Surveillance in contemporary swine herds - the case of porcine

reproductive and respiratory syndrome virus (PRRSV)

by

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DEDICATION

Dedicated to my mother,

Pongsri Sae-Kuai

Who has continually challenged me while providing unconditional love.

Dedicated to my wife, Yaowalak Panyasing,

Who gave me support and inspiration.

Dedicated to my advisor

Professor Jeffrey J. Zimmerman

Who gave me a chance to pursue a PhD in the field of swine ecology and epidemiology.

TABLE OF CONTENTS

ABSTRACT

Surveillance is the primary means to generate information on the presence and/or distribution of pathogens in animal populations. Fulfillment of this function is ultimately dependent on the program's ability to collect, test, aggregate, and analyze data from individual samples. The problem addressed in this dissertation is whether oral fluid specimens could be used to surveillance and monitor individually-based and pen-based for porcine reproductive and respiratory syndrome virus (PRRSV) infection. In particular, but not exclusively, the aim of this research was to evaluate the oral fluid emzyme-linked immunosorbent assay (ELISA) could be used to detect anti-PRRSV antibody isotypes. This problem was addressed in the logical series of experiments described below.

The objective of the Chapter 3 was to evaluate a commercial serum antibody ELISA performance that was modified to detect anti-PRRSV antibodies in pen-based oral fluid specimens. Experimental and field oral fluid samples from defined PRRSV infection status were used to derive the kinetics of detectable concentrations of antibody against PRRSV. IgM and IgA were readily detected in oral fluid specimens from populations in which PRRSV infection was synchronized among all individuals, but not in samples collected in commecial herds. In contrast, IgG was readily detected at diagnostically useful levels in both experimental and field samples for up to 126 days (duration of the study). Estimates of the IgG oral fluid ELISA performance were based on results from testing positive oral fluid samples (n = 492) from experimentally inoculated pigs (n = 251) and field samples (n = 241), and negative oral fluid samples (n = 367) from experimentally inoculated pigs (n = 84) and field samples (n = 283). ROC analysis estimated the diagnostic sensitivity and specificity of the assay as 94.7% (95% CI: 92.4, 96.5) and 100% (95% CI: 99.0, 100.0), respectively, at a sample:positive (S/P) ratio cutoff of ≥ 0.40. The results of this study suggest that the IgG oral fluid ELISA can provide efficient, cost-effective PRRSV monitoring in commercial herds and PRRSV surveillance in elimination programs.

Chapter 4 addressed the repeatability and reproducibility of oral fluid antibody ELISA. The precision of a PRRSV oral fluid antibody ELISA was evaluated by calculating reliability

coefficients for assay repeatability (within laboratory) and assay reproducibility (between laboratories). Randomly ordered oral fluid samples from known ($n = 39$) and unknown ($n = 224$) PRRSV antibody status were tested in 12 diagnostic laboratories. Each laboratory tested the samples twice, first using an antibody ELISA kit and reagents provided to them (Phase 1) and then using an ELISA kit and reagents configured in their laboratory (Phase 2). Repeatability (within laboratory) reliability coefficients calculated using results from samples from known PRRSV antibody status ranged from 0.724 to 0.997 in Phase 1 and 0.953 to 0.998 in Phase 2. Reproducibility (between laboratories) reliability coefficients were calculated for three conditions: Case 1 - samples of unknown status ($n = 224$); Case 2 - samples from known status ($n = 39$), and Case 3 - all samples ($n = 263$). Among the three cases, reliability coefficients ranged from 0.937 to 0.964 in Phase 1 and 0.922 to 0.935 in Phase 2. For Case 3, it was estimated that 96.7% of the total variation in Phase 1 and 93.2% in Phase 2 could be attributed to the oral fluid samples themselves. Overall, the PRRSV oral fluid antibody ELISA was highly repeatable and reproducible. This study supported the routine use of this test in laboratories providing diagnostic service to pig producers.

The study objective outlined in Chapter 5 was to describe and contrast the kinetics of the humoral response in serum and oral fluid specimens during acute PRRSV infection. The study was done in three trials with 24 boars used in each trial. Boars were intramuscularly (IM) inoculated with a commercial modified live virus (MLV) vaccine (Trial 1), a Type 1 PRRSV field isolate (Trial 2), or a PRRSV Type 2 field isolate (Trial 3). Oral fluid samples were collected from individual boars on day post inoculation (DPI) -7 and daily for 21 DPI. Serum samples were collected from all boars on DPI -7, 0, 7, 14, 21 and from 4 randomly selected boars on DPIs 3, 5, 10, and 17. Thereafter, serum and oral fluid were assayed for PRRSV antibody using antibody isotype-specific ELISAs (IgM, IgA, IgG) adapted to serum or oral fluid. Statistically significant differences in viral replication and antibody responses were observed among the three trials in both serum and oral fluid specimens. Anti-PRRSV antibody isotype responses in serum (IgM, IgA, and IgG) were first detected in samples collected on DPI 7, 10, and 10, respectively. Oral fluid IgM, IgA, and IgG were detected in samples collected between DPI 3 to 10, 7 to 10, and 8 to 14, respectively. This study

enhanced the scientific knowledge concerning the pig's the humoral immune response to PRRSV and provided a broader foundation for understanding, interpreting, and developing oral fluid antibody-based diagnostics.

The objective for the study described in Chapter 6 was to assess oral fluid samples use when collected from piglets prior to weaning as a method to monitor sow herd PRRSV antibody and infection status. Samples originated from four ~12,500 sow herds. All four herds were considered endemically infected with PRRSV based on historic diagnostic data. Oral fluid samples were collected from 600 litters prior to weaning and serum samples from their dams after weaning. All samples were randomized and tested for PRRSV antibodies (IgM, IgA, IgG and PRRS X3 Ab Test or PRRS Oral Fluids Ab Test) and PRRSV (RT-qPCR and sequencing). Results were analyzed for associations with sow parity, litter size, and farm by analysis of variance (ANOVA) and correlation analyses. No sow serum samples were positive for PRRSV by RT-qPCR, but 9 of 600 oral fluid samples were confirmed positive by PRRSV RT-qPCR testing at two laboratories. Two were successfully sequenced (ORF5) and identified as wild-type PRRSV. This study showed that piglet oral fluid samples are a useful and sensitive approach for monitoring PRRSV circulation in endemically infected and/or vaccinated herds, as well as for monitoring the PRRSV status in PRRSV negative herds.

CHAPTER 1. INTRODUCTION: DISSERTATION ORGANIZATION

This dissertation consists of 7 chapters. The first chapter is the dissertation organization. Chapter 2 is the general introduction and review of the literature, "Disease surveillance and monitoring systems – challenges to implementing surveillance in contemporary production systems". Chapter 3, "Detection of porcine reproductive and respiratory syndrome virus (PRRSV) antibodies in oral fluid specimens using a commercial PRRSV serum antibody ELISA" was published in the Journal of Veterinary Diagnostic Investigation. Chapter 4, "Ring test evaluation of the repeatability and reproducibility of a porcine reproductive and respiratory syndrome virus (PRRSV) oral fluid antibody enzyme-linked immunosorbent assay" was published in the Journal of Veterinary Diagnostic Investigation. Chapter 5, "Kinetics of the porcine reproductive and respiratory syndrome virus (PRRSV) humoral immune response in swine serum and oral fluids collected from individual boars" was publisehd in BMC Veterinary Research. Chapter 6, "Porcine reproductive and respiratory syndrome virus (PRRSV) surveillance using pre-weaning oral fluid samples detects circulation of wild-type PRRSV" has been submitted for publication in Veterinary Microbiology. References, tables, and the figures associated with each chapter follow the discussion section. The final chapter contains the General Conclusions of the dissertation.

CHAPTER 2. DISEASE SURVEILLANCE AND MONITORING SYSTEMS - CHALLENGES TO IMPLEMENTING SURVEILLANCE IN CONTEMPORARY PRODUCTION SYSTEMS

Apisit Kittawornrat

SUMMARY

Surveillance is the primary means for generating information on the presence and/or distribution of pathogens in animal populations (Hadorn and Stärk, 2008). Fulfilling this function is ultimately depends on the program's ability to collect, test, aggregate, and analyze data from individual samples. Herein the background, requirements, and challenges associated with surveillance sampling from swine populations in field production situations are reviewed.

INTRODUCTION

According to Thrusfield (2005), "monitoring" consists of routine observations on health, productivity, and other characteristics related to them in a population, whereas "surveillance" is a more intensive form of data collection than monitoring. Thrusfield (2005) identified three components in disease surveillance: (1) gathering, recording, and analysis of data, (2) communication of information, and (3) response(s) to actionable thresholds, e.g., intervention(s) for the purpose of disease control. Although monitoring and surveillance are often used interchangeably in common language, the third component (response) differentiates monitoring from surveillance. For simplicity, monitoring and surveillance systems are increasingly referred to as "MOSS" (Salman, 2003).

The first task in designing a MOSS for infectious diseases is to precisely define its objective, e.g., detection, prevalence estimation, process control, etc. The second task is to define the population to be surveilled, e.g., barn, site, system, or region. Thereafter, the design is built

on this foundation: the type of specimen, the collection method, the number of samples, the diagnostic test(s) to be used to analyze the samples, and the methods to be used to analyze the data. For surveillance, actionable thresholds and specific responses must also be defined.

BASIC SAMPLING PRINCIPLES

The idea that a subsample of a population could provide valid estimates of an entire population is relatively new. As reviewed by Kruskal and Mosteller (1980), A.N. Kiaer, Director of the Norwegian Central Bureau of Statistics, proposed the idea of 'representative sampling' at the Berne meeting of the International Statistical Institute in 1895 (Kruskal and Mosteller, 1980). Kiaer believed that national social and economic data could be obtained by studying a subset rather than the entire population. The concept was not well received by the experts of the time, but nevertheless continued to move forward. In 1912, L. von Bortkiewicz introduced a significance test to evaluate the representativeness of the sample subset to the population as a whole. Around the same time, L. March added the concept of probability sampling to the discussion, but nonprobability sampling (purposive methods) remained the norm for a time. As reviewed by Frankel and Frankel (1987), probability sampling began in earnest with a 1934 publication on the topic by J. Neyman and was cemented firmly in place by a large-scale, probability-based study on unemployment in the United States conducted by the Work Projects Administration in 1939.

MOSS sampling methods are either probability or nonprobability based. Nonprobability sampling occurs when some individuals in the population have no chance of selection. Nonprobability sampling methods include convenience sampling, volunteer sampling, judgment sampling, or quota sampling (Dahoo et al, 2009). Nonprobability sampling methods offer simplicity, expediency and low cost, but are susceptible to both random and systematic bias and may not provide data amenable to valid statistical analyses.

Probability sampling requires that every individual in the population have a defined, non-zero chance of being selected and that random selection is conducted at some point in the process.

Probability sampling methods include simple random sampling, systematic sampling, stratified random sampling, cluster sampling, and multistage sampling. Since units from the population are randomly selected and each unit's probability of inclusion can be calculated, statistically valid estimates and inferences can be made about the population. However, probability sampling is usually more complex, time-consuming and expensive than non-probability sampling. As described by Cochran (1977), probability sampling procedures must conform to key statistical principles:

1.   The set of individuals of which the population is composed can be precisely identified and all individuals have an equal probability of selection.
2.   Every individual within the population has a known probability of selection.
3.   Sample selection is conducted by a random process.

In swine populations, sampling the entire population of interest is necessary and practical in some situations, e.g., testing the entire group of replacements due to enter an expected-negative sow farm or boar stud. Pragmatically, sampling an entire swine population is generally impossible and, therefore, it is necessary to determine the appropriate number of individuals to sample. Although there are several approaches for the calculation of sample size, four factors are paramount: (1) the purpose of sampling, e.g., to detect positive animals in an expected negative herd or to estimate herd level prevalence, (2) the desired accuracy and confidence level of test estimates, (3) the cost of collecting and testing samples, and (4) the sensitivity and specificity of the diagnostic test (Chase and Polson, 2000). As an example, the following formula can be used to approximate the sample size required to detect at least one positive animal in an infinite population (Cannon and Roe, 1982):

$$n = \frac{\log(1-c)}{\log(1-p)}$$
Equation 1

where n is the sample size, c is the confidence level, and p is the expected within-herd prevalence. Estimates based on Equation 1 have published in tables, are available in a

variety of software and freeware programs, and have been widely used (Dufresne et al., 2003). Notable, Equation 1 is based the assumption that the diagnostic test has perfect sensitivity (Se) and specificity (Sp). Equation 2 takes actual test performance into consideration when calculating sample size for an infinite population (Martin et al., 1992):

$$n = \frac{\log(1-c)}{\{\log[\text{Sp}(1-p)-(1-Se)p]\}} \qquad \text{Equation 2}$$

where Se and Sp are diagnostic sensitivity and specificity, respectively. Software tools have also been developed to calculate sample size for finite populations and diagnostic tests with imperfect sensitivity and specificity, e.g., FreeCalc® (Cameron and Baldock, 1998).

PROBABILITY SAMPLING IN SWINE POPULATIONS

Swine farms differ from other populations, especially human populations, in three important ways: (1) the rate of population change on farms, (2) the extensive movement of populations between farms, and (3) the physical segregation of groups of animals within the same farm population by age and/or production stage or function. These differences are rarely (if ever) accounted for in current MOSS designs, but have important implications for the accuracy and timeliness of estimates.

Population change consists of "natural change" (total births minus total deaths) plus migration into or out of the population. Population change in humans typically represents a small proportion of the population. For example, the state of Nebraska recorded a population change of +8.4% for the period 1990 to 2000 (Deichert, 2001). This consisted of a natural change of +5.4% and net migration of +3.1%. In contrast, a finishing barn on a typical farm will experience ~250% population change annually as groups of animals enter into the facility, grow, and are sent to market. Extensive movement of animals between sites is part of this process. In particular, pigs are moved from Canadian or US breeding herds into finishing operations located in the Midwestern Corn Belt. As reviewed by Goodell et al. (2013), nearly 40,000,000 live swine were moved across state lines in 2011. Although more

stable than growing pig populations, sow herds are not excluded from rapid population change. Typically, ~40% of females in sow herd populations are replaced annually (PigChamp® Benchmark, 2011).

Thus, in contrast to human populations, swine populations experience extremely high turnover. This has important implications for herd immunity, the ability of pathogens to circulate within them, and the design of sampling protocols that can capture this dynamic. These conditions dictate that MOSS sampling be repeated routinely, if the expectation is accurate and relevant data. This assessment is supported by modeling used to simulate the effect of sampling frequency on the detection of infections in swine populations. Polson and Jordan (2002) developed a model to compare the detection of porcine reproductive and respiratory syndrome virus (PRRSV) infection at three prevalence levels (3%, 5%, and 10%) using 4 combinations of sample size and sampling frequency: 60 samples 4 times per year, 40 samples 6 times per year, 30 samples 8 times per year, and 20 samples 12 times per year. Their results showed that smaller sample size at higher sampling frequency was more likely to result in detection. Rovira et al. (2007) developed a stochastic model to compare the detection of PRRSV in boar studs as a function of sample size (10, 30, and 60), sampling frequency (3 times per week, weekly, and biweekly), and diagnostic procedure (polymerase chain reaction (PCR) testing of semen or serum, antibody ELISA testing of serum, or both PCR and ELISA testing of serum). They found that PCR testing of serum detected PRRSV infection the earliest. Specifically, testing serum samples from 60 boars 3 times per week by PCR resulted in the detection of 95% of the PRRSV introductions within 13 days. In general terms, Thurmond (2003) recommends that sampling intervals should be shorter for pathogens with high effective reproductive rates, for cases in which the sensitivity of the test is low, and/or when the costs associated with failure to detect the disease are significant. However, unlike sample size, there is no published formula for calculating the optimum sampling frequency. Therefore, the decision is usually bounded on one side by the urgency of the need for the information and by cost constraints on the other (Roberts, 2003).

A separate problem in implementing probability sampling on the farm is compliance with the

requirement that animals sampled from the population are randomly selected. In general, farms consist of separate buildings in which animals are segregated by age and/or production stage into rooms and pens. Like sampling frequency, there is no guidance concerning the allocation of samples across these physical strata. Therefore, despite the fact that strata may not be homogenous in terms of disease status, it is common for samples to be selected by convenience and without formal guidelines. Most often sampling is conducted under the unproven assumption that certain strata are most representative of the farm's disease status.

SEEKING PRACTICAL SOLUTIONS

Although the importance of conducting MOSS in compliance with valid statistical principles is rightfully stressed (Salman, 2003), non-statistical considerations, e.g., the availability of personnel and the cost of collecting, testing, and analyzing data, set concrete limits to the procedures that can be implemented on the farm. For this reason, there is a need for approaches that can achieve valid MOSS while satisfying the limits dictated by money and time. Recent work in swine populations has focused on methods of handling specimens that reduce costs while maintaining testing performance. Two examples of this approach are pooling of serum samples from individual pigs and the collection of oral fluid specimens from individuals or groups.

A pooled sample, also known as a "composite sample" in some disciplines, is created by combining equal volumes of two or more sample units into one aliquot for testing (Cameron et al., 2003). The goal of pooling is to detect the presence of a target at a lower cost than testing individual samples (Lovison et al., 1994). This is done in a 2-step process termed the "Dorfman procedure" whereby pooled samples are first tested for the presence of the target. If the pool tests negative, the individual specimens of which the pool is composed are considered negative. If the pool tests positive, the specimens of which the pool is composed are tested individually to determine their status. (Retesting positive pools results is not necessary if determination of population status (positive/negative) is the primary outcome of interest.) The Dorfman procedure was used by the United States Public Health Services during World War II to screen military inductees for syphilis (Dorfman, 1943). Individual

blood samples were drawn, pooled in groups of five, and tested the presence or absence of "syphilitic antigen." When a pool tested positive, each sample in the pool was tested individually to identify which of the members were infected.

Pooling continues to be used extensively in disease detection in swine and other species (Emmanuel et al., 1988; Kline et al., 1989; Maherchandani et al., 2004; Muñoz-Zanzi et al., 2000; Peeling et al., 1998), as well as in human genetics testing (Chowdari et al., 2007), because it offers the advantage of reduced testing costs and faster laboratory turnaround time as a result of the fact that fewer total assays are performed. A core issue for pooling is the fact that the process of combining one or more positive samples with negative samples invariably results in a lower concentration of the analyte (Cameron et al., 2003, Muñoz-Zanzi et al., 2006). The number of individuals that can be pooled while preserving adequate detection sensitivity varies by assay, specimen, and pathogen/analyte characteristics (Cahoon-Young et al., 1989; Dorfman 1943; Kline et al., 1989). Commonly, pool size varies between 5 and 20 samples.

In swine diagnostics, sample identity (animal identification, physical location, sampling date) are typically lost in the process of pooling. This may not impact the utility of the results for producers who are only interested in whether the analyte of interest is present in the population, but further statistical analyses or epidemiological interpretations are often limited or impossible because of the loss of sample unit identification. Therefore, MOSS that rely on testing pooled samples need to accommodate this potential limitation in their design.

The use of pen-based oral fluid specimens in swine medicine is another approach designed to facilitate cost-effective MOSS. "Oral fluid," a combination of serum transudate and saliva, is the liquid collected by placing an absorptive device in the mouth. As reviewed by Prickett and Zimmerman (2010), a variety of pathogens and pathogen-specific antibodies are present in swine oral fluid, e.g., classical swine fever virus (Corthier, 1976; Corthier and Aynaud, 1977), African swine fever virus (Mur et al., 2013), porcine circovirus type 2 (Prickett et al., 2011), PRRSV (Prickett et al., 2008a,b; Kittawornrat et al., 2012), swine influenza virus

(Panyasing et al., 2012), transmissible gastroenteritis virus (DeBuysscher and Berman, 1980), *Actinobacillus pleuropneumoniae* (Loftager et al., 1993), *Escherichia coli* (De Buysscher and Dubois, 1978), and others. In particular, extensive research has been conducted on the use of oral fluids for PRRSV diagnostics. The presence of PRRSV in oral fluids was first reported in 1997 (Wills et al., 1997). In 2008, Prickett and colleagues reported the detection of PRRSV nucleic acid and antibodies in oral fluid specimens. Subsequent research has corroborated and expanded upon these initial observations (Kittawornrat et al., 2010, 2012a,b).

In sampling terminology, an oral fluid sample is best described as a "grab sample" from a sample unit (pen). In this context, "grab sample" is defined as a sample collected "at a specific point in time and at a specific location" (Koerner, 1996) and "sample unit" is the smallest division of the population and the level at which sample size calculations and random sampling procedures are performed (Dohoo et al., 2009; Steele and Torrie, 1980; Toma et al., 1999). Examples of sample units given by Toma et al. (1999) include, "an animal, a group of animals, i.e., a pen, a herd …." Recently, Olsen et al. (2013) reported that pen-based oral fluid samples greatly improved detection over single-animal testing, e.g., among 100 oral fluid samples from pens with PRRSV prevalence between 4 to 36%, 62% were positive for PRRSV RNA and 61% for PRRSV antibody.

CONCLUSIONS

Cost-effective methods of MOSS are needed to support animal health and business decisions at the herd level, improve the quality of field research, and provide timely information on endemic and foreign animal diseases in the national swine herd. The primary long-standing roadblock to this goal is the inconvenience and cost of acquiring the information, i.e., the expense of collecting and testing statistically appropriate numbers of blood, feces, or nasal swab specimens from individual pigs. A cost-effective solution to the current health information vacuum is vital. This will require (1) efficient, low-cost specimen collection and (2) accurate diagnostic assays. In particular, we postulate that a surveillance system for swine infectious diseases based on oral fluid specimens can be developed to achieve the goal

of a comprehensive and integrated swine health surveillance system.

REFERENCES

Cahoon-Young B, Chandler A, Livermore T, Gaudino J, Benjamin R. 1989. Sensitivity and specificity of pooled versus individual sera in a human immunodeficiency virus antibody prevalence study. J Clin Microbiol 27, 1893-1895.

Cameron AR, Baldock FC. 1998. A new probability formula for surveys to substantiate freedom from disease. Prev Vet Med 34, 1-17.

Cameron A, Gardner I, Doherr MG, Wagner B. 2003. Sampling considerations in surveys and monitoring and surveillance systems. In: Salman MD. (Ed.), Animal disease surveillance and survey systems: methods and applications. Blackwell publishing, Ames, Iowa, pp. 47-66.

Cannon RM, Roe RT. 1982. Livestock disease surveys: a field manual for veterinarians. Australian Government Pub. Service, Canberra, Australia.

Chase C, Polson D. 2000. Sampling the herd: When is enough? Proceedings of the American Association of Swine Practitioners, pp. 465-478.

Chowdari KV, Northup A, Pless L, Wood J, Joo YH, Mirnics K, Lewis DA, Levitt PR, Bacanu SA, Nimgaonkar VL. 2007. DNA pooling: a comprehensive, multi-stage association analysis of ACSL6 and SIRT5 polymorphisms in schizophrenia. Genes Brain Behav 6, 229-239.

Cochran WG. 1977. Sampling techniques In: Cochran WG. (Ed.) Wiley, New York.

Corthier G. 1976. Swine fever: influence of passive immunity on pig immune response following vaccination with a live virus vaccine (Thiverval strain). Ann Rech Vet 7, 361-372.

Corthier G, Aynaud J. 1977. Comparison of the immune response in serum and bucco-pharyngeal secretions following immunization by different routes with a live hog cholera virus vaccine (Thiverval strain). Ann Vet Res 8, 159–165.

De Buysscher E, Dubois R. 1978. Detection of IgA anti-Escherichia coli plasma cells in the intestine and salivary glands of pigs orally and locally infected with *E. coli*. Adv Exp Med Biol 107, 593–600.

DeBuysscher E, Berman D. 1980. Secretory immune response in intestinal mucosa and salivary gland after experimental infection of pigs with transmissible gastroenteritis virus. Am J Vet Res 41, 1214–1220.

Deichert J. 2001. Components of population change, Nebraska Counties, 1990-2000. Focus: Nebraska 1(1), 1-10. Center for Public Affairs Research, University of Nebraska at Omaha.

Dohoo I, Martin W, Stryhn H. 2009. Sampling. In: Veterinary Epidemiologic Research (2nd ed.). VER, Inc., Charlottetown, Prince Edward Island, Canada, pp. 33-56.

Dorfman R. 1943. The detection of defective members of large populations. Annals Math Stat 14, 436-440.

Dufresne L, Polson DD, Holck JT, Robert, J. 2003. Serological monitoring in negative and low prevalence populations. In: Zimmerman, JJ, Yoon KJ (Eds.), PRRS Compendium. National Pork Board, Des Moines, Iowa, pp. 87-102.

Emmanuel JC, Bassett MT, Smith HJ, Jacobs JA. 1988. Pooling of sera for human immunodeficiency virus (HIV) testing: An economical method for use in developing countries. J Clin Pathol 41, 582-585.

Frankel MR, Frankel LR. 1987. Fifty years of survey sampling in the United States. Public Opin Q 51, S127-S138.

Goodell C, Wang C, Main R, Roth J, Sundberg P, Wagstrom L, Webb P, Zimmerman J. 2013. Protecting our future – a road map for practical, real-time, on-farm infectious disease surveillance. J Swine Health Prod (in press).

Hadorn DC, Stärk DC. 2008. Evaluation and optimization of surveillance systems for rare and emerging infectious diseases. Vet Res 39, 57.

Kittawornrat A, Prickett J, Chittick W, Wang C, Engle M, Johnson J, Patnayak D, Schwartz T, Whitney D, Olsen C, Schwartz K, Zimmerman J. 2010. Porcine reproductive and respiratory syndrome virus (PRRSV) in serum and oral fluid samples from individual boars: will oral fluid replace serum for PRRSV surveillance? Virus Res 154, 170-176.

Kittawornrat A, Prickett J, Wang C, Olsen C, Irwin C, Panyasing Y, Ballagi A, Rice A, Main R, Johnson J, Rademacher C, Hoogland M, Rowland R, Zimmerman J. 2012. Detection of Porcine reproductive and respiratory syndrome virus (PRRSV) antibodies

in oral fluid specimens using a commercial PRRSV serum antibody enzyme-linked immunosorbent assay. J Vet Diagn Invest 24, 262-269.

Kittawornrat A, Wang C, Anderson G, Ballagi A, Broes A, Carman S, Doolittle K, Galeota J, Johnson J, Lizano S, Nelson E, Patnayak D, Pogranichniy R, Rice A, Scherba G, Zimmerman J. 2012. Ring test evaluation of the repeatability and reproducibility of a Porcine reproductive and respiratory syndrome virus oral fluid antibody enzyme-linked immunosorbent assay. J Vet Diagn Invest 24, 1057-1063.

Kline RL, Brothers TA, Brookmeyer R, Zeger S, Quinn TC. 1989. Evaluation of human immunodeficiency virus seroprevalence in population surveys using pooled sera. J Clin Microbiol 27, 1449-1452.

Koerner CE. 1996. Effect of equipment on sample representativeness. In: Keith LH. (Ed.), Principles of Environmental Sampling (2nd ed.), American Chemical Society, Washington D.C., pp. 155-168.

Kruskal W, Mosteller F. 1980. Representative sampling, IV: The history of the concept in statistics, 1895-1939. Int Stat Rev 48, 169-195.

Loftager M, Eriksen L, Nielsen R. 1993. Antibodies against *Actinobacillus pleuropneumoniae* serotype 2 in mucosal secretions and sera of infected pigs as demonstrated by an enzyme-linked immunosorbent assay. Res Vet Sci 54, 57–62.

Lovison G, Gore SD, Patil GP. 1994. Design and analysis of composite sampling procedures: A review. In: Patil GP, Rao CR. (Eds), Handbook of Statistics Volume 12: Environmental Statistics. Elsevier Science B.V., Amsterdam, The Netherlands, pp. 103-166.

Maherchandani S, Munoz-Zanzi CA, Patnayak DP, Malik YS, Goyal SM. 2004. The effect of pooling sera on the detection of avian pneumovirus antibodies using an enzyme-linked immunosorbent assay test. J Vet Diagn Invest 16, 497-502.

Martin SW, Shoukri M, Thorburn MA. 1992. Evaluating the health status of herds based on tests applied to individuals. Prev Vet Med 14, 33-43.

Munoz-Zanzi C, Thurmond M, Hietala S, Johnson W. 2006. Factors affecting sensitivity and specificity of pooled-sample testing for diagnosis of low prevalence infections. Prev Vet Med 74, 309-322.

Munoz-Zanzi CA, Johnson WO, Thurmond MC, Hietala SK. 2000. Pooled-sample testing as a herd-screening tool for detection of bovine viral diarrhea virus persistently infected cattle. J Vet Diagn Invest 12, 195-203.

