DEVELOPMENT OF A MULTIPLEX FLUORESCENT IMMUNOASSAY FOR THE SIMULTANEOUS DETECTION OF SERUM ANTIBODIES TO MULTIPLE SWINE PATHOGENS

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Abstract

Three economically important swine diseases: Porcine Reproductive and Respiratory Syndrome (PRRS), Porcine Circovirus Associated Disease (PCVAD) and Swine influenza cost the US swine industry more than a billion dollars each year. This study developed a fluorescent microsphere immunoassay (FMIA) to simultaneously detect antibodies to the causative pathogens: PRRSV, porcine circovirus (PCV2) and swine influenza virus (SIV). The results showed that the multiplex assay possessed the predicted specificities. In the case of PRRSV NA, the assay displayed higher sensitivity when compared to a commercially available ELISA. The assay was employed to measure both IgG and IgM responses. The FMIA was found to possess several advantages over standard ELISA which include reduced sample volume, time and cost and provides a new tool for veterinary diagnostics.

The FMIA was applied for swine disease surveillance in Hawaiian and Texan feral swine populations. The antibodies against PCV2 showed the highest prevalence among these three pathogens in both Hawaii and Texas. Hence we consider PCV2 as the most prevalent pathogen in Hawaiian and Texan feral pigs and this pathogen poses the greatest threat to commercial pigs. SIV seroprevelance increased from 2007 to 2010 in Hawaii State, suggesting an increasing risk for commercial pigs. Moreover, yearly surveillance in Texas State shows growth in seropositive response to all pathogens, particularly PCV2.

The development of FMIA for detection of antibodies to multiple swine pathogens in serum samples offers an important alternative for swine disease surveillance in commercial and feral herds.

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Chapter 1 - Introductions

Multiplex Fluorescent Microsphere-based immunoassay

Fluorescent microsphere immunoassay (FMIA) is a protein detection assay in solution that incorporates the use of fluorescent microspheres as the binding surface for antigen-antibody complexes. Multiplex FMIA can detect more than one target proteins in a single sample. In 1997, Gorden and Mcdade from Luminex Corp. published their work regarding a novel assay for the simultaneous detection IgG, IgM and IgA of human serum samples in the same test tube (Gordon & McDade, 1997). At that time, the multiple fluorescent color sets of microspheres/beads carried 64 distinct colors (Kellar & Iannone, 2002). After 15 years of development, Luminex technology now offers up to 500 distinct color-coded beads to detect up to 500 target proteins.

Principle of FMIA xMAP Technology

The FMIA xMAP Technology follows the principles of sandwich immunoassays. The polystyrene microspheres, called bead sets, are given up to 500 distinct color 'addresses' using internal dyes. The xMAP microsphere is a 5.6 micron-sized polystyrene sphere with a carboxylated surface (Figure 1.1A). Internal dyes with differing ratios of two spectrally distinct fluorophores create a family of 500 differentially spectrally addressed bead sets (Figure 1.1B). Different color ratios of red (emission at 658nm) and infrared dyes (emission at 712nm) distinguish one bead set from another (Wilson et al., 2006). The carboxylated surface of microspheres enables the coupling of a capture protein to the beads. The high surface-to-volume ratio of the microsphere provides more binding surface in the assay. Each bead set is coated with a 'capture' protein (antigen) that reacts with the corresponding 'target' protein (antibody) in samples. In a multiplex assay, different bead sets are coated with different capture proteins to make the bead mixture. Subsequently, a secondary antibody labeled with biotin-phycoerythrin (PE) conjugate or directly with PE is reacted with the bead mixture to bind the target protein (antibody). Using an optical detection instrument, the fluorescence signals from internal dyes and PE are measured. The internal dyes identify each bead set. The PE dye acts as 'reporter' dye to measure the amount of target proteins (or antibodies) bound. The analysis software combines the

two signals to give a quantitative result of the different target proteins present in the sample. Using this process, xMAP Technology can perform up to 500 multi-assays within a single sample.

