NPB FINAL RESEARCH GRANT REPORT FORMAT

As a requirement of each research grant, a final report detailing the project results must be provided to the National Pork Board. Please write the industry summary with the producer in mind. The remaining content of the report can be written for a scientific audience. *ALL final reports must be submitted in the following format or the report will be returned for correction. PLEASE DO NOT INCLUDE ANY TYPE OF COVER PAGE.*

Project Title and NPB project identification number: # 21-134, An African Swine Fever (ASF) positive cohort study: Validating various PCR tests and sample types for African Swine Fever Virus (ASFV) detection in boars; and determining the possibility of transmission via artificial insemination.

Principal Investigator: Dr. Darwin Reicks

Institution: Reicks Veterinary Research & Consulting

Date Submitted: October 31, 2022 **FINAL VERSION**

Industry Summary: 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.

Industry Summary:

The objectives of this study were to determine 1) which sample types will detect ASFV in boars early during infection; 2) whether virus is transmissible through artificial insemination (AI) of extended semen; and to test various PCR test kits to provide backup, alternative reagents in the event of an African Swine Fever Virus (ASFV) outbreak.

Research was conducted at the Friedrich-Loeffler-Institut (FLI), Institute of Diagnostic Virology in Germany. Four ASFV naïve, mature boars, Pietrain (n=2) and Large White (n=2) were obtained for the study, along with 14 gilts. Initially, the boars were inoculated oronasally with ASFV strain Estonia 2014. By 4 days after oronasal inoculation, it was determined that despite the inoculum being verified as infectious, the boars had not yet become positive for ASF. Therefore, the four boars were injected intramuscularly (IM) with the same strain of ASFV to ensure infected semen would be available for the synchronized gilts and the study reset to time 0 being the time of injected virus. As boars had been collected four days in a row, boar semen was not collected on day 1. This ensured that infectious semen could be obtained and used for the insemination of the gilts.

Daily blood and semen collection from four boars after IM inoculation with ASFV resulted in detection of ASFV genome as early as day 1 in blood and day 2 in semen and oral fluid. Feces was not a consistent matrix for detecting the virus. All four boars

reached a welfare threshold, determined by severe clinical signs at various times per boar, and were euthanized at 10, 11, 17 and 25 days post inoculation (dpi).

Fourteen gilts were housed in two separate rooms with 7 gilts per room. After insemination with ASFV positive semen with precautions taken to prevent viral contamination of the environment, by 7 days post insemination, 50% of the 14 gilts were ASFV positive. Ten of 14 gilts aborted implanted embryos upon fever development. A proportion of fetuses showed both abnormalities and replication of ASFV in fetal tissues.

Various PCR test kits were utilized, and it was determined that at least 2 of the commercial kits tested would be sensitive and specific for early detection of ASFV in oral fluids, semen and whole blood.

These results are important in that the risk of many sow farms being infected by a boar stud could be significant. Semen shedding of ASFV was early, and in enough quantity to infect half the gilts that were inseminated within 7 days. The infectivity of the semen was additionally verified through virus isolation.

Dr. Darwin Reicks
darwin@rvrcmn.com

Key Findings:

- Please provide 3 to 5 bulleted points that highlight the most important findings of your study
- These can most simply be distilled from the "Discussion" section
- They should be written to convey information to pork producers
- Be brief

Key Findings:

- Boars shed infectious ASFV in semen by day 2 after IM injection.
- Blood, semen, and oral fluids were suitable samples types for early ASFV detection.
- Half of the 14 gilts inseminated with positive semen became infected within 7 days post insemination and the virus was transferred to fetuses.
- Along with automated nucleic acid extraction, at least 2 commercial PCR kits could be utilized for early detection of ASFV in semen, whole blood and oral fluids.

