RESEARCH REPORT
SWINE HEALTH

Title: "Comparison of early immune responses of pigs which are genetically PRRS resistant/tolerant using a swine-specific immune protein (cytokine) multiplex assay." – **NPB #09-244**

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Industry Summary:

PRRS causes major economic losses to the US pig industry, as reflected in debilitating respiratory syndromes, major reproductive losses and unthrifty piglets; economic losses were calculated to be $642 million per year in 2011. Worldwide, PRRS affects pigs at all stages of growth and is easily spread. The studies supported by this NPB grant established the best practices for using a new multiplex Fluorescent Microbead ImmunoAssay (FMIA) to quantitate levels of swine immune proteins (cytokines, chemokines). These proteins help to predict the intensity and speed of the immune response and thus indicate which pigs will resist, or be more susceptible to, PRRSV infection or be protected as a result of PRRS vaccination. The FMIA measures immune proteins involved in 1) early, innate immunity [interleukin-1β (IL-1β), IL-8, interferon-α (IFNα), and tumor necrosis factor (TNF)], 2) the late, adaptive anti-viral responses associated with T helper 1 (Th1) immunity (IL-12, IFNγ), 3) the alternative Th2 immunity (IL-4), 4) regulatory immunity (IL-10) and cell migration (CCL2). The interplay between levels and timing of expression of these immune proteins helps to predict overall immunity.

Our goal was to refine and improve our FMIA test developed with NPB grant #08-189 (Lawson et al. 2010). The test refinements developed through this grant provide for uniform, simultaneous identification and quantification of 8 important immune cytokines and 1 chemokine within a single serum or oral fluid sample. By careful studies the limit of assay detection was established and the minimum dilutions of serum and oral fluids determined. The refined cytokine multiplex test was then used to evaluate cytokine expression in oral fluid samples collected during NPB supported (#10-056) PRRS Host Genetics Consortium (PHGC). Analyses of the cytokine and chemokine data affirmed that PRRSV infection during PHGC trial 6 clearly stimulated innate cytokine (IL-1β, IL-8) expression but not IFNα and IL-12 expression. This appeared to stimulate only a low and slow production of protective IFNγ, and thus likely allowed high viral replication. This oral fluid data will be compared to data that is now being collected on individual pig sera from PHGC trials. That data will enable us to identify the most vigorously responding pigs and identify exactly which cytokines are the best predictors of pigs which will resist PRRSV infection better (have lower viral loads) in a herd.

In addition to our PHGC efforts these FMIAs will provide critical, rapid information on pig immune responses and thus help identify what viral proteins or vaccine constructs stimulate a timely and robust immune response to PRRSV in vitro or in vivo. In fact the refined FMIA will be useful for evaluation of immune responses to various swine infections and serve as an important new tool for comparison of alternate vaccination approaches. Once known, these results should help identify which cytokines to target as positive and negative indicators of efficacy of PRRSV vaccines. Indeed, the importance of this effort has been recognized by the commercial animal health industry. Three different companies are actively involved in establishing FMIAs for swine immune proteins (with advice from this team), thus opening up opportunities to explore swine disease, immune and vaccine responses in more depth in the future.

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Scientific Abstract:

This NPB grant #09-244 has enabled our team to improve the Fluorescent Microbead ImmunoAssay (FMIA), the SDSU BARC FMIA, that was developed with NPB grant #08-189 (Lawson et al. 2010). Overall the use of the FMIA has enabled us to detect immune changes related to PRRSV infection using multiplex assays. This allows for uniform testing of multiple immune proteins (cytokines, chemokines) simultaneously within a small volume of sample and with a broader dynamic range for detection. Compared to past tests using multiple ELISA assays, the multiplex assay is less labor intensive, requires less sample and fewer replicates for each sample, and produces data in a shorter time due to high throughput analytic systems. Our tests have affirmed that cytokine protein levels in culture supernatants of cells, e.g., from PRRSV infected versus vaccinated pigs, can be measured very well with this assay. Thus the FMIA can be used to compare which cytokines in blood, and what tissue cells secrete, in response to different viral and peptide stimulants for vaccination and infection trials. Thus this grant has provided a means of getting much deeper phenotypic data by testing not just 2 but 10 different important cytokines in diverse samples (serum, oral fluids, culture supernatants).

The studies supported by this NPB grant established the best practices for using this new multiplex FMIA to quantitate levels of swine immune proteins (cytokines, chemokines). Immune proteins help to predict the intensity and speed of the immune response and thus indicate which pigs will resist, or be more susceptible to, PRRSV infection or be protected as a result of PRRS vaccination. The FMIA measures immune proteins involved in 1) the early, innate immunity [interleukin-1β (IL-1β), IL-8, interferon-α (IFNα), and tumor necrosis factor (TNF)], 2) the late, adaptive anti-viral responses associated with T helper 1 (Th1) immunity (IL-12, IFNγ), 3) the alternative Th2 immunity (IL-4), 4) regulatory immunity (IL-10) and cell migration (CCL2). The interplay between levels and timing of expression of these immune protein helps to predict overall immunity.

