Use of novel postmortem sample types for detection of African swine fever virus infection after natural consumption

by

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Abstract

African swine fever virus (ASFV) is a recognizable disease by the World Organization for Animal Health (WOAH). Virulent strains can cause 100% mortality in pigs and with no current licensed vaccine or therapeutic, rapid identification, biocontainment, and culling is critical. First discovered in Africa in the 1920s, ASFV has spread rapidly through the continent and into other countries. Since its first introduction into China in 2018, ASFV has rapidly spread across the country and into additional countries such as Germany in 2020. In 2021, the virus was detected in the Dominican Republic, due to its geographical location in respect to the United States, the U.S is on heightened alert. The United States is an ASFV naïve country and relies on mitigation strategies and trade restrictions to maintain that status. Current confirmatory testing for ASFV for postmortem requires spleen, tonsil, gastrohepatic lymph node, renal lymph node, and inguinal lymph node. These samples are not often collected on farms and may be difficult to collect during passive surveillance due to decomposition of a wild boar carcass. It is critical to have validated, consistent practices that can be applied to a wide variety of circumstances. Having more samples validated for the detection of ASFV will improve rapid and reliable detection while also reducing further environmental contamination posed from opening a carcass. This study sought to identify novel sample matrices, that were equivalent to the postmortem sample spleen, for the detection of ASFV Georgia/07 in pigs' that orally consumed ASFV inoculated media. In our experiment, 7-8 week of male pigs (n=10), orally consumed media with 10^4 $TCID_{50}$/ml (tissue culture infectious dose) ASFV Georgia/07, along with controls (n=2), who received sterile non-infectious media. After presentation of clinical signs between 5-7 dpi, pigs were humanely euthanized, and a variety of tissues were immediately collected. This

study compares $\log_{10}$ Starting Quantity (SQ) copy numbers of ASFV Georgia/07 generated from real-time PCR to assess quantity of ASFV DNA present in: swabs (preputial, spleen, muscle, peritoneal fluid, conjunctiva), lymph nodes (mesenteric, gastrohepatic, inguinal, popliteal, submandibular, tracheobronchial, retropharyngeal, sternal), fluid (ocular, urine, feces), and tissues (spleen, tonsil, conjunctiva, muscle, ear notch, tail notch, bone marrow, diaphragm) to the gold-standard postmortem sample of spleen. After collection, samples were processed and stored immediately in -80° C until DNA extraction and PCR was performed. Samples were evaluated using paired t-Test ($p \leq 0.05$) and were individually compared to not only spleen but also other samples with similar mean SQ values and variance for quantity of ASFV DNA present. These multiple comparisons will provide additional information for field veterinarians, hunters, and slaughterhouse staff to select an accessible and available tissue with confidence that it is comparable to the SQ of the spleen or other reliable matrix for the early detection of ASFV.

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Dedication

To the farmers who have lost their livelihoods to African swine fever virus.

Chapter 1 - Literature Review

1.1 Introduction

African swine fever causes high economic losses to pork producers and causes severe disease in pig. After ASFV first detection in Kenya in the 1920s, ASFV quickly became endemic in Africa and has since spread across Europe and Asia (Penrith & Vosloo, 2009). ASFV is a reportable disease designated by the World Organization for Animal Health (WOAH) (Schulz et al., 2017). Between culling to prevent spread and trade restrictions, it is estimated that an ASF incursion into the US would result in a $1.6 billion loss during the first year of introduction (Dee et al., 2018). A 10-year projection estimates damages to be $50 billion and a loss of 140,000 jobs (Carriquiry et al., 2020).

Morbidity and mortality in domestic pigs approach 100% with virulent strains (Galindo & Alonso, 2017). While a vaccine is in the process of evaluation through USDA and Vietnam (Tran et al., 2022), there is no current vaccine commercially available and methods for control are limited to culling and containment practices (Borca et al., 2020). The outbreak in China in 2019 and 2020 has resulted in combined herd culling representing a 32% loss of the annual global pig production (Schambow et al., 2022).

Rapid detection, containment, and surveillance are the best tools to control the spread of this trade-limiting virus. Currently, there are only a few samples validated for postmortem diagnostic testing, most of which are not typically collected on swine farms and require a full necropsy (spleen, tonsil, gastrohepatic lymph node, renal lymph node, and inguinal lymph node)

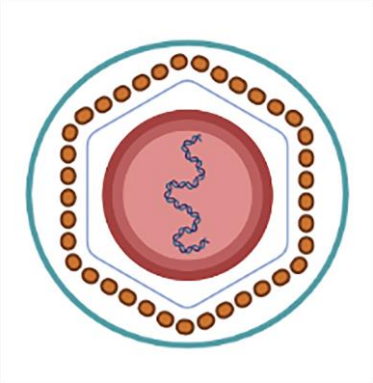
(USDA-APHIS (a), 2022). The purpose of this review is to outline current practices for laboratory diagnosis and surveillance, highlighting how increased sampling matrices may provide an enhanced approach to surveillance in the United States.

1.2 African Swine Fever Virus

ASF is caused by African swine fever virus (ASFV), a highly contagious virus that causes hemorrhages (Liu et al., 2021). ASFV, the only member of the *Asfarviridae* family, is a large, enveloped *ds*DNA virus, comprised of more than 180 kb pairs. ASFV encodes over 150 genes and between 150-200 proteins, assisting in host evasion (Ramirez-Medina et al., 2022; Wang et al., 2019). The virus is icosahedral in shape and contains five layers (Figure 1.1), contributing to ASFV environmental stability: outer envelope, capsid, inner envelope, core shell, and nucleoid (Wang et al., 2019). The conserved capsid protein p72 is the major structural protein that is most often used for serotyping of strains (Wang et al., 2019). ASFV has a specific cellular tropism, replicating in macrophages and monocytes, though specific mechanism of receptor mediated binding remains unclear (Dixon et al., 2019; Wang et al., 2019). The virus has hemadsorption capabilities and readily attaches to erythrocytes (Pikalo et al., 2021).

Figure 1.1.

Structure of ASFV Virion



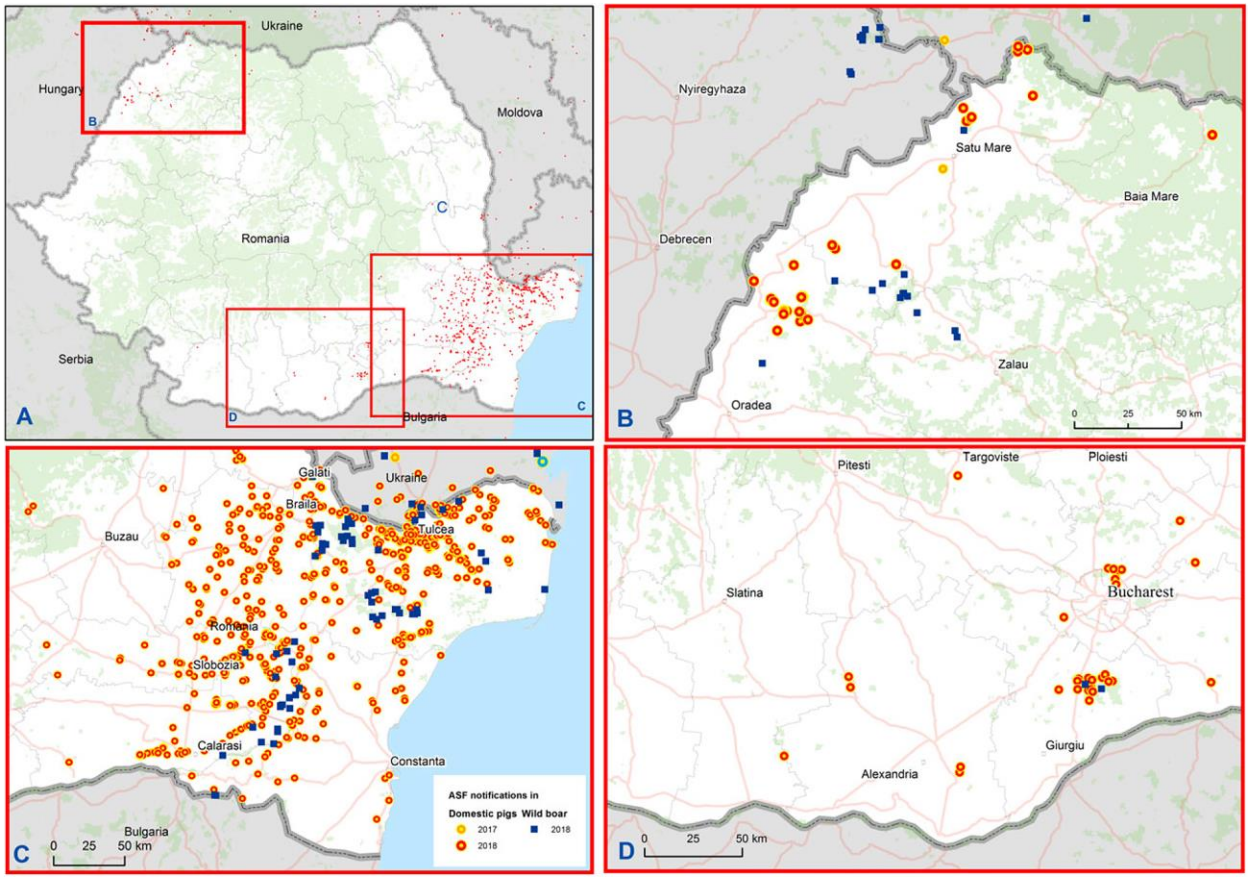
Note. The first layer of the ASFV virion is the nucleoid, shown as the inner most light- red ring. The second layer shown in the dark-red ring is the core shell. The third layer (thin blue icosahedral line) is the inner lipid envelope. The fourth layer shown as the orange dots in an icosahedral shape is the capsid. The fifth and final layer is obtained when the new virus buds from the host cell, the outer envelope shown as the green circle (Image created in Biorender).