Keywords: include at least 5 keywords:

Keywords: semen, artificial insemination (AI), African swine fever virus (ASFV), boar, PCR

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

Scientific Abstract:

The following *<u>was abbreviated but derived from the manuscript</u>* *to be submitted to the journal, Pathogens:*

<u>Artificial insemination as alternative transmission route for African Swine Fever Virus</u>
Virginia Friedrichs, Darwin Reicks, Tobias Hasenfuß, Elisabeth Gerstenkorn, Jeffrey J. Zimmerman, Eric A. Nelson, Tessa Carrau, Paul Deutschmann, Hanna Roszyck, Martin Beer, Jane Christopher-Hennings and Sandra Blome. Pathogens, in preparation

A second manuscript describing the DNA extractions and PCR procedures entitled "Establishment of a suitable pipeline to ensure sensitive detection of African swine fever virus genome in boar semen" is also in preparation for Pathogens.

Rapid spread of African swine fever virus (ASFV), causing severe disease with often high fatality rates in Eurasian suids, prevails as a threat for pig populations and dependent industries world-wide. Although advancing scientific progress continually enhances our understanding of ASFV pathogenesis, major alternative transmission routes for ASFV have yet to be assessed. Here, we demonstrate that ASFV can efficiently be transferred from infected boars to naïve recipient gilts through artificial insemination (AI). Modern pork industries resort to semen acquired from boar studs, which often supply many sow herds spread across the country. This inevitably bears the risk of ASFV being distributed nationwide or even beyond via contaminated semen if a boar stud would be affected. Daily blood and semen collection from four boars after intramuscular (IM) inoculation with ASFV strain 'Estonia 2014' resulted in detection of ASFV genome in the semen as early as 2 days post inoculation (dpi), in blood at 1 dpi while semen quality remained largely unaffected. PCR results and extractions were shown to be sensitive on semen, blood and oral fluid samples using commercial kits. Ultimately, after insemination with extended semen, 7 of 14 gilts were ASFV positive by 7 days post insemination, but all gilts were ASFV positive by 35 days post insemination. Ten of 14 gilts aborted implanted embryos upon fever development. A proportion of fetuses showed both abnormalities and replication of ASFV in fetal tissues. Thus, we do not only present evidence for efficient transmission of ASFV to gilts via AI, but also to implanted embryos, underlining the critical role that boar semen could play in ASFV transmission.

Introduction: An overview of the researchable question and its importance to producers.

Introduction:

African swine fever (ASF) is a devastating viral disease of both wild boar and domestic pigs. Historically, the disease was mainly found in Sub-Saharan Africa. However, after the introduction of ASF into Georgia in 2007, the fatal disease spread to many European and Asian countries and as of July 2021 has now reached the Americas. In the absence

of vaccines or treatment options, early detection of disease incursions is of paramount importance to limit the impact on animal health and the swine industry.

One of the most important avenues for wide-spread transmission of foreign animal diseases is the dissemination of viruses through boars and boar semen. By the millennium, artificial insemination (AI) was used on more than 95% of all sows inseminated which could potentially lead to wide spread virus dissemination. African Swine Fever Virus (ASFV) is found in all relevant gonadal tissues in inoculated boars. The severity of the clinical signs seen with ASFV may decline with age such that ASF could be subclinical in adult animals early in infection while the boar is still being used for semen collections. Therefore, it will be important to detect the virus in boars early in infection for ASF control, especially in high impact breeding animals, (e.g. as long as the boar shows no or very mild clinical signs).

These types of boar studies have been previously performed with endemic viruses such as Porcine Reproductive and Respiratory Syndrome virus (PRRSV)and porcine circovirus (PCV2) and have led to biosecurity protocols to prevent transmission of these viruses from boar studs in the US. However, this is the first study using experimentally ASFV inoculated adult, commercial boars to determine which sample types and PCR procedures were optimum in detecting ASFV in boars and gilts. Additionally, it proved that insemination of ASFV infected semen to naïve gilts resulted in transmission of the virus.

Objectives: From your research proposal.

