NPB FINAL RESEARCH GRANT REPORT FORMAT

Project Title: Evaluation of meat juice and muscle swabs using OIE-approved real time polymerase chain reaction (RT-PCR) from swine with clinically compatible signs of ASF in Ugandan slaughterhouses
NPB Project ID number: 20-150
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Industry Summary:
This research project assessed the African swine fever virus's (ASFV) nucleic acid detection in slaughter expedient samples: diaphragm meat juice, diaphragm muscle swab, spleen, and spleen swab. The collection of samples from the carcass after the pluck is removed is made possible by targeting meat juice and meat swabs and are samples that are easily described and not easily confused with other organs. This study compared meat juice, muscle swabs, spleen, and spleen swabs from 501 pigs with at least two clinical or pathologic signs of ASF collected in slaughterhouses in Kampala, Uganda. The study also assessed pooling of known positive and known negative meat juice, muscle swab, and spleen samples in a ratio of one positive sample being pooled with four negative samples. The samples were tested using DNA extraction and polymerase chain reaction procedures used by and provided by the US Department of Agriculture's (USDA) Foreign Animal Disease Diagnostic Laboratory (FADDL). Meat juice characterized the most pigs as positive, exceeding the number classified as positive by approved USDA sample types. It is unclear if these differences are misclassification or enhanced classification, but given these results, a validation study under experimental settings is warranted. Finally, pooled spleen samples were all classified as positive, while one pooled meat juice sample and 5 pooled muscle swab samples were incorrectly classified as negative.

Key Findings:
- Meat juice (72.9%; 365/501) had the highest detection rate of ASF nucleic acid compared to muscle swabs (44.1% 221/501), spleen (50.7%; 254/501), and spleen swabs (49.3% (247/501).
- Spleen samples had the lowest cycle threshold values, indicating the higher load of viral nucleic acid, which led to more reliability with spleen samples when pooled.
- Meat juice samples require proper collection of meat and not associated tendon and hydrated animals. If tendon was collected or animals were dehydrated the muscle had to be freeze thawed with phosphate buffered saline to collect an adequate sample.

Keywords: African swine fever, diagnostic, meat juice, muscle swab

Scientific Abstract:
This study compared the detection of ASFV nucleic acid from pigs with clinically and pathologically compatible signs between four different sample types: diaphragm meat juice, diaphragm muscle swab, spleen, and spleen swabs. Pigs slaughtered at abattoirs around Kampala, Uganda were evaluated by veterinarians for signs of diarrhea, ecchymosis on the skin, enlarged and hemorrhagic spleens and lymph nodes, and kidney petechiation. If they exhibited at least three of these signs at the time of slaughter, they were included in the study. Diagnostic procedures were done using the US Department of Agriculture's Foreign Animal Disease Diagnostic Laboratory's Standard Operating Procedures. The Qiagen DNeasy kit was used for nucleic acid extraction and a real-time polymerase chain reaction assay previously described by Zsak *et al.* (2004) was used for amplification and detection of the target viral nucleic acid. Of the 501 pigs evaluated, diaphragm meat juice samples classified 365 (72.9%) as positive for ASFV, diaphragm muscle swabs classified 221 (44.1%), spleen samples classified 254 (50.7%) and spleen swabs classified 247 (49.3%) as positive. Meat juice had a statistically greater proportion classified as positive than other samples, and muscle swabs had a

statistically lesser proportion classified as positive than all other samples. Meat juice samples provide a reliable sample type for the diagnosis of ASF when using real time PCR.

