Surveillance in contemporary livestock production systems

by

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A dissertation submitted to the graduate faculty

in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Major: Veterinary Microbiology (Preventive Medicine)

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The student author, whose presentation of the scholarship herein was approved by the program of study committee, is solely responsible for the content of this dissertation. The Graduate College will ensure this dissertation is globally accessible and will not permit alterations after a degree is conferred.

Iowa State University

Ames, Iowa

2017

DEDICATION

To my parents who taught me to never let grass grow under my feet.
Dr. Gary Rotolo and Dianne Rotolo, RN

To my major professor and mentor who challenged me to see myself as more than a student.
Dr. Jeffrey J. Zimmerman

To my steadfast employer and mentor who invested in me and gave me countless opportunities to grow.
Dr. Rodger Main

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ABSTRACT

The objectives of this research were to explore the use of swine oral fluids, a type of aggregate sample, in infectious disease surveillance. In Chapter 2, the uses and surveillance applications of aggregate samples are reviewed. As reported in the refereed literature, bulk tank milk samples from ovine, bovine, and caprine herds have been tested to determine disease status and herd immunity. Likewise, swine producers and veterinarians have used oral fluid testing for disease detection and the evaluation of herd immunity. In Chapter 3, sampling guidelines for oral fluid surveillance in commercial swine herds are presented. These guidelines are the result of field-based research in which oral fluids were collected weekly from 3 barns on one wean-to-finish farm for 9 weeks and tested for porcine reproductive and respiratory syndrome virus (PRRSV) RNA. Results were modeled using a piecewise exponential survival model to provide estimates of the probability of detection by disease prevalence, sample size, and diagnostic assay performance. Notably, this study showed that fixed spatial sampling was as good, if not better, than simple random sampling and that probability of detection on a swine farm improved significantly when multiple barns on the farm were sampled. In Chapter 4, a combined IgM-IgA PRRSV oral fluid ELISA was evaluated for its ability to detect pig-derived antibody produced in response to infection in the presence of maternal antibody. Two studies were performed. In Study 1 (experimental conditions), oral fluid samples were collected daily from 12 PRRSV-negative pigs from days post vaccination (DPV) -7 to DPV 42. Pigs were vaccinated using a modified-live PRRS vaccine on DPV 0. In Study 2 (field conditions), oral fluids were collected weekly from 3 wean-to-finish sites, each with 3 barns, for a total of 9 samplings. Testing of oral fluids from both studies by IgG, IgM, IgA, and IgM-IgA ELISAs showed that the IgM-IgA ELISA was able to detect pig-derived IgM and IgA in the face of circulating maternal antibody and that the combined IgM-IgA assay provided better performance than detection of either IgM or IgA alone.

CHAPTER 1. GENERAL INTRODUCTION

The purpose of disease surveillance and monitoring is to detect infectious agents and identify changes in disease trends within populations, respectively (Paskins, 1999). In order to achieve success in this endeavor, samples need to be collected in an efficient manner and applicable assays must be available for testing. The use of aggregate sampling represents a significant improvement in the effectiveness and efficiency of surveillance because samples representative of multiple animals provide a higher probability of detection at lower cost (Olsen et al., 2013; Thurmond and Perez, 2006). Examples of aggregate samples in livestock surveillance include bulk tank milk samples and oral fluid specimens (Rotolo et al., 2017; Thurmond and Perez, 2006). Therefore, the diagnostic uses of bulk tank milk samples and oral fluid samples were reviewed in Chapter 2 of this dissertation.

While aggregate samples are a highly useful tool in disease surveillance, it must be recognized that sampling guidelines for individual samples do not apply to aggregate samples (Rotolo et al., 2017). Therefore, the development of oral fluid sampling guidelines for disease detection in swine herds was evaluated in Chapter 3 of this dissertation.

Surveillance and monitoring programs cannot function without repeatable, reproducible, and accurate diagnostic assays. No surveillance program can be successful if testing results are ambiguous or unreliable. In the case of the PRRSV oral fluid ELISA assay, a positive result in pigs less than 10 weeks of age may indicate presence of maternal antibody or pig-derived antibody in response to infection (Yoon et al., 1996; Houben et al., 1995). Current PRRSV ELISA tests target IgG antibody. Thus, Chapter 4 of this dissertation reports the evaluation of a combined IgM-IgA ELISA, as well as its ability to detect pig-derived antibody (IgM, IgA) produced in response to infection, despite the presence of maternal IgG.

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CHAPTER 2. HERD-LEVEL INFECTIOUS DISEASE SURVEILLANCE OF LIVESTOCK POPULATIONS USING AGGREGATE SAMPLES

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Modified from a manuscript submitted to Animal Health Research Reviews

Abstract

All sectors of livestock production are in the process of shifting from small populations on many farms to large populations on fewer farms. A concurrent shift has occurred in the number of livestock moved across political boundaries. The unintended consequence of these changes has been the appearance of multifactorial diseases that are resistant to traditional methods of prevention and control. The need to understand complex animal health conditions mandates a shift toward the collection of longitudinal animal health data. Historically, collection of such data has frustrated and challenged animal health specialists. A promising trend in the evolution toward more efficient and effective livestock disease surveillance is the increased use of aggregate samples, e.g., bulk tank milk and oral fluid specimens. These sample types provide the means to monitor disease, estimate herd prevalence, and evaluate spatiotemporal trends in disease distribution. Thus, this article provides an overview of the use of bulk tank milk and pen-based oral fluids in the surveillance of livestock populations for infectious diseases.

Keywords: surveillance, swine oral fluid, bulk tank milk, aggregate sample

Introduction

Globally and locally, achieving the control of historically impactful infectious diseases of livestock continues to frustrate producers and challenge animal health specialists. A core requirement of a successful control program is the on-going collection of disease data from populations. Schwabe (1982) describes this as the process of establishing baseline levels "against which effects of intervention (control) efforts can be measured."

The on-going burden of disease in endemic areas and the expansion of infectious agents into previously free areas exposes the frailty of current surveillance and response/control programs (Saeed et al., 2015; Backer et al., 2009; Lee, 2015; Neira et al., 2017). Foot-and-mouth disease virus (FMDV) was identified in 1897, but 116 years later endemic FMDV losses were estimated at $6.5 to $21 billion dollars annually and only 66 of the 181 (36.5%) OIE-member countries are "FMD free where vaccination is not practiced" (Knight-Jones, Rushton, 2013; Longjam et al, 2011; OIE, 2017a). Classical swine fever virus (CSFV) was identified in 1903 (de Schweinitz, Dorset, 1903), but in 2017, just 32 of the 181 (17.7%) OIE-member countries are considered free of CSFV (OIE, 2017b). This, despite the profound global economic burden of CSFV and the clear benefits of eradication, e.g., the benefit:cost ratio of CSFV eradication in the U.S. was estimated at $\geq$ 13.2 (Pinto et al., 2011; USDA, 1981). Initially identified on the basis of outbreaks of unknown origin in the 1980's porcine reproductive and respiratory syndrome virus (PRRSV) was isolated 1991 and has become endemic in most major pork-producing regions of the world (Wensvoort et al., 1991; Zimmerman et al., 2012). Holtkamp et al. (2013) estimated U.S. pork producers' losses to PRRSV at $664 million annually. Nathues et al. (2017) estimated losses to European producers at €126.79 per sow per year and €3.77 per pig marketed in herds with "slight" PRRS.

A promising trend in the evolution toward more efficient and effective livestock disease surveillance is the increased use of aggregate samples (Gibert et al., 2017; Rotolo, et al., 2017; Strutzberg-Minder et al., 2015; Thurmond, Perez, 2006). By definition, an aggregate sample represents two or more animals at a specific location and time, e.g., bulk tank milk

and pen-based oral fluid samples. The use of aggregate samples in veterinary surveillance has grown in tandem with developments in diagnostic technology, e.g., nucleic acid-based assays and antibody assays specifically adapted to these specimens. The purpose of this article is to review the use of bulk tank milk and pen-based oral fluids in infectious disease surveillance of livestock populations.

Bulk tank milk samples

Bulk tanks are designed to cool, agitate, and store milk in bovine, ovine, and caprine Grade A dairies. Among other requirements of the Pasteurized Milk Ordinance (U.S. Food and Drug Administration, 2015), bulk tanks must chill milk (4.4°C to 7°C) within 2 hours of collection and maintain this range thereafter. The size and number of bulk tanks varies among farms as a function of the number of animals in the herd or flock, but larger operations may have multiple tanks capable of storing thousands of gallons of milk. Milk haulers may collect once a day, more than once a day, or every other day, depending on the farm's storage capacity and milk production levels. Regardless of the collection schedule, bulk tanks must be emptied, cleaned and sanitized at least every 72 hours (Bickett-Weddle et al., 2011; U.S. Food and Drug Administration, 2015).

In the context of disease surveillance, samples from bulk milk tanks represent the lactating cows in the herd (Sekiya et al., 2013). Depending on governmental standards or ordinances, tanks are agitated for ≥ 10 minutes after which samples are collected aseptically from the top of the tank using a sterile pipette, syringe, or sanitized dipper (Bickett-Weddle et al., 2011; U.S. Food and Drug Administration, 2015). Although bulk tank milk samples do not represent dry cows or cows on milk withhold, they provide an economical, convenient, and timely approach for the detection of specific pathogens and/or estimation of herd prevalence (Collins et al., 2017; Lanyon et al., 2014; Olde Riekerink et al., 2006; Sekiya et al., 2013). Economically significant pathogens detectable in bulk tank milk samples and reported in the refereed literature are discussed below and listed in Table 1.

Schmallenberg Virus

Schmallenberg virus (SBV) is an arthropod vector-borne orthobunyavirus first detected in dairy herds in Germany and The Netherlands in 2011 (Balmer et al., 2014; Daly et al., 2015; Gubbins et al., 2014; Johnson et al, 2014). SBV infection causes abortions, congenital malformations, diarrhea, and fever in bovine, ovine and caprine species (Collins et al., 2017; Daly et al., 2015; Johnson et al., 2014). The duration of SBV viremia is relatively short, i.e., an average of 3 to 4 days (Gubbins et al., 2014), but SBV serum neutralizing antibodies can be detected in cattle for as long as 24 months post infection (Elbers et al., 2014). The detection of SBV nucleic acid has not been reported in milk, but antibodies to SBV can be detected in individual cow and bulk tank milk samples using commercial indirect ELISAs (Balmer et al., 2014; Daly et al., 2015; Johnson et al, 2014). Although test performance estimates are not available (diagnostic sensitivity, diagnostic specificity), results of bulk tank milk ELISA testing were predictive of within-herd seroprevalence and herd immunity (Collins et al., 2017). Analyses based on bulk tank milk testing results have been used to assess the spatial distribution, rate of spread, direction of the spread, and effect of farm altitude on the prevalence of SBV (Balmer et al., 2014; Johnson et al., 2014).

Bovine viral diarrhea virus

First described in the 1940's, bovine viral diarrhea virus (BVDV) is a pestivirus transmitted through direct contact or fetal (*in utero*) infection (Goens, 2002). Clinical signs of BVDV include watery and/or bloody diarrhea, dehydration, pyrexia, tenesmus, tachypnea, and ulcers of the muzzle, lips, oral cavity, and/or nares (Goens, 2002).

BVDV antibodies can be detected in bulk tank milk samples using blocking, indirect, or competitive ELISAs (Foddai et al., 2015; Houe, 1999; Hanon et al., 2017; Kramps et al., 1999; Lanyon et al., 2014; Renshaw et al., 2000). A Danish blocking ELISA demonstrated a diagnostic sensitivity of 100% and diagnostic specificity of 62% when testing bulk tank milk samples from herds with a BVDV prevalence of 26% (Foddai et al., 2015). Diagnostic sensitivities and specificities of competitive ELISAs were reported as 97% to 100% and 99%, respectively; whereas the diagnostic sensitivities and specificities of indirect ELISAs

were reported as 94% to 100% and 98% (Hanon et al., 2017). As with Schmallenberg virus, bulk tank milk ELISA results were highly associated with herd seroprevalence (Lanyon et al., 2014).

Persistently infected (PI) animals, the result of fetal infection during the first trimester of pregnancy (immunotolerance), serve as a continuous source of infection (Fray et al., 2000; Houe, 1999; Renshaw et al., 2000). PI cows produce little-to-no BVD antibody, but continuously shed RT-rtPCR-detectable levels of BVDV in milk (Houe, 1999; Kramps, et al., 1998; Radwan et al., 1995; Renshaw et al., 2000). Drew et al., 1999 reported 100% diagnostic sensitivity and specificity for PCR-based detection of BVDV RNA in bulk tank milk samples from herds with PI cows.

Strategically, antibody detection is used to identify herds with circulating BVDV and nucleic acid detection is used to identify herds with PI cattle (Lanyon et al., 2014). Monitoring changes in antibody prevalence has been used to determine whether a BVDV infection is ongoing or recent (Lanyon et al., 2014). ELISA testing has also been used to monitor declining antibody levels after removal of persistently infected cattle (Houe, 1999).

Border disease virus

First reported in England and Wales in 1958 and closely related to BVDV, Border disease virus (BDV) is a pestivirus of ovine and caprine species (Nettleton et al., 1998). BDV is transmitted through direct contact or transplacentally, with infection during early pregnancy resulting in persistently infected offspring (Garcia-Perez et al., 2010). Goats are susceptible to BDV, but infection is rare and typically results in abortion (Nettleton et al., 1998). In sheep, clinical signs of BDV include abortion, stillbirths, and non-viable lambs.

As in the case of BVDV, PI animals shed BDV continuously and do not produce antibodies. Bulk tank milk samples can be tested for BDV by RT-rtPCR, however, estimates of diagnostic performance have not been reported (Berriatua et al., 2006). Immunocompetent animals produce antibodies detectable in bulk tank milk (Garcia-Perez et al., 2010). In one study, the diagnostic sensitivity and specificity of a blocking ELISA for BDV detection in

bulk tank milk samples was reported as 100% and 85.2%, respectively (Corbiere et al., 2012). A high seroprevalence of BDV in lactating animals suggests the presence of persistently infected animals (Berriatua et al., 2006). Thus, ELISA testing of bulk tank milk samples provides the means to estimate the prevalence of BDV in flocks and may indirectly reveal the presence of persistently infected animals (Berriatua et al., 2006; Garcia-Perez et al., 2010).

Foot-and-mouth disease virus

Foot-and-mouth disease virus (FMDV) is a highly impactful picornavirus of cloven-hoofed animals (Knight-Jones, Rushton, 2013; Reid et al., 2006; Thurmond, Perez 2006). FMDV can be transmitted by direct or indirect contact (Bravo de Rueda et al., 2014). Clinical signs of FMDV infection include vesicular lesions, decrease in milk yield in lactating cattle, and pyrexia (Armstrong, Mathew, 2001).

FMDV was detected in milk samples from individual cows by RT-rtPCR for 23 days post-inoculation (Reid et al., 2006). Estimates of the diagnostic sensitivity and specificity of RT-rtPCR for the detection of FMDV in bulk tank milk samples has not been reported, but Thurmond, Perez (2006), predicted that RT-rtPCR testing of bulk tank milk samples would detect FMDV 4 to 7 days earlier than detection based on the recognition/reporting of clinical signs.

FMDV antibodies may be detected in ovine and bovine milk using blocking ELISAs (Armstrong, 1997a;1997b). Estimates for diagnostic sensitivity and specificity of these ELISAs are not available, but Armstrong, Mathew (2001) found a statistically significant correlation ($r = 0.53$) between serum and milk antibody titers. On this basis, these researchers suggested that antibody testing of bulk tank milk samples would be an effective approach for monitoring herd immunity and/or evaluating population susceptibility to FMDV.

Mycobacterium avium subspecies *paratuberculosis*

Mycobacterium avium subspecies *paratuberculosis* (MAP) is the etiologic agent of Johne's disease in ruminants (Mortier et al., 2014). Most commonly acquired via fecal-oral transmission, Johne's disease is characterized by enteritis, decreased milk yield, weight loss, diarrhea, and death (Mortier et al., 2014; Wilson et al., 2010). A causal role for MAP in Crohn's disease has been postulated, but was neither confirmed nor rejected by an assessment of the available data (Feller et al., 2007).

MAP is detectable in milk via culture and PCR testing, but culture of bulk tank milk samples is not practical because the procedure is neither diagnostically sensitive nor timely, i.e., culture can take 18 to 52 weeks (Slana et al., 2008). The most common target of PCR assays is multiple copy insertion sequence *IS900* in the MAP genome (Slana et al., 2008). The analytical sensitivity of the *IS900* PCR is reported as 5 to 6 MAP cells per ml of bulk tank milk versus 83 MAP cells per ml for a PCR targeting *F57*. However, *IS900* PCRs may have issues with analytical specificity because of the homology of this region across mycobacteria species (Cousins et al., 1999; Slana et al., 2008; Tasara et al., 2005). Jayaro et al (2004) reported a diagnostic sensitivity of 21% and diagnostic specificity of 50% for bulk tank milk samples using an *IS900* PCR. No estimates of diagnostic sensitivity and specificity are available for *F57*-based PCRs.

ELISA-detectable MAP antibodies are present in bulk tank milk samples, but interpretation of testing results has not been clearly established (Beaver et al., 2016; Nielsen et al., 2000; van Weering et al., 2007; Wilson et al., 2010). Regardless, some researchers believe that ELISA testing of bulk tank milk samples can be used effectively by monitoring changes over time (Beaver et al., 2016; van Weering et al., 2007). Alternatively, Beaver et al. (2016) suggested the concurrent use of both assays for bulk tank milk monitoring programs for MAP (Beaver et al., 2016). Thus, herds with positive PCR results and high ELISA titers reflected active infection; whereas, herds with positive PCR results but low ELISA titers reflected environmental contamination (Beaver et al., 2016).