Objectives:

1) Determine which sample types will detect ASFV in boars early in infection and throughout a 90-day post inoculation (DPI) time period.
2) Determine which PCR protocols are reliable in early detection of ASFV in boars.
3) Determine whether semen can transmit ASFV via artificial insemination (AI).
4) If ASFV is not transmitted via AI in this *preliminary* study, does the semen still harbor infectious virus?

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

Materials & Methods:

The following <u>was modified but derived from the manuscript</u> to be submitted to the journal, Pathogens:

<u>Artificial insemination as alternative transmission route for African Swine Fever Virus</u>
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A second manuscript is in preparation on comparisons of DNA extractions and PCR reagents. This manuscript is entitled “Establishment of a suitable pipeline to ensure sensitive detection of African swine fever virus genome in boar semen”. Authors are Virginia Friedrichs, Darwin Reicks, Jeffrey J. Zimmerman, Eric A. Nelson, Tessa Carrau, Paul Deutschmann, Martin Beer, Jane Christopher-Hennings and Sandra Blome.

Experimental design

The trial encompassed four adult breeding boars: two adult Large White boars and two Pietrain boars and 14 gilts). All animals were kept under high-containment conditions at the Friedrich-Loeffler-Institut (FLI); isle of Riems, Germany. Synchronization of the gilts was performed using altrenogest (Regumate®, MSD) protocols and estrus induction was facilitated by administration of cloprostenol (Estrumate®, MSD) at the day of transport and pregnant mare serum gonadotropin (Pregmagon®, Ceva) at the FLI one day later.

The boars were were kept in solitary pens. Initially, the breeding boars (n = 4) were inoculated oro-nasally with 10 ml of a spleen suspension containing approximately 10^5 hemadsorbing units 50% (HAU_{50}) per ml of the ASFV strain ‘Estonia 2014’ (genotype II). This virus strain had shown an attenuated phenotype in previous studies and was chosen to allow long-term follow-up of the boars. This initial design had to be modified upon the absence of ASFV genome in all boars at 4 dpi, and IM inoculation was performed with approximately 10^4 HAU_{50} of the same ASFV strain. This modification was inevitable to ensure active viraemia in the time window of the sow synchronization.

After inoculation, the boars were semen collected at close intervals and the semen was analyzed for basic quality parameters and ASFV genome. Prior to AI, boar semen was diluted with BTS (Minitube) at a dilution of 1:5. The semen diluent was pre-warmed to 37°C to ensure viability of spermatozoa. To conduct AI, diluted semen of boars (except #4) was pooled in equal parts and insemination tubes (Minitube) were prepared to contain 3 billion spermatozoa in 60 ml. Most gilts (10 out of 14) were inseminated twice over a period of two days while they showed standing reflex, ultimately utilizing boar semen collected at days 4 and 5 pi. To minimize reflux of semen and subsequent contamination of the pen with ASFV containing semen, super plus size tampons (O.B. tampons super plus, Johnson & Johnson GmbH) were used (3 tampons/gilt with ~20 ml holding capacity each), along with post insemination disinfection of the vulvar area and pen.

A comprehensive clinical scoring (CS) system was utilized to monitor changes in animal behavior and appearance linked to disease progression. Animals were euthanized upon reaching a total of 15 points through scoring or development of other severe clinical signs that classify as insufferable (humane endpoint). Furthermore, rectal temperatures were taken daily and a rectal temperature of ≥ 40.0°C was recorded as fever. After euthanasia, all animals were subjected to a full necropsy.

Viruses and cells

For oro-nasal inoculation of the boars, a porcine spleen acquired from a previous trial to characterize pathogenesis of ‘Estonia 2014’ infections was titrated on macrophages derived from peripheral blood mononuclear cells (PBMCs). Prior to IM inoculation, the virus stock was titrated on PBMC-derived macrophages. For oro-nasal inoculation, a titer

of 10^5 HAU_{50}/ml in a total volume of 10 ml was used, for IM inoculation, 10^4 HAU_{50} in 1 ml have been administered. To ensure accuracy, the inoculum was back titrated in triplicate.