We used this FMIA to assess pig immune responses to PRRSV infection. Our results show that the SDSU BARC FMIA, supplemented with the recently developed commercial Procarta FMIA, enabled us to measure levels of important cytokines and chemokines, in serum and oral fluids (OF) from PRRSV infected pigs using samples collected through the NPB supported (#10-056) PRRS Host Genetics Consortium (PHGC). The PHGC6 OF samples revealed a vigorous innate immune response to PRRSV infection, as evidenced by the high levels IL-1 β and IL-8 cytokines; unfortunately the levels of IL-12 and IFNα remained low. The stimulatory effects needed from IL-12 and IFNα cytokines was not sufficient to prevent high viral loads in these pigs (10^6 – 10^7 viral equivalents/ml serum). Nor could it stimulate a better adaptive immune response to infection, thus, the low levels of OF IFNγ. Thus all pigs are impacted by PRRSV infection. Our detailed PHGC serum analyses are now underway at BARC. They should enable us to compare the exact responses of each PHGC pig using the individually collected sera over 10 timepoints. Statistical analysis of those results will reveal whether there are different cytokine responses in PRRS susceptible versus resistant pigs. They will help determine how viral load influences growth, by comparing responses of sera from pigs with high versus low viral load and growth. It is expected that these studies will highlight the role of cytokine proteins and immune response pathways contributing to different PRRS infection outcomes. Final data will be submitted to the secure, password protected PHGC database http://www.animalgenome.org/lunney/index.php maintained at ISU through NPB grant #10-056. Data is being collected on every PHGC pig and serves as a national resource for further PRRS studies.

As a result of our success with the SDSU BARC FMIA cooperative agreements were established with immune bioassay companies. The technology has been transferred to InVitrogen by SDSU, and samples sent from BARC for their test validation. Beta testing began this winter with SDSU and BARC; the InVitrogen FMIA is expected to be publically available in summer 2012. Our results have also been discussed with Affymetrix/Procarta and BioRad. FMIA test validation samples were sent to both companies from BARC. Interactions with Procarta have resulted in new commercial tests that are already available and expansion of their planned tests. The BioRad tests are expected within a year. Thus the NPB investment has been amplified for swine researchers worldwide who will have validated FMIAs available for their overall disease, vaccine and development work. The current tests provide researchers with tools needed to address pig health and welfare issues. Future availability of a wider range of commercial tests will further expand research options.

Introduction:

PRRS causes major economic losses to the US pig industry, as reflected in debilitating respiratory syndromes, major reproductive losses and unthrifty piglets; economic losses were calculated to be $642 million per year in 2011 (Holtkamp et al., 2012). The NPB first priority for Swine Health is control of PRRS, with one emphasis of “PRRSV Immunology” to “Evaluate and characterize the genetic properties of natural PRRS resistance/tolerance to infection.” The goal of this NPB supported research was to develop improved assays for comparing immune responses to PRRSV infections. To achieve this goal we developed methods to test easily acquired samples, serum and oral fluids, using multiplex assays for immune proteins.

Previous work revealed differences among and within breeds in response to infection with PRRSV but to date no pigs which are completely PRRSV resistant have been identified (Lewis et al., 2007; Lunney and Chen, 2010). This is the basis for

the NPB supported PRRS Host Genetics Consortium (PHGC). The PHGC is co-led by Drs. RRR Rowland at Kansas State Univ. (KSU) and JK Lunney at USDA ARS BARC (BARC) and funded by NPB grants #07-233, #09-208 and #10-056 grants. The PHGC represents the first-of-its-kind approach to food animal infectious disease research. NPB funds for the PHGC support the infection of thousands of pigs, the collection, cataloging, distribution of tens of thousands of samples for analysis by PHGC participants. Blood samples were collected at -6, 0, 4, 7, 10, 14, 21, 28, 35 and 42 days post infection (dpi) for serum and RNA analyses, aliquoted and stored at KSU and BARC. For growth weekly weights were recorded. The NPB grants supported basic phenotyping (infections, sampling, weights, viral and antibody levels at Kansas State Univ; DNA preparations and preliminary analyses of serum samples for 2 immune proteins, interleukin-8 (IL-8), interferon-gamma (IFNγ), at BARC. All data is being stored in the PHGC relational database http://www.animalgenome.org/lunney/index.php led by Dr. J Reecy at Iowa State Univ., ISU). The NPB PHGC investment has been amplified by numerous matches, i.e., pigs from breeding companies, molecular and antibody test resources from animal health companies, and USDA PRRS CAP and Genome Alberta grant funds for genome wide association studies (GWAS) and NIFA funds for functional genomics. The PHGC results revealed the appearance of stratified subpopulations of PRRS resistant/ susceptible animals, exhibiting wide variations in virus load and growth performance (Rowland et al., manuscript in preparation). Most recently, using PHGC data, a genetic marker has been discovered on swine chromosome 4 (SSC4) for reduced susceptibility to PRRS (Boddicker et al., 2011). Importantly this marker is also associated with improved growth of pigs that are infected with PRRSV.

The use of oral fluid to analyze the presence of PRRSV in experimentally infected pigs was first shown by Wills et al., (1997). Methods for oral fluid (OF) collection have recently been improved; OF are now used to determine PRRSV and porcine circovirus (PCV2) levels as detected by qRT-PCR and antibody levels (Chittick et al., 2011; Prickett et al., 2008a,b; Prickett and Zimmerman, 2010; Kittawornrat et al., 2010; 2012). These results indicate that pen-based OF sampling could be an efficient, cost effective approach to PRRSV surveillance in swine populations. Kittawornrat et al. (2010) showed although PRRSV was detected by qRT-PCR in both serum and oral fluid through 21 days post infection (dpi) from individual boars, a comparison of matched samples from individual boars showed that OF was equal to serum for the detection of PRRSV at 7 dpi and more likely than serum to be positive on 14 and 21 dpi.