***Coxiella burnetti* (Q fever)**

Coxiella burnetti (Cb) is an obligate, intracellular rickettsial organism and the cause of Q fever in animals and humans (Kim et al., 2005). Infection with Cb results in reproductive disease, including metritis and infertility in cattle and abortion in goats and sheep (Kim et al., 2005; Rodolakis et al., 2007). Shedding patterns of Cb in milk is species-dependent and varies among cattle, sheep and goats (Rodolakis et al., 2007). Cattle shed Cb in milk for several months, goats shed for a shorter time, and sheep do not reliably shed in milk (Astobiza et al., 2012; Rodolakis et al., 2007). Cb nucleic acid and antibody is detectable in bulk tank milk samples PCR and ELISA (Rodolakis et al., 2007; van den Brom et al., 2012). Muskens et al. (2011) reported diagnostic sensitivity and specificity of 82% and 70%, respectively, when testing bulk tank milk samples by a commercial real-time PCR. The diagnostic sensitivity and specificity of a commercial Cb antibody ELISA for bulk tank milk was reported as 88.2% and 94.6%, respectively, using manufacturer-recommended cutoffs (van den Brom et al., 2012). When used in combination, ELISA testing of bulk tank milk samples can be used to determine herd exposure and estimate prevalence of Cb while PCR testing can be used to determine shedding and prevalence (Astobiza et al., 2012; Muskens et al., 2011).

Detection of bacterial pathogens associated with mastitis

Streptococcus agalactiae is a highly contagious, obligate pathogen of the bovine mammary gland and a cause of subclinical and clinical mastitis (Keefe, 1997; Mweu et al, 2012; Phuektes et al, 2003; Olde Riekerink et al, 2006). *Streptococcus agalactiae* may be detected in bulk tank milk samples by culture or PCR (Keefe, 1997; Phuektes et al., 2003). As reviewed by Phuektes et al. (2003), estimates of the diagnostic sensitivity of culture range from 20% to 84%. Estimates of the diagnostic sensitivity and specificity are not available, but as would be expected, testing multiple bulk tank milk samples was shown to increase the likelihood of detecting *Streptococcus agalactiae* by PCR (Phuektes et al., 2003; Soltau et al., 2017). ELISA-detectable *Streptococcus agalactiae* antibodies have been reported in individual milk samples, but this approach has not been evaluated for bulk tank milk testing (Logan et al, 1982).

Staphylococcus aureus is an opportunistic pathogen and a cause of subclinical and clinical mastitis in cattle, sheep, and goats (Haran et al., 2012; Merz et al, 2016; Olde Riekerink et al., 2006; Zanardi et al., 2012). As reviewed by Olde Riekerink et al. (2010) culture of bulk tank milk for *Staphylococcus aureus* had an estimated diagnostic sensitivity of 21 to 42% and a diagnostic specificity of 100%. Repeated sampling is recognized to improve the probability of detection by culture (Olde Riekerink et al., 2006, 2010). PCR testing of bulk tank milk samples can be used to detect *Staphylococcus aureus*, estimate herd prevalence of the infection, and assess for the presence of methicillin-resistant strains (Haran et al., 2012). The diagnostic sensitivity and specificity of PCR testing for *Staphylococcus aureus* in bulk tank milk samples is reported at 99% and 67%, respectively (Zanardi et al., 2012). Using individual milk, ELISA testing for antibodies against *Staphylococcus aureus* may be used to as a screening tool to detect infected animals (Fox and Adams, 2000).

Mycoplasma bovis is a highly pathogenic mycoplasma causing both mastitis and respiratory disease in adult cattle (Parker et al., 2017a). *Mycoplasma bovis* is detectable in bulk tank milk samples by culture, but the assay can take 7 to 10 days and overgrowth of bacteria is problematic (Parker et al., 2017a;b). The diagnostic sensitivity of *Mycoplasma bovis* culture is reported as 50%, with diagnostic specificity estimates as high as 100% (Justice-Allen et al., 2011; Maunsell et al., 2011). The diagnostic sensitivity and specificity of *Mycoplasma bovis* PCR for individual milk samples is reportedly 100% and 99.3%, respectively, but estimates of PCR performance for bulk tank milk samples have not been reported (Cai et al., 2005). PCR testing allows for more rapid detection of *Mycoplasma bovis* versus culture and herd prevalence estimates can be extrapolated from results (Arcangioli et al., 2011). A commercial antibody ELISA is available for bulk tank milk testing and estimates for diagnostic sensitivity and specificity are 60.4% and 97.3%, respectively (Nielsen et al., 2015). The combination of PCR and ELISA testing can reveal *Mycoplasma bovis* infection in a herd and is an effective approach for surveillance (Nielsen et al., 2015).

Oral fluid samples

Oral fluids are collected from swine or cattle by providing access to a rope suspended in the pen, then recovering the sample for diagnostic testing (Prickett et al., 2008a,b; Prickett et al., 2010; Smith et al., 2004; Stanford et al., 2009). Oral fluid samples are an aggregate sample composed of saliva and transudate originating from capillaries within the buccal and gingival mucosa (Prickett et al., 2008a). Oral fluids contain both local and serum-derived antibodies and pathogens (Prickett et al., 2008a,b; Prickett, Zimmerman, 2010). In addition, viruses, bacteria, and other test analytes in feed, water, or the environment may be present in oral fluids as a result of normal exploratory behavior (Johnson et al., 2012; Kittawornrat and Zimmerman, 2011). This explains the detection of porcine epidemic diarrhea virus in swine oral fluid samples and *Escherichia coli* and salmonella in cattle (Bjustrom-Kraft et al., 2016; Renter et al., 2008; Smith et al., 2005a,b). In cattle, oral fluids have been used in observational studies in feedlot cattle (Renter et al., 2008; Smith et al., 2005a,b), but have not been routinely utilized in surveillance. In contrast, oral fluids have been used extensively for disease surveillance in swine populations. Therefore, the remainder of this section will focus exclusively on this subject.

Oral fluids can be collected from groups or individual pigs (Pepin et al, 2015a,b; White et al., 2014). In group-housed animals, oral fluids offer a higher probability of detection with fewer samples when compared to individual serum samples (Olsen et al., 2013). Sampling guidelines for oral fluid collection at the barn or site level have been published (Rotolo et al., 2017).

Diagnostic assays optimized for swine oral fluid specimens have been available in North American veterinary diagnostic laboratories since 2010 (Bjustrom-Kraft et al., 2017; Olsen et al., 2013). In three North American swine-interest veterinary diagnostic laboratories, the number of oral fluid tests performed increased from 20,963 in 2010 to 369,439 in 2016 (Bjustrom-Kraft et al., 2017). Pathogens detectable in oral fluid samples and reported in the refereed literature are listed in Table 2. Selected pathogens are reviewed below.

Foot-and-mouth-disease virus

Rapid screening of swine herds is critical in the control of FMDV because pigs aerosolize a large amount of virus compared to cattle and promulgate virus transmission (Stenfeldt et al., 2016). Under experimental conditions, FMDV was isolated from swine oral fluids on day post inoculation (DPI) 1 to 5 (Senthilkumaran et al., 2017). By RT-rtPCR, FMDV was detected from one DPI, i.e., prior to the appearance of clinical signs, and up to 21 DPI (Mouchantat et al., 2014; Senthilkumaran et al., 2017). RNA was detected in oral fluids one day earlier than oral or nasal swab samples and continued ~7 days longer (Senthilkumaran et al., 2017). A field-deployable reverse transcription-insulated isothermal PCR (RT-iiPCR) has also been used to detect FMDV RNA in oral fluids (Ambagala et al., 2016). FMDV antigens were detected in oral fluids one to 6 DPI using lateral flow immunochromatographic strip tests and 2 to 3 DPI using a double-antibody sandwich ELISA (Senthilkumaran et al., 2017). FMDV IgA was detected in oral fluids using a solid-phase competitive ELISA beginning at 14 DPI (Senthilkumaran et al., 2017). Pacheco et al. (2010) were not successful in detecting FMDV IgM or IgG in oral fluid samples. Estimates of diagnostic sensitivity and specificity have not been reported for the assays reported in this paragraph. Although FMDV oral fluid assay development is in its early stages, preliminary results support the use of nucleic acid and/or antibody detection as a method to rapidly screen herds (Ambagala et al., 2016; Senthilkumaran et al., 2017).

Classical swine fever virus

Classical swine fever virus (CSFV) is a pestivirus with significant economic consequences resulting from clinical disease, lost export markets, and costs related to control and/or eradication efforts (Fernández-Carrión et al., 2016). CSFV can be transmitted by direct or indirect contact and, depending on the virulence of the strain, causes pyrexia, anorexia, lethargy, conjunctivitis, enlarged and discolored lymph nodes, constipation, and diarrhea in affected pigs (Moennig et al., 2003; Petrini et al., 2017). Under experimental settings, CSFV was detected in oral fluids by RT-rtPCR from 7 up to 30 DPI, with a higher detection rate in oral fluid than blood samples (40% vs 28%) (Dietze et al., 2017; Petrini et al., 2017). Estimates of diagnostic sensitivity and specificity have not been reported for these assays and, overall, research on CSFV oral fluid diagnostics is in its initial phases.

African swine fever virus

Infection with African swine fever virus (ASFV), the only member of family *Asfarviridae*, is a cause of fever, hemorrhage, and mortality in domestic and feral pigs (Gimenez-Lirola et al., 2016; Guinat et al., 2014; Sanchez-Vizcaino, Neira, 2012). Transmitted through direct and indirect contact, ASFV is of particular concern because, since its introduction into Georgia in 2007, it has steadily advanced westwardly into Europe via feral swine and threatens to spread eastwardly into China (Guinat et al., 2014; Vergne et al., 2017).

Under experimental conditions, ASFV was detected in oral fluid 3 to 5 DPI by PCR (Grau et al., 2015; Guinat et al., 2014). ASFV antibodies were detected at 11 DPI in individual oral fluid samples by indirect ELISA under experimental conditions (Mur et al., 2013). The pattern of antibody response in oral fluids was similar to the pattern seen in serum (Mur et al, 2013). ASFV antibodies were also detected using a p30 indirect ELISA in oral fluids (Gimenez-Lirola et al., 2016). Diagnostic sensitivities and specificities for these assays have not been reported. As in the cases of FMDV and CSFV, further studies are needed to optimize ASFV oral fluid assays and assess their use in the field (Grau et al., 2015).

Porcine reproductive and respiratory syndrome virus

Porcine reproductive and respiratory syndrome virus (PRRSV) is an arterivirus transmitted through direct and indirect contact (Zimmerman et al., 2012). Clinical signs of PRRSV vary based on the age of the pig and the virulence of the isolate. In sows, clinical signs include abortion, stillbirths, anorexia and mortality (Zimmerman et al., 2012). PRRSV is often an etiological component of the porcine respiratory disease complex in growing pigs (Zimmerman et al., 2012).

The detection of PRRSV nucleic acid in oral fluids has been extensively documented under field and experimental conditions (Kittawornrat et al., 2010; 2014; Pepin et al., 2015a,b; Prickett et al., 2008a,b; Ramirez et al., 2012; Rotolo et al., 2017). Kittawornrat et al. (2010) reported detection in ~10% of experimentally inoculated boars at 24 hours post-inoculation by RT-rtPCR. Olsen et al. (2013) evaluated test performance as a function of within pen

prevalence. In pens holding 25 pigs, the probability of detecting PRRSV RNA or PRRSV antibody in pens containing ≥ 1 positive (4% prevalence) was 62% and 61%, respectively. PRRSV may also be sequenced from oral fluids (Biernacka et al., 2016).

IgG, IgA and IgM antibody isotypes were detected in oral fluids collected from individual boars using a commercial PRRS serum antibody indirect ELISA modified for oral fluids (Kittawornrat et al., 2013). The pattern of PRRSV antibody ontogeny was similar in serum and oral fluid, with IgM detected in oral fluids at 3 DPI, IgA at 7 DPI, and IgG at 8 DPI (Kittawornrat et al., 2013). Commercial PRRSV oral fluid ELISAs have since become available. Antibodies were also detected in oral fluid using a fluorescent microsphere immunoassay (FMIA) with a reported diagnostic sensitivity of 92% and diagnostic specificity of 91% (Langenhorst et al., 2012).

Testing of oral fluids can be used to assess the effectiveness of PRRSV control and/or elimination programs (Biernacka et al., 2016; Rotolo et al., 2017). A distinct advantage of PRRSV oral fluid-based surveillance is that pen-based oral fluid sampling provides a higher probability of detection than individual animal sampling using either RT-rtPCR or ELISA (Olsen et al., 2013).

Influenza A virus

Influenza A virus (IAV) is an orthomyxovirus of humans, horses, sea mammals, birds, and pigs transmitted via direct and indirect contact (Hughes et al., 2015; Neira et al., 2016). Influenza A virus in commercial swine herds results in chronic, endemic infection with respiratory or reproductive clinical signs, as well as clinically inapparent infections (Goodell et al., 2013; Panyasing et al., 2013). IAV is an important pathogen to surveil in pigs because of its zoonotic potential (Hughes et al., 2015; Vincent et al., 2014).

Under experimental conditions, IAV RNA was detected in swine oral fluids by one DPI and up to 69 DPI (Allerson et al., 2014; Decorte et al., 2015). Decorte et al. (2015) reported the duration of detection in oral fluids as 14 days longer than detection in nasal swabs by RT-rtPCR (Decorte et al., 2015). Compared to individual nasal swabs, the diagnostic sensitivity

and specificity of pen-based oral fluid RT-rtPCR testing was estimated at 80% and 100%, respectively (Romagosa et al., 2012). Although further optimization is necessary, IAV have also been isolated from oral fluids (Goodell et al., 2013). RT-rtPCR testing of oral fluids can be used to track viral circulation and monitor the effect of vaccination and control programs in commercial swine herds (Goodell et al., 2013).

Panyasing et al. (2013) reported the ontogeny of IAV IgM, IgA and IgG in pigs housed under experimental conditions using isotype-specific indirect ELISAs. Serum and oral fluid IgG responses were highly correlated (r = 0.80) (Panyasing et al., 2013). Detection of IAV antibody has also been reported using blocking or competitive ELISA formats (Panyasing et al., 2014; Strutzberg-Minder et al., 2015). Diagnostic sensitivity and specificity estimates have not been established for these assays. Antibody detection in oral fluids allows for the detection of IAV infection in the absence of clinical signs (Panyasing et al., 2013).

Coronaviruses

Porcine epidemic diarrhea virus (PEDV) is an enteric coronavirus transmitted via the fecal-oral route (Bjustrom-Kraft et al., 2016; Crawford et al., 2015). Clinical signs of PEDV infection in swine include watery diarrhea, vomiting, and mortality in neonates (Bjustrom-Kraft et al., 2016). In the field, Bjustrom-Kraft et al. (2016) reported the detection of PEDV nucleic acid in oral fluids from 6 days post exposure (DPE) to 69 DPE. PEDV was detected 15 days longer in oral fluid samples compared to pen fecal samples and, compared to individual rectal swabs, oral fluids demonstrated a higher concentration of detectable virus and higher rate of detection. In the same study, Bjustrom-Kraft et al. (2016) reported the detection of PEDV antibody (IgG and IgA) by 13 DPE in oral fluids. The diagnostic sensitivity and specificity of a PEDV IgG oral fluid ELISA was reported as 69% and 97%, respectively. In contrast, the diagnostic sensitivity and specificity of a PEDV IgA oral fluid ELISA was reported as 100% and 100%, respectively (Bjustrom-Kraft et al., 2016). Although estimates of diagnostic sensitivity and specificity have not been reported, the oral fluid RT-rtPCR is an effective tool to monitor for PEDV presence in herds and IgA antibody testing offers an effective method to evaluate herd level immunity (Bjustrom-Kraft et al., 2016).

Like PEDV, porcine deltacoronavirus (PDCoV) is an enteric coronavirus that causes diarrhea and vomiting in pigs (Homwong et al., 2015). PDCoV can be detected in oral fluids by RT-rtPCR, although estimates of diagnostic sensitivity and specificity are not available (Homwong et al., 2015; Singh et al., 2015; Zhang, 2016). Homwong et al. (2015) reported that the detection of PDCoV nucleic acid in oral fluids was 1.89 times more likely than detection in feces. PDCoV antibody ontogeny in serum and oral fluids have not yet been reported.

Discussion

Globally, the production of livestock - poultry, cattle, swine - is in the process of shifting from small populations on many farms to large populations on fewer farms (Barkema et al., 2015; Gale, 2017; Hoban et al., 1997; Marquer, 2010). Readily accessible USDA data from the dairy and swine industries highlight this trend. In 1982, ~275,000 U.S. dairy farms housed ~11,000,000 dairy cows. By 2012, the number of dairy farms dropped to ~64,000 while animal numbers remained relatively stable at ~9,250,000 (USDA, 2014). Pork production has followed the same trend. In 1982, ~330,000 U.S. farms housed ~55,000,000 pigs in 1982. By 2012, the number of farms with pigs declined to ~63,000 while the number of pigs increased to ~66,000,000 (USDA, 2014). Increases in herd size are important to disease control because herd immunity becomes more difficult to achieve as population increases, which in turn leads to pathogen endemicity (LeBlanc et al., 2006; Pitzer et al., 2016).