Members of the *Suidae* family are the only susceptible species to ASFV; domestic and wild pigs are the most susceptible whereas African warthogs, bush pigs, and ticks in the *Ornithodoros* spp. Are natural reservoirs (Chenais et al., 2019; Liu et al., 2021). Domestic and wild pigs infected with virulent strains often present with acute or peracute forms of ASF, succumbing to the virus 4-15 days post infection (dpi) (Dixon et al., 2020). Clinical signs during the acute phase often present as elevated temperature (40-42°C), lethargy, hemorrhaging of skin and organs, and anorexia (Rathakrishnan et al., 2021; Salguero, 2020). Clinical signs can be variable however and may progress differently depending on many factors such as mode of transmission.

Transmission is through direct contact but can also occur through indirect contact, ingestion, vectors, fomites (Liu et al., 2021), and aerosols over short distances of around 2 meters (Dee et al., 2018; Penrith & Vosloo, 2009). In July of 2017, Romania confirmed two outbreaks in two different backyard operations of domestic pigs. By June of 2018, the Southeast region of Romania (centered around the Danube River) experienced its first outbreak in another backyard operation. A few days later, ASF was confirmed in wild boars. By October, 72 wild boars were confirmed positive (61 found dead and 11 hunted), outbreaks were confirmed in 943 backyard operations, 15 commercial farms, and 1 slaughterhouse (Baños et al., 2022) (Figure 1.2). The Romania outbreak highlights not only the highly infectious nature of ASFV, but also the need to investigate alternate routes of infection such as environmental conditions and natural consumption.

Figure 1.2

Reports of ASFV in Domestic Pigs and Wild Boar in Romania from 2017-2018



Note: ASF notifications in domestic and wild boar in Romania during 2017 and 2018. yellow circles represent 2017 domestic pigs, red circles show 2018 domestic pigs, and blue squares depict 2018 wild boar (Baños et al., 2022).

To investigate if pigs can become infected through natural consumption, Niederwerder (2019) determined the infectious dose of ASFV when liquid is naturally consumed. Using 7 replicates of 6 pigs, each replicate was given 100 ml of RPMI media inoculated with a specific dose of ASFV Georgia/2007. The first replicate began with 10^3 $TCID_{50}$/ml (Tissue Culture Infectious Dose) and each replicate was subsequently adapted to determine the minimum and

median infectious dose. At 10^4 $TCID_{50}$/ml, 100% of the pigs became infected and at 10^0 $TCID_{50}$/ml 37.5% of the pigs became infected. Comparatively, 10^8 $TCID_{50}$/ml was necessary to induce infection in 50% of pigs through natural consumption of plant-based feed. It's possible that saliva proteases may degrade ASFV during consumption of plant-based feed, while liquid medium provides more contact time with the tonsils. Taken together, pigs can become infected through natural consumption of liquid and feed.

Dee et al. (2018), investigated the stability of ASFV in feed using a simulated trans-pacific model. ASFV remained viable in most imported feed matrices tested such as soybean meal and complete feed. The model simulated and tracked feed contamination during manufacturing and processing in Beijing, China, through trans-pacific shipment, and subsequent transport to Des Moines, IA, spanning 37 days from contamination to testing. Imported feed from countries with ASFV may pose a risk of ASFV introduction into the United States (Niederwerder et al., 2021). Surveillance and rapid diagnostics are critical tools to curtail ASFV spread should it be introduced.

1.3 Current Diagnostics

Several diseases can present as ASF, including salmonellosis, erysipelas, classical swine fever and porcine reproductive and respiratory syndrome virus (Chen et al., 2021; Njau et al., 2021; Schulz et al., 2017). Rapid and sensitive testing is required to differentiate causative agents. Diagnostic criteria in the United States are set in accordance with the United States Department of Agriculture-Animal and Plant Health Inspection Service (USDA-APHIS) regulations. In 2019, USDA-APHIS outlined a passive surveillance plan for ASFV. However, active surveillance of clinically sick or dead pigs is recommended, as healthy or subclinical pigs are unlikely to have detectable levels of ASFV DNA. Active surveillance began in 2019 and between June 2019 and June 2022, 31,945 samples had been collected through active surveillance. In July 2022, USDA-APHIS officially added active surveillance to the nations integrated management plan. Sampled pigs include high risk populations of feral swine or domestic outdoor pigs with risk of exposure to feral swine, and commercial herds that are housed indoors(ASF and CSF Executive Summary, 2022) .

To be classified as a confirmed positive, ASFV must test positive for virus isolation OR be identified by two different tests such as antigen and antibody OR two antigen assays (*Swine Hemorrhagic Fevers: African and Classical Swine Fevers Integrated Surveillance Plan*, 2022). Initial testing is typically completed with Real-time PCR (rt-PCR), which detects genetic material. Virulent strains of ASFV cause high viremia, making whole blood the gold standard for antemortem testing. Samples submitted for postmortem testing should follow USDA-APHIS recommendations and include spleen, tonsil, gastrohepatic lymph node, renal lymph node, and inguinal lymph node. Typically, a qualified veterinarian with certification as a Foreign Animal Disease Diagnostician (FADD) performs necropsy collecting these tissues in duplicate.

Collection should be done in accordance with guidelines published in *The Foreign Animal Disease Investigation Manual* (Flannery et al., 2020; *Swine Hemorrhagic Fevers: African and Classical Swine Fevers Integrated Surveillance Plan*, 2022; USDA-APHIS (a), 2022). Apart from FADI, these samples are not often collected on farms, have certain limitations, and require a full necropsy by a trained professional (Table 1.3). Necropsies involve opening the body cavity and potentially risk further contamination of the environment. The ideal sample matrix would be both sensitive to diagnostic tests and would not require opening of the body cavity. To assist in additional sample matrices, various tissues have been investigated as more convenient alternatives (Flannery et al., 2020; Lee et al., 2021).

Table 1.1

Sample Types for the Detection of ASFV and Their Limitations

Sample Matrix	Limitation	Source
Blood EDTA	Labor intensive, active surveillance; unable to collect in carcass	(Flannery et al., 2020)
Serum	Low titers in subclinical animals	(Pikalo J et al., 2021)
Spleen	Requires opening of body cavity and further spread of virus; may not be suitable in decomposed carcass	(Pikalo J et al., 2021; Flannery et al., 2020)
Tonsil	Invasive, not suitable for high throughput active surveillance; requires necropsy; not typically collected on swine farms; requires proper homogenization in lab	(Niederwerder et al., 2022; Pikalo J et al., 2021)
Lymph Node	Invasive, not suitable for high throughput active surveillance; not typically collected on swine farms	(Flannery et al., 2020)

1.4 Blood and Serum

While macrophages and monocytes are the main target of ASFV, the virus readily spreads through regional lymph nodes and blood to replicate in additional tissues (Salguero, 2020). Viremia varies based on several factors such as strain, dose, individual susceptibility and predisposition, and route of infection. Genome copies are typically detected in blood by 2 or 3 dpi (days post infection). The ability to perform early and rapid detection cannot be overstated, which makes blood and serum ideal sample types to be collected from live pigs. Following FADDL guidelines, 10 ml of whole blood should be collected in a green top tube (sodium or lithium heparin) which can be used for virus isolation. PCR has become the industries gold standard as it has high levels of both sensitivity and specificity (Walczak et al., 2022), however heparin can interfere with PCR, thus virus isolation is the preferred confirmatory test. The additional sample of serum is collected in a red top tube (USDA-APHIS (a), 2022), intended for antibody detection. While antibody detection is a robust test, it can take several days for results, limiting its use to a confirmatory test rather than a rapid diagnostic test.

1.5 Swabs as a Novel Sample Matrix

In a study recently published, Lee (2021) was able to detect genomic DNA of the ASFV strain VNUA/HY/Vietnam in rectal, oral, and nasal swabs, increasing viral load was associated with days post infection. DNA was first detected in the standard blood sample at 2.2 ± 0.8 dpi and then in all three swab types at 3.1- 3.6 dpi. By 3 dpi, all eight infected pigs had detectable levels of ASFV at negligible levels. Since early detection is critical to prevent the spread of ASFV, it's paramount to look at the earliest signs of detection, albeit low. At 1 dpi average viral load in blood samples were 1.1×10^2 copies/µl in one infected pig, comparatively, at 2 dpi viral copies in two rectal swabs were 5.3 and 3.9×10^1. All pigs had detectable limits in nasal swabs by 3 dpi, averaging 1.7×10^2 copies/ µl and five oral swabs averaged 2.3×10^2 copies/ µl. At study termination of 8 dpi the highest detections were in blood (9.5×10^6), nasal (5.5×10^3), oral (1.6×10^4), and rectal swabs (5.1×10^3). Similarly, Flannery (2020), in a proof-of-concept design, found mean Ct values of 29.1, 29.5, and 44.0 in oral, nasal, and rectal swab, respectively. It should be noted however that this study had two groups of pigs, each treated with different strains of ASFV, and all samples were collected at 5 dpi except for one pig collected on 4 dpi.