Sample collection and processing

To assess ASFV kinetics in boar semen, semen was collected at days -7, -3, -2, -1, 0, 2, 3, 4, 5, 14, and 20 post IM inoculation. Given the initial trial design with daily collection upon oro-nasal inoculation, semen collection was not carried out on day 1 post IM inoculation. This decision was taken based on the animal welfare permission and to facilitate semen quality for AI.

To enable daily assessment of systemic genome loads, blood samples were collected at days -7, -3, -2, -1, 0, 1, 2, 3, 4, 5, 14, and 20. It is of note that at 1 dpi, capillary blood samples were only obtained from boars #1 and #4. Further, at days 14 and 20 pi, semen was only obtained from boar #3.

For semen collection, each boar was presented a dummy for mounting. The semen was obtained following the principles of the three-glove free-catch semen collection recommended by Reicks et al. In this method, a towel dried clean penis is grasped with clean glove that has had no contact with the environment or prepuce fluids. The tip of the penis was free and clear to allow semen to be directed from the urethra directly in to the collection container. The semen was secured in thermal mugs (Minitube). To prevent bulbourethral secretions or other components from contaminating the semen, specialized collection bags with filters were used (Minitube). During semen collection, anticoagulated blood and native blood for serum acquisition were obtained from each boar through venipuncture of the saphenous vein or neighboring blood vessels using standard collection tubes with aspiration principle (KABE). Upon completion of sample collection, semen and blood samples were processed on the same day. The semen was diluted with BTS as described above and kept at 37°C to ensure viability of spermatozoa.

Spermatozoa of each boar were counted and viability/motility was assessed. Extraction of DNA from whole semen and blood samples allowed detection of ASFV genome in those matrices on the same day.

To allow comprehensive pathogen detection, each boar was presented a cotton chewing rope for the collection of oral fluids after semen collection. Subsequently, the rope was cut and placed in a 50 ml centrifugation tube with 0.5 ml tubes at the bottom and fluids were spun down at 13,000 rpm for 10 min at 4°C. Aliquoted fluids were stored at -80°C until further use. Moreover, dummy swabs (Swiffer) were obtained at 3-5 dpi upon completion of semen collection. In addition, feces and fecal swabs from each boar were obtained on all semen collection days. Swabs were soaked in cell culture medium (Dulbecco's Modified Eagle's Medium, DMEM) without supplements for 2 h, supernatants were stored for further processing. After AI, blood samples from gilts were obtained at 7, 14, 21, and 28 dpi by collecting a few droplets of blood from the caudal vein. All blood samples were processed and ASFV genome loads were assessed on sampling days.

During necropsies, the following samples were acquired from all individuals for subsequent pathogen detection: Blood, serum, tonsil, mandibular lymph node (mnLN), lung, kidney, spleen, liver, gastrohepatic lymph node (ghLN), inguinal lymph node

(ingLN), and popliteal lymph node (pLN). Moreover, samples of the reproductive tracts were obtained. In boars, testis, epididymis, prostate, vesicular glands, and bulbourethral glands were sampled. In gilts, vestibulum, vagina, cervix, uterus, salpinx, and ovaries were sampled.

DNA extraction and qPCR

To extract nucleic acids for downstream molecular tests, 100 µl of each tissue homogenate, blood obtained during necropsies, or serum were processed using the NucleoMag® VET Kit (Macherey-Nagel) on a KingFisher 96 Flex System (Thermo Fisher Scientific) according to manufacturer's instructions. Proven ASFV genome-negative serum was included as extraction control. Since qPCR was carried out utilizing the VetMAX™ African Swine Fever Virus Detection Kit (Thermo Fisher Scientific), the internal control DNA was added to the extraction process according to manufacturer's instructions to assess qPCR performance.

