RESEARCH REPORT
SWINE HEALTH

Title: Serological Approach for Diagnosis and Surveillance of Multiple Agents in Serum and Oral Fluid Samples - **NPB # 10-033**

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Industry Summary

Fluorescent microsphere immunoassay (FMIA), also known as "Luminex," allows for the detection of antibodies to multiple antigens in a single, small volume of sample, and can be adapted for use on non-serum samples, such as oral fluids. The detection of antibodies to multiple pathogens in the same sample is a powerful tool that allows analysis of polymicrobial infections, such as PCVAD and PRDC. The goal of this research is to develop an FMIA to test for antibodies to PRRSV, PCV2, SIV and *M. hyo*, in oral fluid and serum samples. The first step was the expression of several target antigens, including N protein from type 1 and type 2 PRRSV, NP and NS1 from swine influenza virus, CP(43-233) and CP(160-233) from PCV2. All recombinant proteins were expressed, purified and successfully conjugated to polystyrene beads. All viral targets showed reactivity with positive sera. Comparison with the commercial PRRSV ELISA test showed that the Luminex assay possessed increased sensitivity. Another result was the demonstration that the CP(160-233) antigen from PCV2 could be incorporated as a target to differentiate infected from vaccinated animals (DIVA). As part of a collaborative effort, reagents and protocols were distributed to other veterinary diagnostic labs. The next step is to test the implementation of the Luminex technology across the industry.

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Scientific Abstract

Fluorescent microsphere immunoassay (FMIA), or Luminex, is a relatively new technology for serologic diagnosis of infectious disease. Advantages over standard technologies, such as ELISA, include the detection of multiple targets in a small (50 ul) sample with greater sensitivity and specificity, and at a relatively low cost. This technology can be adapted for use on non-serum samples such as oral fluid. Previous Luminex technology, e.g. Bio-plex, incorporated fluorescently labeled polystyrene beads coated with antigen combined with flow cytometry to detect bead types and bound antibody. Just recently, a new instrument, MAGPIX, was introduced by Luminex Corp. The technology incorporates magnetic microspheres and LED detection. The new instrument is lower in cost, possesses a smaller footprint and has simplified sample processing protocols. The magnetic beads can be used in the Bio-Plex instrument. The objectives of this project were to (1) to develop a multiplex fluorescent microsphere immunoassay (FMIA) or "Luminex" for the detection of PCV2, PRRSV, SIV, and *M. hyo*-specific antibodies in serum and oral fluid samples and (2) to validate the FMIA for incorporation into a single multiplex diagnostic platform. A third objective, added during the project, was to adapt the FMIA to the new MAGPIX format. This modification created the opportunity to incorporate a format likely to be used by veterinary diagnostic labs. Viral proteins were expressed as 5xHis-ubiquitin fusion proteins in *E. coli* and affinity purified on a nickel column under native conditions. Purity was verified by SDS-PAGE and total concentration measured by Biorad assay. *M. hyo*. proteins were prepared as a Tween-20 lysate. Except for the Tween-20 lysate, all proteins were successfully conjugated to the MAGPIX beads. The antigens included N protein from type 1 and type 2 PRRSV, NP and NS1 from swine influenza virus, CP(43-233) and CP(160-233) from PCV2. All antigen targets were assembled into a single multiplex and tested against sera known to be positive for antibody, i.e., infected with PRRSV, PCV2 and SIV. All antigens possessed the predicted antibody specificity using sera with known reactivity. Initially, assays were performed with the Bio-Plex and later with the MAGPIX. Results for both instruments were similar. Samples from 200 pigs experimentally infected with PRRSV were tested with both Luminex and IDEXX. The results for days 4, 7 and 11 showed agreement with the two assays, except that the MAGPIX identified more samples as positive, indicating a greater sensitivity for the Luminex assay. Results for the IgM assay showed the presence of PRRSV-specific antibody for the first 21 days after infection.

