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# Exploring alternative methods for population surveillance

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**Exploring alternative methods for population surveillance**

by

**Brent John Pepin**

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

**MASTERS OF SCIENCE**

Major: Veterinary Preventive Medicine

Program of Study Committee:  
Jeffrey Zimmerman, Co-Major Professor  
Alejandro Ramirez, Co-Major Professor  
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Iowa State University

Ames, Iowa

2014

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## ABSTRACT

Swine disease surveillance is of great importance to the swine industry in order to raise and provide healthy animal populations. In order to insure timely disease intervention for optimal animal health, surveillance methods and sampling options need to be readily available. Many of our diagnostic sample collections are based on individual animal samples, i.e. serum, blood swabs, nasal swabs, etc. These individual sample diagnostics are then commonly used to infer information on the population level. In order to understand population status from a collection of individual samples, diagnostic and sampling methods require evaluation.

As reviewed in Chapter 1, the pooling of individual samples is commonly place in various fields of veterinary medicine. In order to properly utilize this diagnostic strategy all the factors that affect pooling results need to be understood. Pooled diagnostics are influenced by the type of analyte, stage of infection, sample dilution, matrix components, the prevalence of disease, and laboratory differences. In order to attain accurate diagnostics and to properly pooling interpret results, these factors must be accounted for.

In continuation of the study of disease surveillance methods, the objective of the first research paper (Chapter 2) was to evaluate different sample collection techniques for the early detection Porcine Respiratory and Reproductive Syndrome Virus (PRRSV) in a boar stud population based in individual sampling techniques. Diagnostic specimens analyzed in this study included serum, oral fluid, blood swabs, frothy saliva, and semen. Semen samples were centrifuged and the seminal supernatant and cell fractions were tested separately. All samples were randomly ordered and tested for PRRSV by real-time quantitative reverse-

transcriptase polymerase chain reaction (rRT-PCR) and PRRSV antibody ELISA. No statistically significant differences were found between serum, blood swabs, and oral fluids in the onset of detection but numerical differences did exist.

The objective of the second study (Chapter 3) was to evaluate if the oral fluid training and collection methods used for the boars in Chapter 2 could be applied to individually housed commercial sows. This study also analyzed the diagnostic reproducibility of PCR and ELISA results from the same animals. To achieve this, oral fluid sample collection was attempted on 513 individually housed; mixed parity sows naive to oral fluid collection. Oral fluid collection was attempted for each animal on two successive days under the same collection conditions. Successful paired oral fluid samples were randomly selected, randomized for submission, and tested by PRRSV PCR and oral fluid ELISA for anti-PRRSV antibodies. Younger sows and the re-sampling of animals were positively associated with collection success and diagnostic results collected on two successive days were correlated.

## INTRODUCTION: THESIS FORMATTING

This thesis contains four chapters. Chapter 1 contains a literature review titled “A review of the issues impacting the diagnostic performance of pooled samples” and will be submitted to the Journal of Swine Health and Production for review for publication. Chapter 2 is a scientific research paper titled “Comparison of specimens for detection of porcine reproductive and respiratory syndrome virus infection in boar studs” and has been published in Transboundary and Emerging Diseases. Chapter 3 is the final scientific research paper titled “Collection of oral fluid from individually housed sows” and has been accepted for publication in the Journal of Swine Health and Production. The final chapter consists of general conclusions for the full thesis.



## CHAPTER I

A REVIEW OF THE ISSUES IMPACTING THE DIAGNOSTIC PERFORMANCE OF  
POOLED SAMPLES

Paper to be submitted to *Journal of Swine Health and Production Medicine*

Brent Pepin, Rodger Main, Alejandro Ramirez, Jeffery Zimmerman

## Summary

A "pooled sample" is a composite sample created by combining discrete samples in equal portions. Testing pooled samples is a method commonly used in swine medicine to determine the infection status of a population, estimate disease prevalence, or identify positive individuals. Pooling has significant strengths and weaknesses. It can improve testing efficiency and reduce testing costs, but it can also lead to incorrect diagnostic results and erroneous conclusions. Inevitably, the question is, "What number of samples will reduce testing costs, but not reduce test performance?" Ultimately, the answer will depend on the purpose of testing and on specific factors that affect the diagnostic accuracy of pooled samples. The purpose of this review is to provide an overview of these factors so that swine practitioners can make informed pooling decisions.

## Introduction

Testing pooled serum, swab, or fecal samples for direct evidence of a pathogen, i.e., by PCR or culture, is commonly used in swine medicine to establish the infection status of a population (Cortey et al., 2011; Muñoz-Zanzi et al., 2006; Rovira et al., 2008; Van Schaik et al., 2007); estimate disease prevalence (Vandenbussche, et al., 2008); or detect positive

individuals (Dorfman, 1943; Muñoz-Zanzi et al., 2000; Schaik et al., 2007). Antibody-based testing of pooled serum samples has also been explored, but it has not become an accepted practice, presents significant issues regarding test performance, and will not be addressed in this review (Lium et al., 2000; Rovira et al., 2008).

A brief review of sampling terminology is necessary to align this discussion. A "composite sample" is a sample created by combining two or more "discrete samples" into one (Patil et al., 2010). "Discrete samples" in veterinary practice could be individual animal samples, e.g., serum or nasal swabs samples, or "aggregate" samples, e.g., bulk milk tank samples, environmental swabs, or air samples (Biom et al., 1978; Lombard et al., 2012; Minkinen and Esbensen, 2009; Schaeffer et al., 1980). Discrete samples are not limited to specimen type, but always have clearly defined source, location, and time identities, i.e., what was sampled, where, and when (Cameron et al., 2003; Patil et al., 2010). Composite samples are created by combining discrete samples in either equal or unequal proportions; as dictated by the goal of testing (Cameron et al., 2003; Patil et al., 2010). A "pooled sample" is a composite sample created by combining discrete samples in equal portions. This implies that the goal is detection of the target in any and all samples contributing to the pool (Cameron et al., 2003; Hathaway et al., 2008; Patil et al., 2010; Rhode, 1976). Composite samples can also be created by combining discrete samples in unequal proportions. This implies that some discrete samples are more important than others (Patil et al., 2010). In monitoring water quality, for example, a larger proportion of the composite sample might come from larger streams vs. smaller streams, if larger streams carry more of the target of interest.

Pooled samples are used in a variety of applications and have been for quite some time. One of the earliest applications of pooling was the screening of World War II draftees for syphilis (Dorfman, 1943; Kim et al., 2009; Muñoz-Zanzi et al., 2006). Dorfman (1943), an economist, wanted to increase testing efficiency by testing pooled samples. For Dorfman, the answer to the question of, "How many individuals to pool?" was driven by the requirement to individually retest all samples in positive pools in order to identify each syphilis-positive draftee. If too many pools were positive, the cost of re-testing would approach the cost of testing the samples individually. Dorfman concluded that cost efficiency was a function of prevalence and that the number of samples in a pool should decrease as prevalence increased (Dorfman, 1943). It is worth noting that Dorfman's considerations did not include the effect of pooling on test performance; perhaps because the concept of test error (diagnostic sensitivity and specificity) did not enter the literature until a few years later (Yerushalmy, 1947).

Pooling samples has significant strengths and weaknesses. It can improve testing efficiency and reduce testing costs, but it can also lead to incorrect diagnostic results and erroneous conclusions. Inevitably, the question is, "What number of samples will reduce testing costs, but not reduce test performance?" Ultimately, the answer will depend on the purpose of testing and on several other factors that affect the diagnostic accuracy of pooled samples. The purpose of this review is to provide an overview of these factors so that swine practitioners can make informed pooling decisions.

## Specimen Factors that Affect the Diagnostic Performance of Pooled Samples

Particularly for PCR-based testing, the more samples are processed and physically manipulated, the greater the risk of cross-contamination and the higher the probability of false-positive results (Carmichael et al., 2010; Muñoz-Zanzi et al., 2006). It follows that the likelihood of false-positive results due to cross-contamination increases as pool size increases (Carmichael et al., 2010; Lanyon et al., 2014; Muñoz-Zanzi et al., 2006). Thus, the first step in assuring the fidelity of results for pooled samples is a critical assessment of the procedures used to collect and process samples in the field and in the clinic. Errors introduced at this level cannot be corrected later.

The "matrix effect" refers to components and/or characteristics of the specimen that reduce test sensitivity. The matrix effect includes both the physical homogeneity of the analyte within the specimen and biological or chemical factors inherent to the specimen that affect target stability or assay performance (Batten et al., 2007). For example, salmonella are known to be unevenly distributed within positive fecal samples (poor sample homogeneity), thus different portions of the same sample may provide discordant testing results (Arnold et al., 2005). If the target is not homogenous within individual samples, pooling can lead even to a high degree of matrix heterogeneity. Therefore, some sampling matrices require homogenization of both the discrete samples and the composite sample in order to produce repeatable and accurate test results (Patil et al., 2010).

Factors inherent to the specimen can also affect assay performance. Although many PCR inhibitors are poorly characterized, known PCR inhibitors found in biological specimens include antibodies, components of blood, bacterial cells, polysaccharides, salts, calcium, sodium, myoglobin, exogenic DNA, and a variety of other substances (Schrader et

al., 2012). Inhibitors in fecal samples include complex polysaccharides, bile salts, lipids, urate, nutritional components, gut flora, and organic debris (Pedersen et al., 2014; Schrader et al., 2012). PCRs are not the only assays to be affected by inherent inhibitory factors. For example, inhibitory factors for bacterial isolation include other nutrient-competing organisms and components of the pigs' immune system produced in response to infection (Arnold et al., 2005).

### The Dilution Effect

The "dilution effect" occurs when the negative samples in a pool dilute the positive sample(s) to the extent that the concentration of the analyte is below the detection threshold (Batten et al., 2007; Muñoz-Zanzi et al., 2006). The dilution effect is well-documented for bacterial culture (Arnold et al, 2005, 2008, 2009; Van Schaik et al., 2007) and for both viral and bacterial PCR-based assays (Batten et al., 2009; Carmichael et al., 2010; Garcia et al., 2013; Johnson et al., 2014; Lee et al., 2014; Polson et al., 2010; Rovira et al., 2007). Rovira et al. (2007) compared the detection of porcine reproductive and respiratory syndrome virus (PRRSV) in blood swab and serum samples on days post-inoculation (DPI) 1 through 15 and found that pooling by 5s resulted in 6% fewer reverse-transcription PCR (RT-PCR) -positive serum samples and 8% fewer RT-PCR-positive blood swab samples compared to individually-tested specimens. Johnson et al. (2014) reported that pooling bronchial and oropharyngeal swabs by 2s, 3s, or 5s for the detection of *Mycoplasma hyopneumoniae* by PCR resulted in 78% to 92% fewer positives than individually-tested samples. Lee et al. (2014) did not find a loss in detection of influenza A virus by RT-PCR when pooling nasal swabs by 3s, but observed significant losses in sensitivity for pools of 5 and 10. Pedersen et

al. (2014) reported a *Lawsonia intracellularis* detection rate of 41.9% for individual fecal samples pooled by 20s versus 53.5% for individual samples.