The Fluorescent Microbead ImmunoAssay (FMIA) was developed at SDSU and BARC with NPB funds [#08-189] and published (Lawson et al. 2010). This SDSU BARC FMIA measures 8 important serum immune proteins, or cytokines. Cytokines are small secreted proteins which mediate and regulate anti-PRRSV humoral and cell-mediated immunity. Unfortunately, there is evidence that PRRSV does not usually induce early, effective cytokine responses thus leading to clinical disease and viral persistence (Darwich et al., 2010; Lunney et al., 2010; Mateu and Diaz , 2008; Murtaugh and Genzow, 2011). Indeed, the immune response to PRRSV occurs later in infection and is weak in comparison to other swine viruses (Bautista and Molitor, 1997; Costers et al., 2009; Nauwynck et al. 2012; Renukaradhya et al., 2010). Cytokine levels have been implicated as key to this poor response, e.g., PRRSV does not stimulate innate interferon-α (IFNα) production yet does stimulate the negative regulator interleukin-10 (IL-10) (Albina et al., 1998; Sang et al. 2011; Subramaniam et al., 2011; Suradhat et al.,2003; Thanawongnuwech et al. 2003). Genetic studies indicate that preinfection serum IL-8 and post infection IFNγ levels are associated with resistance (Petry et al., 2007). Moreover, besides describing PRRSV pathogenesis and how PRRSV activates or deactivates the immune system, the measurement of these cytokines can be useful for genetic resistance screens. In fact, the new cytokine multiplex assay can help determine which viral proteins might be useful, or should be eliminated, from 2nd generation subunit or recombinant vaccines.

The design of the SDSU BARC FMIA involved cytokines active in 1) the earliest, innate immunity [IL-1β, IL-8, IFNα, and tumor necrosis factor (TNF)], 2) the later, adaptive anti-viral responses [T helper 1 (Th1) immunity, IL-12, IFNγ], 3) alternative Th2 immunity (IL-4), 4) regulatory immunity (IL-10) and cell migration (CCL2). Our 2008-9 NPB efforts [NPB grant #08-189] resulted in development of a sensitive 8-plex swine cytokine assay (IL-1β, IL-4, IL-8, IL-12, IFNα, IFNγ, IL-10, TNF), all based on commercially available monoclonal antibody (mAb) reagents. The advantage of mAbs is that they represent a reproducible source of reagents for other labs to prepare their own assays. Unfortunately, certain mAb are no longer available commercially, e.g., an anti- IL-12 and anti-IFNγ mAb, which is why, as we have performed the experiments noted in this grant, that we have had to use recently developed commercial FMIAs to supplement the SDSU BARC FMIA.

Current protocols to detect immune changes related to PRRSV infection involve single ELISA tests for each cytokine measurement; multiplex assays allow for uniform testing of multiple cytokines simultaneously within a small volume of a single-sample and with a broader dynamic range. Compared to multiple ELISA assays, the multiplex assay is less labor intensive, requires less sample and fewer replicates for each sample, and produces data in a shorter time using the BioPlex high throughput system. Thus this grant provided yet another means of getting much deeper phenotypic data by testing not just 2 but 10 different important cytokines in samples collected from PHGC pigs. Data is being collected on every PHGC pig and OF on every pen of PHGC pigs and serves as a national resource for further PRRS studies, such as the anti-PRRSV immune cytokine work reported here. This grant developed protocols for use of the 8-plex cytokine assay to determine timing and levels of OF cytokine responses to PRRSV infection, setting the stage for more detailed analyses.

Objectives:

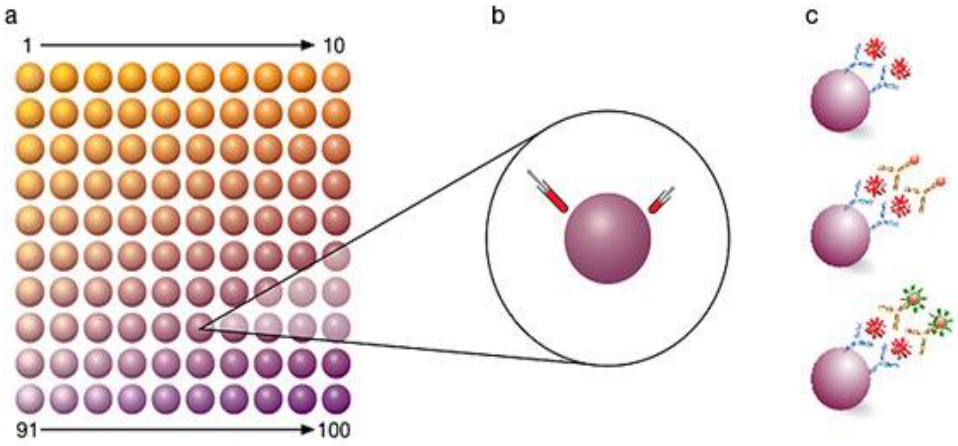
Obj. 1) Use the BioRad/Bioplex/Luminex microsphere, or "multiplex" cytokine assay to quantitate anti-PRRSV responses of PRRS Host Genetics Consortium (PHGC) pigs to determine whether serum cytokine levels predict differences in how genetically PRRS resistant pigs respond to infection as compared to PRRS susceptible or PRRS tolerant pigs.

Obj. 2) Continue optimization of the cytokine multiplex assay for detection and quantification of 8 cytokines simultaneously in both serum and oral fluids.

Materials & Methods:

To monitor PRRS disease and vaccine immune responses sensitive assays for immune proteins, including cytokines and chemokines, are required. For many species recent work has been targeted at multiplex assays developed using the Luminex technology (Luminex Corporation, Austin, TX); 8-50-plex cytokine assays are now routine for mouse, rat or human species (websites: http://www.luminexcorp.com/Applications/index.htm; http://www.panomics.com/index.php?id=product_96; www.bio-rad.com/). The general principles for these Fluorescent Microbead ImmunoAssays (FMIAs) are shown in the diagram in Fig.1 below. Use of different colored beads sets up a bead "address" for each analyte (cytokine or chemokine); specific monoclonal antibody (mAb) reagents then are used to link that address with a given cytokine using a sandwich assay approach. Our FMIA used the Bio-Plex suspension array system; labeled beads are drawn up into the flow-based Bio-Plex array reader, which identifies each specific reaction based on bead color and provides statistical algorithms to quantitate them.