Introduction: Modern disease problems are the frequent result from the interactions of multiple infectious disease agents, such as combinations of PRRSV, PCV2, SIV, and *Mycoplasma hyopneumoniae*. Therefore, producers and veterinarians desire tests that can rapidly detect the presence of multiple agents at the same time and in the same sample. Serology remains the primary tool for detecting infections. Furthermore, the measurement of IgM versus IgG provides an account of prior or acute pathogen exposure. Additional variations employ tests to differentiate infected from vaccinated animals (DIVA). The current standard for performing serology is the ELISA test, developed during the late 1970's. However useful, ELISA suffers from several limitations, including the requirement for multiple ELISA kits to test multiple agents, which sometimes requires a relatively large quantity of serum sample. Fluorescent microsphere immunoassay (FMIA) or Luminex bead-based technology is an alternative that offers several distinct advantages: 1) the detection of antibodies to multiple agents in a single sample (often only 50 ul), 2) improved sensitivity and specificity over ELISA, 3) a semi-quantitative test result without the need for serial dilution of a sample to an endpoint, 4) easy adaptation for use with non-serum samples such as oral fluids, and 5) the flexibility to incorporate additional pathogen detection tests without increasing sample volume. The multiplexing capabilities of the FMIA provide a cost-effective means to embed into routine testing the tracking of emerging pathogens and surveillance for foreign animal diseases (FADs). The proposed FMIA panel described in this project is designed to test for antibodies produced in response to infection with PCV2, PRRSV type 1 (European-like), PRRSV type 2 (North American-like), swine influenza virus (SIV), and the bacterium, *M. hyo.*

FMIA incorporates antigen-coated beads or microspheres, which contain two fluorescent dyes present at different ratios. When beads are excited with a laser excitation, the dye emits a spectrally distinct address within a two dimensional grid. There are 100 distinct sets of beads enabling the identification of 100 unique antigens or pathogens. When stored properly, the antigen-coated beads are stable for several years. For antibody detection, the sample is incubated with antigen-coated beads. If pathogen-specific antibodies are

present, they will bind to the antigen. A reporter tag is employed to identify the presence of bound antibody, either IgG or IgM. The standard instrument for the analysis of FMIA samples is Bio-Plex 200 analyzer, which utilizes flow cytometric techniques for detection of bound antibody. During analysis, one laser excites each bead to identify its address (or antigen). A second laser identifies beads that have pig antibody attached to the antigen. Results are reported as mean fluorescence intensity (MFI), which is proportional to the amount of bound antibody. Recently, Luminex Corp. developed a new technology, which now makes FMIA easier and more affordable. The MAGPIX instrument uses magnetic microspheres, an optics system consisting of light emitting diodes (LEDs) and an imaging system consisting of a CCD camera. The magnetic properties of the beads allow adherence in a monolayer by a magnet within the camera chamber, where the microspheres are excited with red and green LEDs and imaged. Advanced software analyzes the images for internal and surface fluorescence intensity of the beads. The MAGPIX is more accessible to laboratories and academic institutions due to its affordability, relative ease of use and small size, features which will likely make it the instrument of choice in the future. It still retains the speed and efficiency of multiplexing and can measure up to 50 target antigens on a single sample. Additionally, the use of magnetic beads is an advantage because of ease and relatively low cost of equipment needed for wash steps during assays. The materials and protocols developed from the project were distributed to other veterinary diagnostic labs.

Serum is the most common sample used for the detection of swine antibodies. Recently, serological techniques have been successfully adapted for the detection of antibodies in meat juice and in oral fluid samples (Molina et al., 2008; Prickett et al., 2008). As described below, we have adapted FMIA for the detection of PRRSV antibodies in oral fluids. One goal of this project is to measure antibodies to several pathogens in oral fluid samples.

Status of project in regards to stated timeline

The additional time spent on adapting a new Luminex technology to this proposal (see section V. below) has slowed the project slightly relative to the stated timeline. The projected time of six months to achieve the experimental activities associated with test development for each antigen outlined in objective 1 will take slightly longer. Specifically, we still need to develop individual FMIA tests for SIV and M. hyo. and to test all individual antigens with additional experimental samples. The remaining time will be spent validating the assays for incorporation into a multiplex platform; we expect to complete the objectives within the time specified.

Objectives. The overall objective of the project was to work towards the development of a FMIA-based platform that will detect antibodies produced in response to multiple pathogens. The pathogens form polymicrobial interactions that have a significant effect on herd health and production costs. Measurement of IgM and IgG will provide information on the acute versus chronic nature of the antibody response. The application of FMIA to the measurement of antibodies in oral fluids is an adaptation that creates a cost-effective means for performing long-term surveillance.