In the field, the dilution effect is the result of a complex interaction involving the population, the pathogen, and the pig:

1. The prevalence of infection in the population.
2. The level to which the pathogen and/or isolate replicates in the pig, i.e., some pathogens or strains replicate to higher concentrations than others.
3. The type of specimen pooled, i.e., the pathogen is often distributed unequally in the body.
4. The stage of infection.

### **The Prevalence of Infection in the Population**

As the prevalence of an agent declines in a population, the likelihood that a positive animal will be among the individuals sampled also declines. At a 10% prevalence, the probability that a pool will contain 2 positive samples is 0.01 and 0.26 for pools of 2 and 10 samples, respectively (Rovira et al., 2008). If the purpose of testing is detection of the agent in the population, more individuals need to be sampled in low prevalence situations to ensure that a pool contains one or more positives, but the risk of diluting a positive sample with negative samples (dilution effect) is also greatest in low prevalence situations (Batten et al., 2009; Christensen et al., 2000; Muñoz-Zanzi et al., 2006; Van Schaik et al., 2003; Vandenbussche et al., 2008). On the other hand, as prevalence increases, the likelihood that a pool contains one or more positive samples also increases; thereby allowing for a greater number of samples per pool. For this reason, it has been suggested that pool sizes for PCR-

based influenza surveillance in humans should be adjusted to account for changes in prevalence (Van Schaik et al., 2012). If the goal of testing is to determine individual animal infection status, pooling becomes less efficient as disease prevalence increases because of the need to retest each individual sample that formed the pool (Chase and Polson, 2000; Dorfman, 1943; Muñoz-Zanzi et al., 2000). Caution should be exercised if the goal of testing pooled samples is to estimate disease prevalence. It cannot be assumed that all discrete samples from a positive pool were positive and negative pools may have contained positive samples diluted below detectable levels (Hepworth et al., 2009; Muñoz-Zanzi et al., 2006).

### **Effect of Differences among Pathogens and/or Isolates**

Pathogens, and even strains or isolates of a pathogen, differ in the degree in which they replicate in the pig, i.e., some replicate to higher concentrations than others (Johnson et al., 2004; Pepin et al., 2013; Rovira et al., 2007). For example, more virulent PRRS viruses replicate to significantly higher levels than less virulent isolates, especially during the acute phase of the infection (Johnson et al., 2004). As an immediate consequence, PRRSV detection varies marked among isolates (Table 1), especially during the early stages of infection (Johnson et al., 2004; Pepin et al., 2013; Rovira et al., 2007). Therefore, strains that replicate to lower levels are at a greater risk producing false negatives on pooled samples because of the dilution effect (Muñoz-Zanzi et al., 2006; Rovira et al., 2007; Vandebussche et al., 2008).

### **The Effect of Specimen Type on Detection**

Specimen type greatly affects detection because pathogens often have predilections for different "compartments". For example, extreme variation was found in the detection of PRRSV by RT-PCR when testing different specimens from the same animals (Table 2). Notably, serum provided the best detection for the first three days of infection and detection of virus in semen was poor throughout the 7 day observation period (Pepin et al., 2013). Logically, pooling specimens with low levels of the target can only provide for poor testing results. As a general concept, the best samples to pool are those in which the pathogen is associated with cells (Chase and Polson, 2000). Samples like buffy coat can be centrifuged to concentrate the samples to increase the probability of pathogen detection while samples like serum should be made into smaller pools due to risk of false negatives resulting from the dilution effect (Chase and Polson, 2000).

### **Stage of Infection**

Over the course of an infection, the concentration of the pathogen peaks and then declines in the face of the host's immune response. As given in Tables 1 and 2, all sample types (serum, blood swabs, semen and oral fluid) all had lower detectability of virus at early compared to late stages of PRRV infection (Pepin et al., 2013). It is documented that PRRSV detection is the most variable and difficult to detect in the early stages post inoculation or infection (Johnson et al., 2004; Rovira et al., 2007; Kittawornrat et al., 2010; Pepin et al., 2013). In PCR diagnostics, a higher detectable analyte during peak viremic infection stages provides greater detection rates in pooled samples compared to acute disease stages (Batten et al., 2009).



### Laboratory Factors that Affect Pooled Samples?

Differences in laboratory performance can result in large differences in repeatability and reproducibility. For example, a comparison of known PRRSV status samples submitted for PCR testing revealed to great variation in positive or negative diagnostic results (Fetzer et al., 2006; Tryuen et al., 2006). A submission of samples for PCR PRRSV testing to different laboratories revealed great discrepancies between labs as some misclassified positive samples and others misclassified both positive and negative samples (Tryuen et al., 2006). M. hyo Tween-20 ELISA assay has a documented range of diagnostic sensitivity ranging from 0%-43.9% while DAKO H. hyo ELISA assay sensitivity ranges from 46.3%-60% (Erlandson et al., 2005). The misclassification of diagnostic samples results in the accumulation of false-positive and false negative results. This emphasizes an important key point that diagnostic tests are not 100% sensitive and specific and sensitivity and specificity do not take into account testing error induced by pool dilution and matrix effects (Hae-Young et al., 2007; Graff et al., 1972; Vansteelandt et al., 2000). This is important to remember when pooling, as a diagnostic test may not be sensitive or specific enough to accurately detect analyte in a sample diluted below the normal detectable levels (Muñoz-Zanzi et al., 2000; Jordan, 2005)

### Strategies to Improve Diagnostic Performance

#### **Increase Number of Pools Submitted**

Increased diagnostic pooled sensitivity can be attained by increasing the amount of individuals represented in the pooled samples. This is best done by increasing the number of pools submitted and not the number per pool (Lee et al., 2014; Rovira et al., 2007, 2008). Instead of submitting 10 individual serum samples for testing, sample 50 animals in pools of

five provides the same number of diagnostic but allows greater animal representation. This requires more time or money spent on sample collection, but diagnostic costs can be reduced or kept the same (Cortey et al., 2011; Lee et al., 2014; Rovira et al., 2006, 2007, 2008).

When individual animal status is required, positive pools are retested on an individual basis. Pool sizes need to be limited to preserve the pooling cost benefit for individual sample retests (Van Schaik et al., 2003; Dorfman 1943). For example “With 1 positive in 50 you would need to retest all 50 so the total cost would be the initial test along with the 50 retests  $\$20 + 50(\$20)=\$1020$ ...if the testing was done in pools of 10 [there] would be 5 pools and one of these pools would be positive so the cost would be... $5(\$20) + 10(\$20) = \$300$ ” (Chase and Polson, 2000).

### **Repeat Testing – Especially for Surveillance Situations**

Herd retesting, especially in low prevalence situations, increases detection sensitivity. With low disease prevalence, e.g. disease surveillance or very acute herd exposure, pooling too many samples risks a dilution of positive samples below detectable levels. In a boar stud, pooling samples by 3 or 5 for daily testing provides confidence of the detection of acute infections (Reicks, 2005). This concept is further supported in ruminants as weekly collected samples increased pool sensitivity for PCR testing for *Tritrichomonas fetus* and Bluetongue (Vandenbussche et al., 2008; Garcia Guerra et al., 2013). Repeat herd testing also increases the probability of detecting elusive carrier animals that can be otherwise missed (Garcia Guerra et al., 2013).

**Tool to Assist**

A free tool accessible via the internet at <http://epitools.ausvet.com.au> is available to assist in determining pool size and number of pools to submit (Sergeant and Toribio, 2004). Following the link, under “Surveillance utilities” the title “Pooled prevalence calculator” leads to a page with various pooled sample and prevalence related options. On this page the link titled “Pooled testing for demonstration of freedom” provides a calculator for the number of pools required for a given pool size to determine if a population is free of disease. This calculator requires test sensitivity, desired herd-sensitivity, and target prevalence. The “Design (target) prevalence” refers to the minimum prevalence of disease that is likely to occur or the prevalence value at which below the disease is not a concern. “Desired herd-sensitivity” refers to the level confidence desired to be able to detect the disease, if present (Sergeant and Toribio, 2004). This calculator assumes detection of at least one positive pool at the number provided supports a prevalence at or greater than the given target.

Major shortcomings of this calculator are it assumes (1) the test in use has 100% specificity and the calculation will not take into account factors such as sample dilution and matrix effects. (2) In many cases, the sensitivity and specificity of a test may be unknown, especially for pooled samples that are affected by prevalence, dilution, and matrix effects. The answers calculated can provide a starting guideline for the detection of disease with pooled samples and it is up to the veterinarian or diagnostician to determine how to properly utilize the value. It will always be better to provide a greater number of pools (not numbers per pool) to increase diagnostic confidence in disease detection, especially if an accurate test sensitivity is unknown.

## Implications and Conclusions

Optimistic assumptions are often made in relation to test sensitivity, test specificity, and test error in the literature. An early published paper on diagnostic pooling and its statistical modeling assumed the diagnostic test used was "extremely sensitive", which is not a common observation in many veterinary medicine diagnostics (Dorfman, 1943). The assumption of sensitivity was addressed in another study stating "...for many test methods in use there is either a positive probability that a defective item will read good, or a positive probability that a good item will read defective, or sometimes both" (Graff et al., 1972).

Sample pooling for herd diagnostic is often necessary for economic reasons. However, the selection of an appropriate pool and sample size is a difficult and convoluted challenge. When approaching a pooling situation, factors beyond straight economics influence accurate diagnostic interpretations. If pooling hazards are not at least considered, poor methods can lead to misdiagnosed disease statuses and potentially an even greater economic loss. Tools like the epitools website can provide guidance, but the practitioner's final judgment must cogitate all contributing factors beyond what the calculator can evaluate. The ultimate pooling strategy is to approach situations on a case-by-case basis, considering the probable prevalence of infection, financial constraints, the stage of disease, the diagnostic goal, and the acceptable degree of diagnostic uncertainty allowed. Pooled testing is a powerful tool, but must be used wisely and under appropriate restraints for the given situation.

