20.15 to 21.85. Overall, the DNA of ASFv was detected in all spleen samples and bone marrow samples collected from day 0 to day 28, with Ct values ranging from 18.14 to 34.75.

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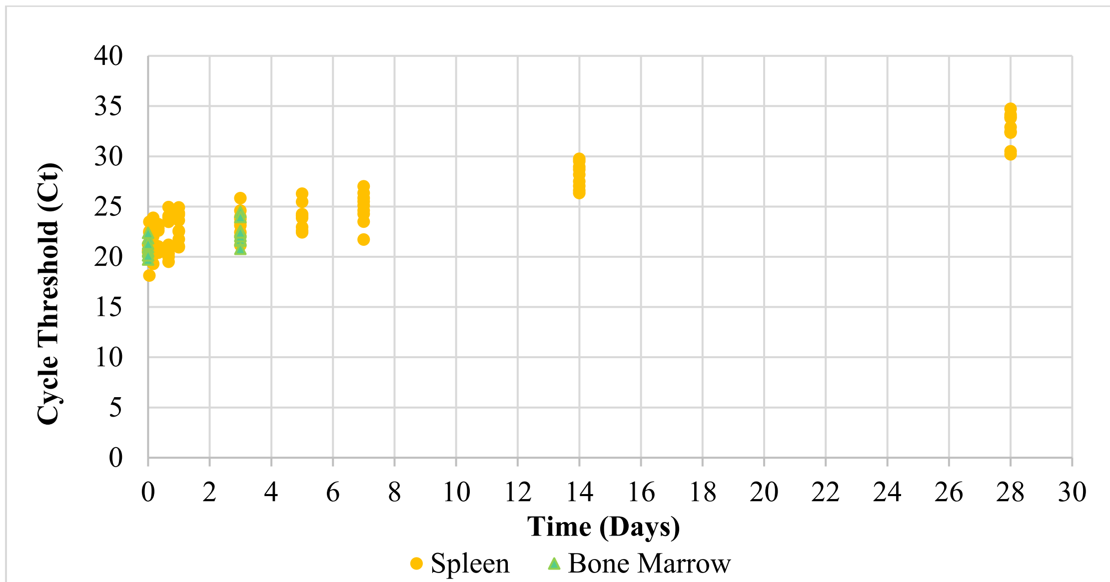

Figure 4. Scatter plot of Ct of post-burial spleen and femur bone marrow samples. Green dots are the Ct values of femur bone marrow samples. Orange triangles are the Ct values of spleen samples.

Viability of ASFv in spleen samples of composted swine carcasses was shown in Fig. 5. The level of ASFv in spleen samples of 3 pigs on day 0 varied from 4.32 to 5 log HAD_{50}. After 1 h after the construction of the compost pile, the virus titer of spleen samples was slightly decreased. Eight hours post-composting, a significant reduction in virus titers was observed in all spleen samples of 3 pigs. The highest reduction of 2.39 log HAD_{50} occurred in spleen samples of pig 3, while the reduction in those of pigs 2 and 1 ranged from 1.32 to 1.34 log HAD_{50}. Surprisingly, the infectious ASFv was not detected after 16 h of composting. For bone marrow samples, the virus was inactivated on day 3.

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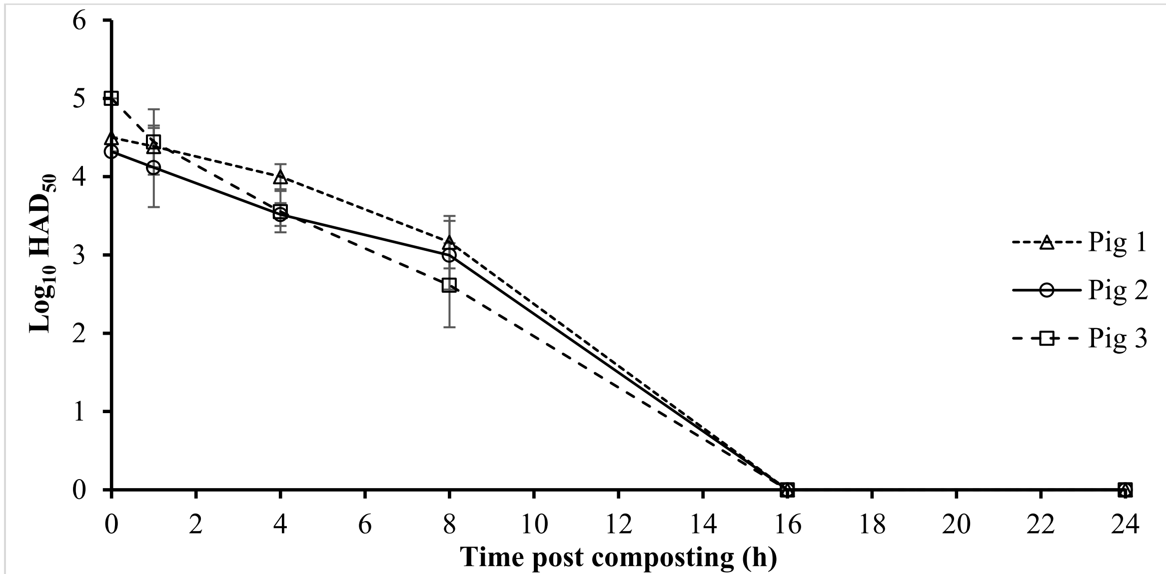

Figure 5. Viability of ASFv in spleen samples of composted swine carcasses

The excavation of compost piles on day 30 revealed the nearly complete decomposition of the whole carcasses. Most of the soft tissue on the carcass had already decomposed, leaving mainly the larger bones exposed, many of which had developed a spongy texture.

Figure 6. The decomposition of carcasses in composting piles on day 30

Bioassay

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The results of the qPCR test showed that the 24 pigs selected for bioassay were negative for ASFv. During the experiment period, only pigs in the positive control group exhibited typical clinical signs of ASF, while those in the negative and experiment groups showed no signs of ASF. The qPCR results also confirm that ASFv was detected in blood samples collected from pigs in the positive control group. On the contrary, the DNA of ASFv was not found in any blood samples of pigs in both the negative and treatment groups on 3, 4, 5, 6, 10, and 25 days after injection. The results of the cell culture were consistent with those of qPCR, indicating that ASFv was only found in blood samples of pigs in the positive control group.

Discussion:

The outbreak of ASFv in recent years has caused serious economic losses in countries with developed pig farming. Meanwhile, the lack of solutions to prevent and treat the disease in pigs, such as vaccines and antiviral drugs, has aggravated the epidemic [17]. Therefore, the most effective measure for control of ASF is to cull infected animals to limit the spread of ASFv into the environment.

Deep burial is one of the main methods used for the disposal of animal carcasses. However, this method may increase the risk of the spread and persistence of pathogens in the environment due to leakage of body fluids from animal carcasses into groundwater and soil. Infected animals must be transported to a suitable burial site, which is a risk factor for disease transmission. In addition, deep burial slows down the decomposition process due to a lack of oxygen, increases the growth of anaerobic microorganisms, and inhibits the destruction of pathogens. Therefore, deep burial is not a sustainable solution for treating animal carcasses [18]. On the other hand, composting is an effective method for destroying viruses, bacteria, fungi, and worm eggs that cause disease [7, 8]. Composting can be carried out in disease-affected areas, limiting movement, thus reducing the risk of ASFv spreading between farms. The process of decomposition of carcasses is rapid, optimizing time, labor, and efficiency in pathogen control [7].