The bead solution is analyzed by a fluorometric array reader: flow cytometric instrument (Luminex 200; Figure 1.2B) or imaging CCD instrument (Magpix; Figure 1.3B). The Luminex 200 (Figure 1.2A) employs flow cytometric technology as the detection system (Sukhanova & Nabiev, 2008). A probe aspirates the bead solution from the assay plate and forces the beads through a 200 micron square flow channel one at a time. When beads pass through a specific position, the 532nm and 635nm wavelength lasers excite the reporter dyes and internal dyes, respectively. The reporter detector collects fluorescent signals between 565nm and 585nm. The bead detector collects fluorescent signals at wavelengths 658nm and 712nm, and the ratio is used to classify each bead set (Sukhanova & Nabiev, 2008).

The newly developed Magpix instrument (Figure 1.3A) employs imaging technology that differs from the flow cytometric technology of Luminex 200. A probe aspirates the solution of magnetic beads and transfers the beads to a magnetic mat. The beads spread out as a monolayer on the magnetic mat. The 511nm and 621nm wavelength Light Emitting Diodes (LEDs) excite reporter dyes and internal dyes, respectively. A CCD imager acts as both reporter and bead detector (Sukhanova & Nabiev, 2008).

The xPONENT software is compatible with both Luminex 200 and Magpix instrument. The functions of this software include converting fluorescent signals to digital data, performing the analysis, and guiding system maintenance routines. The software calculates the mean fluorescence reporter intensity (MFI) for each bead set per sample. At least 100 beads from each set of microspheres are counted per sample. Since the fluorescence from each bead is measured independently, sufficient replicates for statistical analysis are accumulated in every sample.

Comparison with ELISA

The Enzyme-Linked Immunosorbent Assay (ELISA) (Malekzadeh *et al.*, 2012), which is the traditional analysis method for detecting antibody, requires a separate assay for each target protein. In an indirect detection ELISA (sandwich immunoassay), an antigen is attached to the bottom of a well for capturing target primary antibody. A secondary antibody linked to an

enzyme, often horseradish peroxidase (HRP), binds to a different region of the antigen. When the substrate for the enzyme is added, the color signal generated is used for detection of the antibody of interest. The advantages of ELISA are ease-of-use, flexibility, and low cost. The FMIA not only offers benefits of ELISA, but also has several advantages compared to the monoplex ELISA. First, FMIA shows greater specificity and sensitivity for two reasons: type of binding and method of detection. The capture proteins that are covalently bound to beads have higher avidity as opposed to the hydrophobic interaction in ELISA. The higher density of capture protein per surface area on beads and the reduced loss of protein in washing steps increase sensitivity. The bead surface minimizes non-specific binding and, therefore, reduces background. FMIA is based on direct fluorescence detection which improves sensitivity.   A second advantage is that multiple target antibodies can be detected at the same time. And third advantage of FMIA over ELISA is cost and time efficiency for detection of multiple target proteins.

A comparison between ELISA and FMIA in terms of cost and time is presented in Table 1-1 and Table 1-2.

Porcine Reproductive and Respiratory Syndrome virus (PRRSV)

Porcine Reproductive and Respiratory syndrome (PRRS), caused by Porcine Reproductive and Respiratory syndrome virus (PRRSV), is the most economically important infectious disease. The economic impact on the US swine industry was estimated at $560 million dollars per year (Neumann *et al.*, 2005). PRRS symptoms in pigs include severe reproductive failure and respiratory distress. The reproductive failure occurs in sows and gilts including late-term abortions, stillbirth, and premature farrowing. In growing pigs, PRRS can cause increased mortality and decreased growth performance (Corzo *et al.*, 2010). PRRSV is highly infections and can be transmitted by direct contact (oral fluids, nasal secretions, mammary gland secretions and semen) (Bierk *et al.*, 2001) or by indirect routes. The indirect routes include contaminated boots, coveralls, aerosol, and so on (Otake *et al.*, 2002) (Pitkin *et al.*, 2009). PRRSV has been identified in air samples collected at 9.1 km from infected pig barn (Desrosiers, 2011). PRRSV is also an important component of various polymicrobial disease syndromes, such as porcine

respiratory disease complex (PRDC) and porcine circovirus associated disease (PCVAD) (Chand *et al.*, 2012).