Pikalo (2021), investigated the optimization of types of blood swabs in comparison to the gold standard EDTA blood. While the optimization of swabs will not be discussed in this review, it should be noted that swab material can drastically affect both DNA extraction and PCR inhibition. Pikalo (2021) found in both wild boars and domestic pigs infected with the moderately virulent ASFV strain Estonia/2014, that the standard cotton swab performed the worst of the four swabs tested, with an average detection of 10^2 genome copies per run, compared to EDTA blood around 10^5 genome copies per run.

Virulence of ASFV is highly dependent on strain and dose (Dixon et al., 2020; Niederwerder et al., 2019) . The studies referenced here used strains of different virulence, as such, consideration for sample selection should include strain of concern, dpi, and dose. Other considerations should keep in mind diagnostic supplies. The recent outbreak of SARS-CoV-2 has had detrimental effects on the supply chain and specifically, diagnostic supplies, limiting the availability of swab options (Tay et al., 2021). Of the four swabs used by Pikalo et al. (2021), cotton swabs were referenced in this review for their ability to be easily procured. The primary advantage of swabs would allow for surveillance by untrained persons in the field and limit exposure to virus. Care should be taken when collecting blood on swabs as it would increase the chance of spreading ASFV. Sample pooling is an important tool during passive surveillance. The advantages of pooling samples, such as serum that have been shown to carry low levels of DNA, is that it could increase probability of detection and should be considered when assessing surveillance protocols. Thorough consideration should be given in concerns to false negative or false positive when pooling. Oral, nasal, and rectal swabs can all be easily taken at either time of medical treatment such as tail docking and vaccination or at slaughter. These samples as well do not require the opening of the body cavity and can be easily taken in the field by untrained persons such as hunters, increasing capabilities for surveillance.

1.6 Tissues as a Novel Matrix

Current standards for tissue selection include organs that contain myelomonocytic, erythrocytes, and monocytes, cells that have tropism for ASFV, which includes spleen, tonsil, and lymph nodes (Pikalo et al., 2021). Additional samples that are more easily collected and do not require opening the body cavity would be a critical tool for surveillance. One study that investigated alternative tissue matrices including bone marrow and ear biopsy was Flannery (2020). Six ear punches were collected from different locations of the left ear. Bone marrow was collected from the rib and humerus. ASFV was detected in all ear punches with no significant difference between location, Ct values ranged from 24.8 - 31.8. Similarly, bone marrow ranged from 18.2-23.8, with no significant difference between location of rib and humerus.

Bone marrow has proven to be an invaluable matrix, leading to the first identification of ASFV in Germany (Sauter-Louis et al., 2021). In 2020, the carcass of a wild boar was found by a citizen, time of death was estimated to be no older than 2 weeks. The forelimbs were collected, and bone marrow was tested based on WOAH standards and found to have moderate levels of ASFV. Afterwards, the carcass was retested with various other tissues collected for confirmation, all samples showed detectable levels of ASFV. This case highlights the need to submit any sample for testing even if the gold-standard samples are unavailable for collection.

1.7 Lymph Nodes as a Novel Matrix

Lymph nodes possess several advantages for a sampling matrix. ASFV readily travels through lymphoid tissues (Blome et al., 2013), allowing for early diagnostic detection. Several lymph nodes are located directly under the skin such as submandibular, superficial cervical, sub-iliac, and superficial inguinal lymph nodes (SILN). Accessible lymphoid tissues can easily be collected by untrained staff and do not require the body cavity to be opened, reducing environmental contamination. A study conducted by Goonewardene (2022) assessed the utilization of SILN in comparison to spleen in pigs infected with both moderate (Estonia 2014) and virulent (Georgia/07) strains. All six experiments showed high correlation between spleen and SILN genome copies. Pearson correlation analysis showed that Estonia 2014 had an $r = 0.85$, $p \leq 0.0001$ and Georgia/07 was $r = 0.70$, $p \leq 0.0001$. Unpaired t-test revealed no significant differences between genome copies between spleen and SILN however, there was significant differences ($p = 0.0062$) in the highly virulent stains. Notably, there was higher variability in genome copies from SILN taken from pigs infected with moderately virulent strain. These findings were echoed in another study that found wide variability in genome copies in pigs infected with moderately virulent strains of ASFV (Pikalo et al., 2021). It is possible that variability in genome copies could be caused by DNA extraction or PCR inhibition due to fatty tissues attached to SILN (Pikalo et al., 2021; Sajali et al., 2018). As it is well known that fat can inhibit PCR.

1.8 Conclusion

ASFV continues to spread at an unprecedented rate and is edging ever closer to an incursion into the United States. Growing evidence shows that ASFV can remain viable and infective in imported feed. Along with this growing concern in our global economy is the close proximity to the outbreak in the Dominican Republic. Currently, tissues that are approved for collection require full necropsy and rely on the assumption that organs, such as spleen, are present.

There is a lack of approved sample matrices that would be present in a decomposing carcass found in the environment such as bone marrow. Current approved samples include spleen, tonsil, gastrohepatic lymph nodes, renal lymph nodes, and inguinal lymph nodes. All of which pose the risk of environmental contamination during a necropsy. Along with this risk, they are likely to be missing in a found carcass, being easily scavenged by animals.

Samples that are diagnostically sensitive, limit environmental contamination, and are easy to collect will be the most ideal matrix. It is imperative that countries increase sampling matrices for the detection of ASFV, allowing for passive surveillance and increasing testing capacity. Several case studies highlight the need for rapid detection to isolate infected populations and limit the spread in the surrounding population. Adding novel matrices to our already approved sample types will allow for sampling in a multitude of situation, whether they be at a slaughterhouse, a carcass, or in a feed yard.

Chapter 2 - Evaluation of postmortem samples for detection of ASFV

2.1 Introduction

African swine fever virus (ASFV) is a severe trade limiting swine disease. ASFV, the only member of the *Asfarviridae* family, is a large, enveloped *ds*DNA virus. ASFV is comprised of more than 180 kb pairs, encoding over 150 genes and between 150-200 proteins (Ramirez-Medina et al., 2022; Wang et al., 2019). Morbidity and mortality in domestic pigs' approach 100% with virulent strains (Galindo & Alonso, 2017). In the United States, a 10-year projection estimates damages to be $50 billion and a loss of 140,000 jobs (Carriquiry et al., 2020). The outbreak in China has been devastating, in 2019 and 2020, combined herd culling represented a 32% loss of the annual global pig production (Schambow et al., 2022). Due to the extreme infectivity and transmissibility of ASFV, surveillance and rapid diagnostics is a critical tool to curtail disease spread.

Transmissibility in feed was demonstrated by Dee et al. (2018), using a simulated trans-pacific model, ASFV remained viable in a multitude of imported feed matrices such as soybean meal and complete feed. Further research demonstrated that domestic pigs can be infected through consumption of contaminated feed (Niederwerder et al., 2019). Therefore, introduction into the United States could occur through oral-nasal contact from contaminated imported feed (Niederwerder et al., 2021). Despite knowing that ASFV can spread via natural consumption most studies collect samples after inoculation through the more reliable method of intramuscular injection (Lee et al., 2021; Niederwerder & Hefley, 2021). This lack of information leaves a knowledge gap in regards to infection through natural consumption.

At a minimum, samples submitted for postmortem testing should follow USDA-APHIS recommendations and include spleen, tonsil, gastrohepatic lymph node, renal lymph node, and inguinal lymph node. Ideally, a qualified veterinarian who has passed certification for Foreign Animal Disease Diagnostician (FADD) should necropsy 1-10 pigs collecting these tissues in duplicate. This collection should be done in accordance with guidelines published in The Foreign Animal Disease Investigation Manual (Flannery et al., 2020; *Swine Hemorrhagic Fevers: African and Classical Swine Fevers Integrated Surveillance Plan*, 2022). Apart from FADI, these samples are not often collected on farms and require a full necropsy by a trained professional. Necropsies involve opening the body cavity and potentially risk further contamination of the environment.

The ideal sample matrix would be sensitive to diagnostic tests and would not require opening of the body cavity. PCR is the diagnostic test most often used for initial screen of ASFV samples. Results are often presented as cycle threshold (Ct) values, which can be used interchangeably with the term quantification cycle (Cq). The Cq value are directly related to starting quantity (SQ) of the target. Because SQ is analyzed on a log scale, less statistical variation is present, when compared to Cq value (Ruiz-Villalba et al., 2021) This manuscript analyzed Log10 SQ PCR values of additional samples that can be collected quickly, safely, and by non-veterinary staff for the rapid detection of ASFV Georgia/07 in pigs that orally consumed ASFV Georgia/07 inoculated media.

