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Evaluation of porcine respiratory and reproductive syndrome virus (PRRSV) ante-mortem diagnostic techniques

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Evaluation of porcine respiratory and reproductive syndrome virus (PRRSV) ante-mortem diagnostic techniques

by

Abby Rae Patterson

A thesis submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of
MASTER OF SCIENCE

Major: Veterinary Diagnostic and Production Animal Medicine

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ABSTRACT

Objective

The specific objective of this thesis was to address the U.S. pork industry's need for evaluation of alternative ante-mortem diagnostic samples for PRRSV. To accomplish this objective, three trials were undertaken with the following goals:

1. Develop a sampling protocol for the blood swab method.
2. Analyze the diagnostic accuracies of the blood swab, the capillary tube and the jugular sampling methods on two standard PRRS diagnostic tests: quantitative real-time reverse transcriptase polymerase chain reaction (qRT-PCR) and ELISA in known positive and negative animals.
3. Analyze the diagnostic accuracy of the blood swab method for ELISA testing in commercial finishing swine.

Materials and Methods

Trial one

Three trials were conducted as follows: (1) previously collected swine blood was used to compare blood absorption of two brands of swabs, (2) finisher swine in a commercial site were used to compare a 20g x 1/2" needle and a 25g x 5/8" needle using the Fisherbrand swabs or the Puritan swabs and (3) sows were used to compare blood absorption of the two brands of swabs under field conditions.

Trial two

Thirty, mixed gender, seven week old, crossbred, PRRSV negative pigs (21.4 ± 7.6 kg) were used. Fifteen pigs were uninfected controls and 15 pigs were inoculated with PRRSV VR-2332. Blood was collected weekly from each pig for seven weeks post-inoculation (PI) by three methods: (1) jugular vein collection with a 9ml vacutainer tube, (2) auricular vein collection with a sterile polyester swab and (3) auricular vein collection with a capillary tube system. All samples were submitted for qRT-PCR and ELISA analysis. Area under the

receiver-operating characteristic curve (AUC) analysis was used to statistically evaluate continuous qRT-PCR and ELISA data for each of the three sampling methods.

Trial three

One hundred and thirty two, twenty-two week old, commercial swine were sampled as follows: (1) 9 ml venous jugular blood collection using an 18g x 1” bleeding needle and serum separator tube and (2) ear lance with a 20g x 1/2” needle followed by saturation of sterile polyester tipped applicator. BetaBuster freeware software was used to develop prior distributions for PRRSV prevalence within the population and the diagnostic parameters for both sampling methods. WinBUGS freeware software was used to estimate diagnostic parameters for the PRRSV ELISA test for both sampling methods. Using FreeCalc freeware software, a partial budget was constructed to evaluate the economic significance of implementing the blood swab method.

Results

Trial one

Trial 1 showed that under laboratory conditions, the Fisherbrand swabs absorbed a significantly higher volume of blood than the Puritan swabs. Trial 2 showed that when the 20g x 1/2” needle was used, significantly more blood was absorbed than when the 25g x 5/8” needle was used regardless of the swab type. Trial 3 confirmed that when the 20g x 1/2” needle was used, the swab absorbed significantly more blood.

Trial two

For qRT-PCR testing, the sensitivity and specificity for all sampling methods ranged from 93% - 100% for weeks 1-3 PI. Results of ELISA testing depended on cut-off selection. Optimization of ELISA S/P cut-off points for swab sample data was substantially lower (S/P ratio of 0.08 ± 0.05) than the industry standard (S/P ratio of 0.4).

Trial three

The sensitivity and specificity of the swab sampling method was 22.3% (95% probability intervals = 16.0%, 29.2%) and 94.3% (80.1%, 99.8%) respectively when an S/P ratio cut-off of 0.4 was used. The sensitivity and specificity were 94% (89.8%, 97.2%) and 93.5% (77.8%, 99.8%) respectively when an S/P ratio cut-off of 0.08 was used.

Implications

- Fisherbrand swabs absorb a significantly greater blood volume (167 μ l) than Puritan swabs (142 μ l) under ideal sampling conditions.
- Under field conditions, the Fisherbrand swabs absorbed numerically less blood (118 μ l) than under laboratory conditions (167 μ l).
- A Fisherbrand swab and a 20g x 1/2" needle combination would be the best diagnostic sample for sows and finisher pigs when collection time is less than 15 seconds.
- The capillary tube method suffers from neither inadequate volume nor differences in sample type collected (as compared to the blood swab method). This study indicated that the capillary sampling method can be used with ELISA and real-time qRT-PCR testing with diagnostic accuracy equal to the jugular sampling method. The total expense for the capillary sampling method was \$1.44/sample (\$0.94/tube in product and \$0.50/sample in labor cost (calculated for 3 minutes at \$10/hr)). In comparison, the total cost of the jugular sample was \$0.91/sample.
- Early diagnosis (weeks 1-3 PI) of PRRSV infected nursery pigs using real-time qRT-PCR under study conditions can be equivalently accomplished using the capillary, swab, or jugular sampling methods.
- No change in cut-off values for qRT-PCR data dichotomization is necessary for data obtained via any of the sampling methods.
- The diagnostic accuracy of PRRSV ELISA was poor for the swab sampling method when an S/P ratio cut-off of 0.4 was utilized (sensitivity ranged from 20% - 55.6% over weeks 2 - 7 PI).

- When optimal cut-off values are employed, as determined by AUC analysis, all sampling methods are capable of achieving very high diagnostic accuracy on PRRSV ELISA testing. These cut-off values may not be clinically useful.
- Under commercial (field) conditions, the sensitivity of the swab sampling method was low (22.3%) for ELISA results dichotomized at an S/P ratio of 0.4.
- The sensitivity of the swab method improved when a lower S/P cut-off was used (94%) indicating this method may have application in routine ELISA diagnostic monitoring programs.
- In comparison to the jugular sampling method, the sensitivity and specificity of the swab method is lower when used in commercial settings; this will result in more false negative and false positive test results.
- Under the assumptions of the partial budget, the jugular sampling method would cost a 1000-head sow operation \$0.15/pig produced while the swab sampling method would cost \$0.36/pig produced.

CHAPTER 1: GENERAL INTRODUCTION

Thesis organization

This thesis was written in order to provide swine practitioners with information on the diagnostic sensitivity and specificity of alternative blood collection methods, namely sterile polyester-tipped swabs and serum capillary tube systems. The first chapter of this work includes a review of various blood collection techniques presently used in the diagnosis and monitoring of porcine reproductive and respiratory syndrome virus (PRRSV) in swine. This review provides evidence of the need for a reliable, economic, humane, labor-reducing sampling technique for swine. The second, third and fourth chapters are formatted as for journal publication. Chapter two describes the protocol development for blood collection via the two alternative sampling techniques. The third chapter presents diagnostic results from the use of the alternative sampling techniques on swine artificially infected with PRRSV as compared to control animals. The fourth chapter describes the use of the alternative sampling method in a commercial production system. The results of the sampling show the diagnostic sensitivity and specificity of the alternative method. The final chapter includes general discussion on conclusions and results pertaining to the overall hypotheses of the trial.

Review of blood collection techniques in swine

Collection of blood from swine for diagnostic procedures has been accomplished from various vessels. Collection from the anterior vena cava or the jugular vein were among the first methods described for swine and continue to be commonly used today¹⁻⁸. Collection from other vessels has also been described including the tail vessels (via insertion of a needle between the 4th and 5th or 5th and 6th coccygeal vertebrae) described in 1967⁹, collection from the orbital sinus (via insertion of pipette into the medial canthus of the eye) described in 1969¹⁰ and various peripheral vessels including the ear vein¹, cephalic vein⁷, saphenous¹¹ vein, the subcutaneous abdominal vein (cranial superficial epigastric vein)^{12,13} and the femoral artery¹⁴. Additionally, intraosseous collection of blood has been researched¹¹.

While the above methods have been described, the disadvantages of each technique must be considered before implementation. The jugular/anterior vena cava method, while common, is a blind venapuncture into the jugular groove. As a result, damage to the phrenic nerve (leading to severe dyspnea and possible death), the thoracic duct (leading to aspiration of lymph), the thyroid gland, or to the pericardial sac (leading to hemorrhage) can occur - especially if collection is attempted on the animal's left side^{4-8,10,14,15}. Collection of blood from a tail vessel is technically more challenging than from the jugular vein (involves manipulation of the tail and location of the coccygeal vertebrae), results in a small sample volume and may lead to tail biting (which may lead to ascending infections)^{6,9}. Collection of blood from the orbital sinus is also more difficult than the jugular method (technique is more difficult to learn) and the procedure may lead to corneal abrasions¹⁰. Collection from superficial vessels, such as ear vessels, is slow, generally contaminated and may lead to hematoma formation⁶. Intraosseous collection may result in an increased incidence of sample hemolysis, osteomyelitis and lameness¹¹.

Multiple studies have also researched whether the location of the blood sample has an effect on the diagnostic value of the sample. Various factors of interest have been studied including the following: (1) Plasma free amino acid concentration (all sites examined in the study were found to be equivalent)¹⁶, (2) Serum enzyme concentration (enzyme concentrations were found to be dependent on collection technique rather than sample site)^{17,18}, (3) Arterial blood gas values (values were found to be similar between ear and anterior vena cava samples)¹⁹, and (4) glucose concentrations (concentrations were found to be higher in ear samples than venous samples likely due to the stress of restraint)²⁰. No studies were found in which sampling site and subsequent serological or nucleic acid based assays were analyzed. In general, it appears that stressful stimulations (environmental, collection technique/restraint, or other) have a greater influence on the diagnostic value of the sample than the sampling location¹⁷ or the experience of the collector¹⁸. Therefore, stressful stimulations including aversive handling (which negatively affects animal welfare and productivity²¹) and the use of

snares (snaring has been used as a method of stress induction in various endocrine studies²²⁻²⁴) should be minimized during blood sample collection.

In order to maintain a safe environment for the collector and decrease the stress for the animal, various techniques of restraint have been described including racks²⁵, slings²⁶, wire snares⁶ and holding smaller animals in dorsal recumbancy^{4,6}. Multiple studies have also developed various methods of catheterization in order to eliminate the need for restraint during blood collection²⁷⁻³³. Although these methods (exteriorized catheters and vascular access ports) allow sampling from unrestrained animals, they would place economic constraints on a production system as they often involve general anesthesia, veterinary consultation, equipment (access ports, catheters, etc.), and aseptic sample collection techniques after placement and post-surgical care. Additionally, the consequences of this system, such as subcutaneous abscessation, valvular lesions and local or systemic infections³² must be considered. Also, the use of the access port or catheter for extended periods has not been adequately documented.

In boar studs, where semen quality may be negatively influenced by the stress of restraint, a blood collection method in which no restraint is required would be beneficial. As catheterization would greatly increase the cost of sampling, blood collection from a peripheral vein during the time of semen collection appears to be the most economical and least stressful option. As many of the peripheral veins including the cephalic, saphenous and femoral veins are covered with a thick layer of subcutaneous fat or musculature, identification of these vessels is often difficult^{28,34}. In contrast, an ear vein is normally easily located and blood can be collected with either a plastic capillary tube or a sterile polyester swab after lancing the vein with a needle³⁵. Additionally, collection from the ear vein may be less likely to cause pain in the animal. In one study, a 9.6% (5/52) flinch rate was found when the auricular vein was used for blood collection³⁵. Comparatively, a 100% (20/20) flinch rate was found when a perineal vein was used³⁵.

This literature review was completed using the following search terms alone and in various combinations: “blood collection”, “diagnostic samples”, “blood samples”, “swine welfare”, “pain”, “stress”, “stress AND restraint”, “collection AND veterinary”, “collection AND methods”, “collection AND adverse effects”, “swine AND blood”, “swine AND welfare”.

The following databases were used to obtain potential papers: PubMed, AGRICOLCA, CAB abstracts, and the 2006 Swine information CD. Titles of potential papers were examined for relevance based on the inclusion of key words as described above. Abstracts for papers with relevant titles were obtained and examined. All full text articles containing information about collection techniques were collected and reviewed. During the review a subset of articles containing information about surgical and non-surgical catheterization and articles pertaining to stress induction were retained for use in the review. This review was limited to articles in which the text was in English.

Statement of problem

Porcine respiratory and reproductive syndrome virus (PRRSV) is an RNA virus of swine which is a member of the genus *Arterivirus*, family *Arteriviridae*, and order *Nidovirales*³⁶. Although researchers fulfilled Koch’s postulate for porcine respiratory and reproductive syndrome virus (PRRSV) in 1991³⁷, diagnosis by clinical signs alone remains challenging as many other viral and bacterial diseases have a similar clinical presentation. Clinically, PRRS can present in a herd as abortion storms or sporadic abortions, late-term abortions, or as premature farrowing with stillborn fetuses, partially autolyzed fetuses, or mummified fetuses³⁶. Other clinical features may include the following: (1) neonatal pigs - severe dyspnea and tachypnea; (2) sows - anorexia, fever, lethargy, pneumonia, agalactia, red/blue discoloration of the ears and vulva, and delayed return to estrus; (3) weaned pigs - pyrexia, pneumonia, lethargy, failure to thrive, and a marked increase in mortality, and (4) boars - anorexia, pyrexia, coughing, and loss of libido³⁶. While these signs are documented, many other viral and bacterial diseases have a similar clinical presentation making diagnosis based on signs alone challenging. Therefore, virus isolation, serological tests, and various reverse

transcriptase polymerase chain reaction (RT-PCR) assays from clinical samples are utilized to make a clinical diagnosis³⁸.