Figure 1. Fluorescent Microbead ImmunoAssay (FMIA) Technology



http://www.bio-rad.com/evportal/en/US/LSR/Solutions/LUSM0E8UU/Multiplex_Immunoassays

Beads are colored internally with two different fluorescent dyes (red and infrared) (a,b) Ten different concentrations of red and infrared dyes are used to generate 100 distinct bead regions. (c) Each bead region is conjugated to a specific target analyte [blue = mAb anti-swine cytokine], which interacts with standard protein (red) or test sample, followed by binding with a biotinylated detection antibody and a reporter dye streptavidin-conjugated phycoerythrin (SAPE, green).

The results of the FMIA are determined by the intensity of the final fluorescent reporter dye SAPE signal, or mean fluorescent intensity (MFI) for each bead (and thus for each cytokine). The BioPlex software interprets results from the standard curve run for each cytokine to designate the concentration of the tested sample for each cytokine (Fig.2). We used the FMIA for swine cytokines (IFNα, IL-4, IL-8, IL-10, IL-12), developed at SDSU and BARC with NPB funds [#08-189] and published (Lawson et al. 2010). We refer to this test as the "SDSU BARC FMIA."

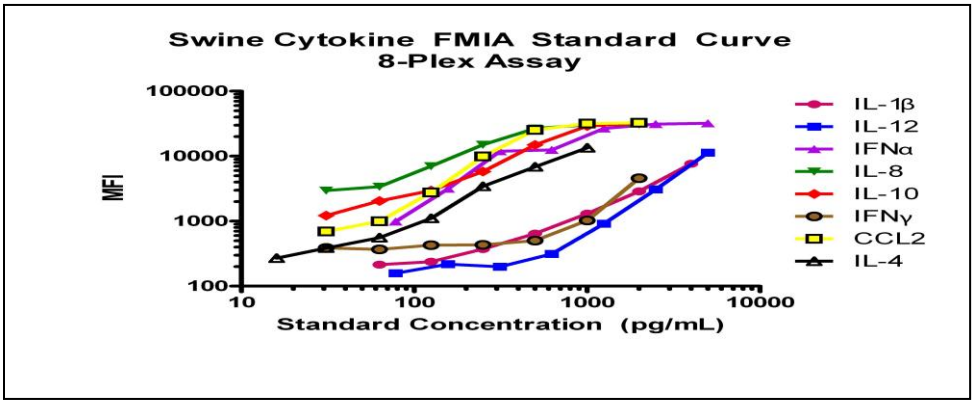


Figure 2. Standard curve for the Fluorescent Multiplex ImmunoAssay (FMIA) for detection of swine cytokines. The FMIA was performed as published by Lawson et al. (2010) and updated as described in this grant report.

Results:

Obj. 1) Use the BioRad/Bioplex/Luminex microsphere, or "multiplex" cytokine assay to quantitate anti-PRRSV responses of PRRS Host Genetics Consortium (PHGC) pigs to determine whether serum cytokine levels predict differences in how genetically PRRS resistant pigs respond to infection as compared to PRRS susceptible or PRRS tolerant pigs.

Obj. 1 a) Perform multivariate analyses on data on pigs from each PHGC trial to assign them into virus/weight categories defining PRRS resistant versus susceptible or tolerant pigs.

The PHGC results revealed the appearance of stratified subpopulations of PRRS resistant/ susceptible animals, exhibiting wide variations in virus load and growth performance (Rowland et al., manuscript in preparation). Based on data from PHGC1-4 a statistical multivariate analysis protocol was designed by Dr. J. P. Steibel, MSU, to identify PRRS resistant/susceptible pigs, as noted in Figure 3 below. For PRRS resistance/susceptibility phenotypic traits, total virus load (VL) from 0-21 dpi was calculated by measuring the area under the curve [viral QTY = log10(V+1) based on ABI RT-PCR for serum viral levels]; weight gain (WG) was computed in total kg gained from 0-42 dpi. To separate four groups of pigs, VL and WG data were standardized and rotated by pre-multiplying them by the inverse of the Choleski decomposition of their variance-covariance matrix. Then extreme data (more than 1 SD above and below the mean of the transformed data) were selected into four quadrants of the bi-variate distribution of AUC and Weight. As illustrated in Figure 3, these statistical analyses categorized pigs into 4 extreme categories including the most desirable, PRRS resistant low virus/high weight gain (Lv/Hg) pigs, the worst, PRRS susceptible high virus/ low weight gain (Hv/Lg) pigs, the PRRS tolerant, high virus/high weight gain (Hv/Hg) pigs, and the less thrifty, low virus/low weight gain (Lv/Lg) pigs.

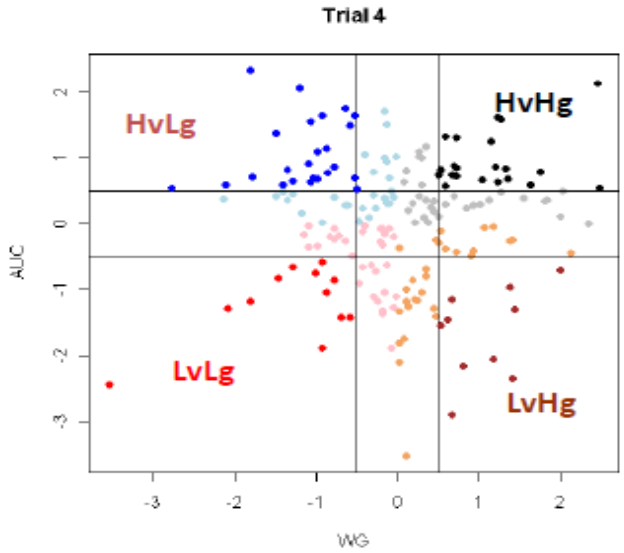


Figure 3. Categorization of PHGC into phenotypic groups. Average daily weight gain in kg (WG) (x-axis) and virus load (serum PRRSV level measured using the AgPath-IDTM commercial PRRS RT-PCR assay) area under the curve (y-axis) were plotted for PHGC4. Key:
high virus/low weight gain (**Hv/Lg**),
high virus/high weight gain (**Hv/Hg**),
low virus/high weight gain (**Lv/Hg**),
low virus/low weight gain (**Lv/Lg**).