2.2 Materials and Methods

ASFV inoculum preparation

Final inoculation dose of ASFV Georgia/07 was 100 ml of 10^4 $TCID_{50}$/ml. 10 ml of 10^6 $TCID_{50}$/ml ASFV Georgia/07 splenic homogenate was diluted into 95 ml RPMI 1640 medium (Gibco) for a final concentration of 10^5 $TCID_{50}$/ml. In a wide-mouth high-density polyethylene bottle (Nalgene, Thermo Fisher Scientific), 90 ml of RPMI media and 10ml of the newly created 10^5 $TCID_{50}$/ml ASFV splenic homogenate were mixed. Once combined, bottles were vortexed for 10 seconds, placed on ice, and immediately delivered to animal rooms for administration. One control virus vial was sent to each room and remained on ice during the duration of inoculation and then returned to the lab to be back titered to confirm dose. Confirmatory testing of infectious titers was determined on control virus vial by performing back-titration on PAMs using endpoint titration assay ($TCID_{50}$/ml) to ensure accurate dosing and no loss of viability occurred during transport. Negative controls were administered 100ml of sterile RPMI media without virus, this was prepared prior to any virus work to ensure no contamination occurred.

Swine Bioassay

Weaned, male pigs (n = 12; 7-8 weeks old) were sourced from a single high-health commercial operation. Pigs were individually housed and placed six per room. Pens were separated by roughly 1.5 m between pens and pens were placed in 3 X 2 configuration. The raised, stainless-steel pens were 1.9 m^2 and were made of slotted fiberglass floor, three sides of the pen were solid and the fourth side, facing the center of the room, was composed of slotted bars and a gate. Each pen was fitted with an attachable and movable feeder and a nipple drinker to provide water. In addition to the drinker, gravity fed ½” X 26” galvanized steel pipe and bracket drinkers

(#270170A, qcsupply) were fitted with a specialized Arato 76 nipples (#8760A, Agworks) and used to deliver challenge inoculum. Efforts were made to reduce aerosols including specialized cleaning protocols of the pens. One out of the six total pigs in each room were designated as the negative control pig to monitor for cross contamination. Pigs were monitored up to twice daily for clinical signs. Pigs were euthanized via Fatal Plus injection after presentation of clinical signs and meeting humane endpoint criteria, necropsy was immediately performed and samples collected. Bioassay was performed under BSL-3Ag conditions located at the Biosecurity Research Institute at Kansas State University. All research was reviewed and approved by the Kansas State University Institutional Animal Care and Use Committee and the Institutional Biosafety Committee. Animal work followed the guidelines and regulations of the Federation of Animal Science Societies Guide for the Care and Use of Agricultural Animals in Research and Teaching, along with the US Department of Agriculture's Animal Welfare Act and Animal Welfare Regulations.

Sample processing

All samples were collected at time of necropsy. Prior to DNA extraction, tissues (spleen, bone marrow, conjunctiva, diaphragm, ear notch, muscle, tail notch, feces, tonsil, lymph nodes) were homogenized to achieve cell lysis and release of viral DNA. Tissues were cut into 2mm sized pieces and placed inside a 2 ml homogenizer tube containing 2.8 mm ceramic beads (#19-628, Omni-International). Tissues were added to homogenizer tubes in a 1:1 ratio with phosphate-buffered saline (PBS) supplemented with penicillin/streptomycin and amphotericin B, 1/3 of the vial was left empty to achieve proper homogenization. Tubes were loaded into the Bead Rupter 4 (#25-010, Omni-International) and ran for four cycles at speed of four with 30 seconds in between cycles. Homogenized tissues were then centrifuged for 30 minutes at 6,000xg and

supernatant collected and stored at -80°C until processed for extraction. Additional swab samples (spleen, peritoneal fluid, muscle, conjunctival, preputial) were collected using a sterile, dry, polyester-tipped swab (#1U054S01, Copan Diagnostics) rotated in site for 10 seconds. Swabs were then placed in a tube containing 3ml universal transport medium (#3C047N, Copan Diagnostics). Swabs were snapped off at the pre-molded break point and stored at 4°C until processing. Swab tubes were then vortexed for 30 seconds and allowed to sit for 5 minutes to allow particulates to settle. Supernatant was then aliquoted into cryovials and stored at -80°C until testing. Fluid samples (urine, ocular fluid) were collected using a sterile syringe and needle, dispensed inside a cryovial, and stored at -80°C until testing.

Necropsy

Necropsies were performed by a trained pathologist and generally followed the guidelines outlined in (Netherton, 2022). Gross pathological lesions were accessed in a similar fashion to (Sánchez-Cordón et.al., 2022). To reduce subjectivity, lesions were noted as either present or absent and severity was noted when applicable. Notations of congestions, enlargement, edematous were noted when present. A total of 15 tissues were observed for gross lesions (skin, oral cavity/upper respiratory, lungs, heart, thymus, liver, stomach, small intestine, large intestine, pancreas, kidneys, spleen, reproductive tract, joints/bone marrow, and central nervous system (CNS). Table 2.1 highlights tissues that were consistently affected across all pigs. Control pigs were included separately in prevalence data. Both control pigs were noted to have pneumonia, as they had nondetectable levels of ASFV on PCR, it is possible this was caused by a secondary infection. One control pig had enlarged sternal LN. No other gross lesions were noted on control pigs.

DNA Extraction

Nucleic acid was extracted using the MagMax™ Core Nucleic Acid Purification Kit (Thermo Fisher Scientific). Fifty microliter of sample homogenate was added to 10 µl of Proteinase K in a 96-well plate or microcentrifuge tube. The 96-well plate was allowed to shake for two minutes then 130 µl of bead mix containing lysis buffer and beads, was added, and again was placed on shaker for three minutes. The 96-well plate or microcentrifuge tube was placed on a magnet for three minutes to capture the beads and the supernatant was collected and discarded. Wells were washed twice with 150 µl of wash buffer 1 and 2, each time magnetic beads were captured, and supernatant discarded. Wells were then eluted with 50 µl of elution buffer and placed on shaker for 3 minutes. The plate was set on a magnetic stand and supernatant was transferred to a nuclease free 96-well plate, sealed, and stored at -20°C until PCR was performed within 24-hours.

ASFV PCR

Extracted ASFV DNA was amplified using primers and probes designed to target conserved regions of the capsid protein p72 as described by (King et al., 2003). PCR protocol was run as previously described by (Niederwerder et al., 2022) according to manufacturer's instructions (VetAlert™African Swine Fever Virus DNA Test Kit, Tetracore®). In summary, 5 ul of samples were combined with 20 ul of master mix (19.25 ul ASF Mastermix and 0.75 ul Enzyme Solution) in a Hard-Shell® 96-well PCR plate (Bio-rad Laboratories). The plate was sealed and quickly centrifuged to remove air bubbles and ran on the CFX96 Real-Time system (Bio-Rad Laboratories). The PCR protocol included the following steps: 48°C for 15 min, 95°C for 2 min,

and then 45 cycles of 95°C for 10 sec and 60°C for 40 sec. Spleen homogenate from a previously tested and confirmed positive pig was used as a positive control. Sterile commercially sourced PBS was used as a negative, No Template Control (NTC). Standard curve was generated from vials provided in VetAlert™African Swine Fever Virus DNA Test Kit. Four vials, each containing a different concentration of ASFV DNA ranging from 10^2-10^5 dilutions to generate the standard curve.

Statistical analysis

Analysis was performed in Microsoft Excel version 2209. Graphs were prepared in GraphPad Prism® 9.5.1 for Windows (La Jolla, CA). This study analyzed both Cq values and $\log_{10}$ SQ. Due to known variability that occurs with Cq in regards to statistical analysis, only $\log_{10}$ SQ was used for primary analysis and Cq values are intended to be supplemental (no provided in this manuscript). Samples with similar Cq and $\log_{10}$ SQ average or variance were analyzed, a total of 73 comparisons were made. Paired t-Test ($p \leq 0.05$) analysis was used between samples to compare mean PCR $\log_{10}$ SQ values. In respect to the small sample size, normality and variance of data was confirmed using Shapiro-Wilk test of normality and f-test, respectively (See Appendix). Threshold criteria for negative Cq values was determined by manufacture standards (*VetAlert*™African Swine Fever Virus DNA Test Kit, Tetracore®), $Cq \leq 38.0$ was considered positive. Negative control samples and two specimens (#80 conjunctival swab, #88 preputial swab) had non-detectable limits of DNA. To reduce variability, these samples, along with negative samples, were assigned a Cq value of 39.0 or a 0 for $\log_{10}$ SQ analysis. Negative control pigs were included in graphical representations of Cq and $\log_{10}$ SQ values but excluded from

sample analyses. Prevalence was calculated for gross necropsy lesions with negative controls included separately from total.

Experimental Design

Study design included two replicates, each replicate containing 6 pigs. Pigs were acclimated to gravity fed nipple drinkers for 3 days prior to challenge material being delivered. To encourage rapid consumption of challenge inoculum, food and water were withheld 10-14 hours prior to delivery of sterile liquid media. During the acclimation period, 100 ml of RPMI media was delivered into the gravity fed drinkers and pigs were observed to confirm all liquid was consumed. If pigs became averse to drinkers, media was delivered in a bowl. After consumption, pigs were given *ad libitum* access to feed and water until the evening when it was once again restricted until the following morning. On the fourth day, 100 ml of 10^4 $TCID_{50}$/ml ASFV Georgia/07 diluted splenic homogenate solution in RPMI was delivered to pigs (n=10). Control pigs (n=2) received 100ml of sterile RPMI media. After challenge, pigs were allowed *ad libitum* access to food and water for the remainder of the study.