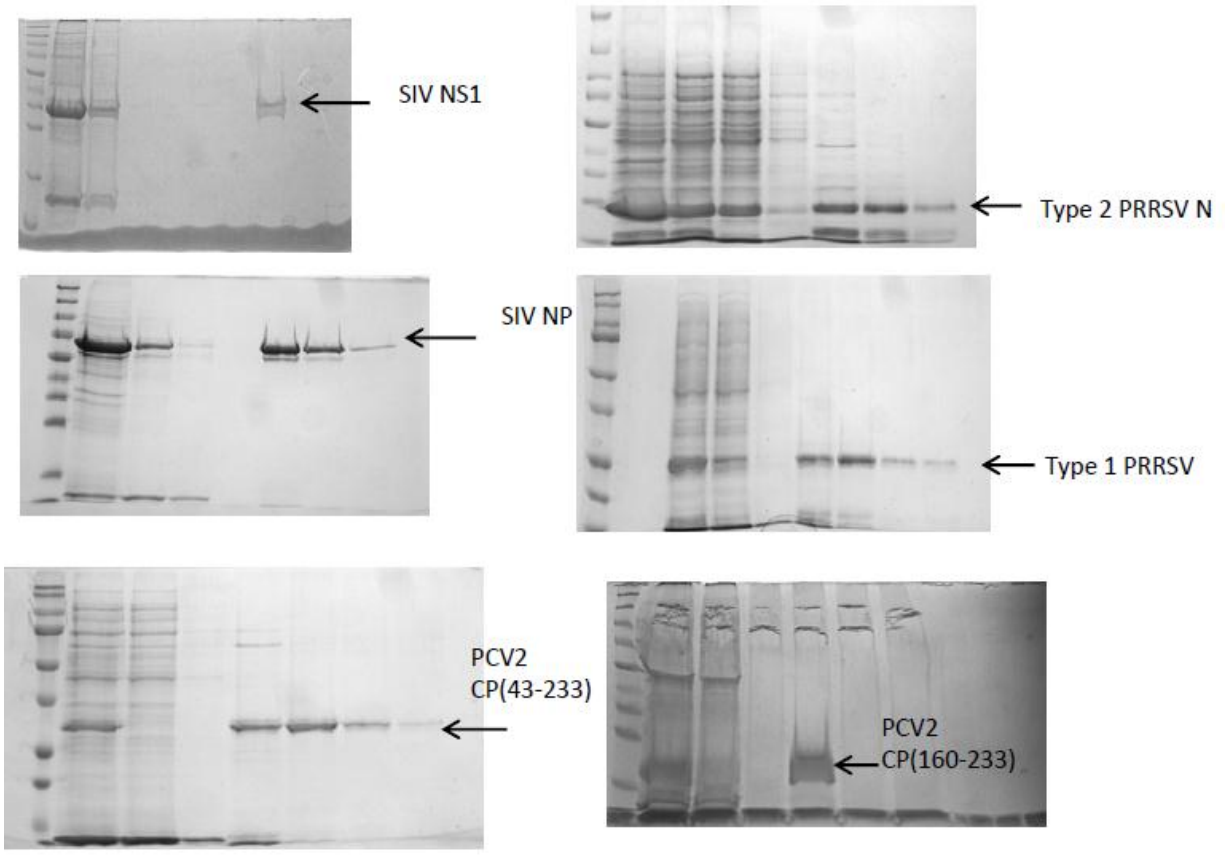
Objective 1. Develop FMIA for the detection of PCV2, PRRSV, SIV, and M. hyo-specific antibodies in serum and oral fluid samples. In the original proposal, pathogen target antigens to be coupled were PCV2 CP(43-233) and CP(160-233), PRRSV type 1 N protein, PRRSV type 2 N protein, SIV NP, and M. hyo Tween 20 lysate (provided by Erin Strait, Iowa State University). Added to this list was SIV NS1. The rationale for CP(160-233) is that it contains an immunodominant epitope associated with disease. Therefore, vaccinated pigs produce antibodies against CP(160-233) and not the smaller CP(160-233) fragment. In a similar manner, antibodies against SIV NP, produced in response to vaccination will not recognize NS1, a nonstructural protein produced during infection. The oral fluid and serum samples used for developing and validating assays for PCV2 and PRRSV are obtained from previous NPB-funded studies, samples from which are archived by the Kansas State Veterinary Diagnostic Laboratory.