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**Table 1. Early detection of PRRSV by PCR in blood and oral fluid specimens collected from boars under experimental conditions\***

Specimen	Virus isolate	Day post inoculation (no. positive/total tested)				Citation
		1	2	3	4	
Serum	MN 30-100	0/20	12/20	16/20	18/20	Reicks et al., 2006a.
	MN 30-100	1/18	-	14/18	-	Rovira et al., 2007a.
	MNB04	10/10	10/10	11/11	10/10	Reicks et al., 2006b.
	PRRS MLV	2/5	4/5	5/5	5/5	Pepin et al., 2013.
	SD-23983	6/6	-	6/6	-	Wasilk et al., 2004.
Blood Swab	PRRS MLV	0/5	1/5	1/5	3/5	Pepin et al., 2013.
	MNB04	9/10	10/10	11/11	10/10	Reicks et al., 2006b.
	MN 30-100	1/18	-	15/18	-	Rovira et al., 2007a.
Oral Fluid	D09-01213	1/21	22/22	22/22	22/22	Kittawornrat et al., 2010 <sup>1</sup>
	MN-184	0/24	17/24	23/24	21/21	Kittawornrat et al., 2010 <sup>1</sup>
	PRRS MLV	2/24	9/22	21/24	24/24	Kittawornrat et al., 2010 <sup>1</sup>
	PRRS MLV	0/15	1/15	10/15	13/15	Pepin et al., 2013.

<sup>1</sup> A. Kittawornrat (personal communication)

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**Table 2. Early detection of PRRSV by PCR as a function of specimen and day post inoculation\***

Specimen	Percent (%) positive by day post inoculation						
	1	2	3	4	5	6	7
Serum	36.5	79.1	89.5	93.8	95.2	97.4	99.9
Blood swab	30.3	73.3	79.4	86.7	87.9	99.9	99.9
Oral fluid	3.6	59.0	89.4	97.6	99.9	99.9	99.9
Whole semen or supernatant	0	12.8	17.1	26.5	22.0	28.9	30.8
Cell fraction semen	0	0	14.7	21.4	18.8	47.1	43.5

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## CHAPTER 2

COMPARISON OF SPECIMENS FOR DETECTION OF PORCINE REPRODUCTIVE  
AND RESPIRATORY SYNDROME VIRUS INFECTION IN BOAR STUDS

Paper published in *Transboundary and Emerging Diseases*

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## Summary

Porcine reproductive and respiratory syndrome virus (PRRSV)-contaminated semen from boars is a route of transmission to females, and early detection of PRRSV infection in boars is a key component in sow farm biosecurity. The purpose of this study was to determine the optimum diagnostic specimen(s) for the detection of acute PRRSV infection in boars. Individually housed boars (n = 15) were trained for semen and oral fluid collection and then vaccinated with a commercial PRRSV modified live virus vaccine. Starting on the day of vaccination and for 14 days thereafter, oral fluid specimens were collected daily from all boars. The 15 boars were subdivided into three groups of 5, and serum, blood swabs and ‘frothy saliva’ were collected at the time of semen collection on a 3-day rotation. Frothy saliva, derived from the submandibular salivary gland, is produced by aroused boars. Semen was centrifuged, and semen supernatant and cell fractions were tested separately. All samples were randomly ordered and then tested by PRRSV real-time quantitative reverse-transcription polymerase chain reaction assay (rRT-PCR) and PRRSV antibody ELISA. In this study, a comparison of serum, blood swab, and oral fluid rRT-PCR results found no statistically significant differences in the onset of detection or proportion of positives, but

serum was numerically superior to oral fluids for early detection. Serum and oral fluid provided identical rRT-PCR results at  $\geq 5$  day post-vaccination. Likewise, the onset of detection of PRRSV antibody in serum, oral fluid and frothy saliva was statistically equivalent, with serum results again showing a numerical advantage. These results showed that the highest assurance of providing PRRSV-negative semen to sow farms should be based on rRT-PCR testing of serum collected at the time of semen collection. This approach can be augmented with oral fluid sampling from a random selection of uncollected boars to provide for statistically valid surveillance of the boar stud.

### Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV) remains one of the most economically important diseases of swine throughout the world, imposing massive losses on American (Sierra et al., 2000; Holtkamp et al., 2013), European (Velasova et al., 2012) and Asian producers (Tian et al., 2007). Porcine reproductive and respiratory syndrome virus epidemiology is complex, and virus shedding in the semen of infected boars, with subsequent transmission to recipient sows, is one of several ways by which PRRSV is maintained in swine populations (Wills et al., 1997; Christopher-Hennings et al., 2001). In contemporary swine production, artificial insemination is standard practice, with semen produced at boar studs and then distributed to sow farms. For this reason, rapid detection of PRRSV-infected boars is paramount to the protection of individual herds, regions and even PRRSV-free countries. This was demonstrated in 2012, when virus-contaminated imported semen resulted in the transmission of PRRSV infection to Switzerland, a PRRSV-free country (OIE, 2012). Elimination of the virus required serological testing of 9500 pigs on

~100 farms in combination with euthanasia of ~1300 piglets and 72 sows that had received contaminated semen (EVD BVET (Eidgenössisches Volkswirtschaftsdepartement, Bundesamt für Veterinärwesen), 2012; OIE, 2012, 2013).

The assurance of PRRSV-free semen is dependent on quickly and accurately identifying PRRSV-infected boars. Because PRRSV may induce minimal clinical signs in boars, routine diagnostic testing is mandatory (Swenson et al., 1994; Prieto and Castro, 2005). Currently, surveillance in boar studs relies on testing serum, blood swabs, oral fluids and/or semen by PRRSV reverse-transcription polymerase chain reaction-based assays (RT-PCR). Serum and semen are the traditional specimens used to test boars for PRRSV. Blood swabs and oral fluids are recent diagnostic innovations (Reicks et al., 2006b; Reicks, 2009; Kittawornrat et al., 2010, 2012). The purpose of the present study was to determine the best specimen (serum, blood swabs, oral fluids, frothy saliva and semen) to use for early detection of PRRSV infection in boars.

## Materials and Methods

### **Experimental Design**

Young boars (n = 15) trained for semen collection were intramuscularly vaccinated with a modified live virus PRRSV vaccine (Ingelvac\_ PPRS MLV; Boehringer Ingelheim Vetmedica, Inc., St. Joseph, MO, USA). Serum, blood swabs, frothy saliva (foam), oral fluids and semen were collected, completely randomized and then tested by PRRSV real-time quantitative reverse-transcription polymerase chain reaction assay (rRT-PCR: VetMAX™ NA and EU PPRS; Applied Biosystems, Carlsbad, CA, USA) and PRRSV antibody ELISA (PPRS X3 Antibody Test; IDEXX Laboratories, Inc., Westbrook, ME,



USA). Data were analyzed in the context of specimen, diagnostic assay and time to PRRSV detection. This study was conducted with the approval of the Iowa State University Office for Responsible Research (#6-12-7398-S).

### **Animals**

Young boars (n = 15) 24–27 weeks of age were individually housed in quarantine facilities equipped with nipple drinkers, fan ventilation and temperature-responsive automatic water misters. Boars were fed a standard corn/soy-based ration at the rate of ~3 kg (6–7 lbs) per day. Animals were acclimated to the facilities and trained to semen collection for 2 weeks prior to the initiation of the study.

### **PRRSV Vaccination**

All boars were intramuscularly inoculated with a modified live PRRSV vaccine (MLV) (Ingelvac\_ PPRS MLV; Boehringer Ingelheim Vetmedica, Inc.) according to the instructions provided by the manufacturer. The vaccine is based on a cell culture-adapted isolate derived from PRRSV ATCC VR2332 (Nielsen et al., 2001; Opriessnig et al., 2002). MLV PRRSV vaccine has been used previously to model PRRSV infection in boars (Christopher-Hennings et al., 1997; Shin et al., 1997; Kittawornrat et al., 2010). The modified vaccine virus produces a lower viral titre and shorter period of viraemia than PRRSV field isolates and consequently stimulates a weaker humoral immune response (Johnson et al., 2004; Kittawornrat et al., 2010).

## **Biological Samples**

The 15 animals were grouped into 3 subsets of 5 boars each. Oral fluid samples were collected from all 15 boars daily from day post-vaccination (DPV) 0–14. Blood, blood swabs, frothy saliva and semen samples were collected on a 3-day rotation. Thus, the first group was collected on DPV 0, 3, 6, 9 and 12; the second group on DPV 1, 4, 7, 10 and 13; and the third group on DPV 2, 5, 8, 11 and 14.

### Serum Samples

Blood was collected from the saphenous vein after the boar had mounted the semen collection dummy using a single-use collection system (EXEL International Medical Products, Los Angeles, CA, USA) and serum separation tubes (Corvac\_; Tyco Healthcare Group LP, Mansfield, MA, USA). In five instances, blood was collected using jugular venipuncture. Samples were centrifuged at 1800 g for 10 min, after which the serum was aliquoted into tubes and stored frozen until assayed.

### Blood Swab Samples

Blood swabs were taken by saturating polyester-tipped sterile applicators (25-806 1PD Puritan Medical, Guilford, ME, USA) with blood that pooled at the puncture site following saphenous vein blood collection. Swabs were immediately placed in 5-ml tubes containing 1 ml of sterile saline solution (Aspen Veterinary Resources Ltd., Liberty, MO, USA). In the event that a sterile swab could not be taken following venipuncture, for example, insufficient blood at the puncture site, blood was accessed by pricking the medial caudal vein at the base of the tail with a sterile needle.

### Semen Samples

During the 2 weeks prior to the start of the trial, boars were trained for semen collection by farm personnel. Semen was collected using the gloved-hand technique while the boar was mounted on the collection dummy. Semen was collected into a container holding semen collection bags with a built-in, tear-away filter to remove the gel portion of the ejaculate (US Bag; Minitube America, Inc., Verona, WI, USA). Collected semen samples were decanted into a 50-ml centrifuge tube, aliquoted into 5-ml plastic tubes and stored frozen. In the diagnostic laboratory, whole semen samples were thawed and then centrifuged at 600 g for 15 min at 4°C. The supernatant was decanted for subsequent testing by rRT-PCR and PRRSV antibody ELISA. The semen pellet (cell fraction) was resuspended 1 : 1 in semen supernatant and subsequently tested by PRRSV rRT-PCR.

