RESEARCH REPORT
SWINE HEALTH

Title: Multi-institutional development and validation of a multiplex fluorescent microsphere immunoassay for the diagnosis of multiple agents in serum and oral fluid – NPB #11-143

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Date Submitted: December 26, 2013

Industry Summary: The overall purpose of this project is to institute a collaboration between Kansas State University (KSU), South Dakota State University (SDSU), and Iowa State University (ISU) to develop and implement a new serological tool, known as Luminex, for the simultaneous detection of PRRSV, PCV2 and SIV antibodies in serum and oral fluid samples. This technology represents a "faster, better and cheaper" alternative to traditional serological tests and can be extended to include the analysis of up to 50 antigens or pathogens in a single sample. Previous projects funded by NPB have demonstrated a proof of concept in the capacity of Luminex to detect antibodies. The goal of this project was to transfer this technology into a workable assay kit for standardization across diagnostic labs. The principal objective was the establishment of samples and standard methodologies for PRRSV, SIV and PCV2 Luminex assays. Many samples were already available in the PI and co-PI labs. However, one specific objective was the creation of a set of samples from pigs infected with a European-like type 1 PRRS virus. As part of the development, the investigators engaged a private biologics company to prepare a commercial kit, which is based on the technology and reagents provided by the project investigators. A commercial test is the best means to establish a standardized test for use by all veterinary diagnostic labs. Recently, USDA recognized the collaborative approach towards the development of a commercial assay as having a high impact.

Objective 1. Establish a panel of serum and oral fluid samples for the standardization of PRRSV, SIV and PCV2 Luminex assays. The purpose of this objective was to create a panel of samples with known antibody reactivity. Standards would include oral fluid and serum for detection of IgM, IgG, and IgA isotypes. Funding was specifically requested to develop a sample set from PRRSV type I genotype-infected pigs.

Objective 2. Determine test reproducibility. The purpose of this objective was to assay the panel of standard samples in Objective 1 and compare results across laboratories (reproducibility).

Objective 3. Conduct a large scale inter-laboratory field validation of PRRSV N-based Luminex assay for the detection of IgM and IgG in serum, and IgM, IgG and IgA in oral fluid samples. The purpose of this objective was to conduct a field validation of PRRSV N-based Luminex assays using serum and oral fluid samples from herds of known PRRSV serological status.

Keywords: include at least 5 keywords
PRRSV, PCV2, SIV, Luminex, fluorescent microsphere immunoassay, FMIA, serology, serum, oral fluid.

Scientific Abstract:
Serologic detection of antibodies remains the primary tool for the diagnosis of infection and for the purpose of conducting disease surveillance in negative populations. Fluorescent microsphere immunoassay (FMIA), also known as "Luminex" technology, represents a major advancement in serology by allowing the detection of antibodies against multiple antigens in a single, small volume of sample. This multiplexing capability has the potential to replace traditional serology-based technologies, such as ELISA. Previous funding was used to establish the utility of the technology in the detection of antibodies against PRRSV, PCV2 and SIV antigens. Based on detection of antibodies against nsp7, PRRS antibody detection was further divided into the detection of type 1 and type 2 genotypes. A recent advancement in technology is the incorporation of magnetic beads and the introduction of the MAGPIX instrument, which is lower in cost, possess a smaller footprint, and improved ease of operation. The goal of this proposal was to implement this technology for everyday use in diagnostic labs. The overall approach was to assemble a multi-institutional effort involving researchers at KSU, SDSU, and ISU. The advantages of the collaborative approach included access to an extensive number of serum and oral fluid samples from field and experimental studies, and the unique expertise of each lab with respect to the test development and standardization. The first objective was to establish a panel of serum and oral fluid samples for the standardization of PRRSV, SIV and PCV2 Luminex assays. SDSU prepared a panel of serum and oral fluid samples from pigs infected with a European-like type 1 PRRSV. The second objective was to determine reproducibility across the three laboratories, which included the development of a standard set of samples and protocols delivered to a commercial company. The third objective was to conduct a large scale inter-laboratory field validation incorporating field samples. Objective 3 identified the presence of false positive results in some pigs. After the engagement of a company, the second and third objectives were modified to include the transfer of all materials for the development of a commercial kit.