2.3 Results

Necropsy Results

All ASFV inoculated pigs displayed congested and/or enlarged submandibular lymph nodes (100%) and 90% of pigs were noted to have congested and/or enlarged tonsils. This is consistent with the literature that viral pathogenesis is largely affected by route of infections (Salguero et. al., 2020; Dixon et al., 2020; Lee et.al., 2021). Following natural consumption of ASFV inoculum, the virus will first contact the tonsils and submandibular lymph nodes. These tissues will receive the most contact time with the virus and not surprisingly, will likely show as the most prevalent gross lesion. As viremia progresses, ASFV is disseminated throughout the lymphatic system. This is represented in the prevalence data from tracheobronchial lymph nodes (80 %, $\log_{10}$ SQ = 5.09), gastrohepatic lymph nodes (50%, $\log_{10}$ SQ = 5.24), inguinal lymph nodes (40%, $\log_{10}$ SQ = 4.98), and retropharyngeal lymph nodes (30%, $\log_{10}$ SQ = 5.63). Individual differences in immunological effects have been documented in the field (Salguero et. al., 2020). Our findings are consistent with this literature as prevalence of gross lesions do not consistently reflect average $\log_{10}$ SQ values of the lymph nodes.

Table 2.1

Prevalence of Gross Lesions in Consistently Affected Tissues of both Control and ASFV Georgia/07 Inoculated Pigs

		Lung	Spleen	Tonsil	Sternal LN	Trach.b. LN	Sub.Man. LN	Inguinal LN	Ret.phar. LN	Gas.hep.LN
		pneumonia (congested and/or edematous)	congested and/or enlarged	congested and/or enlarged	congested and/or enlarged	congested and/or enlarged	congested and/or enlarged	enlarged and/or edematous	congested and/or enlarged	congested and/or enlarged
Number Affected	Control	2/2	0/2	0/2	1/2	0/2	0/2	0/2	0/2	0/2
	Inoculated	7/10	7/10	9/10	7/10	8/10	10/10	4/10	3/10	5/10
% Affected	Control	100%	0%	0%	50%	0%	0%	0%	0%	0%
	Inoculated	70.0%	70.0%	90.0%	70.0%	80.0%	100%	40.0%	30.0%	50.0%

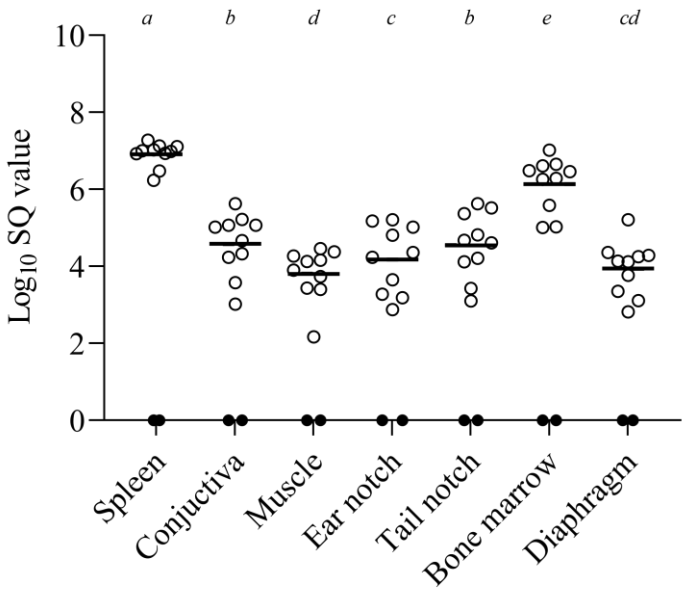
Note. Summary of gross lesions from all twelve pigs (including controls). LN=lymph node, Trach.b = tracheobronchial, Sub.Man.= submandibular, Ret.phar.= retropharyngeal, Gas.hep = gastrohepatic

Comparison of tissues

All tissues had average SQ values within detectable limits, as shown in Figure 2.1 and had 100% sensitivity for detection of ASFV DNA. As expected, not only did spleen have the highest average SQ at 6.91 but also the lowest SD of ± 0.30. As supported by other literature, spleen remains the most sensitive and least variable tissue sample. Paired t-test was performed on all samples in comparison to spleen and analyses showed significant difference ($p \leq 0.05$) between all comparisons (Table 2.3). Indicating that no sample tested is as sensitive as spleen tissue. Bone marrow was the second most sensitive tissues and had an average $\log_{10}$ SQ value of 6.14 (SD ± 0.66). Tail notch and conjunctiva had similar $\log_{10}$ SQ values of 4.55 (SD ± 0.81) and 4.58 (SD ± 0.76), respectively. Paired t-test showed they had no significant difference ($p \leq 0.41$). Indicating that either tail notch or conjunctiva can reliably detect ASFV DNA and be collected depending on the circumstance. Similarly, the $\log_{10}$ SQ values of ear notch (4.18, SD ± 0.83) and diaphragm (3.94, SD ± 0.66) were comparable and showed no significant difference ($p = 0.06$). Diaphragm (3.94, SD ± 0.44) and muscle (3.81, SD± 0.65) also had no significant differences and could be comparable sample matrices ($p = 0.13$).

Figure 2.1

Individual log₁₀ SQ Tissue Values for Control and ASFV Georgia/07 Inoculated Pigs with Averages



Note. Individual values of inoculated pigs represented as white circles with black border. Control pigs are shown as solid black circles. Sample mean is shown as solid black line. Italicized letters at the top represent statistical significance. Samples that have the same letter listed above their group have no significant difference, thus are comparable sample types. Samples that have different letters have significant differences ($p \leq 0.05$) and are not comparable tissue types.

Table 2.2

All Sample Type Comparisons with Similar Average log_{10} SQ Values and Variance with Corresponding p-values

Tissue Comparison		p-value
Tissues compared with spleen		
conjunctiva	spleen	1.34E-07
tail notch	spleen	1.13E-06
muscle	spleen	1.10E-09
bone marrow	spleen	0.000
ear notch	spleen	3.09E-07
tonsil	spleen	0.000
diaphragm	spleen	6.23E-09
Swabs compared with spleen		
preputial swab	spleen	6.24E-07
peritoneal Fld Swb	spleen	6.48E-07
conjunctiva swab	spleen	8.89E-07
spleen swab	spleen	7.58E-05
muscle swab	spleen	1.40E-08
Fluids compared with spleen		
ocular fluid	spleen	1.49E-07
urine	spleen	7.35E-08
feces	spleen	1.80E-07
Lymph nodes compared with spleen		
mesenteric LN	spleen	2.29E-06
submandibular LN	**spleen**	**0.338**
gastrohepatic LN	spleen	0.000
tracheobronchial LN	spleen	1.60E-05
inguinal LN	spleen	5.14E-05
retropharyngeal LN	spleen	0.000
popliteal LN	spleen	0.000
Sternal	spleen	2.46E-05
Tissue with corresponding swab		
spleen swab	spleen	7.58E-05
muscle swab	muscle	0.039
conjunctiva swab	conjunctiva	7.00E-05
Lymph nodes compared with lymph nodes		
submandibular LN	tonsil	0.000
retropharyngeal LN	Sternal LN	0.058
gastrohepatic LN	**sternal LN**	**0.382**
tracheobronchial LN	**inguinal LN**	**0.258**
tonsil	**retropharyngeal LN**	**0.077**
popliteal LN	**gastrohepatic LN**	**0.185**

gastrohepatic LN	**tracheobronchial LN**	**0.250**
tracheobronchial LN	**mesenteric LN**	**0.181**
tonsil	popliteal LN	0.041
popliteal LN	**sternal LN**	**0.116**
gastrohepatic LN	Inguinal LN	0.019
inguinal LN	**mesenteric LN**	**0.397**
retropharyngeal LN	**popliteal LN**	**0.289**
popliteal LN	**tracheobronchial LN**	**0.096**
sternal LN	**tracheobronchial LN**	**0.243**
gastrohepatic LN	**mesenteric LN**	**0.061**
retropharyngeal LN	**gastrohepatic LN**	**0.120**
popliteal LN	inguinal LN	0.016
sternal LN	inguinal LN	0.025
tonsil	Sternal LN	0.004
Fluids compared with fluids		
urine	**feces**	**0.094**
ocular fluid	urine	0.010
ocular fluid	**feces**	**0.335**
Swabs compared with swabs		
peritoneal Fld Swb	**muscle swab**	**0.205**
peritoneal Fld Swb	conjunctiva swab	0.000
muscle swab	conjunctiva swab	0.012
conjunctiva swab	**preputial swab**	**0.336**
Tissues compared with tissues		
conjunctiva	**tail notch**	**0.413**
ear notch	muscle	0.043
conjunctiva	ear notch	0.013
diaphragm	**muscle**	**0.131**
tail notch	ear notch	0.016
ear notch	**diaphragm**	**0.067**
Comparison of samples with comparable SQ values		
submandibular LN	bone marrow	0.001
mesenteric LN	conjunctiva	0.002
feces	**conjunctiva swab**	**0.061**
gastrohepatic LN	**spleen swab**	**0.401**
bone marrow	**tonsil**	**0.442**
muscle	**peritoneal fluid swb**	**0.136**
conjunctiva swab	**ocular fluid**	**0.241**
mesenteric LN	**spleen swab**	**0.329**
bone marrow	retropharyngeal LN	0.042
muscle swab	**urine**	**0.202**
ocular fluid	**preputial swab**	**0.115**
sternal LN	**spleen swab**	**0.420**
muscle swab	**feces**	**0.090**
feces	**preputial swab**	**0.100**