Mur L, Gallardo C, Soler A, Zimmermman J, Pelayo V, Nieto R, Sanchez-Vizcaino JM, Arias M. 2013. Potential use of oral fluid samples for serological diagnosis of African swine fever. Vet Microbiol 165, 135-139.

Olsen C, Wang C, Christopher-Hennings J, Doolittle K, Harmon KM, Abate S, Kittawornrat A, Lizano S, Main R, Nelson EA, Otterson T, Panyasing Y, Rademacher C, Rauh R, Shah R, Zimmerman J. 2013. Probability of detecting porcine reproductive and respiratory syndrome virus infection using pen-based swine oral fluid specimens as a function of within-pen prevalence. J Vet Diagn Invest 25, 328-335.

Panyasing Y, Goodell CK, Wang C, Kittawornrat A, Prickett JR, Schwartz KJ, Ballagi A, Lizano S, Zimmerman JJ. 2012. Detection of influenza A Virus nucleoprotein antibodies in oral fluid specimens from pigs infected under experimental conditions using a blocking ELISA. Transbound Emerg Dis.

Peeling RW, Toye B, Jessamine P, Gemmill I. 1998. Pooling of urine specimens for PCR testing: a cost saving strategy for Chlamydia trachomatis control programmes. Sex Transm Infect 74, 66-70.

PigChamp® Benchmark, 2011. Available at http://www.pigchamp.com/LinkClick.aspx?fileticket=NMdM5F73gKE%3d&tabid=115 Accessed June 30, 2013.

Polson D, Jordan D. 2002. A simulated model approach to sample size determination. Proceedings of the International Pig Veterinary Society Congress, pp. 86

Prickett JR, Kim W, Simer R, Yoon KJ, Zimmerman J. 2008. Oral-fluid samples for surveillance of commercial growing pigs for porcine reproductive and respiratory syndrome virus and porcine circovirus type 2 infections. J Swine Health Prod 16, 86-91.

Prickett JR, Zimmerman J. 2010. The development of oral fluid-based diagnostics and applications in veterinary medicine. Anim Health Res Rev 10, 1-10.

Prickett J, Simer R, Christopher-Hennings J, Yoon KJ, Evans RB, Zimmerman JJ. 2008.

Detection of porcine reproductive and respiratory syndrome virus infection in porcine oral fluid samples: a longitudinal study under experimental conditions. J Vet Diagn Invest 20, 156-163.

Prickett JR, Johnson J, Murtaugh MP, Puvanendiran S, Wang C, Zimmerman JJ, Opriessnig T. 2011. Prolonged detection of PCV2 and anti-PCV2 antibody in oral fluids following experimental inoculation. Transbound Emerg Dis 58, 121-127.

Roberts J. 2003. Serological monitoring in infected sow herds. In: Zimmerman, J.J., Yoon, K.J. (Eds.), PRRS Compendium. National Pork Board, Des Moines, Iowa, pp. 75-86.

Rovira A, Reicks D, Muñoz-Zanzi C. 2007. Evaluation of surveillance protocols for detecting porcine reproductive and respiratory syndrome virus infection in boar studs by simulation modeling. J Vet Diagn Invest 19, 492-501

Salman MD. 2003. Surveillance and monitoring systems for animal health programs and disease surveys. In: Salman, MD. (Ed.), Animal disease surveillance and survey systems: methods and applications. Blackwell publishing, Ames, Iowa, pp. 3-13.

Steel RG, Torrie JH. 1980. Principles and Procedures of Statistics: A Biometrical Approach (2nd ed.), McGraw-Hill Book Company, New York, p. 124, 556.

Thrusfield M. 2005 Veterinary Epidemiology (3rd ed.), Blackwell Scientific, Oxford, p 584.

Thurmond MC. 2003. Conceptual foundations for infectious disease surveillance. J Vet Diagn Invest 15, 501-514.

Toma B, Vaillancourt J-P, Dufour B, Dufour B, Eloit M, Moutou F, Marsh W, Bébet J-J, Sanaa M, Michel P. 1999. Dictionary of Veterinary Epidemiology. Iowa State University Press, Ames, Iowa, pp. 231.

Wills RW, Zimmerman JJ, Yoon KJ, Swenson SL, Hoffman LJ, McGinley MJ, Hill HT, Platt KB. 1997. Porcine reproductive and respiratory syndrome virus: routes of excretion. Vet Microbiol 57, 69-81.

CHAPTER 3. DETECTION OF PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME VIRUS (PRRSV) ANTIBODIES IN ORAL FLUID SPECIMENS USING A COMMERCIAL PRRSV SERUM ANTIBODY ELISA

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ABSTRACT

The purpose of the present study was to evaluate the diagnostic performance of a commercial serum antibody ELISA[a] modified to detect anti-porcine reproductive and respiratory syndrome virus (PRRSV) antibodies in pen-based oral fluid specimens. Experimental and field oral fluid samples of defined status in reference to exposure of swine with PRRSV were used to derive the kinetics of detectable concentrations of antibody against PRRSV. IgM and IgA were readily detected in oral fluid specimens from populations in which PRRSV infection was synchronized among all individuals, but not in samples collected in commecial herds. In contrast, IgG was readily detected at diagnostically useful levels in both experimental and field samples for up to 126 days. Estimates of the IgG oral fluid ELISA performance were based on results from testing positive oral fluid samples (n = 492) from experimentally inoculated pigs (n = 251) and field samples (n = 241), and negative oral fluid samples (n = 367) from experimentally inoculated pigs (n = 84) and field samples (n = 283). ROC analysis estimated the diagnostic sensitivity and specificity of the assay as 94.7% (95% CI: 92.4, 96.5) and 100% (95% CI: 99.0, 100.0), respectively, at a sample:positive (S/P) ratio cutoff of ≥ 0.40. The results of this study suggest that the IgG oral fluid ELISA can provide efficient, cost-effective PRRSV monitoring in commercial herds and PRRSV surveillance in elimination programs.

INTRODUCTION

"Oral fluid," the liquid collected by placing an absorptive device in the mouth[3] is a combination of serum transudate and saliva. Serum transudate enters the mouth from capillaries in the oral mucosa, crevicular gap, and gingival tissues.[5,9] In humans, the ease of collecting oral fluid samples has facilitated the implementation of this approach for large epidemiological studies. For example, in England, oral fluid samples were collected from 11,698 children at home by their parents and mailed to the laboratory for antibody testing.[4]

Oral fluid specimens are used in human medicine and forensics for the diagnosis or detection of a variety of infectious agents,[24] hormones,[20] and drugs.[8] In particular, the recognition that both human immunodeficiency virus (HIV) and anti-HIV antibodies were present in oral fluid specimens[2,12] stimulated assay development. At present, surveillance and monitoring for HIV is commonly done by testing oral fluid specimens using rapid (20 minute) point-of-care assays.[26]

Although oral fluid testing has not been widely applied to livestock health and wellness management, veterinary literature on the presence of antibodies, pathogens, and acute phase proteins in oral fluids from animals reflects the findings in humans.[31] In swine, infectious agents, cortisol, acute phase proteins, and progesterone have all been detected in oral fluid specimens under experimental and field conditions.[13,25,28-30] Increasingly, oral fluid samples are used for the surveillance of porcine reproductive and respiratory syndrome virus (PRRSV) infections in commercial swine operations using polymerase chain reaction (PCR)-based assays.[7,15] While PCR-based assays are useful for detecting the circulation of PRRSV, antibody-based assays are informative regarding herd immunity and history of prior infection. Therefore, the purpose of the present study was to evaluate the diagnostic performance of a commercial serum antibody ELISA[a] modified to detect anti-PRRSV antibodies in oral fluids.

MATERIALS AND METHODS

Experimental design

Oral fluid samples (n = 11) of known PRRSV status ("reference standards") were prepared for use in optimizing the antibody isotype-specific PRRSV ELISAs (IgM, IgA, IgG) and as in-house plate controls. Thereafter, experimental and field samples of defined status were used to describe antibody kinetics (IgM, IgA, IgG) and estimate the diagnostic performance (sensitivity and specificity) of the PRRSV IgG indirect ELISA.

Oral fluid specimens

Animals and animal care   All procedures for pigs intentionally inoculated with PRRSV or vaccinated with licensed modified live virus (MLV) PRRS vaccine were approved by the Institutional Animal Care and Use Committees (IACUC) at Iowa State University (IACUC #10-10-7033-S) and Kansas State University (IACUC #4567). In all cases, oral fluid specimens were collected by allowing pigs access to a short length of cotton rope suspended in the pen. After 20 to 30 minutes of pig interaction (biting) with the rope, oral fluid was extracted by passing the rope through a hand-operated wringer.[b] These procedures are fully described elsewhere.[1]

Reference standards   In brief, 5-week-old pigs (n ~1,100) in one PRRSV-negative, commercial wean-to-finish barn were intramuscularly vaccinated with 2 ml of a type 2 PRRS MLV vaccine.[c] Oral fluid was collected from every pen (n = 37) in the barn at 0, 10, 15, 20, 28, 35, 41, 49, 56, 75, and 91 days post vaccination (DPV). At each collection, the oral fluids were pooled in a single 10 liter container. In the laboratory, the fluid was clarified by centrifugation at 12,000 x *g* for 8 hours at 4°C and stored (-20°C) in 500 ml volumes. After the entire volume had been processed, the oral fluid was placed in a single 10 liter container, stirred at 600 rpm for 30 min to assure homogeneity, decanted into 50 ml centrifuge tubes,[d] and stored at -80°C.

Experimental samples   Oral fluid samples (n = 693) were collected from pigs inoculated with a type 2 PRRSV (NVSL 97-7895, GenBank® accession number AY545985) under

experimental conditions at the Kansas State University BL-2 animal facility. Prior to PRRSV inoculation, pigs were confirmed free of PRRSV infection by testing serum samples using a commercial semi-quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) kit[e] and a commercial ELISA,[a] respectively. In each of 7 trials, approximately 200, 2- to 3-week old pigs (15 or 16 pigs per pen), were challenged with 1 x 10^5 TCID$_{50}$ of PRRSV. One-half of the 3 ml challenge dose was administered intramuscularly and the remainder given intranasally. Thereafter, oral fluid samples were collected at 0, 5, 7, 9, 11, 14, 17, and 21 day post inoculation (DPI) and stored at -80º C.

Field samples   Expected PRRSV antibody-negative oral fluid field samples (n = 283) for assessing IgG ELISA specificity were selected from samples submitted to the Iowa State University Veterinary Diagnostic Laboratory (ISU VDL, Ames, Iowa USA) for PRRSV qRT-PCR testing from herds actively monitored for freedom from PRRSV infection.

Potential PRRSV antibody-positive oral fluid samples were collected from wean-to-finish barns on 10 sites in a longitudinal field study[32] (n = 600). The ten sites were sourced from five sow sources located in four different states (Colorado, Illinois, Oklahoma, and Utah). Most sites (2 to 4 barns per site) were managed all-in all-out and the site fill time was one or two days. Pigs were not vaccinated against PRRSV. At each site, oral fluid samples were collected from the same 6 pens housing approximately 25 pigs per pen at 2-week intervals from placement (~ 3 weeks of age) to slaughter. Following the completion of sample collection, the 600 oral fluid samples (6 pens x 10 barns x 10 sampling points) were randomly ordered and tested by qRT-PCR for PRRSV. Expected PRRSV antibody-positive oral fluid field samples for assessing IgG ELISA sensitivity were defined as all samples collected from a pen after its first PRRSV qRT-PCR positive sample. Among the 600 field samples screened, 241 samples met this definition.

Protocol for PRRSV oral fluid IgG ELISA
The procedure for the commercial indirect ELISA[a] was modified to detect anti-PRRSV antibody in porcine oral fluid specimens.[16] The modified assay required 2 days to complete.

In brief, on day one, oral fluid samples were diluted 1:2 in dilution plates (150µl oral fluid sample + 150µl diluent) using the diluent provided with the test kit. Diluted oral fluid was then transferred to the 96-well PRRSV antigen-coated plates (250 µl per well per sample). Negative and positive kit controls were diluted 1:30 using the kit diluent (10µl kit control + 290µl diluent) and 100µl transferred to the assigned wells on the plates, i.e., A1 and B1 for negative controls; C1 and D1 for positive controls. Thereafter, plates were incubated for 16 hrs at 4°C in a refrigerated incubator.

At the end of the 16 hr incubation period, the coated plates were washed three times with 400µl of 1X 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 (HRP)-conjugated anti-swine IgG$_{FC}$ secondary antibody[f] was added to each well and incubated for 30 min at 22°C. Thereafter, plates were washed 3 times with wash solution, 100 µl of TMB enzyme substrate solution was added to each well, and the plates 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 using an ELISA plate reader[g] controlled by commercial software.[h] The reactions were measured as optical density (OD) and then converted to sample-to-positive ratios (S/P) using the following formula:

Equation 1. $S/P = ((\text{Sample A}(650) - NC\,\bar{x}))/((PC\,\bar{x} - NC\,\bar{x}))$

In Equation 1, NC$\bar{x}$ and PC$\bar{x}$ represented the mean OD of the negative control wells and positive control wells, respectively. For the PRRSV oral fluid IgG ELISA results to be valid, the mean of the kit positive control OD minus the mean of the kit negative control OD had to be ≥ 0.150 and the kit negative control mean OD ≤ 0.150. These validity checks were identical to those required by the manufacturer for the PRRSV serum ELISA.

Preparation of anti-swine IgG$_{FC}$ secondary antibody

The appropriate dilution of horseradish peroxidase (HRP)-conjugated anti-swine IgG$_{FC}$ antibody[f] was calculated for each ELISA kit lot to standardize the strength of the reaction

and assure the repeatability of results. This was achieved by determining the dilution of anti-swine IgG_{FC} that matched the positive control OD value listed in the manufacturer's Certificate of Analysis for the ELISA kit lot. Certificates of Analysis for specific kit lots can currently be obtained using the manufacturers' Certificate of Analysis Request Form [http://www.idexx.com/view/xhtml/en_us/livestock-poultry/certificates-of-analysis.jsf].

In brief, 4 dilutions (1:1000, 1:1500, 1:2000, 1:2500) of anti-swine IgG_{FC} antibody[f] were prepared using the anti-pig IgG:HRPO conjugate provided with the ELISA[a] kit as diluent and then stirred for 48 hours at 4°C. To test the reactivity of the 4 dilutions of anti-swine IgG_{FC}, kit negative and positive controls were diluted 1:30 (10 µl kit control + 290 µl kit sample diluent) and then 100 µl of diluted negative control was transferred into 48 wells, i.e., one-half of the plate, and 100 µl of diluted positive control in each of the remaining 48 wells. Thereafter, the protocol for the PRRSV oral fluid IgG ELISA was conducted, as described above. Negative control OD values were used to detect non-specific reactions. Positive control OD values were used to determine the correct dilution of secondary antibody in two steps: (1) Linear regression (Equation 2) was used to determine the equation of the line that described the relationship between positive control OD response and secondary antibody dilution (1:1000, 1:1500, 1:2000, 1:2500). (2) The appropriate dilution of conjugated antibody dilution was calculated by substituting the mean positive control OD from the Certificate of Analysis for "x" into Equation 2 and then solving for "y".

Equation 2. $y = ax + c$

In Equation 2, "y" is optical density, "a" is the slope of the line, "x" is the dilution of secondary antibody, and "c" is the intercept.

IgM and IgA protocols
The commercial indirect ELISA was also modified to detect PRRSV-specific IgM (or IgA) antibody responses. To detect bound antibody, HRP-conjugated anti-pig IgM antibody[i] or anti-pig IgA antibody[j] was diluted 1:2000 in conjugate diluent provided by the kit

manufacturer. The Reference Standard oral fluid sample collected on day 0 was used as the negative control. S/P values for IgM and IgA ELISAs were calculated as described in Equation 1 using Reference Standard samples collected on DPV 10 and 35 used as positive controls for the IgM and IgA ELISAs, respectively.

For the PRRSV oral fluid IgM and IgA ELISA results to be valid, the mean of the kit positive control OD minus the mean of the kit negative control OD had to be ≥ 0.150 and the kit negative control mean OD ≤ 0.150. These validity checks were identical to those required by the manufacturer for the PRRSV serum ELISA.

Evaluation of IgG ELISA performance

Receiver operator characteristic (ROC) curve analyses were performed using MedCalc® 9.2.1.0.[k] Diagnostic sensitivity, specificity and 95% confidence intervals were estimated for a range of S/P values (≥ 0.20 to ≥ 0.40) using test results from samples of defined status. Positive samples for estimating diagnostic sensitivity included oral fluid specimens from experimentally inoculated pigs (n = 251) collected on DPI 14, 17, and 21 and field samples (n = 241) collected from pens following their first PRRSV qRT-PCR positive oral fluid sample. Negative samples for estimating diagnostic specificity included oral fluid specimens from experimentally inoculated pigs collected on DPI 0 (n = 84) and negative field samples (n = 283). Negative oral fluid field samples were diagnostic samples submitted to the ISU VDL for PRRSV qRT-PCR testing from PRRSV-negative herds with active monitoring programs.

RESULTS

Reference standards   Anti-PRRSV antibody isotype (IgM, IgA, IgG) kinetics in reference standard samples are given in Figure 1 for oral fluid samples collected on DPV 0, 10, 15, 20, 28, 35, 41, 49, 56, 75, and 91. On DPV 10, S/P ratios for IgM, IgA, and IgG were 1.00, 0.31, and 1.22, respectively. On DPV 15, S/P ratios peaked at 1.87, 1.53, and 4.29 for IgM, IgA, and IgG.   IgM declined rapidly, i.e., the S/P ratio was 0.10 at DPV 28. For DPV 20 to 41, IgA S/P ratios ranged from a low of 0.59 (DPV 41) to a high of 1.05 (DPV 28). For

DPV 49 to 91, IgA S/P ratios ranged from a low of 0.33 (DPV 75) to a high of 0.53 (DPV 91). IgG S/P ratios declined relatively slowly and steadily after DPV 15, reaching a low of 2.65 on DPV 75.

Experimental samples   Anti-PRRSV antibody isotype (IgM, IgA, IgG) kinetics in experimental samples are shown in Figure 2 for oral fluid samples collected on DPI 0, 5, 7, 9, 11, 14, 17, and 21. Mean IgM, IgA, and IgG S/P ratios on DPI 7 were 1.14 (95% confidence interval (CI) 1.04, 1.24), 0.09 (95% CI 0.06, 0.12), and 0.59 (95% CI 0.50, 0.68), respectively. The mean IgM S/P ratio peaked at DPI 9 (1.88, 95% CI 1.78, 1.97) and declined rapidly to a mean S/P of 0.10 (95% CI 0.09, 0.13) on DPI 21. Mean IgA and IgG S/P ratios peaked at DPI 11 (1.41, 95% CI 1.29, 1.53) and DPI 14 (3.69, 95% CI 3.52, 3.85), respectively. Mean IgA and IgG S/P ratios remained relatively constant until the end of study at DPI 21.

Field samples   Among the 60 pens on 10 sites from which oral fluid samples were collected over time, 39 pens had one or more PRRSV qRT-PCR-positive oral fluid samples over the course of the 18 week observation period. Expected PRRSV antibody-positive oral fluid field samples were defined as all samples collected from a pen after its first PRRSV qRT-PCR positive sample. Based on this definition, 241 (40%) of 600 oral fluid samples were defined as expected antibody-positive field specimens. Specifically, these samples were from samples collected 2 to 18 weeks following the first qRT-PCR-positive result (Table 1).

Mean oral fluid IgM, IgA, and IgG S/P ratios at the time of the first detection of PRRSV by qRT-PCR were 0.14 (95% CI 0.05, 0.23), 0.23 (95% CI 0.08, 0.38), and 1.98 (95% CI 1.42, 2.53), respectively (Table 1). Peak IgM S/P ratios 0.20 (95% CI 0.07, 0.33) were observed two weeks after the first detection of PRRSV by qRT-PCR and declined thereafter. IgA and IgG S/P ratios were relatively steady, but tended to increase over the course of the observation period, reaching a peak in samples collected 18 weeks after the first qRT-PCR positive sample (Figure 3).

IgG indirect ELISA diagnostic performance   Estimates of IgG indirect ELISA performance were based on samples of defined status. Positive samples (n = 492) for estimating diagnostic sensitivity included samples collected on DPI 14, 17, and 21 (n = 251) from experimentally inoculated pigs and field samples (n = 241) collected from pens after the first PRRSV qRT-PCR positive result. Negative samples (n = 367) for estimating diagnostic specificity included oral fluid specimens from experimentally inoculated pigs collected on DPI 0 (n = 84) and field samples (n = 283) submitted to the ISU VDL for PRRSV qRT-PCR testing from PRRSV-negative monitored herds. The cumulative S/P results for defined positive and negative oral fluid samples are given in Figure 4. Mean S/P ratios of negative and positive samples were -0.001 (95% CI -0.006, 0.002) and 2.751 (95% CI 2.639, 2.864), respectively. Estimates of diagnostic sensitivity and specificity by S/P ratio cutoff obtained through ROC analysis are shown in Table 2 and Figure 5.

DISCUSSION

In this study, the detection of anti-PRRSV antibodies in oral fluid specimens was evaluated using a commercial PRRSV indirect ELISA[a] performed using a procedure adapted to the oral fluid matrix. The validity of this approach was supported by prior evidence that pigs, like humans and other species, produce detectable levels of specific antibodies in oral fluid in response to infection with PRRSV, as well as other pathogens.[31] Essentially, the ELISA procedure was modified to calibrate the reactivity of the assay to the lower concentration of antibody present in oral fluid relative to serum. Specific modifications included larger sample volume, less dilution of the sample, higher secondary antibody concentration, and longer sample incubation.[16] A key step to creating a reproducible test was determining the kit lot-specific dilution of secondary antibody. This was done by titrating the secondary antibody against a 1:30 dilution of positive and negative controls and then calculating the dilution of secondary antibody that matched the OD values for the positive and negative controls in the manufacturer's Certificate of Analysis. This step provided repeatable results and resulted in a cutoff in the oral fluid IgG ELISA that matched the cutoff recommended by the manufacturer for the serum ELISA (S/P $\geq$ 0.40).

Anti-PRRSV IgM, IgA, and IgG responses were evaluated in pen-based oral fluid samples using isotype-specific ELISAs. IgM, IgA, and IgG were readily detected in populations in which the infection was synchronized among all individuals, i.e., reference standards and experimental samples. However, this was not the case in field samples. The reason for the difference in responses in synchronized vs. field samples is unknown, but it may be hypothesized that asynchrony in the spread of PRRSV infection in the field resulted in animals at different stages of the antibody response. Consequently, IgM and IgA from pigs in the early stages of infection would have been diluted by oral fluid from animals not secreting those antibody isotypes. In addition, IgG, both because of its higher concentration and its higher affinity, may have out-competed IgM and IgA for the antigenic sites available in the ELISA plate.[19,23]

In contrast to IgM and IgA, IgG was readily detected at diagnostically useful levels in reference standards, experimental samples, and field samples for an extended period. That is, IgG was consistently detected in reference standards through the termination of sample collection at 91 DPV, and in experimental samples and field samples throughout their respective observation periods, i.e., 21 and 126 days.

Previous reports on anti-PRRSV serum antibody kinetics using indirect immunofluorescence assay (IFA) reported that IgM was first detected 5 to 7 DPI[14,21,27] and IgG was detected 9 to 11 DPI.[35,21] Thus, the kinetics of serum IgM and IgG responses reported in the literature and the findings of this study for oral fluid were similar. In contrast, IgA was first detected by immunoperoxidase monolayer assay (IPMA) in serum at 14 DPI, reached its maximum concentration at 25 DPI, and was undetectable after 35 DPI.[18] That is, the reported detection of IgA in serum was later and shorter compared to the response observed in oral fluid. Overall, the observations for oral fluid are in agreement with our general understanding of antibody kinetics in serum and on mucosal surfaces. Likewise, these data are compatible with previous reports that IgA is locally produced by serum-derived plasma cells in salivary

glands and duct associated lymphoid tissue (DALT), whereas IgM and IgG are primarily derived from serum, with some contribution from local production.[31]

The use of pen-based oral fluid specimens, an unusual sample for an antibody ELISA, requires comment because an understanding of the epidemiological and statistical properties of the sample is central to the interpretation and analysis of the assay results. In sampling terminology, an oral fluid sample is best described as a grab sample from a sample unit (pen), where "grab sample" is defined as a sample collected "at a specific point in time and at a specific location"[17] and "sample unit" is the smallest division of the population and the level at which sample size calculations and random sampling procedures are performed.[10,33-34] Examples of sample units given by Toma et al.[34] include, "an animal, a group of animals, i.e., a pen, a herd …." Oral fluid ELISA results are amenable to diagnostic interpretation because the pathogen-specific epidemic curve and immune responses of the individual pigs within the pen are highly correlated (dependent). The link with specific time and place provides for the statistical analysis of the results, including analysis of geospatial relationships between pens in the context of the larger population.

A pen-based sample should not be confused with a pooled sample. More universally known as a "composite sample",[11] a pooled sample is created by combining two or more sample unit specimens into one aliquot for testing, thereby losing sample unit identity in the process,[5] Pooled samples are typically used to screen populations at a lower cost than testing individual samples using a 2-step process termed the "Dorfman procedure".[22] A pooled sample could be roughly equivalent to pen-based samples if the sample units were members of the same pen, but this is rarely the case. In fact, the geospatial relationships among sample units in pooled samples are usually unknown. Thus, testing of pooled samples may determine whether the defined analyte is present, but further statistical analyses or epidemiological interpretations are usually limited or impossible because of the loss of sample unit identification. This study did not evaluate whether the ELISA procedure described could be used for pooled samples. At any rate, given the ease of collecting pen-based samples and the economic utility of ELISA-based testing, it is doubtful that testing

pooled oral fluid samples could be justified by the loss of statistical and epidemiological interpretability.

Cumulatively, this study showed that a commercial serum antibody ELISA[a] adapted to oral fluid specimens can be used to detect anti-PRRSV antibody. Testing for PRRSV antibody using pen-based oral fluids could provide an efficient, cost-effective approach for monitoring maternal antibody, vaccination compliance, and herd immune parameters in commercial herds and surveiling PRRSV infections in elimination programs. The successful adaptation of one commercial serum antibody ELISA to the oral fluid matrix suggests that antibody assays for other pathogens could also be modified to this purpose.

SOURCES AND MANUFACTURERS

a. HerdChek® PRRS 3X ELISA, IDEXX Laboratories, Inc., Westbrook ME.

b. BL-38, Dyna-Jet Products, Overland Park, KS.

c. Ingelvac® PRRS MLV, Boehringer Ingelheim Vetmedica, Inc., St. Joseph, MO.

d. Becton, Dickinson and Company, Bedford, MA.

e. Applied Biosystems, Inc., Foster City, CA.

f. Pig IgG-Fc Antibody (Cat No. A100-104P), Bethyl Laboratories, Inc., Montgomery, TX.

g. EL800 micro plate reader, Bio Tek® Instruments Inc., Winooski, VT.

h. GEN5™, Bio Tek® Instruments Inc., Winooski, VT.

i. Pig IgM Antibody (Cat No. A100-100P), Bethyl Laboratories, Inc., Montgomery, TX.

j. Pig IgA Antibody (Cat No. A100-102P), Bethyl Laboratories, Inc., Montgomery, TX.

k. MedCalc Software, Mariakerke, Belgium.

DECLARATION OF CONFLICTING INTERESTS

Authors A. Ballagi and A. Rice are employed by IDEXX Laboratories, Inc. The remaining author(s) declare no conflicting interests with respect to their authorship or the publication of this article.

REFERENCES

1. Anon: 2011, Oral fluid collection in pigs booklet (NPB-03623). National Pork Board, PO Box 9114, Des Moines, IA.

2. Archibald DW, Zon L, Groopman JE, et al.: 1986, Antibodies to human T-lymphotropic virus type III (HTLV-III) in saliva of acquired immunodeficiency syndrome (AIDS) patients and in persons at risk for AIDS. Blood 67:831–834.

3. Atkison J, Dawes C, Ericson T, et al.: 1993, Guidelines for saliva nomenclature and collection. Ann N Y Acad Sci 694, xi-xii.

4. Bartington SE, Peckham C, Brown D, et al.: 2009, Feasibility of collecting oral fluid samples in the home setting to determine seroprevalence of infections in a large-scale cohort of preschool-aged children. Epidemiol Infect 137:211-218.