PRRSV was first recognized clinically in pigs in the Netherlands and the United States in 1991 (Wensvoort et al., 1991) (Benfield et al., 1992). PRRSV is classified in the family Arteriviridae, along with equine arteritis virus (EAV), lactate dehydrogenase-elevating virus (LDV), and simian hemorrhagic fever virus (SHFV). The PRRS virus is a positive-sense; single-stranded enveloped RNA virus. The genome is approximately 15kb and encodes ten open reading frames (ORFs) (Firth et al., 2011). The spherical virus particle, which has a diameter of about 50-65 nm, consists of a lipid envelope and a capsid core. The capsid consists of the nucleocapsid (N) protein and the viral genome (Spilman et al., 2009). The envelope includes all structural proteins except the N protein. Multiple N proteins enclose the non-segmented viral RNA (Figure 1.4). PRRSV includes two genotypes: Type I (isolated from European Union) and Type II (isolated form North America). The two PRRSV genotypes show approximately 60% nucleotide identity according to sequence analysis (Hanada et al., 2005).

PRRSV binds the heparin sulphate receptor (Delputte et al., 2002) and sialoadhesin (Delputte et al., 2007) receptor on surface of host cells (Kim et al., 2006). The virus enters cells by clathrin-mediated endocytosis. Host CD163 might be involved in viral uncoating (Van Gorp et al., 2008); however, the mechanisms of fusion and uncoating are not well understood. Virus replication, transcription and translation take place in the cytoplasm by the viral replication-transcription complex. After assembly in the ER and Golgi complex, PRRSV virions are transported to the plasma membrane for release (Knipe & Howley, 2007).

The N protein, encoded by ORF7, is the sole component of the PRRSV capsid, which is 123 and 128 amino acids in length (Type I and Type II, respectively). The NP gene is rarely mutated and is highly conserved (Chang et al., 2002) and immunogenic (Loemba et al., 1996). Antibodies against N protein can be detected as early as 5-7 days post-infection (DPI). The N protein is the earliest detected immunogenic protein compared with other immunogenic proteins, such as Nsp1, Nsp2, Nsp4, Nsp7, Nsp8, GP5, and M proteins. (Brown et al., 2009) (Mulupuri *et al.*, 2008) (Jeong *et al.*, 2010) .

Porcine Circovirus Type 2 (PCV2)

Porcine Circovirus Type 2 (PCV2) is associated with Porcine Circovirus Associated Disease (PCVAD) in pigs. PCVAD encompasses a group of diverse multi-factorial diseases including Post-weaning Multisystemic Wasting Syndrome (PMWS), Porcine Dermatitis and Nephropathy Syndrome (PDNS), porcine respiratory disease complex (PRDC), and reproductive disorders. It is recognized as a global, epizootic disease that causes significant economic losses to pig producers. PCV2 is considered necessary for disease, but the presence of PCV2 alone is not always considered sufficient for development of PCVAD. PCV2 infection and replication in lymphoid tissues can lead to lymphoid depletion, which subsequently leads to immunosuppression. PCV2 is usually found with other swine pathogens, such as PRRSV, or SIV (Opriessnig & Halbur, 2012).