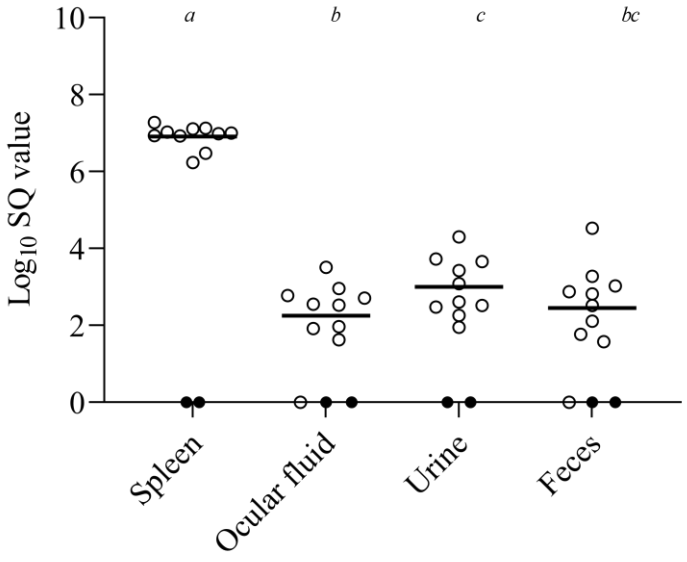
Note. P-values represent one-tailed paired t-Test. Samples in 'Bold' represent sample comparison with no statistically significant differences.

Comparison of fluids

log_{10} SQ values of Ocular fluid (2.26, SD ± 0.92), urine (3.00, SD ± 0.72), and feces (2.45, SD ± 0.11) were grouped as fluids and compared to spleen (Figure 2.2). All fluids had significant differences when compared to spleen ($p \leq 0.0001$). Diagnostic sensitivity for ocular fluid and feces was 90.0%. Whereas urine had 100% sensitivity. No significant difference was found between feces and urine ($p = 0.09$) or feces and ocular fluid ($p = 0.33$). Significant differences were found between urine and ocular fluid ($p = 0.01$).

Figure 2.2

Individual log_{10} SQ Fluid Values for Control and ASFV Georgia/07 Inoculated Pigs with Averages



Note. Individual values of inoculated pigs represented as white circles with black border. Control pigs are shown as solid black circles. Sample mean is shown as solid black line.

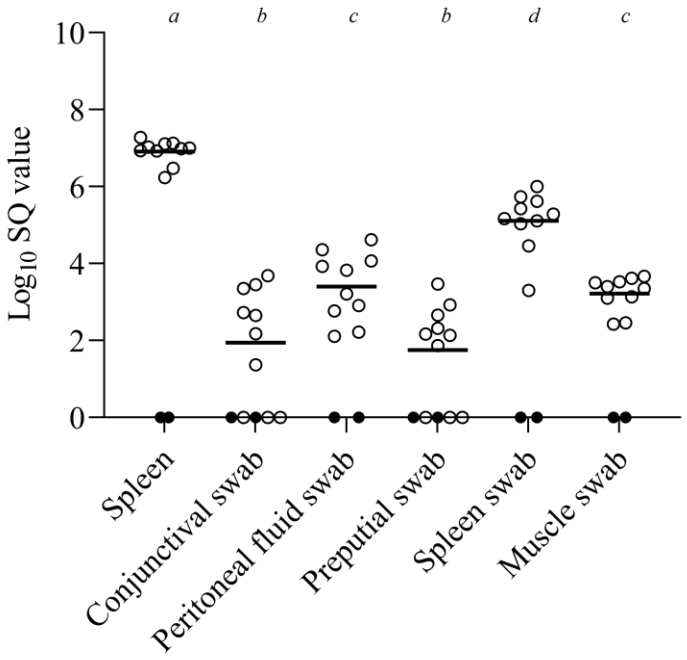
Italicized letters at the top represent statistical significance. Samples that have the same letter listed above their group have no significant difference, thus are comparable sample types. Samples that have different letters have significant differences ($p \leq 0.05$) and are not comparable tissue types.

Comparison of swabs

Peritoneal, spleen, and muscle swab all had 100% sensitivity. Conjunctival and preputial swabs both had 70.0% sensitivity (Figure 2.3). As expected, spleen swab had the highest average $\log_{10}$ SQ value of 5.12 (SD ± 0.73) of all swab samples. However, all swabs had significant differences when compared to spleen sample ($p \leq 7.5E\text{-}05$). Significant difference was also found between tissue and its corresponding swab sample ($p \leq 0.03$). In comparison between swabs, no significant difference was found between peritoneal swab and muscle swab ($p = 0.20$) or conjunctiva swab and preputial swab ($p = 0.33$). Significant differences were found between conjunctiva swab and muscle swab ($p = 0.01$) along with peritoneal fluid swab and conjunctiva swab ($p = 0.0007$). As noted earlier, swab composition can be changed, i.e., polyester vs. flocked (nylon) to potential increase in sensitivity.

Figure 2.3

Individual log₁₀ SQ Swab Values for Control and ASFV Georgia/07 Inoculated Pigs with Averages



Note. Individual values of inoculated pigs represented as white circles with black border. Control pigs are shown as solid black circles. Sample mean is shown as solid black line. Italicized letters at the top represent statistical significance. Samples that have the same letter listed above their group have no significant difference, thus are comparable sample types. Samples that have different letters have significant differences ($p \leq 0.05$) and are not comparable tissue types.

Comparison of lymph nodes

All lymph nodes had 100% sensitivity however they also had one of the highest average SD of ± 0.86. Submandibular LN had the highest average $\log_{10}$ SQ value of 6.88 (SD ± 0.36) and notably, it was the only sample comparable to spleen, showing no significant difference ($p = 0.33$). Inguinal and mesenteric LN had the lowest $\log_{10}$ SQ values of lymph nodes (4.98 and 4.95, respectively). All lymph nodes were significantly different from spleen ($p \leq 0.05$). Comparison between lymph nodes revealed several comparable samples (Table 2.6). No significant differences were found between the following pairs indicating possible replacements if one LN is unavailable: popliteal/sternal ($p = 0.11$), popliteal/tracheobronchial ($p = 0.09$), gastrohepatic/tracheobronchial ($p = 0.25$), gastrohepatic/mesenteric ($p = 0.06$), inguinal/mesenteric ($p = 0.39$), tonsil/retropharyngeal ($p = 0.07$), sternal/tracheobronchial ($p = 0.24$), retropharyngeal/popliteal ($p = 0.28$), popliteal/gastrohepatic ($p = 0.18$), gastrohepatic/sternal ($p = 38$), tracheobronchial/inguinal ($p = 25$), tracheobronchial/mesenteric ($p = 0.18$), retropharyngeal/gastrohepatic ($p = 0.12$).

Table 2.3

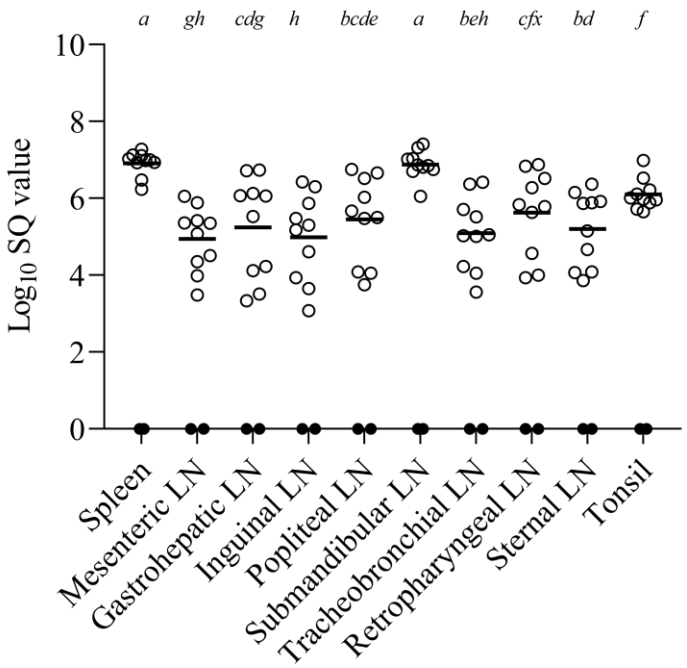
Average log_{10} SQ and p-values for Lymph Node Comparison

Tissue Comparison	Average log_{10} SQ Value	p-value
popliteal LN	5.45	0.11
sternal LN	5.20	
popliteal LN	5.45	0.09
tracheobronchial LN	5.09	
gastrohepatic LN	5.24	0.25
tracheobronchial LN	5.09	
gastrohepatic LN	5.24	0.06
mesenteric LN	4.95	
inguinal LN	4.98	0.39
mesenteric LN	4.95	
tonsil	6.11	0.07
retropharyngeal LN	5.63	
sternal LN	5.20	0.24
tracheobronchial LN	5.09	
retropharyngeal LN	5.63	0.28
popliteal LN	5.45	
popliteal LN	5.45	0.18
gastrohepatic LN	5.24	
gastrohepatic LN	5.24	
sternal LN	5.20	0.38
tracheobronchial LN	5.09	
inguinal LN	4.98	0.25
tracheobronchial LN	5.09	
mesenteric LN	4.95	0.18
retropharyngeal LN	5.63	
gastrohepatic LN	5.24	0.12