Introduction:
African swine fever was diagnosed in the Dominican Republic in July 2021 and Haiti in September 2021, placing it in the Western hemisphere for the first time since the 1980s. This increases the threat of ASF to the United States and the urgency for preparation. Recognition of the need for increased capacity for sampling has driven the development of a Certified Swine Sampling Program and efforts by the USDA to approve more sample types for ASFV diagnosis in the event of an outbreak. Sampling at slaughterhouses may be a viable option for detection and proof of disease freedom. Muscle swabs and meat juice samples will be easy to collect samples that require little training if they provide reliable diagnoses. Limited studies have been published, but there has been some work that found meat juice was a reliable diagnostic sample for classical swine fever (Kaden *et al,* 2009; Lohse *et al,* 2011). As for ASF diagnostics, meat juice has been shown as reliable as spleen when a novel PCR probe was used (McKillen *et al,* 2010). Further, if these samples could be pooled, they could allow for testing of multiple animals in one reaction, which would assist with resource management. Beemer *et al* (2019) highlighted the need for pooled samples by analyzing the impact the use of oral fluids would have on manpower, supplies, and reagents. Overall, the scientific literature is sparse on this topic, despite the ease of access to these samples and there is no apparent work on muscle swab sample comparisons to traditional samples or use in diagnostics. As the threat of ASF importation to the United States grows, improving surveillance capacity at all levels, including at the sample collection level is critical to readiness. This study evaluates meat juice and muscle swab samples against spleen and spleen swab samples from pigs with clinical and pathologic signs consistent for ASF. This report presents findings from 501 pigs that were sampled.

Objectives:
Surveillance for African swine fever (ASF) at a scale to provide surety to disease free zones and compartments will require easily accessible sample and/or aggregate sample types. Currently, sample types include tonsil and spleen, and even pooled spleens, which is critical progress on expanding acceptable sample types for use in the National Animal Health Laboratory Network (NAHLN). Yet, slaughterhouses result in a final carcass separated from its organs and testing by carcass would be simplified if meat juice or muscle swab samples were shown to be reliable for ASF detection. This study aims to:

- Evaluate muscle swabs for at least a 90% sensitivity for detection of ASF virus compared to the traditional spleen sample and spleen swab.
- Evaluate meat juice for at least a 90% sensitivity for detection of ASF virus compared to the traditional spleen sample and spleen swab.
- Evaluate pooled samples in groups of 5 with one positive for at least 90% sensitivity for detection of ASF virus compared to the traditional spleen and pooled samples.

Materials & Methods:

The Pipestone Institutional Animal Care and Use Committee approved this study (IACUC protocol number 2021-4).

Pigs at abattoirs in Kampala, Uganda were visually assessed for lesions that were consistent with signs of African swine fever after they were slaughtered. Live animals were not handled as part of this project. Animals that had at least three of the following clinical signs were enrolled into the project: evidence of diarrhea, skin discoloration or ecchymoses, enlarged and hemorrhagic lymph nodes or spleen, and/or kidney petechiation. The date, location, clinical and pathologic signs were recorded along with a unique identification number for the pig. Samples were collected and included a piece of the diaphragm muscle from the thickest part of the muscle body that was at least 4 cm x 4 cm in size. This was collected and placed in a whirl-pak bag (Lasec, Cape Town, South Africa). A polyester swab on a plastic shaft (Wuxi Nest Biotechnology Co. Ltd, Wuxi, Jiangsu, China), was then used to swab the cut surface of the diaphragm muscle. The swab was vigorously

rubbed on the cut surface and then placed in 2-3 ml of sample collection, transport and storage media (Wuxi Nest Biotechnology Co. Ltd, Wuxi, Jiangsu, China) in a plastic transport tube (Wuxi Nest Biotechnology Co. Ltd, Wuxi, Jiangsu, China), swirled about, the liquid pressed out, and then discarded. An approximately 3 cm x 3 cm piece of spleen was taken and placed in a whirl-pak bag as well. Finally, the spleen swab was collected in the same manner as the muscle swab, but from the cut surface of the spleen. These were transported in a cooler with cool packs to the Makerere University Central Diagnostic Laboratory where they were frozen at -20°C until further analysis.