In 2005, it was estimated that this disease costs the United States approximately \$560.32 million dollars annually from decreased production (sum of farm level production losses from breeding through the grow-finish phase)³⁹. While this study did not include the cost of diagnostic monitoring, frequent testing for PRRSV is a common component of herd health programs due to the economic severity of the disease. Monitoring in negative or low prevalence herds is especially common and has four major objectives as outlined by Dufresne et al. (2003)⁴⁰:

1. “Assess the health status of a group of animals prior to movement and/or introduction into an expected negative population.
2. Confirm the continued (negative) health status of an existing expected negative herd.
3. Monitor the health status of a subpopulation of expected negative animals residing within a known positive (but expected non-shedding) population.
4. Detect and eliminate positive animals in a subpopulation of expected low prevalence in the course of a disease elimination project.”

In order to develop diagnostic monitoring plans which will economically detect disease with reasonable confidence, information about the diagnostic accuracy of the sampling method is necessary. This project will provide producers and practitioners with information on the diagnostic accuracy (sensitivity/specificity) of two alternative sampling methods – the blood swab method and the capillary tube system. Real-time reverse transcriptase polymerase chain reaction (RT-PCR) and enzyme linked immunosorbent assay (ELISA) testing will be utilized in chapter three of this work.

While real-time RT-PCR is used widely as a diagnostic tool and for routine monitoring in boar studs, breeding facilities, nurseries, and at grow/finish sites, it is more expensive than serological methods - roughly five times more expensive. Additionally, the emergence of

PRRSV strains that escape detection by some PCR methods (Dr. Jim Collins at University of Minnesota, personal communication) illustrates the urgent need for additional diagnostic approaches for PRRS monitoring. Therefore, emphasis will be placed on the usefulness of alternative measures for ELISA testing in the fourth chapter of this thesis.

The specific objectives of this thesis were as follows:

4. Develop a sampling protocol for the blood swab method.
 - a. Determine the volume of blood absorbed by various brands of swabs under laboratory conditions.
 - b. Compare laboratory absorption values with absorption values collected under field conditions.
 - c. Evaluate the ease of the collection via the ear vein under field conditions.
5. Analyze the diagnostic accuracies of the polyester-tipped swab, the capillary tube and the jugular sampling methods on two standard PRRS diagnostic tests: real-time RT-PCR (RT-PCR) and ELISA in known positive and negative animals.
 - a. Determine optimal cut-off values for ELISA result dichotomization.
 - b. Determine the sensitivity and specificity of each sampling method for both RT-PCR and ELISA testing.
6. Analyze the diagnostic accuracy of the capillary tube sampling method for ELISA testing in commercial finishing swine.

CHAPTER 2. PROTOCOL FOR USE OF STERILE SWABS FOR ANTEMORTEM SERUM SAMPLING IN SWINE

A paper to be submitted to *The Journal of Swine Health and Production*

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Summary

Objectives: The objective of this study was to determine the volume of blood absorbed by various brands of swabs under laboratory conditions, evaluate the ease of the collection under field conditions, and compare laboratory absorption values with absorption values collected under field conditions.

Materials and Methods: Three trials were conducted as follows: (1) previously collected swine blood was used to compare blood absorption of two brands of swabs, (2) finisher swine in a commercial site were used to compare a 20g x 1/2" needle and a 25g x 5/8" needle using the Fisherbrand swabs or the Puritan swabs and (3) sows were used to compare blood absorption of the two brands of swabs under field conditions.

Results: Trial 1 showed that under laboratory conditions, the Fisherbrand swabs absorbed a significantly higher volume of blood than the Puritan swabs. Trial 2 showed that when the 20g x 1/2" needle was used, significantly more blood was absorbed than when the 25g x 5/8" needle was used regardless of the swab type. Trial 3 confirmed that when the 20g x 1/2" needle was used, the swab absorbed significantly more blood.

Implications: Fisherbrand swabs absorb a significantly greater blood volume (167 μ l) than Puritan swabs (142 μ l) under ideal sampling conditions. Under field conditions, the Fisherbrand swabs absorbed numerically less blood (118 μ l) than under laboratory conditions (167 μ l). A Fisherbrand swab and a 20g x 1/2" needle combination would be the best diagnostic sample for sows and finisher pigs when collection time is less than 15 seconds.

Introduction

Blood can be procured from swine by various routes for ante-mortem diagnosis of disease. Venapuncture of either the anterior vena cava or the jugular vein is among the most commonly used methods today. Blood from either of these vessels is obtained by restraining the animal with a wire snare and inserting a needle into the right jugular fossa, directed slightly toward the opposing shoulder, perpendicular to the neck, at the level of the manubrium sterni^{1,4,5,8,9}. Collection from other vessels has also been described including the tail vessels via insertion of a needle between the 4th and 5th or 5th and 6th coccygeal vertebrae⁷, collection from the orbital sinus via insertion of pipette into the medial canthus of the eye¹¹ and various peripheral vessels including the ear vein⁶, cephalic vein¹⁰, saphenous vein¹², the subcutaneous abdominal vein (cranial superficial epigastric vein)^{13,14} and the femoral artery¹⁵. Additionally, intraosseous collection of blood has been researched¹².

While the above methods have been described, the disadvantages of each technique must be considered before implementation. The jugular/anterior vena cava method, while common, is a blind venapuncture into the jugular groove. As a result, damage to the phrenic nerve (leading to dyspnea), the thoracic duct (leading to aspiration of lymph), the thyroid gland, or the pericardial sac (leading to hemorrhage) can occur - especially if collection is attempted on the animal's left side^{1,5,8-11,15}. Collection of blood from the tail vessels is technically more challenging than a jugular sample, results in a small sample volume and may lead to tail biting (which may lead to ascending infections)^{7,9}. Collection of blood from the orbital sinus is also subjectively more difficult than the jugular method (technique is more difficult to learn) and the procedure may lead to corneal abrasions or damage to other ocular

structures¹¹. Intraosseous collection may result in an increased incidence of sample hemolysis, osteomyelitis and lameness¹². Collection from superficial vessels, such as ear vessels, is slow, generally contaminated and may lead to hematoma formation⁹.

While ear vessels have the above described disadvantages, blood collection from this location, if coupled with a sterile polyester swab, can provide a technically easier, less invasive, and less stressful method (in situations when animals are not snared) of blood collection than the commonly used jugular location. Work by Reicks et al 2006 has indicated that during the first six days post-infection with PRRSV, the blood swab method is diagnostically comparable to the jugular sample when PCR testing is utilized³⁵.

As there has been no published study evaluating various blood swab protocols, the objective of this trial was to determine the volume of blood absorbed by various brands of swabs under laboratory conditions, compare laboratory absorption values with absorption values collected under field conditions, and evaluate the ease of the collection via the ear vein under field conditions.

Materials and Methods

Trial 1: Evaluation of blood absorption under lab conditions

Two brands of polyester swabs, Puritan (product number 25-806 1PD, Puritan Medical Products Company LLC, Guilford, Maine.) and Fisherbrand (product number 14-959-90, Fisher Scientific International, Waltham, Maine), were tested. A standard curve was developed using a 200 µl pipettor (Pipetman P, Gilson Inc., Middleton, Wisconsin), whole blood collected from swine in EDTA tubes (product number 366385, BD, Franklin Lakes, New Jersey), and an analytical scale (PB303, Mettler Toledo Inc., Columbus, Ohio). The following volumes of blood were weighed to create the standard curve: 25, 50, 75, 100, 125, 150, 175, and 200 µl. A second standard curve using the above described procedure was developed after all swabs were weighed. The pre- and post- experiment measurements were averaged to develop the standard curve. For each swab tested, the following procedure was

used: (1) the scale was tared with the swab and weigh boat, (2) the swab was submerged into the blood for 5 seconds, (3) the swab was held above the blood container for 5 seconds, and (4) the swab was reweighed. The weight data collected from the swab was then converted to a volume using a simple linear regression line fitted from the standard curve data. Descriptive statistics analyzed included mean, standard deviation, and range of the volume of blood absorbed by the two brands of swabs. A student's t-test was used to compare the mean volume of blood absorbed by each swab brand using the JMP Statistical Discovery 6.0.0 program (SAS Institute, Cary, North Carolina).

Trial 2: Evaluation of blood absorption on a commercial finisher site

Puritan swabs were placed in 5ml, sterile polystyrene culture tube (product number 352058, BD, Franklin Lakes, New Jersey), labeled, pre-weighed and taken to the finisher site. The commercial finisher site consisted of a 1,000 head barn which was naturally ventilated, had completely slatted floors, and utilized auto-sort scale technology to manage the animals. At the site, the finisher pigs (~ 150 lbs) were restrained and 2ml blood was collected from the jugular vein into EDTA tubes. The left ear was lanced using a 25g x 5/8" needle in either the lateral or medial auricular vein. A pre-weighed swab was used to collect blood from the ear for 15 seconds. This swab was then designated 25 F as it was collected using the 25g needle on the farm. The process was repeated on the right ear using a 20g x 1/2" needle. This swab was then designated 20 F as it was collected using the 20g needle on the farm. After 5-7 samples were taken, the samples were re-weighed on the farm site. The samples were also re-weighed approximately 6 hours after the start of collection in the laboratory. These swab measurements were labeled 20 L and 25 L as the weights were recorded in the laboratory (the number indicates the needle size and L designates the location of the sample weight measurement, namely the laboratory). Volume data was generated using an individual standard curve for each sample (generation of standard curve is described in the previous subsection). A matched pair analysis (JMP Statistical Discovery 6.0.0 program, SAS Institute, Cary, North Carolina) was used to compare the mean volume of blood absorbed by the swabs and the location. This procedure was subsequently repeated at a different finisher site using the Fisherbrand swabs. The second finisher site was a 1,200 head, naturally

ventilated barn with completely slatted floors; at the time of sampling pigs were approximately 100 lbs. The procedure for sampling was the same as previously described except at this site, swabs were only weighed upon return to the laboratory.

Trial 3: Evaluation of blood absorption on a sow site

A 120 sow farrow-to-finish operation was utilized for this study. At this site, gestating sows were housed in completely slatted crates in a naturally ventilated building; a trough watering system was used and the animals were fed by hand twice a day. Before visiting the trial site, 20 gestating sows of unknown parity (not all sows were individually identified and parity records were not kept) were randomly assigned to one of four sample collection methods (n=5 for each group): (1) Puritan swab and 25g x 5/8" needle, (2) Puritan swab and 20g x 1/2" needle, (3) Fisherbrand swab and 25g x 5/8" needle, and (4) Fisherbrand swab and 20g x 1/2" needle. Additionally, swabs were prepared as described for Trial Two prior to visiting the site. At the site, the sows were restrained using a wire snare and blood was collected from the jugular vein into 9 ml Vacutainer EDTA tubes. The right medial or lateral auricular vein was lanced using the designated needle. The designated swab was then used to collect blood from the ear for 15 seconds. The samples were weighed approximately 4 hours after the start of collection in the laboratory. Volume data was generated as previously described. A standard least squares analysis (JMP Statistical Discovery 6.0.0 program, SAS Institute, Cary, North Carolina) was used where needle type, swab type, and their interaction were independent variables and volume of blood absorbed was the dependent variable. For all trials, summary statistics and graphs of the data were analyzed to assess data distribution and accuracy.

Results

Trial 1

Puritan swabs absorbed a greater ($p < 0.0001$) mean volume of blood, 142 μl (95% confidence interval: 136 μl , 148 μl), when compared to the Fisherbrand swabs which absorbed a mean volume of 167 μl (95% confidence interval: 162 μl , 172 μl).

Trial 2

When needle size was compared using only the Fisherbrand swabs, the swabs absorbed a mean volume of 118 μl (95% confidence interval: 108 μl , 128 μl) when the larger needle was used, while the swabs absorbed a significantly smaller ($p < 0.0001$) mean volume, 79 μl (95% confidence interval: 65 μl , 94 μl) when the smaller needle was used. When needle size was compared using only the Puritan swabs, the swabs absorbed a mean volume of 101 μl (95% confidence interval: 87 μl , 115 μl) when the larger needle was used, while the swabs absorbed a significantly smaller ($p < 0.0001$) mean volume, 45 μl (95% confidence interval: 32 μl , 59 μl) when the smaller needle was used. There was no significant difference ($p = 0.85$) in the mean volume of blood absorbed when samples were weighed on the farm and when samples were weighed in the laboratory.

Trial 3

When swab type and needle size were compared together on the sow farm, there was a significant difference ($p < 0.0001$) between needle types, but unlike Trial One no significant difference ($p = 0.86$) was detected between swab types. There was also no significant interaction ($p = 0.32$) between swab type and needle type. Table 2.1 reports the mean volume with 95% confidence intervals for both the Fisherbrand and Puritan swab types for both needle sizes.