Introduction:
A major challenge to pork producers in the management of infectious disease is the complex interaction of PRRSV with multiple infectious agents, such as PCV2, SIV, *Mycoplasma hyopneumoniae* (Myco), and others. Effective disease control and surveillance relies on the rapid diagnostic detection of multiple disease agents in a single sample and at a reduced cost. Fluorescent microsphere immunoassay (FMIA) or Luminex technology represents a significant advancement in serology, because it allows detection of antigen or antibodies to multiple pathogens simultaneously in a single small volume of sample, in a shorter time period, and at a reduced cost (see Table 2 below for cost comparision to ELISA). FMIA incorporates antigen-coated beads or microspheres, which contain differing ratios of two fluorescent dyes. When excited, the combination of dyes emits a spectrally distinct address within a two dimensional grid. For the MAGPIX instrument, there are 50 distinct sets of beads enabling the identification of up to 50 unique antigen (pathogen) targets. For antibody detection, the sample is incubated with antigen-coated beads. If pathogen-specific antibodies are present, they will bind to the antigen. A second reporter tag is employed to identify the presence of bound antibody. For the BioPlex 200 instrument, flow cytometry is used for the detection of bound antibody. During analysis, one laser excites each bead to identify its spectral two dimensional address, which corresponds to an antigen. A second laser identifies those samples that have pig antibody attached to the antigen coated bead. Results are reported as mean fluorescence intensity (MFI), which is directly proportional to the amount of bound antibody. A recent technological advancement is the introduction of the MAGPIX instrument, developed by Luminex Corporation. The MAGPIX relies on the use of magnetic beads with an optics system consisting of light emitting diodes (LEDs) and imaging via CCD camera. Because of lower cost, ease of use, and smaller footprint, it is expected that MAGPIX will increase the accessibility and utility of FMIA for use by veterinary diagnostic labs.

Individual tests for antibodies to PRRSV, PCV2 and SIV in serum and oral fluid were developed with prior NPB funding at Kansas State (KSU), Iowa State (ISU) and South Dakota State University (SDSU). These efforts established a "proof of concept" including preliminary experimental validation. The next step is to determine if Luminex could be developed into a standardized "kit" format and offered to diagnostic lab clientele at a competitive cost. The goal of this proposal was to further validate tests for the detection of antibodies to PRRSV, PCV2 and SIV and develop standardized protocols and proficiency panels.

Objectives:

Objective 1. Establish a panel of serum and oral fluid samples for the standardization of PRRSV, SIV and PCV2 Luminex assays. The purpose of this objective was to create a panel of samples with known antibody reactivity (none, low, medium and high). Samples included oral fluid and serum. Standardization was achieved by the development of a panel derived from experimentally infected pigs. Funding was requested to develop a sample set for PRRSV type I genotype-infected pigs. Samples are made available for the standardization of assays across laboratories and for the purpose of quality assurance when new reagents and/or protocols are instituted.

Objective 2. Determine test reproducibility across the three laboratories. The purpose of this objective was to test available samples and compare results across laboratories.

Objective 3. Conduct a large scale inter-laboratory field validation of PRRSV N-based Luminex assay for the detection of IgM and IgG in serum and IgM, IgG and IgA in oral fluid samples. The purpose of this objective was to conduct a field validation of PRRSV N-based Luminex assay using serum and oral fluid samples from herds of known PRRSV serological status.