Sample processing occurred as follows. Meat juice samples were freeze-thawed and if enough meat juice was not available after the first freeze-thaw, they were freeze-thawed again. If the samples still lacked enough meat juice for analysis (0.5 ml) then 0.5 to 2 ml of phosphate buffered saline (Fisher Scientific, Fair Lawn, NJ, USA) was added and the freeze-thaw repeated a third time. The amount of PBS added was influenced by the amount of juice in the bag as well as the amount of fat on the meat tissue. Samples with a lot of fat required more PBS to provide enough sample for analysis. The target was to obtain at least 0.5 ml of meat juice with PBS for analysis. In total, 33 of the samples had 0.5 ml of PBS added and 43 had 1 ml added, one had 1.5 ml added while two samples had 2 ml added. One gram of a spleen sample was placed into 9 ml of Dulbecco's Modified Eagle Media (Life Technologies Corporation, Grand Island, New York, USA) and homogenized using a Stomacher® 80 Biomaster (Seward Ltd, West Sussex, United Kingdom). This was then centrifuged at 1000 x g for 10 minutes and the supernatant collected and used for DNA extraction.

Meat juice, muscle swabs and spleen samples were also pooled in groups of five to evaluate the impact of pooling on African swine fever virus detection. Seventy-two pooled samples were created for each sample type by combining four negative samples and one unique positive sample. Use of positive samples was not repeated, but negative samples were used more than once in some instances especially when a given sample type had less than 72 samples with a negative result. Negative samples were samples that had an undetermined cycle threshold (Ct) and not samples with Ct values ≥ 40. The 72 samples with the lowest Ct values when tested individually were used for pooling for each of the sample types. These pooled samples were extracted and tested in the same manner that individual samples were handled.

Qiagen DNeasy tissue and blood kits (Qiagen, Hilden, Germany) were used for nucleic acid extraction following standard operating procedures (SOPs) that aligned with manufacturer's instructions and followed the USDA Foreign Animal Disease Diagnostic Laboratory's (FADDL) SOPs. The real time PCR was previously described by Zsak *et al* (2004) and run on a QuantStudio 5 thermocycler (Thermo Fisher Scientific, Waltham, MA, USA) following FADDL's SOPs. The VetMax Xeno internal positive control (IPC) DNA as well as the VetMAX Xeno Internal Positive Control (IPC) - LIZ Assay (Thermo Fisher Scientific, Waltham, MA, USA) were used in all samples for the individual sample testing as per FADDL SOP recommendations, and it were used only in the extraction controls for the pooled sample analysis due to limited supplies.

Analysis included calculation of the percentage of positive samples and sample pools by sample type, calculation of the 95% confidence interval using the Agresti-Coul method (Brown *et al*, 2001) and two-by-two comparisons of detection by sample types using a McNemar chi-squared test for matched data with a Bonferroni adjusted level of significance for six comparisons (alpha =0.0083) was done. Analysis for agreement between sample types was also done using a Cohen's and Brennan and Prediger inter-rater agreement; the latter adjust for prevalence and bias (Byrt, Bishop, and Carlin, 1993; Gwet, 2004) with agreement described as poor, slight, fair, moderate, substantial, and almost perfect (Landis and Koch, 1977). Finally, visual comparison of Ct values was completed by creating scatter plots of Ct values from different samples from the same pig and then fitting the line of best fit. STATA 16.1 IC (Stata Corp, Texas Station, Texas, USA) was used for analysis and Microsoft Excel version 16.63.1 (Microsoft Corporation, Redmond, Washington, USA).

Results:

In total, 98 (19.6%) meat juice samples did not provide enough meat juice after the first freeze-thaw cycle for analysis and were frozen and thawed for a second time. Twenty-seven of the 98 samples (27.6%) provided enough juice for analysis at this step. The remaining samples (71/98; 72.4%) either had little juice that could not be easily pipetted off or had no juice at all. It was observed that these had varying amounts of tendon and or fat tissue that had been collected alongside the meat muscle. These were refrozen with 0.5 to 2 ml of PBS to collect enough meat juice.