Discussion

The first objective in this study was to determine the maximum volume of blood (μl) that could be absorbed by a swab under ideal conditions. This was tested by submerging the swabs in previously collected blood. The current recommended submission for real-time polymerase chain reaction (RT-PCR) at Iowa State University (ISU) Diagnostic Laboratory is 1 ml of serum of which 50 μl are used per RNA extraction (personal communication, Dr. Karen Harmon, Adjunct Assistant Professor and Associate Scientist at ISU)⁴¹. The results of this study indicate that Fisherbrand swabs absorb significantly more blood than Puritan

swabs. As small differences in the initial volume of blood collected on the swab may be magnified during subsequent sample dilution (for maintenance of virus during transportation), the use of the Fisherbrand swabs is recommended in order to achieve the highest initial blood volume of on the swab. Additionally, variation in the blood volume collected may result from a failure to recover an undermined portion of the blood sample from the swab and/or failure to achieve maximum blood absorption under field conditions (poor technique, poor ear vein visualization, etc.). In order to reduce sample dilution, the minimum amount of saline required for coverage of the swab should be used. In general, 0.5 – 1 ml of saline will sufficiently cover the swab. This volume of saline will also provide adequate supernatant (after sample processing) to run multiple diagnostic tests on the sample.

The second objective in this study was to evaluate the ease of collection under field conditions. This was done by (1) setting a time limit of 15 seconds for blood collection to assess the efficiency of the process, (2) using two different needle sizes to compare ease of collection, and (3) analyzing the effect of time of weight measurement for future trial work. The blood swab method is currently used on unrestrained boars during semen collection for routine diagnostic blood collection; therefore, a time limit of 15 seconds was chosen to minimize contact with the boar. With this time limit in place, the two needle sizes were compared to determine if a larger needle size would allow more blood to be absorbed. Since the data indicated that significantly more blood was absorbed by both the Fisherbrand and Puritan swab types when the larger needle was used, it is highly recommended that the larger needle be used when the sampling time is limited. The larger needle allowed a faster flow rate which limits the contact time and decreases the stress of the animal. This increases the safety of the animal and the collector.

While the drying effect on volume of sample has little significance in commercial production settings (where swabs are placed in transport media prior to processing and submission), this was a potential confounding factor during the development of the blood swab protocol. Because Trial One and Two data indicated that there was no significant difference in weights recorded immediately after sampling and weights recorded after samples were returned to the

laboratory, Trial Three did not include weight measurements in the field. There are at least two plausible explanations for the lack of weight difference between recording locations: 1) the swabs were already dried prior to the first sampling or 2) significant drying did not occur during the interim between sampling and weighing.

The third objective in this study was to compare laboratory absorption values with absorption values collected under field conditions. This was done by assigning twenty sows to one of four sampling method groups as described above. Similar to Trial Two, the swabs absorbed more blood when the 20g needle was used compared to the 25g needle. However, there were no significant difference between the volume of blood collected between the Fisherbrand swabs and the Puritan swabs. Swab type blood absorption differences may have been masked as the needle effect was so great in this trial. Additionally, the sample size ($n=5$ per group) may have been too small and therefore may not accurately reflect the true difference in swab type as shown in Trial One. (A power analysis done on the data indicated the power obtained in the trial was 0.92.) It was also observed in this trial that due to the larger size of the medial or lateral auricular vein in the sow, it was not uncommon to fill the swab to capacity in less than 15 seconds when the larger needle was used. This is likely why the swab sample volume collected under field conditions in breeding-sized animals parallels the volume collected under ideal conditions.

Implications

- Fisherbrand swabs absorb a significantly greater blood volume ($167\mu\text{l}$) than Puritan swabs ($142\mu\text{l}$) under ideal sampling conditions.
- Under field conditions, the Fisherbrand swabs absorbed numerically less blood ($118\mu\text{l}$) than under laboratory conditions ($167\mu\text{l}$).
- A Fisherbrand swab and a 20g x 1/2" needle combination would be the best diagnostic sample for sows and finisher pigs when collection time is less than 15 seconds.

Table 2.1. Mean blood volume absorbed with 95% confidence intervals for four combinations of swab and needle type.*

Swab type†	Needle size	Mean volume (μl)	Upper 95% CI (μl)	Lower 95% CI (μl)
Puritan	20g x 1/2"	150	129	171
Puritan	25g x 5/8"	77	11	143
Fisherbrand	20g x 1/2"	138	123	153
Fisherbrand	25g x 5/8"	86	29	142

*Twenty sows from a 120 sow farrow-to-finish operation were randomly assigned to one of four sample collection methods (n=5 for each group). Sows were restrained using a wire snare and blood was collected from the jugular vein into 9 ml Vacutainer EDTA tubes and from the right medial auricular vein (using the appropriate needle to lance the auricular vein and the appropriate swab to collect blood). Sample weight was converted to volume using a standard curve generated from blood collected from each sow. A standard least squares analysis (JMP Statistical Discovery 6.0.0 program, SAS Institute, Cary, North Carolina) was used to evaluate statistical significance ($p < 0.05$) of needle type, swab type, and the needle – swab interaction. A simple distribution analysis was done to obtain mean volume and 95% confidence intervals (CI).

† Two brands of polyester swabs Puritan (product number 25-806 1PD, Puritan Medical Products Company LLC, Guilford, Maine.) and Fisherbrand (product number 14-959-90, Fisher Scientific International, Waltham, Maine) were tested in combination with either a 20g x 1/2" or a 25g x 5/8" needle.

CHAPTER 3. EVALUATION OF ALTERNATIVE ANTEMORTEM DIAGNOSTIC SAMPLES FOR PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME VIRUS (PRRS)

A paper submitted to *The Journal of Swine Health and Production*

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Summary

Objective: To assess the diagnostic accuracy of two minimally invasive methods of blood collection.

Materials and methods: Thirty, mixed gender, seven week old, crossbred, PRRSV negative pigs (21.4 ± 7.6 kg) were used. Fifteen pigs were uninfected controls and 15 pigs were inoculated with PRRSV VR-2332. Blood was collected weekly from each pig for seven weeks post-inoculation (PI) by three methods: (1) jugular vein collection with a 9 ml vacutainer tube, (2) auricular vein collection with a sterile polyester swab and (3) auricular vein collection with a capillary tube system. All samples were submitted for quantitative real-time reverse transcriptase polymerase chain reaction (qRT-PCR) and ELISA analysis. Area under the receiver-operating characteristic curve (AUC) analysis was used to

statistically evaluate continuous qRT-PCR and ELISA data for each of the three sampling methods.

Results: For qRT-PCR testing, the sensitivity and specificity for all sampling methods ranged from 93% - 100% for weeks 1-3 PI. Results of ELISA testing depended on cut-off selection. Optimization of ELISA S/P cut-offs for swab sample data was substantially lower (S/P ratio of 0.08 ± 0.05) than the industry standard (S/P ratio of 0.4).

Implications: Diagnosis of viremic animals (using real-time qRT-PCR) can be equivalently accomplished using the jugular, swab or capillary sampling method. PRRSV ELISA status can be determined using any of the sampling methods provided an alternative cut-off is used to interpret swab data. When a cut-off S/P ratio of 0.4 was utilized, swab sample sensitivity ranged from 20% - 55.6% over weeks 2 - 7 PI.

Key words: swine, PRRSV, antemortem diagnosis

Introduction

Although researchers fulfilled Koch's postulate for porcine respiratory and reproductive syndrome virus (PRRSV) in 1991³⁷, diagnosis by clinical signs alone remains challenging as many other viral and bacterial diseases have a similar clinical presentation. Therefore, diagnosis of infections and monitoring of herd status has historically relied on laboratory testing including virus isolation, immunohistochemistry, reverse transcriptase polymerase chain reaction (RT-PCR)-based assays, and serological tests such as enzyme-linked immunosorbent assays (ELISA), immunofluorescence assays (IFA) and the immunoperoxidase monolayer assay³⁸. While these diagnostic tests have well described advantages and disadvantages³⁸, none is perfectly sensitive and specific. Consequentially, results must be interpreted within the context of the specific situation.

Multiple factors influence the performance of diagnostic tests; one is the composition of the submitted sample. For antemortem diagnosis and monitoring of PRRSV infection, serum samples are commonly utilized. To collect blood or serum, various sites can be sampled⁶

including ear veins, tail vessels, other peripheral vessels, the orbital sinus and vessels (jugular vein or anterior vena cava) in the thoracic inlet (herein referred to as the jugular sampling method). While the jugular sampling method remains commonplace, the potential for injury to the thyroid gland, the phrenic nerve, and the thoracic duct has been noted in the scientific literature^{4,8,10,14,28}. The technique also normally requires restraint of the animal with a wire snare. This manner of restraint has been used as a method of stress induction in various endocrine studies²²⁻²⁴ indicating that it can be stressful to the animal. Additionally, this technique is potentially hazardous to the human handler, especially during collection of blood from boars.

As diagnostic testing of serum samples for PRRSV becomes an increasingly valuable marketing tool for boar studs, alternative methods of serum collection need to be evaluated in order to address personnel safety and animal stress issues. One such method, lancing the ear with a needle and collecting blood with a sterile polyester swab, has recently gained popularity in boar studs^{35,42,43}. During this collection process, whole blood is collected from an ear vein and diluted in sterile saline or phosphate buffered saline (PBS). A recent study advocated the use of this method in negative boar studs as a more appropriate PRRSV monitoring method³⁵. While this study found swab samples to be a reliable alternative to the jugular sample, the use of swabs was only monitored for the first six days post infection (with PRRSV)³⁵. As this sampling method is prone to variation in the amount of sample collected (manuscript in preparation) and variation in subsequent dilution factors across samples, significant variation in diagnostic accuracy of PRRSV testing is likely⁴⁴. Consequently, the diagnostic accuracy of this sampling method throughout the infectious period, especially during later stages of infection, should be assessed before this technique is applied to wider testing protocols. Additionally, diagnostic accuracy of swabs for ELISA testing has not been reported. The emergence of PRRSV strains that escape detection by some PCR methods, (Dr. Jim Collins at University of Minnesota, personal communication), illustrates the urgent need for additional diagnostic approaches for PRRS monitoring.

A potential alternative method of serum collection from the ear uses a device in which a plastic capillary tube is coupled with a microcentrifuge tube containing serum separator gel or anticoagulant⁴⁴. This sampling method provides a serum sample, unlike the swab sample which results in diluted whole blood. The additional expense and labor involved with this method must be weighed against the potential advantages.

The goal of this study was to analyze the diagnostic accuracies of the polyester-tipped swab, the capillary tube and the jugular sampling methods on two standard PRRS diagnostic tests – quantitative real-time RT-PCR (qRT-PCR) and ELISA. As sensitivity and specificity of the ELISA test are dependent on the cut-off point chosen to discriminate between positive and negative pigs, analysis will be conducted to assess the optimal ELISA cut-off values for the study population.

Materials and Methods

Pigs

Thirty, seven week old, crossbred, PRRSV negative barrows and gilts (21.4 ± 7.6 kg, mean \pm standard deviation) were purchased from a commercial vendor which has been historically free of PRRSV infection (based on regular serological and RT-PCR testing). The pigs were transported from the commercial operation to an ISU research facility and randomly assigned to two groups of 15 pigs each upon arrival. Groups were housed separately in identical facilities, were provided with *ad lib* access to commercial diets and water. Additionally, the pigs were cared for according to established criteria⁴⁵. The protocol for this trial was approved by Iowa State's Committee on Animal Care.

Study design

Blood was collected from all pigs on arrival (Day 0) and one week later (Day 6) to confirm PRRS negative status via IFA, qRT-PCR and ELISA. Upon confirmation of negative status, 15 pigs served as an uninfected control group. On Day 7, the remaining 15 pigs were inoculated intramuscularly with 2ml of 10^3 TCID₅₀ of PRRSV VR-2332^{46,47}.

All pigs (n=30) were then sampled once a week for seven weeks post-inoculation. At each sample time, blood was collected from each pig using the following three methods:

1. Jugular sampling method: 9ml venous jugular venapuncture using an 18g x 1” bleeding needle and serum separator tube (Vacutainer SST, BD, Franklin Lakes, New Jersey).
2. Swab sampling method: The medial or lateral auricular vein was lanced using a 20g x 0.5” needle followed by saturation of a sterile polyester tipped applicator (product number 14-959-90, Fisher Scientific International, Waltham, Maine) with blood. The swab was placed in a 5ml, sterile polystyrene culture tube (product number 352058, BD, Franklin Lakes, New Jersey) with 1.0 ml sterile physiological saline (0.9% Sodium Chloride, Hospira, Lake Forest, Illinois).
3. Capillary sampling method: The medial or lateral auricular vein was lanced using a 20g x 0.5” needle followed by collection of approximately 200uL of blood into a commercial capillary blood collection system (SAFE-T-FILL Serum Gel Capillary Collection, RAM Scientific, Yonkers, New York).