### Oral Fluid Samples

Oral fluid was collected daily from each boar. In brief, oral fluid samples were collected with 5/8" (1.6 cm), 3-strand, 100% cotton rope. Ropes were cut to a length of 30 inches (76.2 cm) and tied to the front of each pen at shoulder height and hung for ~20 min. Oral fluid from the boar was absorbed by the rope during mastication. Post-exposure, the wet portion of the rope was inserted into a 1-gallon plastic bag and the bag was sealed. To harvest the sample, the rope was squeezed with gloved hands on the outside of the bag, causing the oral fluid to pool in the bottom of the bag. Thereafter, oral fluids were decanted into 50-ml centrifuge tubes and then aliquoted into 5-ml plastic tubes and frozen.

### Frothy Saliva Samples

Sexually mature boars produce 'frothy saliva' from the submaxillary saliva glands as part of normal reproductive behavior (Pearce et al., 1988). Frothy saliva was collected using two different methods. From DPV 0 to the completion of the trial, frothy saliva was collected by wiping around the outside of each boar's mouth with a sterile sponge pre-hydrated with 10 ml neutralizing buffer (3MTM Hydra-Sponge, St. Paul, MN, USA) while they were collected for semen. The sponge was then returned to its sterile bag, the fluid squeezed from the sponge, and the fluid aliquoted into 5-ml tubes and frozen. On DPV 8, the boars began producing larger quantities of frothy saliva, and a second frothy saliva sample was collected directly into a 50-ml centrifuge tube. Once in the tube, the sample was sprayed once with an anti-foam compound (Thomas Scientific, Swedesboro, NJ, USA) to liquefy the froth. The liquid was then aliquoted into 5-ml plastic tubes and frozen.

### **PRRSV Antibody ELISA Procedures**

All serum samples were tested for anti-PRRSV antibodies using a commercial ELISA (IDEXX PRRS X3 Ab Test, IDEXX Laboratories, Inc.). Samples were assayed according to the manufacturer's instructions. Results with a sample-to-positive (S/P) ratio  $\geq 0.4$  were considered positive.

Oral fluid, frothy saliva, blood swabs and semen supernatant specimens were tested for anti-PRRSV antibodies using a commercial PRRSV antibody ELISA (IDEXX PRRS X3 Ab Test) performed using a modified protocol, as described elsewhere (Kittawornrat et al., 2012). In brief, oral fluid samples were diluted 1:2 in dilution plates using the diluent provided with the test kit and then transferred (250  $\mu$ l) to the 96-well PRRSV antigen-coated

kit plates. Negative and positive kit controls were diluted 1:30 using the kit diluent (100 µl) and run in duplicate on each plate. Plates were incubated for 16 h at 4°C and then washed three times with 400 µl of kit wash solution. To detect bound antibody, reagents were brought to room temperature, and then 100 µl of a solution containing appropriately diluted horseradish peroxidase–conjugated anti-swine immunoglobulin G (IgGFc) secondary antibody (pig IgGFc antibody A100-104P; Bethyl Laboratories Inc., Montgomery, TX, USA) was added to each well and incubated for 30 min at 22°C. Thereafter, plates were washed three times with wash solution, and then 100 µl of tetramethylbenzidine enzyme substrate solution was added to each well and the plates were incubated at 22°C for 15 min. After 15 min, 100 µl of kit stop solution was added to each well, the plates were read at 650 nm and the reactions measured as optical density (OD). OD values were converted to S/P ratios, as described by the kit manufacturer, with S/P ratios  $\geq 0.4$  considered positive.

## **PRRSV Nucleic Acid Extraction and Detection Assays**

### Nucleic Acid Extraction Protocol

RNA extraction was performed using the MagMAX™ Viral RNA Isolation Kit (Life Technologies Corporation, Carlsbad, CA, USA) and a Kingfisher 96 instrument (Thermo Scientific, Waltham, MA, USA). Serum and blood swabs were extracted following the standard lysis procedure using 50 µl of sample added to 130 µl of lysis-binding solution/carrier RNA prepared according to the kit insert, 20 µl magnetic bead mix and 90 µl of elution buffer. Semen cell fraction, semen supernatant, oral fluid and frothy saliva specimens were extracted using a high-volume modified lysis (HVML) procedure (Chittick et al., 2011). The lysis/binding solution for the HVML protocol was prepared using 45 ml

lysis/binding solution with 200 µl carrier RNA without the addition of isopropanol. For the lysis step, 300 µl of sample was added to 450 µl of modified lysis/binding solution, vortexed for 3 min and centrifuged at 2500 g for 6 min. A volume of 600 µl of lysate was added to 350 µl isopropanol with 20 µl magnetic bead mix prior to extraction and elution into 90 µl buffer. The standard lysis protocol used 150 µl of wash solution I and II provided with the kit. The HVML used 300 and 450 µl of wash solutions I and II, respectively. The standard lysis extractions were conducted using the Kingfisher program AM\_1836\_DW\_50\_v3. The HVML extraction was conducted using the Kingfisher AM1836\_DW\_HV\_v3.

#### PRRSV RNA Amplification and Detection

Porcine reproductive and respiratory syndrome virus rRT-PCR was performed on nucleic acid extracts using the MagMAX™ NA and EU PRRSV-specific PCR assay (Life Technologies Corporation). Internal control Xeno™ RNA was included in the master mix to monitor PCR amplification and detection of failed PCR. Two positive extraction controls, one negative extraction control and a negative amplification control were included with each extraction and/or PCR run. Each serum and blood swab reaction included 12.5 µl of 29 RT-PCR buffer, 2.5 µl of 109 PRRSV primer probe mix, 1.25 µl of 209 multiplex RT-PCR enzyme mix, 0.35 µl of internal control RNA at a concentration of 100 copies/µl and 0.4 µl of nuclease-free water. Each semen cell fraction, semen supernatant, oral fluid and frothy saliva reaction used the same volume of reagents described for the serum and blood swabs with the exception of 2.5 l of 209 multiplex RT-PCR enzyme mix and 0.5 µl of nuclease-free water. A final volume of 25 µl consisting of 17 µl mastermix and 8 µl of RNA extract for the standard lysis samples or 18 µl mastermix and 7 µl of RNA extract for the HVML samples

was placed in each well of a 96-well fast PCR plate (Life Technologies). Real-time RT-PCR was performed using an AB 7500 Fast thermocycler with the following cycling conditions: 1 cycle at 45°C for 10 min, 1 cycle at 95°C for 10 min and 40 cycles of 97°C for 2 s, 60°C for 40 s. Amplification curves were analyzed with commercial thermal cycler system software. The 'auto baseline' was used to determine fluorescence baselines, and cycle thresholds were set at 0.1 and 0.05 for NA and EU PRRSV, respectively. Samples with threshold cycle (Ct) values <37 for either strain were considered positive. Internal control Xeno™ RNA Ct values were set at 10% of maximum. Each PCR assay included eight progressive 1:10 dilutions of a known copy number of PRRSV supplied with the MagMAX™ NA and EU PRRSV PCR reagents kit to generate a standard curve for quantification reported as genomic copies per ml.

### **Statistical Analysis**

Data were analyzed using commercial statistical software (SAS\_ Institute Inc., Cary, NC, USA; MedCalc\_ 9.2.1.0, Mariakerke, Belgium). Virus concentration as determined by quantitative rRT-PCR (1.9 ex genome equivalents per ml) and ELISA S/P ratio responses were analysed using a linear mixed model with repeated measures. Tukey's honestly significantly different (HSD) test was used to compare quantitative results over time within specimens. Qualitative differences in rRT-PCR and ELISA results were evaluated using Cochran's Q test. When Cochran's Q test indicated a significant difference ( $P < 0.05$ ) in the proportion of positives among specimens, pairwise comparisons were performed using serum as the reference standard.

## Results

The PRRSV antibody ELISA responses are reported in Tables 1 and 2. Statistically significant increases in mean S/P ratios were detected on DPV 8 in serum, DPV 9 in oral fluid, DPV 10 in frothy saliva, DPV 11 in blood swabs and DPV 13 in semen supernatant sample. Semen supernatant samples were characterized by a high level of non-specific reactivity on the ELISA (Table 1). One semen supernatant sample collected on DPV 1 produced an S/P response of 0.415, whereas the serum S/P response from the same animal on DPV 1 was 0.016. Statistically significant increases in S/P ratios did not directly reflect seropositivity, that is, S/P values were not necessarily above the cut-off ( $S/P \geq 0.4$ ). One of 5 serum samples was ELISA positive on DPV 9 and 5 of 5 on DPV 10. In contrast, no blood swabs were ELISA positive until DPV 13. Five of 5 oral fluid specimens were ELISA positive on DPV 10 and were identical to serum results at all samplings, thereafter. Antibody was detected in frothy saliva samples, but sample collection and antibody detection were more reproducible in oral fluids. One of 5 semen supernatant samples was antibody positive on DPV 13 and 2 of 5 on DPV 14.

The PRRSV rRT-PCR results are reported in Tables 3 and 4. Statistically significant increases in mean genomic equivalents were detected on DPV 2 in serum, DPV 3 in oral fluids, DPV 6 in frothy saliva, DPV 4 in blood swabs, DPV 9 in semen supernatant, and DPV 5 in seminal cell fraction sample. Frothy saliva collected with anti-foam produced greater mean genomic equivalents than frothy saliva specimens collected with a pre-hydrated sponge between DPV 8–14, except for DPV 13 where both were zero. Qualitatively, 2 of 5 serum samples were rRT-PCR positive on DPV 1, 4 of 5 on DPV 2 and 5 of 5 on DPV 3. All 5 oral fluid and blood swab samples were positive on DPV 5 and 6, respectively. In spite of



numerical differences, statistically significant differences between oral fluids and serum qualitative results were not detected, probably as a consequence of sample size. In contrast to oral fluids, detection using frothy saliva samples never achieved more than 60% detection by rRT-PCR. Likewise, detection using semen samples never exceeded 40% detection in semen cell fraction specimens and 20% in semen supernatant.

### Discussion

This study was designed to reexamine PRRSV monitoring in boar studs by comparing diagnostic results on specimens collected from 15 boars inoculated with PRRSV MLV on a daily (oral fluid) or 3-day (semen, serum, blood swab, frothy saliva) schedule. Comparison of these data to published reports on the detection of PRRSV in boars under controlled settings (Tables 5 and 6) showed that the current study was similar in size to the majority and that some of these also used MLV to model PRRSV shedding (Christopher-Hennings et al., 1997; Shin et al., 1997; Kittawornrat et al., 2010). There were few reports with which to compare blood swab results. Reicks et al. (2006b) and Rovira et al. (2007a) found blood swabs to be essentially equal to serum for the detection of PRRSV (Table 5), in contrast to the results of this study. Differences in the level of viremia in individual animals, the swabs used to collect the blood sample or the volume of saline used to resuspend the sample could have contributed to differences in detection rates among the three studies. A systematic effort to optimize the blood swab protocol to the detection of PRRSV infection would be desirable. Likewise, there are few reports with which to compare oral fluid results and no prior reports of testing frothy saliva specimens. In this small study, a comparison of oral fluids and serum rRT-PCR results found no statistically significant differences in the onset of detection or

proportion of positives. Regardless, serum testing was numerically superior to oral fluids for early detection, that is, DPVs 1–4; after which time serum and oral fluid results were nearly identical.

