NPB FINAL RESEARCH GRANT REPORT FORMAT

Evaluation of processing fluids and other novel sample types for the detection and surveillance of less-virulent ASFV strains (NPB #20-152)

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Date Report Submitted: 2023-01-04

Industry Summary:

African swine fever (ASF) is a devastating swine disease that resulted in the death and slaughter of millions of pigs in the past decade. ASF could be introduced to the US breeding herds and gradually spread across swine herds due to its lingering nature. Less potent (low virulent) strains of African swine fever virus (ASFV) could go unnoticed due to mild clinical signs. As a result, it's very important to regularly screen swine herds through active and passive surveillance. Surveillance of large scale swine operations using blood samples is labor intensive. Certain group samples like oral fluids, are ideal for large scale surveillance, but will not be useful in screening breeding herds as well as young piglets. Swine processing fluids (PF) is a group sample (passive sample) that is produced during routine castration and tail docking of piglets. Testing PF for the presence of ASFV genetic material or antibodies against ASFV could indicate the disease status of the tested herd, as well as the breeding animals at a snapshot and help detect infected animals and quickly eliminate them.

The first two objectives of the present study was to evaluate the suitability of PF for early detection of ASF viral genetic material and antibodies to less virulent ASFV strains. The third objective of the study was to evaluate novel sample types such as lymph nodes that are accessible through the skin, skin biopsies, ear notches and muscle swabs that can be collected quickly and safely from dead pigs without a complete post-mortem examination and compare them to the recommended gold standard sample types (whole blood, tonsils, spleen etc.). When experiencing large-scale swine mortalities, performing complete post mortem examinations becomes cumbersome and almost impossible. Moreover, opening up carcasses for the collection of routine sample types increases the risk of contamination of the environment and fomites involved.

In order to fulfill these objectives, we infected two groups of pigs (40 pigs/group) with two moderately-virulent ASFV strains via the oro-nasal route; the natural route of infection. Thereafter, four young piglets were castrated and tail docked under general anesthesia and PF were collected by pooling the testes and tail on an individual basis, daily during the first week of infection. Then, the piglets were humanely euthanized, and the remaining samples were collected starting with superficial lymph nodes followed by skin biopsies, ear notches and meat swabs. Then the carcasses were opened and the body cavity swabs and the internal organs (spleen, tonsils etc.) were collected. Nucleic acid was extracted from all the samples including PF and ASFV specific real time quantitative PCR (RT-qPCR) was performed. In order to detect antibodies in serum and PF, two commercial ELISA kits were used, by following the manufacturer's guidelines.

ASFV genetic material was detected in PF as early as 2 days post-infection in individual PF samples. PF is a group sample and therefore we evaluated the ability to detect ASFV genomic material in pooled PF samples. ASFV was successfully detected in PF pools that contain one sample from one pig at 3-4 dpi and 99 samples from healthy pigs. At this initial detection, the group of pigs didn't display any obvious clinical signs indicative of an ongoing infection. Antibodies were also detected in individual PF samples starting 9-10 dpi and the detection was comparable to that in the corresponding serum samples. ASFV genetic material was also detected in all novel sample types (Superficial lymph nodes, skin biopsies, ear notches, muscle swabs and internal body cavity swabs) starting from 3-4 dpi and continued to detect thereafter. The level of detection was different in each

sample type having the highest amount of viral genome in superficial lymph nodes followed by ear notches, skin biopsies and swabs.

These findings indicate that PF has the capability to serve as an effective passive sample type to screen pig herds for the evidence of an ASF incursion. Alternative sample types such as superficial lymph nodes, skin biopsies, ear notches, meat swabs and nasal wipes can also be used for ASF surveillance based on resource availability.

As stated in the contract, we require an industry summary of the project, suitable for immediate public release by the Board. The purpose of the industry summary is to provide producers with a quick reference to research results supported by Checkoff dollars. The content should include the following: an explanation of the objectives, descriptive narrative of how research was conducted, a discussion of the research findings sufficient to give a thorough understanding of the results, and explain what these findings mean to the industry. This summary is to be written for non-technical audiences. Please include your contact information.