5. Cameron A, Gardner I, Doherr MG, Wagner B: 2003, Sampling considerations in surveys and monitoring and surveillance systems. *In:* Animal Disease Surveillance and Survey Systems, ed. Salman MD, 1st ed., pp. 47-86. Iowa State University Press, Ames, IA.

6. Cameron SO, Carman WF: 2005, The use of the OraSure® collection device for hepatitis virus testing in health care settings. J Clin Virol 34:22-28.

7. Chittick WA, Stensland WR, Prickett JR, et al.: 2011, Comparison of RNA extraction and real-time reverse transcription polymerase chain reaction methods for the detection of porcine reproductive and respiratory syndrome virus in porcine oral fluid specimens. J Vet Diagn Invest 23:248-253.

8. Danhof M, Breimer DD: 1978, Therapeutic drug monitoring in saliva. Clin Pharmacokinet 3:39-57.

9. Delima AJ, Dyke TEV: 2003, Origin and function of the cellular components in gingival crevice fluid. Periodontol 2000 31:55-76.

10. Dohoo I, Martin W, Stryhn H: 2009, Sampling. *In:* Veterinary Epidemiologic Research, 2nd ed., pp. 33-56. VER, Inc., Charlottetown, Prince Edward Island, Canada.

11. Everitt BS: 2006, The Cambridge dictionary of statistics, Cambridge University Press, Cambridge, UK.

12. Groopman J, Salahuddin S, Sarngadharan M, et al.: 1984, HTLV-III in saliva of people with AIDS-related complex and healthy homosexual men at risk for AIDS. Science 226:447-449.

13. Gutiérrez AM, Martínez-Sbiela S, Eckersall, PD, Cerón JJ: 2009, C-reactive protein quantification in porcine saliva: A minimally invasive test for pig health monitoring. Vet J 181:261-265.

14. Joo HS, Park BK, Dee SA, Pijoan C: 1997, Indirect fluorescent IgM antibody response of pigs infected with porcine reproductive and respiratory syndrome virus. Vet Microbiol 55:303-307.

15. Kittawornrat A, Prickett J, Chittick W, et al.: 2010, Porcine reproductive and respiratory syndrome virus (PRRSV) in serum and oral fluid samples from individual boars: Will oral fluid replace serum for PRRSV surveillance? Virus Res 154:170-176.

16. Kittawornrat A: 2011, Procedure for X3 porcine reproductive and respiratory syndrome virus (PRRSV) ELISA – Oral fluid. Document 9.3490. Quality Systems, Veterinary Diagnostic Laboratory, Iowa State University, Ames, Iowa.

17. Koerner CE: 1996, Effect of equipment on sample representativeness. In: Principles of Environmental Sampling, ed. Keith LH, 2nd ed, pp. 155-168. American Chemical Society, Washington, DC.

18. Labarque GG, Nauwynck HJ, Van Reeth K, Pensaert MB: 2000, Effect of cellular changes and onset of humoral immunity on the replication of porcine reproductive and respiratory syndrome virus in the lungs of pigs. J Gen Virol 81:1327-1334.

19. Lee BW, Bey RF, Baarsch MJ, Larson ME: 1995, Class specific antibody response to influenza A H1N1 infection in swine. Vet Microbiol 43:241-250.

20. Lippi G, De Vita F, Salvagno GL, et al.: 2009, Measurement of morning saliva cortisol in athletes. Clin Biochem 42:904-906.

21. Loemba HD, Mounir S, Mardassi H, et al.: 1996, Kinetics of humoral immune response

to the major structural proteins of the porcine reproductive and respiratory syndrome virus. Arch Virol 141:751-761.

22. Lovison G, Gore SD, Patil GP: 1994, Design and analysis of composite sampling procedures: A review. *In*: Handbook of Statistics Volume 12: Environmental Statistics, eds. Patil GP, Rao CR, pp. 103-166. Elsevier Science B.V., Amsterdam, The Netherlands.

23. Mäkelä O, Rouslahti E, Seppälä IJT: 1970, Affinity of IgM and IgG antibodies. Immunochemistry 7:917-932.

24. Malamud D: 1992, Saliva as a diagnostic fluid. Br Med J 305:207-208.

25. Moriyoshi M, Tamaki M, Nakao T, Kawata K: 1996, Early pregnancy diagnosis in the sow by saliva progesterone measurement using a bovine milk progesterone qualitative test EIA kit. J Vet Med Sci 58:737-741.

26. O'Connell RJ, Merritt TM, Malia JA, et al.: 2003, Performance of the OraQuick Rapid Antibody Test for diagnosis of human immunodeficiency virus type 1 infection in patients with various levels of exposure to highly active antiretroviral therapy. J Clin Microbiol 41:2153-2155.

27. Park BK, Joo HS, Dee SA, Pijoan C: 1995, Evaluation of an indirect fluorescent IgM antibody test for the detection of pigs with recent infection of porcine reproductive and respiratory syndrome virus. J Vet Diagn Invest 7:544-546.

28. Parrott RF, Misson BH: 1989, Changes in pig salivary cortisol in response to transport simulation, food and water deprivation, and mixing. Br Vet J 145:501-505.

29. Prickett J, Simer R, Christopher-Hennings J, et al.: 2008, Detection of porcine reproductive and respiratory syndrome virus infection in porcine oral fluid samples: A longitudinal study under experimental conditions. J Vet Diagn Invest 20:156-163.

30. Prickett JR, Kim W, Simer R, et al.: 2008, Oral-fluid samples for surveillance of commercial growing pigs for porcine reproductive and respiratory syndrome virus and porcine circovirus type 2 infections. J Swine Health Prod 16:86-91.

31. Prickett JR, Zimmerman JJ: 2010, The development of oral fluid-based diagnostics and applications in veterinary medicine. Anim Health Res Rev 10:1-10.

32. Ramirez A, Wang C, Prickett JR, et al.: 2011, Efficient surveillance of pig populations

using oral fluids. Prev Vet Med (submitted)

33. Steel RGD, Torrie JH: 1980, Principles and Procedures of Statistics: A Biometrical Approach, 2nd ed., McGraw-Hill Book Company, New York, p. 124, 556.

34. Toma B, Vaillancourt J-P, Dufour B, et al.: 1999, Dictionary of veterinary epidemiology. pp. 231. Iowa State University Press, Ames, Iowa.

35. Yoon K-J, Zimmerman JJ, Swenson SL, et al.: 1995, Characterization of the humoral immune response to porcine reproductive and respiratory syndrome (PRRS) Virus Infection. J Vet Diagn Invest 7:305-312.

Table 1. Detection of PRRSV infection by qRT-PCR and ELISAs (IgM, IgA, IgG) in oral fluid specimens collected over time in 10 wean-to-finish barns on 10 sites

Week*	No. Pens†	No. of PRRSV qRT-PCR positive pens	Mean IgM S/P Ratio (95% CI)	Mean IgA S/P Ratio (95% CI)	Mean IgG S/P Ratio (95% CI)	IgG-positive pens‡
0	39	39 (100%)	0.14 (0.05, 0.23)	0.23 (0.08, 0.38)	1.98 (1.42, 2.53)	27 (69%)
2	37	15 (41%)	0.20 (0.07, 0.33)	0.28 (0.15, 0.42)	1.68 (1.40, 1.96)	37 (100%)
4	35	7 (20%)	0.04 (-0.03, 0.10)	0.38 (0.22, 0.55)	1.53 (1.24, 1.82)	30 (86%)
6	34	4 (12%)	-0.02 (-0.02, -0.01)	0.49 (0.31, 0.67)	1.92 (1.58, 2.56)	31 (91%)
8	27	0 (0%)	-0.02 (-0.03, -0.01)	0.36 (0.18, 0.55)	1.50 (1.16, 1.85)	23 (85%)
10	27	1 (4%)	-0.02 (-0.02, -0.01)	0.51 (0.32, 0.69)	1.83 (1.45, 2.18)	24 (89%)
12	27	1 (4%)	-0.02 (-0.03, -0.01)	0.64 (0.38, 0.89)	1.55 (1.13, 1.97)	23 (85%)
14	22	2 (9%)	-0.01 (-0.02, -0.01)	0.53 (0.24, 0.81)	1.97 (1.47, 2.48)	20 (91%)
16	18	1 (6%)	0.02 (-0.06, 0.11)	0.64 (0.29, 1.00)	2.28 (1.61, 2.93)	15 (83%)
18	14	2 (14%)	0.02 (-0.03, 0.06)	0.80 (0.43, 1.17)	2.51 (2.01, 3.02)	14 (100%)

*Week 0 was defined as the first PRRSV qRT-PCR positive oral fluid sample from a pen. For any pen, the first qRT-PCR positive result may have occurred at any point in the 18-week observation period. Subsequent weeks represent the designated time interval from the first qRT-PCR positive result.

†Number of pens sampled at the designated time interval (weeks) following the first PRRSV qRT-PCR positive oral fluid sample.

‡ IgG positive defined as HerdChek* PRRS X3 oral fluid IgG ELISA S/P ≥ 0.4

Table 2. Diagnostic performance of an ELISA* performed using a protocol modified to detect anti-PRRSV antibodies in swine oral fluids

S/P Ratio	Sensitivity (%) (95% confidence interval)	Specificity (%) (95% confidence interval)
≥ 0.20	95.3 (93.1, 97.0)	99.2 (97.6, 99.8)
≥ 0.25	95.3 (93.1, 97.0)	99.5 (98.0, 99.9)
≥ 0.30	95.3 (93.1, 97.0)	99.7 (98.5, 100.0)
≥ 0.35	95.1 (92.9, 96.9)	100.0 (99.0, 100.0)
≥ 0.40	94.7 (92.4, 96.5)	100.0 (99.0, 100.0)

* HerdChek[®] PRRS X3 Antibody Test Kit, IDEXX Laboratories, Inc., Westbrook, Maine USA
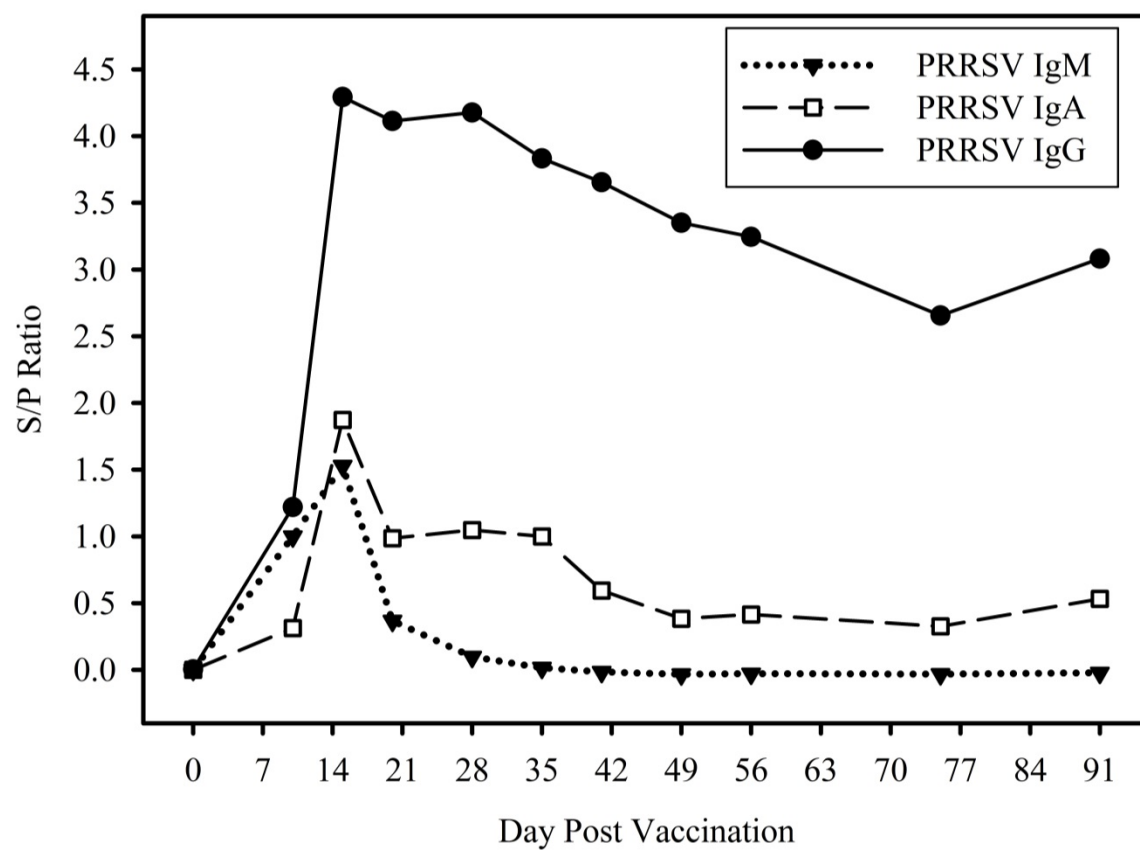
Figure 1.   Kinetics of anti-PRRSV antibody isotypes (IgM, IgA, IgG) in reference standard samples
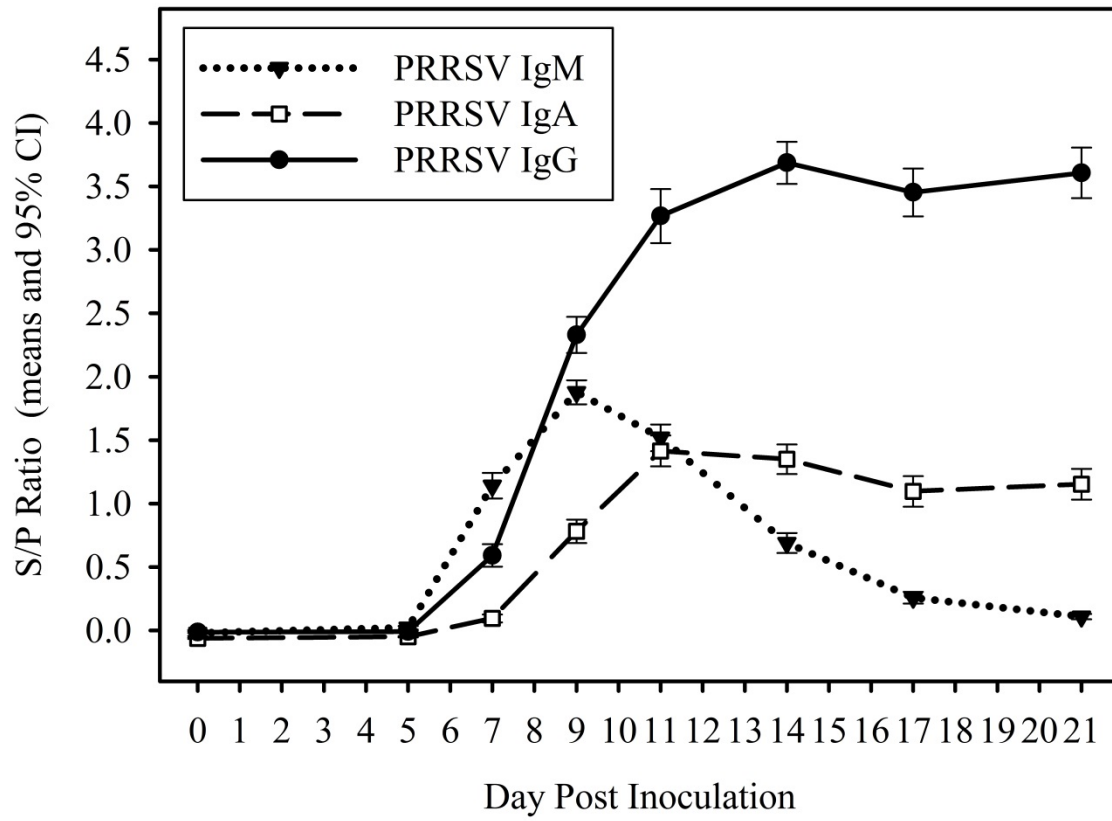
Figure 2. Kinetics of anti-PRRSV antibody isotypes (IgM, IgA, IgG) in experimental samples
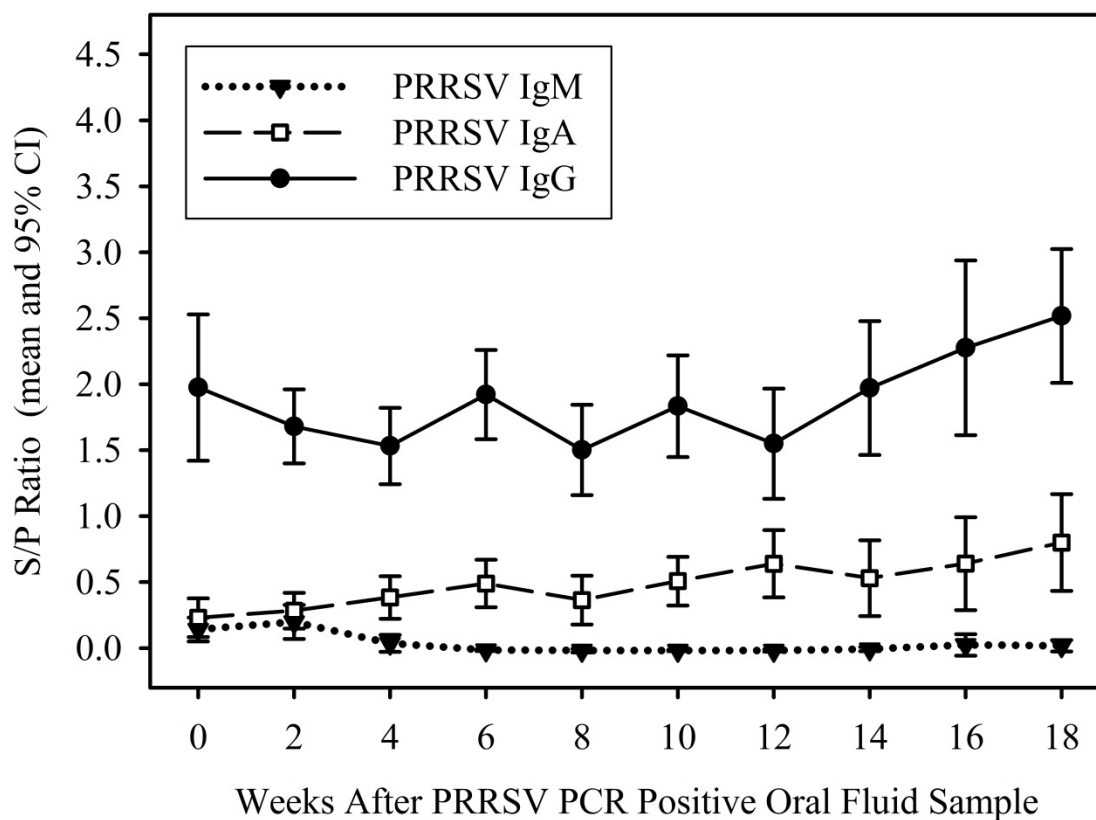
Figure 3. Kinetics of anti-PRRSV antibody isotypes (IgM, IgA, IgG) in field samples where week zero (0) represents the first PRRSV PCR-positive sample from a pen
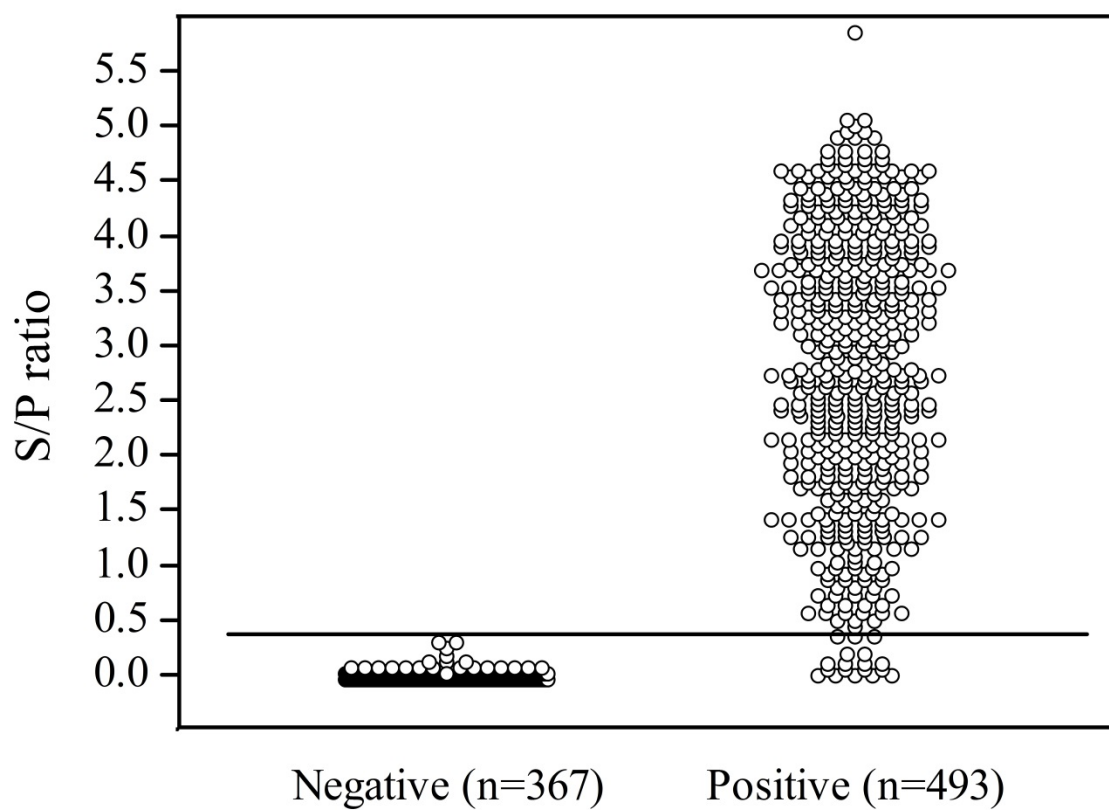
Figure 4. Distribution of PRRSV IgG ELISA results by sample classification (negative, positive) relative to the assay cutoff (S/P ratio = 0.4).
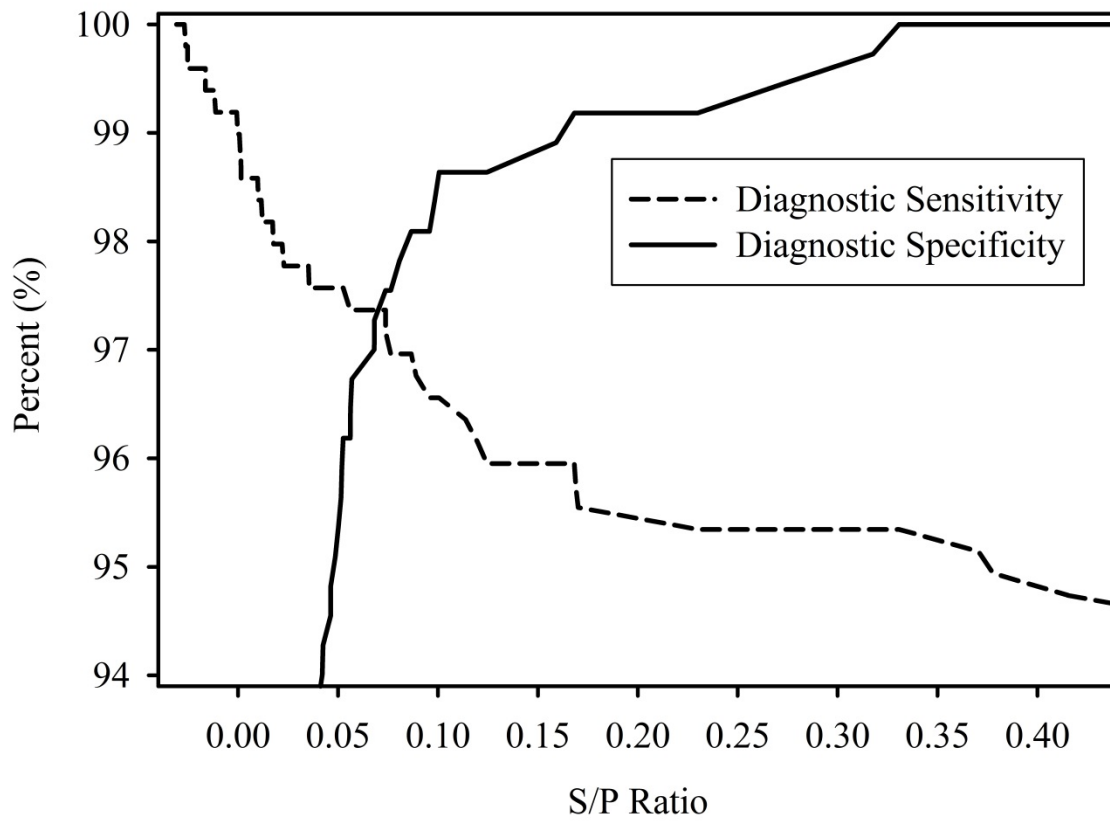
Figure 5. Estimates of PRRSV IgG ELISA diagnostic sensitivity and specificity by S/P ratio cutoff

CHAPTER 4. RING TEST EVALUATION OF THE REPEATABILITY AND REPRODUCIBILITY OF A PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME VIRUS (PRRSV) ORAL FLUID ANTIBODY ENZYME-LINKED IMMUNOSORBENT ASSAY

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ABSTRACT

The precision of a *Porcine reproductive and respiratory syndrome virus* (PRRSV) oral fluid antibody enzyme-linked immunosorbent assay (ELISA) was evaluated by calculating reliability coefficients for assay repeatability (within laboratory) and assay reproducibility (between laboratories). Randomly ordered oral fluid samples of known ($n = 39$) and unknown ($n = 224$) PRRSV antibody status were tested in 12 diagnostic laboratories. Each laboratory tested the samples twice, first using an antibody ELISA kit and reagents provided to them (Phase 1) and then using an ELISA kit and reagents configured in their laboratory (Phase 2). Repeatability (within laboratory) reliability coefficients calculated using results from samples of known PRRSV antibody status ranged from 0.724 to 0.997 in Phase 1 and 0.953 to 0.998 in Phase 2. Reproducibility (between laboratories) reliability coefficients were calculated for three conditions: Case 1 - samples of unknown status ($n = 224$); Case 2 - samples of known status ($n = 39$), and Case 3 - all samples ($n = 263$). Among the three cases, reliability coefficients ranged from 0.937 to 0.964 in Phase 1 and 0.922 to 0.935 in Phase 2. For Case 3, it was estimated that 96.67% of the total variation in Phase 1 and 93.21% in Phase 2 could be attributed to the oral fluid samples themselves. Overall, the PRRSV oral fluid antibody ELISA was highly repeatable and reproducible. This study supports the routine use of this test in laboratories providing diagnostic service to pig producers.

INTRODUCTION

Oral fluid samples are increasingly used for the surveillance of pathogens in commercial swine operations in North America. For example, the specimen type "swine oral fluid" was entered into the Iowa State University Veterinary Diagnostic Laboratory (ISU VDL) information management system in February, 2010. During the remainder of 2010, 10,329 oral fluid specimens were received for testing. This number increased to 32,544 in 2011. In the first 3 months of 2012, the ISU VDL received 13,226 oral fluid samples. Thus, if the current level of submissions is maintained, the laboratory will receive >52,000 oral fluid samples in 2012. The majority of oral fluid samples received at the ISU VDL are tested by PCR-based assays for PRRSV,[4,8-9] influenza virus,[1,10] porcine circovirus type 2,[7] and other respiratory pathogens.

Recently, the first antibody assay for oral fluids became available.[5] Although oral fluid antibody assays are already widely available in human diagnostic medicine for a variety of pathogens,[6] this is the first application in swine diagnostic medicine. Therefore, the objective of this study was to evaluate the repeatability and reproducibility of the PRRSV oral fluid ELISA[a] among 12 laboratories that offer diagnostic services to swine producers and veterinarians.

MATERIALS AND METHODS

Experimental design

Oral fluid samples ($n = 263$) were completely randomized and then distributed to the 12 laboratories participating in the ring trial. All participating laboratories had prior experience with ELISAs and three had previous experience with the PRRSV oral fluid ELISA. Each laboratory received one set of samples, one "pre-configured" commercial PRRSV ELISA kit[a] with pre-diluted reagents and controls, and a copy of the standard operating procedure for performing the assay.[3] Each laboratory tested the samples on the kit provided (Phase 1) and then repeated the testing on a PRRSV ELISA kit[a] configured in their laboratory (Phase 2). Results were evaluated for test repeatability (within laboratory) and reproducibility (between laboratories).