The composting process, with the activity of microorganisms, leads to an increase in temperature, promoting the destruction of pathogens. The results of cell culture in this study showed that ASFv in spleen samples and bone marrow samples rapidly lost their infectivity after 16 h and 3 days of composting, respectively. Consistent with the results, the bioassay confirmed the rapid inactivation of ASFv in spleen and bone marrow samples. The rapid inactivation of ASFv in compost may not result from the effect of temperature but can be the combined effects of high temperature, microbial activity, pH, and by-products of the decomposition process [11, 12]. The effectiveness of pathogen inactivation during composting depends on many factors, including temperature, moisture, aeration, C: N ratio, and the type of mulching material. These factors determine the rate of biodegradation, which in turn affects the duration and extent of ASFv inactivation [7, 10, 19]. Temperature is considered the primary factor contributing to virus inactivation during composting. The U.S. Environmental Protection Agency (USEPA) has established time and temperature criteria used to evaluate the ability of composting to reduce pathogens. The Type A criteria require composting temperatures of 55 ˚C or higher for at least 3 consecutive days. The Type B criteria require composting temperatures of at least 40 ˚C for 5 or more consecutive days with temperatures exceeding 55 ˚C for at least 4 hours during this

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time [20]. In this study, temperatures in the compost piles reached 69 – 70 ˚C after just 2–3 days and remained above 60 ˚C for at least 10 days. This temperature has been reported to be sufficient to kill most viruses and pathogenic bacteria in animal carcasses [7, 8, 21]. Similarly, the ability to inactivate ASFv in compost piles at temperatures of 50 – 60 ˚C within 3 to 7 days was noted in a study by Hutchinson et al. [11], Duc et al. [13], and Gabbert et al. [12]. Vitosh-Sillman et al. [22] recorded PEDv inactivation in pig carcasses incubated at 37 ˚C for 24 hours. Guan et al. [23] reported that the destruction of bovine viral diarrhea virus (BVDv) occurred when the compost pile temperature reached 41 ˚C for one day.

Moisture content affects both the heat generation and the time it takes for high temperatures to be maintained in the compost pile. The optimum moisture for effective composting is recommended to be in the range of 50–70%. Low moisture slows down the decomposition process, while high moisture content causes anaerobic conditions, reduces the heat generation efficiency, and prolongs the survival time of the pathogen [10]. The results of this study showed that the moisture content was maintained in the optimal range by rice hull, thus ASFv was rapidly inactivated, similar to previous reports [11, 12]. Carbon materials play an important role in maintaining temperature, absorbing water, and creating aerobic conditions for the compost pile [7]. In this study, rice hulls were used as a mulching material and showed high efficiency in maintaining a stable temperature and promoting rapid decomposition. Previous studies have demonstrated that other materials, such as sawdust, straw, and wood chips, also have similar effects [11, 12]. Choosing popular, cheap materials like rice hulls also has great practical significance in Vietnam.

In addition to the factors of temperature, moisture content, and composting materials, the inactivation of ASFv during the composting process is also strongly affected by the activity of aerobic microorganisms. During the organic decomposition process, bacteria and fungi use animal carcasses as a substrate source, while generating heat, organic acids, ammonia, and many decomposing enzymes. These by-products not only increase the temperature of the compost pile but also directly break down the protein and nucleic acid structures of pathogens, thereby promoting virus inactivation [19, 24, 25]. For ASFv, Hutchinson et al. [11] showed that microbial activity in aerated static compost helped raise the temperature and maintain unfavorable conditions for ASFv, contributing to accelerating the virus inactivation process. Overall, the results of the study provide clear scientific evidence that the composting process with rice hull material is capable of inactivating ASFv in pig carcasses quickly and effectively. Factors such as temperature, moisture content, composting material, and microorganisms play a decisive role in the efficiency of the composting process.

Topic #2 – Assessing the viability of ASFv in the deep burial environment

Industry Summary:

African swine fever virus (ASFv) remains a serious threat to swine producers around the world. When pigs die from the disease, it is essential to dispose of carcasses in a way that prevents the virus from spreading to other animals or farms. This study examined how long ASFv remains active in pigs buried two meters deep. Results showed that the virus could still be detected for several weeks, but by day 54, it was no longer infectious. Laboratory testing and bioassays confirmed that ASFv was inactivated

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at that point. However, researchers also found that soft tissue was still present in the carcasses even after six months, indicating that full decomposition takes longer in deep burial. For producers, this means that while deep burial can eventually inactivate the ASF virus, it takes much longer than composting or shallow burial with carbon. Deeper burial may be appropriate only when space, soil type, or biosecurity concerns make composting or shallow burial impractical. Proper site selection, burial depth, and management remain critical to ensure both virus inactivation and environmental protection.

Key Findings:

- ASFV DNA was detected in spleen samples up to 54 days post-burial.
- Infectious ASFV was present in buried carcasses for up to 28 days.
- Bioassay confirmed complete inactivation of ASFV on day 54.
- Carcass decomposition was slow under anaerobic deep burial conditions.

Keywords: African Swine Fever Virus; Deep Burial; Inactivation; Carcass decomposition; Bioassay

Scientific Abstract:

Introduction:

African swine fever (ASF) is a highly fatal infectious haemorrhagic fever of pigs. It is caused by the African swine fever virus (ASFv), a double-stranded DNA virus with a complex molecular structure. It is the only member of the family Asfarviridae and the only DNA virus transmitted by arthropods, soft ticks of the genus Ornithodoros [1,26]. Since it was first detected in Kenya in 1920, ASFv has spread to countries in Africa, Europe, the Americas, and Asia over the decades [3]. However, there is currently no effective vaccine or treatment for ASFv, so control relies on isolation and culling of infected or contact pigs to reduce the risk of virus transmission [6]. Carcass disposal methods currently used in many countries include incineration, composting, and deep burial [18,27–29]. Although burning can quickly destroy pathogens with high temperatures, it requires fuel, incinerators, and especially pollutes the environment through gas emissions [30]. Composting is a method of treating animal carcasses with carbon materials, creating conditions for aerobic microorganisms to produce high temperatures during the natural decomposition process, helping to neutralize pathogenic microorganisms [19]. This method is recognized as effective and economical for the treatment of animal carcasses [11,13]. Nevertheless, potential drawbacks include the risk of pathogen leakage, unpleasant odors, and disturbance of the compost pile by insects and scavenging animals such as rodents [7].

Deep burial shows the main advantages of being able to handle large amounts of animal carcasses and minimizing the spread of pathogens [31]. However, its effectiveness depends on several factors, including depth, soil biogeochemical characteristics, groundwater level, temperature, moisture, pH, and the use of additional materials such as lime powder or carbon material coating [11,32–35]. Previous studies have demonstrated that ASFv is inactivated in burial pits within six months [29,36]. On the other hand, a limitation of the deep burial method is the possibility of releasing leachate and pathogens from decomposing carcasses, contaminating agricultural land and groundwater near the burial area [18]. Pathogens in animal carcasses can survive

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for extended periods in deep burial pits due to favorable environmental factors such as low temperature and anaerobic conditions [37].

Although deep burial is the traditional method and is most widely used in African swine fever outbreaks in Vietnam, limited information is available regarding the survival of pathogens, especially ASFv, within burial pits. Therefore, assessing the survival of ASFv in deep burial environments is necessary to provide scientific evidence for the development of safe destruction procedures, contributing to effective disease control, limiting the risk of pathogen spread to the environment, and ensuring biosafety.

Objectives: To determine the survival and inactivation period of ASFV in pig carcasses buried at a depth of two meters under field conditions.

Materials & Methods:

Burial pit construction

Forty-eight pigs with typical symptoms of ASF were used for this study (Figure 1). Before burial, spleen samples were carefully collected for a Real-time PCR (qPCR) test to confirm ASFv infection. Eleven burial pits with dimensions of 2 m wide × 3 m long × 2 cm deep were constructed for the burial of 44 pigs. Four ASF-infected adult pigs were placed at the bottom of each pit. Afterward, the excavated soil was returned to the pits.

Figure 1. Four swine carcasses were placed in each burial pit, facing the same direction

Sample processing

The spleen samples of the buried pigs were collected for the detection and isolation of ASFv over the short term (Days 1, 3, 5, 7, 14, 28, and 54) and the long term (6, 12, 18, and 24 months) using qPCR và cell culture. Each sampling day corresponded

to one burial pit, with each pit being opened only once for sample collection. Although 4 pigs at day 0 were not buried in the pit, their spleen was also collected to determine the initial ASFv titers. The carcass decomposition was assessed through carcass exhumation.