PCV2 is a member of the Circoviridae family. Sequence analysis shows that PCV2 isolates can be classified into two main genotypes; PCV2a and PCV2b (Segalés et al., 2008). Porcine circoviruses possess an ambisense single-stranded DNA genome in the form of a covalently closed circle. The genomes of PCV2a and PCV2b are 1,768, and 1,767 nucleotides (nt) in length, respectively. The genome sequences of PCV2a and PCV2b share an identity of approximately 95% (Fenaux et al., 2004). The PCV2 genome is composed of ORF1 (encodes two replicase proteins Rep and Rep'), ORF2 (encodes a capsid protein), and ORF3 (encodes an apoptosis protein). The 233-amino-acid capsid protein (CP), encoded by ORF2, is the only structural protein, which constitutes the outer protein of the PCV2 virus like particle (VLP). PCV2 VLP spans ~17nm and is composed of 60 capsid protein molecules arranged into 12 pentameric units (Figure 1.5).

PCV2 can infect multiple cell types, including epithelial cells and monocyte/macrophage cells. The mechanisms of PCV2 entry are not well understood. It is believed that PCV2 uses a relatively common cell receptor to attach to and enter cells (Misinzo et al., 2006). After entry and

uncoating, the viral genome localizes to the nucleus, the site of virus replication. CP contains an arginine rich basic N-terminus responsible for nuclear localization.

Recently, an epitope in the C-terminal region of CP, CP(169-180), was identified as an immunodominant epitope. Antibodies targeted to the CP(169-180) region were detected only in PCVAD affected pigs associated with relatively low neutralizing antibody (NA). In the monomeric form, CP(169-180) is exposed on an outer loop, and acts as decoy epitope directing the main antibody response towards this region. However, the CP(169-180) is hidden within the VLP structure (Trible et al., 2011). Low levels of neutralizing activity associated with high levels of anti-PCV2 CP(169–180) antibody in PCVAD affected pigs explains the absence of protection following virus infection (Trible et al., 2012). However, vaccinated pigs almost exclusively recognize the polypeptide CP(43-233). Therefore, the measurement of antibodies against CP(169-180) provides the basis for diagnostic methods that can differentiate infected from protected animals .

Swine Influenza Virus (SIV)

Swine influenza, caused by swine influenza A virus (SIV), is an acute respiratory disease affecting pigs of all ages. Clinical signs include the sudden onset of fever, sneezing and a barking cough. Infected pigs may exhibit breathing problems as well as loss of appetite. SIV is highly contagious among pigs and may cause considerable economic losses in an infected herd (Neumann & Kawaoka, 2011). Swine influenza (SI) was first recognized clinically in pigs in the Midwestern U.S., in 1918. With the increasing number of novel subtypes and the presence of multiple co-circulating strains, the prevention of SIV has become increasingly difficult. In commercial swine populations, influenza is an important component of the porcine respiratory disease complex (PRDC) (Wei et al., 2010).

SIV type A is one of the five genera in the family Orthomyxoviridae. This virus is a negative-sense, single-stranded RNA virus with 8 segments. The spherical virus particle, which has a diameter of about 80-120 nm, consists of a lipid bilayer envelope, an inner shell of matrix proteins, and a nucleocapsid surrounding the viral genome (Figure 1.6). The envelope is

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comprised of hemagglutinin (HA), neuraminidase (NA), and M2 proteins. A layer of M1 protein acts as the inner shell. The core of the virus particle, the ribonucleoprotein complex (RNP complex), includes the viral RNA segments, the nucleoprotein (NP), and the three subunits of viral polymerase (PB1, PB2, and PA). NP is the major viral protein in the RNP complex and multiple NPs coat individual segments of RNA. PB1, PB2, and PA are situated at the ends of the RNP complex. Subtypes are classified by antigenic and genetic properties of surface proteins haemagglutinin (HA) and neuraminidase (NA). Frequent mutations occur in the HA and NA regions of the virus resulting in "antigenic drift". So far there are 16 hemaglutinin (H 1-H16) subtypes and 9 neuraminidase (N1-N9) subtypes (Knipe & Howley, 2007). Three virus subtypes (H1N1, H3N2 and H1N2) are most frequently identified in pigs and are spreading within swine populations worldwide (Vijaykrishna et al., 2011).