The order of sampling was randomized by pig; this designated sampling order was then used for the remainder of the trial. Sample one (jugular method) was centrifuged at 8050 x g for 10 minutes; serum was removed from the tube and submitted in sterile polystyrene culture tubes. Sample two (blood swab method) was vortexed for 15 seconds then centrifuged at 4°C at 1,738 x g for 10 minutes. This protocol is similar to one reported in Chung et al. 2005⁴⁸. Approximately 0.5 ml of supernatant was submitted for testing in sterile polystyrene culture tubes. Sample three was centrifuged at 4,000 x g for 5 minutes; serum was removed from the tube and submitted in sterile microcentrifuge tubes. All samples were refrigerated overnight and submitted the following morning to Iowa State University Veterinary Diagnostic Lab for qRT-PCR and ELISA analysis using established protocols^{41,49}. Samples were submitted following a blinded protocol such that technicians performing the qRT-PCR and ELISA testing were unaware of which samples came from control and which came from infected study animals. As the jugular and the blood swab samples were submitted in a sterile polystyrene culture tube and the capillary sample in a microcentrifuge tube, complete

blinding of sample type was not achieved. An industry standard cut-off point of 0.4 S/P ratio was used for ELISA test dichotomization. For PCR testing, a set of standards, each of which contained a known virus titer [fluorescent focus forming unit (FFU)/ml], was included in each qRT-PCR run with clinical samples to determine the validity and reproducibility of the assay. The amount of PRRSV in each sample was estimated by converting the threshold cycle (C_t) value (the cycle where the PCR amplicon of the target genetic material is first detected⁴⁸) to virus titer (FFU/ml) using a standard curve. Samples with a C_t value higher than 35 were considered to be negative for PRRSV.

Statistics

Receiver Operating Characteristic (ROC) curve analysis was used to assess the diagnostic accuracy of three sampling methods. This method was chosen as it utilizes the entire range of test data providing a comprehensive overview of sample diagnostic accuracy independent of prevalence⁵⁰. Specifically, this methodology was utilized to (1) select a cut-off value (a specific S/P ratio value) for each sampling method which optimized sample sensitivity and specificity when used to dichotomize data, (2) use optimal cut-off values to evaluate the change in diagnostic sensitivity and specificity of all sampling methods over weeks 1-7 PI and (3) perform statistical pairwise comparison of areas under the ROC curve (AUC) among sampling methods for each time point.

An AUC value is interpreted as the “probability that a randomly drawn individual from the positive reference sample has a greater test value than a randomly drawn individual from the negative reference sample”⁵¹. An AUC of 0.80, for example, would indicate that the a randomly selected known PRRSV positive pig will have a S/P ELISA ratio greater than a randomly selected known PRRSV negative pig 80% of the time⁵⁰. AUC values range from 0.5 to 1 where 1 indicates that using a given sample, the diagnostic test would perfectly discriminate between PRRSV positive and negative pigs and 0.5 indicates there is no discrimination between groups⁵⁰. Confidence intervals (95%) are given for AUC values. When the confidence interval contains 0.5, there is evidence the test is not discriminating between groups⁵⁰. Pair-wise comparisons of ROC curves (MedCalc Version 9.1.0.1,

Mariakerke, Belgium) were analyzed at each sampling point (weekly) to determine if there were significant differences ($p < 0.05$) among sampling methods. Optimization of S/P cut-off values for continuous ELISA data for each sampling method were compared using an analysis of variance and a Student-Newman-Keuls test using $p < 0.05$ to indicate significant differences (MedCalc Version 9.1.0.1, Mariakerke, Belgium).

Results

All 30 pigs were visually monitored daily for lameness and clinical signs of respiratory disease; all completed the entire trial. During the nine week trial, a total of four pigs were treated for respiratory conditions and/or lameness (two control and two infected animals), one pig was treated for rectal prolapse (control animal) and one pig was treated for a tail bite (control animal) according to recommendations by the university veterinarian. No adverse reactions, including ear hematomas lasting longer than two days post collection, were noted at the site of blood collection.

Real-time qRT-PCR results (Detection of viremia)

ROC curve analysis illustrated AUC values ranging from 0.82 to 1.0 (AUC values of 1 indicate perfect discrimination) for all samples for the first four weeks post inoculation (Table 1). After this time, AUC values for all samples (including the jugular sample) dropped substantially (range 0.50 to 0.83) indicating poor discrimination between known positive and negative animals (Table 1). Pair-wise comparison of ROC curves was significant only when capillary tube and swab samples were compared on week five post-inoculation ($p = 0.02$). Optimization of C_t cut-off points for continuous qRT-PCR data was not significantly different among the three sample methods ($p = 0.74$). The mean C_t cut-off values for the jugular, capillary and swab samples were 34.7, 35.0, and 36.4, respectively. The sensitivity, calculated using optimized cut-off values, of all sampling methods was high for weeks 1 – 3 PI, but decreases thereafter (Figure 1). Specificity of the sampling methods remained high throughout the testing period (Figure 2).

ELISA results (Detection of seropositive animals)

Swab sample sensitivity was highly dependent on cut-off point selection. When the industry standard cut-off S/P ratio (i.e. 0.4) was utilized, sensitivity ranged from 20% - 55.6% over weeks 2 - 7 PI (Table 2). In contrast, jugular sample sensitivity was 100% over weeks 2 – 7 PI and capillary sample sensitivity ranged from 93.3% - 100% over weeks 2 – 7 PI when the industry standard cut-off S/P ratio was used.

These results are substantially different than when an optimized cut-off S/P ratio was used. Sensitivity for all sampling methods, generated using cut-off points optimized through AUC analysis, was 100% for weeks 2 - 7 PI. Specificity, calculated with AUC analysis, was 100% for weeks 3 – 7 PI. Specificity on week 2 PI was 100% and 86% for jugular and swab samples respectively. The swab sampling method had a significantly lower ($p < 0.05$) average cut-off point in comparison to either the jugular or capillary tube sampling method.

ROC curve analysis revealed that all sampling methods were able to perfectly distinguish ($AUC = 1$) between known positive and negative animals for weeks 3-7 post-inoculation. Week 1 PI AUC values were 0.693 and 0.569 for the jugular and swab samples respectively. Week 2 PI AUC values were 1, 0.937, and 1 for jugular, swab, and capillary samples respectively. Pair-wise comparison revealed no significant differences among sampling methods at any time point (all p -values were > 0.05).

Discussion

When swab samples are submitted, they are known to be diluted; a dilution factor of 10 to 20 fold for a swab sample submitted for PRRSV testing has been reported in previous publications^{35,43}. This dilution likely results from both the inability to recover the entire volume of blood from the swab and from placing the swab into sterile saline for transport. These and other unidentified factors result in a clinically significant decrease in the amount of serum obtained from a swab sample. Additionally, after the swab sample is processed

(vortexed and centrifuged), the sample drawn off the top is not serum, as can be collected from the jugular sampling method, but contains remnants of red blood cells which may interfere with the ELISA test (Dr. John Johnson at Iowa State University, personal communication). Therefore, when the swab is submitted to the laboratory and further diluted (40:1) for ELISA testing (standard laboratory protocol), a significantly lower amount of antibody is expected to be recovered compared to serum collected from a jugular sample.

Because dilution of the swab sample results in a lower PPRSV antibody concentration in the submitted sample, it was hypothesized that a lower cut-off point may be necessary to adequately discriminate between positive and negative animals. In order to analyze what the optimal PRRSV ELISA cut-off would be, ROC analysis was performed to (1) select a cut-off value (a specific S/P ratio value) for each sampling method which optimized sample sensitivity and specificity when used to dichotomize data, (2) use optimal cut-off values to evaluate the change in diagnostic sensitivity and specificity of all sampling methods over weeks 1-7 PI and (3) perform statistical pairwise comparison of areas under the ROC curve (AUC) among sampling methods for each time point.

Evaluation of AUC values indicated that for each sampling method, a cut-off value was identified which enabled perfect distinction ($AUC = 1$) between positive and negative animals at times when the nursery pigs were expected to be seropositive (week 2 - 7PI). A significant difference ($p < 0.05$, ANOVA followed by Student-Newman-Keuls test) was noted between the optimized cut-off value for the swab sampling method as compared to the jugular and capillary sampling methods. The average optimized cut-off S/P ratio for the swab sampling method for weeks 1 - 7 PI was 0.08. This ratio implies that samples with an S/P ratio < 0.08 are considered negative and those > 0.08 are considered positive. Additionally, it was noted that for all sampling methods, a range of cut-off values would provide optimal diagnostic sensitivity and specificity. For the swab sampling method, the range of optimal cut-off values was substantially smaller as compared to the other sampling methods.

Optimized cut-off values were also used to assess the diagnostic accuracy of the sampling methods over the course of a PRRSV infection (1 – 7 weeks PI). The results indicate that using the optimized cut-off S/P ratio of 0.08, swab samples will be diagnostically equivalent to serum from a jugular sample for diagnosis of PRRSV for weeks 2-7 PI. The sensitivity of the swab sampling method was 100% for all times PI when PRRSV antibodies are expected to be present (weeks 2-7 PI). Additionally, no statistical differences in AUC values were noted at any time point between sampling methods (p-values > 0.05 using a pair-wise comparison of AUC curves). Conversely, when the industry standard ELISA S/P cut-off of 0.4 was used to dichotomize data from the swab samples there was a decrease in diagnostic sensitivity (range of 20% - 55.6 % depending on the time after inoculation). These results provide evidence that the swab sampling method does require the use of a significantly lower S/P ratio cut-off to dichotomize PRRSV ELISA results accurately.

In contrast to the swab samples, capillary tube samples suffer from neither inadequate volume, as it is not diluted in saline, nor differences in sample type, as serum can be collected. This study indicated that the capillary sampling method can be used with ELISA testing with diagnostic accuracy equal to the jugular sampling methods (under laboratory conditions for weeks 2 – 7 PI) for PRRSV diagnosis using the industry standard cut-off S/P ratio of 0.4.

Comparison of real-time qRT-PCR results indicated that any of the sampling methods can accurately diagnose animals expected to be viremic in this study. These results agree with a previous report in which swab samples were shown to have a high proportion of agreement (90 – 100%) to serum samples for 6 days post-infection³⁵. One exception was a significant difference (p-value<0.05 on a pairwise comparison) between the ROC curves on week 5 PI for swab and capillary tubes. The difference of 20% in sensitivity between swab and jugular samples (when individual samples are tested at 5 weeks PI) was observed and should be carefully considered within the context of the testing situation; especially in situations where the exact time of PRRSV infection is unknown. The data presented in this study indicate that late in the infection (weeks 5 – 7 PI), all three samples are relatively poor at detecting

viremic animals. This is due to the dynamic properties of sensitivity and can be explained by fully understanding the assumptions of the statistical model. For the purposes of AUC analysis, animals were determined to be positive or negative based on infection status. As infection progresses, viremia is cleared and no longer corresponds to initial infection status. Therefore, regardless of sample type, virus testing would be expected to be less reliable as an indicator of infection than antibody testing as time elapses from exposure. After week 5 PI, regardless of sample type, qRT-PCR was not predictive of infection in this study.

Overall, this study has evaluated the diagnostic accuracy, defined as “the ability to correctly classify subjects into clinically relevant subgroups”, of three sampling methods for the diagnosis of PRRSV⁵⁰. Given this definition, the *quality* of information provided by the various samples not the *usefulness* (or clinical practicality) was analyzed⁵⁰. In future studies, the usefulness of these sampling methods under field conditions should be assessed. Combining information from such a study with farm specific economic and labor considerations will enable practitioners to effectively develop PRRSV testing protocols.

Implications

- Early diagnosis (weeks 1-3 PI) of PRRSV infected nursery pigs using real-time qRT-PCR under study conditions can be equivalently accomplished using the capillary, swab, or jugular sampling methods.
- No change in cut-off values for qRT-PCR data dichotomization is necessary for data obtained via any of the sampling methods.
- The diagnostic accuracy of PRRSV ELISA was poor for the swab sampling method when the industry standard cut-off (0.4) was utilized (sensitivity ranged from 20% - 55.6% over weeks 2 - 7 PI).
- When optimal cut-off values are employed, as determined by AUC analysis, all sampling methods are capable of achieving very high diagnostic accuracy on PRRSV ELISA testing. These cut-off values may not be clinically useful.

Table 1. Summary of ROC analysis of continuous real-time qRT-PCR capillary tube (C), swab (S) and jugular (J) sample data. Fifteen seven week old, crossbred, PRRSV negative barrows and gilts (21.4 ± 7.6 kg) were inoculated with PRRSV and fifteen animals were left as controls. All pigs were sampled weekly for seven weeks post-inoculation (PI) by a swab, capillary and jugular method; samples were analyzed weekly using qRT-PCR. Weekly ROC statistical analysis was performed on data to generate area under the ROC curve (AUC) values. AUC values range from 0.5 to 1 where 1 indicates that using a given sample, the diagnostic test would perfectly discriminate between PRRSV positive and negative pigs and 0.5 indicates there is no discrimination between groups. Confidence intervals (95%) are given for AUC values. When the confidence interval contains 0.5, there is evidence the test is not discriminating between groups.