Materials & Methods:

Antigens were prepared and coated onto beads by KSU and SDSU. Antigens included type 1 PRRSV N proteins, type 2 PRRSV N protein, type I nsp7, type 2 nsp7, PCV2 capsid, and SIV nucleoprotein. Protein labeling of microspheres (Luminex) was performed according to kit instructions. The serum and oral fluid samples used for validation came from experimental samples derived from studies performed at SDSU, ISU and KSU. Since sufficient amounts of standard serum and oral fluid samples were not available from type I PRRSV-infected pigs, funding was requested for the generation of these samples in Dr. Fang's laboratory. Briefly, 25 pigs were infected with the SD-08 type 1 laboratory isolate. Oral fluid samples were collected every other day for the first 28 days and then weekly thereafter. Serum samples were collected on a weekly basis and pooled to obtain sufficient quantities for inclusion in the panel.

For the Luminex assay, samples were diluted in PBS with 5% goat serum (PBSG). Affinity-purified biotin-labeled anti-swine IgG or IgM was added at an optimal dilution. The plate was incubated with gentle shaking for 30 minutes at room temperature followed by three washes with assay buffer. Washes were accomplished by adding buffer to each well, and then placing the 96 well plate in a magnetic holder. R-phycoerythrin-conjugated streptavidin diluted in assay buffer was added, incubated at room temperature for 30 minutes, washed three times, and assayed on the Bio-Plex 200 or MagPix instrument. The median fluorescence intensity (MFI) for at least 50 beads per antigen was recorded for each well.

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Results:

Objective 1. Objective 1. Establish a panel of serum and oral fluid samples for the standardization of PRRSV, SIV and PCV2 Luminex assays. Samples collected from PRRSV 1ype 1 infected pigs.

Defined samples for type II PRRSV, SIV, and PCV2 were available from previous studies, thus these were not produced as part of this objective. Therefore, funding for this objective was directed at infection of pigs with a

type 1 PRRSV isolate. Briefly, a total of 25 six-week old pigs were used in the experiment. Pigs were divided into two groups. Group1 pigs (n=20) were infected with type I PRRSV SD 01-08 and Group 2 pigs (n = 5) were mock-infected. Oral fluid samples were collected every other day for the first 28 days post infection (dpi) and then collected once per week until 84 dpi. Serum samples were collected on a weekly basis.

For the assay, two sets of internal control standards were established for type I PRRSV using the serum or oral fluid collected from experimental animals (Table1). Both serum and oral fluid standards were established as "high positive", "low positive" and "negative" standards for each antigen.

Table 1. Control standards for type I PRRSV N protein based-FMIA

Sample	Reactivity	Type I PRRSV N protein (MFI*)
Serum	**High**	25,000-29,000
	Low	12,000-15,000
	Negative	800-1,200
Oral fluid	**High**	25,000-29,000
	Low	12,000-15,000
	Negative	800-1,200

*MFI: mean fluorescence intensity.

GRAPH ROC software was used for ROC analysis of type I PRRSV FMIA to determine an optimized cutoff that maximized both the diagnostic specificity and diagnostic sensitivity of the assay. Oral fluid samples from a known positive population and oral fluid samples from a known negative population were analyzed. As a comparison, serum samples from a known positive population and serum samples from a known negative population were analyzed. The type I PRRSV oral fluid-based FMIA showed a diagnostic cut off of 0.31 with 95% sensitivity and 95.7% specificity, while the serum-based FMIA showed a diagnostic cut off of 0.54 with 100% sensitivity and 99.7% specificity. The precision of type I PRRSV FMIA was determined using internal control standards. For serum standard, the intra-assay repeatability was 1.7% and inter-assay repeatability is 3.4%. For oral fluid standard, the intra-assay repeatability was 1.5% and inter-assay repeatability was 1.4%. The data indicate that these FMIAs are highly repeatable in diagnostic applications.