Key Findings:

- ASF genomic material was detected in swine processing fluids as early as 3-4 dpi
- One ASF infected pig among 100 pigs could be successfully detected by testing pooled PF
- Antibodies were detected in PF starting from 9-10 dpi
- PF are helpful to screen breeding herds
- Novel sample types, particularly superficial inguinal lymph nodes are highly suitable to screen dead pigs for ASF, instead of performing a complete post mortem exam
- Skin biopsies/ear notches can also be used for ASFV genome detection reliably, when other samples types are not accessible
- Meat swabs can also be used as an alternative sample types to detect ASFV in meat samples (meat shops, illegal meat) and carcasses (processing plants)
- The level of ASFV genomic material in skin and swab samples are lower than what was detected in superficial lymph nodes

Keywords: ASF, moderately virulent, swine processing fluids, novel sample types, surveillance

Scientific Abstract: This should be a scientific description limited to one page in length to describe your project and its results.

African swine fever (ASF) is one of the most important viral disease of pigs in the world at present, and the North American swine industry is striving hard to prevent its entry to our region. Moderately and low virulent strains of ASFV pose a higher threat of entering and slowly spreading while remaining undetected in North American swine herds, particularly in breeding herds. Processing fluids (PF) are serosanguineous tissue fluid passively recovered from castration and tail docking of young piglets which is an industry practice called “piglet processing”. Currently, PF is being used to detect other viral pathogens of pigs in breeding herds The first two objectives of this study were to evaluate the utility of PF to detect ASFV genomic material, as well as ASFV specific antibodies following an ASF incursion in young piglets. The third objective of this study was to evaluate novel sample types such as superficial lymph nodes, skin biopsies, ear notches as well as muscle swabs for the early detection of ASFV genetic material in comparison to standard samples like whole blood and spleen.

A total of 80, 3 weeks old, intact male piglets were subject to two independent experiments conducted back to back. The first group of 40 piglets were infected with moderately-virulent ASFV Estonia/2014 and the second group of 40 with ASFV Malta’78 by oro-nasal route. Following infection, sampling was conducted daily up to 5 days post inoculation (dpi) and at pre-planned sampling points or humane end points thereafter. At each sampling point, four piglets were processed (castrated and tail docked), under general anaesthesia, blood samples were collected and

euthanized to perform post mortem examinations and sample collection. The testicles and tails from each pig were pooled in sampling bags and processing fluids were collected on an individual basis. Following humane euthanasia of the piglets, complete post-mortem examinations were performed while collecting samples; from the most superficial ones first to the internal ones last. Total nucleic acid extraction was performed directly on PF, 10% tissue homogenates and other novel swab samples. ASFV genomic detection was performed using real time PCR. Furthermore, antibody detection in PF and corresponding serum samples was performed using two commercial indirect ELISA assays.

ASFV genome was detected in PF as early as 2-3 dpi at relatively lower levels, reached higher detection levels thereafter and continued at the same levels till the end of the study. Dilution of positive PF samples with known negative PF samples revealed that up to 100 PF samples can be pooled without loosing the detection of ASFV genomic material in the samples. Testing PF with two commercial indirect ELISA assays showed that antibodies to ASFV can be detected in processing fluids as early as 9-10 days post-infection, as observed in serum samples. ASF genome was detected in all novel sample types (superficial lymph nodes, skin biopsies, ear notches, meat swabs, abdominal and thoracic cavity swabs and nasal wipes) as early as 3-4 dpi and the detection continued thereafter with 100% specificity parallel to the detection in whole blood and spleen.

Based on the data from this study, we can suggest that PF is suitable to screen young swine herds for ASF. Unconventional, superficial samples such as superficial lymph nodes, skin biopsies, ear notches and muscle swabs can also serve as suitable alternative samples to detect the presence of ASF during large scale dying events, without having to perform complete post mortem examinations.

Introduction: African swine fever (ASF) could get introduced to breeding herds and spread rapidly due to the lingering nature of the virus as well as subtle and non-specific clinical signs caused by lesser potent strains. As a result, it's very important to perform surveillance; screening of breeding herds and litters by testing for African swine fever virus (ASFV), before it spreads extensively across North America.

Surveillance based on individual sampling such as blood in commercial pig farms is labor-intensive and costly. Passively collected group samples such as oral fluids (OF) and processing fluids (PF) are practical and very useful in screening large herds, to identify potentially infected groups of animals that could be further confirmed by testing individual blood. Oral fluid collection may not be practical in breeding herds and new born piglets. Comparatively, processing fluids is a sample that gets passively generated during routine piglet processing (castration and tail docking of piglets), that could also serve as an indicator of disease status in the breeding herd.

Passive surveillance of sick or dead pigs is an integral part of the ASFV surveillance plan. However, performing a complete post-mortem examination on every dead pig is labour-intensive, requires technical expertise and therefore would be practically impossible especially during a large die off. Complete post-mortem examinations also increase the risk of contamination of the environment, instruments and personnel involved.