Note. LN = lymph node; p-value represents one-tailed paired t-Test

Figure 2.4

Individual log$_{10}$ SQ Lymph Node Values for Control and ASFV Georgia/07 Inoculated Pigs with Averages



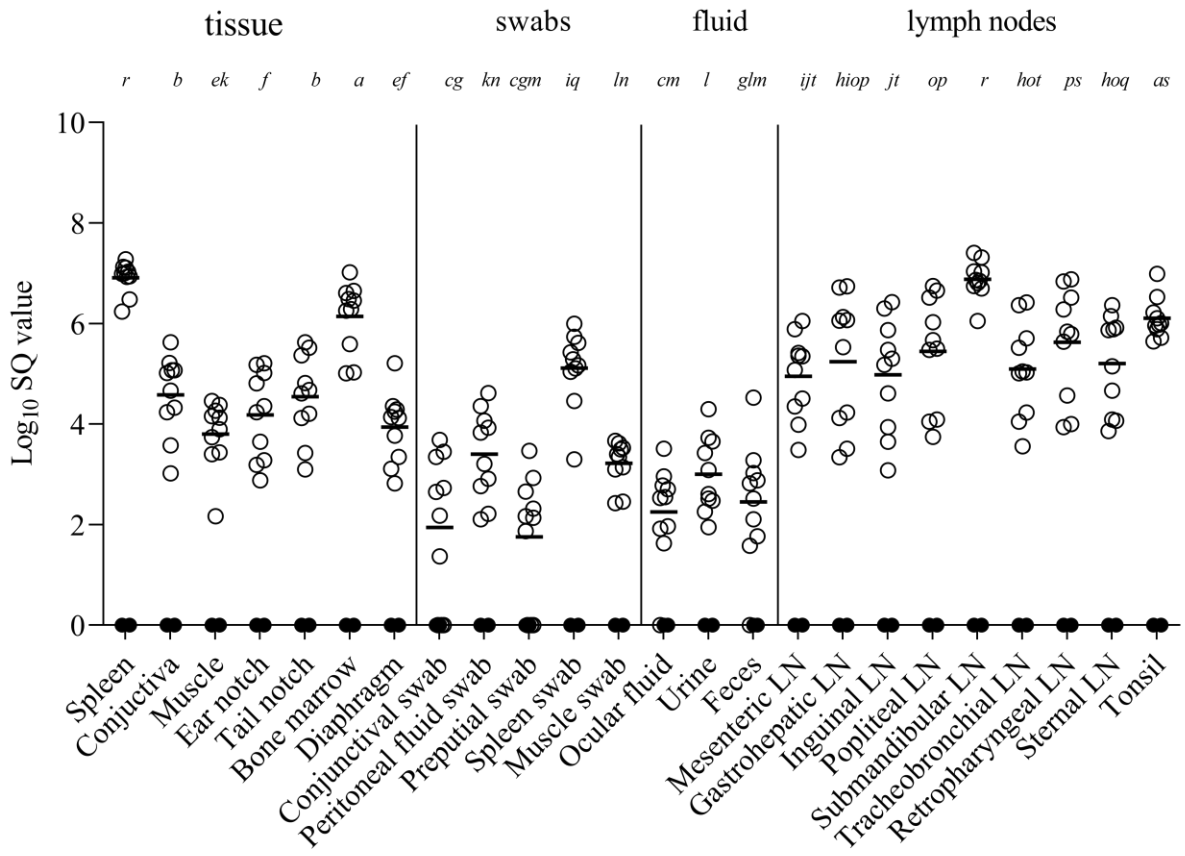
Note. Individual values of inoculated pigs represented as white circles with black border. Control pigs are shown as solid black circles. Sample mean is shown as solid black line. *Italicized* letters at the top represent statistical significance. Samples that have the same letter listed above their group have no significant difference, thus are comparable sample types. Samples that have different letters have significant differences ($p \leq 0.05$) and are not comparable tissue types.

General comparisons

Samples with similar average $\log_{10}$ SQ values were compared and no significant differences were found between the following pairs: gastrohepatic LN/ spleen swab ($p = 0.40$), mesenteric LN/spleen swab ($p = 0.32$), peritoneal swab/muscle ($p = 0.13$), urine/muscle swab ($p = 0.20$), conjunctiva swab/ocular fluid ($p = 0.24$), preputial swab/ocular fluid ($p = 0.11$), tonsil/bone marrow ($p = 0.44$), sternal LN/ spleen swab ($p = 0.41$), feces/conjunctiva swab ($p = 0.06$), feces/preputial swab ($p = 0.10$), muscle swab/feces ($p = 0.09$) (Table 2.3). It is important to note that while these samples may have comparable $\log_{10}$ SQ values, some do differentiate on SD and sensitivity. For example, conjunctival swab and ocular fluid were statistically comparable based on average $\log_{10}$ SQ values, however their sensitivity differed by 14%.

Figure 2.5

Individual log₁₀ SQ Values for all Samples Including Control and ASFV Georgia/07 Inoculated Pigs with Averages



Note. Individual values of inoculated pigs represented as white circles with black border. Control pigs are shown as solid black circles. Sample mean is shown as solid black line. *Italicized* letters at the top represent statistical significance. Samples that have the same letter listed above their group have no significant difference, thus are comparable sample types. Samples that have different letters have significant differences ($p \leq 0.05$) and are not comparable tissue types.

2.4 Discussion

African swine fever virus continues to pose an imminent risk to the United States. Currently, approved samples for the detection of ASF require either a full necropsy or are not typically collected on swine farms. Furthermore, available literature focusing on the expansion of tissue sensitivity for the detection of ASFV do not expand on the natural consumption model. Even though, it has been shown that ASF can remain infective after transoceanic shipment of imported feed. This study provides invaluable insights on novel sample sensitivity for the detection of ASFV after inoculation through natural consumption.

While only one sample, submandibular lymph node, had the level of genomic material comparable to that of spleen, detectable $\log_{10}$ SQ values were seen in the majority of sample types. Field conditions are rarely as ideal as the research setting. For example, hunters participating in passive surveillance may come across a decomposed carcasses and may be unable to collect an approved sample type. Samples such at bone marrow, tail notch and ear notch could serve as a sample matrix and are quickly and easily collected by untrained persons such as hunters or swine farm workers.

A highlight of this study was the use of sample collection via swab, reducing the need to fully open the body cavity and risking further contamination of the environment. Swabs pose a significant advantage and are the more pragmatic approach compared to collection of tissues. Swabs samples are quick to collect, staff can easily be trained on techniques, and they can be employed in a variety of settings such as swine farms, slaughterhouses, or processing plants. While the swab samples in this study did have significantly lower $\log_{10}$ SQ values and reduced sensitivity, the advantages should be weighed depending on the circumstances.

Currently, no scientific literature has employed a study design such as this study to compare different tissue types utilizing a natural consumption model. Multiple comparisons were made between tissue types to allow the industry to select a sample for diagnostic testing depending on what is available in the animal in conjunction with the training of their staff. Statistically, feces and urine had comparable $\log_{10}$ SQ values, allowing for increased surveillance on swine farms. In our experience, popliteal lymph node was difficult to excise due to its small size and requires trained persons to locate. Analyses performed by herein showed that sternal and tracheobronchial lymph nodes are comparable tissue types. Understanding how pigs may have become infected along with assessing gross lesions will be tools to guide lymph node selection. Pigs in this study were inoculated via natural consumption of liquid, ergo gross lesions and high $\log_{10}$ SQ values are seen most prominently in tonsil and submandibular lymph nodes. If pigs were infected through a tick bite, one might see more prominent lesions nearest the bite site such as inguinal or popliteal.

The small sample size of this study was a limitation we attempted to reduce by using a repeated measurements design to increase power (see Appendix). This design not only increased confidence in analysis but also allowed within subject comparison which increases confidence of suitable sample type replacements. Further research is needed to expand on sample types with reduced sensitivity such as conjunctiva and preputial swabs. It may be possible to optimize sample quality through use of different swab types. The supply chain disruption triggered by the SARS-CoV-2 epidemic has brought forth multiple studies which expand on different swab types and their ability to increase sensitivity in PCR testing. This study used dry, polyester-tipped swabs; it is possible that sensitivity could be increased with use of a different swab type. Cell

culture assay would greatly increase the confidence of these matrices along with providing invaluable data on potential infectivity and should certainly be expanded upon.

While it has been well documented that foreign animal diseases such as ASFV remain infective in feed after trans-oceanic shipment, there continues to be limited research expanding on sample sensitivity after inoculation via natural consumption. It is paramount to increase the duality of sample matrices that provide both reliable, robust results and can be pragmatically collected by untrained staff. Results reported in this study provides additional postmortem samples and their comparisons which will significantly increase our nations surveillance plan.

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Appendix A - Parametric statistical analysis

The paired t-Test, also known as the students t-Test, is a parametric test used to compare means between two groups. This test is often used to assess independent variables and differences in the same group, pre and post a certain event. The null hypothesis states that both means are statistically equal while the alternative hypothesis states that both means are statistically different(Mishra et al., 2019) (Equation A.1)

Equation A.1.