Over the course of the project we developed a cost estimate comparing Luminex with standard ELISA. The results, presented in Table 2 below, show estimates for the two Luminex formats, the standard Bioplex format, and for the newer MAGPIX instrument. As the number of targets goes up, the cost of Luminex decreases compared to ELISA. For example, using the MAGPIX format for the detection of three antigens the cost is estimated at $22.55. This compares to $62.94 for three ELISA tests.

Table 2. Estimated cost comparison between ELISA and Luminex (cost per 96 well plate)

		Cost per 96 Well Plate		
			Luminex format	
Item	Cost	ELISA	Magpix	Bioplex
96-well plate		$1.73	$3.66	$3.66
Magplex beads	$630/ml	NA*	$2.00	$4.00
Antigen for detection	$20/100ug	$8.00	$0.4	$0.8
Anti-swine IgG conjugate-biotin	$96/2ml	NA	$0.45	$0.45
Goat serum for blocking	$204/500ml	$1.63	$3.76	$3.76
Streptavidin-PE	$270/ml	NA	$5.40	$5.40

Anti-swine IgG conjugate-peroxidase	$96/2ml	$0.30	NA	NA
TMB-Substrate	$153/250ml	$6.12	NA	NA
Plate sealing film		$0.82	$0.88	$0.88
Stop solution	$27.10/1L	$0.38	NA	NA
Buffers, tips. Etc		$2.00	$2.00	$2.00
	TOTAL	$20.98	$18.55	$20.95
	Two antigens	$41.96	$20.55	$24.95
	Three antigens	$62.94	$22.55	$28.95
*NA, not applicable				

Objective 2. Determine test reproducibility.
Over the course of the project, the investigators engaged a private company as a means to develop a kit as a means to standardize the Luminex multiplex assay. An initial MTA was signed by all parties and the following materials transferred.

1. Detailed protocols to set up and perform an 8-plex Luminex using magnetic beads.
2. Recombinant proteins, including protocols for expression and purification.
    a. nsp 7 protein from PRRSV type 1 and 2
    b. ORF7 derived protein from PRRSV type 1 and 2
    c. SIV nucleoprotein (NP)
    d. PCV2 capsid protein (CP)
3. Panels of defined negative and positive serum and oral fluid samples for assay validation
    a. 100 negative samples and 100 positive samples for type II PRRSV
    b. 100 negative samples and 100 positive samples for type I PRRSV
    c. 50-100 positive samples and 50-100 negative samples for SIV
    d. 100 positive samples and about 50-100 negative samples for PCV2.

Objectives 3. Conduct a large scale inter-laboratory field validation of PRRSV N-based Luminex assay for the detection of IgM, IgG in serum and oral fluid samples.

One goal of the Luminex test validation was to examine samples from different age pigs. Analysis of results from experimentally infected animals by ISU showed a good correlation between Luminex and the PRRS IDEXX ELISA X3 (κ= 0.80). However, the analysis of 90 field samples from 17 farms with unknown PRRSV status showed that rates of PRRSV antibody increased with age. Significant differences between PRRSV Luminex and the IDEXX PRRSV ELISA X3 were identified in adult pigs, potentially indicating a high false positive rate. In a subsequent analysis of 205 field samples from negative herds, the false positive rate was 1/205 for IDEXX versus 37/205 for Luminex. Similar results were obtained when the same assays were performed at KSU. There were at least two possibilities to explain the false positive results. The first is the presence of a cross-reactive antibody that recognized the PRRSV antigen. The second possibility is the presence of antibody that was non-specific; i.e., bound to the Luminex bead surface. To test these possibilities, we included a blank bead in the assay. False-positive samples possessed a high binding affinity for the blank bead, indicating that antibody reactivity was non-specific. As a means to reduce the false positive rate, a blank background binding bead has now been included in the assay.

Discussion:
The project developed a set of reagents and protocols that are being incorporated into a commercial assay kit. If successful, the kit will deliver to veterinary diagnostic laboratories a standard multiplex serological assay for major respiratory pathogens. The inclusion of background beads creates the means to eliminate false positive results.

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