For daily qPCR, DNA from 80 µl whole semen or blood was manually extracted with the QIAamp Viral RNA Mini kit (Qiagen). As described above, ASFV genome-negative serum was included as extraction control. After extraction, qPCR was performed utilizing the virotype® ASFV PCR kit (Indical). To validate optimal qPCR performance, the internal control provided by the manufacturer was included. All qPCR reactions were carried out on a Bio-Rad C1000TM thermal cycler, equipped with the CFX96TM Real-Time System (Bio-Rad).

Virus isolation

To assess the presence of infectious virions in semen, blood, spleen, and samples of the reproductive tracts of boars and gilts, HATs were performed. As a blind passage, PBMCs were seeded into a 24-well plate and macrophages were differentiated as described above. Subsequently, 200 µl of tissue homogenate or 200 µl of diluted EDTA blood (1:10 in PBS) was added to differentiated macrophages in duplicates. After 72 h, the plate was frozen at -80°C for at least 24 h to ensure throughout freezing and lysis of cells. Ultimately, the supernatant was subjected to a HAT to assess the presence of infectious ASFV particles. Each technical duplicate of the blind passage was assessed in technical quadruplicates in HAT.

Antibody detection

To assess seroconversion of boars and gilts after inoculation, samples of all animals were subjected to various testing methods, routinely used in ASFV serological testing. We included two complementary ELISA kits: (I) ID Screen® ASF Indirect (ID.vet), detecting antibodies targeting ASFV p32, p62, p72, and (II) Ingezim PPA COMPAC (Ingenasa), detecting p72 targeting antibodies. Assays were performed according to manufacturer's instructions. All serum samples obtained from boars were subjected to these tests. Of gilts, blood swabs were soaked in PBS and served as matrix for serology. Final serum samples obtained during necropsy were collected from all animals and subjected to ELISA. Furthermore, all final serum samples and positive samples in ELISA were additionally tested via Immunoperoxidase test (IPT). Since the IPT is currently the most sensitive test in ASFV serology, these results served as reference for ELISA results.

Comparison of nucleic acid extraction methods and PCRs

The performance of three commercially available nucleic acid extraction kits was compared using both blood and semen samples: (I) the NucleoMag® VET kit (Macherey-Nagel), (II) the MagMAX™ Pathogen RNA/DNA kit (ThermoFisher), and (III) the MagMAX™ 96 Viral RNA isolation kit (ThermoFisher). All samples were extracted in triplicates and all kits were used according to manufacturer's instructions on the automated extraction platform KingFisher™ 96 flex (ThermoFisher). Performance was compared based on triplicate results obtained using the VetAlert ASFV DNA test kit (Tetracore) on a Bio-Rad C1000TM thermal cycler, equipped with the CFX96TM Real-Time System (Bio-Rad).

After choosing the extraction method, five different PCR protocols were compared using the samples mentioned above, namely (I) the VetAlert™ ASFV DNA Test Kit (Tetracore), (II) Virotype ASFV 2.0 PCR Kit (Indical), (III) the VetMax™ ASFV Detection Kit (ThermoFisher), (IV) ASF System 1 (WOAH, King et.al.), and (V) the RealPCR ASFV DNA Test (IDEXX). All methods were performed according to manufacturer's instructions.

Results: Report your research results by objective.

Results:

1) Determine which sample types will detect ASFV in boars early in infection and throughout a 90-day post inoculation (DPI) time period.

Whole blood (in EDTA) was the first sample type detected as PCR and virus isolation (VI) positive in 2 of 2 boars collected by 1 dpi. Screening of oral fluids collected from ropes allowed ASFV detection at 2 dpi and rectal swabs and fecal samples were positive for ASFV at 5 and 4 dpi (traces of viral genome), respectively. Serum testing resulted in ASFV genome detection in 2 of 4 boars collected by 2 dpi. Environmental swabs of the mounting dummy were PCR positive on the days tested (3-5 dpi). Due to severe clinical signs boars could not be maintained through the proposed 90 dpi time frame. Boars were euthanized at 10, 11, 17 and 25 dpi when they they reached their humane endpoint. ASFV genome was detected in all organs tested at those times.
To complete the picture, serology was carried out. From day 10 onwards, first positive results were seen in all boars, though #2 remained negative in the confirmatory IPT. All gilts were positive upon necropsy. Blood swabs for some sows became positive from 28 dpi.