Oral fluid specimens

Oral fluid specimens tested ($n = 263$) included samples of unknown ($n = 224$) and known ($n = 39$) PRRSV antibody status. Oral fluid field specimens of unknown PRRSV antibody status samples were collected over a 2-month period from among diagnostic samples submitted to the Iowa State University Veterinary Diagnostic Laboratory (ISU VDL) for either PRRSV real-time reverse-transcription polymerase chain reaction (rRT-PCR) or PRRSV antibody testing. ISU VDL records indicated that specimens were collected on 42 different production sites located in 7 states (Colorado, Iowa, Illinois, Indiana, Missouri, North Carolina, and Ohio). Samples were included on the basis of sufficient sample volume and without knowledge of testing results in the ISU VDL. Each sample was assigned a random number, divided into 12 aliquots of 1.25 ml, and stored at $-20°C$.

Oral fluid samples of known PRRSV antibody status originated from pigs inoculated with a type 2 PRRSV modified-live virus vaccine[b] and collected on 0, 10, 15, 20, 28, 35, 41, 49, 56, 75, and 91 days post vaccination (DPV). These samples were used to create 13 samples that were run on every plate. Specifically, the day 0 sample was used as in-house negative control; the day 75 sample was used to create high (1:5), medium (1:10), and low (1:20) positive controls using the PRRS ELISA kit[a] sample diluent. The remaining 9 samples, i.e., DPV 10, 15, 20, 28, 35, 41, 49, 56, 91, were split into 3 aliquots and then placed within the sample set such that all 9 were run on every plate.

Oral fluid ELISA procedures

Oral fluid samples were tested on a commercial PRRSV ELISA using a protocol designed to detect PRRSV antibody in oral fluid.[3,5] In Phase 1, all 12 laboratories used the same kit lot (40959–W721). The 11 laboratories that participated in Phase 2 used 5 different kit lots: 5 used lot 40959–EG288, 3 used lot 40959–EG233, and 1 laboratory each used lots 40949–W721, 40949–W791, and 40949–X861. Phase 1 and Phase 2 were completed before the data were analyzed.

To perform the test, oral fluid samples were diluted 1:2 in dilution plates using the diluent provided with the test kit. 250 µl of diluted oral fluid was then transferred to the 96-well PRRSV

antigen-coated plates. Negative and positive kit controls were diluted 1:30 using the kit diluent and 100 μl transferred to the assigned wells on the plates (i.e., A1 and B1 for negative controls; C1 and D1 for positive controls). Thereafter, plates were incubated for 16 hr at 4°C. Three laboratories used refrigerated incubators; all other laboratories used standard refrigerators. At the end of the 16 hr incubation period, the plates were washed 3 times with 400 μl of 1× 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 (HRP)–conjugated anti-swine immunoglobulin G (IgG$_{FC}$) secondary antibody[c] was added to each well and incubated for 30 min at 22°C. Thereafter, plates were washed 3 times with wash solution, 100 μl of tetramethylbenzidine enzyme substrate solution was added to each well, and the plates 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 measured as optical density (OD).

All laboratories provided results as both OD and sample-to-positive (S/P) ratios. S/P was calculated as:

$$S/P = \frac{[\text{Sample A}(650) - \text{NC}]}{(\text{PC} - \text{NC})} \qquad (1)$$

where NC and PC represented the mean OD of the two negative control wells and two positive control wells, respectively. S/P ratios ≥ 0.40 were considered positive for PRRSV antibody (Kittawornrat et al., 2012).

Statistical analysis

The consistency of the PRRSV oral fluid ELISA was quantified by estimating reliability coefficients (ρ) for assay repeatability (within laboratory) and assay reproducibility (between laboratories) using the results from oral fluid samples of known ($n = 39$) and unknown ($n = 224$) PRRSV antibody status, respectively.[2,10]

For calculation of repeatability reliability coefficients, the model and associated parameters were based on n samples with d repeated measurements of a continuous variable x and denoted by x_{ij},

the jth measurement made on the ith subject (for $i = 1, \dots , n$ and $j = 1,\dots, d$):

$$x_{ij} = \tau_i + \beta_j + e_{ij} \qquad (2)$$

where τ_i is the sample effect, β_j is the plate effect, and e_{ij} is the measurement error. For within laboratory data, the d repeated measurements referred to results derived from repeated testing of the same samples by the same laboratory, i.e., the 13 samples tested on every plate. The equation for calculating assay repeatability reliability coefficients is given as:

$$\rho = \frac{\sigma_\tau^2}{\sigma_\tau^2 + \sigma_\beta^2 + \sigma_e^2} \qquad (3)$$

where σ_τ^2 is the variance due to the oral fluid sample, σ_β^2 is the variance due to the plate, and σ_e^2 is the variance due to measurement error.

Reproducibility reliability coefficients were estimated for three conditions (Case 1: samples of unknown status, Case 2: samples of known status, and Case 3: all oral fluid samples) using equation 4:

$$x_{ijk} = \tau_i + \alpha_k + \gamma_{jk} + \omega_{ik} + e_{ijk} \qquad (4)$$

In equation 4, x_{ijk} is the measurement made on the ith subject in jth kit lot number by kth laboratory (for $i = 1, \dots , n, j = 1,\dots, d$, and $k = 1, \dots, m$), τ_i is the effect of the ith oral fluid, α_k is the effect of the kth laboratory, γ_{jk} is the effect of kit lot number jth within laboratory k (where kit lot is nested within laboratory), ω_{ik} is the interaction between oral fluid i and laboratory k and e_{ijk} is the measurement error. The reproducibility reliability coefficient may be calculated as:

$$\rho = \frac{\sigma_\tau^2}{\sigma_\tau^2 + \sigma_\alpha^2 + \sigma_\gamma^2 + \sigma_\omega^2 + \sigma_e^2} \qquad (5)$$

where the variance σ_τ^2 is nominator and the total variance components, i.e., sum of variances of

subject error, laboratory error, kit lot error, the interaction between oral fluid and laboratory and measurement error (denominator).

Variance estimates for equations 2 and 4 were calculated from random effect models solved using commercial statistical software.[d] S/P ratios within linear random effects models were analyzed using the GLIMMIX procedure. Oral fluid samples, laboratory, kit lot number, and interaction between oral fluid and laboratory were treated as random effects.

RESULTS

A total of 3,156 PRRSV oral fluid ELISA results were produced by the 12 laboratories that participated in Phase 1 (Figure 1). Among these 1,581 (50.1%) were positive at a S/P cutoff of ≥ 0.40. In Phase 2 (Figure 2), the 11 participating laboratories produced 2,885 results with 1,438 positives. As illustrated in Figures 1 and 2, the dispersion of the results increased as the magnitude of the S/P response increased. The number of samples tested, mean ELISA S/P ratios, and S/P standard deviation are shown in Table 1 by laboratory. Among the samples of unknown PRRSV antibody status ($n = 224$), discordant results were produced in 7 samples in Phase 1 and 5 samples in Phase 2 (Table 2).

Repeatability (within laboratory) reliability coefficients calculated from linear random effects models are shown in Table 3. The within laboratory agreement ranged from 0.942 to 0.997 in Phase 1 and 0.953 to 0.998 in Phase 2. Laboratory 6 reported one false positive result in Phase 1, which resulted in a within laboratory reliability coefficient of 0.724 for that laboratory. No statistically significant difference in repeatability was detected for any laboratory when comparing Phase 1 vs. Phase 2 reliability coefficients.

Reproducibility (between laboratories) reliability coefficients were calculated using results from 224 samples of unknown status and 39 samples of known status samples tested in all laboratories. As shown in Table 3, this allowed for the calculation of reproducibility reliability coefficients for three conditions: Case 1 - samples of unknown status ($n = 224$); Case 2 - samples of known status ($n = 39$), and Case 3 - all samples ($n = 263$). Table 4 lists the variance components estimates used to calculate the reproducibility reliability coefficients for Case 3, i.e.,

oral fluid samples, laboratory, ELISA kit lot, ELISA plate, and measurement error. Among these sources of variation, oral fluid samples accounted for 95.67% of the total variation in Phase 1 and 93.21% in Phase 2.

DISCUSSION

A recent analysis of the PRRSV oral fluid ELISA reported test sensitivity of 94.7% (95% CI: 92.4, 96.5) and specificity of 100% (95% CI: 99.0, 100.0).[5] Further evaluation of this ELISA was motivated by the fact that the protocol differs significantly from the protocol for the PRRSV serum antibody ELISA. That is, in contrast to the serum antibody assay, the PRRSV oral fluid ELISA uses a sample volume of 250 µl (100 µl for serum), a 16 hr incubation period at 4° C (30 min at 22°C for serum), and requires the operator to purchase and correctly prepare the appropriate concentration of anti-swine IgG$_{FC}$ secondary antibody.[3,5] To fully evaluate the precision of the assay, the study was conducted in a ring test involving various North American laboratories expected to offer this test to swine clientele on a routine basis.

Visual analysis of the cumulative results (Figure 1) showed greater dispersion of S/P ratios in Phase 2, i.e., laboratories tested the samples on a total of 5 ELISA kit lots using reagents they had prepared, as compared with Phase 1, i.e., samples were tested on a single kit lot with pre-diluted reagents. However, as shown in Table 2, relatively few discordant qualitative results were seen either in Phase 1 (7 discordant samples) or Phase 2 (5 discordant samples). With one exception, discordant results were single events. The one exception was a sample (#193) which was reported positive in 3 of 12 laboratories in Phase 1 and 2 of 11 laboratories in Phase 2. The frequency of discordant results for this sample can be explained by the fact its mean S/P value lies close to the positive cutoff (S/P ≥ 0.40). Thus, small differences in quantitative measurements produced divergent categorical results.

Reliability coefficients were used as quantitative summary measures of agreement. By definition, the repeatability reliability coefficient is a measure of intra-laboratory assay agreement and the reproducibility reliability coefficient is a measure of inter-laboratory agreement.[12] Shoukri and Pause[11] have suggested that coefficients > 0.75 indicate excellent reliability. The limitation of this approach is that reliability coefficients reflect both the assay

and the distribution of the analyte in the samples tested. That is, given the same assay, reliability coefficient estimates will be affected as the concentration and/or prevalence of the analyte in the population of samples tested varies.[2]

Repeatability reliability coefficients were calculated using 13 samples tested on each ELISA plate in every laboratory (Table 3). Coefficients ranged from 0.724 to 0.997 (Phase 1) and 0.953 to 0.998 (Phase 2). No statistically significant differences were detected in Phase 1 vs. Phase 2 repeatability reliability coefficients in the 11 laboratories participating in both phases. Based on the reliability coefficient criteria described by Shoukri and Pause,[11] the results were shown to be highly repeatable in the participating laboratories.

Reproducibility reliability coefficients were calculated for three cases (Table 3): samples of unknown PRRSV antibody status (Case 1, $n = 224$), samples of known status (Case 2, $n = 39$), and all samples tested in every laboratory, regardless of PRRSV antibody status (Case 3, n = 263). Among the three cases, reliability coefficients ranged from 0.937 to 0.964 in Phase 1 and 0.922 to 0.935 in Phase 2, indicating excellent reproducibility across laboratories.[11] Based on overall samples results, the variance components estimated that 95.7 % of the total variation in Phase 1 and 93.2 % in Phase 2 could be attributed to the oral fluid samples themselves (Table 4), with the remainder of the variation allocated to laboratory, ELISA kit lot, ELISA plate (laboratory), and measurement error.

Cumulatively, the current study showed that a commercial serum antibody ELISA adapted to oral fluid samples was highly reproducible within and between laboratories responsible for swine health testing. These results support the routine use of this test in laboratories providing diagnostic service to pig producers. Thus, herd monitoring based on oral fluid antibody testing could be one part of a PRRSV control and/or elimination program. The successful ring trial of one commercial serum antibody ELISA to the oral fluid diagnostic suggests that antibody assays for other pathogens could also be modified to this approach.

SOURCES AND MANUFACTURERS

DECLARATION OF CONFLICTING INTERESTS

FUNDING

REFERENCES

1. Detmer SE, Patnayak DP, Jiang Y, et al.: 2011, Detection of Influenza A virus in porcine oral fluid samples. J Vet Diagn Invest 23:241–247.

2. Jordan D, Kirkland P, Morris S, et al.:2012, Describing the within laboratory and between laboratory agreement of a serum ELISA in a national laboratory network. Prev Vet Med 104(3–4):240-248.

3. Kittawornrat A: 2011, Procedure for X3 porcine reproductive and respiratory syndrome virus (PRRSV) ELISA – Oral fluid. Document 9.3490. Quality Systems, Veterinary Diagnostic Laboratory, Iowa State University, Ames, Iowa.

4. Kittawornrat A, Engle M, Johnson J, et al.:2010, Porcine reproductive and respiratory syndrome virus (PRRSV) in serum and oral fluid samples from individual boars: will oral fluid replace serum for PRRSV surveillance? Virus Res 154:170-176.

5. Kittawornrat A, Prickett J, Wang C, et al.:2012, Detection of porcine reproductive and respiratory syndrome virus (PRRSV) antibodies in oral fluid specimens using a commercial PRRSV serum antibody ELISA. J Vet Diagn Invest 24:262-269.

6. Malamud D: 2011, Saliva as a Diagnostic Fluid. Dent Clin North Am 55:159-178.

7. Prickett JR, Johnson J, Stone S, et al.:2011, Prolonged detection of PCV2 and anti-PCV2 antibody in oral fluids following experimental inoculation. Transbound Emerg Dis 58:121-127.

8. Prickett JR, Kim W, Simer R, et al.:2008, Oral-fluid samples for surveillance of commercial growing pigs for porcine reproductive and respiratory syndrome virus and porcine circovirus type 2 infections. J Swine Health Prod 16:86-91.

9. Prickett J, Simer R, Christopher-Hennings J, et al.: 2008, Detection of porcine reproductive and respiratory syndrome virus infection in porcine oral fluid samples: A longitudinal study under experimental conditions. J Vet Diagn Invest 20:156-163.

10. Rousson V, Gasser T, Seifert B: 2002, Assessing intrarater, interrater and test–retest reliability of continuous measurements. Stat Med 21:3431-46.

11. Shoukri MM, Pause CA: 1999, Statistical analysis of measurements reliability. In: Statistical Methods for Health Sciences, 2nd ed., pp. 30-53. CRC Press, Boca Raton.

12. United Nations: 2009, Glossary of terms for quality assurance and good laboratory practices. United Nations Office on Drugs and Crime. Laboratory and Scientific Section, Vienna Austria.

Table 1. Summary of results used to estimate test variance components in the PRRSV oral fluid ELISA ring trial

Lab	No. of samples	Phase 1 Test Results[*]				Phase 2 Test Results[†]			
		Mean S/P	SD	No. Positive[‡]	No. Negative[‡]	Mean S/P	SD	No. Positive[‡]	No. Negative[‡]
1	39[§]	2.47	0.58	36	3	2.51	0.57	36	3
	224[¦]	1.03	1.32	96	128	0.92	1.17	95	129
2	39	2.86	0.61	36	3	2.34	0.59	36	3
	224	0.98	1.32	95	129	0.83	1.06	95	129
3	39	2.53	0.63	36	3	3.10	0.66	36	3
	224	0.84	1.13	95	129	0.92	1.28	95	129
4	39	2.99	0.59	36	3	3.10	0.55	36	3
	224	1.14	1.50	95	129	1.17	1.52	98	126
5	39	3.07	0.58	36	3	2.68	0.60	33	2
	224	1.13	1.45	96	128	1.01	1.27	96	128
6	39	2.96	0.66	37	2	Laboratory did not participate in Phase 2 testing			
	224	0.91	1.20	95	129				
7	39	2.65	0.61	36	3	2.39	0.54	36	3
	224	0.94	1.18	95	129	0.73	0.93	95	129
8	39	2.74	0.64	36	3	2.52	0.62	36	3
	224	0.97	1.29	97	127	0.83	1.07	95	129
9	39	2.69	0.61	36	3	2.37	0.56	33	2
	224	0.93	1.25	96	128	0.78	1.06	95	129
10	39	2.49	0.60	36	3	2.19	0.66	36	3
	224	0.88	1.17	97	127	0.69	0.93	95	129
11	39	2.84	0.68	36	3	2.32	0.68	36	3
	224	0.87	1.18	95	129	0.68	0.92	94	130
12	39	2.54	0.60	36	3	2.33	0.65	36	3
	224	0.87	1.11	96	128	0.77	1.03	95	129

[*] Laboratories were provided PRRSV ELISA kit (same lot number) and all reagents needed to test the samples.

[†] Laboratories provided their own PRRSV ELISA kit and prepared all reagents needed to test the samples. Note: 4 known-status samples in laboratories 5 and 9 were of insufficient volume to test in Phase 2

[‡] Samples with S/P ≥ 0.40 were classified as positive (Kittawornrat et al., 2012)

[§] S/P ratios from samples with known PRRSV antibody status

[¦] S/P ratios from samples with unknown PRRSV antibody status

Table 2. Summary of PRRSV oral fluid ELISA discordant results among 224 oral fluid samples of unknown PRRSV antibody status (S/P cutoff ≥ 0.40)

Phase	Sample random number	Laboratories participating in the PRRSV oral fluid ELISA ring test (S/P ratios)											
		1	2	3	4	5	6	7	8	9	10	11	12
Phase 1	4	-0.05	-0.03	0.00	-0.02	0.02	0.00	0.02	-0.03	-0.02	0.01	-0.04	**1.21**
	16	0.05	-0.01	0.00	-0.01	0.01	0.04	0.04	0.03	0.00	**0.72**	0.00	0.01
	62	0.07	-0.04	-0.01	0.00	0.02	0.02	0.04	0.00	0.07	**0.96**	0.00	0.02
	112	-0.03	0.00	-0.02	-0.05	0.00	-0.08	0.03	**0.51**	-0.01	0.00	-0.06	-0.02
	151	**1.19**	-0.03	-0.04	-0.06	0.00	-0.04	-0.01	0.08	-0.03	-0.02	-0.04	0.04
	175	-0.03	-0.01	-0.04	-0.05	0.00	-0.04	0.02	-0.01	**3.03**	-0.02	-0.02	0.00
	193	0.27	0.26	0.26	0.39	**0.45**	0.27	0.33	**0.41**	**0.40**	0.26	0.26	0.25
Phase 2	2	0.98	0.86	0.68	1.60	1.07	NA	0.83	0.96	0.80	0.61	NA	**0.31**
	12	0.17	0.22	0.12	**0.43**	0.25	NA	0.20	0.19	0.14	0.14	0.12	0.15
	53	-0.03	0.00	-0.02	**0.51**	0.01	NA	0.00	0.01	-0.07	0.01	-0.01	0.00
	193	0.32	0.32	0.27	**0.56**	**0.51**	NA	0.30	0.28	0.30	0.17	0.20	0.22
	231	0.81	0.51	0.51	0.69	0.70	NA	0.43	0.47	0.62	0.53	**0.33**	0.48

* PRRSV ELISA kit and all reagents required to test the samples were provided to each laboratory.

† Laboratories provided their own PRRSV ELISA kit and prepared all reagents required to test the samples.

Table 3. PRRSV oral fluid antibody ELISA reliability coefficients*

Repeatability reliability coefficients	Phase 1[†]	Phase 2[‡]
Laboratory 1	0.991	0.995
Laboratory 2	0.986	0.989
Laboratory 3	0.996	0.994
Laboratory 4	0.967	0.986
Laboratory 5	0.990	0.998
Laboratory 6	0.724	(no results provided)
Laboratory 7	0.942	0.953
Laboratory 8	0.950	0.987
Laboratory 9	0.994	0.967
Laboratory 10	0.969	0.956
Laboratory 11	0.997	0.997
Laboratory 12	0.991	0.960
Reproducibility reliability coefficients	**Phase 1[†]**	**Phase 2[‡]**
Case 1: samples of unknown status	0.964	0.935
Case 2: samples of known status	0.937	0.922
Case 3: all samples	0.957	0.932

* Repeatability reliability coefficients were calculated using the data from 13 samples tested on each plate in each laboratory. Reproducibility reliability coefficients were calculated for 3 cases: all samples ($n = 263$), field samples of unknown PRRSV antibody status ($n = 224$), and samples of known PRRSV antibody status ($n = 39$). Shouki and Pause (1999) interpret reliability coefficients as excellent (> 0.75), good (0.40 to 0.75), and poor (< 0.4).

† PRRSV ELISA kit and all reagents required to test the samples were provided to each laboratory.

‡ Laboratories provided their own PRRSV ELISA kit and prepared all reagents required to test the samples.

Table 4. Estimates of the variance components used to calculate reproducibility reliability coefficients for the PRRSV oral fluid antibody ELISA*

Variation by source	Phase 1[†]		Phase 2[‡]	
	Variance (95% CI)	% of model variance	Variance (95% CI)	% of model variance
Oral fluid sample	1.768 (1.494 – 2.126)	95.67	1.403 (1.185 – 1.688)	93.21
Laboratory	0.007 (0.003 – 0.048)	0.39	0.022 (0.009 – 0.093)	1.43
ELISA kit lot-to-lot	Does not apply because all laboratories used kits from the same lot		0.007 (0.001 – 158.2)	0.60
ELISA plate (laboratory)	0.010 (0.006 – 0.023)	0.54	0.003 (0.002 – 0.008)	0.21
Measurement error	0.063 (0.060 – 0.066)	3.40	0.071 (0.067 – 0.075)	4.70

* Variance components were estimated in a linear random effects model using the S/P data from all oral fluid samples (n = 263).

† PRRSV ELISA kit and all reagents required to test the samples were provided to each laboratory.

‡ Laboratories provided their own PRRSV ELISA kit and prepared all reagents required to test the samples.
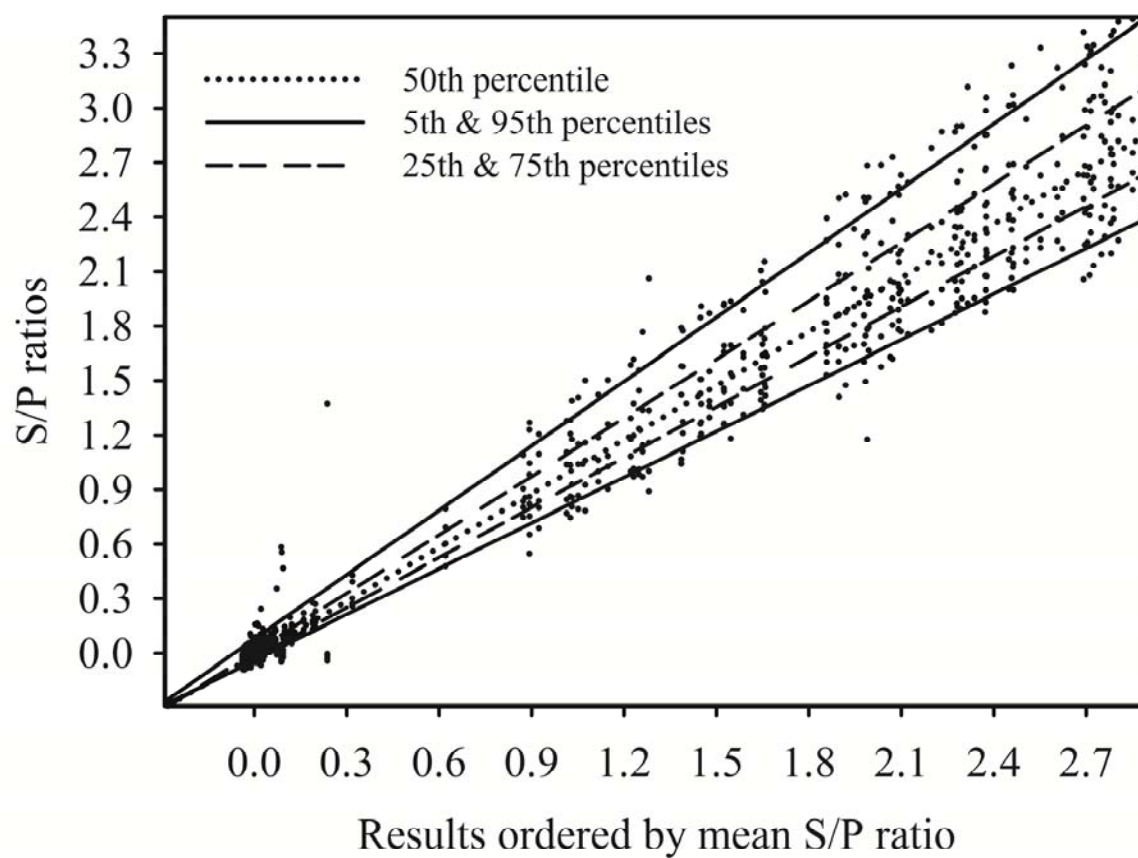
Figure 1. Results from 224 samples tested in each of 12 laboratories. All laboratories using the same PRRSV oral fluid antibody ELISA kit lot and reagents (Phase 1).
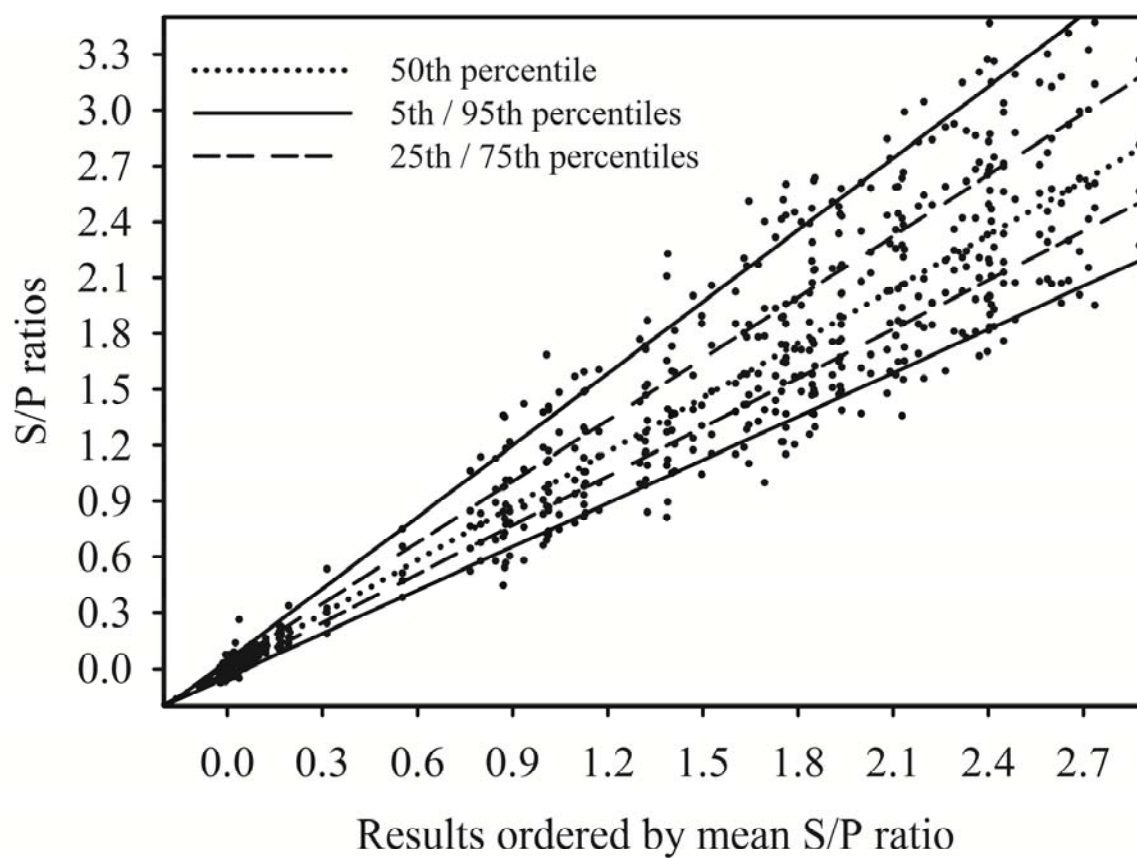
Figure 2. Results from 224 samples tested in each of 11 laboratories on a total of 5 PRRSV ELISA kit lots using reagents they had prepared (Phase 2).

CHAPTER 5. KINETICS OF THE PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME VIRUS (PRRSV) HUMORAL IMMUNE RESPONSE IN SWINE SERUM AND ORAL FLUIDS COLLECTED FROM INDIVIDUAL BOARS

Apisit Kittawornrat, Mark Engle, Yaowalak Panyasing, Chris Olsen, Kent Schwartz, Anna Rice, Sergio Lizano, Chong Wang, Jeffrey Zimmerman

ABSTRACT

Background: The object of this study was to describe and contrast the kinetics of the humoral response in serum and oral fluid specimens during acute porcine reproductive and respiratory syndrome virus (PRRSV) infection. The study involved three trials of 24 boars each. Boars were intramuscularly inoculated with a commercial modified live virus (MLV) vaccine (Trial 1), a Type 1 PRRSV field isolate (Trial 2), or a Type 2 PRRSV field isolate (Trial 3). Oral fluid samples were collected from individual boars on day post inoculation (DPI) -7 and 0 to 21. Serum samples were collected from all boars on DPI -7, 0, 7, 14, 21 and from 4 randomly selected boars on DPI 3, 5, 10, and 17. Thereafter, serum and oral fluid were assayed for PRRSV antibody using antibody isotype-specific ELISAs (IgM, IgA, IgG) adapted to serum or oral fluid.