This statistical categorization of pigs from each PHGC trial provides a critical basis for selecting pigs and samples for detailed analyses of processes that control proteomic responses to PRRSV infection challenge as proposed in this grant.

Obj. 1b) Use the cytokine multiplex assay to quantitate cytokines important for determining PRRSV resistance/tolerance, i.e., the innate (IL-1a, IL-8, IFNa, TNF); regulatory (IL-10), Th1 (IL-12, IFNa) and Th2 (IL-4) cytokines.

The swine cytokine FMIA has been used to quantitate cytokines for effects on PRRS disease responses. The first studies of these responses were performed on sets of sera from PHGC1 pigs with samples selected from each of the 4 anti-PRRS response groups based on the Steibel clustering (Obj.1a). The early timepoints after PRRSV infection, at 0, 4, 7, 10, 14, and 21 dpi, were targeted. Serum samples from 6 pigs in each of the 4 PRRSV response groups (144 sera total) were arrayed for FMIA analysis. Data on PHGC1 samples have been collected; results show distinct patterns of responses for the sera from different PRRSV response sets (data not shown). However, as these data were being collected it became apparent that there were non-specific inhibitory effects of undiluted serum on the cytokine standard curve (Fig.4).

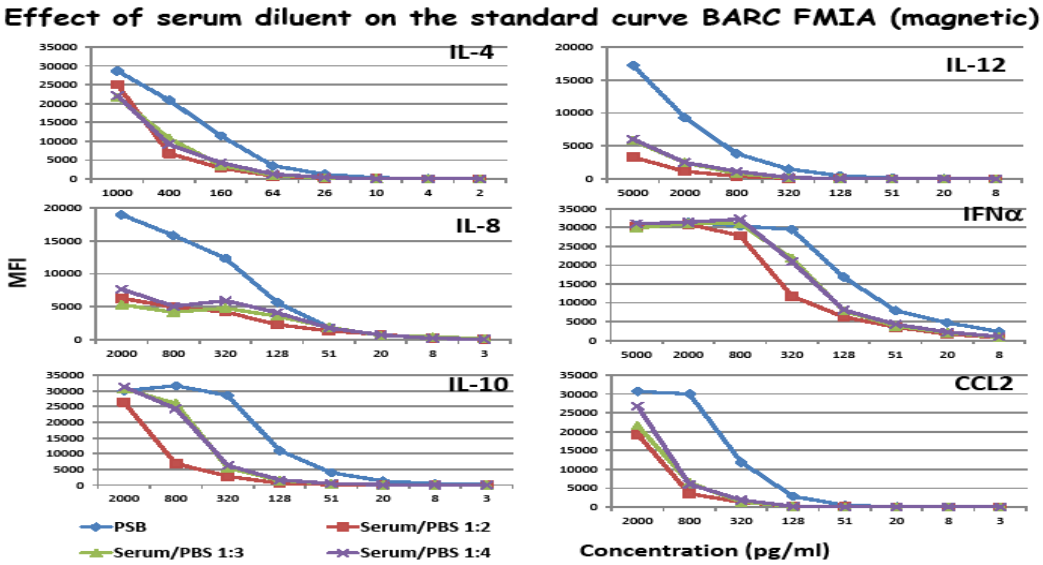


Figure 4. Comparison of effects of normal serum on SDSU BARC FMIA results. The FMIA was performed using cytokines diluted in Procarta serum diluents (PSD) (**blue**), per advice from Affymetrix personnel, or in normal pig serum diluted 1:2 in phosphate buffered saline (Serum/ PBS 1:2) (**red**), 1:3 (**green**), or 1:4 (**purple**). Further tests of 1:4 and 1:5 dilutions showed no increase in FMIA sensitivity (Souza et al., unpublished data).

The data in Figure 4 above clearly show that normal pig serum causes a substantial decrease in mean fluorescent intensity (MFI) for each cytokine. Without serum (blue graph) most assays were sensitive to ~100pg/ml cytokine. For IFNα this decreases by ~50% whereas for IL-4, IL-10 and CCL2 it decreased to ~20 with 1:3 or 1:4 normal serum diluents, and was worse with 1:2 diluted serum samples. For IL-8 and IL-12 the serum inhibition substantially decreased the signal/noise ration. As a result of these non-specific inhibitory effects of undiluted serum on the cytokine standard curve all FMIA tests of PHGC sera were reevaluated. A new higher intensity Streptavidin Phycoerythrin (SAPE) is being used. All PHGC serum samples are now being diluted 1:3 in PBS and assay results compared to the cytokine standard diluted 1:3 in normal pig serum with PBS (Souza et al., unpublished data). These tests are now underway at BARC. Separate tests have affirmed that culture supernatants of cells from PRRSV infected pigs can be measured very well with this assay with no problems of inhibitory effects. Thus this assay can be used to compare which cytokines blood and tissue cells secrete in response to different viral and peptide stimulants as will be planned for vaccination and infection trials.