Over the same time period, a shift occurred in the movement of livestock across political boundaries. In 1960, 13,500,000 live cattle crossed U.S. state lines for feeding or breeding purposes (Hennessy et al., 2005). By 2015, this number had risen to 20,500,000 (USDA, 2017). Similarly, ~2,500,000 pigs were moved across U.S. stateliness in 1960, in contrast to ~52,500,000 moved in 2016 (Shields, Mathews, 2003; USDA, 2017). Similar patterns have emerged in Europe. For example, Denmark, France, Germany, Italy, Netherlands, Poland, and Spain cumulatively imported ~910,000 live pigs and exported ~937,000 live pigs in 1961 (FAO, 2017). In contrast, these countries imported ~22,000,000 imported live pigs and

exported ~27,000,000 in 2013 (FAO, 2017). Trends in livestock movement are important because of the well-established role of animal transport in the spread of disease spread, e.g., the 2001 FMDV outbreak in the United Kingdom and, more recently, spread of PEDV throughout the Western Hemisphere (Davies, 2015; Guinat et al., 2016).

The unintended consequences of changes in the structure and management of livestock populations have manifested themselves in the appearance of multifactorial diseases resistant to traditional methods of prevention and control, e.g., bovine and porcine respiratory disease complexes (Bochev, 2007; Edwards, 2010; Gardner et al., 2002; Hagglund et al., 2006; LeBlanc et al., 2006; Pitzer et al., 2016; Schwabe, 1982). The need to understand complex animal health conditions mandates a shift toward the collection of longitudinal animal health data. New intervention strategies or unanticipated events, e.g., the introduction of an exotic pathogen, can then be evaluated in the context of their impact on baseline values.

Cumulatively, peer-reviewed research supports the conclusion that aggregate samples offer the opportunity to expand the scope of applied surveillance. Testing of bulk tank milk samples provides bovine and small ruminant practitioners and producers the means to monitor disease and estimate herd prevalence and provides animal health researchers the means to evaluate the spatial distribution and rate of disease transmission (Balmer et al., 2014; Berriatua et al., 2006; Collins et al., 2017; Garcia-Perez et al., 2010; Johnson et al., 2014). Swine oral fluids offer a more analytically sensitive detection system than individual pig samples, and at a lower cost (Goodell et al., 2013; Olsen et al., 2013). Continued progress toward the goal of effective surveillance using aggregate sampling requires research in two areas: 1) continued development and adaption of diagnostic technology for the most globally impactful diseases of animals and humans (zoonoses); 2) continued development of statistically valid sampling guidelines for farm and regional surveillance.

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Tables

Table 1. Pathogens detected in bulk tank milk

Nucleic acid detection	**Reference**
Border disease virus	Berriatua et al., 2006
Bovine viral diarrhea virus	Drew et al., 1999; Houe, 1999; Kramps, et al., 1998; Lanyon et al., 2014; Radwan et al., 1995; Renshaw et al., 2000
Coxiella burnetti	Astobiza et al., 2012; Muskens et al., 2011; Rodolakis et al., 2007
Foot-and-mouth disease virus	Reid et al., 2006; Thurmond and Perez, 2006
Mycobacterium avium subspecies *paratuberculosis*	Cousins et al., 1999; Jayaro et al., 2004; Slana et al., 2008; Tasara et al., 2005
Mycoplasma bovis	Arcangioli et al., 2011; Justice-Allen et al., 2011; Maunsell et al., 2011
Staphylococcus aureus	Haran et al., 2012; Zanardi et al., 2012
Streptococcus agalactiae	Phuektes et al., 2003; Soltau et al., 2017
Antibody detection	
Border disease virus	Berriatua et al., 2006; Corbiere et al., 2012; Garcia-Perez et al., 2010
Bovine viral diarrhea virus	Foddai et al., 2015; Houe, 1999; Hanon et al., 2017; Kramps et al., 1999; Lanyon et al., 2014; Renshaw et al., 2000
Coxiella burnetti	Muskens et al., 2011; van den Brom et al., 2012
Foot-and-mouth disease virus	Armstrong et al., 1997a,b; Armstrong, Mathew, 2001
Mycobacterium avium subspecies *paratuberculosis*	Beaver et al., 2016; Nielsen et al., 2000; van Weering et al., 2007; Wilson et al., 2010
Mycoplasma bovis	Nielsen et al., 2015
Schmallenberg virus	Balmer et al., 2014; Collins et al., 2017; Daly et al., 2015; Johnson et al., 2014
Culture or Isolation	
Mycobacterium avium subspecies *paratuberculosis*	Slana et al., 2008
Mycoplasma bovis	Justice-Allen et al., 2011; Maunsell et al., 2011; Parker et al., 2017 a,b
Staphylococcus aureus	Olde Riekerink et al., 2006, 2010
Streptococcus agalactiae	Keefe, 1997

Table 2. Pathogens detected in oral fluid

Nucleic acid detection	**Reference**
African swine fever virus	Grau et al., 2015; Guinat et al., 2014
Classical swine fever virus	Dietze et al., 2017; Petrini et al., 2017
Foot-and-mouth disease	Ambagala et al., 2016; Mouchantat et al., 2014; Senthilkumaran et al., 2017
Influenza A virus	Allerson et al., 2014; Decorte et al., 2015; Goodell et al., 2013; Romagosa et al., 2012
Porcine deltacoronavirus	Homwong et al., 2015; Singh et al., 2105
Porcine epidemic diarrhea virus	Bjustrom-Kraft et al., 2016
Porcine reproductive and respiratory syndrome virus	Biernacka et al., 2016; Kittawornrat et al., 2010; 2014; Pepin et al., 2015a,b; Prickett et al., 2008a,b; Olsen et al., 2013; Ramirez et al., 2012; Rotolo et al., 2017
Antigen Detection	
Foot-and-mouth disease	Senthilkumaran et al., 2017
Antibody detection	
African swine fever virus	Gimenez-Lirola et al., 2016; Mur et al, 2013
Influenza A virus	Panyasing et al., 2013;2014; Strutzberg-Minder et al., 2015
Porcine epidemic diarrhea virus	Bjustrom-Kraft et al., 2016
Porcine reproductive and respiratory syndrome virus	Kittawornrat et al., 2013; Langenhorst et al., 2012
Culture or Isolation	
Influenza A virus	Goodell et al., 2013

CHAPTER 3. SAMPLING GUIDELINES FOR ORAL FLUID-BASED SURVEYS OF GROUP-HOUSED ANIMALS

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Modified from a manuscript published in Veterinary Microbiology (2017) 209: 20-29.

Abstract

Formulas and software for calculating sample size for surveys based on individual animal samples are readily available. However, sample size formulas are not available for oral fluids and other aggregate samples that are increasingly used in production settings. Therefore, the objective of this study was to develop sampling guidelines for oral fluid-based porcine reproductive and respiratory syndrome virus (PRRSV) surveys in commercial swine farms. Oral fluid samples were collected in 9 weekly samplings from all pens in 3 barns on one production site beginning shortly after placement of weaned pigs. Samples (n = 972) were tested by real-time reverse-transcription PCR (RT-rtPCR) and the binary results analyzed using a piecewise exponential survival model for interval-censored, time-to-event data with misclassification. Thereafter, simulation studies were used to study the barn-level probability of PRRSV detection as a function of sample size, sample allocation (simple random sampling vs fixed spatial sampling), assay diagnostic sensitivity and specificity, and pen-level prevalence. These studies provided estimates of the probability of detection by sample size and within-barn prevalence. Detection using fixed spatial sampling was as good as, or better than, simple random sampling. Sampling multiple barns on a site increased the probability of detection with the number of barns sampled. These results are relevant to

PRRSV control or elimination projects at the herd, regional, or national levels, but the results are also broadly applicable to contagious pathogens of swine for which oral fluid tests of equivalent performance are available.

Introduction

As reviewed by Christensen (2001), various definitions of surveillance and monitoring appear in the literature, with the primary difference that surveillance implies that an action will be taken in the case of a positive result. However, as discussed in the FAO "Manual on Livestock Disease Surveillance and Information Systems" (Paskins, 1999), “surveillance” is often used interchangeably with “monitoring” (even by epidemiologists) and, in practice, the distinction between the two is often blurred. Paskins (1999) goes on to define surveillance as, *“All regular activities aimed at ascertaining the health status of a given population with the aim of early detection ...."* and monitoring as *“All activities aimed at detecting changes in the epidemiological parameters of a specified disease”.* Consistent with this approach, the assumption in this paper is that the purpose of surveillance is to detect infectious agents and the purpose of monitoring to detect changes in pathogens' trends in populations. Regardless of the purpose for which samples are collected, the sampling guidelines reported herein apply equally to both.

Beginning in the 20th century and continuing into the present, pig production moved from relatively small, extensive, labor-dependent enterprises into larger, intensive, technified production systems. In these farms, animals are segregated by age, production stage, and/or function - with little interaction between groups. Both breeding and growing pig populations turn over rapidly, but non-uniformly, as animals finish the production cycle and are replaced by others – often of differing infectious and/or immune status. Thus, the size and structure of contemporary production systems leads to instability in herd immunity and promotes the circulation of agents. Schwabe (1982) recognized the impact of these changes on the expression of disease and prescribed continuous monitoring as a means of discovering the levels and patterns of pathogen circulation and evaluating the effectiveness of interventions.

In conjunction with these changes and particularly in North America and Europe, large numbers of young pigs are moved from breeding farms to finishing farms located in proximity to the areas where crops are produced. Thus, not exclusively, but primarily for this reason, 27,500,000 live animals entered the state of Iowa USA between December 1, 2014 and December 1, 2015 (NASS, 2016). While it is more cost-effective to bring the pigs to the feed (rather than the reverse), this management practice effectively connects distant farms and rapidly moves infectious agents between them. Ultimately, movement of large numbers of pigs compromises the ability of veterinary health authorities to control the spread of infectious diseases at the regional and national levels. This is of particular concern for transboundary and OIE-listed pathogens.

Cumulatively, these recent developments drive the need to collect infectious disease information more rapidly and efficiently. Historically, swine surveillance has been based on individual animal sampling, e.g., serum, nasal swabs, tonsil biopsies, etc., but aggregate specimens, such as oral fluids, offer specific advantages. In particular, oral fluid specimens can be collected by a single person, can be collected frequently (even daily) without stress to pigs or people, and can provide a higher probability of analyte detection with fewer samples than serum (Olsen et al., 2013). This approach provides for an inexpensive, practical, and welfare-friendly method to surveil pig populations. Detection of nucleic acids or antibodies in oral fluids have been reported for most swine pathogens, including *Actinobacillus pleuropneumoniae* (Loftager et al., 1993), African swine fever virus (Greig and Plowright, 1970; Giménez-Lirola et al., 2016), classical swine fever virus (Corthier and Aynaud, 1977), foot-and-mouth disease virus (Eblé et al., 2004; Senthikumaran et al., 2016a; Vosloo et al., 2015), influenza A virus (Goodell et al., 2013; Panyasing et al., 2013), porcine circovirus type 2 (Prickett et al., 2011), porcine epidemic diarrhea virus (Bjustrom-Kraft et al., 2016), porcine reproductive and respiratory syndrome virus (Kittawornrat et al., 2010, 2012, 2013; Prickett et al., 2008a,b), swine vesicular disease virus (Senthilkumaran et al., 2016b), vesicular stomatitis virus (Stallknecht et al., 1999), and others.

The general need for a new surveillance approach reflects the requirement to adapt to the population structure and production practices in use on contemporary swine farms and the

availability of new sampling/testing methods. The specific objective of the present study was to develop sampling guidelines for oral fluid-based porcine reproductive and respiratory syndrome virus (PRRSV) surveillance or monitoring in commercial swine farms. Estimates for probability of detection are needed to expedite on-farm data collection and aid in PRRSV control and/or eradication efforts.

Materials and methods

Experimental design

Oral fluid samples were collected in 9 weekly samplings from all occupied pens (~25 pigs per pen, 36 pens per barn) in 3 commercial wean-to-finish (WTF) barns on one production site in the Midwest USA. The Iowa State University Office of Responsible Research reviewed and approved the on-farm sampling procedures. After the final collection, the 972 oral fluid samples (36 pens x 3 barns x 9 samplings) were randomized and tested for PRRSV RNA by real-time reverse transcription polymerase chain reaction (RT-rtPCR). Longitudinal binary diagnostic test outcomes were analyzed using a piecewise exponential survival model for interval-censored, time-to-event data with misclassification. The model and the parameters estimated from analyses of field data were then used in simulations (10,000) to study the barn-level probability of PRRSV RNA detection in the context of sample size, sample allocation (fixed spatial vs simple random sampling), assay diagnostic sensitivity and specificity, and the number of positive pens. The effect of disease spread on probability of detection by time was evaluated using simulation studies for three scenarios; the observed spread of the infection (β_1, β_2), one-half the observed spread of the infection ($\beta_1/2, \beta_2/2$), and twice the observed spread of the infection ($2\beta_1, 2\beta_2$).

Animals and animal care

The study was conducted on one swine farm with three curtain-sided, wean-to-finish barns (13.4 m × 61.0 m) sited parallel to each other and spaced 10 m apart. Barns used split-zone ventilation, with independent control of curtains and ridge ventilation by zone. Manure was collected in shallow pits beneath each barn and moved to an outdoor above-ground slurry storage tank via a scraper system. The site functioned on an all-in-all-out basis, with

buildings cleaned and disinfected between groups. Animal veterinary care, housing, handling, and feeding were under the supervision of production system veterinarians.

Each barn contained 40 pens with 20 pens on either side of a central walkway. Pens (3 m × 6 m) were built with solid concrete walls and partial slats. At the time of the study, 36 pens in each barn were occupied, with ∼25 pigs in each pen. Barns were filled with weaned pigs (∼21 days of age) sourced from the same PRRSV-endemic breeding herd over the course of approximately one week. Commercial modified-live PRRS vaccines were administered to replacement gilts in the breeding herd, but PRRS vaccine was not administered to sows or pigs.

Sample collection

Oral fluid samples were collected weekly from each of the 36 occupied pens in each of the 3 barns, i.e., 108 samples per week, using a procedure described elsewhere (Prickett et al., 2008a, 2008b). In brief, oral fluid samples were collected by hanging one 100% cotton rope in each pen, with the end of the rope hanging at the height of the pigs' shoulder. One day before the first sample was collected, pigs were “trained” by providing access to ropes for 60 min (White et al., 2014). For routine sampling, ropes were hung for 20–30 min. Thereafter, the wet portion of the rope was inserted into a one gallon plastic bag and severed from the remainder of the rope. Oral fluid was extracted by passing the rope, still within the bag, through a chamois wringer. Samples were decanted into 50 mL centrifuge tubes and placed on crushed ice for transport to the laboratory. At the laboratory, samples were aliquoted into cryovials (4 mL) and stored at −20 °C. Prior to testing, samples were placed in random order to control for systematic bias. Sampling began one week after pigs were placed in the facility and continued for 8 weeks thereafter (total of 9 samplings).

PRRSV RT-rtPCR

All samples were tested for the presence of PRRSV RNA at the Iowa State University Veterinary Diagnostic Laboratory (ISU VDL) using standard protocols. Extraction of the oral fluids was performed using the MagMAX™ viral RNA isolation kit (Life Technologies, Carlsbad, CA, USA) and a Kingfisher 96 magnetic particle processor (Thermo-Fisher

Scientific, Waltham, MA, USA) using a high-volume modified lysis (HVML) procedure. A modified lysis/binding solution was prepared with 120 µL lysis/binding solution, 2 µL carrier RNA, 120 µL isopropanol and 2 µL Xeno™ RNA template at 10,000 copies/µL. At the lysis step, 240 µL of the prepared lysis/binding solution was added to 20 µL of magnetic bead mix prior to extraction and elution into 90 µL buffer. An additional modification for the HVML procedure was an increase in volume of wash I and II solutions, i.e., the procedure used 300 µL in wash I and 450 µL in wash II. The extraction was performed using Kingfisher AM1836_DW_HV_v3, provided by Thermo Fisher Scientific.

Samples were assayed using a commercial PRRSV real-time rtPCR kit (EZ-PRRSV MPX 4.0 assay, Tetracore©, Rockville, MD, USA). For each run, one positive control for PRRSV Types 1 and 2 and a negative amplification control were included. For each control well, 17.25 µL of EZ-PRRSV MPX 4.0 Reagent was added. The EZ-PRRSV MPX 4.0 Reagent includes buffer, primer and probes, 0.75 µL Enzyme Blend, 0.25 µL IC and 7 µL of positive control (Type I or 2 IVT) or negative control (1x TE). Specifically for oral fluid samples, each well contained 17.25 µL of the EZ-PRRS MPX 4.0 Reagent, which included buffer, primer, probes, 0.75 µL Enzyme Blend and 7 µL of the oral fluid extract. Plates were loaded onto the thermal cycler (7500 Fast Real-Time PCR System, Applied Biosystems©, Foster City, CA, USA) and the following cycling conditions were used: one cycle at 48 °C for 15 min, one cycle at 95 °C for 2 min, 45 cycles of: 95 °C for 5 s, and 60 °C for 40 s. Samples with Ct values <45 for Type 2 PRRSV were considered positive.

