RESEARCH REPORT
SWINE HEALTH

Title: Diagnostic Characteristics of Oral Fluid for Detection of PRRS - **NPB #07-129**

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Date Submitted: March 13, 2009

Abstract:

Simple and effective surveillance methods are critical for control and elimination of PRRS. Current methods for PRRSV herd surveillance are based on statistical sampling of random individuals repeatedly over time. The methods are well characterized but require significant labor and capital while remaining subject to failure. Zimmerman and colleagues recently described a simple, pen-based community sampling method of oral fluid that is a promising method for low-cost, routine monitoring. The basic evaluation of this method using standard ELISA and PCR methods in experimental and field conditions is promising. Here, we optimize ELISA conditions for testing of anti-PRRSV protein antibodies in oral fluids, and characterized the time course of anti-PRRSV antibody responses. The study, carried out in three replicates, showed that assay conditions must be optimized for oral fluid samples to increase sensitivity, and that anti-PRRSV antibodies appear in oral fluids at the same time or later than in serum. Interestingly, differences were observed in the dominant isotypes present in oral fluids depending on sampling method. IgG was more abundant in sampling of individual pigs with absorbent wicks, whereas IgA was more abundant in pen sampling with rope. The findings support the value of pen-based sampling and suggest that multiple mechanisms regulate antibody secretion into the oral cavity

Introduction

Serum is the standard diagnostic sample for disease monitoring in the swine industry. Serum profiling is used routinely for health monitoring and for detection of pathogens, especially viral agents including PRRSV and PCV2. However, current testing protocols could be improved if diagnostic samples could be collected non-invasively and with less stress to the animals and human personnel, so long as test sensitivity and specificity is unaffected.

Oral fluid (the term here is used to include saliva, capillary transudate and fluids from any other source that reside in the oral cavity) collected from PRRSV-infected pigs contains PRRSV (Wills et al 1997, Cho et al 2006) and anti-PRRSV antibodies. The levels of PRRSV observed in oral fluid was substantially lower than were present in serum (Cho et al. 2006). Thus, the relative insensitivity of oral fluid as a diagnostic sample for PRRSV detection with current PCR technology suggests that diagnostic testing for anti-PRRSV antibodies may be desirable. Human studies show that antibody-based assays that are optimized for oral fluid samples yield levels of sensitivity in oral fluid samples that are equivalent to serum (Cameron and Carman 2005).

Zimmerman and colleagues recently provided exciting data that oral fluids could be easily collected from groups of pigs by chewing on an absorbent rope, followed by extraction and ELISA detection of specific antibodies. Here, we took advantage of our expertise in production of recombinant PRRSV proteins and characterization of protein-specific antibody responses to PRRSV (Johnson et al. 2007) and in oral fluid collection and antibody assessment (Foss and Murtaugh 1999) to characterize anti-PRRSV antibodies in oral fluids of individual pigs to shed light on group results obtained by Zimmerman and colleagues.

Objectives and Research Question

1 To characterize the antibody titers in saliva to structural and nonstructural PRRSV proteins. Q: What are the kinetics and relative levels of antibody secretion in oral fluids of pigs infected with PRRSV over time?

2 To determine the subclass distribution of antibodies to PRRSV proteins. Q: What are the relative

amounts of anti-PRRSV IgA, IgM and IgG in oral fluids compared to serum of PRRSV-infected pigs?

3 To optimize the ELISA conditions for detection of anti-PRRSV antibodies in oral fluids. Q: What are the optimum conditions of antigen coating and fluid incubation time on sensitive and specific detection of anti-PRRSV antibodies?

Materials & Methods

Animals. Groups of 20 6-8 week-old pigs that were free of PRRSV were moved into the Swine Disease Eradication Center research farm (Appleton, MN) and exposed to natural infection with PRRSV MN-184. Under these conditions, animals routinely become infected with PRRSV within one week of entry, as confirmed by 20 replicates conducted between May 2006 and March 2007.

Oral Fluid Sampling and ELISA. Oral fluids were collected at intervals with absorbent wicks (Polyfiltronics, Rockland MA) by placing between the teeth and cheek wall and swabbing in the oral cavity. Wicks were immediately frozen on dry ice and stored at -70° until use. Wicks were extracted with 1 ml saline containing 5% nonfat dry milk and protease inhibitors (10 µM leupeptin, 1 µmg/ml aprotinin, 50 µm PMSF, and 5 µM bestatin) (Foss and Murtaugh 1999). Pigs also were bled at 7 day intervals for serum. Diluted fluids were centrifuged to remove debris and assayed for antibodies to PRRSV nsp1, nsp2, GP5, M, and N proteins as described (Johnson et al. 2004, Johnson et al. 2007) using two-fold dilutions and detection for IgG, IgM and IgA using specific goat anti- porcine Ig subclass conjugated to HRP (Bethyl Labs, Montgomery TX).