Week (PI)	Sample	AUC	L 95% CI [†]	U 95% CI [Ⓟ]
1	C	1.000	0.883	1.000
2	C	1.000	0.883	1.000
3	C	1.000	0.883	1.000
4	C	0.858	0.682	0.957
5	C*	0.900	0.734	0.978
6	C	0.567	0.374	0.745
7	C	0.573	0.381	0.751
1	S	1.000	0.883	1.000
2	S	1.000	0.867	1.000
3	S	0.967	0.827	0.994
4	S	0.822	0.640	0.936
5	S*	0.663	0.439	0.800
6	S	0.533	0.343	0.716
7	S	0.502	0.315	0.689
1	J	1.000	0.883	1.000
2	J	1.000	0.883	1.000
3	J	1.000	0.883	1.000
4	J	0.920	0.761	0.986
5	J	0.833	0.653	0.943
6	J	0.633	0.439	0.800
7	J	0.644	0.450	0.809

* ROC curves were significantly different ($p=0.016$, pairwise ROC curve comparison)

[†]U 95% CI = upper 95% confidence intervals for AUC

[Ⓟ]L 95% CI = lower 95% confidence intervals for AUC

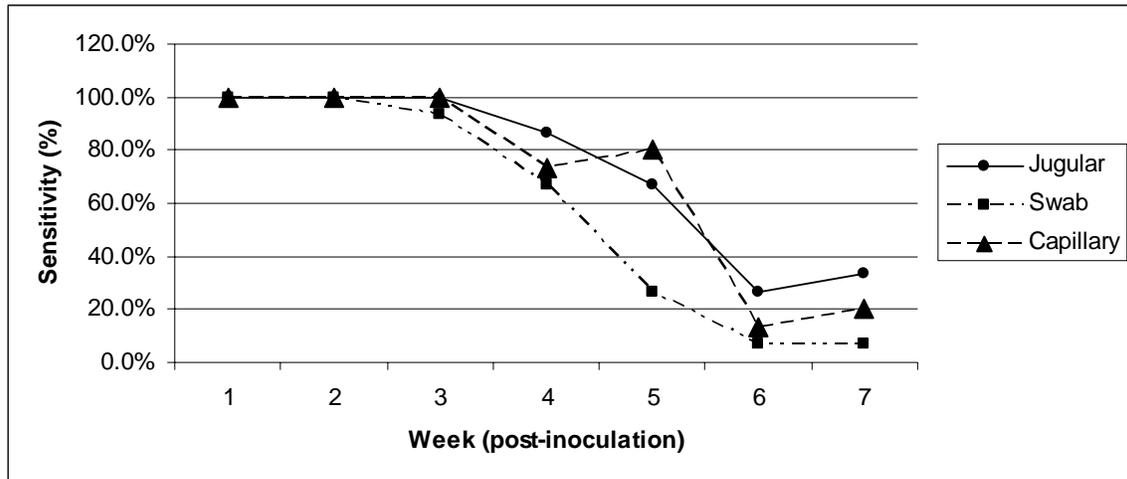


Figure 1. Diagnostic sensitivity of three sampling methods for PRRSV diagnosis via real-time qRT-PCR. Sensitivity was determined using receiver operating characteristic (ROC) statistical analysis in which sensitivity data is generated based on optimized cut-off values for data dichotomization. Samples were taken from crossbred barrows and gilts which were initially seven weeks old with a mean weight \pm standard deviation of 21.4 ± 7.6 kg. Fifteen of the animals were inoculated intramuscularly with PRRSV and fifteen animals were left as controls. All pigs were sampled weekly for seven weeks post-inoculation (PI) by a swab, capillary and jugular method; samples were analyzed weekly using qRT-PCR.

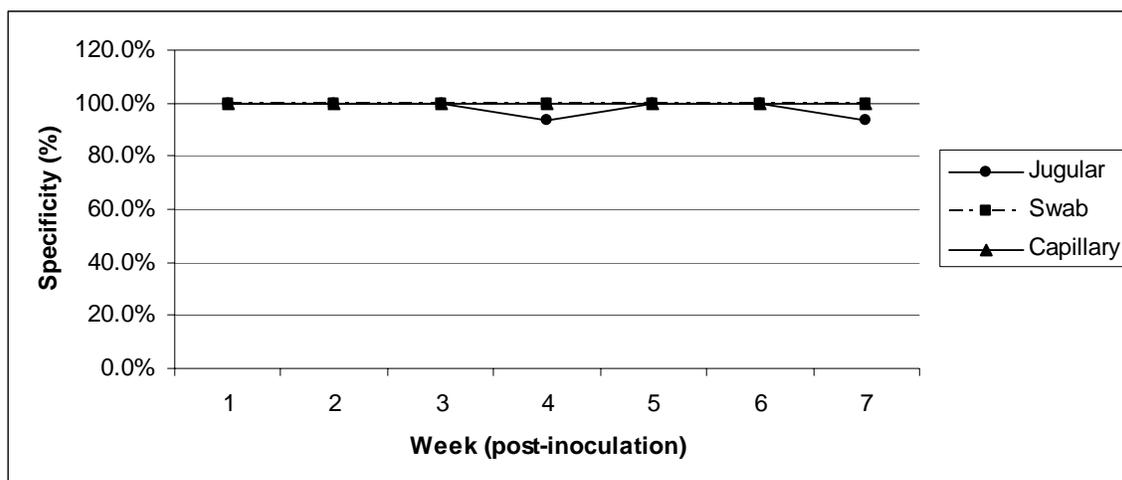


Figure 2. Diagnostic specificity of three sampling methods for PRRSV diagnosis via real-time qRT-PCR. Specificity was determined using receiver operating characteristic (ROC) statistical analysis in which specificity data is generated based on optimized cut-off values for data dichotomization. Samples were taken from crossbred barrows and gilts which were initially seven weeks old with a mean weight \pm standard deviation of 21.4 ± 7.6 kg. Fifteen of the animals were inoculated intramuscularly with PRRSV and fifteen animals were left as controls. All pigs were sampled weekly for seven weeks post-inoculation (PI) by a swab, capillary and jugular method; samples were analyzed weekly using qRT-PCR.

Table 2. Diagnostic sensitivity and specificity of the swab sampling method for PRRSV diagnosis via PRRSV ELISA analysis when a cut-off S/P ratio of 0.4 or an optimized cut-off S/P ratio is applied to dichotomize continuous data.*

Week (PI)	Swab ELISA S/P ratio cut-off[†]	Se	Sp
7	0.06	100.0%	100.0%
	0.4	20.0%	100.0%
6	0.05	100.0%	100.0%
	0.4	46.7%	100.0%
5	0.02	100.0%	100.0%
	0.4	40.0%	100.0%
4	0.1	100.0%	100.0%
	0.4	46.7%	100.0%
3	0.09	100.0%	100.0%
	0.4	46.7%	100.0%
2	0.15	100.0%	85.7%
	0.4	55.6%	100.0%

*Samples were taken from crossbred barrows and gilts which were initially seven weeks old with a mean weight \pm standard deviation of 21.4 ± 7.6 kg. Fifteen of the animals were inoculated intramuscularly with PRRSV and fifteen animals were left as controls. All pigs were sampled weekly for seven weeks post-inoculation (PI) by the swab sampling method. This sample was collected by lancing the medial or lateral auricular vein using a 20g x 0.5” needle followed by saturation of a sterile polyester tipped applicator with blood. The swab was placed in a 5ml, sterile polystyrene culture tube with 1.0 ml sterile physiological saline. The swab sample was vortexed for 15 seconds then centrifuged at 4°C at 1,738 x g for 10 minutes. Approximately 0.5 ml of supernatant was submitted for ELISA testing in sterile polystyrene culture tubes.

[†]Two cut-off S/P ratios were used to dichotomize the continuous ELISA data for each week PI. The S/P ratio of 0.4 is the current industry standard cut-off; the other S/P ratio for each week was generated by receiver operating characteristic (ROC) analysis. The cut-off generated by ROC analysis was optimized for both diagnostic sensitivity and specificity and varies by week PI.

CHAPTER 4. EVALUATION OF THE BLOOD SWAB METHOD FOR PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME VIRUS (PRRSV) ELISA MONITORING

A paper to be submitted to *The Journal of Swine Health and Production*

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Summary

Objective: To determine the diagnostic sensitivity and specificity of the blood swab method and evaluate the economic implications of using this method for PRRSV ELISA herd monitoring.

Materials and methods: 132 twenty-two week old, commercial swine were sampled as follows: (1) 9ml venous jugular blood collection using an 18g x 1” bleeding needle and serum separator tube and (2) ear lance using a 20g x 1/5” needle followed by saturation of sterile polyester tipped applicator. BetaBuster freeware software was used to develop prior distributions for PRRSV prevalence within the population and the diagnostic parameters for both sampling methods. WinBUGS freeware software was used to estimate diagnostic parameters for the PRRSV ELISA test for both sampling methods. Using FreeCalc freeware software, a partial budget was constructed to evaluate the economic significance of implementing the blood swab method.

Results: The sensitivity and specificity of the swab sampling method was 22.3% (97.5% probability intervals = 16.0%, 29.2%) and 94.3% (80.1%, 99.8%) respectively when a S/P

ratio cut-off of 0.4 was used. The sensitivity and specificity were 94% (89.8%, 97.2%) and 93.5% (77.8%, 99.8%) respectively when an S/P ratio cut-off of 0.08 was used.

Implications: In agreement with previous work, the sensitivity of the swab sampling method was low for ELISA results dichotomized at an S/P ratio of 0.4. The sensitivity of the swab method improved when a lower S/P cut-off was used. Partial budget analysis indicated that the jugular sampling method would cost a 1000-head sow operation \$0.15/pig produced while the swab sampling method would cost \$0.36/pig produced.

Key words: swine, PRRSV, antemortem diagnosis

Introduction

Porcine respiratory and reproductive syndrome virus (PRRS), a member of the genus *Arterivirus*, family *Arteriviridae*, and order *Nidovirales*, is an RNA virus of swine initially described in 1987³⁶. In 2005, it was estimated that this disease costs the U.S. pork industry approximately \$560.32 million dollars annually from decreased production (sum of farm level production losses from breeding to the grow-finish phase)³⁹. While this study did not include the cost of PCR and ELISA-based disease monitoring, these protocols are an economically significant part of production.

Disease monitoring programs are developed by combining sampling number and frequency in order to achieve the highest statistical probability of identifying disease within the economic constraints of the production system. In order to reduce expenses related to labor, increase ease of collection, and increase employee safety, the blood swab method³⁵ has been implemented in diagnostic monitoring programs. Recently, the diagnostic accuracy of this method was evaluated for potential use in ELISA testing³⁵. While this study showed that the sensitivity of swab samples for ELISA testing ranged from 20% - 55.6% over weeks 2 - 7 post-inoculation (PI) using an S/P cut-off of 0.4, little information is available on test performance under commercial production settings³⁵.

The Bayesian method of assessing the diagnostic accuracy of tests in the absence of a “gold standard” is gaining popularity in veterinary medicine. Recent articles including Enoe et al. 2001⁵², Frossling et al. 2003⁵³ and Klement et al. 2005⁵⁴ among others have employed this method. When the standard 2 x 2 factorial analysis of a new diagnostic test versus an imperfect gold standard test is utilized, biased estimates of the new test parameters are generated as the results depend on the accuracy of the gold standard test^{55,56}. In this situation, a new test that is more accurate than the gold standard test will appear to have relatively poor diagnostic parameters. Therefore, a statistical method which does not rely on a gold standard test will generate more accurate parameter estimates.

The Bayesian approach involves three basic steps: (1) Development of prior distributions for unknown parameters (beta distributions generated from either expert knowledge or data from previous trials), (2) calculation of posterior distributions using iterative sampling (posterior distributions are the product of a prior distribution and the likelihood functions generated from the study data) and (3) generation of parameter estimates with probability intervals from the posterior distribution^{56,57}. A 95% probability interval is defined as the “interval which contains the parameter with a 95% certainty”⁵⁶. This is in contrast to a 95% confidence interval which is defined as the interval containing the true parameter estimate 95% of the time (contingent on performing the same sampling procedure and statistical analysis)⁵⁶.

The Bayesian method utilizes prior distributions which account for the uncertainty of unknown parameters (such as the sensitivity and specificity of a new sampling method) instead of assuming that these parameters are exactly known. This fact accounts for the increased accuracy of parameter estimates generated with this method⁵⁷. Prior distributions are based on beta distributions which are “flexible”, meaning that many possible distribution shapes can be created by manipulating the distribution parameters⁵⁶. Consequentially, confidence intervals are improved as exact posterior distributions instead of normal distribution approximations are used (as data rarely conforms to normal distributions)⁵⁷.

Additionally, Bayesian inferences can be made without using large sample theory assumptions as posterior distributions are not required to have normal distributions⁵⁶.

Despite the advantages, the method has been criticized as it relies on the use of prior information (from experts or previous published information) in combination with likelihood functions (calculated from trial data) to generate estimates for unknown parameters. While the prior information must be chosen carefully, posterior distributions for test parameters can be reported over a range of prior distributions (sensitivity analysis)⁵⁶. This allows practitioners to interpret the parameter estimate based on knowledge of the clinical situation and prior experience with the test. Additionally, all parameter estimates are accompanied by true probability confidence intervals (posterior credible intervals) which can be used to evaluate the quality of the estimate⁵⁶.

In order to increase practitioners' ability to evaluate PRRSV ELISA diagnostic test results when the blood swab method is utilized, this report describes the use of Bayesian statistics to provide the diagnostic sensitivity and specificity of this method. These diagnostic parameters are then used in a partial budget analysis to evaluate the economic implications of using the blood swab method in a diagnostic monitoring program for a 1,000 head sow production unit.