Statistical analysis

Longitudinal binary diagnostic test outcomes, $u_{ij} = (u_{ij1},...u_{ijt})$, for pen *j* in barn *i* and sampling time *t* were analyzed using a piecewise exponential survival model for interval-censored time-to-event data with misclassification (Sun, 2017). The corresponding unobserved true infection status, $y_{ij} = (y_{ij1},...y_{ijt})$, was modeled through a binary latent survival process that followed a piecewise exponential model. The hazard of the onset of infection for pen *j* in the survival model, λ_{ijt},was modeled as a function of the infection status of the other pens *j'* in the building and the distance ($d_{jj'}$) between pens *j* and *j'* within the same barn:

$$\lambda_{ijt} = \exp\{-[\beta_0 + \sum_{j' \neq j}(\beta_1 + \beta_2 \frac{1}{d_{jj'}})y_{ij',t-1}]\} \quad (1)$$

Here β_0 is the baseline negative log-hazard and β_1 and β_2 are parameters quantifying the spread of the infection. Specifically, β_1 represents the change in log-hazard for each additional positive pen in the same barn regardless of distance and β_2 represents the change in log-hazard for each additional positive pen in the same barn per $1/d_{jj'}$.

Diagnostic test outcomes were modeled conditional on the latent disease process using Bernoulli distribution parametrized through the assay's diagnostic sensitivity (se) and specificity (sp):

$$u_{ijt} \mid (y_{ijt}=1) \sim \text{Bernoulli(se)}, u_{ijt} \mid (y_{ijt}=0) \sim \text{Bernoulli(1-sp)}. \quad (2)$$

These test outcomes are correlated over space and time as a result of the model structure. Since the pens were sampled at pre-determined time points, *t* (weekly), the true infection onset time can be viewed as interval-censored. The model parameters β_0, β_1, and β_2 were estimated through a hierarchical Bayes approach utilizing non-informative priors. The model and the parameters estimated from analyses of field data were then used in simulation studies to study the effect of sample size, sample allocation (simple random sampling or fixed spatial sampling), and sampling frequency on the probability of detecting PRRSV infection while controlling for assay diagnostic sensitivity and specificity, prevalence (proportion of positive pens), and spread of the virus. For any selected sample size, pen samples were either randomly selected using software *R* 3.2.2 (R Development Core Team, 2010) or selected using a fixed spatial sampling approach. Fixed spatial sampling was based on selecting pens equidistant to each other and on alternate sides of the center alleyway over the length of the barn.

Probability of detection in a single barn (single sampling)

The probability of detection in a single barn at a single sampling was evaluated for a range of relevant criteria, i.e., diagnostic sensitivities and specificities, sample sizes (1 to 36), sample allocation (simple random sampling vs. fixed spatial sampling), and prevalence (0 to 36 positive pens). Simulations were carried out in *R* 3.2.2. In each simulation study, the true infection status of the 36 pens in each of the 3 barns was simulated over time using the estimated model parameters ($\beta_0, \beta_1, \beta_2$). For each set of sampling criteria, the probability of detection was calculated as the proportion of simulations (out of 10,000 runs) with ≥ 1 positive pens among the total pens sampled.

Infection status and sample test outcomes were generated using simulation studies over the sampling period of 8 weeks, $t = 0, 1, \ldots, 8$. For each pen j in barn i, the true infection status, y_{ij0}, at the initial sampling point was generated from the Bernoulli distribution with probability p_0., the initial prevalence at week 0. If the result was $y_{ij0} = 1$, the pen was classified positive at sampling point 0 and all subsequent sampling periods. If the result was $y_{ij0} = 0$, the time to positive pen status t_{ij1} was simulated from an exponential distribution with parameter λ_{ij1} defined as in (1), where $y_{ij'0}$ was the true infection status for pen j' at sampling time 0. If $t_{ij1} \leq 1$, then the true infection status for pen j at time 1 was $y_{ij1} = 1$, thus $y_{ij2} = \ldots = y_{ij8} = 1$. If $t_{ij1} > 1$, t_{ij2} was generated from an exponential distribution with parameter λ_{ij2}, as defined in (1), where $y_{ij'1}$ was the true infection status for pen j' at sampling time 1. If $t_{ij2} \leq 1$, then the true infection status for pen j at sampling time 2 was $y_{ij2} = 1$, thus $y_{ij3} = \ldots = y_{ij8} = 1$. If $t_{ij2} > 1$, t_{ij3} was generated from an exponential distribution with parameter λ_{ij3}, as defined in (1), where $y_{ij'2}$ was the true infection status for pen j' at sampling time 2. Similarly, the true infection status for each pen at each sampling point was generated through this procedure.

After simulation of infection status, diagnostic test outcomes were simulated with the number of pens sampled (1 to 36) allocated using either simple random sampling or fixed spatial sampling. For any predetermined level of diagnostic sensitivity or specificity, the test outcome, u_{ijt}, was generated conditionally on y_{ijt} from (2). At each prevalence level, the

probability of detection was calculated as the proportion of simulations (out of 10,000 simulations) with ≥ 1 positive pen among the total pens sampled. The probability of detection was calculated for both simple random sampling and fixed spatial sampling and the results compared using McNemar's test for paired proportions.

Effect of the spread of infection on the probability of detection

As shown in Equation (1), the spread of infection was controlled by β_1, β_2 such that larger values of β_1, β_2 resulted in faster spread among pens within a barn, while smaller values of β_1, β_2 produced slower spread. The effect of spread on the probability of detection by time in a single barn was explored by changing the values of these parameters in simulation studies. Fixed spatial sampling was used with sample sizes 2, 4, and 6 while allowing prevalence to change over time. For simplicity, diagnostic sensitivity and specificity were assumed to be 100%.

The effect of the spread of infection on the probability of detection was evaluated for three scenarios while keeping p_0, β_1 constant: the observed spread (β_1, β_2), one-half the observed spread ($\beta_1/2, \beta_2/2$), and twice the observed spread of infection ($2\beta_1, 2\beta_2$). Simulation studies were carried out and the true infection status at each sampling point was generated using the methods described above through the end of the sampling period (8 weeks). Test outcomes were generated conditional on the true infection status. At each sampling point, the probability of detection was calculated as the proportion of simulations (out of 10,000 simulations) with ≥ 1 positive pen among the total pens sampled.

Effect of sampling 2 or more barns on a site

The approach described above estimates the probability of detecting infection in one barn. Assuming independence among barns, the overall probability of detecting infection on one production site by sampling ≥ 2 barns can be calculated as:

$$P = (1 - (1 - p_1)(1 - p_2)(1 - p_3) \ldots (1 - p_k)). \quad (3)$$

In equation 3, p_i is the probability of detection in the ith (i = 1,2, …, k) barn. When the k barns are similar in design and are sampled with same scheme, then all p_i can be assumed equal to a common p of detection and the formula simplifies to:

$$P = (1 - (1 - p)^k). \quad (4)$$

Results

Oral fluid samples were completely randomized prior to testing for PRRSV nucleic acid and then tested in batches of ~252 samples to optimize laboratory throughput. RNA extraction (Life Technologies) and RT-rtPCR (Tetracore, Inc.) were each performed using a single production lot. Samples were tested once, i.e., no retests were performed. A total of 425 samples tested positive (Ct ≤ 45) and 547 samples tested negative. The mean Ct among positives was 30.7 (95% confidence interval 30.4, 30.9). Table 1 provides a spatiotemporal perspective of the results. Descriptively, the 3 barns differed by the week at which they reached ≥ 4 PCR-positive pens (≥ 11% positivity): Barn A at week 1, Barn B at week 3, and Barn C at week 6. Likewise, barns differed in the time it took for PRRSV to spread from ≥ 4 positive pens to ≥ 32 (≥ 89%) positive pens: Barn A 4 weeks, Barn B 3 weeks, and Barn C 1 week.

Test results were used to estimate model parameters p_0, β_0, β_1, and β_2 through a hierarchical Bayes approach using non-informative priors with JAGS Version 4.0.0 (Plummer, 2007). Posterior means, standard errors, and 95% credible intervals are given in Table 2. The 95% credible intervals did not include 0, indicating that the parameters' estimates were statistically significant and that the constructed model effectively represented the spread of infection. The parameter estimates were then used in simulation studies, as described in section 2.5.1 and section 2.5.2, to evaluate the effect of sample size, sample allocation (simple random sampling vs fixed spatial sampling), and time on the probability of detecting PRRSV infection in a single barn while controlling for assay diagnostic sensitivity and specificity, prevalence (proportion of positive pens), and spread of infection.

Simple random sampling and fixed spatial sampling were compared in terms of the probability of detecting ≥ 1 positive samples over a range of sample sizes and number of positive pens in a single barn. For simplicity, the data presented in Figure 1 assume that diagnostic sensitivity and diagnostic specificity are both 100%. The results for each set of parameters were based on 10,000 simulations, i.e., the standard errors for each estimate should be smaller than 0.005. Comparisons of the results showed that the probability of detection using fixed spatial sampling was equal to, or greater than, the probability of detection using simple random sampling (McNemar's test, $p < 0.05$). Therefore, the remainder of the analyses reported herein were based on fixed spatial sampling.

The effect of diagnostic sensitivity on the probability of detecting PRRSV infection in a single barn was evaluated for fixed spatial sampling as a function of sample size and number of positive pens (Table 3). Diagnostic specificity was assumed to be 100% for each level of diagnostic sensitivity. Conversely, the effect of diagnostic specificity on the probability of producing a false positive result is given in Table 4.

The effect of β_1 and β_2 on the probability of detection is shown in Figure 2 for 2, 4, and 6 samples collected using fixed spatial sampling from one barn. Three separate scenarios were analyzed: one-half the observed spread of infection ($0.5 \times (\beta_1, \beta_2)$), the observed spread of infection ($1.0 \times (\beta_1, \beta_2)$), and twice the observed spread of infection ($2.0 \times (\beta_1, \beta_2)$). The number of positive pens by week were derived from the simulations and, therefore, vary slightly from the field data reported in Table 3. Table 5 reports the probability of ≥ 1 true positive results in 1, 2, or 3 barns as a function of the spread of infection (β_1, β_2), the number of barns sampled, the number of pens sampled within barns using a fixed spatial sampling, and the number of positive pens in the barns. The probabilities for 2 or 3 barns reported in Table 5 were calculated using Equation 4.

Discussion

Cannon and Roe (1982) introduced the concept of statistical sampling to an earlier generation of livestock health specialists by presenting sample size guidelines based on perfect tests in a highly readable and widely disseminated pamphlet. The first wholesale application of statistical sampling to the livestock industry may have been the U.S. Aujeszky's disease (Pseudorabies) eradication program initiated in 1989 and successfully concluded in 2002 (Anderson et al., 2008). Subsequently, Cameron and Baldock (1998) developed formulas to calculate sample sizes for surveillance based on imperfect diagnostic tests and Cannon (2001) derived fast approximation formulas for this calculation. Such work provided a strong theoretical basis for surveillance based on individual animal samples, e.g., serum, but did not provide guidance for surveillance based on aggregate samples, e.g., oral fluids.

In this study, a piecewise exponential survival model was used to model 'time-to-infection' at the pen level using PRRSV RT-rtPCR results on oral fluid samples collected weekly (1, 2, … *t*). Since sampling occurred at seven-day intervals, pen-level 'time-to-infection' was treated as interval-censored. The piecewise exponential model has previously been used for interval-censored time-to-event data where a constant hazard is assumed in each time interval. Covariate effects, if present, can be accommodated using proportional hazards (Friedman, 1982; Lindsey and Ryan, 1998). Simulation studies were then used to determine the effect of sampling allocation (simple random sampling vs. fixed spatial sampling), sample size, prevalence, time, and test performance (diagnostic sensitivity and specificity) on the probability of PRRSV detection in a single barn.

Independent of test performance, the probability of detection increased as sample size and/or PRRSV prevalence increased (Table 3); whereas, the probability of false positive results increased with larger sample size and/or with declining prevalence (Table 4). The overall trends observed were generally as expected, with estimates for specific conditions provided by the simulation studies.

Somewhat unexpectedly, fixed spatial sampling was found to be equal to, or better than, simple random sampling in terms of the probability of detecting infection (Fig. 1). Simple random sampling assumes that the characteristic of interest is independent and spatially distributed (Cochran, 1977), but in infectious diseases, observations in proximity with each other are likely to be of similar status as a result of pathogen spread. Although rarely used in veterinary medicine, spatially-based sampling is widely used in other fields, where it is considered to offer advantages in terms of cost and efficiency (Wang et al., 2013). Fixed spatial sampling provides for a surveillance sampling design that is easily described and easily implemented in pig barns. Results of repeated sampling from the same pens over time provide a coherent picture of the infectious process and/or immune responses that can be easily juxtaposed with temporal productivity or clinical parameters.

Currently, farm- or herd-level surveillance is challenged by the larger population size and heterogeneous hierarchies (sites, barns, animals) common to contemporary production sites. A design based on sampling individual barns provides flexibility in tailoring surveillance to farms ranging widely in size and complexity. Furthermore, sampling across multiple barns on a site is a powerful approach for detecting infection. For example, assuming fixed spatial sampling, within-barn prevalence of 25%, and test sensitivity/specificity of 95/100%, the probability of detecting PRRSV infection in one barn using 2 oral fluid samples is 43% (Table 3). Under these same assumptions, if 2 oral fluid samples were collected from each of 3 barns on one site, the probability of detection is 81%. This may be calculated using Equation (4): $P = (1 - (1 - p)^k) = (1 - (1–0.43)^3) = 0.81$. If prevalence is thought to differ among barns on a site, Table 3 and Equation (3) can be used to estimate the probability of detection by sample size. This approach assumes independence among barns. If this assumption does not hold, the piecewise exponential survival model can be generalized to include the pathogen's spread among barns and the overall chance of detection in multiple barns generated using simulations.

Sample size addresses the probability of detection at a single point in time, whereas the combination of sample size and frequency address the probability of detection as a pathogen spreads over time. The pattern of PRRSV spread observed in this study was in agreement

with a previous report (Dufresne et al., 2003), but given that barns and pens-within-barns vary in design and size, it is possible that the parameters for the spread of infectious agents may differ somewhat among production sites. This concept has not been widely explored, but using a modeling approach, Maurice et al. (2016) predicted the spread of encephalomyocarditis virus to be faster in a barn with gated pens as opposed to concrete walls. The impact of spread on detection was addressed by modeling detection at 0.5, 1, and 2 times the observed spread of infection (Fig. 2, Table 5). From this analysis it can be seen that frequent sampling is mandatory, if early detection is the objective.

The first step in developing a sampling design is to establish a clear objective: surveillance vs. monitoring. To that end, the primary purpose of this study was to provide sampling guidelines for commercial pig farms. Given that perfect tests do not exist, a clear strategy for addressing unexpected results, e.g., suspected false positives, should be in place before sampling is initiated. Tables 3–5, provide the probabilities of detection for various scenarios and serve to guide sample size decisions. These tables describe the number of samples to collect in a barn as a function of the probability of detection. The number of pens in a barn is not an issue in selecting sample size. If the barn is designed with many pens, samples will likely be collected from separate pens. If the barn is designed with few pens, more than one sample per pen could be collected. The key feature is a fixed spatial approach: space samples equally over the length of the barn.

The purpose of surveillance is to assure animal health and welfare, improve producer profitability, and protect a valuable national asset. The specific objective of the present study was to develop sampling guidelines for oral fluid-based PRRSV surveillance or monitoring in commercial swine farms. These results will have immediate application to PRRSV control and/or elimination projects at the herd, area, and regional levels. The analysis was based on PRRSV infection in commercial swine production facilities detected using PRRSV RT-rtPCR testing, but the results are expected to be broadly applicable to swine pathogens for which oral fluid tests of equivalent performance are available.

Funding

This study was funded in part by Checkoff Dollars administered through the National Pork Board (Grant #13-157), Des Moines, Iowa (USA).

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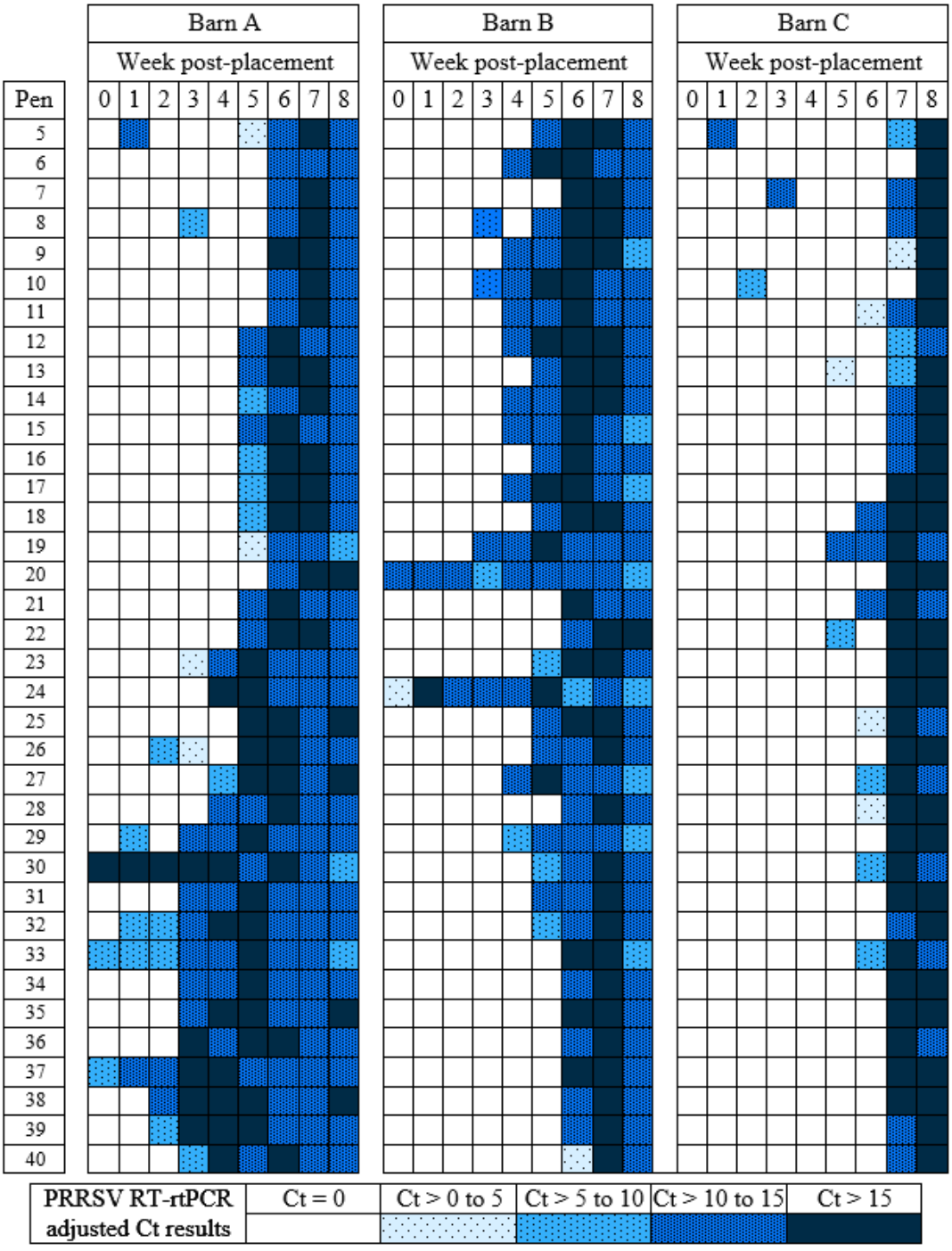
Tables

Table 1. Spatiotemporal patterns of PRRSV spread in three wean-to-finish barns as revealed by weekly RT-rtPCR testing of pen-based oral fluids beginning one week post-weaning.