Objective 2. Validate assays for incorporation into a single diagnostic platform: Once the individual tests are developed, the beads are combined into a single, multiplex assay. The tests are validated using experimental and field samples. QA/QC protocols are developed and the test offered to clients. The viral antigens and protocols are made available for use by other diagnostic labs upon request.

Materials & Methods. The methods described in the project are routinely performed in the PI's lab, including the production of purified recombinant proteins, development of techniques, adaptation of assays for oral fluids, interpretation of results, and quality controls. This project is a continuation of these activities.

Objective 1. Development of FMIA for the detection of PCV2, PRRSV, SIV, and M. hyo specific antibodies in porcine serum and oral fluid samples.

1.a. Production of recombinant proteins as target antigens: For bacterial expression, cDNA was PCR amplified and cloned into pHUE, which yields a 6xHis-ubiquitin fusion protein (Catanzariti et al., 2004). Expressed proteins were affinity purified on a nickel (Ni) column and protein purity assessed by SDS PAGE. Protein concentration was measured using Protein Assay dye reagent (Bio-Rad) with bovine serum albumin (BSA) as a standard. Examples of protein expression results are shown below.



1.b. Preparation of antigen-coated beads and FMIA procedure. Protein labeling of the polystyrene beads (Luminex) is according to the manufacturer's instructions. For the FMIA, a 96-well 1.2 µm-pore-size filter plate is blocked and washed with PBS, 1% BSA, and 0.02% sodium azide (PBN buffer). Diluted sera or oral fluid samples are added to each well of the filter plate. Approximately 2,500 beads are incubated with the sample in 50 µl of PBN buffer. Plates are incubated for 30 min at room temperature with gentle shaking. Following a wash with PBS-T, affinity-purified biotin-labeled goat anti-swine IgG, IgM is added. Plates are incubated with gentle shaking for 20 minutes at room temperature followed by three washes with PBS-T. R-phycoerythrin-conjugated streptavidin, diluted in PBN, is added, the plate incubated at room temperature for 15 minutes, washed three times, and assayed on the Bio-Rad Bio-Plex multiplex system. The median fluorescence intensity (MFI) for at least 50 beads per antigen is recorded for each well. For sample preparation, the unique feature in the use of the magnetic beads is the use of a magnet to hold the beads on the bottom of the 96-well plate during washing.

1.b. Development of tests. Tests were developed using sera and oral fluid samples from previous NPB-supported studies, archived samples, and from collaborators. Samples with known negative activity serve as

controls. Originally, assays were to be developed individually and then combined. A significant modification was the development of an assay with the other bead sets present. For example, the assay for PCV2 was tested simultaneously with beads containing PRRSV antigens. The only test not using a bacterially expressed recombinant protein was the M. hyo assay, which consisted of a Tween-20 lysate prepared from bacteria. The lysate was conjugated to beads using the same methodology and performed with the technical assistance of Luminex personnel.

Objective 2. Validate assays for incorporation into a multiplex platform. Under this objective, the individual bead set for each of the assays was to be combined into a single multiplex. As indicated above, the tests were developed by adding each new test to the existing platform.

Results

Objective 1. Development of FMIA for the detection of PCV2, PRRSV, SIV, and M. hyo specific antibodies in porcine serum and oral fluid samples.

1. Detection of PRRSV-specific IgG amd IgM antibodies in serum samples from experimentally infected pigs. An example of the detection of IgG and IgM in serum is shown in Fig. 1. The results show that IgM peaks at about 11 days after infection and decays towards background levels by 28 days. IgG peaks later and reached a plateau at about 14 days. It would be expected that IgM should appear sooner. We believe that the anti-IgG antibody cross-reacts with IgM. Therefore, both antibodies appear at the same time. These results illustrate the use of IgM for the detection of acute infection.