SIV binds the sialic acid-containing receptor on surface of cells followed by clathrin-mediated endocytosis for virus entry. At the low pH within the endosome, HA mediates fusion of the viral membrane with the endosomal membrane. M2 protein allows H+ ion influx into the virus particle, which disrupts protein-protein interaction between the core RNP complex and outer shell. This results in release of free RNP complex into the cytoplasm. NPs in RNP complex carry nuclear localization signals (NLSs) that bind with host importin α protein for transport across the nuclear membrane. Virus replication in the nucleus occurs via the viral polymerase complex. After assembly, the export occurs via a host export receptor in a Crm1-dependent manner.

The nucleoprotein (NP), which is 498 amino acids in length, coats and protects the virus RNA against degradation by various enzymes (Li et al., 2009). The NP gene is rarely mutated and has more than 90% homology at nucleotide level among H1N1, H2N2, H3N2 and other subtypes of influenza type A virus (Ohba et al., 2007). For example, the nucleoprotein (NP) of seasonal H1N1 virus contains 9 B-cell epitopes, four of which were conserved in 2009 pandemic H1N1 (Combadière et al., 2010). Serum antibody responses have been ascribed to the HA, NA, M2, NP, and M1 proteins (Couch, 2003) . The antigenic drift and shift of the surface antigens make antibody responses against HA and NA frequently variable. The high conservation of NP antigen and the known longevity of antibody responses (Amanna et al., 2007) suggest it as a

suitable antigen for detection of virus-specific antibodies of all subtypes of SIV (LaMere et al., 2011).

Figures and Tables

Figure 1.1 The xMAP microsphere

(A) Carboxylated surface of microsphere; (B) Luminex internally color-codes microspheres with precise concentrations of various fluorescent dyes yielding up to 500 distinctly colored bead sets. (www.luminexcorp.com)
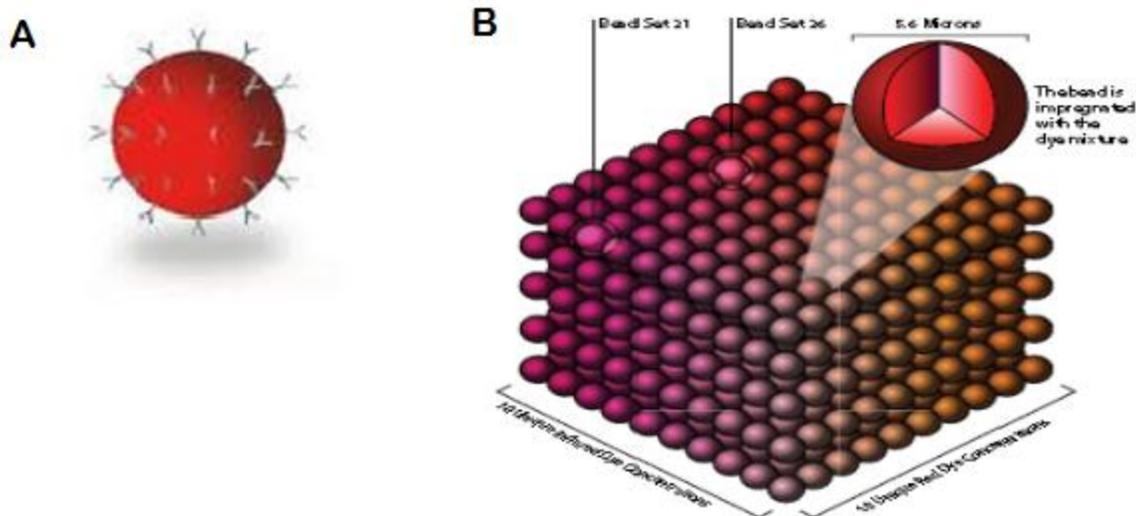
Figure 1.2 Schematic of Luminex 200