[a]Adjusted Ct is calculated as follows: Cutoff – Result = Adjusted Ct: Example: 45- 30 = 15. The higher the adjusted Ct, the higher the concentration of virus detected.

Table 2. Model parameter estimates, standard errors, and 95% credible intervals

	p_0	β_0	β_1	β_2
Estimate	0.032	3.980	-0.063	-1.286
Standard error	0.0167	0.3118	0.0427	0.0962
95% credible interval	[0.008, 0.073]	[3.339, 4.440]	[0.117, 0.031]	[-1.435, -1.082]

Table 3. Probability of ≥ 1 true positive results in one barn at one sampling. Probability (%, *in italics*) is a function of the number of positive pens in the barn, the number of pens sampled using a fixed spatial approach, and test diagnostic sensitivity*

Test	No. of samples	Number of positive pens among a total of 36 pens in the barn									
		1	**2**	**3**	**4**	**5**	**6**	**9**	**18**	**27**	**36**
Diagnostic sensitivity 60%	1	*2*	*3*	*5*	*7*	*9*	*10*	*16*	*34*	*52*	*60*
	2	*3*	*7*	*10*	*14*	*16*	*20*	*30*	*56*	*74*	*84*
	3	*5*	*10*	*15*	*20*	*24*	*29*	*40*	*70*	*86*	*93*
	4	*6*	*12*	*19*	*25*	*30*	*36*	*50*	*80*	*93*	*97*
	5	*9*	*17*	*23*	*31*	*37*	*42*	*58*	*86*	*96*	*99*
	6	*10*	*19*	*28*	*36*	*43*	*50*	*66*	*91*	*98*	*100*
	9	*15*	*28*	*39*	*49*	*56*	*64*	*80*	*97*	*100*	*100*
	18	*29*	*51*	*66*	*77*	*84*	*89*	*97*	*100*	*100*	*100*
	27	*46*	*70*	*83*	*91*	*96*	*98*	*100*	*100*	*100*	*100*
	36	*60*	*83*	*94*	*97*	*99*	*100*	*100*	*100*	*100*	*100*
Diagnostic sensitivity 70%	1	*2*	*4*	*6*	*8*	*10*	*13*	*19*	*38*	*60*	*69*
	2	*4*	*8*	*11*	*16*	*19*	*23*	*33*	*63*	*82*	*91*
	3	*6*	*12*	*17*	*22*	*27*	*32*	*46*	*76*	*92*	*97*
	4	*8*	*15*	*23*	*28*	*34*	*40*	*57*	*87*	*97*	*99*
	5	*10*	*19*	*27*	*35*	*42*	*49*	*65*	*92*	*98*	*100*
	6	*10*	*22*	*31*	*41*	*49*	*55*	*71*	*95*	*99*	*100*
	9	*17*	*32*	*45*	*56*	*64*	*72*	*86*	*99*	*100*	*100*
	18	*35*	*58*	*73*	*84*	*90*	*94*	*99*	*100*	*100*	*100*
	27	*54*	*77*	*90*	*95*	*98*	*99*	*100*	*100*	*100*	*100*
	36	*70*	*91*	*97*	*99*	*100*	*100*	*100*	*100*	*100*	*100*
Diagnostic sensitivity 80%	1	*3*	*4*	*7*	*9*	*11*	*14*	*21*	*46*	*69*	*80*
	2	*4*	*9*	*13*	*17*	*21*	*26*	*38*	*70*	*90*	*96*
	3	*7*	*13*	*19*	*25*	*31*	*36*	*52*	*82*	*97*	*99*
	4	*9*	*17*	*25*	*33*	*40*	*46*	*63*	*91*	*99*	*100*
	5	*11*	*21*	*31*	*39*	*47*	*54*	*71*	*95*	*100*	*100*
	6	*13*	*25*	*36*	*45*	*53*	*61*	*78*	*98*	*100*	*100*
	9	*20*	*37*	*50*	*61*	*70*	*77*	*90*	*100*	*100*	*100*
	18	*42*	*65*	*80*	*88*	*94*	*97*	*100*	*100*	*100*	*100*
	27	*60*	*84*	*93*	*98*	*99*	*100*	*100*	*100*	*100*	*100*
	36	*80*	*96*	*99*	*100*	*100*	*100*	*100*	*100*	*100*	*100*
Diagnostic sensitivity 90%	1	*2*	*5*	*8*	*10*	*13*	*15*	*24*	*50*	*78*	*90*
	2	*5*	*10*	*15*	*20*	*25*	*29*	*42*	*77*	*95*	*99*
	3	*7*	*15*	*22*	*29*	*35*	*41*	*57*	*89*	*98*	*100*
	4	*10*	*20*	*28*	*36*	*44*	*51*	*67*	*95*	*100*	*100*
	5	*12*	*24*	*34*	*43*	*52*	*59*	*76*	*98*	*100*	*100*
	6	*14*	*28*	*40*	*49*	*59*	*66*	*83*	*99*	*100*	*100*
	9	*23*	*41*	*55*	*66*	*74*	*82*	*94*	*100*	*100*	*100*
	18	*45*	*71*	*85*	*92*	*97*	*98*	*100*	*100*	*100*	*100*
	27	*68*	*90*	*97*	*99*	*100*	*100*	*100*	*100*	*100*	*100*
	36	*89*	*99*	*100*	*100*	*100*	*100*	*100*	*100*	*100*	*100*

Table 3. Cont'd

Test	No. of samples	Number of positive pens among a total of 36 pens in the barn									
		1	**2**	**3**	**4**	**5**	**6**	**9**	**18**	**27**	**36**
Diagnostic sensitivity 95%	1	*3*	*5*	*8*	*10*	*13*	*16*	*25*	*53*	*81*	*95*
	2	*5*	*11*	*16*	*20*	*25*	*30*	*43*	*79*	*96*	*100*
	3	*8*	*16*	*23*	*30*	*36*	*42*	*58*	*90*	*99*	*100*
	4	*11*	*21*	*30*	*39*	*46*	*53*	*71*	*96*	*100*	*100*
	5	*12*	*25*	*36*	*46*	*55*	*63*	*80*	*99*	*100*	*100*
	6	*15*	*30*	*42*	*52*	*62*	*69*	*85*	*99*	*100*	*100*
	9	*23*	*43*	*58*	*68*	*77*	*84*	*95*	*100*	*100*	*100*
	18	*47*	*73*	*87*	*94*	*97*	*99*	*100*	*100*	*100*	*100*
	27	*73*	*93*	*98*	*100*	*100*	*100*	*100*	*100*	*100*	*100*
	36	*94*	*100*	*100*	*100*	*100*	*100*	*100*	*100*	*100*	*100*
Diagnostic sensitivity 98%	1	*2*	*5*	*8*	*11*	*14*	*17*	*26*	*54*	*83*	*98*
	2	*5*	*10*	*16*	*22*	*26*	*31*	*45*	*81*	*97*	*100*
	3	*7*	*15*	*23*	*30*	*36*	*42*	*60*	*91*	*100*	*100*
	4	*12*	*21*	*30*	*39*	*46*	*54*	*71*	*97*	*100*	*100*
	5	*14*	*26*	*37*	*47*	*46*	*62*	*79*	*99*	*100*	*100*
	6	*16*	*30*	*42*	*53*	*62*	*69*	*85*	*99*	*100*	*100*
	9	*24*	*43*	*59*	*70*	*78*	*85*	*96*	*100*	*100*	*100*
	18	*49*	*74*	*88*	*94*	*98*	*99*	*100*	*100*	*100*	*100*
	27	*74*	*94*	*99*	*100*	*100*	*100*	*100*	*100*	*100*	*100*
	36	*98*	*100*	*100*	*100*	*100*	*100*	*100*	*100*	*100*	*100*
Diagnostic sensitivity 100%	1	*3*	*5*	*8*	*12*	*14*	*17*	*26*	*55*	*83*	*100*
	2	*6*	*11*	*16*	*22*	*27*	*32*	*46*	*80*	*98*	*100*
	3	*8*	*16*	*23*	*30*	*38*	*45*	*62*	*92*	*100*	*100*
	4	*11*	*21*	*31*	*40*	*47*	*54*	*72*	*97*	*100*	*100*
	5	*13*	*26*	*36*	*46*	*55*	*63*	*80*	*99*	*100*	*100*
	6	*17*	*31*	*43*	*54*	*63*	*70*	*85*	*100*	*100*	*100*
	9	*25*	*45*	*60*	*72*	*80*	*86*	*96*	*100*	*100*	*100*
	18	*49*	*75*	*89*	*95*	*98*	*99*	*100*	*100*	*100*	*100*
	27	*75*	*94*	*99*	*100*	*100*	*100*	*100*	*100*	*100*	*100*
	36	*100*	*100*	*100*	*100*	*100*	*100*	*100*	*100*	*100*	*100*

* Data for Table 3 were derived from the field data (Table 1) and simulation studies described in Section "Probability of detection in a single barn (single sampling)". Field data were derived from barns with 36 pens. Diagnostic specificity was assumed to be 100% to generate the data in Table 3.

Table 4. Probability of ≥ 1 false positive results in one barn at one sampling. Probability (%, *in italics*) is a function of the number of positive pens in the barn, the number of pens sampled using a fixed spatial approach, and test diagnostic specificity*

Test	No. of samples	Number of negative pens among a total of 36 pens in the barn									
		1	**2**	**3**	**4**	**5**	**6**	**9**	**18**	**27**	**36**
Dx specificity = 98%	1	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*1*	*1*	*2*
	2	*0*	*0*	*0*	*0*	*0*	*0*	*1*	*2*	*3*	*4*
	3	*0*	*0*	*0*	*0*	*0*	*1*	*1*	*3*	*4*	*6*
	4	*0*	*0*	*0*	*0*	*1*	*1*	*2*	*3*	*6*	*8*
	5	*0*	*0*	*0*	*1*	*1*	*2*	*2*	*4*	*7*	*9*
	6	*0*	*1*	*1*	*1*	*1*	*2*	*3*	*5*	*9*	*11*
	9	*0*	*1*	*2*	*2*	*3*	*4*	*5*	*8*	*13*	*17*
	18	*1*	*2*	*2*	*4*	*5*	*5*	*10*	*16*	*24*	*30*
	27	*1*	*2*	*3*	*4*	*5*	*7*	*11*	*22*	*34*	*43*
	36	*2*	*4*	*6*	*7*	*10*	*11*	*16*	*31*	*42*	*53*
Dx specificity = 99%	1	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*1*	*1*
	2	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*1*	*2*	*2*
	3	*0*	*0*	*0*	*0*	*0*	*0*	*1*	*2*	*2*	*3*
	4	*0*	*0*	*0*	*0*	*0*	*0*	*1*	*1*	*3*	*4*
	5	*0*	*0*	*0*	*0*	*1*	*1*	*1*	*3*	*4*	*5*
	6	*0*	*0*	*0*	*1*	*1*	*1*	*1*	*3*	*4*	*6*
	9	*0*	*1*	*1*	*1*	*1*	*1*	*2*	*4*	*6*	*9*
	18	*0*	*1*	*2*	*2*	*3*	*3*	*4*	*8*	*12*	*16*
	27	*1*	*1*	*1*	*2*	*3*	*4*	*5*	*11*	*19*	*24*
	36	*1*	*2*	*3*	*3*	*5*	*6*	*8*	*16*	*24*	*31*
Dx specificity = 99.9%	1	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*
	2	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*
	3	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*
	4	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*
	5	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*
	6	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*1*
	9	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*1*	*1*
	18	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*1*	*1*	*2*
	27	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*1*	*2*	*2*
	36	*0*	*0*	*0*	*0*	*0*	*1*	*1*	*2*	*2*	*4*

* Data for Table 4 were derived from the field data (Table 1) and simulation studies described in Section "Probability of detection in a single barn (single sampling)". Field data were derived from barns with 36 pens. Diagnostic sensitivity was assumed to be 0% to generate the data in Table 4.

Table 5. Probability of ≥ 1 true positive results in 1, 2, or 3 barns. Probability (%, *in italics*) is a function of the spread of infection (β_1, β_2) the number of barns sampled, the number of pens sampled within barns using fixed spatial sampling, and the number of positive pens in the barn(s)*

	Sampling		Number of positive pens predicted over time (week)								
	Barns	**Pens**	**1 (0)**	**3 (1)**	**5 (2)**	**8 (3)**	**12 (4)**	**17 (5)**	**23 (6)**	**28 (7)**	**32 (8)**
Spread = 0.5 × (ß1, ß2)	1	2	*5*	*16*	*28*	*42*	*57*	*72*	*85*	*94*	*98*
		4	*11*	*30*	*48*	*65*	*80*	*90*	*96*	*99*	*100*
		6	*17*	*42*	*63*	*79*	*90*	*96*	*99*	*100*	*100*
	2	2	*10*	*29*	*48*	*66*	*82*	*92*	*98*	*100*	*100*
		4	*21*	*51*	*73*	*88*	*96*	*99*	*100*	*100*	*100*
		6	*31*	*66*	*86*	*96*	*99*	*100*	*100*	*100*	*100*
	3	2	*14*	*41*	*63*	*80*	*92*	*98*	*100*	*100*	*100*
		4	*30*	*66*	*86*	*96*	*99*	*100*	*100*	*100*	*100*
		6	*43*	*80*	*95*	*99*	*100*	*100*	*100*	*100*	*100*
	Barns	**Pens**	**1 (0)**	**3 (1)**	**7 (2)**	**15 (3)**	**26 (4)**	**34 (5)**	**36 (6)**	**36 (7)**	**36 (8)**
Spread = 1.0 × (ß1, ß2)	1	2	*6*	*18*	*37*	*65*	*90*	*98*	*100*	*100*	*100*
		4	*10*	*32*	*58*	*83*	*96*	*100*	*100*	*100*	*100*
		6	*16*	*44*	*72*	*91*	*99*	*100*	*100*	*100*	*100*
	2	2	*12*	*33*	*60*	*88*	*99*	*100*	*100*	*100*	*100*
		4	*19*	*54*	*82*	*97*	*100*	*100*	*100*	*100*	*100*
		6	*29*	*69*	*92*	*99*	*100*	*100*	*100*	*100*	*100*
	3	2	*17*	*45*	*75*	*96*	*100*	*100*	*100*	*100*	*100*
		4	*27*	*69*	*93*	*100*	*100*	*100*	*100*	*100*	*100*
		6	*41*	*82*	*98*	*100*	*100*	*100*	*100*	*100*	*100*
	Barns	**Pens**	**1 (0)**	**4 (1)**	**15 (2)**	**33 (3)**	**36 (4)**	**36 (5)**	**36 (6)**	**36 (7)**	**36 (8)**
Spread = 2.0 × (ß1, ß2)	1	2	*6*	*22*	*64*	*97*	*100*	*100*	*100*	*100*	*100*
		4	*11*	*39*	*83*	*99*	*100*	*100*	*100*	*100*	*100*
		6	*16*	*52*	*91*	*99*	*100*	*100*	*100*	*100*	*100*
	2	2	*12*	*39*	*87*	*100*	*100*	*100*	*100*	*100*	*100*
		4	*21*	*63*	*97*	*100*	*100*	*100*	*100*	*100*	*100*
		6	*29*	*77*	*99*	*100*	*100*	*100*	*100*	*100*	*100*
	3	2	*17*	*53*	*95*	*100*	*100*	*100*	*100*	*100*	*100*
		4	*30*	*77*	*100*	*100*	*100*	*100*	*100*	*100*	*100*
		6	*41*	*89*	*100*	*100*	*100*	*100*	*100*	*100*	*100*

* Data for Table 5 were derived from the field data (Table 1) and simulation studies described in Section "Effect of the spread of infection on the probability of detection". The probabilities for 2 and 3 barns were calculated using Equation 4. Diagnostic sensitivity and specificity were assumed to be 100%.