This is the first report of PRRSV detection by rRT-PCR in frothy saliva. Frothy saliva is produced by the submandibular salivary gland (*glandula submandibularis*) and is associated with pheromone release in sexually aroused boars (Pearce et al., 1988 and Marchese et al., 1998). The boars used in this trial were under 10 months, the age at which the submaxillary gland achieves adult pheromone productivity (Kirkwood et al., 2012). Perhaps for this reason, the boars in this study produced low volumes of frothy saliva. In particular, it was not possible to collect frothy saliva into tubes for treatment with anti-foam until DPV 8, and collection was still problematic thereafter (Table 2). Neither of the two methods of frothy saliva collection was as diagnostically sensitive as serum, blood swabs or oral fluids. Thus, although it is a convenient sample to collect in mature boars, there are no data to support the diagnostic use of frothy saliva.

This study provided the opportunity to simultaneously examine the kinetics of the PRRSV ELISA-detectable antibody response in a variety of specimens, including serum, blood swabs, oral fluid, frothy saliva and semen supernatant. The presence of PRRSV antibodies has previously been reported in oral fluid (Kittawornrat et al., 2012) and semen (Kaiser et al., 2000; Oleksiewicz et al., 2001). A report of the presence of plasma cells producing antibody against transmissible gastroenteritis virus (TGEV) in the submandibular salivary gland, that is, the source of frothy saliva, justified the inclusion of this specimen in the evaluation (DeBuysscher and Berman, 1980).

Overall, serum and oral fluid proved to be the most reliable in terms of sample collection and PRRSV antibody detection (Table 2). However, in both specimens, antibodies were not detected in 100% of boars sampled until DPV 10. Specifically, 1 of 5 boars was serum ELISA positive on DPV 9 and a 5 of 5 were positive in both serum and oral fluids on DPV 10 (Table 2). Antibody detection in other specimen types was essentially ineffective. In contrast, 2 of 5 boars were serum rRT-PCR positive on DPV 1, 4 of 5 on DPV 2 and 5 of 5 on DPV 3 through 10.

While antibody detection was not an effective approach for early detection of PRRSV infection, antibody detection may hold value for screening incoming boars during quarantine and as an alternative to serum for monitoring boars not being collected for semen.

The PRRSV rRT-PCR results on serum, oral fluid, semen supernatant and semen cell fraction specimens in this study were consistent with previous reports of PRRSV detection in boars under experimental conditions (Tables 5 and 6). It should be noted that MLV replicates to a lower level in pigs than most wild-type viruses and stimulates a correspondingly lower antibody response (Johnson et al., 2004). Thus, the current results may be considered conservative estimates of early detection. The relevance of this observation is illustrated in Table 5 where, depending on the study and the virus isolate used, detection in serum by rRT-PCR at 1 DPI was between 0% and 100% (Christopher-Hennings et al., 1995a; Wasilk et al., 2004; Reicks et al., 2006b; and Rovira et al., 2007a).

The data in Tables 5 and 6 were subsequently used to calculate the cumulative predicted probability of early detection of PRRSV in boars by PCR using a binomial logistic regression model. As shown in Table 7, the highest likelihood of detecting PRRSV infection on DPI 1 by PCR was provided by testing serum (36.5% of animals positive), followed by

blood swabs (30.3%) and oral fluids (3.6%). This ranking continued on DPI 2, with serum (79.1%) leading blood swabs (73.3%) and oral fluids (59%). On DPIs 3 through 7, serum and oral fluids were essentially equivalent, with the detection rate in blood swabs slightly lower. In all studies, semen samples were the poorest specimen for detecting early PRRSV infection by PCR.

Currently, prevention of PRRSV transmission via semen relies on rRT-PCR testing of semen and/or serum for PRRSV detection in boar stud units (Rovira et al., 2007b; Reicks, 2009). Based on these results and an evaluation of the literature, it is apparent that detection of PRRSV infection in the first 48 h following exposure is best achieved using serum collected via venipuncture. Serum is easily collected from the saphenous vein during semen collection (Reicks et al., 2006b; Broes et al., 2007; Reicks, 2009), and individuals monitoring boar studs should master this technique.

In practice, samples to be tested for PRRSV are typically obtained from boars at the time they are collected for semen (Dee and Deen, 2001; Reicks et al., 2006b; Reicks, 2009). That is, true surveillance based on a random sampling of animals in the population is rare, largely because restraining boars (snaring) for blood collection is stressful for both humans and animals (Dee and Deen, 2001). This lapse is justified by the need to find a balance between the production of PRRSV-free semen and the animal welfare/human safety risks created by frequent blood collections from adult boars (Dee and Deen, 2001), but this omission compromises the ability of boar studs to assure their freedom from PRRSV. Alternatively, oral fluid were shown to be equivalent to serum for PRRSV detection by DPI 3, and oral fluid samples are easily collected from boars – even on a daily basis (Kittawornrat

et al., 2010, 2012). This approach would provide for the random selection of a larger subset of individual boars for statistically valid population surveillance.

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**Table 1. Quantitative PRRSV Antibody ELISA Responses in Boars<sup>1</sup> Following PRRSV Vaccination**

Day post vaccination <sup>2</sup>	PRRSV antibody response <sup>3</sup> [LS mean sample:positive (S/P) ratios]					
	Serum	Blood swab <sup>4</sup>	Oral fluid	Frothy saliva <sup>5</sup>	Frothy saliva <sup>6</sup>	Semen supernatant
0	0.03 <sup>d</sup>	0.00 <sup>d</sup>	0.01 <sup>d</sup>	0.00 <sup>c</sup>	No sample	0.16 <sup>c</sup>
1	0.02 <sup>d</sup>	0.00 <sup>d</sup>	0.03 <sup>d</sup>	0.00 <sup>c</sup>	"	0.13 <sup>c</sup>
2	0.02 <sup>d</sup>	0.00 <sup>d</sup>	0.03 <sup>d</sup>	0.00 <sup>c</sup>	"	0.11 <sup>c</sup>
3	0.05 <sup>d</sup>	0.00 <sup>d</sup>	0.01 <sup>d</sup>	0.00 <sup>c</sup>	"	0.17 <sup>c</sup>
4	0.02 <sup>d</sup>	0.00 <sup>d</sup>	0.01 <sup>d</sup>	0.00 <sup>c</sup>	"	0.18 <sup>c</sup>
5	0.01 <sup>d</sup>	0.00 <sup>d</sup>	0.01 <sup>d</sup>	0.00 <sup>c</sup>	"	0.24 <sup>c</sup>
6	0.03 <sup>d</sup>	0.00 <sup>d</sup>	0.02 <sup>d</sup>	0.00 <sup>c</sup>	"	0.18 <sup>c</sup>
7	0.07 <sup>d</sup>	0.00 <sup>d</sup>	0.04 <sup>d</sup>	0.00 <sup>c</sup>	"	0.17 <sup>c</sup>
8	0.12 <sup>c</sup>	0.00 <sup>d</sup>	0.13 <sup>d</sup>	0.00 <sup>c</sup>	0.04 <sup>d</sup>	0.22 <sup>c</sup>
9	0.28 <sup>c</sup>	0.02 <sup>c, d</sup>	0.49 <sup>c</sup>	0.01 <sup>c</sup>	0.04 <sup>d</sup>	0.16 <sup>c</sup>
10	0.80 <sup>b</sup>	0.17 <sup>b, c, d</sup>	0.76 <sup>c</sup>	0.09 <sup>a</sup>	0.87 <sup>a, b</sup>	0.18 <sup>c</sup>
11	0.89 <sup>b</sup>	0.18 <sup>b, c</sup>	1.27 <sup>b</sup>	0.02 <sup>b, c</sup>	0.47 <sup>b, c, d</sup>	0.24 <sup>b, c</sup>
12	0.95 <sup>b</sup>	0.13 <sup>c, d</sup>	1.52 <sup>b</sup>	0.01 <sup>b, c</sup>	0.38 <sup>c, d</sup>	0.20 <sup>b, c</sup>
13	1.30 <sup>a</sup>	0.42 <sup>a</sup>	2.26 <sup>a</sup>	0.06 <sup>a, b</sup>	1.26 <sup>a</sup>	0.39 <sup>a</sup>
14	1.32 <sup>a</sup>	0.34 <sup>a, b</sup>	2.31 <sup>a</sup>	0.03 <sup>b, c</sup>	0.75 <sup>b, c</sup>	0.38 <sup>a, b</sup>

<sup>1</sup> The 15 boars in the study were divided into 3 groups of 5 boars and sampled on a 3-day rotation. Data for each day post vaccination represents 5 boars except for frothy saliva (antifoam) on DPV 9 (4 boars), DPV 10 (4 boars), and DPV 13 (4 boars).

<sup>2</sup> Ingelvac® PPRS MLV, Boehringer Ingelheim Vetmedica, Inc., St. Joseph, Missouri USA.

<sup>3</sup> PRRS X3 Antibody Test, IDEXX Laboratories, Inc., Westbrook, Maine USA.

<sup>4</sup> Blood swabs were taken using saturating polyester tipped sterile applicators (25-806 1PD Puritan Medical, Guilford, Maine USA) and then placed in tubes containing 1 ml of sterile saline solution (Aspen Veterinary Resources Ltd., Liberty, Missouri USA).

<sup>5</sup> Frothy saliva collected around the boar's mouth using a sterile sponge pre-hydrated with 10 ml neutralizing buffer (3M™ Hydra-Sponge, St. Paul, Minnesota USA) as they were collected for semen.

<sup>6</sup> Frothy saliva collected into a tube and then sprayed with antifoam (Thomas Scientific, Swedesboro, New Jersey USA).

<sup>a, b, c, d</sup> Within columns, means with the same letter are not significantly different (Tukey's Honestly Significantly Different test, p<0.05).