(A) The red laser excites the internal dyes to distinguish the microsphere set and the green laser excites the fluorescent dye on the reporter molecules for the assay output. (B)Picture of luminex 200 machine from www.luminexcorp.com
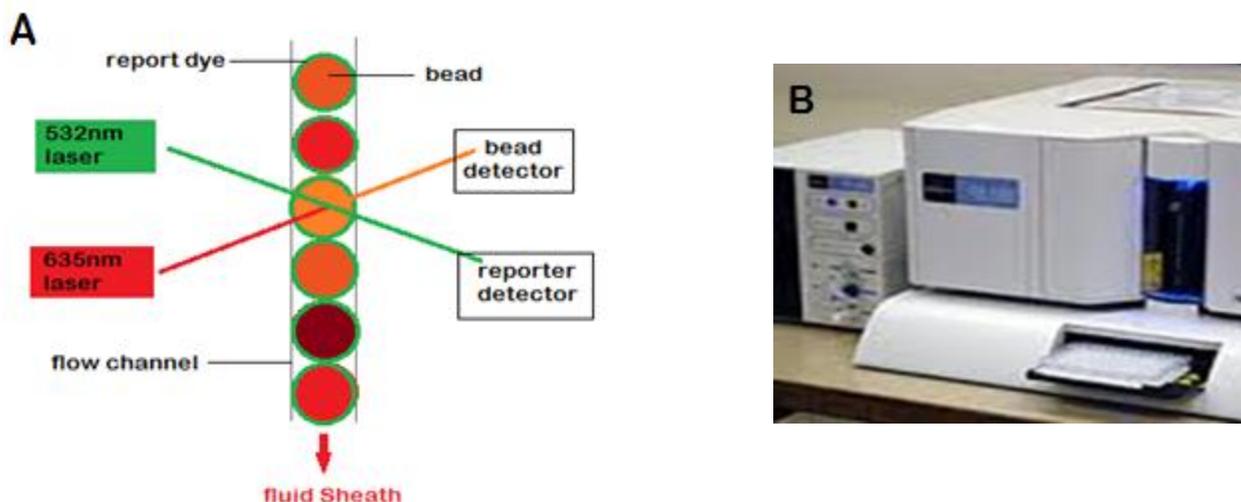
Figure 1.3 Schematic of Magpix

(A) Magpix holds beads in a monolayer with a magnetic mat. The 511 nm (green) and 621 nm (red) LEDs excited reporter dyes and internal dyes respectively. CCD camera takes an image of the beads in the magnetic mat. (B) Picture of Magpix machine form www.luminexcorp.com
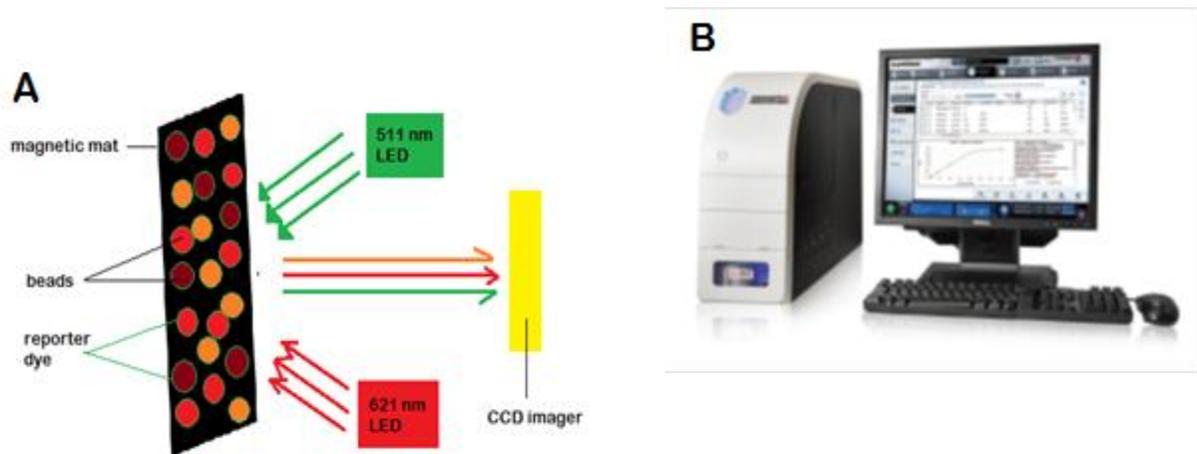
Figure 1.4 Schematic representation of PRRSV particle