Detection of African Swine Fever Virus

The extraction of ASFv DNA was carried out using a MagMAX™ Viral/Pathogen Nucleic Acid Isolation Kit (Thermo Fisher, MA, USA) following the manufacturer's instructions. Next, qPCR was performed according to the previous method described by Tignon et al. (2011) [16] using a TaqMan probe (5′-FAM-TTCCATCAAAGTTCTGCAGCTCTT-TAMRA-3′) and primer pair (Forward, 5′-TGCTCATGGTATCAATCTTATCG-3′; Reverse, 5′-CCACTGGGTTGGTATTCCTC-3′) [12]. The PCR mixture (25 µL) consisted of 5 µL of nuclease-free water, 12.5 µL of PCR master mix 2X (Invitrogen Superscript III qRT-PCR, Thermo Fisher, USA), 2.5 µL of the primer-probe mix (forward primer (0.6 µM), reverse primer (0.6 µM), a TaqMan probe (0.3 µM)), and 5 µL of DNA template. A thermal cycling machine (CFX96, BioRad Laboratories, Hercules, CA, USA) was used to carry out a PCR amplification program consisting of 1 cycle at 95 °C for 2 min, 45 cycles at 95 °C for 15 s, and 60 °C for 45 s.

Isolation of African Swine Fever Virus

Porcine Alveolar Macrophages (PAMs) were thawed in a water bath at 37 °C before centrifuging at 2000 rpm for 10 min at room temperature. The pellet was collected and then washed with 5 mL of PBS buffer, mixed with 10 mL of culture medium (RPMI 1640, 10% Fetal bovine serum) to obtain a cell concentration of 5×10^6 cells/mL. An aliquot of cell suspension was transferred into a flat-bottom microplate and incubated for 16–24 h at 37 °C in 5% CO2. Next, PAMs were infected with 100 µL of 10-fold serial dilutions of the prepared sample and incubated for 30 min under the same conditions as mentioned above. Wells uninoculated with the sample were used as negative controls, while wells spiked with haemadsorbing ASFv were used as a positive control. Following the incubation, the suspension was discarded. The wells were added with 200 µL of fresh RPMI supplemented with 10% fetal bovine serum, 1% antibiotics and antifungus, and 10% swine erythrocytes and incubated at 37 °C in 5% CO_2. The PAM cells in the plate were observed under a microscope daily for 7 days for the presence of cytopathic effect (CPE) or haemadsorption (HAD). Virus concentration results are reported as HAD_{50}.

Bioassay

Bioassays contained experiment groups, a positive control group, and a negative control (four pigs for each group). Pigs in the experiment group were injected with supernatant produced by a pool of spleen samples from 4 pigs whose spleens were demonstrated as negative for ASFv using cell culture (collected 54 days post-burial), while those in the positive control group received supernatant from a pool of spleen samples from 4 pigs at day 0. Ringer's lactate was used instead of the supernatant of the spleen samples for pigs in the negative control. The injected pigs were monitored daily for clinical symptoms of ASF. Blood

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samples of the pigs were collected on days 3, 4, 5, 6, 10, and 25 after injection for the detection of ASFv using Real-time PCR and cell culture.

Results:

Detection and Viability of African Swine Fever Virus

The results of qPCR indicated that all 44 pigs used for deep burial were positive for ASF, with Ct values of spleen samples varying from 18.04 to 23.10. After burial, Ct values tend to increase from day 1 to day 54 post-burial, ranging from 20.49 to 30.05. In general, the DNA of ASFv was detected in all samples collected from day 1 to day 54 post-burial (Fig. 2).

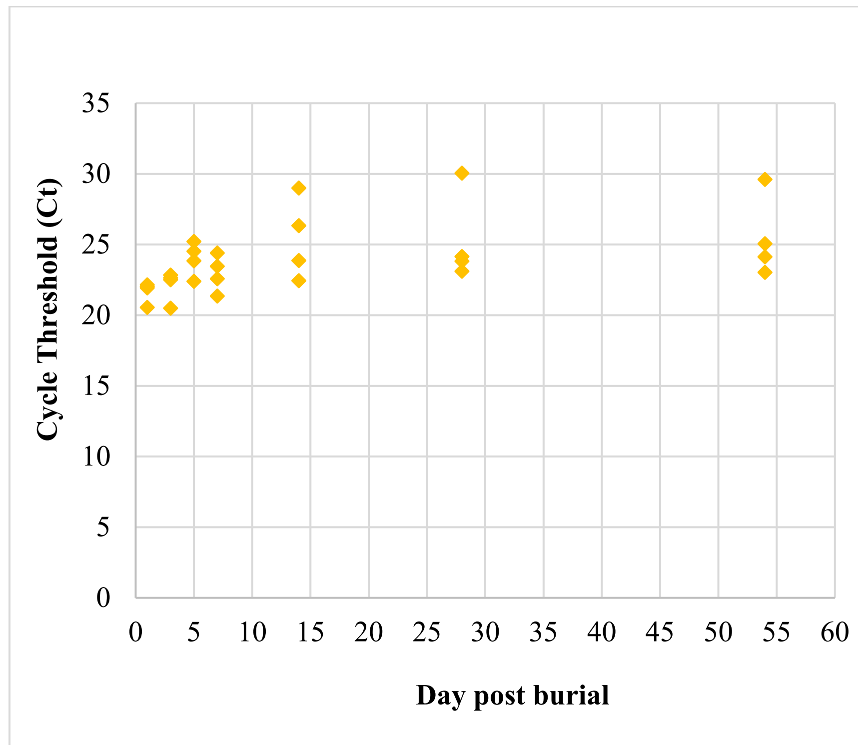

Figure 2. Scatter plot of Ct of post-burial spleen samples

Virus isolation results showed that the titer of ASFv recovered from spleen samples of buried pigs reduced gradually over time (Fig. 3). On day 14 post-burial, infectious ASFv particles were detected in only 1 out of 4 spleen samples, with a titer of $10^{2.17}$ HAD_{50}. On day 28, one of the four spleen samples remained positive for ASFv at a low concentration.

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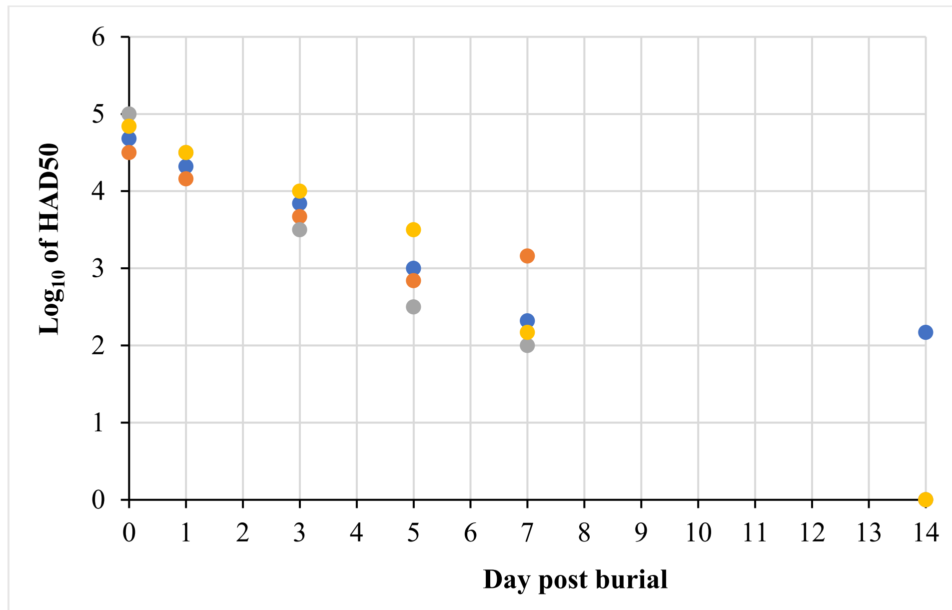

Figure 3. Viability of ASFv in spleen samples of buried swine carcasses

Carcass Decomposition

The exhumation of buried pigs revealed progressive decomposition of carcasses over time. The muscle tissue of buried pigs on day 1 was still fresh. From day 3 to day 5, signs of decomposition began to appear, with a slight foul odor. The muscle tissue did not look as fresh as it did on day 1, and gas was generated in the abdomen. On days 5 and 7, the muscle tissue color began to fade, remained elastic, and the organs began to decompose. Starting from day 14, the muscle tissue was observed to darken and emit a strong, foul odor, become flabby with partial peeling, and the internal organs lost their structural integrity. By day 21, partial decomposition of tissues and organs is observed, while some muscle patches (in the thigh and shoulder areas) remain. By day 56, the muscle tissue and organs had largely disintegrated into a crushed form. However, the tissue still retained a pink color, making it difficult to identify the organs. After 6 months, the color of the muscle tissue started to change to gray. Most of the superficial muscle tissue had decomposed, leaving only the deeper layers intact. The bones had also begun to separate from their original positions.