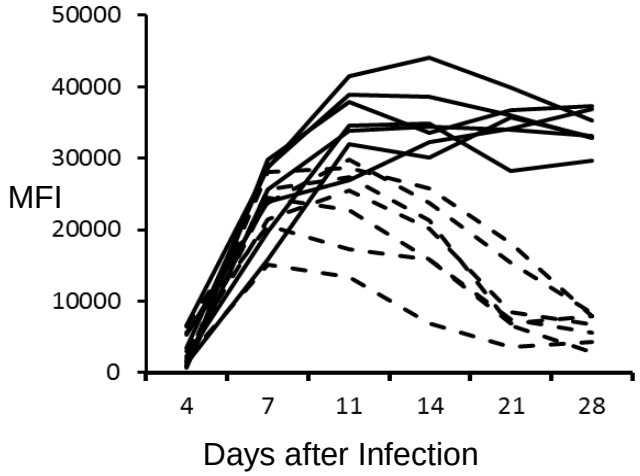


Fig. 1. IgM and IgG responses in sera following infection with PRRSV. Data are from eight pigs experimentally infected with PRRSV. Data are obtained using the MAGPIX, The dashed and solid lines show the results for IgM and IgG, respectively.

2. Comparison of Luminex and ELISA. Serum samples from 200 experimentally infected pigs, taken at 4, 7 and 11days after infection with PRRSV were assayed using the standard IDEXX ELISA and Luminex. The results, in Fig. 2 show that 95% of the pigs were positive for PRRSV by ELISA on day 11. All samples were positive by Luminex. On day 7, 40% of samples were positive by IDEXX versus greater than 80% of pigs positive by Luminex. This result demonstrates the increased sensitivity of Luminex over the standard ELISA.

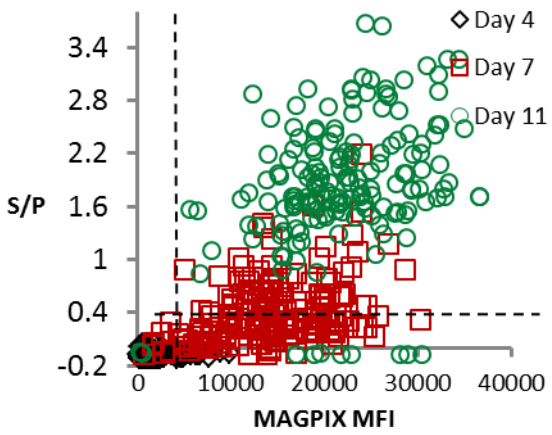


Fig. 2. ELISA vs. Luminex. The results above are from assays on 600 PRRSV-positive samples, collected between 4 and 11 days after infection of 200 pigs with PRRSV. The dashed line shows the positive and negative cutoff values for ELISA and Luminex. The red squares in the lower right quadrant show samples positive by Luminex and negative by ELISA.

3. Incorporation of Luminex to differentiate infected from vaccinated animals (DIVA). In previous work, we identified an epitope in the CP(160-233) region of PCV2 CP that is immunodominant and recognized by a subpopulation of infected pigs and by pigs with PCVAD. Vaccinated pigs fail to recognize this epitope. An example of the use of CP(160-233) as a marker for immunity is shown in Fig.3. Luminex assays were performed using CP(43-233) and CP(160-233) as antigen targets. Data were obtained from approximately 100 PCV2 antibody-positive feral pigs (as a source of natural infection) and 20 three week-old antibody-positive pigs derived from vaccinated dams. Results were reported as the MFI CP(160-233) divided by MFI CP(43-233). High ratios indicate a preferred recognition of the decoy epitope. The results in Fig. 3 show that about half of the naturally infected pigs possessed ratios greater than 0.5; whereas, samples from vaccinated pigs possessed ratios less than 0.5. These results demonstrate the capacity of a serological assay to provide information on the antibody status of a vaccinated herd. For example, high CP(160-233)/CP(43-233) ratios would indicate that pigs are infected with PCV2