Results: Statistically significant differences in viral replication and antibody responses were observed among the three trials in both serum and oral fluid specimens. PRRSV serum IgM, IgA, and IgG were first detected in samples collected on DPI 7, 10, and 10, respectively. Oral fluid IgM, IgA, and IgG were detected in samples collected between DPI 3 to 10, 7 to 10, and 8 to 14, respectively.

Conclusions: This study enhanced our knowledge of the PRRSV humoral immune response and provided a broader foundation for the development and application of oral fluid antibody-based diagnostics.

BACKGROUND

The presence of systemic and locally-produced antibodies in oral fluid has led to its use as a diagnostic specimen for a variety of infectious diseases. In humans, oral fluids have been used in the diagnosis of human immunodeficiency virus (HIV), Hepatitis A, B, and C viruses, measles, mumps and other infectious diseases [1]. In swine, antibodies against a variety of economically significant pathogens have been reported in oral fluids, including classical swine fever virus [2-3], porcine circovirus type 2 [4], porcine reproductive and respiratory syndrome virus [5-6], swine influenza virus [7], transmissible gastroenteritis virus [8], *Actinobacillus pleuropneumoniae* [9], and *E. coli* [10]. In large measure, the kinetics of the antibody response against individual agents has not been described. Therefore, the purpose of present study was to describe and contrast the ontogeny of PRRSV IgM, IgA, and IgG in oral fluids and serum specimens collected from individually housed boars during acute PRRSV infection.

RESULTS

PRRSV antibody isotypes in serum

The PRRSV antibody isotype (IgM, IgA, and IgG) responses in serum samples are shown in Figure 1 and Table 1. Estimates for DPI -7, 0, 7, 14, 21 were based on data from 72 boars, whereas estimates for DPIs 3, 5, 10, and 17 were based on a subset of 12 animals randomly selected from the 72 boars. Based on pairwise comparisons, statistically significant levels of IgM were detected by DPI 7, peaked at DPI 14, and remained stable through DPI 21. In contrast, statistically significant levels of IgA and IgG were detected by DPI 10, after which they remained stable (IgA) or increased significantly (IgG) through DPI 21. Further analysis showed that IgM and IgA responses were associated with DPI (IgM: $p < 0.0001$, IgA: $p < 0.0001$), trial (IgM: $p < 0.0001$, IgA: $p < 0.0149$), and the interaction between trial and DPI (IgM: $p < 0.0001$, IgA: $p < 0.0001$). In contrast, DPI ($p < 0.0001$) was the only factor associated with the IgG response. Neither the age of the boar at the time of inoculation nor the quantity of oral fluid collected from each boar had a significant effect on IgM, IgA, or IgG.

PRRSV antibody isotype in oral fluid samples

The PRRSV antibody isotype (IgM, IgA, and IgG) responses in oral fluid samples are shown in Figure 2 and Table 1 for oral fluid samples collected on DPI -7, 0-14, 17, and 21. Estimates were based on ≥66 oral fluid samples at each sampling point. IgM S/P ratios were statistically significant on DPI 7, peaked at DPI 11, and declined thereafter. Levels of IgA and IgG were significant on DPI 8 and increased thereafter through the end of the experiment (DPI 21). Factors significantly associated with IgM and IgA S/P ratios included trial (IgM: p < 0.0001, IgA: p = 0.0273), DPI (IgM: p < 0.0001, IgA: p < 0.0001), oral fluid volume (IgM: p = 0.0002, IgA: p < 0.0001), and the interaction between trial and DPI (IgM: p < 0.0001, IgA: p < 0.0001). Factors associated with IgG included DPI (p < 0.0001) and the interaction of trial by DPI (p < 0.0001). Regardless of isotype, boar age at the time of inoculation had no significant effect on antibody response.

Comparison of PRRSV antibody responses in serum and oral fluid

A comparison of the qualitative antibody response in serum vs. oral fluid found no significant difference in the proportion of ELISA positive results on DPI 0 – 14, 17, and 21 (Table 2). No significant difference was detected in the number of positive results for each pair-wise sample combination (serum vs. oral fluid) by trial, DPI, or trial by DPI. On DPI 21, 100% of serum and oral fluid samples were positive with mean S/P ratios of 1.69 (95% Confidence Interval [CI]: 1.58, 1.79) and 2.60 (95% CI: 2.34, 2.86), respectively. An analysis of the quantitative antibody isotype responses in serum and oral fluid samples using test results from samples collected on DPI -7, 0 – 14, 17, 21 estimated Pearson's correlation coefficient as r = 0.84, 0.78, and 0.90 for IgM, IgA, and IgG responses, respectively (Figure 3).

Comparison between viremia levels and antibody responses

All serum samples from DPI -7 and 0 (n = 144) were PRRSV qRT-PCR negative, whereas all serum samples from boars tested on DPI 3 (n = 12) and DPI 7 (n = 72) were positive. PRRSV was detected in oral fluids from 7 of 69 boars at DPI 1, 52 of 68 boars at DPI 2, 66

of 70 at DPI 3, and all boars were PRRSV qRT-PCR positive at DPI 4. A comparison of matched samples from individual boars showed that oral fluid was equal to serum for the detection of PRRSV at DPI 7 and more likely to be positive than serum on DPI 14 and 21. These data are reported in detail elsewhere [11].

To evaluate the association between viremia and antibody responses, cumulative serum and oral fluid PRRSV qRT-PCR (log_e geq/µl) and antibody isotype (IgM, IgA, IgG) responses over time were re-expressed as area under the curve (AUC) (MedCalc®) prior to performing the analyses. The mean qRT-PCR and antibody isotype AUCs for serum and oral fluid are given in Table 3. Statistical analysis (ANOVA) of main effects showed significant differences among trials ($p < 0.001$), sample type ($p < 0.001$), and quantitative responses (PRRSV, IgM, IgA, IgG; $p < 0.001$). With the exception of serum IgG, statistically significant differences in means were detected among trials both in the replication of PRRSVs and in antibody responses (Tukey's Honestly Significant Difference (HSD) test). However, at the individual boar level, correlation analysis found a weak association between PRRSV viremia AUC and serum IgM, IgA, or IgG AUCs (r = 0.3762, 0.2915, and 0.0005). Likewise, the correlation was weak between PRRSV oral fluid AUC and oral fluid IgM, IgA, or IgG AUCs (r = 0.3147, 0.2671, and 0.2137).

DISCUSSION

By definition, oral fluid is a mixture of saliva, oral mucosal transudate, and gingival crevicular fluid recovered from the buccal cavity using an absorptive device [12]. Among a variety of other constituents, oral fluid contains both locally-derived and systemic antibodies [13]. Thus, pathogen-specific IgM, IgA, and IgG for PRRSV [5], influenza A virus [7], and porcine circovirus type 2 [4] could be detected in oral fluid samples collected from groups of pigs (pens) under either experimental or field conditions. The largest proportion of locally produced antibody consists of dimeric secretory IgA (SIgA) produced by plasma cells in salivary glands and duct-associated lymphoid tissue (DALT) [14]. IgM and IgG are also produced in these tissues, but the majority of IgM and IgG in oral fluid is derived from serum via the gingival crevicular fluid [15].

In the present experiment, the collection of paired oral fluid and serum samples from 72 individually-housed boars inoculated with three different PRRSV isolates allowed for a more comprehensive evaluation of the onset and magnitude of serum and oral fluid antibody isotype responses, as well as the variation therein. Statistically significant differences in viral replication and antibody responses were observed among the three trials in both serum and oral fluid specimens (Table 3). Since each trial included only one virus isolate, statistically valid comparisons of the effects of virus isolates on PRRSV replication and antibody responses were not possible. Nevertheless, the statistically significant differences in virus replication and antibody response observed among trials was consistent with previous reports of virus isolate-dependent differences in the magnitude of replication in pigs [16] and corresponding differences in antibody response [16-17]. However, at the individual boar level, the correlation between virus replication and antibody response was relatively weak. Thus, what was true for the group of boars in a trial did not necessarily apply to an individual boar.

The purpose of this study was to describe and contrast the kinetics of PRRSV antibody in oral fluids and serum. PRRSV serum IgM, IgA, and IgG were first detected in samples collected on DPI 7, 10, and 10, respectively. These results were compatible with prior reports describing the detection of PRRSV serum IgM between DPI 5 and 7 [18-20], IgG between DPI 9 and 11 [19, 21], and IgA at DPI 14 [19]. PRRSV oral fluid IgM, IgA, and IgG appeared concurrently with serum antibodies, but collection of daily oral fluid samples provided more precise estimates. That is, oral fluid IgM, IgA, and IgG were detected in samples collected between DPI 3 to 10, 7 to 10, and 8 to 14, respectively. There are no prior reports on PRRSV oral fluid antibody kinetics in individual animals with which to compare these results. However, we previously reported the detection of PRRSV oral fluid IgM and IgG in pen-based oral fluid samples from experimentally inoculated animals on DPI 7 [5]. Thus, the PRRSV serum and oral fluid antibody responses observed in this study were in agreement with prior observations on PRRSV and our general understanding of the humoral immune response as reflected in these sample matrices [14, 22]. Most significantly, this

study provided a broader foundation for understanding, developing, and interpreting oral fluid antibody-based diagnostics in the context of the humoral immune response.

CONCLUSIONS

This study demonstrated that anti-PRRSV antibody isotypes can be detected in oral fluid specimens. These results were compatible with prior reports describing the detection of anti-PRRSV antibody in both serum and oral fluid. Detection of PRRSV antibody in oral fluids collected from individual boars could provide an effective approach for monitoring PRRSV infection in boar studs. Successful oral fluid collection and testing from individual boars suggests that approach could also be applied to population in swine production systems, i.e. pen-housed sows, farrowing crates, etc.

METHODS

Experimental design

A total of 72 boars ranging from 6 months to 3.6 years of age under the ownership of PIC North America (Hendersonville, TN, USA) were used in this study. Housing, feed rations, animal care guidelines, and sampling protocols were approved and supervised by the PIC USA Health Assurance and Welfare department. In 3 trials of 24 boars each, animals were intramuscularly (i.m.) inoculated with either modified-live virus (MLV) PRRSV vaccine (Trial 1), a Type 1 field isolate (Trial 2), or a Type 2 field isolate (Trial 3). Serum and oral fluid samples were collected from all boars beginning 7 days prior to inoculation and continuing through 21 days post inoculation (DPI). After the completion of Trial 3, samples were completely randomized and tested for PRRSV IgM, IgA, and IgG antibody isotypes. Descriptive and comparative statistical analyses were conducted to describe and compare PRRSV antibody responses in serum and oral fluid and evaluate differences among individual boars and between trials.

Animals and animal care

Boars were obtained from two Midwest USA breeding stock sources documented to be free of PRRSV infection. Culled boars ($n = 24$) ranged from one year to 3.6 years of age and select boars ($n = 48$) ranged from 5 to 6 months of age. The boars were housed in a commercial production facility equipped with nipple drinkers, concrete slatted flooring, curtains, and tunnel ventilation. Feeder space, water delivery, square footage per animal, sanitation, and ventilation parameters met or exceeded PIC North America health assurance and welfare requirements. Upon arrival, animals were housed individually in crates (Hog Slat, Inc., Newton Grove, NC USA) and fed a commercial corn/soy swine diet (Land O' Lakes® Farmland Feed, Roland, IA USA) at a rate of 4 pounds per animal per day for acclimation/training and 7 pounds per animal per day thereafter.

Porcine reproductive and respiratory syndrome viruses

In Trial 1, 24 boars were inoculated i.m. with 2 ml of a commercial MLV vaccine (Ingelvac® PRRS MLV, Boehringer Ingelheim Vetmedica, Inc., St. Joseph, MO 64506) rehydrated and administered according to the instructions provided by the manufacturer. In Trial 2, 24 boars were inoculated i.m. with 2 ml of a Type 1 PRRSV (isolate D09-012131) at an estimated concentration of 1 x $10^{5.5}$ median tissue culture infectious dose ($TCID_{50}$) per ml. Isolate D09-012131 was isolated from serum samples submitted to the University of Minnesota Veterinary Diagnostic Laboratory (St. Paul, MN USA) in March 2009 as part of a routine monitoring program in a sow herd located in Illinois USA and propagated on pulmonary alveolar macrophage cells, as described elsewhere [11]. In Trial 3, 24 boars were IM inoculated with 2 ml of a Type 2 PRRSV isolate (MN-184, GenBank accession no. AY656992) at a concentration of 1 x $10^{4.5}$ $TCID_{50}$ per ml. Isolate MN-184 (kindly provided by Dr. Scott Dee, University of Minnesota) was propagated on MARC-145 cells [23].

Sample collection

Oral fluid collection

Oral fluid samples were collected daily from individually-housed boars beginning 7 days prior to inoculation and continuing through 21 days post inoculation (DPI) using a procedure

described by Kittawornrat et al. [11]. In brief, oral fluid samples were collected by allowing boars to chew on 1.6 cm (5/8") cotton rope (Web Rigging Supply, Inc., Lake Barrington, IL USA). Prior to collection, ropes were soaked with a solution of sucrose and apple juice (unsweetened apple juice with 50% (v/v) sucrose) and then air-dried. To collect oral fluid samples, ropes were placed in "rope holders" fixed at the front of each pen for 20 minutes. Oral fluids were deposited as the boars chewed on the rope. To recover the oral fluid specimens, the bottom 15 cm (~6") of the rope (wet portion) was inserted into a 3.8 liter (one gallon) re-sealable plastic bag and severed from the dry portion of the rope. The bag with the wet rope inside was passed through a wringer (Dyna-Jet Products, Overland Park, KS USA), causing the fluid to pool in the bottom of the bag. Samples were then decanted into a 50 ml centrifuge tube and the volume recorded. Thereafter, samples were centrifuged at 1,000 x g for 10 minutes at 4°C, aliquoted into 5 ml plastic tubes (Becton, Dickinson and Company, Bedford, MA USA), and stored at -80°C until assayed.

Serum collection

In each trial, serum samples were collected from all boars on DPI -7, 0, 7, 14, and 21. Additional serum samples were collected on DPI 3, 5, 10, 17 from a subset of boars ($n = 4$) randomly selected at the beginning of each trial. Blood was collected by jugular venipuncture using serum separation tubes (Corvac®, Tyco Healthcare Group LP, Mansfield, MA USA). Samples were centrifuged at 1,000 x g for 10 minutes and the serum was aliquoted into 5 ml plastic tubes (Becton, Dickinson and Company) and stored at -80ºC until assayed.

PRRSV antibody ELISAs

Commercial PRRSV serum antibody ELISA

All serum samples were assayed for PRRSV antibodies using a commercial indirect ELISA (PRRS X3 Ab Test, IDEXX Laboratories, Inc., Westbrook, ME USA) performed according to the manufacturer's instruction. As recommended by the manufacturer, a positive result was defined as a sample-to-positive (S/P) ratio ≥ 0.4. Modifications to the commercial serum ELISA for the detection of antibody isotypes in serum and oral fluid are described below and

listed in Table 4.

PRRSV antibody isotypes in serum

The commercial indirect ELISA (PRRS X3 Ab Test) was modified to detect PRRSV-specific IgM, IgA, and IgG antibody isotypes in serum. In brief, serum samples were diluted 1:40 (5µl serum sample + 195µl kit diluent) for IgM and IgG and 1:5 (40µl serum sample + 160 µl kit diluent) for IgA. 100 µl of diluted serum was then transferred to the PRRSV antigen-coated plates and incubated for 30 minutes at 22°C. After washing 3 times with 1X kit wash solution (400 µl), appropriately diluted horseradish peroxidase (HRPO)-conjugated anti-pig immunoglobulin (Ig) antibody (IgM (A100-100P), IgA (A100-102P), or IgG_{Fc} (A100-104P) (Bethyl Laboratories, Montgomery, TX USA) was added to each well and incubated for 30 minutes at 22°C. Thereafter, plates were washed three times with kit washing solution, after which 100µl of tetramethylbenzidine (TMB) was added to each well and the plates incubated at 22°C for 15 minutes. At precisely 15 minutes, 100 µl of kit stop solution was added to each well. The plates were read at 650 nm using an ELISA plate reader (EL800 micro plate reader, Bio Tek® Instruments Inc., Winooski, VT) controlled by commercial software (Gen5™ Bio Tek® Instruments Inc., Winooski, VT USA) and the reactions measured as optical density (OD).

PRRSV antibody isotypes in oral fluid

Modification of the commercial PRRSV ELISA for the detection of PRRSV-specific IgM, IgA, and IgG antibody in swine oral fluid has previously been described [5]. In brief, oral fluid samples were diluted 1:2 (150 µl oral fluid sample + 150 µl kit diluent). 250 µl of diluted oral fluid was then transferred to PRRSV antigen-coated plates and incubated for 16 hours at 4°C. Thereafter, the plates were washed three times with 400 µl of 1X kit wash solution. To detect the reaction, 100 µl of a solution containing appropriately diluted HRPO-conjugated anti-pig Ig (M, A, or G) was added to each well and the plates incubated for 30 minutes at 22°C. The procedure for determining the optimal dilution of secondary antibody is described in preparation of secondary antibody section. After washing three times, 100 µl of TMB was added to each well and the plates incubated at 22°C for 15 minutes. Finally,

100 µl of kit stop solution was added to each well. As described in preparation of secondary antibody section, the plates were read at 650 nm and the reactions measured as optical density (OD).

Preparation of secondary antibody

To assure assay repeatability, the concentration of anti-pig Ig (M, A, or G) was standardized using the positive control OD value listed in the manufacturer's Certificate of Analysis as the benchmark. The general procedure for calculating the conjugate dilution was as follows: 4 dilutions of anti-pig Ig (M, A, or G) were prepared in bottles wrapped in aluminum foil using diluent provided by manufacturer (IDEXX Laboratories, Inc., Westbrook, MA USA) and then stirred for 48 hours at 4°C. The reactivity of the 4 dilutions was determined using negative and positive kit controls. Specifically, kit negative control was dispensed into 48 wells (one-half plate) and kit positive control in each of the remaining 48 wells (Figure 4). Negative control OD values were used to screen for non-specific reactions and positive control OD values were used to determine the equation of the line:

$$y = ax + c \qquad\qquad (1)$$

where (y) is the anti-pig Ig (M, A, or G) OD response, (a) is the slope of the line, (x) is the dilution of anti-pig Ig (M, A, or G), and (c) is the intercept (Figure 5). Thereafter, the correct dilution of anti-pig Ig (M, A, or G) was calculated by substituting the mean positive control OD from the Certificate of Analysis for "y" in Equation 1 and solving for "x".

The general procedure for preparing the appropriate dilution of anti-pig Ig (M, A, or G) was modified for serum and oral fluid antibody ELISAs. In this study, all serum and oral fluid specimens were tested on a single lot of ELISA kits. Thus, the appropriate dilution of anti-pig Ig was calculated specifically for the PRRSV indirect ELISA (PRRS X3 Ab Test) kit lot (#99-40959-W721).

For the serum ELISA, the correct dilution of anti-pig IgG$_{Fc}$ was determined by titrating 4

dilutions of anti-pig IgG$_{Fc}$ (1:14,000, 1:14,500, 1:15,000, 1:15,500) against 100 µl of kit positive control and generating the equation of the line, as described above. The appropriate dilution of anti-pig IgM was determined by titrating 4 dilutions of secondary antibody (1:4,000, 1:4,500, 1:5,000, 1:5,500) against 100 µl of a positive control consisting of a 1:40 dilution of a pool of serum from 72 boars at DPI 7. For anti-pig IgA, 4 dilutions (1: 1,000, 1:2,500, 1:3,000, 1:3,500) were titrated against 100 µl of a positive control consisting of a 1:5 dilution of a pool of serum from 72 boars at DPI 21. Diluted conjugate was simultaneously titrated against negative controls, i.e., kit negative control for anti-pig IgG$_{Fc}$ and a pool of serum from 72 boars at DPI 0 for anti-pig IgA (1:5) and anti-pig IgM (1:40).

The protocol for preparing the optimal secondary antibody dilution for the PRRSV oral fluid ELISA has been described elsewhere [5]. To prepare anti-pig IgG$_{Fc}$, 4 conjugate dilutions (1:1,000, 1:1,500, 1:2,000, 1:2,500) were titrated against 100 µl of kit negative and positive controls diluted 1:30 (10 µl kit control + 290 µl kit sample diluent). Controls for anti-pig IgM and anti-pig IgA consisted of oral fluid samples from PRRSV-negative pigs vaccinated with 2 ml of PRRS modified live virus (MLV) vaccine (Ingelvac® PRRS MLV, Boehringer Ingelheim Vetmedica, Inc., St. Joseph, MO USA). The sample collected immediately prior to vaccination was used as negative control. Samples collected on 10 and 56 days post vaccination were used as positive controls for anti-pig IgM and anti-pig IgA conjugates, respectively. Four dilutions of anti-pig IgM (1: 3,000, 1:3,500, 1:4,000, 1:4,500) and anti-pig IgA (1:1,000, 1:1,500, 1:2,000, 1:2,500) were used to calculate appropriate dilution, as described previously.

Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Detailed qRT-PCR protocols for serum and oral fluids are reported elsewhere [11, 24]. In brief, nucleic acid extraction from serum and oral fluid samples was performed using a commercial RNA extraction kit (Ambion® MagMax™-96 Viral RNA isolation kit, Applied Biosystems™, Foster City, CA USA). Real-time PCR was performed with commercial reagent sets (TaqMAN® NA and EU PRRSV Reagents and TaqMAN® NA and EU PRRSV Controls, Applied Biosystems™) using the following cycling conditions: 1 cycle at 45 ºC for

10 minutes, 1 cycle at 95 ºC for 10 minutes, 40 cycles of: 97 ºC for 2 seconds, 60 ºC for 40 seconds. Eight 10-fold serially-diluted (10^0 to 10^7 copies/µl) plasmid-derived commercial standards (TaqMAN® NA and EU PRRSV RNA controls, Applied Biosystems™) were run on each PCR plate and their Ct values used to derive a standard curve. Samples were quantified as genome equivalents per µl (geq/µl) by fitting the sample Cts to the standard curve using the AB7500 Fast System SDS Software (Applied Biosystems™).

Statistical analysis

All optical density (OD) data were converted to sample-to-positive (S/P) ratios prior to statistical analysis using the following formula:

$$S/P = [Sample\ A\ (650) - NC] / (PC - NC) \qquad (2)$$

where NC and PC represented the mean OD of the two negative control wells and two positive control wells, respectively. Statistical analyses were performed using SAS® Version 9.2 (SAS® Institute Inc., Cary, NC USA) and MedCalc® 12.3.0.0 (MedCalc Software, Mariakerke Belgium).

Initially, descriptive and comparative analyses were conducted to describe the onset, magnitude, and duration of PRRSV antibody isotype S/P ratios in serum and oral fluid. Thereafter, S/P results were analyzed in a linear mixed model with repeated measures (SAS® PROC GLIMMIX) using trial (1, 2, 3), DPI, boar age (month), oral fluid volume (ml), and their pairwise interactions as fixed effects and "boar" as the subject of repeated measures. Tukey's Honestly Significantly Difference (HSD) test was used to detect statistically significant differences between S/P ratios in each trial by DPI. Pearson's correlation coefficient (SAS® PROC CORR) was used to evaluate the overall quantitative relationship between IgM, IgA, and IgG S/P ratios in serum and oral fluid samples. Defining an S/P ratio ≥ 0.4 as positive (Kittawornrat et al., 2012), McNemar's test (SAS® PROC FREQ) for paired samples was used to determine whether the proportion IgG ELISA positive serum and oral fluid samples were significantly different by trial (1, 2, 3) and DPI.

The association between the level of PRRSV replication and the strength of the humoral response was evaluated in individual boars. The virus concentration ($\log_e$ geq/µl) and antibody isotype S/P responses (IgM, IgA, IgG) in serum and oral fluid over the course of the experiment were re-expressed as the area under the curve (AUC; MedCalc®) and evaluated using

Pearson's Correlation Coefficient (SAS® PROC CORR). In addition, the AUC data were evaluated for statistically significant differences among sample types (serum, oral fluid), trials (1, 2, 3), and quantitative responses (PRRSV, IgM, IgA, IgG) by analysis of variance (ANOVA). Sample type, trial, response, and their interaction were used as fixed effects in the model. Thereafter, Tukey's Honestly Significant Differences (HSD) test was used to test for statistically significant differences among trial means.

COMPETING INTERESTS

AUTHORS' CONTRIBUTIONS

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REFERENCES

1. Madar R, Straka S, Baska T: Detection of antibodies in saliva--an effective auxiliary method in surveillance of infectious diseases. *Bratisl Lek Listy* 2002, 103:38-41.

2. Corthier G: Swine fever: influence of passive immunity on pig immune response following vaccination with a live virus vaccine (Thiverval strain). *Ann Rech Vet* 1976, 7: 361-372.

3. Corthier G, Aynaud J: Comparison of the immune response in serum and bucco-pharyngeal secretions following immunization by different routes with a live hog cholera virus vaccine (Thiverval strain). *Ann Vet Res* 1977, 8:159–165.

4. Prickett JR, Johnson J, Murtaugh MP, Puvanendiran S, Wang C, Zimmerman JJ, Opriessnig T: Prolonged Detection of PCV2 and Anti-PCV2 Antibody in Oral Fluids Following Experimental Inoculation. *Transbound Emerg Dis* 2011, 58:121-127.

5. Kittawornrat A, Prickett J, Wang C, Panyasing Y, Ballagi A, Rice A, Main R, Johnson J, Rademacher C, Hoogland M, Rowland R, Zimmerman J: Detection of porcine reproductive and respiratory syndrome virus (PRRSV) antibodies in oral fluid specimens using a commercial PRRSV serum antibody ELISA. *J Vet Diagn Invest* 2012, 24:262-269.

6. Prickett J, Simer R, Christopher-Hennings J, Yoon KJ, Evans RB, Zimmerman JJ: Detection of Porcine reproductive and respiratory syndrome virus infection in porcine oral fluid samples: a longitudinal study under experimental conditions. *J Vet Diagn Invest* 2008, 20:156-163.

7. Panyasing Y, Goodell CK, Wang C, Kittawornrat A, Prickett JR, Schwartz KJ, Ballagi A, Lizano S, Zimmerman JJ: Detection of Influenza A Virus Nucleoprotein Antibodies in Oral Fluid Specimens From Pigs Infected Under Experimental Conditions Using a Blocking ELISA. *Transbound Emerg Dis*, in press.

8. De Buysscher E, Berman D: Secretory immune response in intestinal mucosa and salivary gland after experimental infection of pigs with transmissible gastroenteritis virus. *Am J Vet Res* 1980, 41:1214–1220.

9. Loftager M, Eriksen L, Nielsen R: Antibodies against Actinobacillus pleuropneumoniae serotype 2 in mucosal secretions and sera of infected pigs as demonstrated by an enzyme-

linked immunosorbent assay. *Res Vet Sci* 1993, 54:57–62.

10. De Buysscher E, Dubois R: Detection of IgA anti-Escherichia coli plasma cells in the intestine and salivary glands of pigs orally and locally infected with E. coli. *Adv Exp Med Biol* 1978, 107:593–600.

11. Kittawornrat A, Prickett J, Chittick W, Wang C, Engle M, Johnson J, Patnayak D, Schwartz T, Whitney D, Olsen C, Schwartz K, Zimmerman J: Porcine reproductive and respiratory syndrome virus (PRRSV) in serum and oral fluid samples from individual boars: Will oral fluid replace serum for PRRSV surveillance? *Virus Res* 2010, 154:170-176.

12. Atkinson JC, Dawes C, Ericson T, Fox PC, Gandara BK, Malamud D, Mandel ID, Navazesh M, Tabak LA: Guidelines for saliva nomenclature and collection. *Ann N Y Acad Sci* 1993, 694:xi-xii.

13. Ebersole JL: Humoral immune responses in gingival crevice fluid: local and systemic implications. *Periodontol* 2003, 31:135-166.

14. Brandtzaeg PER: Do Salivary Antibodies Reliably Reflect Both Mucosal and Systemic Immunity? *Ann N Y Acad Sci* 2007, 1098:288-311.