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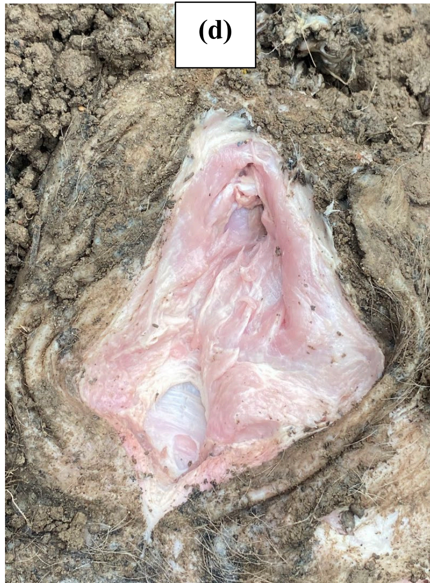

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(e) (f)

(g) (h)

Figure 3. The decomposition of carcasses in burial pits on days 1 (a), 3 (b), 5 (c), 7 (d), 14 (e), 28 (f), 56 (g), and 6 months (h)

Bioassay

Bioassay was conducted to verify the inactivation of ASFv in spleen samples of pigs buried for 54 days. During 25 days of experiment, only pigs in control group showed signs of ASF (Fig. 6). In contrast, the pigs in both negative and experiment groups still stayed healthy at the end of experiment. The results of PCR test were in agreement with clinical observation,

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indicating that DNA of ASFv was only found in pigs in control group, while it was not detected in those in negative and experiment groups thoroughout the experiment period.

Among 4 pigs in control group, 3 pigs were dead on day 13 and 15 post injection. Interestingly, pig 1 was postive for ASFv on day 3, 4, 5, 6, and 10 but negative on day 25.

The DNA concentration of ASFv in blood samples of the pigs in the positive control group was represented in Table 1. The lowest Ct values were observed on day 10 with pig 2, pig 3, and pig 4, while that was on day 5 with pig 1.

Table 1: Detection of DNA of ASFv in blood samples of pigs in bioassay

Days	**Ct values**			
	Pig 1	**Pig 2**	**Pig 3**	**Pig 4**
3	30.83	30.17	28.81	27.48
4	29.35	28.33	29.13	25.88
5	21.01	27.2	24.86	25.92
6	26.4	25.11	23.7	26.89
10	27.69	23.06	18.48	21.37
13	NT	Dead	Dead	NT
15	NT			Dead
25	Negative			

NT: not tested

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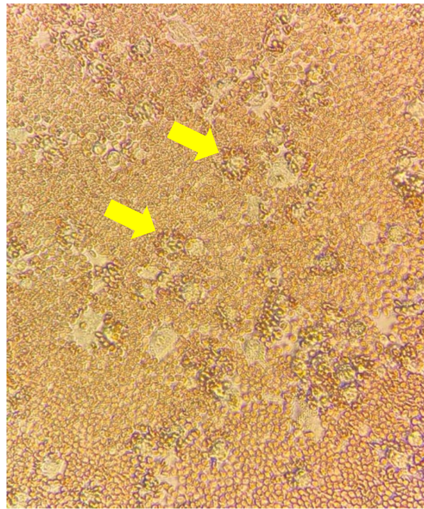

Figure 4. Hemadsorption in ASF virus-infected cells. The arrow indicates HAD rosettes

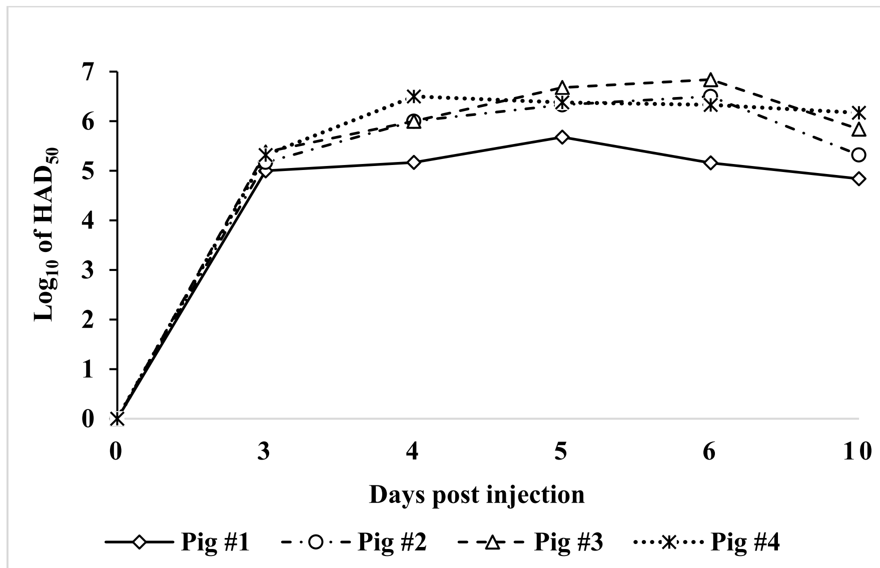

Figure 5. The titer of ASFv in the blood samples of pigs in the positive control group

Overall, the results of the cell culture indicated that the level of ASFv in blood samples increased after injection. On day 3, the ASFv level in the blood of pigs varied from 5 to 5.38 log HAD_{50}. From day 4 to day 10, ASFv titers ranged from 4.84 to 6.84. The highest ASFv concentration in blood samples was observed in pig 3 on day 6. Although the pigs 2, 3, and 4 were dead on days 13 and 15, the titers of ASFv in their blood samples were lower on day 10 compared to those on day 6.

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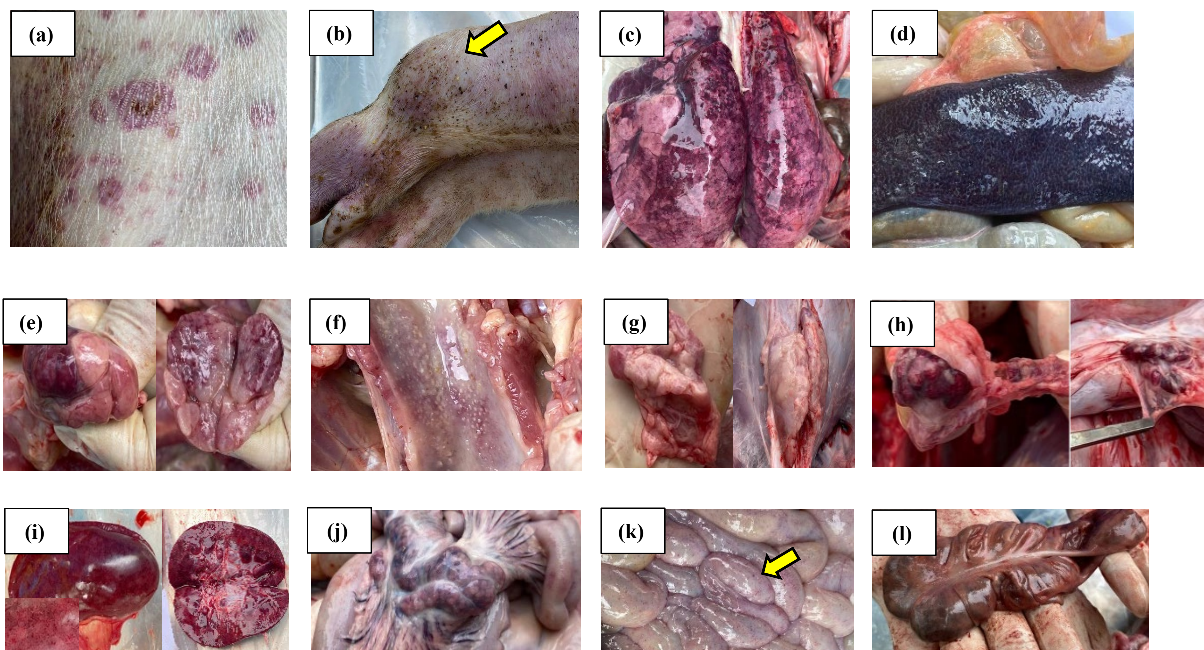