**Table 2. Qualitative PRRSV Antibody Responses in Boars<sup>1</sup> Following PRRSV Vaccination**

Day post vaccination <sup>2</sup>	Antibody response <sup>3</sup> (percent positive)						Cochran's Q <sup>7</sup> <i>p</i> -value
	Serum	Blood swab <sup>4</sup>	Oral fluid	Frothy saliva <sup>5</sup>	Frothy saliva <sup>6</sup>	Semen supernatant	
0	0%	0%	0%	0%	No sample	0%	No sample
1	0%	0%	0%	0%	"	20%	0.406
2	0%	0%	0%	0%	"	0%	No sample
3	0%	0%	0%	0%	"	0%	"
4	0%	0%	0%	0%	"	0%	"
5	0%	0%	0%	0%	"	0%	"
6	0%	0%	0%	0%	"	0%	"
7	0%	0%	0%	0%	"	0%	"
8	0%	0%	0%	0%	0%	0%	"
9	20%	0%	0%	0%	0%	0%	0.406
10	100%	0%*	100%	0%*	75%	0%*	<0.001
11	80%	0%	80%	0%	80%	0%	0.003
12	100%	0%*	100%	0%*	40%	0%*	<0.001
13	100%	60%	100%	0%*	100%	20%	0.005
14	100%	20%	100%	0%*	100%	40%	0.004

<sup>1</sup> The 15 boars in the study were divided into 3 groups of 5 boars and sampled on a 3-day rotation. Data for each day post vaccination represents 5 boars except for frothy saliva (antifoam) on DPV 9 (4 boars), DPV 10 (4 boars), and DPV 13 (4 boars).

<sup>2</sup> Ingelvac® PPRS MLV, Boehringer Ingelheim Vetmedica, Inc., St. Joseph, Missouri USA.

<sup>3</sup> PRRS X3 Antibody Test, IDEXX Laboratories, Inc., Westbrook, Maine USA.

<sup>4</sup> Blood swabs were taken using saturating polyester tipped sterile applicators (25-806 1PD Puritan Medical, Guilford, Maine USA) and then placed in tubes containing 1 ml of sterile saline solution (Aspen Veterinary Resources Ltd., Liberty, Missouri USA).

<sup>5</sup> Frothy saliva collected around the boar's mouth using a sterile sponge pre-hydrated with 10 ml neutralizing buffer (3M™ Hydra-Sponge, St. Paul, Minnesota USA) as they were collected for semen.

<sup>6</sup> Frothy saliva collected into a tube and then sprayed with antifoam (Thomas Scientific, Swedesboro, New Jersey USA)

<sup>7</sup> Cochran's Q was used to test for significant differences ( $p < 0.05$ ) among sample types by day post vaccination. Within DPV, (\*) indicates a significant difference in sample type vs. serum results.

**Table 3. Quantitative PRRSV rRT-PCR Results in Boars<sup>1</sup> Following PRRSV Vaccination**

Day post vaccination <sup>2</sup>	PRRSV qRT-PCR LS means ( $1 \times e^x$ genome equivalents per ml)						
	Serum	Blood swab <sup>3</sup>	Oral fluid	Frothy saliva <sup>4</sup>	Frothy saliva <sup>5</sup>	Semen supernatant	Semen cell fraction
0	0 <sup>e</sup>	0 <sup>d</sup>	0 <sup>g</sup>	0 <sup>c</sup>	No sample	0 <sup>b</sup>	0 <sup>c</sup>
1	1.92 <sup>e</sup>	0 <sup>d</sup>	0 <sup>g</sup>	0 <sup>c</sup>	"	0 <sup>b</sup>	0 <sup>c</sup>
2	5.17 <sup>d</sup>	0.98 <sup>c, d</sup>	0.20 <sup>g</sup>	0 <sup>c</sup>	"	0 <sup>b</sup>	0 <sup>c</sup>
3	6.79 <sup>b, c, d</sup>	0.68 <sup>c, d</sup>	2.36 <sup>e, f</sup>	0 <sup>c</sup>	"	0 <sup>b</sup>	1.37 <sup>a, b, c</sup>
4	7.83 <sup>a, b, c, d</sup>	2.30 <sup>b, c</sup>	4.39 <sup>c, d</sup>	1.70 <sup>a, b, c</sup>	"	0 <sup>b</sup>	0.86 <sup>a, b, c</sup>
5	7.47 <sup>a, b, c, d</sup>	1.45 <sup>c, d</sup>	6.24 <sup>a</sup>	0.90 <sup>b, c</sup>	"	0 <sup>b</sup>	2.49 <sup>a, b</sup>
6	8.92 <sup>a</sup>	4.42 <sup>a</sup>	6.51 <sup>a</sup>	2.59 <sup>a, b</sup>	"	0.94 <sup>a, b</sup>	3.36 <sup>a</sup>
7	8.56 <sup>a, b</sup>	4.27 <sup>a, b</sup>	5.78 <sup>a, b</sup>	2.90 <sup>a</sup>	"	0 <sup>b</sup>	0 <sup>c</sup>
8	7.59 <sup>a, b, c, d</sup>	2.50 <sup>a, b, c</sup>	4.75 <sup>b, c</sup>	0 <sup>c</sup>	1.36 <sup>b</sup>	0 <sup>b</sup>	0 <sup>c</sup>
9	8.00 <sup>a, b, c</sup>	3.79 <sup>a, b</sup>	4.28 <sup>c, d</sup>	0.76 <sup>b, c</sup>	1.20 <sup>b</sup>	1.49 <sup>a</sup>	1.95 <sup>a, b, c</sup>
10	7.17 <sup>a, b, c, d</sup>	1.41 <sup>c, d</sup>	3.78 <sup>c, d</sup>	0.88 <sup>b, c</sup>	2.01 <sup>b</sup>	0 <sup>b</sup>	0 <sup>c</sup>
11	5.43 <sup>d</sup>	0.79 <sup>c, d</sup>	4.03 <sup>c, d</sup>	0 <sup>c</sup>	2.35 <sup>a, b</sup>	0 <sup>b</sup>	0 <sup>c</sup>
12	6.13 <sup>d</sup>	1.44 <sup>c, d</sup>	3.29 <sup>d, e</sup>	0.85 <sup>b, c</sup>	1.93 <sup>b</sup>	0 <sup>b</sup>	0.95 <sup>a, b, c</sup>
13	0.55 <sup>e</sup>	0 <sup>d</sup>	1.74 <sup>f</sup>	0 <sup>c</sup>	0 <sup>b</sup>	0 <sup>b</sup>	0 <sup>c</sup>
14	1.67 <sup>e</sup>	0 <sup>d</sup>	1.59 <sup>f</sup>	1.05 <sup>a, b, c</sup>	1.49 <sup>b</sup>	0 <sup>b</sup>	0 <sup>c</sup>

<sup>1</sup> The boars in the study were divided into 3 groups of 5 boars and sampled on a 3-day rotation. Data for each day post vaccination represents 5 boars except for frothy saliva (antifoam) on DPV 9 (4 boars), DPV 10 (4 boars), and DPV 13 (4 boars).

<sup>2</sup> Ingelvac® PPRS MLV, Boehringer Ingelheim Vetmedica, Inc., St. Joseph, Missouri USA.

<sup>3</sup> Blood swabs were taken using saturating polyester tipped sterile applicators (25-806 1PD Puritan Medical, Guilford, Maine USA) and then placed in tubes containing 1 ml of sterile saline solution (Aspen Veterinary Resources Ltd., Liberty, Missouri USA).

<sup>4</sup> Frothy saliva collected around the boar's mouth using a sterile sponge pre-hydrated with 10 ml neutralizing buffer (3M™ Hydra-Sponge, St. Paul, Minnesota USA) as they were collected for semen.

<sup>5</sup> Frothy saliva collected into a tube and then sprayed with antifoam (Thomas Scientific, Swedesboro, New Jersey USA).

<sup>a, b, c, d, e, f, g</sup> Within specimen type, means with the same letter are not significantly different (Tukey's Honestly Significantly Different (HSD) test,  $p > 0.05$ ).

**Table 4. Qualitative PRRSV rRT-PCR Results in Boars<sup>1</sup> Following PRRSV Vaccination**

Day post vaccination <sup>2</sup>	PRRSV qRT-PCR (percent positive)							Cochran's Q <i>p</i> -value <sup>6</sup>
	Serum	Blood swab <sup>3</sup>	Oral fluid	Frothy saliva <sup>4</sup>	Frothy saliva <sup>5</sup>	Semen supernatant	Semen cell fraction	
0	0%	0%	0%	0%	No sample	0%	0%	-
1	40%	0%	0%	0%	"	0%	0%	0.075
2	80%	20%	20%	0%*	"	0%*	0%*	0.017
3	100%	20%	60%	0%*	"	0%*	20%	0.004
4	100%	40%	80%	40%	"	0%*	20%	0.010
5	100%	40%	100%	20%	"	0%*	40%	0.004
6	100%	100%	100%	60%	"	20%	40%	0.010
7	100%	100%	100%	60%	"	0%*	0%*	0.001
8	100%	60%	100%	0%*	20%	0%*	0%*	0.001
9	100%	80%	100%	20%	25%	20%	20%	0.007
10	100%	40%	80%	20%	50%	0%*	0%*	0.004
11	80%	20%	100%	0%	60%	0%	0%	0.001
12	100%	40%	100%	20%	40%	0%*	20%	0.005
13	20%	0%	40%	0%	0%	0%	0%	0.152
14	40%	0%	20%	20%	20%	0%	0%	0.352

<sup>1</sup> The 15 boars in the study were divided into 3 groups of 5 boars and sampled on a 3-day rotation. Data for each day post vaccination represents 5 boars except for frothy saliva (antifoam) on DPV 9 (4 boars), DPV 10 (4 boars), and DPV 13 (4 boars).

<sup>2</sup> Ingelvac® PPRS MLV, Boehringer Ingelheim Vetmedica, Inc., St. Joseph, Missouri USA.

<sup>3</sup> Blood swabs were taken using saturating polyester tipped sterile applicators (25-806 1PD Puritan Medical, Guilford, Maine USA) and then placed in tubes containing 1 ml of sterile saline solution (Aspen Veterinary Resources Ltd., Liberty, Missouri USA).

<sup>4</sup> Frothy saliva collected around the boar's mouth using a sterile sponge pre-hydrated with 10 ml neutralizing buffer (3M™ Hydra-Sponge, St. Paul, Minnesota USA) as they were collected for semen.

<sup>5</sup> Frothy saliva collected into a tube and then sprayed with antifoam (Thomas Scientific, Swedesboro, New Jersey USA).

<sup>6</sup> Cochran's Q was used to test for significant differences ( $p < 0.05$ ) among sample types by day post vaccination. Within DPV, (\*) indicates a significant difference in sample type vs. serum results.