Sample detection and agreement is summarized in Table 1. Of the samples tested from 501 pigs, meat juice had the largest proportion of samples test positive for African swine fever virus (365/501, 72.9%) and muscle swabs had the smallest proportion (221/501, 44.1%). There was an association between sample type and outcome for all pairwise comparisons with that included meat juice, but the comparison between spleen samples and spleen swabs and muscle swab and spleen swab when considering the Bonferroni adjustment did not have significant associations. Further, when considering agreement between the sample types the percent agreement ranged from 61.5% to 69.7% and showed fair agreement with Cohen's kappa scores ranging from 0.225-0.39 and Brennan & Prediger Kappa ranging from 0.23 to 0.39) for pair-wise comparisons and moderate levels of agreement overall with kappas of 0.41. The Cohen and Brennan & Prediger kappa statistics did not differ, suggesting no bias associated with prevalence or test bias.

Table 1: Summary of African swine fever virus nucleic acid detection and comparison of sample type from slaughterhouse pigs with compatible clinical and pathologic signs in Kampala, Uganda

Meat juice			**Muscle swab**			**Spleen**			**Spleen swab**			
n	# positive (%)	95% CI	n	# positive (%)	95% CI	n	# positive (%)	95% CI	n	# positive (%)	95% CI	
501	365 (72.9%)	(68.8%, 76.6%)	501	221 (44.1%)	(39.8%, 48.5%)	501	254 (50.7%)	(46.3%, 55.1%)	501	247 (49.3%)	(44.9%, 53.7%)	

Pair-Wise Comparison Agreement, McNemar Chi-Squared P-value

	% Agreement	*McNemar P-value*	*Cohen's Kappa*	*Brennan & Prediger Kappa*		*% Agreement*	*McNemar P-value*	*Cohen's Kappa*	*Brennan & Prediger Kappa*
Meat juice vs muscle swab p-value	64.1%	<0.0001	0.32	0.28	***Muscle swab vs spleen p-value***	69.1%	0.008	0.38	0.38
Meat juice vs spleen p-value	61.5%	<0.0001	0.225	0.23	***Muscle swab vs spleen swab p-value***	69.7%	0.035	0.39	0.39
Meat juice vs spleen swab p-value	64.5%	<0.0001	0.29	0.29	***Spleen vs spleen swab p-value***	70.3%	0.5663	0.41	0.41
Overall	66.5%	<0.0001	0.34	0.33					

Agresti-Coul confidence intervals were calculated.
The significance level of the McNemar p-value, that assessed if there was difference in detection between sample types, was adjusted using a Bonferroni adjustment and was set at 0.0083.
All kappa results were statistically significant at a significance level of 0.05.

Scatter plots on ordered data reveal the relationship between cycle thresholds (Ct) of different samples from the same animal. Meat juice had a higher Ct than spleen samples and spleen swabs, muscle swabs had a higher Ct value than meat juice and muscle swab, and spleen swabs had a higher Ct than spleen. The slopes differ based on the compared sample types with meat juice to muscle swab and spleen to spleen swab having the closest relationship of a 1:1 change ratio.