Figure 6. Some lesions of pigs in the positive control group: (a) petechial hemorrhage on the skin; (b) swollen joints with fluid accumulation; (c) pulmonary hemorrhage; (d) splenic congestion; (e) hemorrhage of the submandibular lymph nodes; (f) tonsillar lymph node lesions; (g) hemorrhage of the superficial inguinal lymph nodes; (h) hemorrhage in the ileal Peyer's patches; (i) petechial hemorrhage on the kidneys (pinpoint hemorrhage); (j) hemorrhage of the mesenteric lymph nodes; (k) petechial hemorrhage in the small intestine; (l) hemorrhage of the cecum

Discussion:

The emergence and rapid spread of ASF has become a significant threat to the global swine industry [38]. Controlling ASFv requires the destruction and disposal of large numbers of infected/exposed pig carcasses in a biosecure manner. However, different methods of handling animal carcasses during ASF outbreaks have their advantages and disadvantages [18]. The choice of animal carcass disposal method varies depending on the policies and regulations of each country. In Europe, the burial of animal carcasses has been banned due to concerns that pathogens could spread into the environment, contaminate water sources, and enter the food chain [18]. However, this method has been recommended for use during ASF outbreaks in Vietnam because it can handle large amounts of animal carcasses and minimize the spread of pathogens, reduce direct human and animal contact with pathogens, and lower the risk of further disease spread [31]. Moreover, it requires less investment in infrastructure and operational costs compared to incineration. However, disadvantages include slow decomposition rates, early pathogen survival, and the risk of pathogen-containing leachate leaking into the environment if the landfill's location or design is not suitable [18]. Furthermore, this approach requires substantial land resources and is unsuitable for regions with high groundwater tables.

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In this study, the decomposition of pig carcasses occurred slowly under anaerobic conditions at a depth of 2 m. During the first 7 days, muscle tissue and internal organs remained relatively intact, with only a slight odor. From day 14 onward, visible signs of decomposition were observed in several organs, accompanied by a strong odor. By day 56, most of the soft tissue had decomposed into a paste, but the deep muscle layers and bone structure remained intact. Even after six months, deep muscle tissue still existed, and bones began to move away from their original position. The anaerobic environment inhibited the activity of microorganisms that decompose animal carcasses, resulting in a slower process compared to the composting method [11,13,18].

The results of qPCR showed that the DNA of ASFv was still detected in spleen tissue up to 54 days after burial. However, virus isolation and bioassay indicated that ASFv was no longer infectious after 54 days of burial. These findings are consistent with previous reports indicating that viral DNA may persist considerably longer than infectious virus particles. For example, Zani et al. [29] reported the absence of viable ASFv in spleen and bone marrow samples collected from exhumed pig carcasses between 18 to 440 days post-burial. However, most of the exhumed pig carcasses were PCR positive for the ASFv gene. In the study of Arzumanyan, ASFv in bones and soft tissues in burial pits did not survive more than 6 months [36]. This suggests that the risk of ASFv spreading from animal carcasses in burial pits through the environment to pig farms is relatively low. In this study, the inactivation of ASFv was observed after approximately two months of deep burial, likely due to the combined effects of environmental factors such as temperature, pH, carcass decay, and microbial activity [33,39]. Virus inactivation in deep burial pits occurs much more slowly than in compost piles; it could be because soil temperatures tend to be lower than compost temperatures. In addition to temperature, many other factors influence the rate of decomposition and the survival of viruses: microbial communities, as well as fluctuations in soil pH, may either accelerate or delay virus inactivation [18,40]. Regional differences in climate and soil biochemistry further contribute to variability in the inactivation process. In addition, the inactivation rate depends on the characteristics and habitat of the ASFv strains [33,41].

Topic #3 – Assessing the viability of ASFv in manure slurry and the surface water environment

Industry Summary:

African swine fever virus (ASFv) is highly contagious and can spread easily through infected animals, carcasses, and contaminated materials. Understanding how long the virus survives in manure, slurry, and other waste products is critical for producers working to protect their herds. This study examined how long ASFv remains active in different waste materials under various temperature conditions. Infectious virus particles were detected in manure and urine shortly after infection, but not in oral fluids. The virus survived for up to 24 hours in manure stored at cool (4°C) and room temperature (24°C) conditions, but it became inactive after 24 hours at 37°C (roughly body temperature). In artificial slurry, ASFv DNA could still be detected for up to 48 hours, but the virus itself was no longer infectious after one day. For producers, these results highlight that temperature plays a major role in ASF virus survival. Warm conditions speed up virus breakdown, while cooler environments allow it to persist

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longer. This means that proper manure and slurry management—especially in cooler climates or shaded areas—is critical to reducing the risk of ASF spread. Effective cleaning, disinfection, and storage practices remain essential to protect herds and neighboring farms.

Key Findings:

- ASF virus (ASFv) was detected in blood from day 3 post-infection, while in oral fluid, urine, fecal swabs, and manure from days 5–7.
- Infectious ASFv particles were found in blood, urine, and manure, but not in oral fluid.
- ASFv remained viable in manure for up to 24 h at 4 °C and 24 °C, but was inactivated after 24 h at 37 °C.
- In artificial swine slurry, ASFv DNA was detected up to 48 h, but the virus lost infectivity after 24 h.

Keywords: African swine fever virus (ASFv); Survival; Manure; Slurry; Swine

Scientific Abstract:

Introduction:

African swine fever virus (ASFv) of the family Asfarviridae causes the most dangerous infectious disease in pigs, leading to heavy economic losses in the pig farming industry [42]. ASFv poses a threat to pigs of all ages, with clinical manifestations of ASF that can vary depending on the virulence of the virus strains, with the disease occurring in subacute, chronic, and subclinical forms. In the acute form, ASFv causes several clinical manifestations, such as high fever, anorexia, cyanosis, and hemorrhage, leading to a high mortality rate, which can be up to 100% [43,44]. However, there is currently no effective vaccine or treatment to control this virus, leading to a global outbreak. Therefore, the World Organization for Animal Health (WOAH) has included ASF in the list of notifiable diseases [45]. ASFv is transmitted through multiple routes, both direct and indirect, which complicates its prevention and control. In particular, ASFv is transmitted through direct contact with blood, urine, nasal secretions, saliva, and manure, as well as through aerosol exposure [46]. In addition, ASFv can be transmitted via intermediate hosts such as soft ticks, rodents, and humans [43,47]. However, increased ambient temperature significantly shortened the survival time of ASFv [7]. The survival of ASFv in waste is also influenced by other factors, including microorganisms, enzymes, and pH [49,50].

Determining the survival time of ASFv in clinical samples supports more effective diagnosis and control of the disease in swine herds [51,52]**.** ASFv shedding is directly related to the ASFv load in secretions and waste samples [53,54]. The blood of infected animals has the highest concentration of ASFV; therefore, blood sampling is a basic method used in veterinary surveillance. However, it can be harmful to the animals and is labor-intensive [55]. Therefore, non-invasive sampling strategies have been investigated, and new approaches to sample collection during veterinary surveillance have been developed [53,55,56]. Enhanced surveillance for early detection is essential for the successful control and eradication of ASFv incursions in pig farms [53].