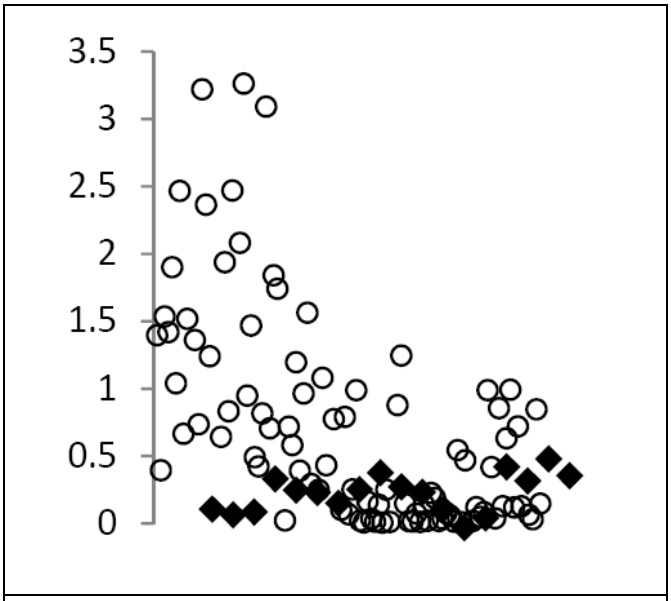


Fig. 3. CP(160-233)/CP(43-233) ratio in naturally infected (open circles) and vaccinated pigs (solid diamonds). Samples were obtained from approx.100 feral pigs and 20 three week-old pigs derived from vaccinated dams.

4. Multiplex detection of antibodies against PRRSV N, SIV NP and PCV2. The utility in the use of FMIA or Luminex is the capacity to perform multiple assays on a single small sample. Adding a new assay is as simple as adding a new set of antigen-coated beads. The actual cost for adding a new bead to the assay is as low as 15 cents. An illustration of assay multiplexing is found in Fig. 4, which shows results for the simultaneous detection of IgG and IgM antibodies against PRRSV, PCV2 and SIV. The results show that within this population of 204 feral pigs, there are pigs that possess detectable levels of IgG against all viruses at different rates of infection; i.e. approximately 5% positive for SIV, 30% for PCV2 and 20% for PRRSV. For IgM, the results show a much smaller percentage of positive pigs: approximately 2% for SIV, 10% for PCV2, and 5% for PRRSV. Besides presenting results on the infection rate of feral pigs to common pathogens, the results demonstrate the utility of Luminex for measuring antibodies to multiple pathogens.

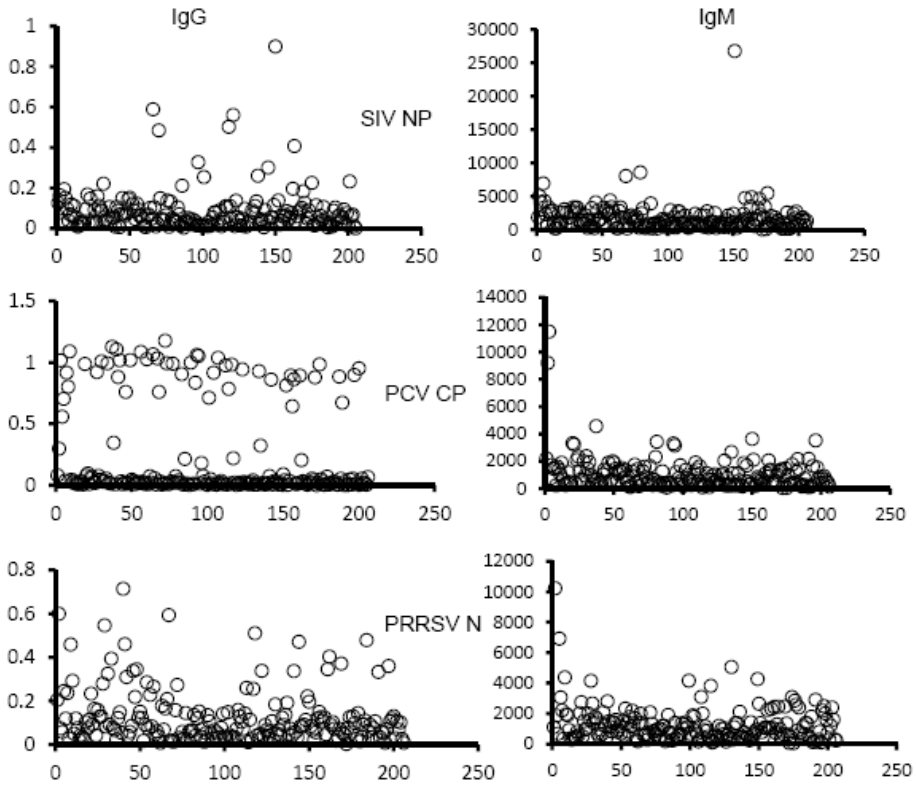


Fig.4. Detection of IgG and IgM in sera from 204 feral pigs. Results for IgG are reported as a signal/positive ratio. Results for IgM are reported as the raw MFI values.