**Table 5. Early Detection of PRRSV by PCR in Blood and Oral Fluid Specimens Collected from Boars under Experimental Conditions**

Specimen	Virus isolate	Day post inoculation (no. positive/total tested)							Citation
		1	2	3	4	5	6	7	
Serum	D09-012131	-	-	4/4	-	-	-	22/22	Kittawornrat et al., 2010
	MN 30-100	0/20	12/20	16/20	18/20	17/20	19/20	-	Reicks et al., 2006a.
	MN 30-100	1/18	-	14/18	-	18/18	-	-	Rovira et al., 2007a.
	MN-184	-	-	4/4	-	-	-	24/24	Kittawornrat et al., 2010.
	MNB04	10/10	10/10	11/11	10/10	10/10	10/10	-	Reicks et al., 2006b.
	PRRS MLV	2/5	4/5	5/5	5/5	5/5	5/5	5/5	(current study)
	PRRS MLV	-	-	4/4	-	-	-	24/24	Kittawornrat et al., 2010.
	PRRS MLV	-	3/3	-	-	-	3/3	-	Shin et al., 1997.
	PRRS MLV	-	-	-	4/5	-	-	-	Christopher-Hennings et al., 1997.
	SD-23983	6/6	-	6/6	-	6/6	-	-	Wasilk et al., 2004.
	SD-23983	-	-	-	8/8	-	-	8/8	Christopher-Hennings et al., 2001.
	VR-2332	4/4	-	4/4	-	4/4	-	4/4	Christopher-Hennings et al., 1995a.
	VR-2332	-	3/3	-	-	-	-	3/3	Shin et al., 1997.
VR-2332	-	2/2	-	-	-	-	-	Christopher-Hennings et al., 1997.	
Blood Swab	PRRS MLV	0/5	1/5	1/5	3/5	2/5	5/5	5/5	(current study)
	MNB04	9/10	10/10	11/11	10/10	10/10	10/10	-	Reicks et al., 2006b.
	MN 30-100	1/18	-	15/18	-	17/18	-	-	Rovira et al., 2007a.
Oral Fluid	D09-01213	1/21	22/22	22/22	22/22	20/20	20/20	22/22	Kittawornrat et al., 2010 <sup>1</sup>
	MN-184	0/24	17/24	23/24	21/21	23/23	22/22	23/23	Kittawornrat et al., 2010 <sup>1</sup>
	PRRS MLV	2/24	9/22	21/24	24/24	24/24	24/24	24/24	Kittawornrat et al., 2010 <sup>1</sup>
	PRRS MLV	0/15	1/15	10/15	13/15	15/15	15/15	15/15	(current study)

<sup>1</sup> A. Kittawornrat (personal communication)

**Table 6. Early Detection of PRRSV by PCR in Whole Semen or Supernatant and Cell Fraction Semen Specimens Collected from Boars under Experimental Conditions**

Specimen	Virus isolate	Day post inoculation (no. positive/total tested)							Citation
		1	2	3	4	5	6	7	
Whole Semen or supernatant	MN 30-100	0/20	0/19	0/20	1/19	0/20	1/20	-	Reicks et al., 2006a.
	MNB04	0/10	4/10	6/10	8/10	6/10	8/10	-	Reicks et al., 2006b.
	PRRS MLV	0/5	0/5	0/5	0/5	0/5	1/5	0/5	(current study)
	PRRS MLV	-	0/3	-	-	-	1/3	-	Shin et al., 1997.
	VR-2332	0/4	-	1/4	-	1/4	-	2/4	Christopher-Hennings et al., 1995a.
	VR-2332	-	1/2	-	-	-	-	2/2	Shin et al., 1997.
	VR-2402	-	-	0/2	-	2/2	-	0/2	Christopher-Hennings et al., 1995b.
Cell Fraction Semen	MN 30-100	0/20	0/19	-	1/9	-	4/10	-	Reicks et al., 2006a.
	MN 30-100	0/17	-	0/17	-	2/17	-	-	Rovira et al., 2007a.
	PRRS MLV	0/5	0/5	1/5	1/5	2/5	2/5	0/5	(current study)
	PRRS MLV	-	0/5	-	0/5	-	-	2/5	Christopher-Hennings et al., 1997.
	SD-23983	-	-	-	3/7	-	-	5/7	Christopher-Hennings et al., 2001.
	SD-23983	0/6	-	1/6	-	2/6	-	-	Wasilk et al., 2004.
	VR-2332	0/4	-	1/4	-	0/4	-	1/4	Christopher-Hennings et al., 1995a.
	VR-2332	-	0/2	-	1/2	-	2/2	-	Christopher-Hennings et al., 1997.
VR-2402	-	-	2/2	-	-	-	2/2	Christopher-Hennings et al., 1995b.	



**Table 7. Early Detection of PRRSV by PCR as a Function of Specimen and Day Post Inoculation<sup>1</sup>**

Specimen	Percent (%) positive by day post inoculation						
	1	2	3	4	5	6	7
Serum	36.5	79.1	89.5	93.8	95.2	97.4	99.9
Blood swab	30.3	73.3	79.4	86.7	87.9	99.9	99.9
Oral fluid	3.6	59.0	89.4	97.6	99.9	99.9	99.9
Whole semen or supernatant	0	12.8	17.1	26.5	22.0	28.9	30.8
Cell fraction semen	0	0	14.7	21.4	18.8	47.1	43.5

<sup>1</sup> Probability calculated from the data listed in Tables 5 and 6 using a binomial logistic regression model with estimate values obtained using the least square methods.

## CHAPTER 3

## COLLECTION OF ORAL FLUID FROM INDIVIDUALLY HOUSED SOWS

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## Summary

Oral-fluid sampling was attempted on 513 individually housed, mixed-parity sows. Younger sows ( $P < .01$ ) and re-sampling ( $P < .001$ ) were associated with successful collection. Diagnostic results on samples collected on 2 successive days were correlated. Oral-fluid sampling in breeding herds would facilitate surveillance and animal welfare.

## Introduction

Testing oral-fluid samples by antibody-based assays or polymerase chain reaction- (PCR-) based assays is an effective and efficient method to survey for a variety of infectious agents, including porcine reproductive and respiratory syndrome virus (PRRSV),<sup>1-5</sup> influenza A virus,<sup>6-9</sup> porcine circovirus type 2,<sup>10</sup> and others.<sup>11-13</sup> Oral fluids are commonly collected from pens of animals,<sup>14</sup> but can also be collected from individual animals. Thus, it has been reported that most boars could be trained for oral-fluid collection by providing the boars repeated exposure to the collection process.<sup>1,5</sup>

The premise of this study was that collection of oral fluid on commercial sites of individually housed sows could facilitate breeding-herd surveillance for infectious diseases and improve animal and worker welfare by reducing the need to restrain sows for sample collection. However, to the knowledge of the authors, there is no published data on the

collection of oral-fluid samples from individually housed sows and, likewise, there is little data on the repeatability of test results on successive oral-fluid samples collected from same individual in commercial settings. Therefore, the purpose of this study was not only to evaluate the concept that oral-fluid collection in breeding herds is plausible, but also to provide basic collection parameters in relation to parity, a training effect, and diagnostic repeatability.

### Materials and Methods

The study was conducted with the approval of the Iowa State University Institutional Animal Care and Use Committee.

The study involved 513 individually housed, mixed-parity, gestating sows on two separate commercial farms. No criteria or specifications were used to select animals for participation. The only requirement was that oral fluids had not previously been collected from any of these animals, ie, they were "untrained" for rope collection. Three parameters were of interest: the relationship between sow age (parity) and successful oral-fluid collection, the effect of re-sampling ("training") on collection, and the repeatability of diagnostic test results on two successive oral-fluid samples collected from the same animal. The study was carried out by attempting oral-fluid collection on 2 successive days under the same conditions, ie, ropes were placed at approximately 7:00 am prior to feeding. Oral fluids were collected by hanging a 5/8-inch (1.59-cm) diameter 100% cotton rope at the front of each crate for 30 to 45 minutes. To harvest the oral fluid, the rope was first gathered in a plastic bag and then grasped tightly while pulling the rope from the bag. A volume of  $\geq 1.0$  mL was defined as a successful collection. After sampling was completed, paired oral-fluid

samples (days 1 and 2) from 48 animals were randomly selected by a random number generator based on the sow sequence number from the order in which the ropes were placed of the successfully collected animals. The selected samples were then completely randomized using a random number generator, submitted to the Iowa State University Veterinary Diagnostic Laboratory (ISU-VDL), and tested for PRRSV by real-time reverse transcriptase PCR (RT-PCR) (TetraCore, Inc, Rockville, Maryland) and anti-PRRSV antibodies (HerdChek X3 oral fluid ELISA; Idexx Laboratories, Inc, Westbrook, Maine) using procedures routinely performed in the laboratory. The effect of sow age (parity) and re-sampling (training) on successful oral-fluid collection was analyzed using a logistic regression model,  $\text{logit}(p) = \alpha + \beta_1 \times 1 + \beta_2 \times 2 + \beta_3 \times 1 \times 2$ , where  $p$  = probability of successful oral-fluid collection;  $\alpha$  = intercept;  $\beta_1$  = regression coefficient for day;  $\beta_2$  = regression coefficient for parity; and  $\beta_3$  = regression coefficient for interaction of parity and day (SAS version 9.2; SAS Institute Inc, Cary, North Carolina). In this model, day, parity, and the interaction of parity and day are fixed effects and sow ID is a random effect. This logistic regression model was also used to predict oral-fluid collection success from the collected data. Logistic regression was used in the analysis because the logit link provided the means to evaluate the probability of successful oral-fluid collection (yes or no) in the context of the covariates that could affect this probability. This approach factored in the influence of day, sow parity, the interaction of day and parity, and the random effects of individual animals while accounting for the uneven distribution of sows in each parity level, providing a better prediction of success rates by parity than the raw field data alone. To analyze the diagnostic repeatability of diagnostic test results, a Pearson's correlation coefficient was used. A  $P < .01$  was considered statistically significant.

## Results

Oral fluids were collected on Day 1 from 119 of 513 individually housed sows (23.2%). On Day 2, samples were collected from 245 of the same 513 animals (47.8%). Only four animals that provided a successful collection on Day 1 did not provide a sample on Day 2. Parity was associated with oral-fluid collection (logistic regression,  $P < .01$ ), with lower collection success observed at higher parities (Table 1). The total number of animals from which an oral-fluid sample was collected was significantly higher on Day 2 than on Day 1 (logistic regression,  $P < .001$ ). This increase in response was observed at all parity levels.