Figures

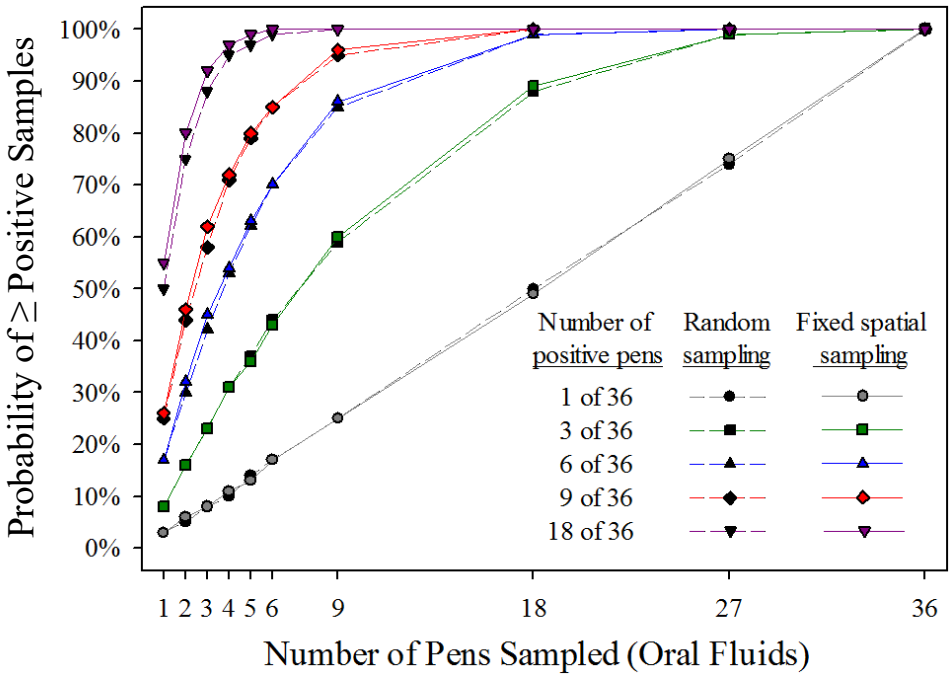


Figure 1. Probability of detecting PRRSV in a single barn using pen-based oral fluids tested by RT-rtPCR as a function of sample allocation (simple random sampling vs. fixed spatial sampling), sample size, and prevalence.

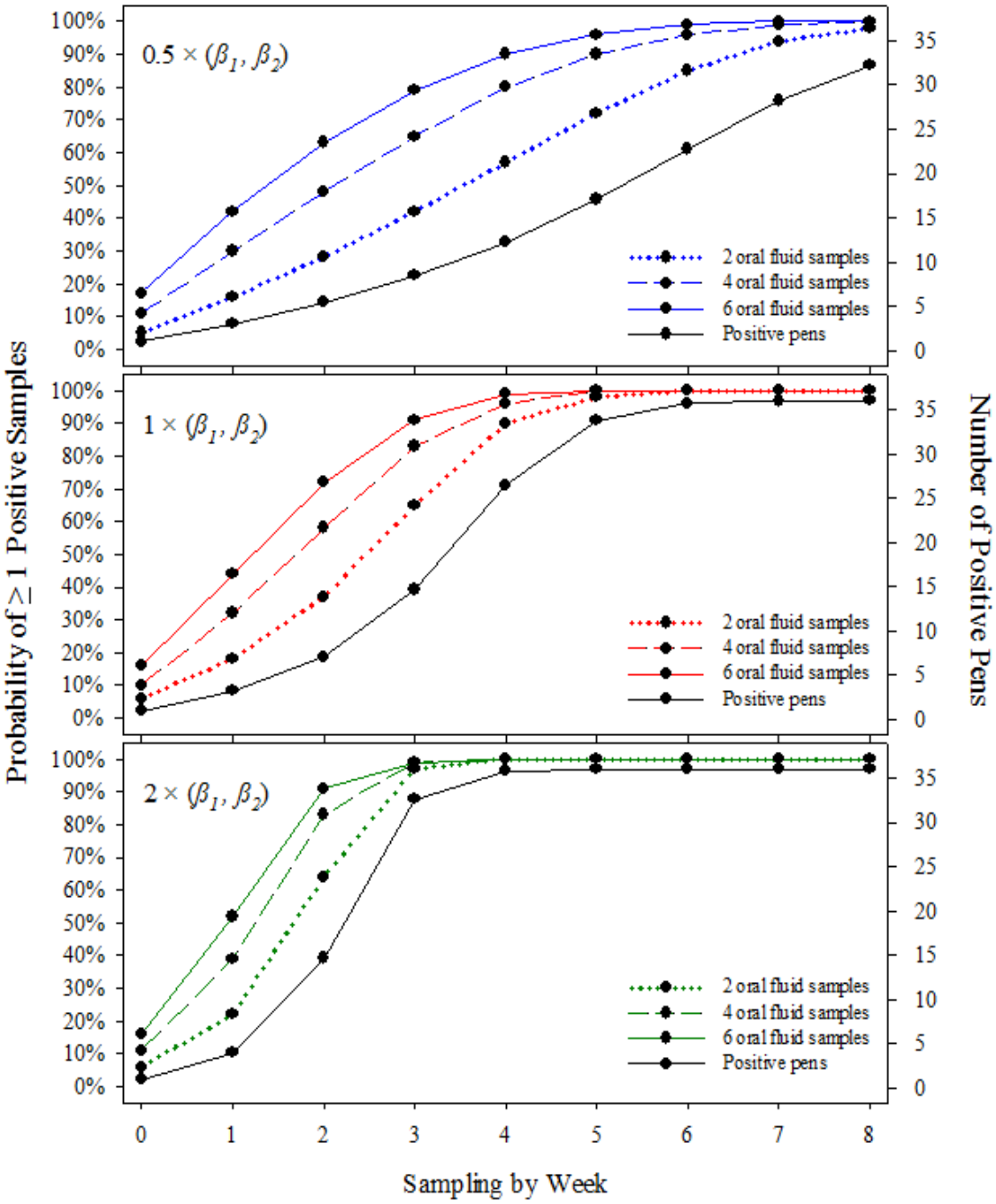


Figure 2. Effect of spread of infection on the probability of detection by time in a single barn modeled by changing the values of $\boldsymbol{\beta_1, \beta_2}$ in simulation studies. Fixed spatial sampling was used with sample sizes 2, 4, and 6 while allowing prevalence to change over time. For simplicity, diagnostic sensitivity and specificity were assumed to be 100%.

CHAPTER 4. DETECTION OF PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME VIRUS (PRRSV)-SPECIFIC IGM-IGA IN ORAL FLUID SAMPLES REVEALS PRRSV INFECTION IN THE PRESENCE OF MATERNAL ANTIBODY

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Modified from a manuscript accepted for publication in Veterinary Microbiology

Abstract

The ontogeny of PRRSV antibody in oral fluids has been described using isotype-specific ELISAs. Mirroring the serum response, IgM appears in oral fluid by 7 days post inoculation (DPI), IgA after 7 DPI, and IgG by 9 to 10 DPI. Commercial PRRSV ELISAs target the detection of IgG because the higher concentration of IgG relative to other isotypes provides the best diagnostic discrimination. Oral fluids are increasingly used for PRRSV surveillance in commercial herds, but in younger pigs, a positive ELISA result may be due either to maternal antibody or to antibody produced by the pigs in response to infection. To address this issue, a combined IgM-IgA PRRSV oral fluid ELISA was developed and evaluated for its capacity to detect pig-derived PRRSV antibody in the presence of maternal antibody. Two longitudinal studies were conducted. In Study 1 (modified-live PRRS vaccinated pigs), testing of individual pig oral fluid samples by isotype-specific ELISAs demonstrated that the combined IgM-IgA PRRSV ELISA provided better discrimination than individual IgM or IgA ELISAs. In Study 2 (field data), testing of pen-based oral fluid samples confirmed the findings in Study 1 and established that the IgM-IgA ELISA was able to detect antibody

produced by pigs in response to wild-type PRRSV infection, despite the presence of maternal IgG. Overall, the combined PRRSV IgM-IgA oral fluid ELISA described in this study is a potential tool for PRRSV surveillance, particularly in populations of growing pigs originating from PRRSV-positive or vaccinated breeding herds.

Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV) was first identified in 1991 (Wensvoort et al., 1991). Assays for the detection of PRRSV serum antibody became widely available shortly thereafter, including immunoperoxidase monolayer (Wensvoort et al., 1992), immunofluorescence (Benfield et al., 1992), serum-virus neutralization (Benfield et al., 1992), and ELISA (Albina et al., 1992). The detection of PRRSV serum antibody using fluorescent microsphere-based assays has been reported under experimental conditions (Langenhorst et al, 2012). At the present time, the ELISA is the most common format for PRRSV antibody detection and commercial PRRSV antibody ELISA kits are widely available for serum and swine oral fluid specimens (Pejsak et al., 2017).

The ontogeny of PRRSV antibody in serum and oral fluids has been described using isotype-specific ELISAs. Kittawornrat et al. (2013), using paired samples collected over time post inoculation, showed that the temporal appearance of antibody isotypes in serum and oral fluid was essentially identical in animals inoculated with viable, replicating PRRSV. That is, IgM was detectable by 7 days post inoculation (DPI), IgA after 7 DPI, and IgG by 9 to 10 DPI. Because of the higher concentration of IgG relative to other isotypes, commercial ELISA kits usually target the detection of IgG, although detection of IgM and IgA has been used in human diagnostic medicine.

In addition to antibody produced in response to PRRSV infection or vaccination, younger animals may also have ELISA-detectable PRRSV-specific passive antibody, primarily IgG, in serum and oral fluid (Biernacka et al, 2016; Goyal, 1993; Ramirez et al, 2012). In oral fluid-based testing, maternally-derived antibody creates a challenge in discerning whether a positive ELISA is the result of infection, vaccination, or maternal antibody. Therefore, the

goal of this study was to develop a PRRSV combined IgM-IgA oral fluid ELISA and evaluate its performance under experimental and field conditions.

Materials and methods

Experimental design

Two longitudinal studies were conducted to evaluate PRRSV oral fluid antibody ontogeny using isotype-specific ELISAs (IgM, IgA, IgG, IgM-IgA). In Study 1 (experimental data), oral fluid antibody isotype responses were evaluated in individual pigs following administration of a modified-live PRRSV vaccine. In Study 2 (field data), PRRSV antibody isotype responses were monitored in oral fluid samples collected from PRRS unvaccinated, group-housed pigs in commercial wean-to-finish farms in Iowa USA. In Study 2, wild-type PRRSV infection was determined by PRRSV real-time reverse-transcriptase PCR (RT-rtPCR) testing and sequencing. Studies were conducted with the authorization of the Iowa State University Office for Responsible Research and the permission of the producers.

Animals and animal care

Study 1 was an experimental study conducted in a biosafety level 2 research facility located at Iowa State University and accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). The facility was designed with a single-pass, non-recirculating ventilation system, i.e., unidirectional flow from low contamination areas to high contamination areas. Each room was ventilated separately and humidity and temperature was strictly controlled. Zones of negative pressure prevented airborne contamination from area-to-area or room-to-room. Pigs were housed in individual pens (1.52 m x 1.83 m) in one room. Partitions with evenly-spaced vertical bars allowed interactions between pigs in adjacent pens. Animal care, housing, and feeding were under the supervision of the research facility staff.

Pigs (n = 12; 50 kg) were sourced from a PRRSV-naïve commercial herd. To confirm PRRSV-naïve status, pigs were tested for PRRSV serum antibody at 19 and 11 days prior to arrival at the research facility and again prior to vaccination. Pigs were acclimated in the

facilities for 5 days and then vaccinated with a modified-live PRRS vaccine on Day 0 of the study (Ingelvac PRRS® MLV, Boehringer Ingelheim Vetmedica Inc., St. Joseph Missouri). Individual oral fluid samples were collected twice daily from each of the 12 pigs from Day -7 through Day 42 using a protocol described elsewhere (Prickett et al., 2008a, b). In brief, one 100% cotton rope was hung in each pen for 30 minutes, during which time the pigs interacted (chewed on) the rope. Thereafter, the wet portion of the rope was inserted into a plastic bag and severed from the remainder of the rope. Oral fluid was extracted by passing the wet rope, still within the bag, through a portable towel wringer (Dynajet, Nürtingen, Germany). Samples were decanted into 50 ml centrifuge tubes and placed on crushed ice. The morning and afternoon oral fluid samples from each day were combined, aliquoted into 5 ml cryovial tubes and stored at -80°C.

Study 2 was a field study conducted on three separate farms in one production system. Each farm (A, B, C) consisted of three curtain-sided, wean-to-finish barns (1, 2, 3) sited parallel to each other and spaced 10 m apart. Barns (13.4 m x 61.0 m) were designed with split-zone ventilation, independent control of curtains, and ridge ventilation by zone. Manure was collected in shallow pits beneath each barn and moved to an outdoor above-ground slurry storage tank via a scraper system. All farms were managed on an all-in-all-out basis, with buildings cleaned and disinfected between groups. Animal housing, handling, feeding, and health care were implemented by producers and with the assistance of production system veterinarians. For the purpose of implementing this study, producers and veterinarians did not vaccinate or move pigs between pens or barns during the 2-month sampling period.

Each barn contained 40 pens arranged as 20 pens on either side of a central walkway. On Farm A, pens (3 m x 6 m) were built with solid concrete walls and partial slats. On Farm B and C, pens (3 m x 6 m) were built with gated walls and partial slats. During the collection period, all occupied pens held ~25 pigs. Barns were populated with weaned pigs (~21 days of age) sourced from one PRRSV-endemic breeding herd over the course of 7 to 14 days, but each farm's pigs came from a different sow herd. For all breeding herds, commercial modified-live PRRS vaccines were administered to replacement gilts during quarantine, but not to sows or pigs in other phases of production.

Oral fluid samples were collected weekly from every occupied pen (n = 36) in every barn (n = 3) on each farm (n = 3) using the procedure described above. Samples were decanted into 50 ml centrifuge tubes and placed on crushed ice for transport to the laboratory. Samples were aliquoted into 4 ml cryovials in the laboratory and stored at -20°C.

After weekly oral fluid collection, blood samples were collected from 20 pigs in each barn by sampling 10 pigs from each of two pens. Pens selected were approximately 1/4 of the distance from each end of the barn on opposite sides of the walkway. The same pens were sampled each week, but not necessarily the same pigs (convenience sampling). Blood samples were collected using a single-use vacutainer system with 10 ml serum separation tubes (Becton, Dickinson and Company, Franklin Lakes, NJ, USA). Blood samples were placed on ice for transport to the laboratory. In the laboratory, samples were centrifuged (1000 x *g* for 10 min), aliquoted into 4 ml cryovials, and stored at -20°C.

PRRSV ELISAs

Serum samples were tested for PRRSV antibodies using a commercial PRRSV ELISA (IDEXX PRRS X3 Ab Test, IDEXX Laboratories, Inc., Westbrook ME USA) following the instructions provided by the manufacturer. Sample-to-positive (S/P) results ≥ 0.4 were considered positive for PRRSV antibody.

Oral fluid samples were tested for PRRSV antibodies using a commercial PRRSV oral fluid antibody (IgG) ELISA (IDEXX PRRS OF Ab Test, IDEXX Laboratories, Inc.) following the instructions provided by the manufacturer. S/P results ≥ 0.4 were considered positive for PRRSV antibody. IgM, IgA, and IgM-IgA oral fluid ELISAs were performed as instructed by the manufacturer for the PRRSV OF Ab ELISA (IgG) with the following exceptions: the kit IgG conjugate was replaced with goat anti-pig IgM (A100-100P Bethyl Laboratories) diluted 1/5,000 in IDEXX conjugate diluent; or goat anti-pig IgA (A100-102P Bethyl Laboratories) diluted 1/3,000 in IDEXX conjugate diluent; or dual mixture of IgM (1/5,000)-IgA (1/3,000). Plate positive controls for the IgM, IgA, or IgM-IgA ELISAs were based on oral fluid samples of known positive PRRSV status diluted in kit sample diluent to produce

optical density (OD) values between of 0.6 and 0.7. Tests were performed as recommended by the manufacturer and results reported as S/P ratios. Cutoffs for the IgM, IgA, and IgM-IgA oral fluid ELISAs were determined by receiver operator characteristic curve (ROC) analysis, as described in section 2.6 (statistical analysis).

PRRSV RT-rtPCR

All samples were tested for PRRSV RNA at the Iowa State University Veterinary Diagnostic Laboratory (ISU VDL) using standard protocols. Systematic bias was addressed by randomizing samples order prior to submission.

In Study 2 (field data), serum samples from the same pen were pooled by five and tested by PRRSV RT-rtPCR. Nucleic acid extraction was performed using the MagMAX™ viral RNA isolation kit (Life Technologies, Carlsbad, CA, USA) and a Kingfisher 96 magnetic particle processor (Thermo-Fisher Scientific, Waltham, MA, USA) using a standard lysis procedure. A lysis/binding solution was prepared with 65 µL lysis/binding solution, 1 µL carrier RNA, 65 µL isopropanol and 2 µL Xeno™ RNA template at 10,000 copies/µL. At the lysis step, 130 µL of the prepared lysis/binding solution was added to 20 µL of magnetic bead mix prior to extraction and elution into 90 µL buffer. The standard lysis procedure used 150 µL in wash I and 150 µL in wash II. The extraction was performed using the Kingfisher AM1836_DW_50_v3 program (Thermo-Fisher Scientific).