To accurately assess seroconversion of all animals, all samples of boars and gilts were analyzed by two commercially available ELISA kits. In the competition assay (Ingezim PPA COMPAC), boar #1 was positive on the day of necropsy with an S/P% of 68.8% (11 dpi), boar #2 remained negative until necropsy (37.6% at 10 dpi). Boar #3 gave a positive result at day 14 pi (57.4%) and remained positive until the day of necropsy (86.9%). Boar #4 was positive at the day of necropsy (80%). In general, results were comparable in the indirect assay (ID Screen®), with exception of boar #2, which was positive at 10 dpi

(40.4%). Validation via IPT revealed that all samples beginning 10 dpi were positive, with exception of boar #2.

In gilts, early necropsied animals #539 and #567 rendered negative results in all as-says tested. Gilts euthanized along the course of the trial (#534, #536, #627) partly showed opposing results in different assays. Gilt #534 was positive in the indirect (54.5%) and competition assay (71.9%), while gilt #536 gave a positive result in the competition (56.5%), but a questionable result in the indirect assay (37.3%). Gilt #627, euthanized at 33 dpi, was positive in competition (69.2%), but questionable in indirect ELISA (33.4%). Ultimately, the IPT revealed that all samples are negative, with exception of gilt #627. In the competition assay, all remaining gilts were negative until day 28 pi. Here, only gilts that were positive in PCR at day 7 pi turned positive: #530 (54.5%), #533 (57.1%), #576 (61.7%), and #610 (50.2%). All gilts were positive on days of necropsy (35, 36 dpi). In the indirect assay, all gilts were negative until the days of necropsy. The IPT confirmed that all samples tested were positive starting day 28 pi. Generally, a reduced suitability of blood swab samples compared to serum samples was observed for both ELISA methods.

2) Determine which PCR protocols are reliable in early detection of ASFV in boars.

To define the nucleic acid extraction kit with the highest precision in extracting ASFV genome from boar semen, all semen samples were extracted in triplicates. Blood samples of boars were included as reference, since higher genome loads were expected in blood and performance of all kits on blood samples was validated previously. Three criteria were employed to define assay efficiency and ultimately the best-suited extraction method for boar semen samples: (I) successful detection in all true positive samples, (II) accurate detection of ASFV genome in semen early after inoculation (2-3 dpi), and (III) preference for lower Cq values. True positive samples were defined by manual extraction and increasing the number of replicates, which was beneficiary for detection of samples with low amounts of ASFV genome. All blood samples (n = 18), and 14/17 semen samples (n = 14) contained ASFV genome.
Extraction of samples with the NucleoMag® VET Kit resulted in detection of 18/18 positive blood samples and 11/14 positive semen samples by the VetAlert ASFV DNA Test kit. Extraction with the MagMAX™ Pathogen RNA/DNA Kit resulted in successful detection of 16/18 and 7/14 positive samples, while extraction with the MagMAX™-96 Viral RNA Isolation Kit resulted in 14/18 and 0/14 positive samples. Extraction using the MagMAX™-96 Viral RNA Isolation Kit was carried out three times, verifying these negative results. Of all samples obtained early after inoculation (n = 8, of which 5 were true positive), extraction with the NucleoMag® VET Kit resulted in detection in 2/5 true positive samples and 1/5 using the MagMAX™ Pathogen RNA/DNA Kit. Overall, extraction with the NucleoMag® VET Kit rendered lower Cq values for both blood and semen samples. The mean Cq value of blood samples was 20.3 for the NucleoMag® VET Kit, 23.6 for the MagMAX™ Pathogen RNA/DNA Kit, and 24.6 for the MagMAX™-96 Viral RNA Isolation Kit. Samples with low amounts of ASFV genome, e.g. blood samples of boars #2 (Cq 35.2) and #3 (Cq 38.1) at 1 dpi were not detected by any of the MagMAX™ kits. Comparable results were obtained with semen samples, the mean Cq value after

extraction with the NucleoMag® VET Kit was 34.1 and 36.7 using the MagMAX™ Pathogen RNA/DNA Kit.