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To control ASFv, early detection by laboratory diagnosis is essential. Depending on the virulence of the strains and the immune system of the host, clinical symptoms and pathological conditions vary widely; therefore, clinical diagnosis is not feasible [57]. Several diagnostic techniques for ASFv are recommended and applied worldwide by WOAH: hemoadsorption assay (HAD), conventional and Real-time polymerase chain reaction (PCR), antigen detection by fluorescent antibody test, Enzyme-Linked Immunosorbent Assay (ELISA), and indirect fluorescent antibody test [58]. Among these methods, molecular diagnosis by Real-time PCR is the most reliable, providing higher sensitivity and specificity, and can even determine the amount of ASFv DNA [59]. In addition, cell culture (HAD) is also used to determine the amount of viable virus particles in samples, but usually in scientific research [60]. There is limited information regarding the survival of ASFv in manure and slurry. Therefore, this study was conducted to provide data on the viability of ASFv in manure and slurry.

Objectives: This study aimed to investigate the viability of African swine fever virus (ASFv) in different sample types, particularly manure and artificial swine slurry, under various environmental conditions and time intervals, to provide data supporting biosecurity management and disease control in pig farms.

Materials & Methods:

Experiment design

Eight market-size hogs were artificially infected with a known concentration of ASFv (10^2 HAD_{50}/mL). Samples of oral swabs, urine, fecal swabs, manure, and blood were collected on days 1, 3, 4, 5, 6, 7, 14, and 21. The survival of ASFv in various sample types was determined using qPCR and cell culture.

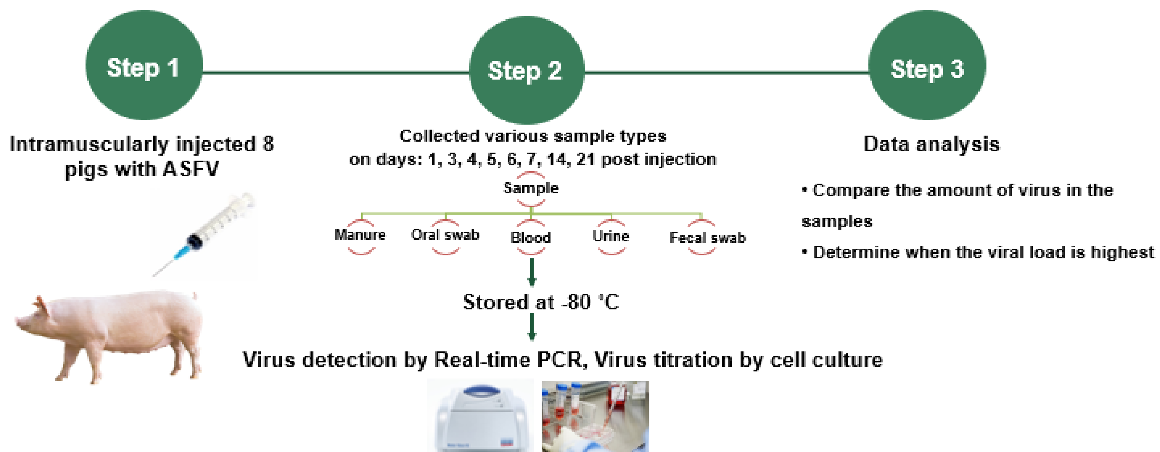

Figure 1. Experimental Design

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Figure 2. Collect samples of fecal swabs, urine, manure, blood, and oral swabs samples

Sample collection

Individual samples (blood, fecal swabs) are collected according to National Centre for Foreign Animal Disease (NCFAD) standards [61]. These samples were collected individually for each pig on days 1, 3, 4, 5, 6, 7, 14, and 21. For blood samples, 2 mL of blood from each pig was collected into tubes containing K2-EDTA. The procedure for collecting fecal swabs was as follows: gently hold the pig's tail to expose the anus. Use a sterile cotton swab to insert 2–3 cm into the rectum, rotate gently for 5–10 seconds to collect the manure. Then, place the sample in a tube containing Phosphate-Buffered Saline (PBS) and close the tube tightly. Urine samples are collected by stimulating pigs to urinate naturally, according to the instructions of K. Stærk et al. [62]. Pooled urine samples were collected per pen. Samples of oral swabs were collected according to the method described by K. B. Goonewardene et al. [56]. A cotton rope was used to collect oral fluid. The oral fluid is subsequently extracted from the rope and transferred into a centrifuge tube for further use. Fresh manure was collected on day 1, day 3, day 4, day 5, day 6, day 7, day 14, and day 21 after injection and kept at 4 °C to favor the survival of ASFv. Each sample was thoroughly homogenized in PBS using Retsch MM400 (Retsch, Dusseldorf, Germany) and then centrifuged at 3000 rpm for 10 min. The supernatant was collected for viral DNA detection and virus isolation using qPCR and cell culture.

Detection of African Swine Fever Virus

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The extraction of ASFv DNA was carried out using a MagMAX™ Viral/Pathogen Nucleic Acid Isolation Kit (Thermo Fisher, MA, USA) following the manufacturer's instructions. Next, qPCR was performed according to the previous method described by Tignon et al. (2011) [16] using a TaqMan probe (5′-FAM-TTCCATCAAAGTTCTGCAGCTCTT-TAMRA-3′) and primer pair (Forward, 5′-TGCTCATGGTATCAATCTTATCG-3′; Reverse, 5′-CCACTGGGTTGGTATTCCTC-3′). The PCR mixture (25 μL) consisted of 5 μL of nuclease-free water, 12.5 μL of PCR master mix 2X (Invitrogen Superscript III qRT-PCR, Thermo Fisher, USA), 2.5 μL of the primer-probe mix (forward primer (0.6 μM), reverse primer (0.6 μM), a TaqMan probe (0.3 μM)), and 5 μL of DNA template. A thermal cycling machine (CFX96, BioRad Laboratories, Hercules, CA, USA) was used to carry out a PCR amplification program consisting of 1 cycle at 95 °C for 2 min, 45 cycles at 95 °C for 15 s, and 60 °C for 45 s.

Isolation of African Swine Fever Virus

Porcine Alveolar Macrophages (PAMs) were thawed in a water bath at 37 °C before centrifuging at 2000 rpm for 10 min at room temperature. The pellet was collected and then washed with 5 mL of PBS buffer, mixed with 10 mL of culture medium (RPMI 1640, 10% Fetal bovine serum) to obtain a cell concentration of 5 × 10^6 cells/mL. An aliquot of cell suspension was transferred into a flat-bottom microplate and incubated for 16–24 h at 37 °C in 5% CO_2. Next, PAMs were infected with 100 μL of 10-fold serial dilutions of the prepared sample and incubated for 30 min under the same conditions as mentioned above. Wells uninoculated with the sample were used as negative controls, while wells spiked with haemadsorbing ASFv were used as a positive control. Following the incubation, the suspension was discarded. The wells were added with 200 μL of fresh RPMI supplemented with 10% fetal bovine serum, 1% antibiotics and antifungus, and 10% swine erythrocytes and incubated at 37 °C in 5% CO_2. The PAM cells in the plate were observed under a microscope daily for 7 days for the presence of cytopathic effect (CPE) or haemadsorption (HAD). Virus concentration results are reported as HAD_{50}.

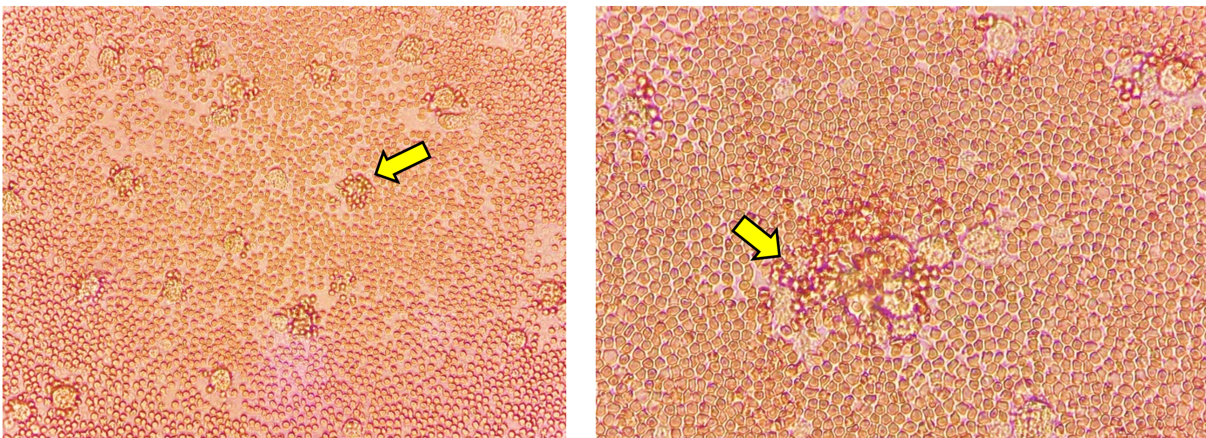

Figure 3. Hemadsorption in ASF virus-infected cells. The arrow indicates HAD rosettes