Objectives:

The first objective of this proposed research was to evaluate the efficacy of detecting ASFV genomic material and in PF collected from piglets infected with moderately-virulent ASFV strains; ASFV Malta'78 and ASFV Estonia 2014, and explore their detection limits as pooled samples.

The second objective was to explore the ability to detect the subsequent antibody response in PF parallel to that in serum.

The third objective of this study was to evaluate novel sample types that can be collected quickly and safely from dead pigs without a complete post-mortem examination and compare their suitability to the gold standard sample types (whole blood, tonsils, spleen etc.). Novel sample types included superficial lymph nodes that are accessible through the skin, skin biopsies, ear notches and muscle swabs.

Materials & Methods: This section should include experimental design, methods and procedures used, number of animals, etc.

A total of 80, 3 weeks old, intact piglets were subject to two experiments conducted back to back. One pig died during the acclimatization period. One group of 39 was infected with moderately virulent ASFV Estonia 2014 and the other 40 with ASFV Malta'78 in the oro-nasal route. Following infection, sampling was conducted daily up to 5 days post inoculation (dpi) and at pre-planned sampling points or humane end points thereafter. Four piglets were processed (castrated and tail docked), under general anaesthesia, blood samples were collected and euthanized to perform post mortem examination and sample collection daily. The testicles and tails from each pig were pooled in sampling bags and processing fluids were collected after leaving for few hours in 4C temperature. Tissues and other post mortem sample types were collected starting from superficial lymph nodes first and then moving onto the more internal samples. Muscle swabs were collected using a commercially available sterile sponge (VWR), soaked in 10ml of sterile PBS and by wiping the musculature just below the skin in the thoracic region. Thereafter, the swab contents were squeezed into a sterile vial. In the ASF Malta'78 experiment only, internal thoracic and abdominal cavities were swabbed in similar manner using the same commercial sterile sponges. The pigs were observed for pathogenesis and clinical disease progression at the same time.

The tissue samples were homogenized into 10% tissue suspensions. Exudates were collected from the muscular bases of the whole ears severed from the carcasses (ear juice) and diaphragms (meat juice) by freezing the tissues in -20^{0}C and thereafter thawing overnight in 4^{0}C.

In order to test the detection cut off in pooled PF, 6 PF samples from each experiment were individually subject to 10 fold dilution series, using clean PF obtained from the industry. Total nucleic acids were extracted from PF, swab material and tissue homogenates using Magmax™ Core nucleic acid purification kit. ASF genome was detected using a quantitative real time PCR (qRT-PCR) assay (Modified Zsak assay) developed by Wang et. al.

For antibody detection in serum and PF, two commercial indirect ELISA assays were used; ID screen® ASF indirect ELISA kit (ID-Vet, Grabels, France) and AsurDX™ ASF Antibody test (Biostone™, Dallas, TX, USA). The tests were performed according to manufacturer's guidelines. Since PF was a novel sample type that has never been tested by these ELISA assays, the sample dilutions were optimized for each assay.

Results: Report your research results by objective.

Objective 1: Evaluating the efficacy of detecting ASFV genomic material in piglets infected with ASFV Estonia 2014 and ASFV Malta'78

ASF genomic material was first detected in the PF of individual pigs as early as 2-3 dpi at low levels (average Cq ASFV Estonia 2014 = 36.92 and ASFV Malta'78 = 34.83). In ASFV Estonia 2014 infected pigs, individual PF contained high levels of ASFV genomic material starting from 3 dpi (average Cq 23.69), and continued to detect at the same level or higher till the end of the experiment. Comparatively, the detection of ASF genomic material in ASFV Malta'78 infected pigs'

individual PF appeared a day later, with high detections starting at 4 dpi (average Cq 23.98) and continuing till the end of the experiment. This genomic detection occurred concurrently to the onset of viremia and rising rectal temperatures. According to the clinical picture, ASFV Estonia 2014 infection caused early pathogenesis and clinical disease progression compared to ASFV Malta'78.

Upon dilution with clean PF, it was evident that ASFV genomic material can be easily detected in PF pools of 100 pigs each.

Objective 2: Evaluating the ability to detect subsequent antibody response in PF following an ASF incursion

Antibodies were detected in PF 9-10 dpi. There was a subtle difference in the earliest detection points between the two commercial ELISA kits when the pigs were beginning to sero-convert. The antibody detections in PF were further confirmed by Immunoperoxidase test (IPT).