Materials and Methods

Animals

Twenty-two week old, crossbred finishing pigs were sampled. The pigs were housed in three conventional 1,000 - head confinement buildings at a site located in north central Iowa. The pigs originated from two 3,800 – head PRRSV positive sow farms. Gilts were vaccinated with one dose of PRRSV-MLV (Ingelvac PRRS MLV, Boehringer Ingelheim, St. Joseph, Missouri) at 18-23 kg. The sow herd was not routinely vaccinated. Piglets from the sow herds were commingled at weaning into multiple 2,300 – head nursery sites. Multiple nursery sites within the system were vaccinated with MLV PRRSV vaccine at 6 weeks of

age. The finishing site was managed as an all-in-all-out (by site), process verified production system.

Sampling techniques

The following procedures were approved by the Iowa State University Institutional Animal Care and Use Committee. A total of 132 pigs from the previous described finisher barn were sampled once. Each pig was sampled using the following two methods:

1. Jugular sampling method: 9ml venous jugular venapuncture using an 18g x 1” bleeding needle and serum separator tube (Vacutainer SST, BD, Franklin Lakes, New Jersey).
2. Swab sampling method: The medial or lateral auricular vein was lanced using a 20g x 0.5” needle followed by saturation of a sterile polyester tipped applicator (product #14-959-90, Fisher Scientific International, Waltham, Maine) with blood. The swab was placed in a 5ml, sterile polystyrene culture tube (product #352058, BD, Franklin Lakes, New Jersey) with 1.0 ml sterile physiological saline (product #1590-02, Hospira, Lake Forest, Illinois).

Statistics

The statistical method used in the study was adapted as follows from Branscum et al. 2005⁵⁸. Prior distributions were developed for the prevalence of PRRSV within the population (the 1,000 head finisher barn filled from a PRRSV vaccinated nursery) and for the diagnostic parameters for both the jugular and swab sampling methods (sensitivity and specificity for each method). To obtain prevalence estimates, three experts in the field of swine medicine were provided with background information on the population being sampled (Baker RB, Ramirez A, Holtkamp D., personal communication). A detailed outline of the background material presented to the experts appears in the appendix. Each expert generated a prior distribution for PRRSV prevalence in the population using BetaBuster, a freeware program available at <http://www.epi.ucdavis.edu/diagnostictests/betabuster.html>. Graphical representations of the prior distributions appear in Figure 1. Expert opinions were combined in the WinBUGS program, a freeware program available at

bsu.cam.ac.uk/bugs/winbugs/contents.shtml (unpublished WinBUGS code data). The prior distributions for the jugular sampling method were generated in BetaBuster from previous published information⁴⁹. Prior distributions for the swab sampling method were generated in BetaBuster using information from previous research by the author (manuscript submitted for publication) as described below^{59,60}. A sensitivity of 42.6% and specificity of 100% were assumed for calculations when ELISA S/P ratios were dichotomized using an S/P ratio cut-off of 0.4⁵⁹. A sensitivity of 93% and specificity of 93% was assumed for calculations when ELISA S/P ratios were dichotomized using a 0.08 cut-off⁵⁹. The beta distribution parameters (a,b) were derived from the following formulas:

$$a = s + 1$$

$$b = n - s + 1$$

where s = the number of animals found to be test positive (negative) and n = the number of truly infected (uninfected) animals for the sensitivity (specificity)⁶⁰. The prior distributions are shown in Figure 2. The prior distributions were entered into the WinBUGS code for 2 dependent tests, 1 population, no gold standard statistical model^{61,62}. A sensitivity analysis for the prior distributions generated for swab diagnostic parameters was done to assess the effects of variable prior information. For this analysis, a prior distribution with the same point estimate but a diffuse beta distribution (sensitivity \sim beta(1.25,1.25), specificity \sim beta(1.25,1.25)) was used.

The WinBUGS program was used to estimate diagnostic parameters for the PRRSV ELISA test for both the jugular and the swab sampling method. This program creates a joint posterior distribution by calculating the product of likelihood functions derived from a 2 x 2 table of ELISA test results and the prior distributions⁶³. Gibbs sampling⁵⁷ is then used to reconstruct parameter estimate distributions (i.e. marginal posterior distributions); 100,000 iterations were ran for each model. For further explanation of this Bayesian method readers are referred to Branscum et al 2005⁶³.

Economic analysis

A partial budget was constructed to evaluate the economic significance of implementing the blood swab method in a 1000 head commercial sow operation. In this example, the number of pigs produced/year (23,046) was estimated using the upper 10th percentile national average for litters/sow/year (2.3) and pig weaned/litter (10.02)⁶⁴. The freeware software program FreeCalc version 2 (available at http://www.ausvet.com.au/content.php?page=res_software#freecalc) was used to calculate the required sample size per testing period and the number of cutpoint reactors based on inputs of test sensitivity, test specificity, population size and the minimum expected prevalence. Test sensitivity and specificity of the jugular and swab sampling methods were assumed from previous research (manuscript submitted for publication)⁵⁹. A confidence level of 95% and a minimum expected prevalence of 10% were assumed in this example as producers and practitioners are generally familiar with these values and the implications. Assumptions for the cost of supplies are outlined in Table 1. In this example, it was assumed that ELISA testing (at \$4.00/sample) would be performed every four weeks and samples would not be pooled.

Results

Estimates for the diagnostic accuracy parameters for the jugular and swab sampling methods are provided in Table 2. Using the same model as for estimation of diagnostic accuracy parameters, the point estimate for prevalence was determined to be 98.3% with a 95% probability interval of (0.9615, 0.995). The results of the sensitivity analysis are also provided in Table 2.

Based on the certain economic, statistical and herd assumptions, the jugular sampling method would cost \$0.15/pig produced while the swab sampling method would cost \$0.36/pig produced. The full partial budget is shown in Table 3.

Discussion

In agreement with previous work, the sensitivity of the swab sampling method was low (22.3%) when a S/P ratio cut-off of 0.4 was used. When a significantly lower cut-off (S/P ratio of 0.08) was used to dichotomize ELISA results, the sensitivity improved to 94%. As this remains lower than the sensitivity reported for the jugular sampling method (98.5%), the cost and labor involved in each of the methods relative to the specific monitoring situation deserves consideration. In order to facilitate the decision, a partial budget (Table 3) is provided. This budget presents findings for a 1000 head sow operation but could be easily converted for use in other diagnostic monitoring plans with various disease inputs.

It is essential to understand the implications of the economic analysis provided in this study. In the example provided a confidence level of 95% was used. Assuming this, if a random sampling of 56 animals was duplicated 100 times, test results would be expected to show a prevalence of at least 10% in 95 of the sample sets. The example provided also assumes a minimum expected prevalence of 10%. Consider the situation in which a random sample of 56 animals is taken and 2 (or fewer) samples are reported as ELISA positive by the diagnostic laboratory. A common, erroneous interpretation of these results is that the herd is negative. This is significantly different from the correct interpretation, which is that the true prevalence of PRRS in the herd would be equal to or less than 10%. This is a critical differentiation as a PRRS herd prevalence of 9% would not be considered equivalent to a negative or naïve herd.

The objective of this study was to estimate the diagnostic accuracy of the swab sampling method under field conditions without relying on a gold standard sampling method. To meet this objective, a Bayesian statistical model developed for two dependent tests used on one population was applied. While controversy exists on this model's reliance on prior information, Dendukuri and Joseph 2001 summarize the alternative concisely – “Constraining any of the parameters at a fixed value gives us a solution for a simpler, identifiable problem but not the one with which we are presented⁶⁵.” In this study, prior

information on the prevalence of PRRSV in the population was elicited from three experts in the field of swine medicine. After background information was provided independently to each expert, very similar beta densities were developed. The action of using newly developed WinBUGS code to randomly incorporate each experts estimate into the calculations also strengthens the reliability of the prevalence prior distribution. Prior information for the jugular sample was taken from diagnostic accuracy data published for the IDEXX HerdCheck PRRS 2XR product⁴⁹. As this product is USDA licensed and has been validated in randomized controlled trials, this data is considered reliable and a sensitivity analysis was not preformed. Prior information for the swab sample was taken from a randomized controlled trial specifically designed to provide an estimate of the diagnostic accuracy of the swab sampling method⁵⁹. Although this trial produced reliable estimates, the sample size of this trial was small. Therefore, a sensitivity analysis was preformed to show the effect of the prior information on estimates. The sensitivity analysis showed wider confidence intervals, a slight decrease in sensitivity, and a substantial decrease in specificity. The decrease in specificity is likely explained by the study population. Because the finisher barn that was sampled had a PRRSV prevalence of 98.3%, little information on the specificity of the test was available (as there were very few negative animals). As a result, the statistical method relied on the prior distribution to generate an accurate estimation for specificity. When this prior distribution generated from previous trial data was replaced with a non-informative prior distribution (specificity \sim beta(1.25,1.25)), a specificity estimate was generated which was illogical in comparison to previous trial information. This illustrates the value of incorporating previous trial information with current trial data to generate accurate parameter estimates.

Implications

- In agreement with previous work, the sensitivity of the swab sampling method was low (22.3%) for ELISA results dichotomized at an S/P ratio of 0.4.

- The sensitivity of the swab method improves when a lower S/P cut-off is used (94%) indicating this method may have application in routine ELISA diagnostic monitoring programs.
- In comparison to the jugular sampling method, the sensitivity and specificity of the swab method is lower; this will result in more false negative and false positive test results.
- Under the described assumptions, the jugular sampling method would cost a 1000-head sow operation \$0.15/pig produced while the swab sampling method would cost \$0.36/pig produced.

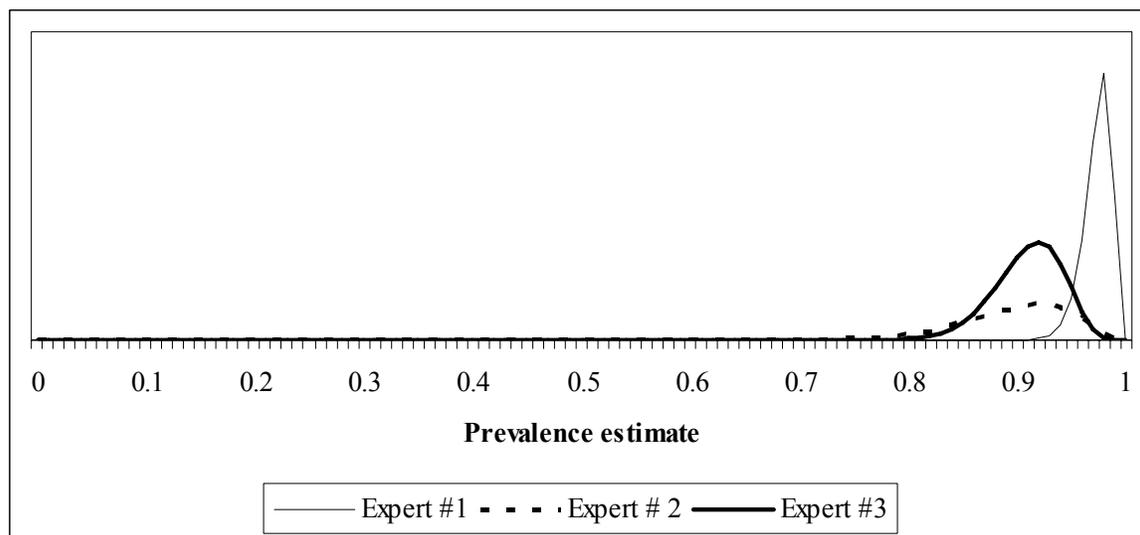


Figure 1. Graphical representation of beta distributions for the prevalence of PRRSV on a 1,000-head crossbred grow-finish population of 22 week old pigs originating from a PRRSV vaccinated nursery. The x-axis represents the PRRSV prevalence estimate; the y-axis represents an arbitrary scale. Each prevalence estimation[†] was developed by one of three swine experts (Baker RB, Ramirez A, Holtkamp D., personal communication) using the freeware computer program BetaBuster* after being presented with specific information on the population (information included site location, number of finisher spaces within a 3-mile radius, management procedures for the site and source population, animal source, PRRSV vaccination protocols and site layout). The beta distribution (prevalence \sim beta(a,b)) generated by each expert was combined and incorporated into a Bayesian statistical model[‡] as prior distributions for prevalence.

[†]The prevalence estimate information used to generate a beta distribution in BetaBuster for Expert #1 was a mode of 0.98 with a 95% confidence that the prevalence was greater than 0.95; for Expert #2 a mode of 0.92 with a 95% confidence that the prevalence was greater than 0.8 was used; for Expert #3 a mode of 0.92 with a 95% confidence that the prevalence was greater than 0.85 was used.

*The freeware program BetaBuster is available online at:
<http://www.epi.ucdavis.edu/diagnostictests/betabuster.html>.