15. Challacombe SJ, Russell MW, Hawkes J: Passage of intact IgG from plasma to the oral cavity via crevicular fluid. *Clin Exp Immunol* 1978, 34:417-422.

16. Johnson W, Roof M, Vaughn E, Christopher-Hennings J, Johnson CR, Murtaugh MP: Pathogenic and humoral immune responses to porcine reproductive and respiratory syndrome virus (PRRSV) are related to viral load in acute infection. *Vet Immunol Immunopathol* 2004, 102:233-247.

17. Martínez-Lobo FJ, Díez-Fuertes F, Segalés J, García-Artiga C, Simarro I, Castro JM, Prieto C: Comparative pathogenicity of type 1 and type 2 isolates of porcine reproductive and respiratory syndrome virus (PRRSV) in a young pig infection model. *Vet Microbiol* 2011, 154:58-68.

18. Joo HS, Park BK, Dee SA, Pijoan C: Indirect fluorescent IgM antibody response of pigs infected with porcine reproductive and respiratory syndrome virus. *Vet Microbiol* 1997, 55:303-307.

19. Loemba HD, Mounir S, Mardassi H, Archambault D, Dea S: Kinetics of humoral

immune response to the major structural proteins of the porcine reproductive and respiratory syndrome virus. *Arch Virol* 1996, 141:751-761.

20. Park BK, Joo HS, Dee SA, Pijoan C: Evaluation of an indirect fluorescent IgM antibody test for the detection of pigs with recent infection of porcine reproductive and respiratory syndrome virus. *J Vet Diagn Invest* 1995, 7:544-546.

21. Yoon KJ, Zimmerman JJ, Swenson SL, McGinley MJ, Eernisse KA, Brevik A, Rhinehart LL, Frey ML, Hill HT, Platt KB: Characterization of the humoral immune response to porcine reproductive and respiratory syndrome (PRRS) virus infection. *J Vet Diagn Invest* 1995, 7:305-312.

22. Prickett JR, Zimmerman JJ: The development of oral fluid-based diagnostics and applications in veterinary medicine. *Anim Health Res Rev* 2010, 11:207-216.

23. Kim HS, Kwang J, Yoon IJ, Joo HS, Frey ML: Enhanced replication of porcine reproductive and respiratory syndrome (PRRS) virus in a homogenous subpopulation of MA-104 cells. *Arch Virol* 1993, 133:477−483.

24. Chittick WA, Stensland WR, Prickett JR, Strait EL, Harmon K, Yoon KJ, Wang C, Zimmerman JJ: Comparison of RNA extraction and real-time reverse transcription polymerase chain reaction methods for the detection of porcine reproductive and respiratory syndrome virus in porcine oral fluid specimens. *J Vet Diagn Invest* 2011, 23:248-253.

Table 1. Porcine reproductive and respiratory syndrome virus (PRRSV) antibody isotypes (IgM, IgA, and IgG) in oral fluid and serum samples collected from boars over day post inoculation (DPI)

DPI	Serum (mean S/P ratios)[a]				Oral fluid (mean S/P ratios)[a]			
	Samples	IgM	IgA	IgG	Samples	IgM	IgA	IgG
-7	72	0.10 [d]	0.35 [b]	0.03 [d]	70	0.02 [g]	0.15 [g]	0.01 [i]
0	72	0.19 [d]	0.31 [b]	0.03 [d]	68	0.02 [g]	0.08 [g]	0.01 [i]
1	-	-	-	-	69	0.04 [g]	0.12 [g]	0.01 [i]
2	-	-	-	-	68	0.04 [g]	0.13 [g]	0.02 [i]
3	12	0.05 [d]	0.23 [b]	0.01 [d]	70	0.07 [g]	0.16 [g]	0.02 [i]
4	-	-	-	-	68	0.13 [g]	0.24 [g]	0.01 [i]
5	12	0.12 [d]	0.30 [b]	0.02 [d]	67	0.05 [g]	0.11 [g]	0.01 [i]
6	-	-	-	-	66	0.09 [g]	0.12 [g]	0.01 [i]
7	72	0.82 [c]	0.58 [b]	0.05 [d]	69	0.30 [f]	0.23 [g]	0.03 [i]
8	-	-	-	-	66	1.28 [e]	0.41 [f]	0.17 [h]
9	-	-	-	-	66	2.32 [c]	0.95 [e]	0.59 [g]
10	12	3.56 [b]	1.70 [a]	0.87 [c]	70	2.72 [b]	1.45 [b, c]	0.99 [f]
11	-	-	-	-	70	2.95 [a]	1.75 [a]	1.41 [e]
12	-	-	-	-	70	2.70 [b]	1.59 [a, b]	1.78 [d]
13	-	-	-	-	69	2.64 [b]	1.64 [a]	2.05 [c]
14	72	4.02 [a]	1.91 [a]	1.60 [b]	68	2.32 [c]	1.56 [a, b]	2.28 [b]
17	12	3.51 [b]	1.94 [a]	1.69 [a, b]	69	1.53 [d]	1.25 [c,d]	2.63 [a]
21	72	3.18 [b]	1.70 [a]	1.76 [a]	68	1.02 [e]	1.14 [d, e]	2.60 [a]

[a] Within columns, different superscripted letters denote statistically significant differences between means ($p < 0.05$)

Table 2. Porcine reproductive and respiratory syndrome virus (PRRSV) serum[1] and oral fluid[2] ELISA qualitative results[3] by day post inoculation (DPI)

Trial (virus isolate)	Sample	DPI 0 positive / tested	DPI 7 positive / tested	DPI 14 positive / tested	DPI 21 positive / tested
Trial 1: (Ingelvac® PRRS MLV)	Oral fluid	0 / 24	0 / 24	24 / 24	24 / 24
	Serum	0 / 24	0 / 24	24 / 24	24 / 24
Trial 2: (Type 1, D09-012332)	Oral fluid	0 / 22	0 / 22	17 / 21	22 / 22
	Serum	0 / 24	0 / 24	22 / 24	24 / 24
Trial 3: (Type 2, MN-184)	Oral fluid	0 / 24	0 / 23	24 / 24	22 / 22
	Serum	0 / 24	0 / 24	24 / 24	24 / 24
TOTAL	Oral fluid	0 / 70	0 / 69	65 / 69	68 / 68
	Serum	0 / 72	0 / 72	70 / 72	72 / 72

[a] PRRS X3 Ab Test (IDEXX Laboratories, Inc, Westbrook, Maine, USA) performed according to the manufacturer's instruction.

[b] PRRS X3 Ab Test (IDEXX Laboratories, Inc, Westbrook, Maine, USA) modified to detect anti-PRRSV antibody in oral fluid specimens (Kittawornrat et al., 2012).

[c] Samples with S/P ratio ≥ 0.4 were classified as positive

Table 3. Comparison of cumulative quantitative reverse transcription polymerase chain reaction (qRT-PCR) and antibody responses (IgM, IgA, IgG) for 21 days following PRRSV inoculation

Trial (virus isolate)	Serum (mean AUC and 95% confidence intervals)				Oral fluid (mean AUC and 95% confidence intervals)			
	qRT-PCR	IgM	IgA	IgG	qRT-PCR	IgM	IgA	IgG
Trial 1: (Ingelvac® PRRS MLV)	21.6[b]	46.6[b]	18.5[b]	17.4[a]	21.0[b]	27.6[b]	12.3[b]	25.1[b]
	(18.8-24.5)	(43.1-50.2)	(14.6-22.6)	(15.9-18.9)	(17.0-25.1)	(23.9-31.5)	(7.7-17.0)	(20.6-29.6)
Trial 2: (Type 1, D09-012332)	23.8[b]	39.5[b]	30.0[a]	18.3[a]	21.2[b]	17.6[c]	17.9[b]	21.9[b]
	(21.2-26.6)	(31.1-48.0)	(23.2-36.9)	(16.5-20.2)	(18.1-24.3)	(12.0-23.2)	(10.9-24.9)	(16.7-27.2)
Trial 3: (Type 2, MN-184)	38.1[a]	53.7[a]	31.6[a]	18.4[a]	31.6[a]	35.4[a]	26.2[a]	30.1[a]
	(34.9-41.5)	(48.1-59.3)	(24.7-38.5)	(17.1-19.85)	(28.4-34.9)	(28.6-42.3)	(19.1-33.5)	(25.3-34.9)

[1] Individual boar PRRSV ($\log_e$ geq/µl) and antibody (IgM, IgA, IgG) S/P responses over time were summarized as the area under the curve (AUC) prior to performing the statistical analysis.

[abc] Superscripts within columns indicate statistically significant differences among means (Tukey's Honestly Significant Differences test, $p < 0.05$).

Table 4. Summary of porcine reproductive and respiratory syndrome virus (PRRSV) serum and oral fluid antibody enzyme linked-immunosorbent assay (ELISA) condition

	IgM	IgA	IgG	Commercial ELISA
Serum ELISAs				
Sample dilution	1:40	1:5	1:40	1:40
Sample volume	100 µl	100 µl	100 µl	100 µl
Conjugate dilution	1:5,000[b]	1:1,000[c]	1:15,000[d]	Provided with kit
Negative control	100 µl of pooled negative serum diluted 1:40	100 µl of pooled negative serum diluted 1:5	100 µl of kit negative control	100 µl of kit negative control
Positive control	100 µl of pooled serum from DPI 7 diluted 1:40	100 µl of pooled serum from DPI 21 diluted 1:5	100 µl of kit positive control	100 µl of kit positive control
Oral fluid ELISAs				
Sample dilution	1:2	1:2	1:2	
Sample volume	250 µl	250 µl	250 µl	
Conjugate dilution	1:3,800[b]	1:2,000[c]	1:2,400[d]	
Negative control	250 µl of reference standard oral fluid[e] DPI 0 diluted 1:2	250 µl of reference standard oral fluid[e] DPI 0 diluted 1:2	100 µl of kit negative control diluted 1:30	
Positive control	250 µl of reference standard oral fluid[e] DPI 10diluted 1:5	250 µl of reference standard oral fluid[e] DPI 91 diluted 1:2	100 µl of kit positive control diluted 1:30	

[a] Oral fluid ELISA conditions represent modifications to a commercial PRRSV serum antibody ELISA protocol (IDEXX PRRS X3 Ab Test, IDEXX Laboratories, Inc., Westbrook, ME, USA)

[b] Anti-pig IgM: HRPO conjugate (A100-100P, Bethyl Laboratories, Montgomery, TX, USA)

[c] Anti-pig IgA: HRPO conjugate (A100-102P, Bethyl Laboratories, Montgomery, TX, USA)

[d] Anti-pig IgG_{Fc}: HRPO conjugate (A100-104P, Bethyl Laboratories, Montgomery, TX, USA)

[e] Reference standard oral fluid samples have been described by Kittawornrat et al (2012).
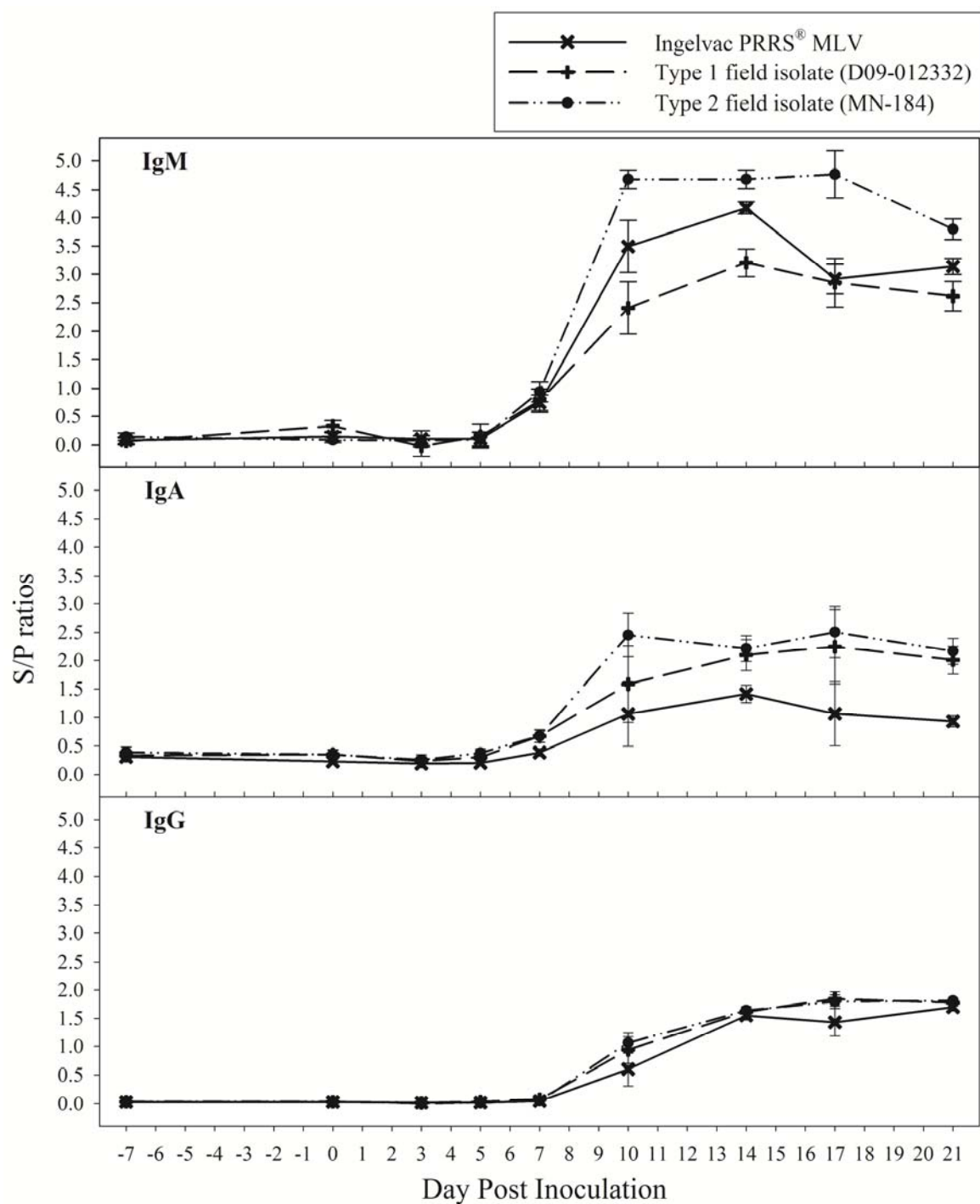
Figure 1. Kinetics of PRRSV antibody isotypes (IgM, IgA, and IgG) in serum based on responses in 72 boars inoculated with 3 different PRRSV isolates.
Results are reported as mean sample-to-positive (S/P) ratios and standard errors.
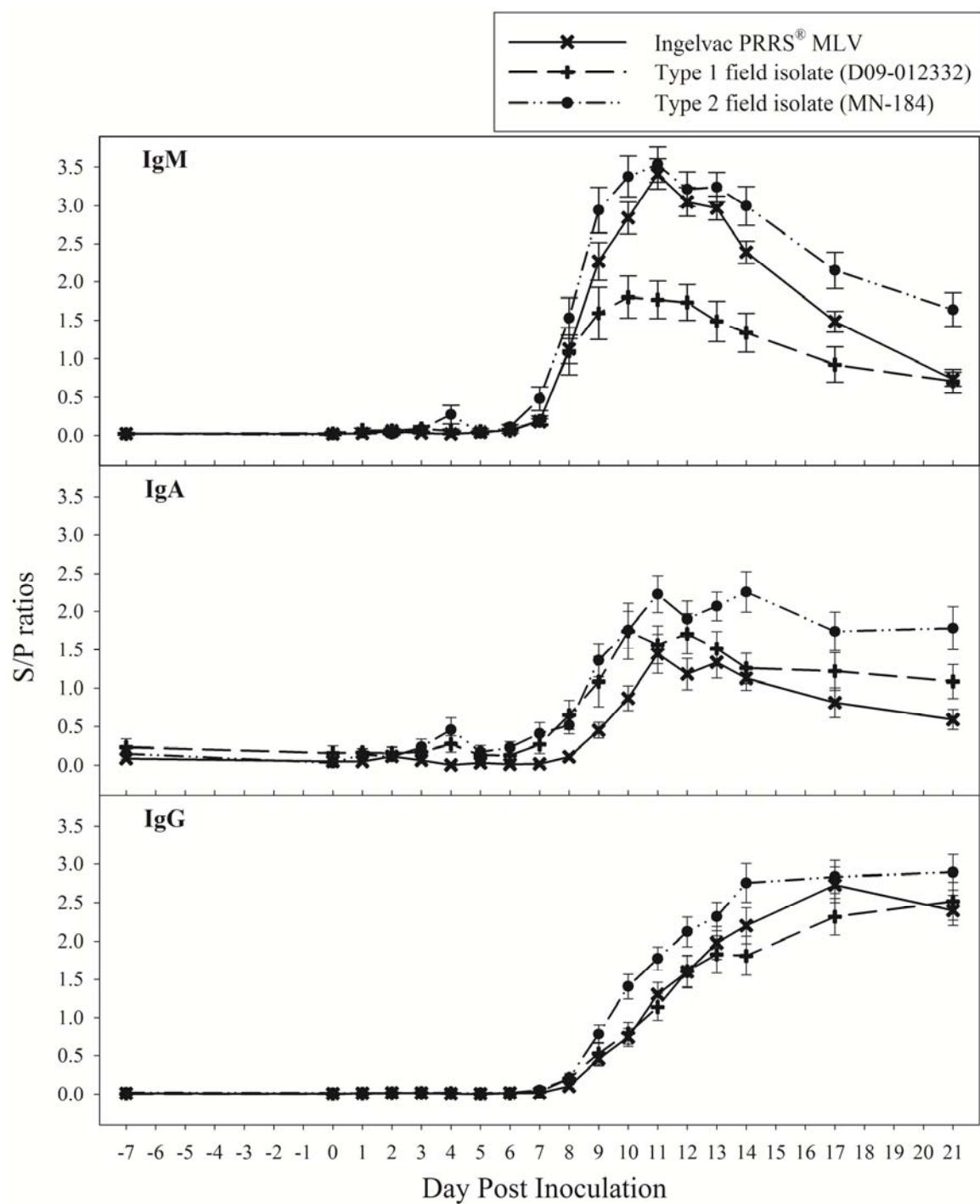
Figure 2. Kinetics of PRRSV antibody isotypes (IgM, IgA, and IgG) in oral fluid based on responses in 70 boars inoculated with 3 different PRRSV isolates.

Results are reported as mean sample-to-positive (S/P) ratios and standard errors.
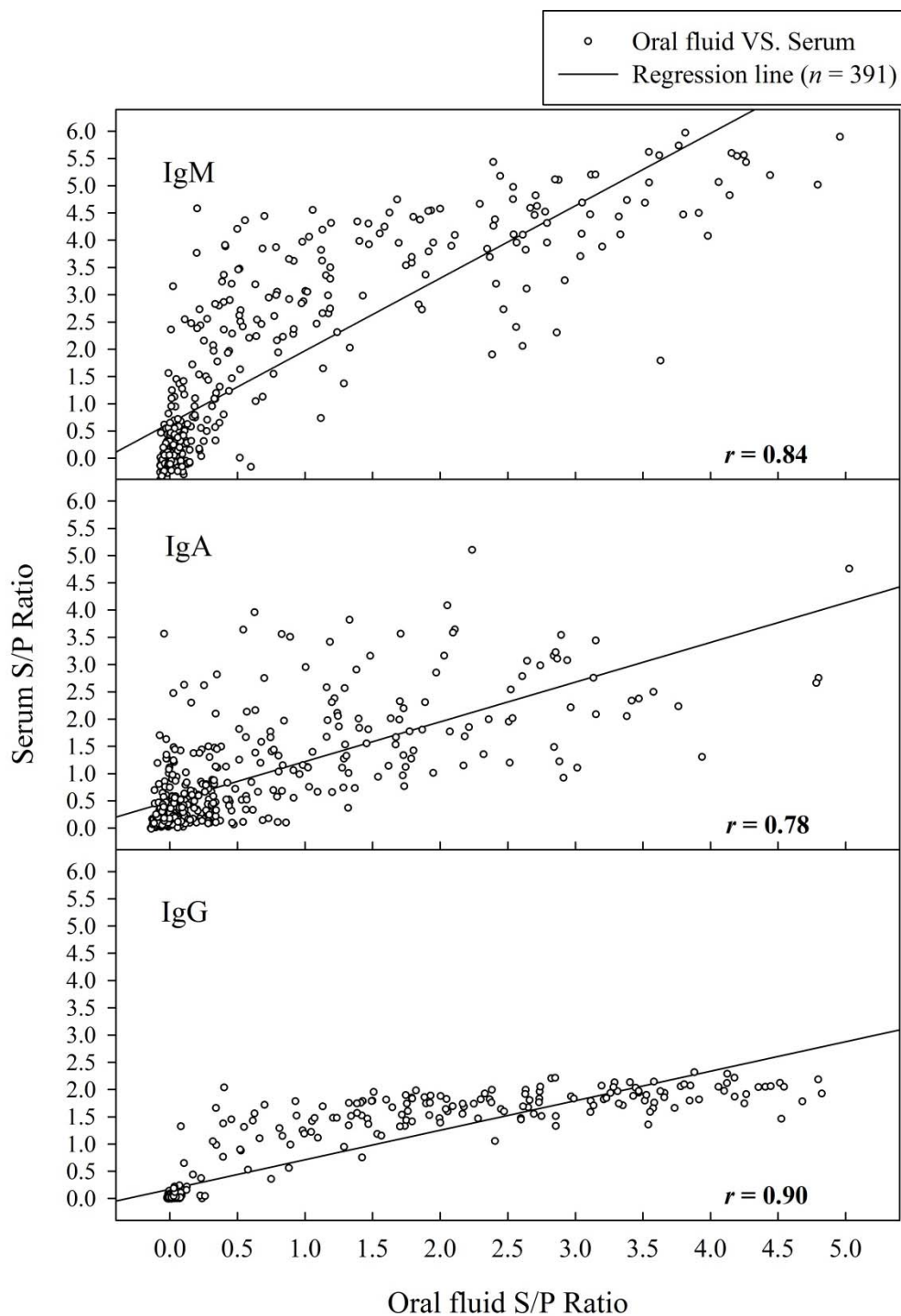
Figure 3. Correlation between serum and oral fluid PRRSV antibody isotypes (IgM, IgA, and IgG) based on results from individual boars

Dilutions of anti-pig immunoglobulin											
Dilution 1			Dilution 2			Dilution 3			Dilution 4		
1	2	3	4	5	6	7	8	9	10	11	12

		1	2	3	4	5	6	7	8	9	10	11	12
Negative Control	A	1	5	9	1	5	9	1	5	9	1	5	9
	B	2	6	10	2	6	10	2	6	10	2	6	10
	C	3	7	11	3	7	11	3	7	11	3	7	11
	D	4	8	12	4	8	12	4	8	12	4	8	12
Positive Control	E	13	17	21	13	17	21	13	17	21	13	17	21
	F	14	18	22	14	18	22	14	18	22	14	18	22
	G	15	19	23	15	19	23	15	19	23	15	19	23
	H	16	20	24	16	20	24	16	20	24	16	20	24

Figure 4. Microtitration plate map for titrating anti-pig immunoglobulin (M, A, or G) antibody.

The reactivity of the 4 dilutions was determined using negative and positive controls. Negative control OD values were used to screen for non-specific reactions. Positive control OD values were used to calculate the equation of the line (y = ax + c), where (y) is the anti-pig immunoglobulin (M, A, or G) OD response, (a) is the slope of the line, (x) is the dilution of anti-pig Ig (M, A, or G) antibody, and (c) is the intercept (Figure 2). The correct dilution of anti-pig Ig (M, A, or G) was calculated by substituting the mean positive control OD from the ELISA kit Certificate of Analysis for "y" in Equation 1 and then solving for "x".
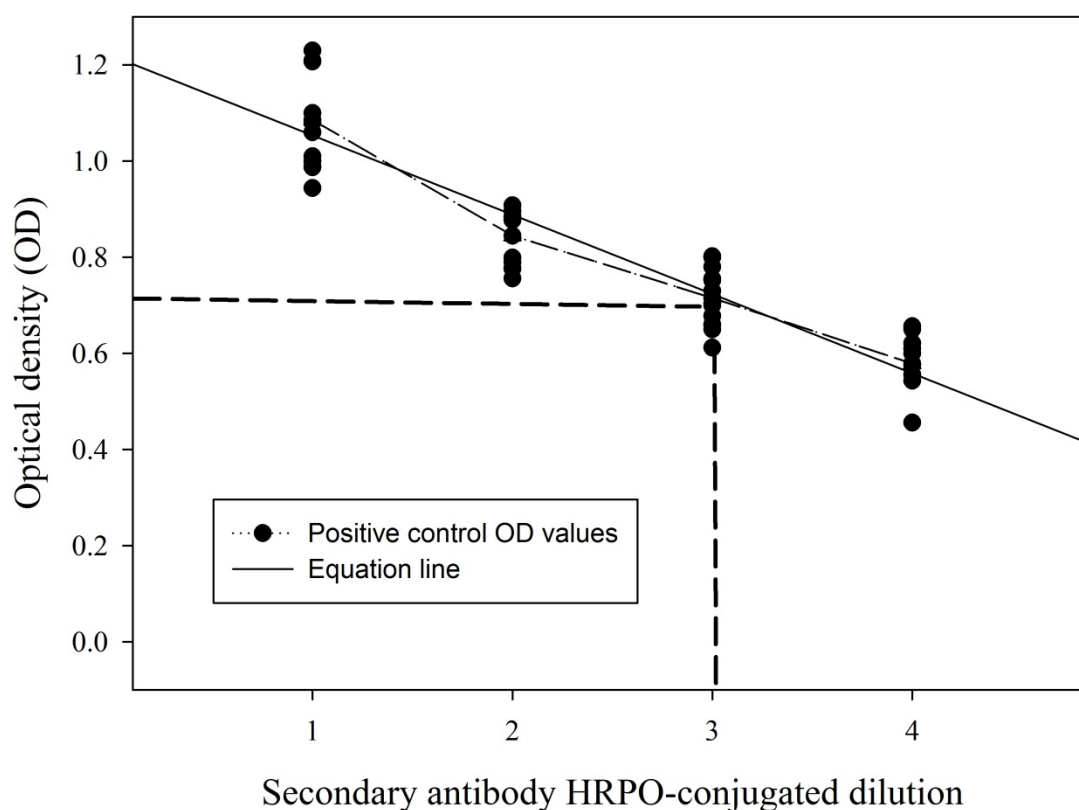
Figure 5. Calculation of the optimal dilution of anti-pig secondary antibody for PRRSV IgM, IgA, IgG ELISAs.

The relationship between the positive control optical density (OD) and dilution of anti-pig secondary antibody was plotted as $y = ax + c$, where (y) is the secondary antibody OD response, (a) is the slope of the line, (x) is the dilution of secondary antibody, and (c) is the intercept. The appropriate dilution corresponds to the positive control value provided in the manufacturer's Certificate of Analysis.

CHAPTER 6. PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME VIRUS (PRRSV) SURVEILLANCE USING PRE-WEANING ORAL FLUID SAMPLES DETECTS CIRCULATION OF WILD-TYPE PRRSV

Apisit Kittawornrat, Yaowalak Panyasing, Christa Goodell, Chong Wang, Phil Gauger, Karen Harmon, Rolf Rauh, Luc Desfresne, Ian Levis, Jeffrey Zimmerman

ABSTRACT

Oral fluid samples collected from litters of piglets (n = 600) one day prior to weaning were evaluated as a method to surveil for porcine reproductive and respiratory syndrome virus (PRRSV) infections in four ~12,500 sow herds. Serum samples from the litters' dam (n = 600) were included for comparison. All four herds were endemically infected with PRRSV and all sows had been vaccinated ≥ 2 times with PRRSV modified-live virus vaccines. After all specimens had been collected, samples were randomized and assayed for PRRSV nucleic acid by real-time reverse transcription polymerase chain reaction (RT-qPCR) and anti-PRRSV antibodies using four PRRSV ELISA assays (IgM, IgA, IgG, and Commercial Kit). All sow serum samples were negative by PRRSV RT-qPCR, but 9 of 600 oral fluid samples tested positive at two laboratories. Two of the 9 positive oral fluid samples were sequenced (open reading frame 5) and the presence of nucleic acid from wild-type viruses was detected. A comparison of antibody responses in RT-qPCR positive vs. negative oral fluid samples showed significantly higher IgG S/P ratios in RT-qPCR-positive oral fluid samples (mean S/P 3.46 vs. 2.36; $p = 0.02$). Likewise, sow serum samples from RT-qPCR-positive litter oral fluid samples showed significantly higher serum IgG (mean S/P 1.73 vs. 0.98; $p < 0.001$) and Commercial Kit (mean S/P 1.97 vs. 0.98; $p < 0.001$) S/P ratios. Overall, this study showed that pre-weaning litter oral fluid samples could provide an efficient and sensitive approach to surveil for PRRSV in infected, vaccinated, or presumed-negative pig breeding herds.