Obj. 1c) Evaluate statistical value of each cytokine in predicting PRRS response phenotype.

Statistical analysis of these results will be performed once the data on PHGC samples is collected. Based on the analyses the exact timepoints for future tests of sera from other trials will be determined. In addition we are evaluating whether samples from all 4 anti-PRRS response groups, or only from selected sets of pigs, e.g., from only HvHg versus LvHg pigs, should be tested. Additional details such as whether to use individual samples or pools of animals would be determined based on the results obtained from the current FMIA experiments.

It is expected that these studies will highlight the role of cytokine proteins and immune response pathways contributing to different PRRS infection outcomes. Final data will be submitted to the secure, password protected PHGC database maintained at ISU: http://www.animalgenome.org/lunney/index.php. Comparisons of this protein expression data with gene expression data may reveal important genes or pathways that are utilized in PRRS resistant as compared to PRRS susceptible pigs. This data will be used to affirm genotyping efforts underway as part of the overall PHGC program.

Obj. 2) Continue optimization of the cytokine multiplex assay for detection and quantification of 8 cytokines simultaneously in both serum and oral fluids.
Obj. 2a) Finalize optimization of cytokine multiplex assay for 8 cytokines in serum.

With NPB #08-189 grant funding SDSU and BARC completed the development of the FMIA for 8 cytokines (IL-1β, IL-4, IL-8, IL-10, IL-12, IFNα, IFNγ, TNF) (Lawson et al., 2010). This SDSU BARC FMIA was supplemented by a new FMIA for swine chemokine CCL2. This was developed as part of the US Veterinary Immune Reagent Network (VIRN) (www.vetimm.org) with funds from a separate USDA NIFA grant (#2006-35204-16880). Dr. Lunney is the Swine Species Chair for US VIRN and is tasked with developing new immunological reagents to advance veterinary immunology and disease control. Developemnt of the CCL2 FMIA involved several labs in the VIRN team. First swine CCL2 was cloned by our VIRN collaborators at Univ. Massachusetts Amherst and expressed in yeast at Kingfisher Biotech http://www.kingfisherbiotech.com/. This protein was proven to be bioactive at BARC and then used to immunize mice and prepare a panel of anti-CCL2 mAbs at Cornell Univ. Two of these newly developed mAb were selected for the final CCL2 FMIA (clone 5-2 for bead coupling and clone 18-1 for detection). The new CCL2 FMIA was shown to detect both recombinant and native porcine CCL2 (Lunney et al. manuscript in preparation). It is expected that more FMIA tests will be

developed by VIRN. Swine IL-6 and IL-17A have been cloned, expressed and shown to be bioactive. They are now targets for mAb production and if mAb are developed new FMIA will be developed.

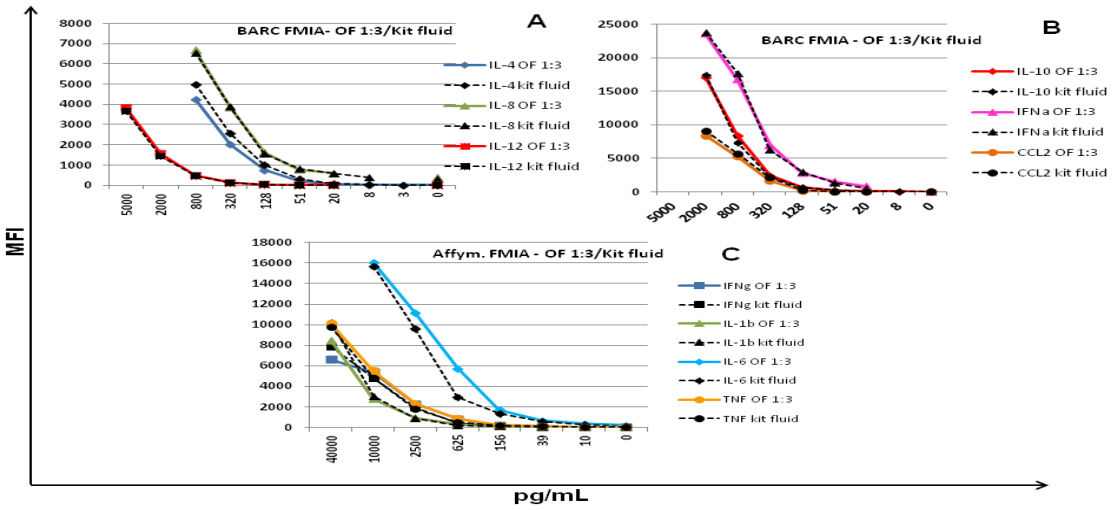


Figure 1

Figure 5. Comparison of FMIA standard curves for oral fluid (OF) samples. Full standard curves for each cytokine and chemokine were obtained in SDSU BARC FMIA (A, B) and Procarta FMIA (C) using pooled pre infection OF (1:3 PBS-BN) and compared to the normal kit diluents (Araujo et al. manuscript in preparation).

The expanded SDSU BARC FMIA has been used for many of our cytokine/chemokine analyses. We modified some protocols developed by Lawson et al. (2010). For biotinylating mAb we used the LinkTM Chromophoric Biotin labeling Kit (Kirkegaard & Perry Laboratories, Inc. KPL®) so that coupling efficiency could be quantitated. Since magnetic beads became available in 2011 all FMIAs were switched to use magnetic carboxylated microspheres (Bio-Plex Pro™ Magnetic COOH Beads) to take advantage of the Bio Plex®200 instrument with associated wash station and software. This resulted in lower backgrounds for many of the individual FMIAs. Additionally new Streptavidin Phycoerythrin (SAPE) conjugates have been utilized to enhance the MFI signal.