Based on these results, PCR comparison was done using nucleic acids obtained with the NucleoMag® VET Kit. All qPCR kits detected 18/18 true ASFV positive blood samples, Cq-values were, however, considerably higher using the RealPCR ASFV DNA Test. The mean Cq values were as follows: 20.3 for the VetAlert™ ASFV DNA Test Kit, 21.5 for the Virotype ASFV 2.0 PCR Kit, 21.3 for the VetMax™ ASFV Detection Kit, 20.4 for ASF System 1 (WOAH, King et.al.), and 23.8 for the RealPCR ASFV DNA Test. Results obtained with semen samples were more heterogenous.
Considering that early detection of low amounts of ASFV genome in boar semen is critical, performance of all methods with semen samples at 2-3 dpi was crucial. Here, the VetAlert™ ASFV DNA Test Kit detected 2/5 true positive samples, the Virotype ASFV 2.0 PCR Kit detected 1/5, the VetMax™ ASFV Detection Kit detected 3/5, ASF System 1 detected 2/5, and the RealPCR ASFV DNA Test detected 1/5 positive samples. Overall, mean Cq values were comparable and ranged between 33.6 (Virotype ASFV 2.0 PCR Kit) and 35.9 (RealPCR ASFV DNA Test).

In conclusion, highest performance with semen was seen when combining the NucleoMag® VET Kit with either the VetMax™ ASFV Detection Kit or the VetAlert™ ASFV DNA Test Kit. Similar results were seen when using the WHOA recommended King protocol. No major differences were observed when testing blood samples.

Further details will be presented in the refereed manuscript.

3) Determine whether semen can transmit ASFV via AI

AI was found to transmit ASFV to gilts and VI was also performed on the samples to verify that infectious ASFV was in the pooled extended semen collected from boars at 4 and 5 dpi (this pool consisted of semen from all boars except #4).

After insemination with extended semen, 7 of 14 gilts were ASFV positive by 7 days post insemination, but all gilts were ASFV positive by 35 days post insemination. Thirteen sows implanted embryos after AI, but 10 aborted after developing a high fever. The morphology of fetuses and amniotic fluid differed among the offspring of one gilt which was still pregnant upon completion of the study at 35 days post insemination. Deformed/abnormal fetuses obtained during necropsy were all highly positive in qPCR and VI.

Infectious ASF virions could be isolated from the reproductive organs of boars and sows, independent of the day of necropsy.

4) If ASFV is not transmitted via AI in this *preliminary* study, does the semen still harbor infectious virus?

(see notes above in #3 since it was proven that ASFV can be transmitted via AI)

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

Please add page numbers at bottom center of each page. Do not include any other headers or footers. All tables, figures and graphics must be included in **one** Word document only and must be submitted electronically to beveritt@pork.org. Final reports will be published on our Web Site exactly as submitted **no later** than 12 months after receipt, but the industry summary will be released immediately.

Any publications, presentations or abstracts of the project results, need to recognize proper funding credit. A statement such as this would be sufficient: "Funding, wholly or in part, was provided by the National Pork Checkoff" (Or in the case of state funding "on behalf of the [state] Pork Producers Association"). Thank you for your attention to these instructions. Please contact Bev Everitt (phone-515/223-2750 or E-mail: grants@pork.org if you have any questions.