Investigation of the survival of ASFv in manure in swine pen conditions

Collected manure was stored in the container that is kept in the corner of the swine pen. Samples were subjected to qPCR

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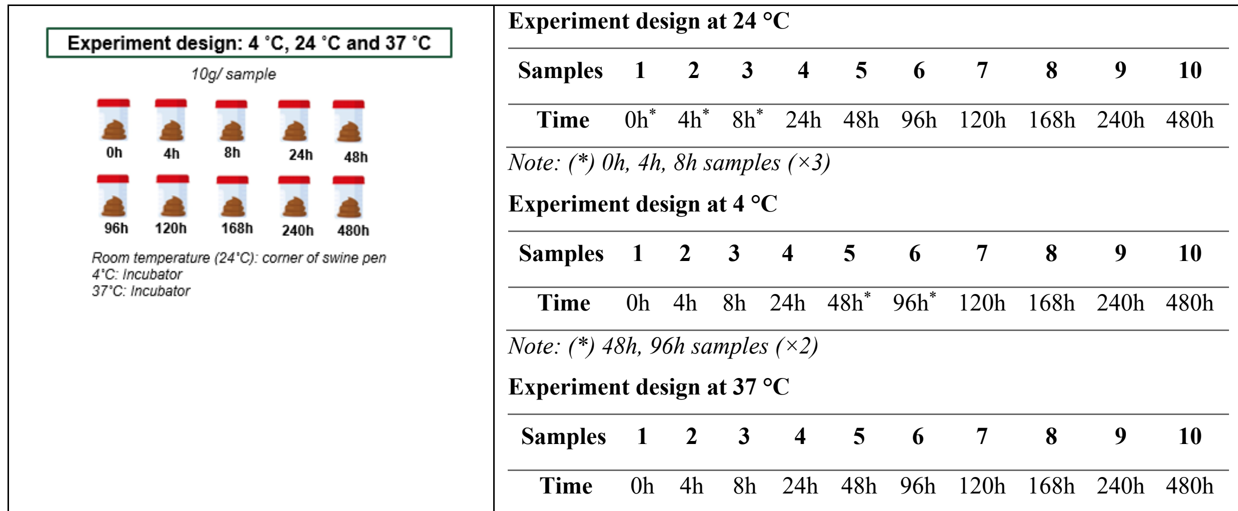
and cell culture at 0, 4, 8, 24, 48, 96, 120, 168, 240, and 480 h post-exposure in the swine pen environment. At the same time, determine the moisture content of the manure sample at different times. When cell culture indicated that ASFv particles were inactivated at a specific time point, the corresponding sample was used for bioassay.



Experiment design at 24 °C

Samples	1	2	3	4	5	6	7	8	9	10
Time	0h*	4h*	8h*	24h	48h	96h	120h	168h	240h	480h

Note: (*) 0h, 4h, 8h samples (×3)

Experiment design at 4 °C

Samples	1	2	3	4	5	6	7	8	9	10
Time	0h	4h	8h	24h	48h*	96h*	120h	168h	240h	480h

Note: (*) 48h, 96h samples (×2)

Experiment design at 37 °C

Samples	1	2	3	4	5	6	7	8	9	10
Time	0h	4h	8h	24h	48h	96h	120h	168h	240h	480h

Figure 4. Experimental design to determine the viability of ASFv in manure under temperature conditions

Each bioassay consisted of three groups: experimental, positive control, and negative control, with 4 pigs per group. Pigs in the experiment group were intramuscularly injected with 1 mL of supernatant produced from a manure sample negative for cell culture, while those in the control group received 1 mL of supernatant generated from a manure sample collected on day 0. Ringer lactate was used instead of the manure supernatant for the pigs in the negative control. The pigs in the 3 groups were then monitored daily for clinical signs of ASF. On days 3, 4, 5, 6, 10, and 25 post-injection, blood samples were collected for the detection of ASFv using qPCR and cell culture.

Experiment 2. Investigation of the survival of ASFV in artificial swine slurry (swine lagoon)

Collected manure (6 kg) were mixed with ASFv-free water (14.4 L; collected from pig farm) to produce artificial swine slurry (assuming that 90% of fresh manure is water and 96% of swine slurry is water) (The concentration of ASFv in artificial swine slurry in this proposal is supposed to be equal or higher than swine slurry in real condition that allows us to predict the maximum time the virus can survive in slurry, thereby giving the best/safe advice to a farmer to deal with ASFv infected slurry. In other words, using a higher ASFv concentration in the artificial swine slurry helps avoid underestimating the virus's survival in the slurry. The artificial swine slurry was divided into 10 one-liter subsamples. The subsamples were then kept in containers that were exposed to the stagnant environment (*The bottle of sample was attached with a string and kept in the swine lagoon).* After 0, 4, 8, 24, 48, 96, 120, 168, 240, 480 h of exposure, the subsamples were withdrawn and subjected to qPCR and cell culture, as shown in Table 1

Table 1. Experimental design to determine the viability of ASFV in swine lagoon

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Subsample Number	1	2	3	4	5	6	7	8	9	10
Time from sample collection:	Immediately	4 hrs.	8 hrs.	24 hrs. (1 day)	48 hrs. (2 days)	96 hrs. (4 days)	120 hrs. (5 days)	168 hrs. (7 days)	240 hrs. (10 days)	480 hrs. (20 days)

The inactivation of ASFv in a first subsample negative for cell culture was confirmed using bioassay, as described above.

Results:

The viability of ASFv in different sample types

In blood samples, the virus DNA was first detected on day 3 post-infection (dpi) with a Ct value of 31.20. The Ct value then gradually decreased, reaching a lowest value of 20.34 on day 7. For oral swabs, urine, fecal swabs, and manure, the DNA of ASFv was first detected on day 5 with a Ct value of 37.15, 32.56, 32.71, and 35.45, respectively. On day 14, the DNA of ASFv was only detected in blood samples (Table 2).

Table 2. The survival of ASFv in different sample types

Sample	Ct value							Log_{10} HAD_{50}						
	1 dpi	3 dpi	4 dpi	5 dpi	6 dpi	7 dpi	14 dpi	1 dpi	3 dpi	4 dpi	5 dpi	6 dpi	7 dpi	14 dpi
Oral swabs	-	-	-	37.15	38.87	35.58	-	-	-	-	-	-	-	-
Urine	-	-	-	32.56	30.48	31.05	-	-	-	-	2.17	2.32	2.32	-
Fecal swabs	-	-	-	32.71	31.71	30.31	-	-	-	-	2.25	2.44	2.92	-
Manure	-	-	-	35.45	31.16	30.98	-	-	-	-	-	2.32	2.84	-
Blood	-	31.20	28.74	26.16	23.12	20.34	23.02	-	4.29	4.79	5.51	6.21	6.86	4.71

The results of virus isolation showed that infectious ASFv particles were detected in blood samples from day 3 to day 14, ranging from 4.29 to 6.86 log HAD_{50}. Whereas, infectious ASFv particles were not found in any oral swab samples. In urine and fecal swab samples, titers of the virus from days 5 to 7 varied from 2.17 to 2.92 log HAD_{50}. For manure samples, ASFv maintained its infectivity on days 6 and 7, with levels of 2.32 and 2.84 log HAD_{50}, respectively.

The survival of ASFv in manure

The survival of ASFv in swine manure at different temperature conditions is shown in Table 3. At 4 °C, the infectious ASFv was detected at 0 h, 4 h, 8 h, and 24 h after incubation with levels ranging from 2.32 to 2.83 log HAD_{50}. Similarly, at 24 °C, ASFv remained active until 24 h post-treatment, with titers ranging from 2.17 to 2.73 log HAD_{50}. At 37 °C, the virus was inactivated

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after 24 h of incubation. The results of the bioassay confirmed that the ASFv in manure lost its infectivity when incubated at 4°C and 24°C for 48 h and 37°C for 24 h.