Results

Initial Findings. As reported in the interim report (May, 2008), the initial findings of replicate one showed little evidence of anti-PRRSV IgG or IgA in oral fluids of 20 growing pigs in a 17 day period after the animals were moved into a barn with acutely infected swine. It appeared that infection did not occur rapidly in these animals since anti-nucleocapsid (N) IgGs were only detected in serum at day 17 and showed a wide range of values. Anti-N IgM and IgA was not observed in serum or oral fluids and anti-N IgG was not observed in oral fluids. The result was surprising since IgA was expected to be the dominant Ig subclass in oral fluids. Specific porcine IgA levels in oral fluids were determined by ELISA and were estimated to be in

the range of 100 ng/ml prior to infection, and about 150 ng/ml at 17 days of infection. The reproducibility if this finding was variable, so further investigation was not pursued. Also, we elected to focus on characterization of antibody subclasses involved in the PRRSV response. Therefore, only N protein was used as the target antigen since it is widely used and known to elicit antibody responses induced in response to all strains of PRRSV.

A second replicate was performed with saliva and serum sampling carried out for 28 days. Modifications were made to the ELISA assay to increase sensitivity by reducing the oral fluid dilution factor from 1/50 to 1/3 – 1/10. Coating of N protein on the plate was maintained at 100 ng/well, and detection antibody diluted 1/100,000 and the incubation time extended to 2 hr. Antibody responses observed here were consistent with rapid infection of pigs shortly after entry. Anti-IgM was detected in oral fluids on day 9 (Fig. 1), and in serum on day 7 (Fig. 2). IgG antibodies were detected on day 13 in both serum and oral fluids. Levels of anti-N IgG in oral fluids were low at day 13, but increased to a peak at day 25. There was no evidence of an anti-PRRSV response in the IgA subclass in either serum or oral fluids. Because this finding was unexpected we collaborated with Jeffery Zimmerman, Iowa State University, to assess the Ig subclass responses to PRRSV infection in a group of pigs using samples collected on rope. Here, the response to IgM appeared first, at 10 days, followed by IgA, which demonstrated two peaks of expression, and IgG, which was similar to IgA but at lower levels. This result suggests that the source of oral fluids may be from a different secretory compartment in pigs that are actively masticating or salivating during interaction with the rope, compared to resting pigs that are swabbed in the oral cavity. We also noted a substantial difference in the intensity of ELISA color reactions, indicating that the anti-PRRSV antibody concentrations were much higher in serum than in the oral fluids.

A third replicate was performed in which the pigs were rapidly exposed to a severe infection, marked by 20% mortality at day 22 and 40% mortality at day 28, when the study was terminated. Serum and and oral fluids were tested for anti-N IgG, IgA and IgM, and each plate was run with positive and negative controls. Antibody responses to PRRSV were evident in IgM and IgG subclasses in serum at day 14, indicating that

pigs were rapidly exposed and seroconverted (Fig. 4). No IgA was detected in serum. Antibodies to PRRSV N were not detected in oral fluids (Fig. 4). Analysis of individual responses over time confirmed that all animals seroconverted between days 7 and 14 (Fig. 5). High background levels of nonspecific reactivity were observed in serum samples analyzed for anti-PRRSV IgM (Figures 4 and 5). Thus, serum samples were retested at a dilution of 1/200 rather than 1/50. Absorbance values were reduced, but there was no difference in the results.

Discussion

We show here that naïve pigs housed with pigs that are acutely infected with PRRSV rapidly seroconvert to a PRRS-positive status, but could not be detected by individual sampling of oral fluids, while at the same time they were readily detected by testing of serum. By contrast, change in antibody status from PRRS-negative to PRRS-positive was readily detected in oral fluid samples collected on a pen basis by pigs interacting with an absorbent rope. We conclude that there is regional localization of antibody secreting B cells specific for PRRSV in lymphoid tissues of the oral cavity, including tonsils, salivary glands, and parotid gland. Under normal conditions when animals are not feeding or otherwise active in gustatory stimulation, the secretion of antibodies from these tissues appears to be minimal, whereas under conditions of interaction with a rope the secretory activity is stimulated. The differences observed here do not appear to be due to marked differences in sampling technique. Individaul animal sampling was performed by inserting a wick between cheek and gum, by moving it around, and by relocating it with swabbing motions inside the oral cavity. In addition, it is likely that normal musculoskeletal activity mixes the oral cavity contents so that regionally secreted antibodies would be mixed and redistributed throughout the oral cavity.