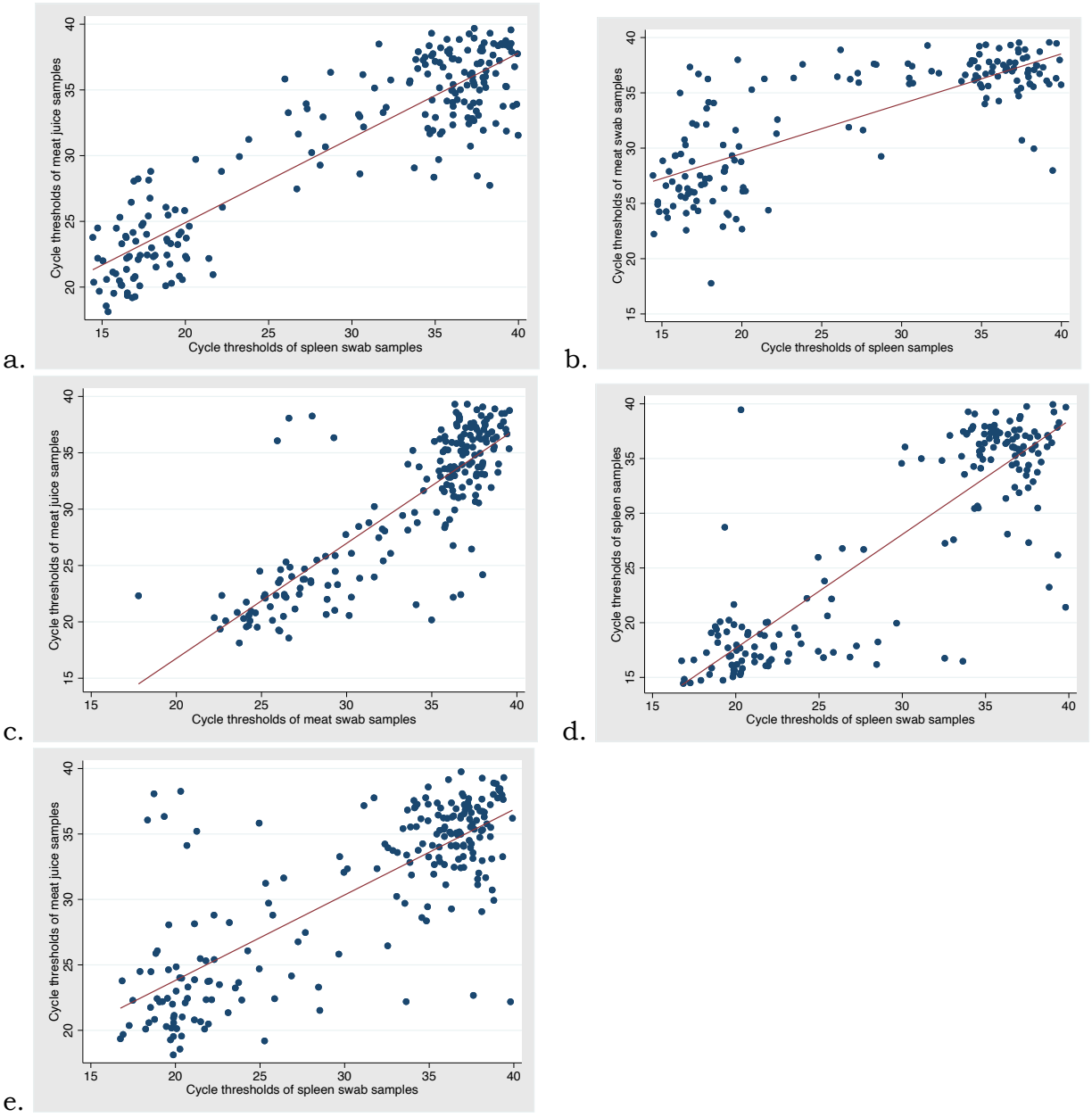

Figure 1a-e: Scatter plots with lines of best fit of cycle threshold values of different sample types from slaughterhouse pigs with compatible clinical and pathologic signs tested using a real-time PCR assay to detect African swine fever virus. a) compares meat juice and spleen samples; b) compares muscle swab to spleen; c) compares meat juice to muscle swab; d) compares spleen to spleen swab; and e) compares meat juice to spleen swab.

Table 2 summarizes the results from efforts to detect African swine fever virus in pooled samples (one positive:4 negative samples). Pooled samples had 100% of spleen pools showing ASFV detection with a median Ct of the positive sample of 17.9. For meat juice, 98.6% of the pools (71/72) were positive and had a median Ct of 23.2. The undetected pool of meat juice used a positive sample with a Ct of 25.9. Muscle swab pools had a 93.1% detection rate with a mean Ct of 26.7 and the 5 undetected pools had a mean Ct of 28.9. When the negative pooled samples were re-tested using Xeno as an internal positive control in the sample, they were positive, but the entire plate of samples was not re-tested as not enough Xeno IPC was available.