[‡]The freeware program Winbugs used in this trial is available online at: <http://www.mrc-bsu.cam.ac.uk/bugs/winbugs/contents.shtml>

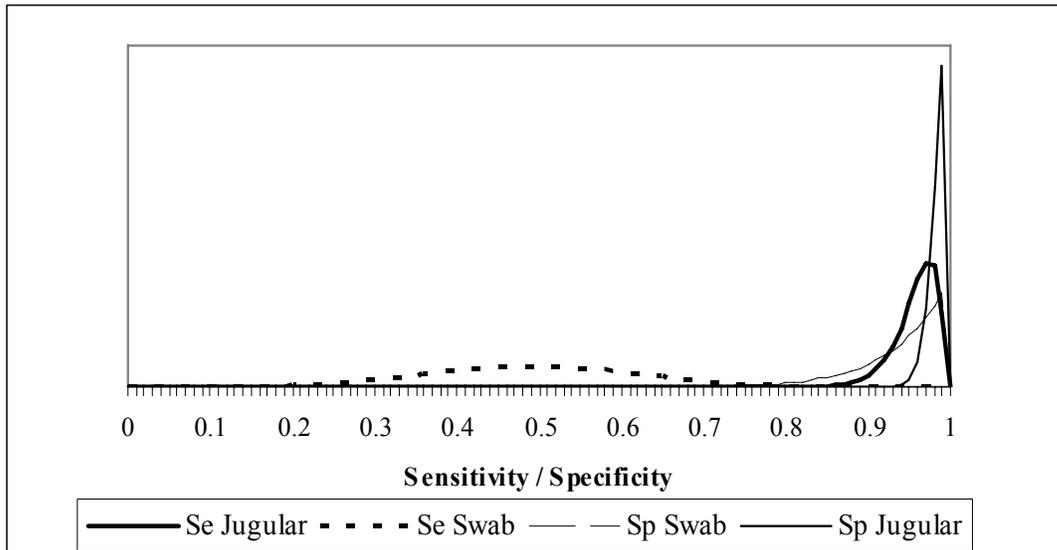


Figure 2. Graphical representation of beta distributions for the sensitivity (Se) and (Sp) of the jugular and swab sampling methods. The x-axis represents the sensitivity or specificity estimate for the specified sampling method; the y-axis represents an arbitrary scale. These beta distributions were developed using the freeware computer program BetaBuster^{*}. Each beta distribution was generated from previous trial information (swab sampling method)[†] or from published data (jugular sampling method)[‡]. The beta distribution (\sim beta(a,b)) generated for each parameter was incorporated into a Bayesian statistical model[§] as a prior distribution for the specified parameter.

*The freeware program BetaBuster is available online at:

<http://www.epi.ucdavis.edu/diagnostictests/betabuster.html>.

[†]Beta density information for the swab sampling method: sensitivity \sim beta(7.39,7.61), specificity \sim beta(16,1).

[‡]Beta density information for the jugular sampling method: sensitivity – mode of 0.974 with a 97% confidence that the mode was greater than 0.90, specificity – mode of 0.996 with a 97% confidence that the mode was greater than 0.978.

[§]The freeware program Winbugs used in this trial is available online at: <http://www.mrc-bsu.cam.ac.uk/bugs/winbugs/contents.shtml>.

Table 1. Cost assumptions for products used in a partial budget analysis^Φ of PRRSV ELISA monitoring with either the jugular[‡] or the swab[†] sampling method.

Product name	Product information [†]	\$/item [§]
Fisherbrand swabs	Product #14-959-90, Fisher Scientific International, Waltham, Maine	\$ 0.13
9 ml vacutainer tubes	Product #367988, BD, Franklin Lakes, New Jersey	\$ 0.29
5ml, sterile polystyrene culture tube	Product #352058, BD, Franklin Lakes, New Jersey	\$ 0.25
Needles for ear lance	Product # 305175, BD, Franklin Lakes, New Jersey	\$ 0.06
Needles for jugular venapuncture	Product #367215, BD, Franklin Lakes, New Jersey	\$ 0.12
Labor (\$10/hr for 3 min)	N/A*	\$ 0.50
Total swab collection cost		\$ 0.94
Total jugular cost		\$ 0.91

^ΦPartial budget analysis was performed to evaluate the economic implications of using the blood swab method in a diagnostic monitoring program for a 1,000 head sow production unit.

[‡]Jugular sampling method: 9ml venous jugular venapuncture using an 18g x 1" bleeding needle and serum separator tube.

[†]Swab sampling method: The medial or lateral auricular vein was lanced using a 20g x 0.5" needle followed by saturation of a sterile polyester tipped applicator with blood. The swab was placed in a 5ml, sterile polystyrene culture tube with 1.0 ml sterile physiological saline.

[†]Product number, manufacturer, city, state

[§]Actual cost of an individual piece of equipment calculated by dividing the total cost paid for the product by the number of individual pieces per package.

*N/A = not applicable

Table 2. Diagnostic sensitivity and specificity estimates for the swab[§] and jugular* sampling methods. Parameter estimates were generated using a Bayesian statistical analysis which involved the following steps: (1) Development of prior distributions for unknown parameters (beta distributions generated from either expert knowledge or data from previous trials), (2) calculation of posterior distributions using iterative sampling (posterior distributions are the product of a prior distribution and the likelihood functions generated from the study data) and (3) generation of parameter estimates with probability intervals from the posterior distribution.

Parameter	Swab Method[‡] (0.4 cut-off)	Swab Method[†] (0.08 cut-off)	Jugular Method[‡] (0.4 cut-off)	Sensitivity Analysis^{θ‡} (0.4 cut-off)	Sensitivity Analysis^{θ†} (0.08 cut-off)
Sensitivity	0.223	0.940	0.985	0.198	0.93
Sensitivity 95% PI**	(0.160, 0.292)	(0.898, 0.972)	(0.963, 0.997)	(0.137, 0.269)	(0.877, 0.963)
Specificity	0.943	0.935	0.992	0.578	0.426
Specificity 95% PI**	(0.801, 0.998)	(0.778, 0.998)	(0.977, 0.999)	(0.075, 0.969)	(0.031, 0.929)

[§]Swab sampling method: The medial or lateral auricular vein was lanced using a 20g x 0.5” needle followed by saturation of a sterile polyester tipped applicator with blood. The swab was placed in a 5ml, sterile polystyrene culture tube with 1.0 ml sterile physiological saline.

*Jugular sampling method: 9ml venous jugular venapuncture using an 18g x 1” bleeding needle and serum separator tube.

**PI = probability interval (the interval which contains the true parameter value with a 95% probability)

[‡]Diagnostic parameter estimates are based on dichotomization of ELISA S/P ratios using the industry standard 0.4 cut-off.

[†]Diagnostic parameter estimates are based on dichotomization of ELISA S/P ratios using a 0.08 cut-off.

^θDiagnostic parameter estimates for the swab method were generated using the following diffuse beta prior distributions: swab sensitivity ~ beta(1.25,1.25), swab specificity ~ beta(1.25,1.25).

Table 3. Partial budget developed to compare the costs associated with the swab[§] and jugular^{*} sampling methods as part of a PPRSV diagnostic monitoring program.

Assumptions	Sampling Method	
	Jugular [*]	Swab [§]
Sample submitted	Jugular [*]	Swab [§]
ELISA S/P ratio cut-off used for data dichotomization ^Φ	0.4	0.08
Confidence level	95%	95%
Sample method sensitivity [†]	98.5%	94.0%
Sample method specificity [†]	99.0%	93.5%
Number sows in herd	1000	1000
Minimum expected prevalence	10%	10%
Required sample size/testing period	56	130
Cutpoint # of reactors ^{**}	2	13
Cost of PRRSV ELISA test	\$ 4.00	\$ 4.00
Total cost of sampling supplies/labor	\$ 0.91	\$ 0.94
Pooled in "x"	1	1
Total samples submitted/testing period	56	130
Number of sampling times per week	1	1
Weeks between sampling times	4	4
Total test dates/year	13	13
Total samples submitted per year	728	1690
Total herd sampling cost per year	\$ 3,570.84	\$ 8,345.22
Number pigs produced/year	23,046	23,046
Cost/pig produced/year	\$ 0.15	\$ 0.36

[§]Swab sampling method: The medial or lateral auricular vein was lanced using a 20g x 0.5" needle followed by saturation of a sterile polyester tipped applicator with blood. The swab was placed in a 5ml, sterile polystyrene culture tube with 1.0 ml sterile physiological saline.

^{*}Jugular sampling method: 9ml venous jugular venapuncture using an 18g x 1" bleeding needle and serum separator tube.

^ΦAn S/P ratio cut-off of 0.08 was used to dichotomize ELISA data generated from the swab sampling method. This cut-off was the average of the weekly cut-offs generated by using receiver operating characteristic (ROC) analysis on 15 pigs infected with PRRSV and 15 control pigs sampled for 7 weeks post-inoculation.

[†]Sample method sensitivity and specificity are taken from a Bayesian statistical analysis performed to generate parameter estimates.

^{**}Cutpoint # of reactors: if the number of test positive animals is less than or equal to this number, then there is a 0.95 probability that the prevalence of disease in the tested population is less than or equal to 10%.

CHAPTER 5. GENERAL CONCLUSIONS

General Discussion

The specific objective of this thesis was to address the U.S. pork industry need for evaluation of alternative ante-mortem diagnostic samples for PRRSV. To accomplish this objective, three trials were undertaken with the following goals:

7. Develop a sampling protocol for the blood swab method.
8. Analyze the diagnostic accuracies of the polyester-tipped swab, the capillary tube and the jugular sampling methods on two standard PRRS diagnostic tests: real-time RT-PCR (RT-PCR) and ELISA in known positive and negative animals.
9. Analyze the diagnostic accuracy of the blood swab method for ELISA testing in commercial finishing swine.

The previous chapters have presented the protocols and individual conclusions developed for each objective. The following discussion serves to combine the results of the individual conclusions in order to address the overall usefulness of implementing an alternative ante-mortem diagnostic sample into a herd diagnostic monitoring plan.

As previously discussed, the capillary tube method suffers from neither inadequate volume nor differences in sample type collected (as compared to the blood swab method). Therefore, as expected, this study indicated that the capillary sampling method can be used with ELISA and real-time qRT-PCR testing with diagnostic accuracy equal to the jugular sampling method. Because of this, cost and ease of use are the main factors to be considered before inclusion of this sampling method into a diagnostic monitoring plan. Direct cost may be compared as follows. The total expense for the capillary sampling method was \$1.44/sample (\$0.94/tube in product and \$0.50/sample in labor cost (calculated for 3 minutes at \$10/hr)). In comparison, the total cost of the jugular sample was \$0.91/sample (calculations are presented in chapter 4). Ease of use must be considered for the specific situation and specific employee. Application of this method in boar studs would likely have safety benefits (for both animal and handler), while this might not be relevant in finisher barns where animals

must be snared to collect either sample. Additionally, employees who are already trained to collect a jugular sample may find this method easier and quicker than the capillary sampling method. The remainder of this discussion will focus on the blood swab method and factors that must be considered prior to its implementation in a diagnostic monitoring plan.

Prior to beginning evaluation of the blood swab method, a number of factors were identified which decrease the probability that the blood swab will provide an adequate sample. First, small volumes of sample are used in both real-time qRT-PCR (50 μ l) and ELISA (10 μ l) testing (personal communication, Dr. Karen Harmon, Adjunct Assistant Professor and Associate Scientist at ISU and Sheila Norris, Research Associate at ISU, 2007)^{41,49}. Second, for routine ELISA testing, the ISU diagnostic lab dilutes the sample 1:40 prior to testing (personal communication, Dr. John Johnson Clinician/Serology Section Leader at ISU)⁴⁹. Third, current blood swab protocols involve placing swab samples in sterile saline prior to submission which leads to dilution. Lastly, it is unknown what volume of blood is actually recovered from the swab after processing. Therefore, to develop a protocol for the blood swab method, the amount of blood absorbed by the swab under ideal sampling conditions needed to be quantified. Once this volume was known, it was compared to volumes collected under field conditions (where maximum blood absorption may not occur due to poor technique, poor ear vein visualization, etc.). This enabled evaluation of protocols without specifically quantifying the volume of blood in the diluted sample that is submitted.

At the time of the study, there were two brands of commercially available sterile polyester tipped swabs available commercially. When the swabs were tested, it was found that the Fisherbrand swabs absorbed significantly more blood than the Puritan swabs. The average volume collected by the Fisherbrand swabs was 167 μ l under ideal sampling conditions. As expected, the volume of blood absorbed under field conditions was less than under ideal conditions. Additionally, sampling technique, sampling time and the age of animal (relevant to the visualization of the auricular vein) were important factors in the volume of blood collected. Based on the results of this study, a Fisherbrand swab and a 20g x 1/2" needle

followed by dilution into 0.5 to 1 ml of sterile saline was an adequate sample for both ELISA and real-time qRT-PCR testing.

Once a protocol for the blood swab method was in place, the diagnostic accuracy was evaluated using pigs of known status, in a randomized, controlled trial. As stated previously, diagnostic accuracy is defined as “the ability to correctly classify subjects into clinically relevant subgroups”⁵⁰. A relatively recent method of assessing the diagnostic accuracy of tests in veterinary medicine, receiver operating characteristic (ROC) analysis, was used in this trial. Based on this analysis, the following conclusions were made concerning the blood swab sampling method:

1. Early diagnosis (weeks 1-3 PI) of infected animals using real-time qRT-PCR under laboratory conditions can be equivalently accomplished using any of the sampling methods.
2. No change in cut-off values for qRT-PCR data dichotomization is necessary for data obtained via any of the sampling methods.
3. The diagnostic accuracy of PRRSV ELISA was poor for swabs when a S/P ratio cut-off of 0.4 was utilized.
4. When optimal cut-offs are employed, as determined by AUC analysis, all sampling methods are capable of achieving very high diagnostic accuracy on PRRSV ELISA testing.