In the last year commercial swine cytokine assays have become commercially available. The first of these was a Procarta/Affymetrix® immunoassay (http://www.panomics.com/index.php?id=product_35), which we refer to as the "Procarta FMIA." We used a 4 plex Procarta FMIA to measure IL-1β, IL-6, IFNγ and TNF cytokines for our OF data. Our approach was to use the BARC SDSU FMIA for most assays unless a problem occurred. For IL-6, IFNγ and TNF that was the lack of availability of mAbs to prepare our BARC SDSU FMIA; for IL-1β it was the improved signal/noise provided by the Procarta FMIA.

Unlike the problem with serum samples oral fluids when tested at 1:3 dilutions (dashed versus solid lines) did not substantially alter the FMIA standard curves (Fig.5 A,B,C) (Araujo et al. manuscript in preparation). Thus we were able to proceed with testing levels of cytokines and chemokines in OF collected from PRRSV infected or vaccinated pigs (Obj.2b below).

Obj. 2b) Develop protocols for determining cytokine levels in oral fluid samples with the cytokine multiplex assay. Use oral fluid samples collected during PRRSV infection of boars to compare timing and level of expression of serum and oral fluid cytokines.

In her NPB#09-234 project "Development of diagnostic assays for detecting PRRSV infection using oral fluid samples as an alternative to serum-based assays" final report Dr. Y. Fang noted that a total of 534 serum and 1110 oral fluid samples were sent to SDSU from Dr. Zimmerman's laboratory in January, 2010. They were processed and distributed to UMN (Murtaugh) and BARC (Lunney) in March 2010. As part of this NPB grant #09-244 qRT-PCR assays were performed to quantitate PRRSV nucleic acid presence in every one of these OF and serum samples. The PRRSV quantitation was completed at the ISU Diagnostic Lab in 2010.

Scientists at SDSU developed standard diagnostic assay protocols for testing OFs using FMIAs (NPB#09-234 project final report; Langenhorst et al., 2012). These standardized assay conditions were then applied for handling oral fluids for FMIA assays for cytokine levels at BARC. This included incubating beads with the OF samples overnight before completing the assay. As noted in Obj.1a above the OF samples could be tested at 1:3 with no inhibition. For their antibody tests Langenhorst et al. typically used much higher OF dilutions because of the sensitivity of their assays and the levels of antibodies. Unfortunately cytokines are expressed at pg/ml levels and so more concentrated samples had to be used.

For our cytokine analyses we tested several sets of OF samples. Tests are still underway for some of them. Figure 6 shows results of analyses of cytokine levels in OFs collected during PHGC trial 6. Pigs from PHGC trial 6 were acclimated to their pens after shipping at weaning to Kansas Stare Univ. Collection of OFs started prior to their being infected at ~22 days age with PRRSV isolate NVSL 97-7985 (Osorio et al., 2002). Oral fluids from individual pens (10-15 pigs/pen) were collected at -1, 8, 15, 20, 28, 35, 40 dpi and samples analyzed by FMIAs. The levels of IL-1β, IL-4, IL-6, IL-8, IL-10, IL-12, CCL2, IFNα, IFN γ and TNF were measured using the SDSU BARC and Procarta FMIAs with OF samples diluted 1:3.

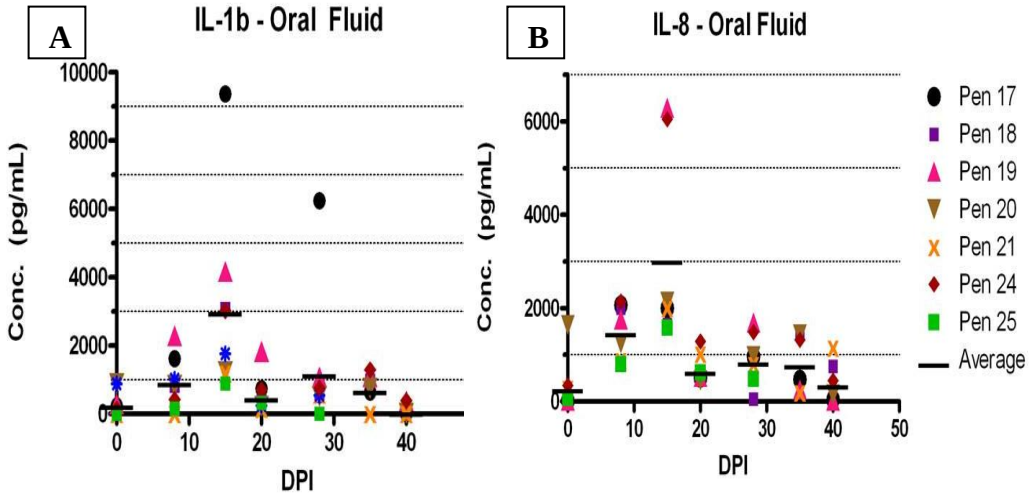


Figure 6. Differences in OF cytokine levels following PRRSV infection. Increased expression of (A) IL-1β and (B) IL-8 were found at 7 and 14 dpi in OFs collected from individual pens of PRRSV infected pigs during PHGC trial 6.