Objective 3: Evaluation of novel sample types that can be collected quickly and safely from dead pigs without a complete post-mortem examination

All novel sample types displayed the presence of ASFV genomic material as early as 3-4 dpi. In the submandibular lymph nodes (SMLN), ASFV Estonia 2014 low level of detection started appearing at 2 dpi however, higher detection levels were observed at 3 dpi and beyond. Viremia began from 2 dpi in the ASFV Estonia 2014 pigs, whereas the Malta'78 infected pigs started viremia a day later, at 3 dpi. Superficial inguinal lymph nodes (SILN) from both experiments contained the ASF genomic material from 3 dpi on. ASFV genome was detected in skin biopsies, ear notches and muscle swabs starting 2-3 dpi onwards.

The ASF genomic detection in ear notches was much early compared to the skin biopsies although that early detection was at pretty low levels. Out of the two exudate samples, ear juice showed earlier ASF genomic detection compared to meat juice which was generated from the diaphragms. Lower level of detection in meat juice started at 2-3 dpi. In the ASF Estonia 2014 inoculated pigs, ear juices had relatively higher levels of viral genome detection during the early points such as 3-4 dpi. Thereafter, the detection levels were quite similar and equally high. Comparatively, in ASF Malta'78 infected pigs, both exudates had similar level of detection throughout the study.

Considering the novel swab types that were collected using a sterile sponge hydrated with sterile PBS, and the nasal wipes, both showed early lower level of detection as early as 2-3 dpi. However, overall detection level was higher in meat swabs. Thoracic and abdominal cavity swabs were only collected in ASF Malta'78 experiment. Both those swab types displayed early detection at 3 dpi however, the detection of ASF genomic material was inconsistent when compared to the meat swabs.

Table 1: The earliest and the highest points of ASF genomic DNA detections in each sample type collected in the study. The new sample types other than the ones originally proposed are in **bold**.

a. ASF Estonia 2014

Sample type	Earliest detection point			Point of highest detection	
	DPI	# of positive pigs	Average Cq	DPI	Average Cq
Whole blood	2	4/4	31.74	9	17.59
Processing Fluids	2	3/4	36.91	8	18.52

Spleen	2	4/4	32.84	5	16.31
Tonsils	2	2/4	36.36	7	17.57
SILN	3	4/4	29.02	8	17.50
SMLN	2	2/4	33.21	7	17.99
PFLN	3	4/4	29.88	7	18.21
PLN	3	4/4	29.48	9	16.89
SCLN	3	4/4	30.12	7	16.46
GHLN	2	3/4	36.05	7	17.31
Skin Biopsy	3	4/4	33.22	9	22.96
Ear Notches	2	1/4	36.64	9	19.49
Meat Swab	3	4/4	27.4	11	19.38
Nasal Wipes	**2**	**3/4**	**35.66**	**9**	**18.74**
Ear Juice	2	4/4	32.78	5	17.68
Meat Juice	3	4/4	29.48	9	16.89

b. ASF Malta'78

Sample Type	**Earliest detection point**			**Point of highest detection**	
	DPI	**# of positive pigs**	**Average Cq**	**DPI**	**Average Cq**
Whole blood	3	4/4	32.59	10	18.37
Processing Fluids	3	4/4	34.82	6	19.78
Spleen	2	2/4	37.51	5	14.48
Tonsils	3	1/4	36.51	6	17.06
SILN	3	2/4	36.35	6	19.41
SMLN	3	3/4	32.83	6	18.9
PFLN	3	2/4	37.08	6	19.47
PLN	3	3/4	32.20	7	19.63
SCLN	3	2/4	37.06	6	19.39
GHLN	3	4/4	35.56	6	17.80
Skin Biopsy	4	4/4	29.33	6	23.15
Ear notches	3	1/4	37.10	6	23.38
Meat Swab	3	2/4	35.8	6	20.99
Thoracic cavity Swab	**3**	**1/4**	**34.73**	**7**	**25.36**
Abdominal cavity Swab	**3**	**2/4**	**32.63**	**7**	**26.08**
Nasal Wipes	**2**	**2/4**	**35.92**	**7**	**22.73**
Ear Juice	2	3/4	34.87	5	19.78
Meat Juice	3	4/4	33.74	6	18.35

Discussion: Explain your research results and include a summary of the results that is of immediate or future benefit to pork producers.