Revised 10/2019

Discussion:

The following was modified but derived from the manuscript to be submitted to the journal, Pathogens:

Artificial insemination as alternative transmission route for African Swine Fever Virus
Virginia Friedrichs, Darwin Reicks, Tobias Hasenfuß, Elisabeth Gerstenkorn, Jeffrey J. Zimmerman, Eric A. Nelson, Tessa Carrau, Paul Deutschmann, Hanna Roszyck, Martin Beer, Jane Christopher-Hennings and Sandra Blome. Pathogens, in preparation.*

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Since the modern pork industry largely relies on AI, boar semen is purchased on demand to inseminate numerous sows at once. Considering that it is unfeasible to distribute boar semen frozen, quality control measurements are conducted thoroughly, but rapid to ensure viability of spermatozoa. Possible consequences of ASFV dissemination via boar semen originating from boar studs, even if contact infection is not established, is provided by classical swine fever virus (CSFV): Here, over 1,000 sow farms had to be closed after potentially CSFV-containing semen was distributed across the Netherlands from affected boar studs. Considering the lack of an ASFV vaccine and the ongoing pandemic spread, the magnitude of consequences for global pig populations and associated economies might be even more devastating. Therefore, we evaluated the possibility of ASFV transmission via semen of infected boars and report an infection rate of 50% during the act of AI, which is crucial for risk mitigation strategies. Although our approach comprised only 18 pigs in total and therefore allows only limited derivation of generally applicable statements for venereal

transfer of ASFV, we provide thorough evidence that ASFV can efficiently be transferred to gilts via AI.

Daily monitoring of semen samples allowed comprehensive insight into ASFV kinetics and effects on boar semen. We observed the presence of ASFV genome and infectious virus in semen as early as day 2 pi upon IM inoculation. Strikingly, even acute viraemia had no effects on spermatozoa; count, motility and shape were unaffected by the infection. It is of note that modern boar studs conduct quality control on each batch of semen and routinely check for (I) erythrocytes in semen, (II) motility of spermatozoa, (III) deformations, and (IV) spermatozoa count. Since neither was altered by ASFV infection in any of the boars included in this study, routine testing for ASFV genome in boar semen should likely be considered in risk assessment procedures. However, as early detection of ASFV infection even before manifestation of clinical signs is key for effective disease control, it is of importance to note that high Cq values require replicates for accurate data interpretation. To ensure detection of low genome loads and therefore early detection of ASFV infection, a detection pipeline with high sensitivity needs to be defined. Under our settings, highest diagnostic performance with semen was seen when combining the NucleoMag® VET Kit with either the VetMax™ ASFV Detection Kit or the VetAlert™ ASFV DNA Test Kit. Similar results were seen when using the WHOA recommended King protocol. No major differences were observed when testing blood samples.

As quick dispatch of semen samples is essential for optimal viability and offspring numbers, we assessed the suitability of other matrices for early detection of ASFV infection in boar studs, though at very limited scale. It was previously described that blood samples show the highest accuracy in ASFV genome detection; however, especially larger boar studs favor non-invasive strategies. Thus, oral fluids and rectal samples were examined for suitability. We report that rectal swabs and fecal samples are inadequate matrices for early detection, as only few samples rendered positive in qPCR as also described previously. Oral fluids, obtained from cotton ropes, allowed early detection of ASFV genome at day 2 pi. However, similar to semen samples obtained early after inoculation, early oral samples contained low amounts of ASFV genome. Therefore, increasing the number of replicates during testing is likely beneficial to ensure precise detection. This in conjunction with tests of high sensitivity is crucial to prevent ASFV dissemination via boar semen.

Two commercial PCR tests (see above) were sufficient to detect early ASFV in semen, whole blood and oral fluids, along with a commercial DNA extraction, allowing backup, alternative reagents in the event of an ASFV outbreak.

In terms of timing and detectability of the virus in various organs and body fluids, including semen, IM infection represents a worst-case scenario. Studies using other strains of ASFV show that the incubation period can be several hours to days shorter and is much more efficient. This route of inoculation does not correspond to the natural route of transmission, which is usually oronasal in the absence of the tick vector. In the presented study with a complex and interlinked design, the IM scenario was accepted to ensure that the following study parts could be conducted. Thus, proof of concept was provided, but future studies should target the natural route and its shedding kinetics.