Acknowledgements. The work was performed with the generous help and assistance of Scott Dee and Satoshi Otake, University of Minnesota Swine Eradication Center, St. Paul MN, Suzanne Stone and Craig Johnson who performed the immunological assays, and by Jeff Zimmerman, Iowa State University, Ames IA, who generously shared advice, expertise and samples to compare pen-based group sampling to individual animal sampling.

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Lay Interpretation

Oral fluid sampling for health monitoring by assessment of antibody levels specific for pathogens is an exciting new concept in swine management. The work here shows that sampling of individual animals that are serologically positive for anti-PRRSV antibodies are not detected by analysis of oral fluids. Failure to detect anti-PRRSV antibodies is not a failure of the assay or lack of sensitivity, since the same method detects all forms of antibodies in oral fluids collected by pen-based group sampling with absorbent rope. We conclude that interaction with the rope stimulates secretion of antibodies that are not normally present in the oral cavity. The findings also suggest that the levels of antibody are low as compared to serum and may benefit from further optimization of the assay and a better understanding of the underlying biology.

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Figure 1. Anti-N IgG, IgM and IgA in oral fluids of pigs (n=20) exposed to PRRSV infection.

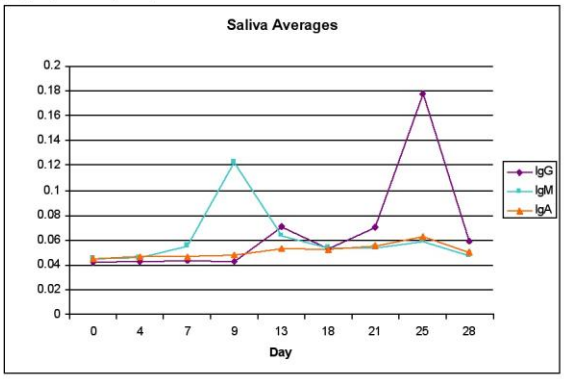


Figure 2. Anti-N IgG, IgM and IgA in serum of pigs (n=20) exposed to PRRSV infection.

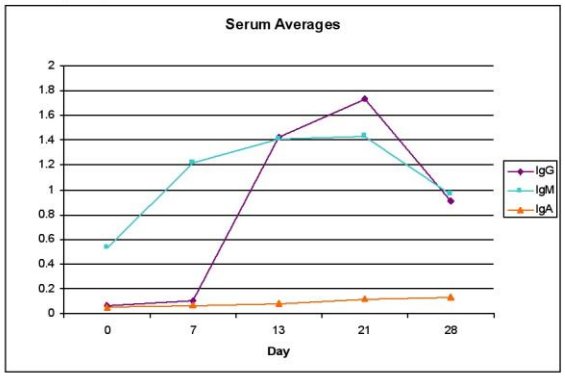
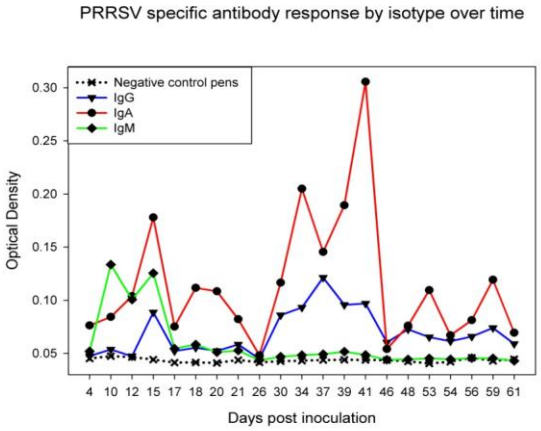
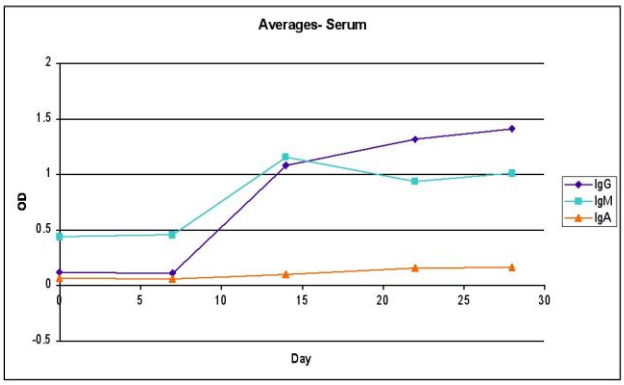