Table 3. The survival of ASFv in swine manure at different temperature conditions

Incubation time	Temperature					
	4 °C		24 °C		37 °C	
	Ct	*Log_{10} HAD_{50}*	*Ct*	*Log_{10} HAD_{50}*	*Ct*	*Log_{10} HAD_{50}*
0 h	30.89	2.83	30.77	2.73	31.03	2.68
4 h	31.87	2.83	31.49	2.56	30.85	2.50
8 h	32.42	2.63	30.83	2.44	33.36	2.32
24 h	34.16	2.32	32.75	2.17	-	-
48 h	31.74	-	-	-	-	-
96 h	33.51	-	-	-	-	-
120 h	-	-	-	-	-	-
168 h	-	-	-	-	-	-
240 h	-	-	-	-	-	-
480 h	-	-	-	-	-	-

The survival of ASFv in artificial swine slurry

Table 4 revealed the survival of ASFv in artificial swine slurry. The results of qPCR showed that the DNA of ASFv was detected until 48 h of treatment. While the results of virus isolation indicated that the titer of ASFv in artificial swine slurry was very low, the virus was inactivated after 24 h post-treatment. Bioassay verified the inactivation of ASFv in artificial swine slurry after 24 h of treatment. The DNA and infectivity of ASFv were detected only in pigs in the positive control, but not in pigs in the negative and treatment controls.

Table 4. The survival of ASFv in artificial swine slurry

Times	Ct	*Log_{10} HAD_{50}*
0 h	34.85	+
4 h	35.09	+
8 h	33.31	+
24 h	36.85	-
48 h	35.49	-

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96 h	-	-
120 h	-	-
168 h	-	-
240 h	-	-
480 h	-	-

Discussion:

ASFv load during disease progression, from incubation, latent to infectious stages, is an important epidemiological parameter to describe virus dynamics and assess the ability of infected pigs to shed the pathogen into the environment [63]. The incubation, latency, and infectious period depend on many factors, the most important of which are the viral dose and the characteristics of the infecting ASFv strain [64]. Accurate determination of ASFv load and the timing of viral excretion at different stages of disease progression enables prediction of the risk of pathogen dissemination through various excretory routes, including blood, oral fluids, manure, and urine.

The blood of infected animals usually has the highest ASFv concentrations; therefore, blood sampling is a fundamental method used in veterinary surveillance [55,65]. In this study, the virus was detected in blood as early as 3 days post-infection (dpi). The results of this study are consistent with previous reports, showing that ASFv can persist and remain at high density in blood.

Several previous studies have suggested that during infection, pigs excrete ASFv at higher concentrations orally than nasally and rectally [40,66]. The results of this study showed that ASFv DNA appeared in oral swabs and fecal swabs from 5 – 7 dpi, with Ct values decreasing from 38.87 to 30.31. In contrast, for oral swabs, only DNA was detected, but no virus was isolated. This was also reported in the study by H. C. de Carvalho Ferreira et al. [21], and A. S. Olesen et al. [28,30]. ASFv DNA can persist much longer than live virus particles, leading to a positive PCR result when the actual risk of infection has already disappeared. Studies by A. S. Olesen et al. [24] and Walczak et al. [53,68] only detected ASFv DNA in oral and rectal swabs. On the other hand, Guinat et al. [69] reported ASFv in oral swabs and fecal swabs after approximately 5.4 days and 4.9 days of infection, respectively. Goonewardene et al reported that a pooled oral fluid sample may be suitable for ASFv surveillance [56]. Similarly, the study by Ramirez-Medina et al. [70] showed that ASFv in nasal swabs remained undetectable in samples taken from all animals on 2 and 4 dpi; however, low to moderate viral titers were recorded in all animals on day 6 dpi.

In the case of oral swabs and fecal swabs, low ASFv levels and qPCR inhibitors in samples may lead to false-negative results. Oral fluid is a mixture of salivary gland secretions and non-salivary components, such as blood and serum components from oral lesions, bacteria and their products, viruses, fungi, desquamated epithelial cells, and other cellular components, as well as food debris [55]. The failure to detect ASFv in the oral fluid of pigs may be due to its reduced stability due to anti-viral

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antibodies (IgA) in the oral cavity, and the effects of feeding pigs [69,71]. Low virus levels in oral and rectal samples have been reported previously [68,70].

ASFv excretion in manure and urine plays an important role in ASFv dissemination [40]. Pigs infected with ASFv often develop hemorrhagic enteritis, which contributes to the high infectivity of their manure. The presence of ASFv in urine may be due to the virus passing from the blood through the urinary system, causing bladder hemorrhage and excretion in the urine [72]. In this study, although the amount of virus detected in blood was relatively high at 5–7 dpi, the amount of viable ASFv excreted in manure and urine was low. Previous studies have shown that ASFv persists longer in urine than in manure and oral fluid samples, suggesting that urine is the most stable medium tested for ASFv survival [48]. This may explain that the intestinal microbiota stimulates the mucosal immune system (secreting IgA and IgA +), which affects the persistence of ASFv in manure [50]. In addition, enzyme concentrations, including protease or lipase, have been shown to inactivate viruses in samples [48].

ASFv genetic material is detected as early as 3–5 days after infection and 2–7 days before death of infected pigs [61]. After infection, pigs typically begin shedding virus into the blood during a latent period of 1–7 days. The highest ASFv loads are usually detected between 3 and 7 dpi, which is regarded as the period of greatest transmission risk. Viral shedding may persist for as long as 70 dpi in infections caused by low-virulence ASFv strains [40]. The timing of sampling may influence the results of this study. ASFv was first detected in blood samples of pigs during the incubation period (3 dpi). During the onset of fever (5 dpi - 7 dpi), the proportion of positive samples increased, including oral swabs, fecal swabs, manure, and urine samples. Although pigs died at different times, the ASFv load in the samples between pigs did not differ significantly. This suggests that conducting veterinary surveillance of symptomatic animals or frequent sampling provides higher accuracy in disease diagnosis [73]. However, ASFv detection in non-invasive specimens is later compared to blood samples, creating a risk that even clinically symptomatic animals may test negative when using oral swabs, fecal swabs, manure, or urine. The presence of ASFv in clinical specimens such as oral swabs, fecal swabs, manure, and urine is usually at lower levels than in blood, which is entirely appropriate [53]. Since ASFv belongs to a group of viruses that target myeloid cells mainly found in blood or lymphoid organs [38]. In addition, ASFv exhibits high stability in blood [74].

Manure and artificial slurry from ASFv-infected pigs are potential sources of pathogen dissemination to the environment. Evaluating the viability of the virus in these wastes under different temperature conditions is important for disease prevention, as temperature is one of the key environmental factors affecting the rate of virus inactivation.

The results of this study showed that ASFv survival in fecal samples was significantly affected by ambient temperature and fecal moisture. At 4 °C and 24 °C conditions, ASFv was still successfully isolated up to 24 h after incubation. In contrast, at 37 °C, the shelf life of the virus decreased, and it was inactivated after 24 h. This demonstrates that high temperature plays an important role in virus inactivation. Guinat et al. (2014) [69] also showed that the half-life of ASFv in manure decreased sharply when the temperature increased from 4 °C to 37 °C, consistent with the results recorded in this study. According to Davies et al. (2017) [48], the half-life of infectious ASFV ranged from 0.65 days in feces stored at 4 °C to 0.29 days in feces stored at 37 °C.

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However, the shelf life of ASFv in our study was shorter than that in the previous study by Davies et al. (2017), possibly due to our lower initial viral load.

In this experiment, an artificial slurry was used to simulate optimal conditions for the survival virus (absence of light, high moisture content, and stable ambient temperature) in the slurry; however, the shelf life of ASFv was found to be relatively short. This may be explained by the relatively low initial concentration of ASFv in the fecal sample. In addition, the rapid inactivation of the virus in artificial slurry may be related to physicochemical factors characteristic of this environment [75].

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