In this study, two moderately virulent ASFV strains were used. Compared to ASFV Estonia 2014, Malta'78 exhibited a slightly slower disease progression. Both groups developed fever starting 3-4 dpi. In both experiments ASFV genome was detected in PF as early as 3-4 days post infection at higher positive levels. Although in ASF Estonia 2014 experiment, the viral genome was detected in individual pigs' PF at very low level as early as 2 dpi, it could go unnoticed in a pooled PF sample. With the level of detection at 3-4 dpi, 1 infected pig in 100 non-infected pigs can be identified confidently by testing pooled PF. This provides data to support that PF could be a very helpful passive sample type to screen new litters during the first week of life. It would be particularly beneficial in screening, identifying and preventing low and moderately virulent ASF strains, as these strains produce minimum clinical signs that can go unnoticed.

Antibody detection in PF could assist in detecting previous exposure to ASF by the sows or the piglets at birth. Depending on the time of ASF incursion, either trans-placental during gestation or just after birth, if the piglets survived, they would likely seroconvert. In reality, piglet processing is performed in the first week of life. Depending on whether the piglets seroconverted after an early ASF infection or they passively received anti ASF antibodies via colostrum from convalescent sows, those antibodies maybe reflected in processing fluids. However, the utility of PF for antibody detection may vary and needs to be studied further. Within our experimental conditions, we were able to successfully detect antibodies in PF, 9-10 days following infection. There was a noticeable difference in sensitivity between the two ELISA kits detections; ID screen® ASF indirect ELISA being rather less sensitive compared to the AsurDX™ ASF Antibody test by Biostone™ where the latter detected early seroconverting pigs in both serum and PF at least 3 days prior to the other kit. Another clinical observation was that, ASF Estonia 2014 infection caused acute disease that resulted in rapid reaching of humane end points. As a result, the number of sero-converted pigs were very few in the ASF Estonia 2014 infected group. Pigs infected with Malta'78 had rather slower disease progression so that the experiment concluded by 18 dpi resulting in relatively more seroconverted pigs. Regardless, the antibody positivity in PF resembled that of corresponding serum. The antibody detections in PF was further confirmed by performing an Immunoperoxidase test (IPT).

Considering the novel and alternative sample types, both ASF strains were detected in all superficially collected sample types (superficial lymph nodes, skin biopsy, ear notches and meat swabs) within 3-4 days following oro-nasal infection, with high levels and continued to detect consistently.

Out of the superficial lymph nodes, SILNs are the most superficially located lymph node that can be collected first and foremost from a carcass prior to opening it. In an outbreak situation, staff can be quickly trained for its collection due to the superficial location under the skin, and the minimal chance to cause contamination of the premise.

The other sample types also help to detect the presence of ASF genomic material as early as 3 dpi. Particularly, skin biopsies and ear notches are superficial sample types that are easy to collect with minimum tools for both outbreaks as well as to sample dead or hunted pigs in the wild. Muscle swabs are an easy to collect sample type from meat at the store or plants, when it's not possible to collect a piece of meat for meat exudate collection. In the experiment #2, we further collected swabs from internal body cavities: abdominal and thoracic cavity which are more of an appropriate sample type to collect from carcasses in the processing plants when the carcasses are being processed in the conveyer line. The sterile sponge swab we used for swabbing meat and body cavities is commonly used for bacteriological sample collection in the field.

Samples such as ear notches, ear juices and nasal wipes indicated the presence of ASF genomic material as early as the induction of viremia. The reason for such early detection could be due to contamination of these sample types from the inoculum given to pigs oro-nasally. Young pigs are highly social animals and they play, bite and rub each other with their snouts resulting in matting each other's' bodies with virus containing bodily fluids. We noticed that the meat swabs displayed high levels of genomic materials even at the early stages. When the skin is dissected to swab the muscles, blood escaping from small capillaries could have contributed these low Cq values, that were comparable to the corresponding blood samples Once a pig is succumbed to ASF, the chances are that its body is already covered with virus contaminated material so, there could be viral genomic detection in most of the superficially collected samples. Nasal wipes can be easily collected from live pigs making it a more suitable sample type for live pig surveillance.

Considering the clinical picture of ASF, it is evident that these novel sample types evaluated in the current study are particularly going to be helpful in the early detection and prevention of low to moderately virulent ASF infections due to their slower progression with minimal and less specific clinical signs compared to a highly virulent strain. PF is a passive sample generated during the common industry practice of piglet processing, which makes it a convenient and useful early diagnostic sample to assess piglet health and to perform ASF surveillance at the same time. All alternative sample types are relatively easy to collect without a complete post mortem examination. Staff can be trained promptly to collect them with minimum contamination of premises and fomites. Those positive aspects of the proposed alternative samples would certainly assist us to detect ASF early and reduce its spread in the North American region during any potential ASF incursion. However, due to inconsistency and low levels of ASFV genome in some of the alternative samples, negative laboratory results must be interpreted carefully.