In Study 2, oral fluid samples were tested individually (not pooled) for PRRSV RT-rtPCR testing. Nucleic acid extraction was performed using the MagMAX™ viral RNA isolation kit and a Kingfisher 96 magnetic particle processor using a high-volume modified lysis (HVML) procedure. A modified lysis/binding solution was prepared with 120 µL lysis/binding solution, 2 µL carrier RNA, 120 µL isopropanol and 2 µL Xeno™ RNA template at 10,000 copies/µL. At the lysis step, 240 µL of the prepared lysis/binding solution was added to 20 µL of magnetic bead mix prior to extraction and elution into 90 µL elution buffer. An additional modification for the HVML procedure was an increase in wash I and II solutions, i.e., the procedure used 300 µL in wash I and 450 µL in wash II. The extraction was performed using the Kingfisher AM1836_DW_HV_v3 program.

Both serum and oral fluid samples were assayed using a commercial PRRSV real-time RT-rtPCR kit (EZ-PRRSV MPX 4.0 assay, Tetracore©, Rockville, MD, USA). For each run, positive controls for PRRSV Types 1 and 2 and a negative amplification control were included. For each control well, 17.25 µL of EZ-PRRSV MPX 4.0 Reagent was added. The EZ-PRRSV MPX 4.0 Reagent includes buffer, primer and probes, 0.75 µL Enzyme Blend, 0.25 µL IC and 7 µL of positive control (Type I or 2 IVT) or negative control (1 x TE). Each well contained 17.25 µL of the EZ-PRRS MPX 4.0 Reagent, which included buffer, primer, probes, 0.75 µL Enzyme Blend and 7 µL of the oral fluid extract. Plates were loaded onto the thermal cycler (7500 Fast Real-Time PCR System, Applied Biosystems©, Foster City, CA, USA) and the following cycling conditions were used: one cycle at 48°C for 15 min, one cycle at 95°C for 2 min, 45 cycles of: 95°C for 5 s, and 60°C for 40 s. Samples with Ct values < 45 for Type 2 PRRSV were considered positive.

PRRSV sequencing

In Study 2 (field data), one RT-rtPCR-positive pooled serum sample (pool of 5) from each barn was selected for PRRSV sequencing each week. Approximately 1,082 base pairs of open reading frame (ORF)5 region and the flanking regions within 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-rtPCR Kit (Quanta Biosciences, Gaithersburg, MD USA). The serum 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. The final PCR volume of 25 µl consisted of 21 µl of master mix and 4 µl of RNA extract. One positive extraction control, one negative extraction control, and one negative amplification control were included with the reaction. The PCR was performed (Applied Biosystems® 2720 thermal cycler, Life Technologies Corporation) with the following cycling conditions: one cycle at 48°C for 20 min, one cycle at 94°C for 3 min, 45 cycles of 94°C for 30 s, 50°C for 50 s, and 68°C for 50 s. The final elongation step was 68°C for 7 min. Detection of the RT-rtPCR 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 USA) 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, USA).

Statistical analysis

Receiver operating characteristic curve (ROC) analyses were performed in R 3.3.0 (R Core Team, 2013) with the objective of calculating the cutoffs and associated diagnostic sensitivity and specificity for each oral fluid antibody isotype-specific ELISA assay. PRRSV IgM, IgA, and IgM-IgA ELISA ROC analyses were done separately for Study 1 and Study 2.

In Study 1 (experimental data), sample status (positive/negative) was defined relative to the day of vaccination (Day 0, Ingelvac PRRS® MLV). For the PRRSV IgM and IgM-IgA ELISA ROC analyses, samples from days -7 to 5 were considered negative and samples from days 10 to 28 as positive. For the PRRSV IgA and IgG ELISA ROC analyses, days -7 to 7 were classified as negative and days 11 to 28 as positive.

In Study 2 (field data), the infection status for pen-based samples was determined by RT-rtPCR testing. For the ROC analyses, oral fluid samples from a pen were considered IgM and IgA antibody negative up to, and including, the first positive PRRSV RT-rtPCR result from the pen. Thereafter, samples were considered positive for IgM beginning with the next weekly sampling and for four subsequent weekly samplings. For IgA and IgM-IgA ELISA ROC analyses, samples were considered positive for all weekly samplings after the first RT-rtPCR positive result. The IgG ELISA results were not analyzed due to the presence of maternal PRRSV antibody.

For both Study 1 and 2, transformation of S/P values ($x^{3/7}$) was performed to fulfill the assumption of normality for the IgM, IgA, and IgM-IgA data. Thereafter, a linear mixed model was fitted to the data, with PRRSV infection status as the explanatory variable and pen as the random effect. After obtaining the fixed parameter estimates and standard deviation, point estimates, variance, and confidence intervals for diagnostic sensitivity and specificity

were calculated based on the Normal model. Because of the correlated structure of the data, i.e., repeated measures at the sampling level, binomial exact confidence intervals were calculated using model-based estimation of diagnostic sensitivity and specificity. Asymptotic logit transformation was used to avoid confidence intervals beyond [0, 1]. That is, diagnostic sensitivity and specificity point estimates were first logit transformed, then confidence intervals were calculated, after which the final confidence intervals were obtained by back transformation.

Results

All samples were randomized prior to PRRSV ELISA or RT-rtPCR testing. All samples were tested once and no retests were performed.

Study 1 (experimental data)

Study 1 followed the PRRSV oral fluid antibody isotype response of 12 pigs following vaccination with a modified-live PRRSV vaccine (Ingelvac PRRS® MLV, Boehringer Ingelheim Vetmedica, Inc.) under experimental conditions. All pigs were confirmed free of PRRSV infection by PRRSV RT-rtPCR and PRRSV ELISA testing. From DPV -7 to 42, oral fluid samples were collected twice daily from individual pigs for a total of 600 oral fluid samples. At the end of the trial, all samples were tested for PRRSV antibody using isotype-specific ELISAs (IgM, IgA, IgG, and IgM-IgA). The oral fluid isotype-specific ELISA mean S/P values and the percent of IgM-IgA ELISA samples with S/P ratios ≥ 0.40 by DPV are shown in Figure 1.

Study 2 (field data)

Study 2 was conducted on 3 commercial farms (A, B, C) in one production system, each with 3 wean-to-finish barns (1, 2, 3). Oral fluid samples were collected weekly from the 36 occupied pens (~25 pigs per pen) in each of the 3 barns, i.e., 108 samples per week, for a total of ~972 oral fluid samples per farm. In addition, 20 serum samples from two pens in each barn were collected at each weekly sampling for a total of 1,620 serum samples per farm. The PRRSV status of barns and farms was determined on the basis of RT-rtPCR

testing and reflected the endemic circulation of virus in the production system. No PRRSV vaccine was used in the pigs or their dams during the production cycle.

The PRRSV RT-rtPCR results for oral fluid samples are shown in Figure 2. In Farm A, PRRSV RT-rtPCR-positive oral fluid samples were observed in one or more of the three barns at every sampling period (week 0 through 8). Of the 972 oral fluid samples collected on Farm A, 425 samples were positive. At the last sampling, all oral fluid samples collected from all Farm A (n = 108) were positive. No RT-rtPCR-positive oral fluid samples were observed in Farm B and in Farm C one oral fluid sample tested positive at week 8.

PRRSV RT-rtPCR-positive serum samples (pooled by fives) were found in Farm A on sampling weeks 4 through 8. No RT-rtPCR-positive serum samples were detected at any time in Farms B and C. In Farm A, PRRSV sequencing was attempted on RT-rtPCR-positive pooled serum samples collected weeks 4 through 8. To optimize sequencing success and collect sequencing data over time, the pool with lowest Ct was selected each week from each barn. A total of 14 pooled serum samples were submitted for sequencing and 10 ORF 5 sequences were obtained. Sequence analysis provided evidence of the circulation of wild-type PRRSV (Figure 3).

All oral fluid samples collected in Study 2 (n = 2,916) were tested for PRRSV antibody using isotype-specific ELISAs (IgM, IgA, IgG and IgM-IgA). The mean S/P values for the IgG ELISA and IgM-IgA ELISA are shown in Figure 4. The percent of IgM-IgA ELISA samples with S/P ratios ≥ 0.40 by sampling week are given in Figure 2.

ROC analysis

For both Study 1 (experimental data from individual pigs) and Study 2 (field data from pens of pigs), the diagnostic sensitivities, specificities, and 95% correlated confidence intervals were calculated for the IgA, IgM, and IgM-IgA ELISAs over a range of S/P cutoffs of 0.20, 0.30, 0.40, 0.50, and 0.60 (Table 1). For Study 1, the oral fluid isotype-specific ELISA mean S/P values and the percent of IgM-IgA ELISA samples with S/P ratios ≥ 0.40 by DPV are shown in Figure 1. Two of 156 samples collected between DPV -7 to 5 had S/P values $\geq$

0.40. Specifically, one sample collected on DPV -3 had an S/P value of 0.46 and one sample collected on DPV -1 had an S/P value of 0.53. These values were accounted for in the ROC analysis (Table 1), resulting in a diagnostic specificity of 99%.

Discussion

Various economic studies have uniformly shown that PRRSV inflicts major losses on swine health and productivity (Holtkamp et al., 2013; Nathues et al, 2017; Neumann et al, 2005; Nieuwenhuis et al., 2012; Zhang et al, 2012). In Europe and North America, the cost of PRRSV to the industry in terms of hog marketed has been estimated at $6.25 to $15.25 per pig (Holtkamp et al., 2013; Nathues et al., 2017). On-going losses at this level are unacceptable in terms of animal welfare and the public's perception of swine production, but the solution to this dilemma is not apparent. Calvin Schwabe in 1982 recommended that veterinary practitioners use surveillance to understand the patterns of disease and establish baselines against which the effect of control interventions could be measured. Schwabe's vision was never realized, but the complex, dynamic, global nature of contemporary swine production mandates that on-going, near-real-time surveillance be part of the PRRSV solution.

PRRSV surveillance can be based on the detection of nucleic acid and/or antibody: each has its strengths and weaknesses. The time to RT-rtPCR-detectable viremia differs among PRRSV isolates, but the majority of animals are viremic within 48 hours (Pepin et al., 2015). Under experimental conditions, ~50% of pigs were still nucleic acid-positive at ~50 DPI and ≤ 10% of animals remained positive at ~100 DPI (Horter et al., 2002; Molina et al. 2008). This unusually long duration of viremia makes nucleic acid detection a viable option for PRRSV surveillance. However, serum-RT-rtPCR-negative animals can still harbor infectious PRRSV. That is, after the immune response clears virus from the circulatory system, infectious PRRSV can still be recovered from lymphoid tissues, e.g., tonsils of the soft palate (Horter et al., 2002; Wills et al., 2003).

The humoral immune response against a variety of PRRSV proteins has been described (Molina et al., 2008) and a variety of serum antibody detection platforms are available in diagnostic laboratories, e.g., ELISA, IFA, IPMA and neutralizing antibody assays (Decorte et al, 2014; Ouyang et al, 2013; Pejsak et al., 2017; Yoon et al, 1995). ELISA is compatible with high-throughput laboratories, is technically simple, and is a widely used assay for PRRSV antibody detection (Pejsak et al., 2017). Kittawornrat et al., (2012b) in a study involving 12 laboratories found that the results produced by a PRRSV oral fluid ELISA were highly repeatable within laboratories and highly reproducible between laboratories. When used in surveillance, PRRSV serum and oral fluid ELISAs can provide useful data concerning herd immunity and exposure history. However, PRRSV maternal antibody may be present in pigs up to 10 weeks old (Yoon et al., 1996; Houben et al., 1995). The presence of maternal IgG antibody complicates the interpretation of ELISA results because, in younger pigs, a positive result may represent maternal antibody or antibody produced by the pig in response to PRRSV infection.

Prior research demonstrated that inoculation with type 1, type 2, or MLV PRRS viruses produced detectable levels of PRRSV IgM and IgA in both serum and oral fluid (Kittawornrat et al, 2013). The goals of the present research were to develop a combined IgM-IgA oral fluid ELISA, evaluate its performance using experimental and field samples, and determine whether the assay could detect PRRSV-specific IgM and IgA produced by pigs in response to infection, even in the presence of maternal IgG antibody.

Using samples from MLV-vaccinated pigs and type 2 field virus-infected pigs, the results confirmed prior reports of IgM, IgA, and IgG antibody ontogeny in oral fluids following exposure to the virus. Testing of oral fluids from pigs originating from sow herds endemically infected with PRRSV found abundant PRRSV IgG, but no evidence of maternally-derived IgM or IgA in oral fluid specimens.

A comparison of IgM, IgA, and IgM-IgA ELISAs showed that the combined IgM-IgA assay provided better performance than detection of either isotype alone (Table 1). The authors were unable to locate other examples of combined antibody isotype ELISAs with which to

compare these data, but there are examples of combining the results of two different isotype assays to establish infection status. For both Dengue virus and Crimean-Congo hemorrhagic fever virus, performing IgM and IgG ELISAs in parallel and interpreting a positive result on either as indicative of infection significantly improved diagnostic sensitivity (Dowall et al., 2011; Vaughn et al. 1999). While the combined IgM-IgA oral fluid ELISA demonstrated adequate diagnostic sensitivity and high diagnostic specificity, higher performance may be achievable through further assay optimization and/or the removal of IgG. Removal of IgG from specimens using anti-IgG or protein G has been described as a method to improve detection of IgM and IgA (Ankerst et al., 1974; Martins et al., 1995). For example, Dowall et al. (2011) showed that removal of IgG from diagnostic samples resulted in increased both the diagnostic sensitivity and specificity of a Crimean-Congo hemorrhagic fever virus IgM ELISA.

Overall, the combined PRRSV IgM-IgA oral fluid ELISA described in this proof-of-concept study is a promising tool for PRRSV surveillance, particularly in populations of growing pigs originating from PRRSV-positive or vaccinated breeding herds because of its ability to detect pig-derived IgM and IgA antibody in the presence of maternal IgG.

Funding

This study was funded in part by Checkoff Dollars administered through the National Pork Board (Grant #13-157, #15-158), Des Moines, Iowa (USA).

Conflict of interest statement

The authors declare no conflicts of interest with respect to the conduct, authorship, and/or publication of this study. Co-authors Baum, Giménez-Lirola, and Zimmerman have consulted with IDEXX Laboratories, Inc. on areas of diagnostic medicine independent of this research.

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Tables

Table 1. PRRSV isotype-specific ELISA diagnostic sensitivity and specificity based on experimental and field data

		Study 1 (Experimental data from individual pigs)		Study 2 (Field data from pens of pigs)	
PRRSV ELISA antibody target	**Cutoff (S/P)**	**Diagnostic sensitivity (95% CI)**	**Diagnostic specificity (95% CI)**	**Diagnostic sensitivity (95% CI)**	**Diagnostic specificity (95% CI)**
IgA	0.2	0.78 (0.76, 0.80)	0.87 (0.86, 0.88)	0.76 (0.75, 0.76)	0.94 (0.94, 0.94)
	0.3	0.63 (0.59, 0.66)	0.97 (0.97, 0.97)	0.69 (0.68, 0.70)	0.97 (0.97, 0.97)
	0.4	0.48 (0.44, 0.52)	0.99 (0.99, 0.99)	0.66 (0.65, 0.67)	0.98 (0.98, 0.98)
	0.5	0.40 (0.37, 0.44)	0.99 (0.99, 0.99)	0.63 (0.62, 0.64)	0.99 (0.99, 0.99)
	0.6	0.34 (0.31, 0.38)	0.99 (0.99, 0.99)	0.60 (0.59, 0.61)	0.99 (0.99, 0.99)
IgM	0.2	0.69 (0.67, 0.71)	0.99 (0.99, 0.99)	0.63 (0.62, 0.64)	0.99 (0.99, 0.99)
	0.3	0.61 (0.58, 0.63)	0.99 (0.99, 0.99)	0.56 (0.55, 0.57)	1.0 (0.99, 1.0)
	0.4	0.54 (0.51, 0.57)	1.0 (NA*)	0.50 (0.49, 0.52)	1.0 (0.99, 1.0)
	0.5	0.47 (0.44, 0.50)	1.0 (NA*)	0.45 (0.43, 0.46)	1.0 (0.99, 1.0)
	0.6	0.40 (0.38, 0.43)	1.0 (NA*)	0.42 (0.41, 0.44)	1.0 (0.99, 1.0)
Combined IgM-IgA	0.2	0.93 (0.93, 0.94)	0.85 (0.83, 0.86)	0.80 (0.80, 0.81)	0.95 (0.95, 0.95)
	0.3	0.84 (0.83, 0.85)	0.96 (0.96, 0.96)	0.77 (0.77, 0.77)	0.98 (0.98, 0.98)
	0.4	0.77 (0.75, 0.79)	0.99 (0.99, 0.99)	0.74 (0.73, 0.74)	0.99 (0.99, 0.99)
	0.5	0.68 (0.66, 0.71)	0.99 (0.99, 0.99)	0.72 (0.72, 0.73)	0.99 (0.99, 0.99)
	0.6	0.64 (0.61, 0.67)	1.0 (NA*)	0.69 (0.68, 0.70)	1.0 (0.99, 1.0)

*Confidence intervals not calculable.