Testing showed that all oral-fluid samples ( $n = 96$  from 48 animals) were negative for PRRSV by RT-PCR, but positive for PRRSV antibody by oral-fluid ELISA. Therefore, the analysis of diagnostic repeatability on paired samples (Day 1 versus Day 2) was based only on the sample-to-positive (S:P) ratios of the PRRS ELISA. The analysis of the ELISA S:P ratios (Figure 1) revealed a strong correlation between Day 1 and Day 2 results (Pearson's correlation coefficient = 0.82) and no significant difference between days (paired  $t$  test,  $P > .05$ ).

## Discussion

The routine collection of oral-fluid samples from individually housed boars has been documented in both experimental and field studies.<sup>1,5</sup> In these studies, individual boars were trained for oral-fluid collection by hanging the rope at the front of the pen for 20 minutes daily for 2 or 3 days. Thereafter, most boars were compliant with oral-fluid collection. Although the assurance of PRRSV-free semen requires testing by RT-PCR serum samples or blood swabs from boars at the time of semen collection, oral-fluid sampling from non-donor

boars provides a mechanism for disease monitoring while avoiding the necessity of collecting blood.<sup>1,5</sup> This decreases the frequency of restraining animals for sample collection and increases worker safety.<sup>1,15</sup>

Although this is a "proof of concept" study, the findings suggested that the behavior seen in boars also applies to individually housed sows in commercial herds. In particular, repeated exposure of sows to the rope produced a measurable training effect regardless of animal age. It was also observed that younger females were more likely to interact with the rope, which is supported by both the observed and the statistically predicted oral-fluid successful collection rates. This suggests the possibility of training animals prior to entry into the breeding herd during isolation or quarantine. Of course, the advantages of oral-fluid collection in boars also apply to sow herds for more consistent and safer disease monitoring.

Accurate surveillance depends on the repeatability and reproducibility of the diagnostic assays used. In this study, quantitative analysis of testing results showed a strong correlation (Pearson's correlation coefficient of 0.82) between samples collected from the same individuals on 2 consecutive days. This further increases confidence in the process of surveillance in sows using oral-fluid samples.

These baseline results suggest that oral-fluid samples can be collected from individually housed sows, but that further studies on the optimization of oral-fluid collection in the sow unit (gestation and farrowing) would be of value. Potential future studies include further evaluation of training methods and an assessment of the duration of the training effect. Regardless of the approach, more extensive surveillance of the sow herd will be necessary if we are to achieve control of agents such as PRRSV and porcine epidemic diarrhea virus.

### Implications

- Oral-fluid collection is most likely to be successful in younger sows.
- Regardless of age, improved collection success on re-sampling suggests that sows could be trained for oral-fluid collection, eg, during quarantine.
- The strong correlation ( $r = 0.82$ ) observed between PRRS oral fluid antibody test results on different samples from the same animal strengthens the validity of oral-fluid testing.
- The use of oral fluids for monitoring PRRSV in breeding herds is plausible and could improve the current level of surveillance in most breeding herds by facilitating sample collection from animals and reducing the need to collect blood samples.

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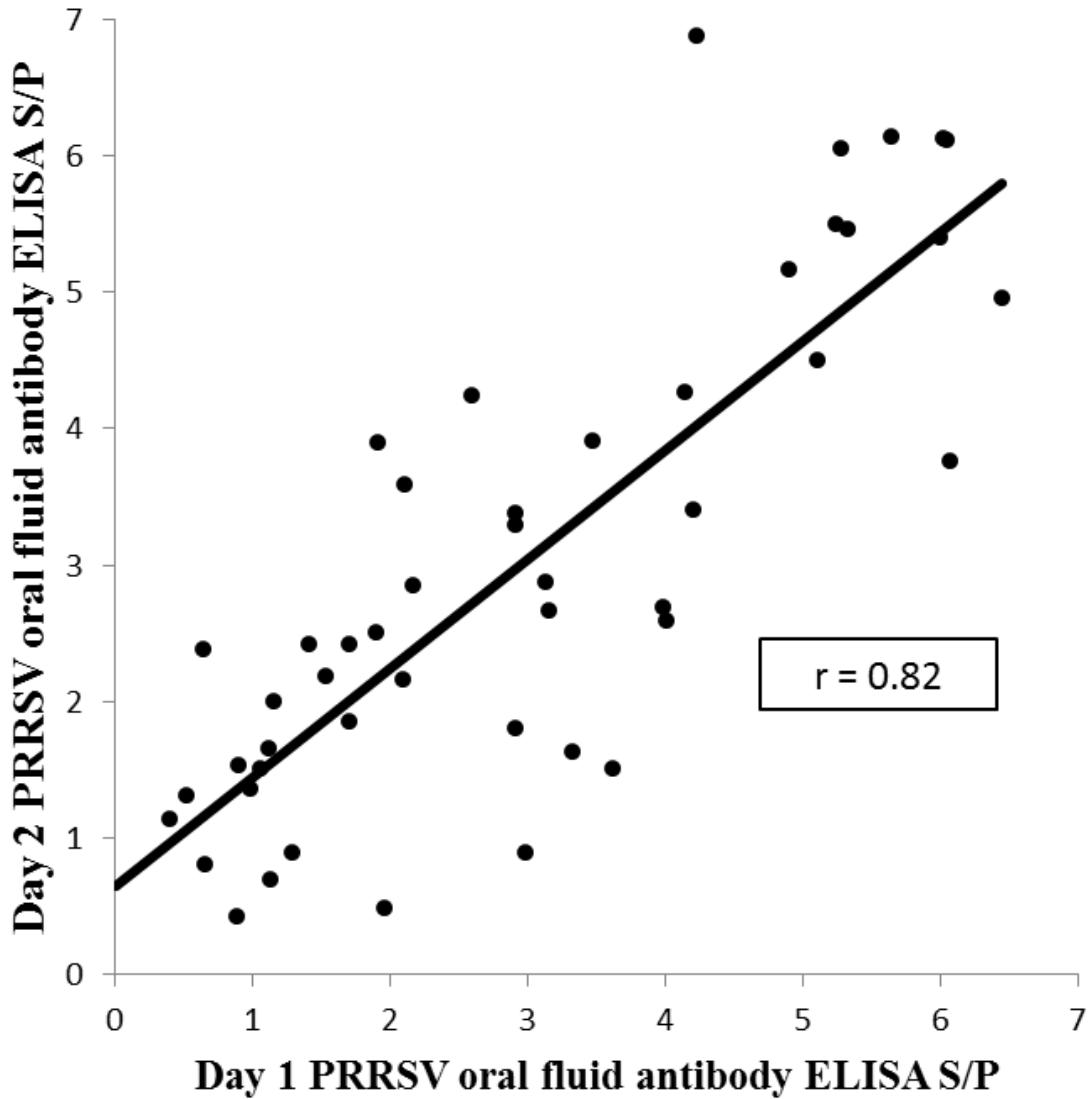
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**Figure 1.** The random selection of 48 sows from the study participants that provided consecutive oral-fluid samples for the 2 days of the study showed a strong correlation (Pearson correlation,  $r = 0.82$ ) in porcine reproductive and respiratory virus (PRRSV) antibody enzyme-linked immunosorbent assay (ELISA) sample-to-positive (S:P) values with repeat testing on the same individual animals. Each data point represents the S:P response from one animal on day 1 and day 2 of the study.

**Table 1. Percent Success of Oral Fluid Collection from Individual Sows in Individual Housing by Parity and by Day 1 and Day 2 of Collection**

Parity‡	Sowsα	Actual collection data (% successful collection)*		Predicted oral fluid collection† (% successful collection)	
		Day 1	Day 2§	Day 1	Day 2
0	41	14.6	36.6	29.5	61.8
1	89	34.8	67.4	25.1	57.2
2	94	25.5	50.0	21.3	52.4
3	71	33.8	56.3	17.8	47.5
4	72	16.7	47.2	14.9	42.8
≥ 5	146	15.1	33.6	12.3	38.1

\* Success was defined as being able to collect an oral-fluid volume  $\geq 1.0$  mL.

† Predicted oral-fluid collection success was based on analysis of the field collection data using a logistic regression model,  $(\text{logit}(p) = \alpha + \beta_1 \times_1 + \beta_2 \times_2 + \beta_3 \times_1 \times_2)$ , where  $P$  = probability of successful oral-fluid collection;  $\alpha$  = intercept;  $\beta_1$  = regression coefficient for day;  $\beta_2$  = regression coefficient for parity, and  $\beta_3$  = regression coefficient for interaction of parity and day.

‡ Parity was significantly associated with sampling success (logistic regression,  $P < .01$ ).

§ Collection rate on Day 2 was significantly higher than on Day 1 (logistic regression,  $P < .001$ ).

α Sows were individually housed in conventional gestational confinement and oral fluid samples were collected on an individual animal basis. Cotton ropes for oral fluid collection were hung directly in front of each sow from the front of their individual confinements. Each sow in the study was positioned next to another animal in the study. Each individual had their own feeder and watering system.

## GENERAL CONCLUSIONS

Due to current large scale production standards, the presence of pig dense areas, and the constant movement of people and animals worldwide, the potential for disease transmission is high. To minimize disease impact, timely and accurate detection is required. Disease surveillance is vital for early detection and intervention to mitigate the effect swine pathogens. Research in the area of swine disease surveillance is required to continuously improve and expand our knowledgebase and methods to ensure health of our swine populations.

In spite of the gap in literature, pooling is used regularly for diagnostic testing and pathogen surveillance. Sample pooling was developed to save on testing costs by allowing more individuals animals to be represented in a single test. Though, potentially financially beneficial, many pooling downfalls need to be accounted for to provide accurate diagnostic interpretations. From studies on swine pathogens, along with pathogens of other species, we are aware of the loss in sensitivity pooling creates when done improperly.

Sample pooling is dependent on the ability to collect and test individually collected samples. For routine surveillance methods, sample collection needs to be easy to accomplish and be diagnostically accurate. In boar stud populations, where animals are commonly housed individually, disease surveillance methods are important to keep sow farms safe from pathogens that can be spread in semen. Due to individual housing, disease detection in the boar stud is dependent on individual sample collection. Though serum in the first study of this project proved the best early detection, boars trained for oral fluid provides a simple way

to get diagnostic samples and allows the sampling of animals not being collected for semen that day.

To the knowledge of the authors, the methods of training individual animals for oral fluid rope collection was not previously documented in individually housed sows prior to the second research study of this project. This study documented the feasibility of applying oral fluid collection to commercial sow herds using the training methods utilized in boar studs. The potential ease in sample collection that oral fluid samples can provide to breeding herds could increase current disease detection and surveillance practicality.

Overall, the importance of early disease detection and surveillance methods in the swine industry justifies the need for more research for proper application.