Table 2: Comparison of African swine fever virus detection by a real-time PCR assay in pooled samples by sample types collected from slaughterhouse pigs with compatible clinical and pathologic signs in Kampala, Uganda

	Spleen		Meat Juice		Muscle Swab	
	# (%)	Median Ct (Range)	# (%)	Median Ct (Range)	# (%)	Median Ct (Range)
Positive pools	72 (100%)	17.9 (14.4, 28.8)	71 (98.6%)	23.2 (18.1, 30.0)	67 (93.1%)	26.7 (17.8, 33.6)
Negative pools	0 (0%)	--	1 (1.4%)	25.9 (-,-)	5 (6.9%)	28.9 (25.9, 33.3)

Discussion:

This study compared meat juice and muscle swabs for the diagnosis of African swine fever in clinically and pathologically compatible pigs to spleen and spleen swab samples. The meat juice sampled categorized the most pigs as positive. There can be two reasons why there were more positive results with meat juice than with spleen swabs, both of which need further investigation.

The first reason that more samples tested positive with meat juice was that meat juice was a more reliable sample. Other work has shown that meat juice was a reliable sample, but in these studies, meat juice did not exceed the detection of other well-characterized samples. For example, Lohse *et al* (2011) showed that meat juice had a lower sensitivity than serum for detecting classical swine fever. Another study evaluated meat juice against whole blood in inoculated animals with ASFV strains of varying virulence, and all samples were positive in pigs inoculated with the highly and moderately virulent strains, but the Ct values were lower for the whole blood (Onyilagha *et al*, 2021). A recent study evaluating foot-and-mouth disease virus (FMDV) detection showed that meat juice samples from the biceps femoris muscle of experimentally infected animals could be used as reliably as sera in detecting FMDV using real-time reverse transcriptase PCR techniques (Yeo et al, 2020). Yet, there were some challenges with the sample type even if its detection was better. Diaphragm muscle had tendons and fat associated with it, and when present these reduced the amount of meat juice recovered. Dehydration of the animal also likely impacted meat juice recovery. A validated method is needed for samples that return limited meat juice after an initial freeze-thaw. We added PBS at volumes of 0.5 to 2 ml. The limitation of the sample was the need to freeze and then thaw the sample to collect the exudate for testing. It was relatively easy to collect, but it may limit high throughput sample testing because of this freeze-thaw step. This is exacerbated for samples that fail to provide adequate exudate and require a second freeze-thaw with additional PBS. The second freeze thaw could also reduce the diagnostic capability of the assay. Yet, the overall processing was simpler than tissues in that no dilution and homogenization was needed.

The second option is that meat juice results have a high number of false positive results and the specificity of the assay is impacted when meat juice is a sample type. Myoglobin is a known PCR inhibitor, as is hemoglobin, and their impact is related to iron that impacts DNA synthesis (Rådström *et al*, 2004). This may be addressed by using a different polymerase than the Taq polymerase (Belec *et al*, 1998; Rådström *et al*, 2004). Yet, if the issue was inhibition, one would expect more false negatives relative to the USDA approved sample types of spleen and spleen swab, as was seen with the muscle swab. Therefore, prior to use of meat juice samples, further work including a large-scale negative cohort study should be done to determine if the results from this study are due to false positive results or better detection. Also, a full characterization of testing against positive samples should be done to determine the ranges in detectability based on the virulence of the infecting ASF variant.

Pooling of five samples by type showed the most reliable sample type for detection when pooled was spleen samples. Among the individual samples, the lowest Ct values were found in spleen samples, then spleen swabs, then meat juice, and finally muscle swabs. As a result, the positive samples used for the pooled spleen samples had the lowest mean Ct of 18.671, followed by meat juice samples (Ct=23.511), and finally muscle swab samples (27.248). The lower Ct values may be most predictive of success in pooling.

Meat juice and muscle swabs were simple to collect, and easier to process than tissues if the first freeze-thaw cycle was effective. Collection requires careful sampling of the diaphragm muscle pillar. Meat juice provided more positive results than spleen samples and may provide an easy to collect sample type for routine monitoring at slaughterhouses. A complete validation study to include a large-

scale negative cohort study would be worthwhile based on these results to fully characterize the potential sensitivity and specificity of this sample type.

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