Figures

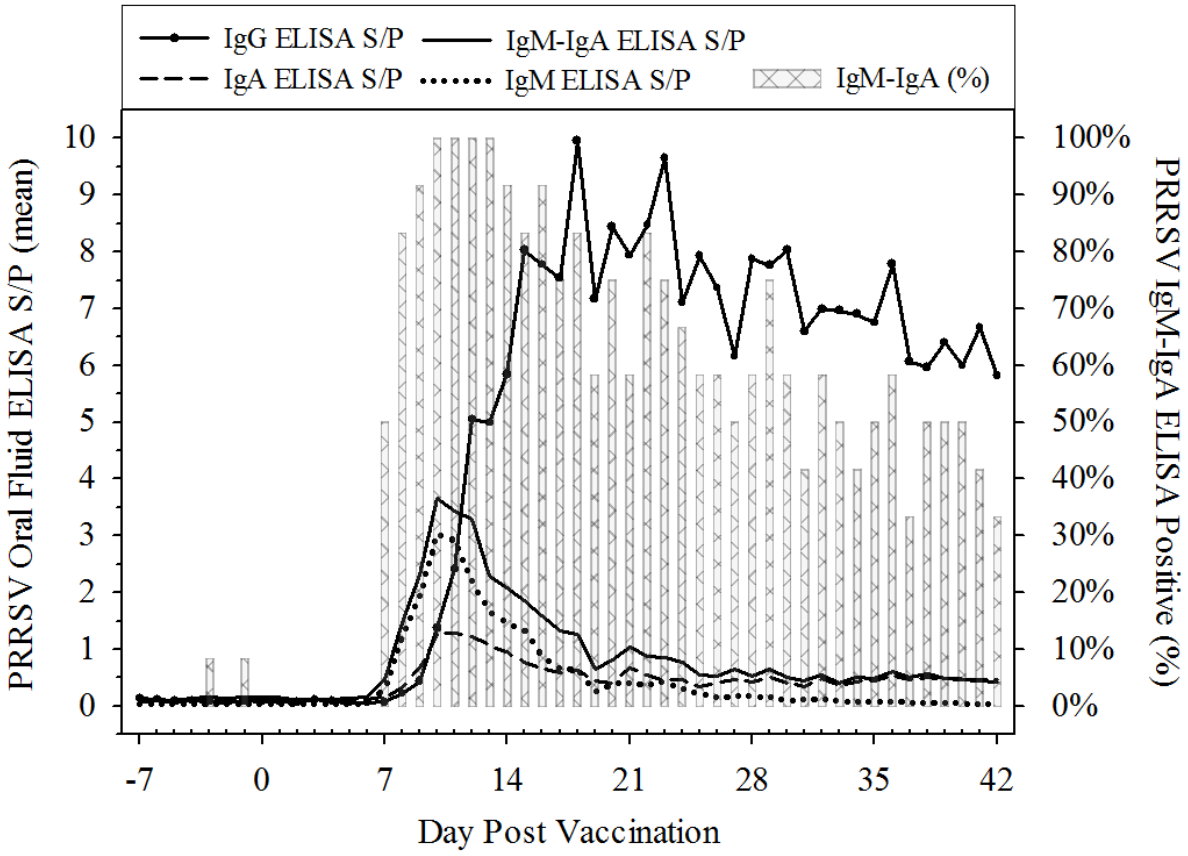

Figure 1. PRRSV antibody ontogeny in oral fluid samples collected from 12 pigs vaccinated with a modified-live virus vaccine over the course of 49 days (Study 1)

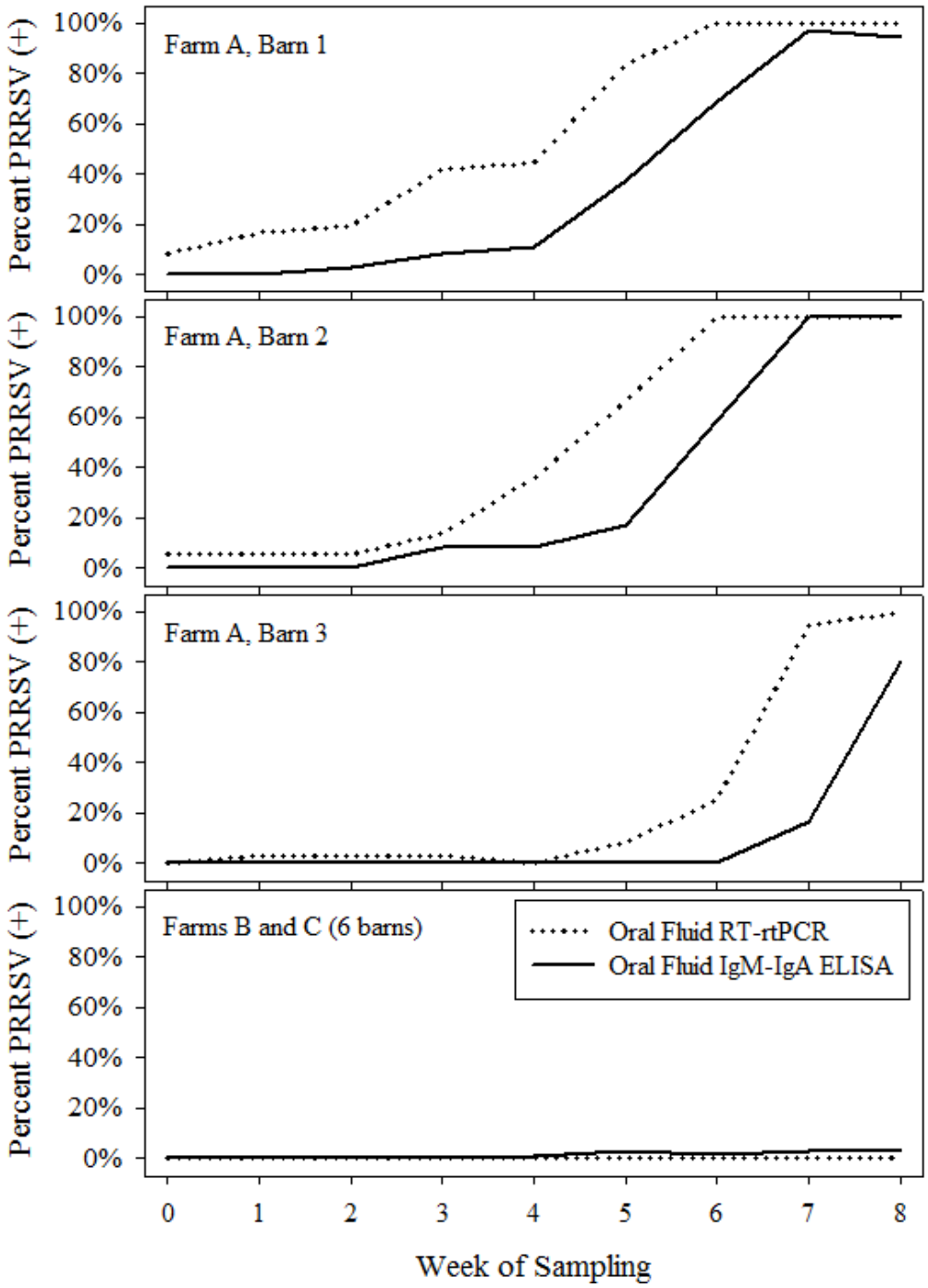

Figure 2. Oral fluid testing results (% positive) in PRRSV-positive (A) and PRRSV-negative (B, C) wean-to-finish farms (Study 2).

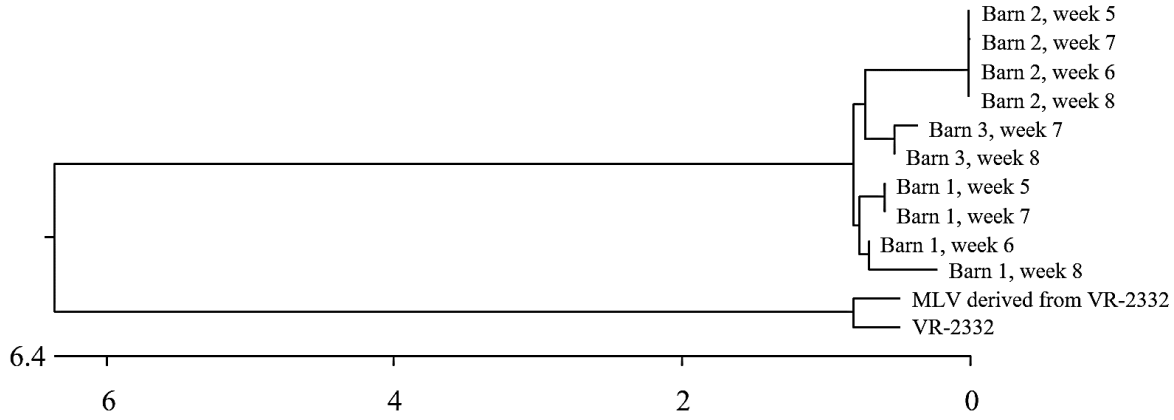

Figure 3. Phylogenetic analysis (ORF 5 nucleotide level) of wild-type PRRS viruses circulating in Farm A (Study 2) reported as nucleotide substitutions per 100 residues

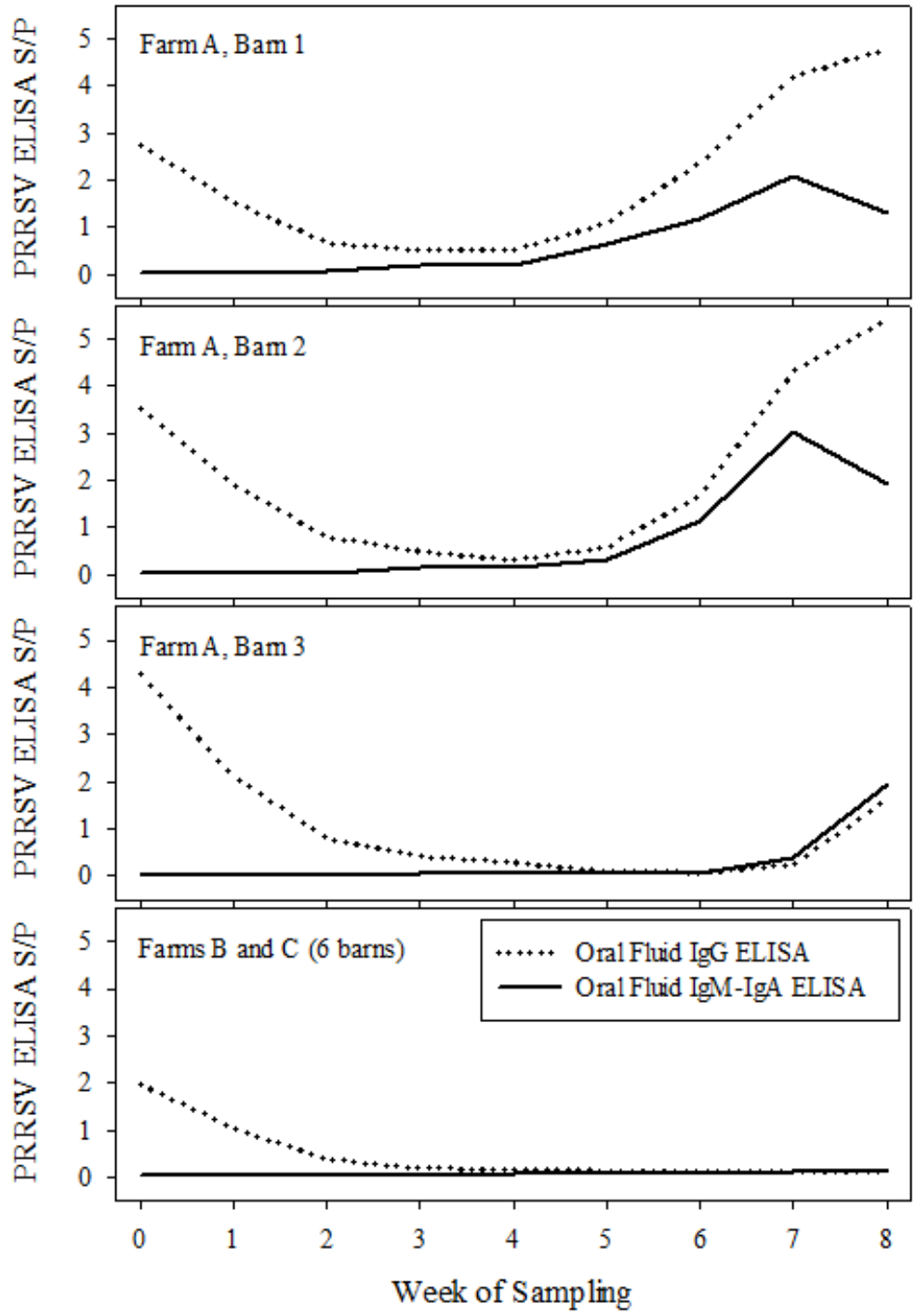

Figure 4. Oral fluid testing results (mean S/P) in PRRSV-positive (A) and PRRSV-negative (B, C) wean-to-finish farms (Study 2).

CHAPTER 4. GENERAL DISCUSSION

Current efforts to control and/or eradicate diseases in pig populations have been complicated by two recent changes in husbandry: the emergence of larger herds and the increased movement of pigs between farms (multi-site production), often across local, regional, or national borders, as a core management strategy.

Throughout the world, pork production is rapidly transitioning from smaller, "extensive" farms to larger, "intensive" farms (Gale, 2017; Marquer, 2010; USDA, 2005). For example, backyard production accounted for 94% of pig inventory in China in 1983 but by 2009, family farms and large systems accounted for 61% of pig inventory (Xiao et al., 2012). This change from small to large farms is important vis-à-vis disease control because larger farms are more likely to become infected due to the greater number of potential "transmission events", i.e., movement of employees, trucks, and animals, and because larger populations are less able to achieve and maintain herd immunity (Gardner et al., 2002; Haggett, 2000).

Increased movement of pigs between farms is important because it serves to physically connect distant populations (metapopulations) (Dorjee et al., 2013; Relun et al., 2017). This is an old problem, but it is occurring on a greater scale than ever before. Previously, the movement of rinderpest via infected animals led to the founding of the World Animal Health Organisation (OIE) and the Food and Agriculture Organization of the United Nations (Roeder, 2011). But current levels of pig movement far exceed historic patterns. In the United States, 4,317,000 pigs crossed state borders in 1990, but by 2016 this number had increased to 52,555,000 pigs, i.e., roughly 1,000,000 per week (USDA NASS). In England and Wales, 61,937,855 pigs were moved between farms from 2009 to 2013 (Guinat et al., 2016).

Thus, fundamental changes in pig rearing have impacted efforts to detect, prevent, control and/or eliminate infectious disease and have accelerated the speed at which pathogens are dispersed among farms. Current approaches to improve on-farm disease control rely on more stringent biosecurity protocols and enhancement of herd immunity through the use of

vaccines or intentional exposure (Pitzer et al., 2016). While biosecurity and herd immunity play primary roles in animal disease control, on-farm disease surveillance and monitoring must be in place to verify their effectiveness. In addition, efficient, effective regional surveillance must be in place for the detection of foreign animal diseases (FAD).

The research presented in this dissertation aimed to begin the work of developing new methods for livestock surveillance based on aggregate samples, specifically the use of oral fluid samples in swine populations. Guidelines for oral fluid sampling will provide estimates for probability of detection to guide the development of surveillance and monitoring programs. In addition, the development of a combined IgM-IgA oral fluid ELISA, i.e., a test that allows for the detection of PRRSV infection in the presence of maternal antibody, is an indicator of the potential for new and improved diagnostic technology.

Future work should focus on the continued development and/or adaptation of aggregate sample-based diagnostic assays to the detection of endemic and exotic animal diseases, e.g., foot-and-mouth disease virus, classical swine fever virus, and African swine fever virus. Simultaneously, additional work is required to develop statistically-valid methods for applying these tests to the detection of disease in populations, farms, and regions.

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ACKNOWLEDGEMENTS

Behind every PhD student, there is a village of people who support him or her as they grow their career and pursue their passions. I would not be where I am today without the support of my village.

My sincerest thank you to Dr. Jeffrey Zimmerman and Dr. Rodger Main. Both Jeff and Rodger have mentored me and invested in my future from the very beginning of my academic journey at Iowa State University. To Jeff and Rodger, thank you for taking a chance on the veterinary student who had never worked with pigs so many years ago.

I am very grateful to my program of study committee: Dr. Chong Wang, Dr. Phillip Gauger, Dr. David Baum and Dr. Paulo Arruda for their support and guidance. Each of my committee members has contributed to my education and helped me advance my research and my future.

I extend my sincere thanks to my colleagues in the swine ecology laboratory: Dr. Luis Gimenez-Lirola, Dr. Ronaldo Magtoto, Jordan Bjustrom-Kraft, Dr. Wendy Gonzalez, Dr, Chris Olsen, Dr. Melisa Spadaro, Dr. Alexandra Henao Díaz, Dr. Ting-Yu Cheng, and Dr. Juan Carlos Mora Diaz for their friendship, for their patience, for listening to me practice talks, letting me steal their meeting times with Jeff, and providing friendly faces in the audience of conferences.

To Dr. Darin Madson and Dr. Eric Burrough, thank you for giving me the opportunity to work with you on countless research trials and teaching me the in-and-outs of running a successful research trial. To Dr. Drew White, thank you for giving me a crash course in all things pigs, from snaring, to sorting and how to effectively use a panel board.

To Dr. Katie Woodard and Dr. Brent Pepin, I would not have survived these past few years without your counsel. Thank you for your friendship, advice and for never allowing me to give up.

To Dr. Joshua Ydstie and Dr. Dr. Paisley Canning, thank you for your friendship and reminding me to take a break every now and again.

To my family, thank you for understanding when my crazy schedule interfered with family events. Thank you for all of your support during my academic journey. Thank you for instilling in me a work ethic that made me capable of pursuing my dreams. I love you.