Figure 3. Anti-N IgG, IgM and IgA responses to PRRSV infection. Group samples were collected in a pen by interaction with a rope (Zimmerman and colleagues, Iowa State University).





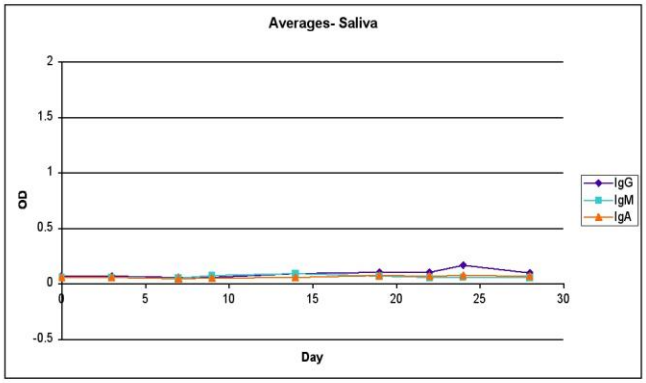


Figure 4. Anti-N IgG, IgM and IgA responses to PRRSV infection in replicate 3. Assays were performed the same as for replicate 2. Data are the means of 20 pigs through day 14, 16 pigs on days 19 and 22, 13 pigs on day 24, and 12 pigs on day 28.

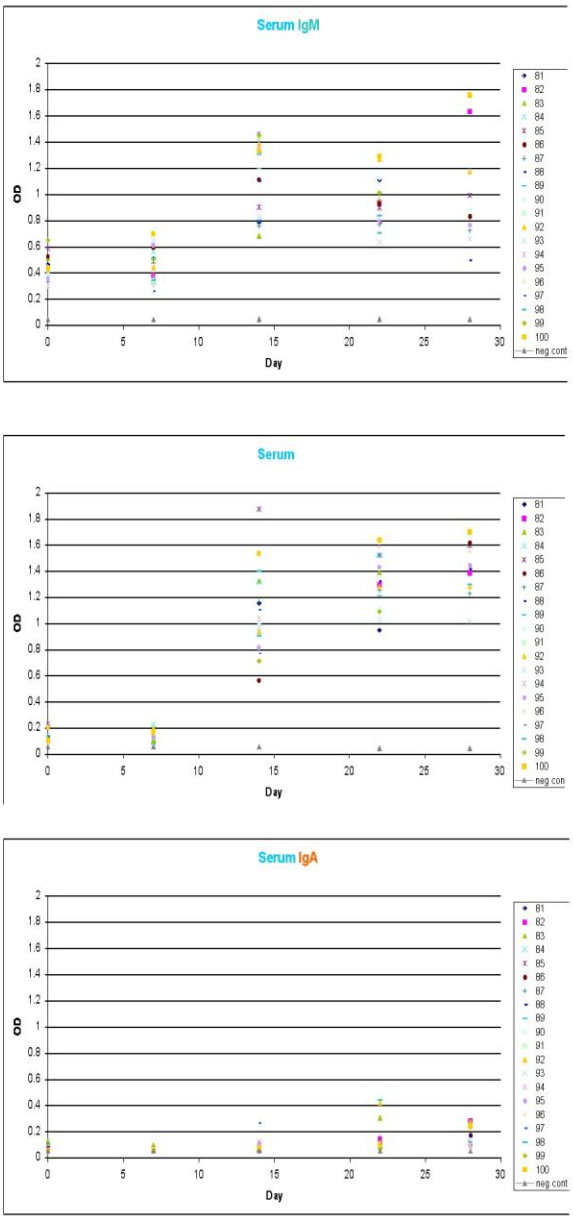


Figure 5. Individual pig antibody responses in serum to PRRSV N. Top panel: IgM, middle panel: IgG, bottom panel; IgA. Data are the individual values from which the means shown in Figure 4 were calculated.