5. Comparison of MAGPIX with Bio-Plex. One modification to the project was the opportunity to incorporate the new MAGPIX technology. Significant time and effort was devoted to comparing results on the MAGPIX to the standard BioPlex 200 instrument. As illustrated in the figure below (Fig. 5), the MAGPIX data largely correspond to data obtained from the BioPlex 200. In some cases, the MAGPIX instrument provided a superior result.

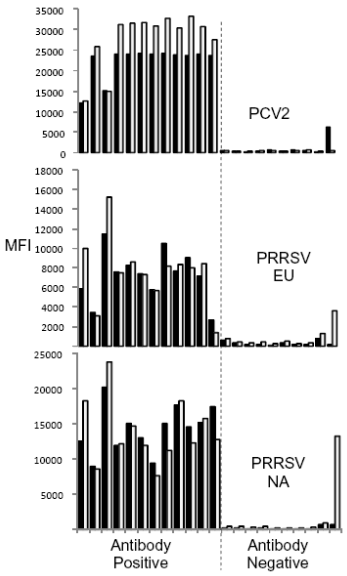


Fig. 5. Comparison of results for the same serum samples analyzed on the MAGPIX (light bars) and the Bio-Plex (dark bars) instruments. The results are for samples that are known to be antibody positive or negative for PRRSV or PCV2. The results for PRRSV nucleocapsid antigen are from pigs infected with a North American virus and sera reacted with either a North American (NA) or European (EU) nucleocapsid protein.

Discussion. The results from this project, along with similar work being performed at South Dakota State University and Iowa State University, contribute to the development of Luminex technology for routine veterinary diagnostic lab use. The deliverables from this work are summarized in Table 1. Much of the material, including recombinant proteins and protocols were transferred to Iowa State.

Two goals of the study, not achieved, were the adaption of all targets for use with oral fluid samples and the development of an assay for the detection of antibodies against *M. hyo*. The M. *hyo* antibody target was whole cell lysate prepared from bacteria. Several attempts were made to conjugate the lysate material to beads, without success. One alternative to the lysate approach is the incorporation of recombinant bacterial proteins. Once these become available, then future efforts will be made to develop the *M. hyo* assay. The results from this project evolved into a new NPB proposal integrating the Luminex development activities of the South Dakota State and Iowa State diagnostic labs. The proposal is titled "Multi-institutional development and validation of a multiplex fluorescent microsphere immunoassay for the diagnosis of multiple agents in serum and oral fluid samples." The first objective is to establish a panel of serum and oral fluid samples for the standardization of PRRSV, SIV and PCV2 Luminex assays across all diagnostic labs. The second objective is to determine reproducibility or precision across the three laboratories. The third objective is to conduct a large scale validation of a PRRS Luminex assay. Common reagents, standard operating procedures, and quality control materials will be developed throughout the study and distributed to interested laboratories. The ultimate outcome is to provide the swine industry with standardized cost-effective multiplex FMIA test for the simultaneous detection of PRRS, PCV2, SIV and other agents in serum and oral fluids.

Table 1. Summary of assays developed from this project.

Virus	Target	IgG	IgM	Serum	Oral	Diagnostic Application
PRRSV Type 1	N	+	+	+		Preferential detection of type 1 PRRS
PRRSV Type 2	N	+	+	+	+	Preferential detection of type 2 PRRS
PCV2	CP (43-233)	+	+	+	+	Detection of PCV2 infection
PCV2	CP (160-233	+	+	+		DIVA epitope for detection of naturally infected pigs
SIV	NP	+	+	+		Detection of SIV infection
SIV	NS1	+		+		DIVA for detection of naturally infected pigs