Figure 6 shows that major differences in IL-1 β and IL-8 cytokine levels are clearly apparent following PRRSV infection with maximal levels at 15 dpi ($p<0.05$) for most pens with a 2nd lower peak in some pens a 28 dpi. OFs collected from some days showed substantial variation between pens in IL-1 β and IL-8 levels. Other cytokines were secreted at 10-100 fold lower levels in PHGC6 OFs (data not shown). After PRRSV infection the average OF levels of IL-1β were 381 pg/mL at 1 dpi, increased to 751 pg/mL at 8 dpi ($p<0.05$ significant increase compared to pre infection levels) reaching a peak of 2190 pg/mL at 15 dpi ($p<0.05$), declined at 20 dpi. There was a 2nd IL-1β peak (1062 pg/mL at 28 dpi) that was followed by decreases back to pre infection expression levels (Fig.5A). For IL-8 the average post infection IL-8 levels showed a similar pattern, starting at 350 pg/mL on 1 dpi, increasing to 1640 at DPI 8 ($p<0.01$), peaking at 2959 pg/mL at 15 dpi ($p<0.01$), with a 2nd peak (1262 pg/mL) at 28 dpi and decreasing thereafter. The OF levels of other cytokines were substantially lower. For the chemokine CCL2 and cytokine IL-6 OF levels were 41 and 20 pg/mL at 1 dpi, respectively. After PRRSV infection levels of IL-6 and CCL2 in PHGC6 OF samples were significantly increased, reaching a peak at 15 dpi (227 and 70 pg/mL at 15 dpi, respectively) ($p > 0.05$); TNF (121 pg/mL at 1 dpi) increased after infection with highest level at 15 dpi (242 pg/mL $p > 0.05$) but, unlike other cytokines, stayed elevated until experiment termination. Levels of IFNγ (17 pg/mL at 1 dpi) peaked later to ~40pg/mL at 28 and 35 dpi ($p<0.05$) and decreasing at 42 dpi. The levels of IL-4, IL-10, IL-12 and IFNα remained low (below detectable levels) throughout the 42 day trial.

Overall the OF cytokine levels follow the viremia found in PHGC6 pigs. Serum viremia was apparent at 4 dpi and increased to highest levels a 7, 11 and 14 dpi depending on the individual pig (Rowland et al., unpublished). All PHGC6 pigs that survived were clearing the PRRSV infection by 21 dpi. Thus the inflammatory cytokines IL-1β and IL-8 were highest in OFs at peak viremia and decreased as serum viremia resolved. This was similar for the chemokine CCL2 and cytokine IL-6, albeit at substantially lower levels whereas the other inflammatory cytokine, TNF, remained high well past peak viremia. Levels of OF IFNγ were low and increased only moderately as the serum viremia was being resolved.

Discussion:

This NPB grant #09-244 has enabled our team to apply the Fluorescent Microbead ImmunoAssay (FMIA), that was developed with NPB grant #08-189 (Lawson et al. 2010), to assess pig immune responses to PRRSV infection. From the results shown above the SDSU BARC FMIA, supplemented with the recently developed commercial Procarta FMIA, enabled us to measure levels of important immune proteins, cytokines and chemokines, in serum and OF from PRRSV infected pigs. As shown in Figure 6 it is clear that there was a vigorous innate immune response of pigs from PHGC trial 6 to PRRSV infection, as evidenced by the high levels IL-1 β and IL-8 cytokines in OFs; unfortunately the levels of IL-12 and IFNα remained low. The stimulatory effects needed for IL-12 and IFNα cytokines was not sufficient to prevent high viral loads in these pigs ($10^6 – 10^7$ viral equivalents/ml serum). Nor could it stimulate a better adaptive immune response to infection, thus, the low levels of OF IFNγ. Separate tests have affirmed that cytokine protein levels in culture supernatants of cells from PRRSV infected and vaccinated pigs can be measured very well with this assay with no problems of inhibitory effects. Thus this assay can be used to compare which cytokines blood and tissue cells secrete in response to different viral and peptide stimulants as will be planned for vaccination and infection trials.

Our PHGC serum analyses, which are now underway at BARC, will enable us to compare the exact responses of each PHGC pig using the individually collected sera over 10 timepoints. Statistical analysis of those results will reveal whether there are different cytokine responses in PRRS susceptible versus resistant pigs, by comparing Hv to Lv pigs. They will help determine how viral load influences growth, by comparing responses of sera from LvHg to LvLg pigs and HvHg to HvLg pigs. It is expected that these studies will highlight the role of cytokine proteins and immune response pathways contributing to different PRRS infection outcomes. Final data will be submitted to the secure, password protected PHGC database

http://www.animalgenome.org/lunney/index.php maintained at ISU through NPB grant #10-056. Comparisons of protein expression data with USDA grant funded gene expression data may reveal important genes or pathways that are utilized in PRRS resistant as compared to PRRS susceptible pigs. This data will be used to affirm and extend the genotyping efforts underway as part of the USDA PRRS CAP funded PHGC program.

Overall the use of the FMIA has enabled us to detect immune changes related to PRRSV infection using multiplex assays. This allows for uniform testing of multiple cytokines simultaneously within a small volume of a single-sample and with a broader dynamic range. Compared to multiple ELISA assays, the multiplex assay is less labor intensive, requires less sample and fewer replicates for each sample, and produces data in a shorter time using the BioPlex high throughput system. Thus this grant has provided a means of getting much deeper phenotypic data by testing not just 2 but 10 different important cytokines in samples collected from PHGC pigs. Data is being collected on every PHGC pig and serves as a national resource for further PRRS studies.

As a result of our success with the SDSU BARC FMIA cooperative agreements were established with immune bioassay companies. The technology has been transferred to InVitrogen by SDSU, and samples sent from BARC for their test validation. Beta testing began this winter with SDSU and BARC; the InVitrogen FMIA is expected to be publically available in summer 2012. Our results have also been discussed with Affymetrix/Procarta and BioRad. FMIA test validation samples were sent to both companies from BARC. Interactions with Procarta have resulted in new commercial tests that are already available and expansion of their planned tests. The BioRad tests are expected within a year. Thus the NPB investment has been amplified for swine researchers worldwide who will have validated FMIAs available for their overall disease, vaccine and development work. The current tests provide researchers with tools needed to address pig health and welfare issues. Future availability of a wider range of commercial tests will further expand research options.

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