INTRODUCTION

Cost-effective methods of disease surveillance are needed to provide timely information on the presence and/or distribution of pathogens in swine herds, support animal health and business decisions at the herd level, and improve the quality of field research. Current methods of surveillance in pig populations are primarily based on labor-intensive sampling methods, i.e., blood collection from individual animals. As a result, routine herd surveillance is cost-prohibitive and generally under-utilized.

The current body of literature provides evidence that oral fluid sampling could provide a cost-effective and welfare-friendly alternative to serum-based surveillance. That is, a variety of pathogens and pathogen-specific antibody can be detected in pig oral fluids, e.g., classical swine fever virus (Corthier, 1976; Corthier and Aynaud, 1977), porcine circovirus type 2 (Prickett et al., 2011), swine influenza virus (Panyasing et al., 2012), transmissible gastroenteritis virus (DeBuysscher and Berman, 1980), *Actinobacillus pleuropneumoniae* (Loftager et al., 1993), *E. coli* (De Buysscher and Dubois, 1978), *Erysipelothrix rhusiopathiae* (Giménez-Lirola et al., 2013), and others. For PRRSV, research has described the pattern of viral shedding (Kittawornrat et al., 2010; Prickett et al., 2008) and the ontogeny of the anti-PRRSV humoral response (Kittawornrat et al., 2012a,b; 2013) in oral fluids.

The need for efficient, effective surveillance is particularly true for porcine reproductive and respiratory syndrome virus (PRRSV), which remains one of the most costly diseases of swine worldwide, imposing significant losses on North American (Holtkamp et al., 2013; Sierra et al., 2000), European (Velasova et al., 2012) and Asian producers (Tian et al., 2007). Based on our cumulative global experience, it would seem that achieving control of PRRSV will require that animal health specialists develop the capacity to easily, efficiently, and continuously surveil herds for PRRSV. Therefore, the purpose of the present study was to evaluate a method to detect PRRSV circulation in the breeding herd and growing pig populations based on collection and testing of oral fluid samples prior to weaning.

MATERIALS AND METHODS

Experimental design

In four PRRSV vaccinated commercial swine herds, oral fluid samples were collected from 600 litters 24 hours prior to weaning and serum samples from their dams two days post weaning. Thereafter, samples were completely randomized and tested for PRRSV (RT-qPCR and sequencing) and PRRSV antibodies using commercial ELISA Kits (PRRS X3 Ab Test and PRRS Oral Fluids Ab Test, IDEXX Laboratories, Inc., Westbrook, ME) and antibody isotype-specific assays (IgM, IgA, and IgG). In addition, PRRSV ORF5 sequencing was attempted on RT-qPCR-positive samples. Virus and antibody assay results were analyzed for associations with farm, sow parity, litter size, time, and infection status.

Animals and animal care

The study was performed in four ~12,500 sow breeding herds. Animal housing, feeding, handling, and veterinary care were under the supervision of Seaboard L.L.C. Health Assurance and Welfare personnel. Sample collection protocols were approved by the Iowa State University Institutional Animal Care and Use Committee (#8-11-7202-S).

All four herds were considered to be endemically infected with PRRSV on the basis of diagnostic history and on-going surveillance. Commercial PRRSV vaccines were used to control clinical losses, with some differences in the vaccination protocols among sites. In all four herds, replacement gilts were vaccinated with a commercial modified-live (MLV) PRRSV vaccine (Ingelvac MLV[®], Boehringer Ingelheim Vetmedica, Inc., St. Joseph. MO) at weeks 0 and 2 of quarantine, i.e., prior to entering the sow farm. Once entered into the sow farms, all gilts and sows were routinely vaccinated with either PRRSV MLV or PRRSV ATP vaccines (Ingelvac ATP[®,] Boehringer Ingelheim Vetmedica, Inc., St. Joseph. MO) according to a schedule based on the weeks of the calendar year. Animals in Herd 1 were vaccinated with PRRSV ATP vaccine at weeks 9 and 13. Herd 2 animals were vaccinated with PRRSV ATP at weeks 1, 4, 47, and 51 and PRRSV MLV at week 5. Herd 3 used PRRSV ATP at weeks 2, 20, and 24, and Herd 4 vaccinated all animals with PRRSV ATP at week 1 and MLV at week 5.

Sample collection

Oral fluid specimens were collected from litters of pigs to be weaned and serum from their dams over a period of four months, as shown in Table 1. Oral fluid samples were collected from litters of piglets by farrowing room personnel one day prior weaning. Piglets averaged 17 days of age (range: 15 to 19 days) at the time of collection. The oral fluid collection procedure has been fully described by Kittawornrat et al. (2010). In brief, oral fluid samples were collected in the morning (when piglets were active) by suspending 1.25 cm (0.5 inch) unbleached cotton rope (Web Rigging Supply, Inc., Lake Barrington, IL USA) over the heat mat area, thereby providing access to the piglets, but not the sow. The ropes were hung until they were wet (15 minutes to 2 hours), after which the wet portion of the rope was inserted into a single-use re-sealable plastic bag and the fluid extracted by manually squeezing the rope inside the plastic bag. The oral fluid sample was then decanted into a tube, immediately chilled, refrigerated overnight, and shipped to the laboratory on the following day. At the laboratory, oral fluid samples were aliquoted into 5 ml cryogenic vials and stored at -80ºC. Within 48 hours of collecting oral fluids, serum samples were collected from sows using a single-use blood collection system (Corvac®, Tyco Healthcare Group LP, Mansfield, MA USA). Samples were transported to the laboratory in blood tubes. At the laboratory, samples were centrifuged at 1,000 x g for 10 minutes, after which the serum was aliquoted into 5 ml plastic tubes and stored at -80ºC. When all samples had been collected, they were placed in random order and submitted for testing.

PRRSV antibody ELISAs

Commercial Kit (serum)

All serum samples were assayed for PRRSV antibodies using a commercial indirect ELISA (PRRS X3 Ab Test, IDEXX Laboratories, Inc., Westbrook, ME) performed according to the manufacturer's instruction. Using the formula provided by the manufacturer, a positive result was defined as a sample-to-positive (S/P) ratio ≥ 0.4.

PRRSV antibody isotypes in serum

The commercial indirect ELISA (PRRS X3 Ab Test) was modified to detect PRRSV-specific

IgM, IgA, and IgG antibody isotypes in serum. In brief, serum samples were diluted 1:30 (5μl serum sample + 145μl kit diluent) for IgM, 1:20 (10μl serum sample + 190 μl kit diluent) for IgA and 1:40 (5μl serum sample + 195 μl kit diluent) for IgG. 100 μl of diluted serum was then transferred to the PRRSV antigen-coated plates and incubated for 30 minutes at 22℃. After washing 3 times with 1X kit wash solution (400 μl), appropriately-diluted horseradish peroxidase (HRPO)-conjugated anti-pig IgM antibody (1:5,000; A100-100P, Bethyl Laboratories, Montgomery, TX), anti-pig IgA antibody (1:1,000; A100-102P), or anti-pig IgG$_{Fc}$ antibody (1:18,000; A100-104P) was added to each well and incubated for 30 minutes at 22℃. Thereafter, plates were washed three times with kit washing solution, after which 100μl of tetramethylbenzidine (TMB) was added to each well and the plates incubated at 22℃ for 15 minutes. At precisely 15 minutes, 100 μl of kit stop solution was added to each well. Negative and positive serum controls were created by pooling serum from boars of precisely known PRRSV infection status (Kittawornrat et al., 2013). Negative control serum for IgM and IgA assays consisted of pooled serum collected on day post inoculation (DPI) 0. Pooled serum samples collected on DPI 7 and 21 were used as positive controls for serum IgM and IgA assays, respectively. The IgG assay used the negative and positive controls provided by the manufacturer. The plates were read at 650 nm using an ELISA plate reader (EL800 micro plate reader, Bio Tek® Instruments Inc., Winooski, VT) controlled by commercial software (Gen5^{TM}, Bio Tek® Instruments Inc.) and the reactions measured as optical density (OD). Based on previous work (Kittawornrat et al., 2012), a positive results for the IgG assay was defined as a S/P ratio $\geq$ 0.4.

Commercial Kit (oral fluid)

All oral fluid samples were assayed for PRRSV antibodies using a commercial indirect ELISA (PRRS Oral Fluids Ab Test) performed according to the manufacturer's instruction. In brief, oral fluid samples were diluted 1:2 (100 μl oral fluid sample + 100 μl kit diluent). 100 μl of diluted oral fluid was then transferred to PRRSV antigen-coated plates and incubated for 2 hours at 22℃. Thereafter, the plates were washed 4 times with 400 μl of 1X kit wash solution. To detect the reaction, 100 μl of kit conjugate was added to each well and the plates incubated for 30 minutes at 22℃. After washing 4 times, 100 μl of TMB substrate

N.12 was added to each well and the plates incubated at 22℃ for 15 minutes. Finally, 100 µl of kit stop solution was added to each well. The plates were read under dual-wavelength setting at 450-650 nm and the reactions measured as OD. Using the formula provided by the manufacturer, a positive result was defined as a sample-to-positive (S/P) ratio ≥ 0.4.

PRRSV antibody isotypes in oral fluid

Modification of the commercial PRRSV ELISA for the detection of PRRSV-specific IgM, IgA, and IgG antibody in swine oral fluid has previously been described (Kittawornrat et al., 2012). In brief, oral fluid samples were diluted 1:2 (150 µl oral fluid sample + 150 µl kit diluent). 250 µl of diluted oral fluid was then transferred to PRRSV antigen-coated plates and incubated for 16 hours at 4℃. Thereafter, the plates were washed 4 times with 400 µl of 1X kit wash solution. To detect the reaction, 100 µl of a solution containing appropriately-diluted HRPO-conjugated anti-pig IgM antibody (1:4,000), anti-pig IgA antibody (1:2,000), or anti-pig IgG antibody (1:2,500) was added to each well and the plates incubated for 30 minutes at 22℃. After washing 3 times, 100 µl of TMB was added to each well and the plates incubated at 22℃ for 15 minutes. Finally, 100 µl of kit stop solution was added to each well. Oral fluid antibody assay controls consisted of oral fluid samples collected from PRRSV-negative pigs intramuscularly inoculated with 2 ml of PRRS modified live virus (MLV) vaccine (Ingelvac® PRRS MLV, Boehringer Ingelheim Vetmedica, Inc., St. Joseph, MO USA). The sample collected immediately prior to vaccination was used as the negative control. Samples collected on 10 and 56 days post vaccination were used as positive controls for IgM and IgA assays, respectively (Kittawornrat et al., 2012). The IgG assay used the negative and positive controls provided by the manufacturer. As described in 2.6.2, the plates were read at 650 nm and the reactions measured as OD. Based on previous work (Kittawornrat et al., 2012), a positive results for the IgG assay was defined as a S/P ratio ≥ 0.4.

PRRSV real-time reverse transcription polymerase chain reaction (RT-qPCR)
PRRSV RT-qPCR #1

All samples (600 serum samples, 600 oral fluid samples) were tested at the Iowa State

University - Veterinary Diagnostic Laboratory (ISU-VDL) for the presence of PRRSV. RNA extraction was performed using the MagMAX™ Viral RNA Isolation Kit (Life Technologies Corporation, Carlsbad, CA) and a Kingfisher 96 instrument (Thermo Scientific, Waltham, MA). Serum was 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. Oral fluids were extracted using a high volume modified lysis (HVML) procedure. 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 minutes and centrifuged at 2,500 x g for 6 minutes. 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.

RT-qPCR was performed on nucleic acid extracts using a commercially available NA and EU PRRSV-specific PCR assay (VetMAX™ TaqMan® NA and EU PRRSV Reagents, Life Technologies Corporation). The assay contained multiple primers and probes to detect and differentiate North American (Type II) and European (Type I) strains. Internal control RNA (Xeno™, Life Technologies Corporation) was included in the master mix to monitor PCR amplification and detection of PCR inhibition. Two positive extraction controls, one negative extraction control, and a negative amplification control were included with each extraction and/or PCR run. Each serum reaction included 12.5 μl of 2X RT-PCR buffer, 2.5 μl of 10X PRRSV primer probe mix, 1.25 μl of 20X 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 oral fluid reaction used the same volume of reagents described for serum, with the exception of 2.5 μl of 20X 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

serum samples, or 18 µl mastermix and 7 µl of RNA extract for the oral fluid samples, was placed in each well of a 96-well fast PCR plate (Life Technologies Corporation). RT-qPCR was performed (7500 Fast Real-Time PCR System, Applied Biosystems®, Foster City, CA) with the following cycling conditions: one cycle at 45°C for 10 min, one cycle at 95°C for 10 min, and 40 cycles of 97°C for 2 sec, and 60°C for 40 sec. 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. Eight progressive 1:10 dilutions of a PRRSV standard supplied with the commercial PRRSV PCR kit were used to generate a curve for virus quantification (genomic copies per ml) on each plate.

PRRSV RT-qPCR #2

Samples initially tested at the ISU-VDL were submitted for confirmatory testing at a second laboratory (Tetracore, Inc., Rockville, MD). The sample set included 30 litter oral fluid samples (9 PRRSV RT-qPCR-positive samples and 21 blindly selected negatives) and 30 blindly selected RT-qPCR-negative sow serum samples. RNA extraction was performed using the MagMAX™ Pathogen RNA/DNA Kit (Life Technologies Corporation) and BioSprint® 96 Workstation particle processor (Qiagen, Valencia, CA). Serum was extracted following the manufacturer instructions for low-cell-content samples. Oral fluid samples were extracted using the protocol for "all other sample types". 300 µl of sample (serum or oral fluid) was used in the reaction and reagents were prepared accordingly, with the exception that 8 µl of in-house control was used in the lysis/binding solution, rather than 2 µl of Xeno™ RNA (Life Technologies Corporation). Extraction of both sample types was completed using program 4462359_DW_HV.

Real time RT-PCR was performed using an in-house PRRSV RT-PCR assay (Tetracore®, Rockville, MD). The assay covers two target regions of the PRRSV Type 1 and 2 genes using FAM as a reporter dye for the detection of the Type 1 PRRSV and TAMRA as a

reporter dye for the detection of the Type 2 PRRSV. CY5 was used as a reporter dye for the detection of the extraction/inhibition control. Serum component volumes per well included 19.25 µl of master mix (includes buffer, primer and probes), 0.25 µl of enzyme 1, 0.5 µl of enzyme 2, and 8 µl of extracted serum sample or internal control. Oral fluid component volumes per well included 16.25 µl of master mix (includes buffer, primer and probes), 0.25 µl of enzyme 1, 0.5 µl of enzyme 2, and 8 µl of extracted oral fluid sample or internal control. Plates were briefly vortexed (10 sec) and centrifuged before being loaded onto the thermal cycler (7500 Fast Real-Time PCR System, Applied Biosystems®, Foster City, CA). The reaction was completed using the following thermal cycling conditions: one cycle at 48 °C for 15 min, one cycle at 95 °C for 2 min, 45 cycles of: 95 °C for 5 seconds, and 60 °C for 40 seconds. Samples with Ct values <38 for either strain were considered positive.

PRRSV ORF5 sequencing

Oral fluid samples were extracted for open reading frame 5 (ORF5) sequencing as described above in the nucleic acid extraction protocol for ISU-VDL. Approximately 1082 base pairs of the ORF5 region of the PRRSV genome were amplified using forward primer 5'-AAG GTG GTA TTT GGC AAT GTG TC-3' and reverse primer 5'-GAG GTG ATG AAT TTC CAG GTT TCT A-3' and the qScript™ Custom One-Step RT-PCR Kit (Quanta Biosciences, Gaithersburg, MD). The oral fluid sequencing PCR setup reaction used 320 nM of each primer with 12.5 µl 2X qScript One-Step master mix, 0.5 µl qScript One-Step reverse transcriptase and 7.2 µl nuclease-free water. A final volume of 25 µl consisted of 21 µl mastermix and 4 µl of RNA extract. One positive extraction control, one negative extraction control and one negative amplification control were included with the reaction. Polymerase chain reaction was performed (Applied Biosystems® 2720 thermal cycler, Life Technologies Corporation) with the following cycling conditions: one cycle at 48°C for 20 minutes, one cycle at 94°C for 3 minutes, 45 cycles of 94°C for 30 seconds, 50°C for 50 seconds, and 68°C for 50 seconds. The final elongation step was 68°C for 7 minutes. Detection of the RT-PCR product of the correct size (1082 bp) was performed on a QIAxcel® capillary electrophoresis system (Qiagen) using a DNA screening cartridge and the AM420 method and purified with ExoSAP-IT® (Affymetrix, Santa Clara, CA) following the manufacturer's

recommendations. Samples were submitted to the Iowa State University DNA Facility for sequencing and commercial software was used to compile sequences (Lasergene®, DNAStar, Madison, WI).

Statistical analysis

Statistical analyses were performed using SAS® 9.2 (SAS® Institute Inc., Cary, NC USA) and MedCalc® 9.2.1.0 (MedCalc Software, Mariakerke, Belgium). Serum and oral fluid S/P ratios were analyzed for significant associations with sow parity, litter size, herd, sampling time point, and their pairwise interactions by analysis of variance (ANOVA). The overall quantitative relationship between IgM, IgA, IgG, and Kit S/P ratios in serum versus oral fluid samples was evaluated by correlation analysis. Qualitative (pos/neg) differences in ELISA performance by specimen type were assessed using Cochran's Q test. Descriptive and comparative analyses were used to evaluate the quantitative (Ct) and qualitative (pos/neg) RT-qPCR results in serum and oral fluid samples. PRRSV ORF5 nucleotide percent identity of wild type sequences recovered during the study were compared to wild type isolates recovered from the farms either before or after the study, the North American prototype PRRSV (VR-2332), and modified live vaccines (Ingelvac MLV® and Ingelvac ATP®) using BioEdit 7.1.11 (Ibis Biosciences, Carlsbad, CA).

RESULTS

PRRSV RT-qPCR results

Testing of pre-weaning oral fluid samples (n = 600) and sow serum samples (n = 600) using Testing of pre-weaning oral fluid samples (n = 600) and sow serum samples (n = 600) using PRRSV RT-qPCR #1 resulted in 9 positive oral fluid samples and no positive serum samples. The 9 positive samples, 21 blindly-selected oral fluid samples, and 30 blindly-selected serum samples were re-tested using PRRSV RT-qPCR #2. The results of RT-qPCR #2 exactly matched the results reported for PRRSV RT-qPCR #1 (Table 4). Among the 9 PRRSV RT-qPCR-positive samples, 3 samples had been exhausted. The remaining 6 samples were submitted for PRRSV ORF5 sequencing and sequences were obtained on 2 of the 6 (Table 4 and 5). As indicated in Table 5, the sequence from Farm 2 shared 99.0%, 98.1%, and 98.6%

ORF5 nucleotide identity with 3 viruses isolated from sow serum samples collected in association with clinical PRRSV between 11/2010 and 03/2012. Likewise, the sequence from Farm 3 shared 99.0% ORF5 nucleotide identity with a virus isolated 8 months earlier in conjunction with reproductive disease.

A statistical analysis based on PRRSV RT-qPCR quantitative results (Ct values) detected no statistically significant associations with farm, sow parity, or their interactions. However, an analysis of antibody responses showed significantly higher IgG S/P ratios in RT-qPCR-positive versus negative oral fluid samples (mean S/P 3.46 vs. 2.36; $p = 0.02$). In contrast, a comparison between these two groups detected no significant difference in Commercial Kit mean S/P responses (mean S/P 6.19 vs. 4.60; $p = 0.07$). Sow serum samples from RT-qPCR-positive litter oral fluid samples showed significantly higher serum IgG (mean S/P 1.73 vs. 0.98; $p < 0.001$) and Commercial Kit (mean S/P 1.97 vs. 0.98; $p < 0.001$) S/P ratios, but no significant difference in IgM or IgA responses.

PRRSV antibody responses in serum and oral fluid samples
The manufacturer of the PRRSV antibody ELISA provides validity criteria in the kit insert. Specifically, for each plate, the mean of the two positive controls minus the mean of the two negative controls should be ≥ 0.15 and the mean of the two negative controls must be ≤ 0.15. All plates met these criteria with the exception of the serum IgM plates in which the mean of the negative control ranged from 0.20 to 0.25.

Qualitative results for the PRRSV Commercial Kit and PRRSV IgG ELISAs are shown in Table 2. A total of 443 (73.8%) sow serum samples were PRRSV antibody positive on the Commercial Kit compared to 473 (78.8%) using the serum IgG assay. In contrast, 594 (99.0%) litter oral fluid samples were antibody positive on the PRRSV oral fluid Commercial Kit vs. 579 (96.5%) on the IgG antibody assay. Pairwise comparisons detected no statistically significant differences between the results produced by the oral fluid Commercial Kit and oral fluid IgG ELISAs, but significant differences ($p < 0.05$) were detected between the two serum antibody assays and between the serum and oral fluid test results (Table 2).

The quantitative results of testing the two sample types (sow serum and litter oral fluids) by four PRRSV ELISAs (IgM, IgA, IgG, and Commercial Kit) are presented in Tables 3 and 4. No statistically significant differences in serum antibody ELISA S/P ratios were detected among farms, but significant differences among parities were observed with the IgG and Commercial Kit ELISAs. Among the oral fluid antibody ELISAs, statistically significant differences in IgA S/P ratios were detected among farms and among sows by parity. Likewise, differences among S/P ratios were detected among farms using the oral fluid Commercial Kit. As shown in Figures 1 and 2, correlation analyses of the association between sample types (sow serum and oral fluid) S/P ratios estimated Pearson's correlation coefficients for each of the four assays: IgM ($r = 0.23$), IgA ($r = 0.39$), IgG ($r = 0.73$), and Commercial Kit ($r = 0.68$).

DISCUSSION

The purpose of surveillance is to determine the presence and/or distribution of target pathogens in a population over time (Hadorn and Stark, 2008). When implemented on commercial swine farms, surveillance should inform regarding the health status of the population and guide targeted, proactive animal health and business decisions. Despite the potential benefits, routine, continuous on-farm surveillance is rare because of the inconvenience and cost of collecting and testing individual pig serum samples.

Previous studies have shown a correlation between serum and swine oral fluid diagnostic results for PRRSV and other pathogens (Ramirez et al., 2012). Prickett et al. (2008a,b) reported the detection of PRRSV by RT-qPCR in oral fluid samples collected from pens of pigs under both research and field conditions. Kittawornrat et al. (2010, 2013) described the pattern of PRRSV shedding and kinetics of the PRRSV antibody responses in oral fluid specimens from individually housed boars over time. Olsen et al. (2013) found that pen-based oral fluid testing by either antibody ELISA or RT-qPCR greatly improved PRRSV detection compared to individual pig serum sampling across a range of within-pen prevalences (4% to 36%). Cumulatively, these studies suggested that surveillance based on

oral fluids could be practical in commercial swine herds because of the ease of sample collection and the diagnostic performance of assays optimized for the oral fluid matrix.

The purpose of this study was to evaluate the feasibility of a system of PRRSV surveillance based on testing oral fluid samples collected from litters of piglets prior to weaning. For comparison, serum samples were collected from each litter's dam. In total, 600 litter oral fluid and 600 litter-matched sow serum samples were collected in four commercial sow herds over a period of 4 months. All samples were tested by PRRSV RT-qPCR and for PRRSV antibody using four different antibody ELISAs (IgM, IgA, IgG, and Commercial Kit).

PRRSV RT-qPCR testing found no positive sow serum samples (n = 600), i.e., there was no evidence of PRRSV viremia among the sows tested. However, 9 of the litter oral fluid samples (n = 600) were RT-qPCR positive, indicating that one or more of the piglets in these 9 litters was infected with PRRSV. Given the small number of positives and the absence of clinical signs suggestive of PRRSV infection, it was necessary to confirm that the original RT-qPCR results were true positive reactions. Therefore, the 9 positive oral fluid samples and a set of negative control samples consisting of 21 blindly-selected negative oral fluid samples and 30 blindly-selected serum samples were submitted for PRRSV RT-qPCR testing at a second laboratory. Retesting at the second laboratory exactly matched the initial results. Thereafter, ORF5 sequencing from oral fluids was attempted on the 6 positive samples with sufficient volume still remaining for testing. Of the 6 samples submitted, ORF5 sequencing was successful on 2. Subsequent analysis of the 2 ORF5 sequences showed $\geq 98\%$ nucleotide identity with wild-type PRRS viruses isolated from these herds before and/or after the study.

The observed RT-qPCR results showing an extremely low rate of PRRSV transmission among litters of suckling piglets were compatible with previous field studies on PRRSV infections in pre-weaned piglets. Graham et al. (2013) tested pre-wean piglet serum samples (n = 454 piglets in 44 litters) collected in a commercial herd by PRRSV RT-qPCR and found 2 positive litters; one litter with one positive piglet and one with two positive piglets.

Similarly, Cano et al. (2008) detected 13 PRRSV RT-qPCR positive litters among 42 litters tested. Of the 13 positive litters, 8 contained one positive piglet, 2 had 2 positive piglets, and 3 had $\geq$ 4 positive piglets.

Evaluation of antibody responses in sow serum and piglet oral fluid samples from PRRSV RT-qPCR positive litters provided insight into the RT-qPCR results. Previous research has described the appearance of anti-PRRSV IgM, IgA, and IgG in serum and oral fluid specimens under experimental conditions, but IgM and IgA have not been reported in samples collected under field conditions (Kittawornrat et al., 2012a, 2013). In the present study, failure to detect IgM and IgA may be explained by the extensive use of PRRSV MLV vaccines and the fact that IgG, both because of its higher concentration and its higher affinity, may have out-competed IgM and IgA for antigenic sites in the antibody ELISAs (Lee et al., 1995; Makela et al., 1970). Nevertheless, the significantly higher S/P ratios observed in sow serum and oral fluid specimens from PRRSV RT-qPCR positive litters suggests that the animals were responding to recent PRRSV infections. Although all sows had been vaccinated against PRRSV, protection against infection with heterologous PRRSV isolates is recognized as inconsistent (Benson et al., 2000). The likelihood of this scenario is reinforced by the detection of nucleic acid from wild-type viruses in oral fluid samples. It was not possible to determine the source of infection in this study, but vertical transmission between the dam and her piglets can occur either by direct contact or transplacental transmission (Christianson et al., 1992; Lager et al., 1997a,b, 1999). In addition, Wagstrom et al. (2001) demonstrated that PRRSV-naïve dams inoculated late in gestation shed virus in colostrum and milk, although females with prior exposure to PRRSV did not. Transmission could also have occurred through cross-fostering of infected piglets into negative litters or other human interventions, e.g., exposure of animals via virus-contaminated fomites, but the low prevalence and sporadic pattern of infection in the population did not reveal a specific pattern suggestive of a particular procedure or event.

Cumulatively, the data suggest that PRRSV herd endemnicity is maintained, at least in part, by a cycle that involves subclinical PRRSV infection in suckling piglets, with transmission to

the population of growing pigs occurring as pigs are moved and mixed post-weaning. The fact that the neonatal infection occurred at a very rate, i.e., 9 of 600 litters, illustrates the challenge of on-farm PRRSV surveillance. Even under these circumstances, however, it was possible to detect the presence of the virus and thereby demonstrate the efficiency and effectiveness of PRRSV surveillance using pre-weaning oral fluid samples from litters of piglets.

DECLARATION OF CONFLICTING INTERESTS

Authors S. Lizano and R. Rauh are employed by private industry. The remaining author(s) declare no conflicting interests with respect to their authorship or the publication of this article.

REFERENCES

Benson, J.E., Yaeger, M.J., Lager, K.M., 2000. Effect of porcine reproductive and respiratory syndrome virus (PRRSV) exposure dose on fetal infection in vaccinated and nonvaccinated swine. Swine Health Prod. 8, 155-160.

Cano, J.P., Dee, S.A., Rovira, A., Muñoz-Zanzi, C., Anil, S.S., Morrison, R., 2008. PRRSV vertical transmission dynamics in an endemically infected sow-herd. Proceedings Seminar of American Association of Swine Veterinarians. San Diego, California, p. 105.