While the real-time qRT-PCR results agreed with previous work³⁵, no previous research has been published illustrating the use of the blood swab method with ELISA testing. As the average optimized ELISA S/P cut-off found under a randomized controlled trial was very low (S/P ratio < 0.08 was considered negative), the clinical applicability or usefulness of these results was questioned. In order to address this concern, a field study was designed using Bayesian methods and WinBUGS freeware computer programming to assess the diagnostic accuracy of the blood swab method under field conditions⁵⁸. The results of this trial (presented in chapter 4) agreed with results from the previous trial, indicating the sensitivity of the swab sampling method was low (22.3%) for ELISA results dichotomized at

an S/P ratio of 0.4 but substantially improved (sensitivity = 94%) when a lower S/P cut-off (0.08) was used.

In order to evaluate the usefulness of the blood swab method further, a partial budget was developed. This budget allows practitioners to consider both the economics of testing while accounting for the level of statistical confidence in the sample set. Under the described assumptions, the jugular sampling method would cost a 1000-head sow operation \$0.15/pig produced while the swab sampling method would cost \$0.36/pig produced. The swab sampling method in this situation would be more expensive, require employee training, have a lower diagnostic accuracy, and would not reduce animal stress (as snaring of the animal is still necessary). As such, it is this author's opinion that the jugular sampling method be used in this situation. In other situations, such as a boar stud where the sample can be collected without the use of a snare, the ease of sample collection and increased safety to workers likely outweigh the additional cost and decreased diagnostic accuracy of the swab sample.

It is important to use the results of this study with caution as they do not imply that the ELISA test is a poor diagnostic tool. Rather, the results of this trial would indicate that when the blood swab sample is used, a lower cut-off can be used to achieve accurate results. It is critical to remember that when used with a serum sample (the sample with which the ELISA test achieved USDA validation), the ELISA test provides reliable, accurate results. The use of blood swabs, as outlined in this thesis, is not an approved, USDA validated method. This implies that if the use of a lower cut-off results in negative economic or health changes in a production system, there is no legal protection for the veterinarian who recommended the cut-off value.

In conclusion, this thesis was written to provide practitioners with scientifically sound information concerning the diagnostic accuracy and economic feasibility of implementing alternative ante-mortem diagnostic samples into their diagnostic monitoring plans. While the thesis achieved this objective further research must be done to address the fact that both alternative methods utilize an open sampling system (both of these methods involve pricking

the ear with a needle and subsequent collection of a blood sample) as opposed to the closed, jugular collection method. Inherently, there is a higher probability of environmental contamination of the sample in both of these methods depending on the cleanliness of the animal, environment and employee. Future studies in this area may be warranted before either method is routinely implemented.

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*Denotes a non-refereed article

APPENDIX

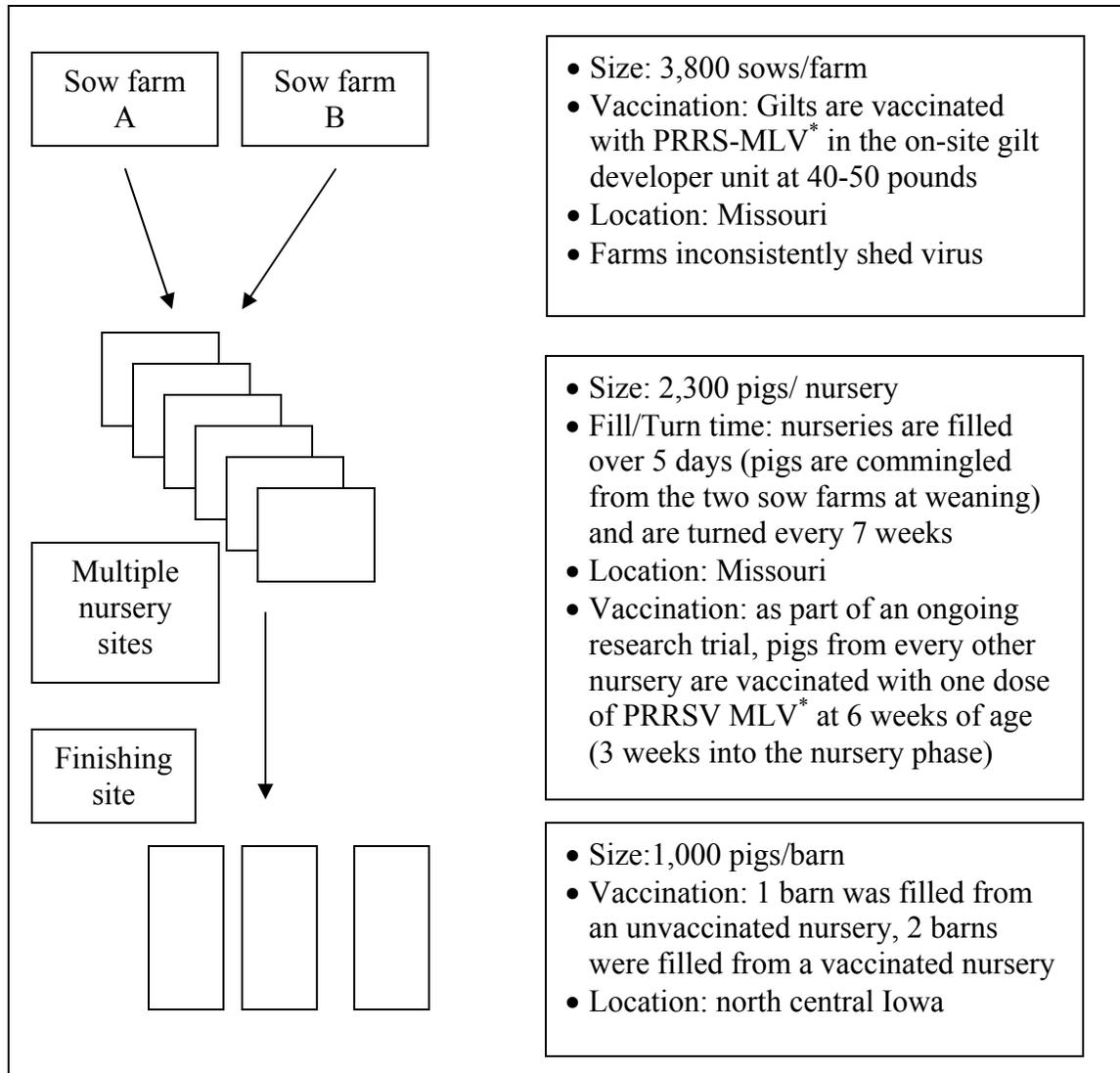


Figure 1. Diagram outlining the pig flow (farrow-to-finish) for the production system used in trial three[†]. This information was provided to swine experts[‡] as background information in order to elicit PRRSV prevalence estimates.

[†]In trial three, blood was collected from 132 pigs from one PRRSV vaccinated finisher barn in order to determine the diagnostic accuracy of two sampling methods.

[‡]Three swine experts (Baker RB, Ramirez A, Holtkamp D., personal communication) developed prevalence estimates using the freeware computer program BetaBuster (available online at: <http://www.epi.ucdavis.edu/diagnostictests/betabuster.html>) after being presented with specific information on the population.

*PRRSV-MLV - Ingelvac PRRS modified live vaccine, Boehringer Ingelheim, St. Joseph, Missouri.

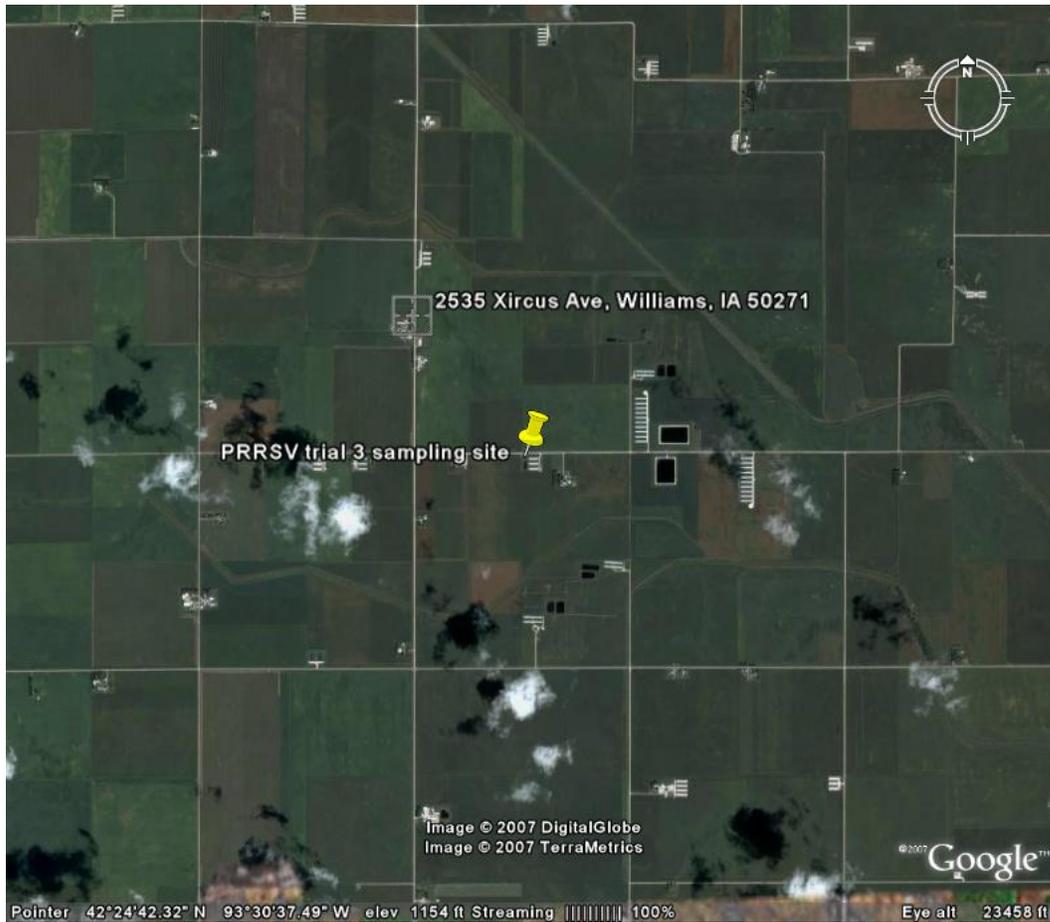


Figure 2. Google earth™ image of the sampling location and surrounding area for trial three[†]. This information was used to assess the number of finishing spaces surrounding the trial site. It was provided to swine experts[‡] as background information in order to elicit PRRSV prevalence estimates.

[†]In trial three, blood was collected from 132 pigs from one PRRSV vaccinated finisher barn in order to determine the diagnostic accuracy of two sampling methods.

[‡]Three swine experts (Baker RB, Ramirez A, Holtkamp D., personal communication) developed prevalence estimates using the freeware computer program BetaBuster (available online at: <http://www.epi.ucdavis.edu/diagnostictests/betabuster.html>) after being presented with specific information on the population.



Figure 3. Google earthTM image of the sampling site trial three[†]. This information was used to establish the production system layout. It was provided to swine experts[‡] as background information in order to elicit PRRSV prevalence estimates.

[†]In trial three, blood was collected from 132 pigs from one PRRSV vaccinated finisher barn in order to determine the diagnostic accuracy of two sampling methods.

[‡]Three swine experts (Baker RB, Ramirez A, Holtkamp D., personal communication) developed prevalence estimates using the freeware computer program BetaBuster (available online at: <http://www.epi.ucdavis.edu/diagnostictests/betabuster.html>) after being presented with specific information on the population.

Table 1. Additional information regarding the management of the production system used in trial three[†]. This information was provided to swine experts[‡] as background information in order to elicit PRRSV prevalence estimates.

Additional information:

- There are 10,000 pig spaces (same production company) within a mile of the site and 20,000 spaces (different production company) to the east.
- The site is operated by a husband and his wife; neither of them have other exposure to pigs.
- Dead animals are removed by a common rendering truck (one truck services all buildings in the area).
- Feed is supplied from a mill in Algona, Iowa; there is no hierarchy for use; plastic boots are not utilized by the drivers while they are outside of the vehicle.
- Transport trucks are site-dedicated trucks based out of Missouri; they are clean for the first cut but not for subsequent load outs; trucks are cleaned and disinfected but not dried/baked.
- Sites are all-in-all out; facilities are well cleaned and managed; rodent control is excellent (according to the production company veterinarian).
- The site is process verified with routine audits by the production company veterinarian.
- Service employees do not shower in and out between sites within the production company.

[†]In trial three, blood was collected from 132 pigs from one PRRSV vaccinated finisher barn in order to determine the diagnostic accuracy of two sampling methods.

[‡]Three swine experts (Baker RB, Ramirez A, Holtkamp D., personal communication) developed prevalence estimates using the freeware computer program BetaBuster (available online at: <http://www.epi.ucdavis.edu/diagnostictests/betabuster.html>) after being presented with specific information on the population.