Equation of Null and Alternative Hypothesis for Paired t-Test

$$H_0 = 0$$

$$H_1 \neq 0$$

The paired t-test has a few assumptions that need to be ascertained prior to using to ensure that a nonparametric test is not more suitable. As stated, the paired t-test is a parametric test, meaning the population needs to be normally distributed and have equal variance. A limitation of this study was its small sample size. Normal distribution may not be captured on a histogram plot due to the small sample size. If sample results are not centered around the mean, then an accurate comparison cannot be made using this parametric t-Test. Because of this, confirmatory testing was used to ensure normality and variance (Mishra et al., 2019). The Shapiro-Wilks test (Equation A.2) was used to assess normality of the population and f-test was used to confirm variance.

Equation A.2

Equation for Shapiro-Wilks Test to Assess Normality

$$W = \frac{\left(\sum_{i=1}^{N} a_i f_i\right)^2}{\sum_{i=1}^{N} (f_i - \bar{f})^2}.$$

N is equal to the number of observations in the population. f_i is the value of the ordered sample. a_i is a tabulated coefficient found in normality charts. Data is first entered into a table in ascending order (smallest to largest). Mean is then calculated for the population. Then the difference of the individual sample is taken from the mean and squared $(f_i - \bar{f})^2$. While a_i is readily found from coefficient tables for Shapiro-wilks test only. a_i is then multiplied by its corresponding sample value. Sum values are then taken for $(f_i - \bar{f})^2$ and $a_i f_i$. *W* represents the test statistic. To compare the test statistic *W*, the *W* numerator and *W* denominator first need to be found. *W* numerator is simply the sum of a_i f_i squared. The *W* denominator is $(f_i - \bar{f})^2$. p-value is determined by using a coefficient table and finding the test statistic that corresponds to the p-value and sample size, which was 0.842 for this study. If *W* is greater than 0.842 then the p-value is greater than 0.05, we retain the null hypothesis and determine that the sample is normally distributed (Flynn, 2010). As seen with the segment $(f_i - \bar{f})^2$, variation from the mean is a large part of this analysis. As such, variance also needs to be confirmed. This study utilized the f-test for variance.

The f-Test for variance does as the name suggests, tests two populations for variance. As seen in the equation for paired t-Test and Shapiro-Wilks test, variance carries heavy weight that could skew the sample analysis, increasing the rate of type I or type II error. It is then critical to confirm that sample data has approximately normal variance.

Equation A.3

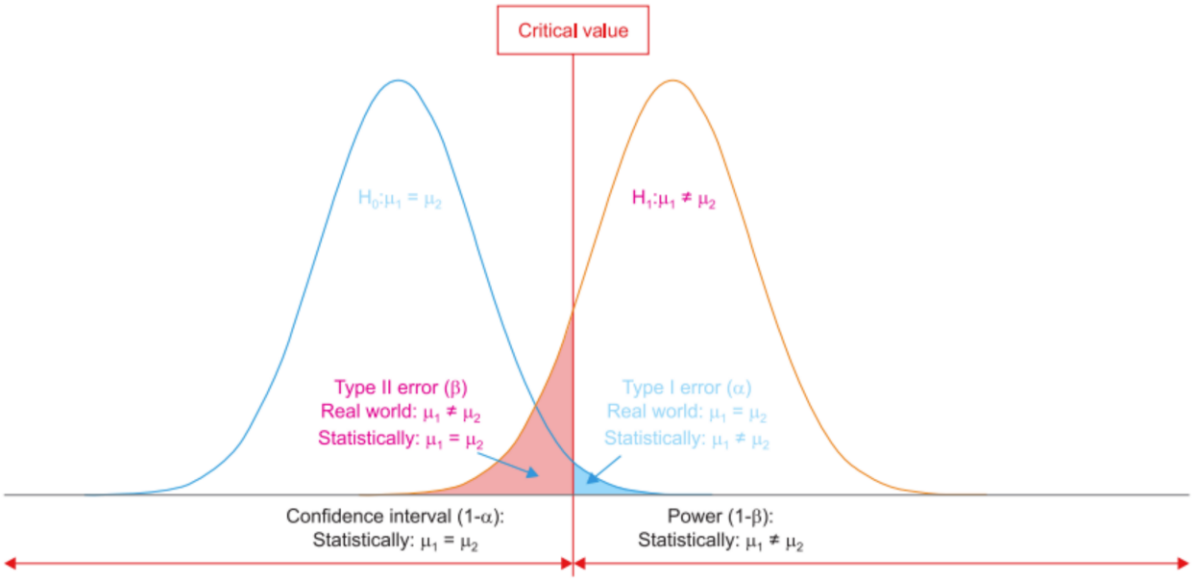
Equation for f-test to Confirm Variance of Sample Population

$$F = \sigma_1^2 \, / \, \sigma_2^2$$

Where σ_1^2 represents the standard deviation from sample 1 squared and σ_2^2 represents the standard deviation from sample 2 squared. Critical values for F are readily found online. If *F* value is smaller than *F*-critical, then we can say that the two standard deviations are statistically similar. The f-test was run on every tissue comparison, 73 in total. Overall, most sample comparisons did have significantly different variance. This deviation from the requirements of a pair t-test can be partially overcome by the study design. We used a repeated measure design which allowed multiple comparisons. Using repeated measures adds additional power, reducing the likelihood of Type I error.

Appendix A Figure A.1.

Graphical Representation of Hypothesis Testing

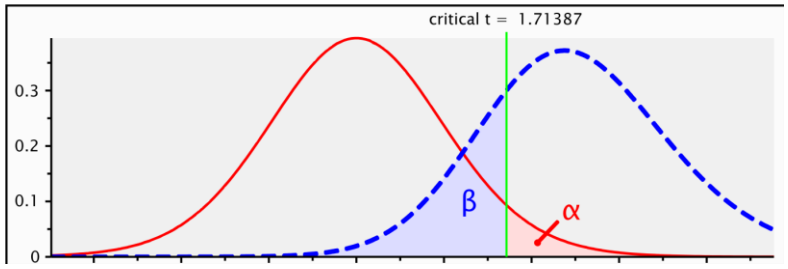


Note. H0: null hypothesis, H1: alternative hypothesis, μ1 and μ2: mean values of two groups (Kim & Park, 2019)

Post-hoc analysis was completed using G*power version 3.1.9.6 (Germany). Figure A.2 shows that the α – value is set inside the alternative hypothesis (the blue curve). This overlap conveys that with a total sample size of 24 (sample 1 n=12, sample 2 n=12), α (p-value) = 0.05, and effect size set at 0.5, there is a 76% chance that our paired t-test will detect a difference, if that difference exists. Figure A.3 shows us that if we want the desired 95% confidence rate, we require a sample size of 45 postmortem samples.

Appendix A Figure A.2

Post-hoc Analysis to Determine Power of Sample Size

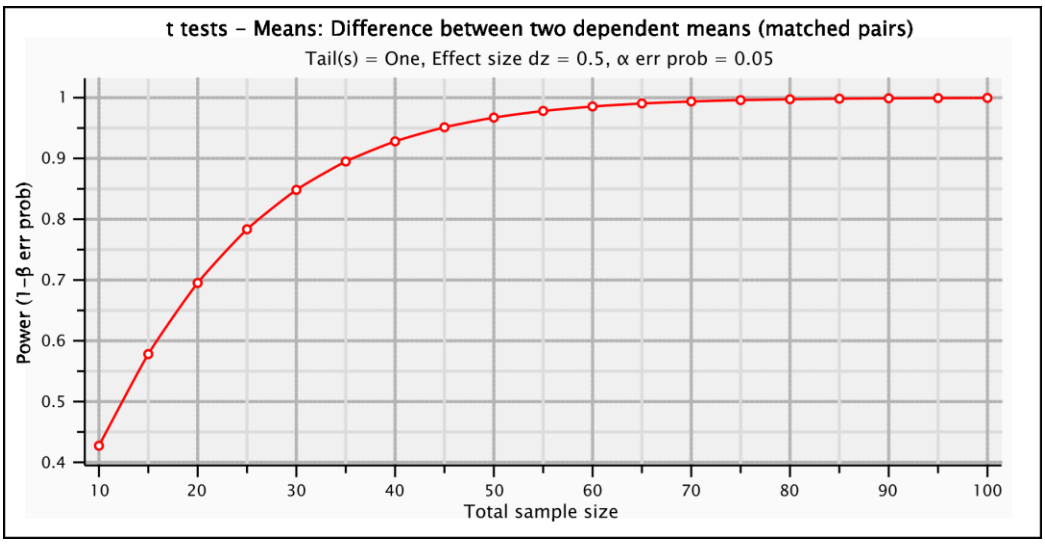


t tests – Means: Difference between two dependent means (matched pairs)

Analysis:	Post hoc: Compute achieved power		
Input:	Tail(s)	=	One
	Effect size dz	=	0.5
	α err prob	=	0.05
	Total sample size	=	24
Output:	Noncentrality parameter δ	=	2.4494897
	Critical t	=	1.7138715
	Df	=	23
	Power (1−β err prob)	=	0.7677848

Appendix A Figure A.3

Plot of Necessary Postmortem Sample Size to Achieve Statistical Power



Taken collectively, these statistics should be considered when evaluating the p-values obtained in this study but should not necessarily be grounds for exclusion. We understand that immunological differences occur in ASFV infections and variations can occur through human error in DNA extraction and PCR assay. These sources for variation could partially account for the variations found in the Shapiro-wilks test and f-Test.