Christianson, W.T., Collins, J.E., Benfield, D.A., Harris, L., Gorcyca, D.E., Chladek, D.W., Morrison, R.B., Joo, H.S., 1992. Experimental reproduction of swine infertility and respiratory syndrome in pregnant sows. Am. J. Vet. Res. 53, 485-488.

Corthier, G., 1976. Swine fever: influence of passive immunity on pig immune response

following vaccination with a live virus vaccine (Thiverval strain). Ann. Rech. Vet. 7, 361-372.

Corthier, G., Aynaud, J.M., 1977. Comparison of the immune response in serum and bucco-pharyngeal secretions following immunization by different routes with a live hog cholera virus vaccine (Thiverval strain). Ann. Rech. Vet. 8, 159-165.

DeBuysscher, E., Berman, D., 1980. Secretory immune response in intestinal mucosa and salivary gland after experimental infection of pigs with transmissible gastroenteritis virus. Am. J. Vet. Res. 41, 1214-1220.

DeBuysscher, E., Dubois, R., 1978. Detection of IgA anti-Escherichia coli plasma cells in the intestine and salivary glands of pigs orally and locally infected with E. coli. Adv. Exp. Med. Biol. 107, 593-600.

Gimenez-Lirola, L.G., Xiao, C.T., Zavala, M., Halbur, P.G., Opriessnig, T., 2013. Improving ante mortem diagnosis of Erysipelothrix rhusiopathiae infection by use of oral fluids for bacterial, nucleic acid, and antibody detection. J. Microbiol. Methods. 92, 113-121.

Graham, J., Rademacher, C., Swalla, R., 2013. Use of oral fluid sampling in suckling pigs for PRRSV monitoring. Proceedings Seminar of American Association of Swine Veterinarians. San Diego, California, p. 83.

Hadorn, D.C., Stark, K.D., 2008. Evaluation and optimization of surveillance systems for rare and emerging infectious diseases. Vet. Res. 39, 57.

Holtkamp, D. J., Kliebenstein, J.B., Neumann, E.J., Zimmerman, J.J., Rotto, H.F., Yoder, T.K., Wang, C., Yeske, P.E., Mowrer, C.L., Haley, C.A., 2013: Assessment of the economic impact of porcine reproductive and respiratory syndrome virus on United States pork producers. J. Swine Health Prod. 21, 72–84.

Kittawornrat, A., Engle, M., Panyasing, Y., Olsen, C., Schwartz, K., Rice, A., Lizano, S., Wang, C., Zimmerman, J., 2013. Kinetics of the porcine reproductive and respiratory syndrome virus (PRRSV) humoral immune response in swine serum and oral fluids collected from individual boars. BMC Vet. Res. Doi: 10.1186/1746-6148-9-61.

Kittawornrat, A., Prickett, J., Chittick, W., Wang, C., Engle, M., Johnson, J., Patnayak, D., Schwartz, T., Whitney, D., Olsen, C., Schwartz, K., Zimmerman, J., 2010. Porcine reproductive and respiratory syndrome virus (PRRSV) in serum and oral fluid samples from individual boars: will oral fluid replace serum for PRRSV surveillance? Virus Res. 154, 170-176.

Kittawornrat, A., Prickett, J., Wang, C., Olsen, C., Irwin, C., Panyasing, Y., Ballagi, A., Rice, A., Main, R., Johnson, J., Rademacher, C., Hoogland, M., Rowland, R., Zimmerman, J., 2012a. Detection of Porcine reproductive and respiratory syndrome virus (PRRSV) antibodies in oral fluid specimens using a commercial PRRSV serum antibody enzyme-linked immunosorbent assay. J. Vet. Diagn. Invest. 24, 262-269.

Kittawornrat, A., Wang, C., Anderson, G., Ballagi, A., Broes, A., Carman, S., Doolittle, K., Galeota, J., Johnson, J., Lizano, S., Nelson, E., Patnayak, D., Pogranichniy, R., Rice, A., Scherba, G., Zimmerman, J., 2012b. Ring test evaluation of the repeatability and reproducibility of a Porcine reproductive and respiratory syndrome virus oral fluid antibody enzyme-linked immunosorbent assay. J. Vet. Diagn. Invest. 24, 1057-1063.

Lager, K.M., Mengeling, W.L., Brockmeier, S.L., 1997a. Duration of homologous porcine reproductive and respiratory syndrome virus immunity in pregnant swine. Vet. Microbiol. 58, 127-133.

Lager, K.M., Mengeling, W.L., Brockmeier, S.L., 1997b. Homologous challenge of porcine reproductive and respiratory syndrome virus immunity in pregnant swine. Vet. Microbiol. 58, 113-125.

Lager, K.M., Mengeling, W.L., Brockmeier, S.L., 1999. Evaluation of protective immunity in gilts inoculated with the NADC-8 isolate of porcine reproductive and respiratory syndrome virus (PRRSV) and challenge-exposed with an antigenically distinct PRRSV isolate. Am. J. Vet. Res. 60, 1022-1027.

Lee, B.W., Bey, R.F., Baarsch, M.J., Larson, M.E., 1995. Class specific antibody response to influenza A H1N1 infection in swine. Vet. Microbiol. 43, 241-250.

Loftager, M., Eriksen, L., Nielsen, R., 1993. Antibodies against Actinobacillus pleuropneumoniae serotype 2 in mucosal secretions and sera of infected pigs as demonstrated by an enzyme-linked immunosorbent assay. Res. Vet. Sci. 54, 57-62.

Mäkelä, O., Rouslahti, E., Seppälä, I.J.T., 1970. Affinity of IgM and IgG antibodies. Immunochemistry 7, 917-932.

Olsen, C., Wang, C., Christopher-Hennings, J., Doolittle, K., Harmon, K.M., Abate, S., Kittawornrat, A., Lizano, S., Main, R., Nelson, E.A., Otterson, T., Panyasing, Y., Rademacher, C., Rauh, R., Shah, R., Zimmerman, J., 2013. Probability of detecting Porcine reproductive and respiratory syndrome virus infection using pen-based swine oral fluid specimens as a function of within-pen prevalence. J. Vet. Diagn. Invest. 25, 328-335.

Panyasing, Y., Goodell, C.K., Wang, C., Kittawornrat, A., Prickett, J.R., Schwartz, K.J., Ballagi, A., Lizano, S., Zimmerman, J.J., 2012. Detection of Influenza A Virus Nucleoprotein Antibodies in Oral Fluid Specimens From Pigs Infected Under Experimental Conditions Using a Blocking ELISA. Transbound. Emerg. Dis. doi: 10.1111/tbed.12019.

Prickett, J.R., Johnson, J., Murtaugh, M.P., Puvanendiran, S., Wang, C., Zimmerman, J.J., Opriessnig, T., 2011. Prolonged Detection of PCV2 and Anti-PCV2 Antibody in Oral Fluids Following Experimental Inoculation. Transbound. Emerg. Dis. 58, 121-127.

Prickett, J., Simer, R., Christopher-Hennings, J., Yoon, K.-J., Evans, R.B., Zimmerman, J.J., 2008a. Detection of Porcine reproductive and respiratory syndrome virus infection in porcine oral fluid samples: a longitudinal study under experimental conditions. J. Vet. Diagn. Invest. 20, 156-163.

Prickett J, Simer R, Yoon K-J, Kim W-I and Zimmerman J., 2008b. Surveillance of commercial growing pigs for PRRSV and PCV2 infections using pen-based oral fluid samples: A pilot study. Swine Health Prod. 16, 86–91.

Prickett, J.R., Zimmerman, J.J., 2010. The development of oral fluid-based diagnostics and applications in veterinary medicine. Anim. Health Res. Rev. 11, 207-216.

Ramirez, A., Wang, C., Prickett, J.R., Pogranichniy, R., Yoon, K.-J., Main, R., Johnson, J.K., Rademacher, C., Hoogland, M., Hoffmann, P., Kurtz, A., Kurtz, E., Zimmerman, J., 2012. Efficient surveillance of pig populations using oral fluids. Prev. Vet. Med. 104, 292-300.

Sierra, N., Ramirez, R., Mota, D., 2000. Aislamiento del virus de PRRS en México: Estudio clínico, serológico y virológico. Arch. Med. Vet. 32, 1-9.

Tian, K., Yu, X., Zhao, T., Feng, Y., Cao, Z., Wang, C., Hu, Y., Chen, X., Hu, D., Tian, X., Liu, D., Zhang, S., Deng, X., Ding, Y., Yang, L., Zhang, Y., Xiao, H., Qiao, M., Wang, B., Hou, L., Wang, X., Yang, X., Kang, L., Sun, M., Jin, P., Wang, S., Kitamura, Y., Yan, J., Gao, G.F., 2007. Emergence of fatal PRRSV variants: unparalleled outbreaks of atypical PRRS in China and molecular dissection of the unique hallmark. PLoS One 2, e526.

Velasova, M., Alarcon, P., Williamson, S., Wieland, B., 2012. Risk factors for porcine reproductive and respiratory syndrome virus infection and resulting challenges for effective disease surveillance. BMC Vet. Res. 8, 184.

Wagstrom, E.A., Chang, C.C., Yoon, K.J., Zimmerman, J.J., 2001. Shedding of porcine reproductive and respiratory syndrome virus in mammary gland secretions of sows. Am. J. Vet. Res. 62, 1876-1880.

Wills, R.W., Zimmerman, J.J., Yoon, K.J., Swenson, S.L., McGinley, M.J., Hill, H.T., Platt, K.B., Christopher-Hennings, J., Nelson, E.A., 1997 Porcine reproductive and respiratory syndrome virus: a persistent infection. Vet. Microbiol. 55, 231-240.

Table 1. Summary of sow serum and litter oral fluid sampling by farm over time

---------------------------- Calendar week and month ----------------------------

	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	
Farm[1]	May			June					July				August			*n*
1[2]	11	•	•	•	33	33	•	40	36	•	•	•	•	•	•	153
2[3]	18	5	17	•	•	15	•	•	42	24	24	•	•	•	•	145
3[4]	•	•	•	•	•	•	•	•	•	•	•	51	52	32	17	152
4[5]	•	•	•	•	•	•	•	•	•	•	30	30	35	42	13	150
n	29	5	17	0	33	48	0	40	78	24	54	81	87	74	30	600

[1] All gilts were vaccinated twice prior to placement in the sow farm with "MLV" (Ingelvac MLV®, Boehringer Ingelheim Vetmedica, Inc., St. Joseph. MO)

[2] Vaccinated at calendar weeks 9 and 13 with "ATP" (Ingelvac ATP®, Boehringer Ingelheim Vetmedica, Inc., St. Joseph. MO)

[3] Vaccinated with "ATP" at calendar weeks 1 and 4, and then "MLV" at calendar week 5

[4] Vaccinated with "ATP" at calendar weeks 2, 20, and 24

[5] Vaccinated with "ATP" at calendar week 1 and "MLV" at calendar week 5

Table 2. Comparison of qualitative results between assays and sample types

PRRSV antibody assay (sample matrix) n = 600	Negative S/P < 0.4	Positive S/P ≥ 0.4	% Positive	Statistically significant pairwise comparisons (Cochran's Q, $p < 0.05$)
1. Kit[1] (sow serum)	157	443	73.8%	2,3,4
2. Kit[2] (litter oral fluid)	6	594	99.0%	1,3
3. IgG[3] (sow serum)	127	473	78.8%	1,2,4
4. IgG[3] (litter oral fluid)	21	579	96.5%	1,3

[1] PRRS X3 Ab Test (IDEXX Laboratories, Inc., Westbrook, ME)

[2] PRRS Oral Fluids Ab Test (IDEXX Laboratories, Inc., Westbrook, ME)

[3] PRRS X3 Ab Test (IDEXX Laboratories, Inc., Westbrook, ME) modified to detect anti-PRRSV antibody IgG in serum and oral fluid specimens (Kittawornrat et al., 2012)

Table 3. Sow serum and litter oral fluid anti-PRRSV antibody testing results

			Mean serum antibody ELISA S/P ratio (SEM)				Mean oral fluid antibody ELISA S/P ratio (SEM)			
		n	IgM[1]	IgA[1]	IgG[1]	Commercial kit[2]	IgM[1]	IgA[1]	IgG[1]	Commercial kit[3]
Farm	1	153	0.21 (0.02)	0.25 (0.04)	0.99 (0.05)	0.98 (0.06)	0.00 (0.01)	0.05 (0.01)[a]	2.73 (0.12)	5.27 (0.21)[a]
	2	145	0.24 (0.03)	0.21 (0.04)	1.10 (0.05)	1.09 (0.07)	0.01 (0.01)	0.05 (0.01)[a]	2.61 (0.11)	5.06 (0.21)[a,b]
	3	152	0.20 (0.02)	0.17 (0.02)	0.92 (0.05)	0.91 (0.06)	0.00 (0.01)	0.02 (0.01)[b]	2.00 (0.11)	3.86 (0.21)[c]
	4	150	0.23 (0.02)	0.16 (0.02)	0.97 (0.06)	0.99 (0.07)	0.00 (0.01)	0.05 (0.01)[a]	2.19 (0.11)	4.30 (0.21)[b,c]
Sow parity	1	169	0.23 (0.02)	0.15 (0.02)	0.99 (0.06)[a,b,c]	1.04 (0.07)[a]	0.00 (0.01)	0.06 (0.01)[a]	2.31 (0.12)	4.49 (0.21)
	2	113	0.22 (0.02)	0.19 (0.03)	0.86 (0.06)[c]	0.83 (0.07)[c]	0.00 (0.01)	0.05 (0.01)[a,b]	2.25 (0.13)	4.46 (0.24)
	3	91	0.20 (0.03)	0.16 (0.02)	1.06 (0.06)[a,b]	1.05 (0.08)[a,b]	0.00 (0.01)	0.01 (0.01)[c]	2.46 (0.16)	4.70 (0.29)
	4	93	0.20 (0.03)	0.22 (0.06)	0.91 (0.05)[b,c]	0.86 (0.06)[b,c]	0.00 (0.01)	0.02 (0.01)[b,c]	2.34 (0.14)	4.51 (0.25)
	5	57	0.21 (0.03)	0.29 (0.06)	1.21 (0.07)[a]	1.21 (0.08)[a]	0.00 (0.01)	0.04 (0.01)[a,b,c]	2.62 (0.18)	5.12 (0.34)
	≥6	77	0.26 (0.05)	0.26 (0.08)	1.06 (0.08)[a,b,c]	1.05 (0.09)[a,b,c]	0.00 (0.01)	0.04 (0.01)[a,b,c]	2.45 (0.16)	4.82 (0.28)
Mean		600	0.22 (0.01)	0.20 (0.02)	0.99 (0.03)	0.99 (0.03)	0.00 (0.01)	0.04 (0.01)	2.38 (0.06)	4.62 (0.11)

[1] PRRS X3 Ab Test (IDEXX Laboratories, Inc., Westbrook, ME) modified to detect anti-PRRSV antibody isotypes in serum and oral fluid specimens (Kittawornrat et al., 2012)

[2] PRRS X3 Ab Test (IDEXX Laboratories, Inc., Westbrook, ME)

[3] PRRS Oral Fluids Ab Test (IDEXX Laboratories, Inc., Westbrook, ME)

[a,b,c] Within columns, different superscripted letters denote statistically significant differences between means ($p < 0.05$)

Table 4. Follow-up testing on matched pairs (sow serum and litter oral fluid) of PRRSV qRT-PCR-positive oral fluid samples

Farm	Calendar week	Sow parity	Litter size	Serum Ct and S/P ratios (sow)						Pre-weaning oral fluid testing results (litter)					
				PCR1[1]	PCR2[2]	IgM[3]	IgA[3]	IgG[3]	Kit[4]	PCR1[1]	PCR2[2]	IgM[3]	IgA[3]	IgG[3]	Kit[5]
1	24	1	11	Neg	Neg	0.32	0.09	0.85	0.92	34.3	34.9	0.03	0.31	3.38	7.75
1	25	1	12	" "	" "	0.72	0.19	0.33	0.31	36.5	37.1	0.01	0.03	0.58	1.24
1	27	2	9	" "	" "	0.47	1.54	1.76	1.75	27.2	27.2	0.05	0.14	5.44	8.83
1	27	1	10	" "	" "	0.00	0.28	2.08	2.68	34.6	36.1	0.05	0.22	4.13	6.98
2	20	5	11	" "	" "	0.08	0.05	2.09	2.14	33.6	36.4	0.00	0.04	4.85	8.26
2[6]	30	5	10	" "	" "	0.08	1.26	2.22	2.91	*29.0*	*30.7*	*0.04*	*0.20*	*2.14*	*4.22*
3	31	5	11	" "	" "	0.39	0.10	1.02	1.11	33.6	34.7	0.01	0.01	1.26	1.92
3[6]	32	1	9	" "	" "	0.94	0.07	2.36	2.75	*27.9*	*29.1*	*0.08*	*0.39*	*5.97*	*8.21*
3	33	1	11	" "	" "	0.22	0.14	2.21	2.61	29.6	30.6	0.01	0.06	2.81	6.84

[1] Cycle threshold (Ct) value ≥37 considered negative (tested by Iowa State University Veterinary Diagnostic Laboratory, Ames, IA)

[2] Cycle threshold (Ct) value ≥38 considered negative (tested by Tetracore Inc., Rockville, MD)

[3] PRRS X3 Ab Test (IDEXX Laboratories, Inc., Westbrook, ME) modified to detect anti-PRRSV antibody isotypes in serum and oral fluid specimens (Kittawornrat et al., 2012)

[4] PRRS X3 Ab Test (IDEXX Laboratories, Inc., Westbrook, ME)

[5] PRRS Oral Fluids Ab Test (IDEXX Laboratories, Inc., Westbrook, ME)

[6] PRRSV ORF5 sequences obtained from litter oral fluid samples (see Table 5)

Table 5. Pair-wise comparison of PRRSV isolates based on ORF5 nucleotide percent identity

Isolate	Farm 2 11/2010	Farm 2[1] 07/2011	Farm 2 11/2011	Farm 2 03/2012	Farm 3 12/2010	Farm 3[1] 08/2011	ATP[2]	MLV[3]	VR2332
Farm 2 11/2010		99.0	98.6	99.3	86.5	86.0	89.0	87.3	87.3
Farm 2 07/2011	99.0		98.1	98.6	86.2	85.7	88.3	86.7	86.7
Farm 2 11/2011	98.6	98.1		99.3	86.4	86.2	88.3	86.4	86.4
Farm 2 03/2012	99.3	98.6	99.3		86.5	86.4	88.7	86.7	86.7
Farm 3 12/2010	86.5	86.2	86.4	86.5		99.0	89.3	86.0	86.0
Farm 3 08/2011	86.0	85.7	86.2	86.4	99.0		88.8	85.5	85.5
ATP[2]	89.0	88.3	88.3	88.7	89.3	88.8		90.2	90.5
MLV[3]	87.3	86.7	86.4	86.7	86.0	85.5	90.2		99.3
VR2332	87.3	86.7	86.4	86.7	86.0	85.5	90.5	99.3	

[1] Sequences from Farm 2 isolate 07/2011 and Farm 3 isolate 08/2011 were derived from litter oral fluid samples collected in the study. Other Farm 2 and Farm 3 viruses were derived from sow serum samples collected in association with abortions and submitted for routine testing at veterinary diagnostic laboratories.

[2] Ingelvac ATP®, Boehringer Ingelheim Vetmedica, Inc., St. Joseph. MO

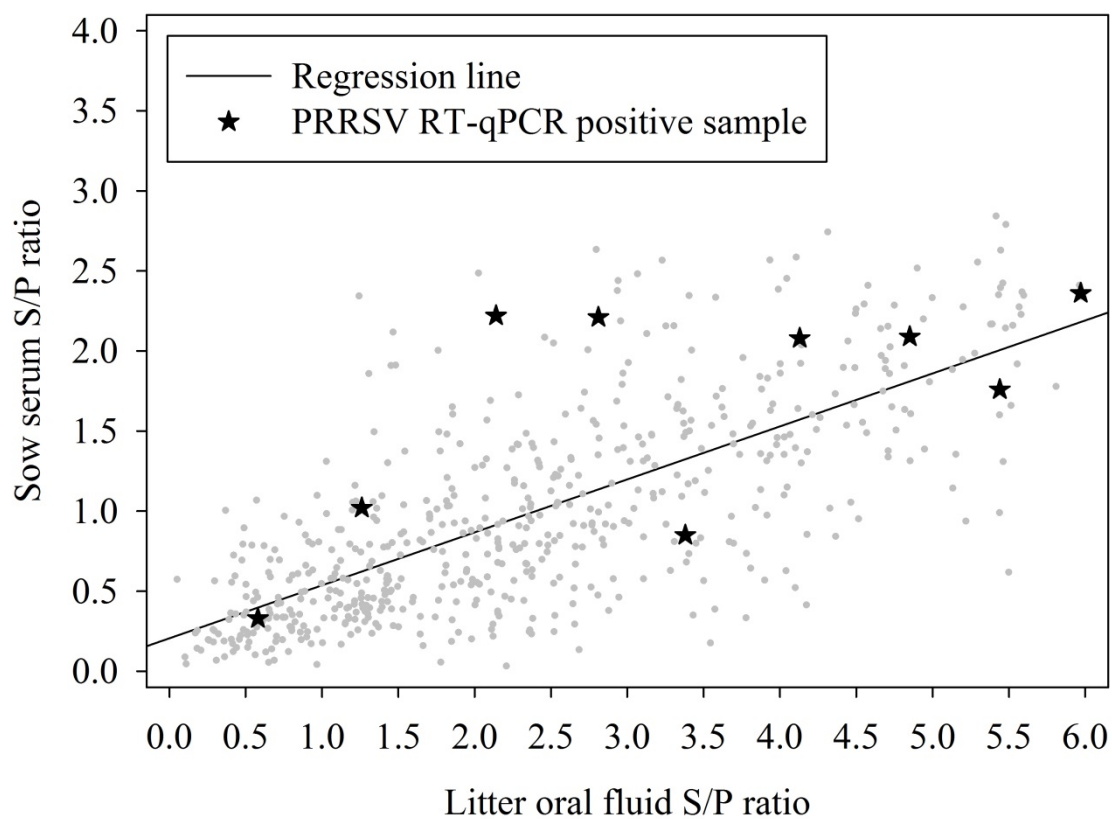[3] Ingelvac MLV®, Boehringer Ingelheim Vetmedica, Inc., St. Joseph. MO

Figure 1. Correlation between paired sow serum and litter oral fluid sample S/P ratios. Samples were tested using a PRRSV IgG ELISA, with procedures adapted to each sample matrix.
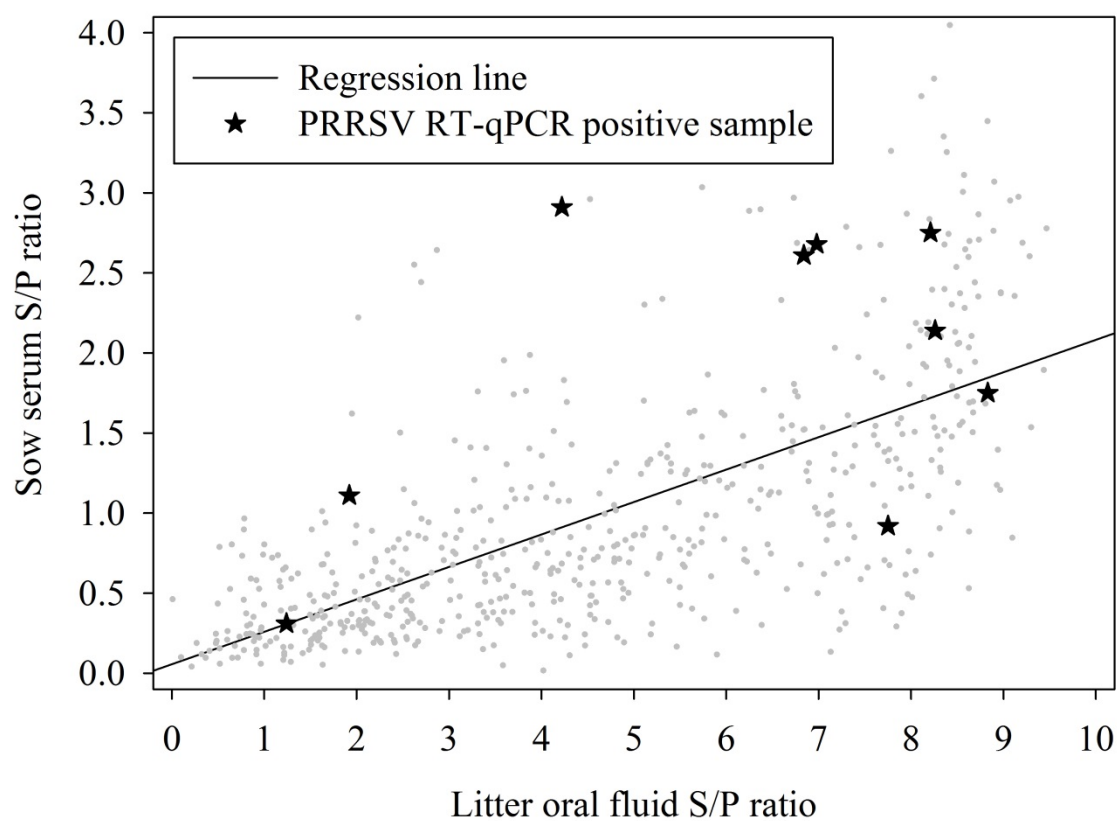
Figure 2. Correlation between paired sow serum and litter oral fluid sample S/P ratios. Samples were tested using commercial ELISA kits, i.e., PRRS X3 Ab Test (serum) or PRRS Oral Fluids Ab Test (oral fluid), IDEXX Laboratories, Inc., Westbrook, ME.

CHAPTER 7. GENERAL CONCLUSIONS

Current surveillance and monitoring systems (MOSS) in swine populations are primarily based on blood sampling - a labor-intensive sampling method. As a result, routine surveillance is generally cost-prohibitive and under-utilized. Cost-effective methods of disease monitoring are needed to support animal health and business decisions at the herd level, improve the quality of field research, and provide timely information on endemic and foreign animal diseases in the national swine herd.

Oral fluid samples are increasingly used for the surveillance of pathogens in commercial swine operations in North America. For example, the specimen type "swine oral fluid" was entered into the Iowa State University Veterinary Diagnostic Laboratory (ISU VDL) information management system in February, 2010. During the remainder of 2010, 10,329 oral fluid specimens were received for testing. The oral fluid sample submissions increased to 32,544 in 2011 60,050 in 2012, respectively. If the current level of submissions is maintained, the laboratory will receive >80,000 oral fluid samples in 2013.

The research described in this dissertation confirmed earlier research on the detection of porcine reproductive and respiratory syndrome virus (PRRSV) in oral fluids and expanded on earlier work by demonstrating that a commercial serum antibody ELISA could be adapted to oral fluid specimens and used routinely in commercial diagnostic laboratories for the detection of anti-PRRSV antibody (Chapter 3). Importantly, the protocol that was developed for the PRRSV oral fluid IgG ELISA was highly repeatable, reproducible, and compatible with the routine performance of the assay in high throughput diagnostic laboratories (Chapter 4). Furthermore, the diagnostic performance of the assay suggested the possibility of a cost-effective method to routinely monitor commercial swine populations for maternal antibody, vaccination compliance, and herd immune parameters using oral fluid sampling. Subsequent work (Chapter 5) provided a foundation for understanding assay performance in the context of PRRSV replication, shedding and the anti-PRRSV antibody response. This work provided the basis of a commercial PRRSV oral fluid antibody ELISA that is now available globally (PRRSV Oral Fluids Ab ELISA, IDEXX Laboratories, Inc., Westbrook, ME). In addition,

we began the process of transitioning this research to the field in this dissertation research. Specifically, as shown in Chapter 6, PRRSV surveillance was conducted easily, effectively, and cheaply using pre-weaning oral fluid samples from litters of piglets. This provided evidence that on-farm, real-time surveillance could be conducted using this approach, thereby achieving the goal of cost-effective methods of MOSS.

The research presented in this dissertation advanced the use of oral fluid specimens for PRRSV surveillance in swine production systems, but many issues, questions, and topics remain to be addressed. These generally fall into two categories: application and development. The application category contains questions that are simple to express, but will require hard data to answer: "How many oral fluid samples to collect?", "How often should testing be repeated", and "Which barn or room or pen should be collected". The development category represents the future of oral fluid testing. That is, having successfully produced one commercial oral fluid antibody ELISA, what other tests could be developed and commercialized to detect antibodies, pathogens, hormones, or drugs in oral fluids? Although there is much to be done, oral fluid sampling in swine populations would seem to be poised to fulfill the need to obtain the real-time data crucial to the health, productivity, and management of pig herds.

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