(Provided by Benjamin Trible)
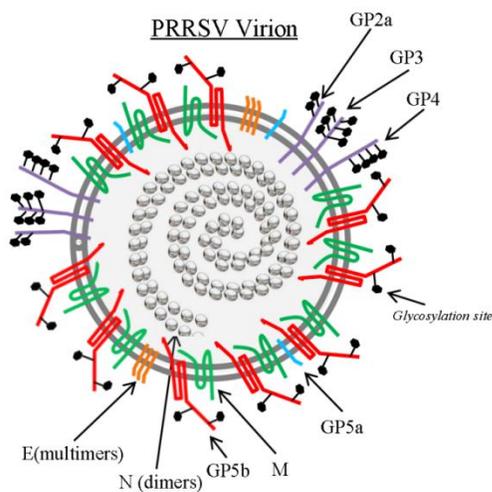
Figure 1.5 PCV2 CP subunit model structure and assembly into a viral capsid.
(A) The ribbon model of the CP subunit with helices, loops and sheets shown in green, blue and red, respectively. (B) A CP subunit placed in the context of viral capsid. The remaining CP subunits are depicted in gray. Both models are based on the data of Khayat et al. (Khayat et al., 2011) and reproduced using the UCSF Chimera computer program (Pettersen et al., 2004). (Figure provided by Benjamin Trible).
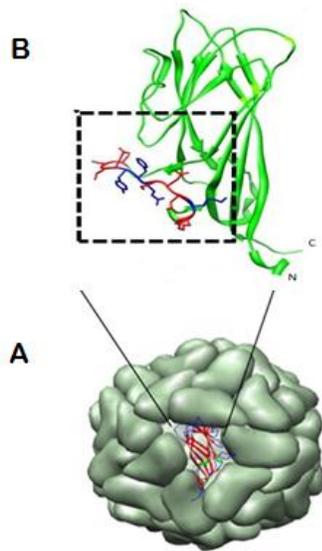
Figure 1.6 Schematic representation of SIV
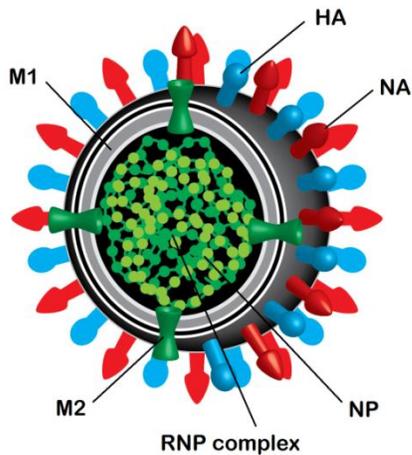
Table 1-1 Cost comparison of FMIA vs. ELISA.

Example is for the detection of swine antibody

Item	Cost	Cost per 96 Well Plate		
		ELISA	Magpix	Bioplex
96-well plate		$1.73	$3.66	$3.66
Magplex beads	$630/ml	NA*	$12.60	$25.20
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	$29.15	$41.75
	Two tests	$41.96	$41.79	
	Three tests	$62.94	$54.43	
*NA, not applicable				

Table 1-2 Time comparison of FMIA vs. ELISA

FMIA (Magpix)		ELISA	
Step	Time (Min)	Step	Time (Min)
		Block plate	65
Prepare standards and samples	40	Prepare standards and samples	40
Prepare beads, Pipette 50 uL to each well	10	Pipette200 µL sample or standard to each well	10
Pipette 50 µL of sample or standard to each well	10	Mixing	2
Incubation	40	Incubation	60
Wash step (3x) using magnetic plate	10	Wash step (3x)	5
Prepare detection antibody solution	2	Prepare detection antibody	2
Pipette 50 µL per well of antibody	3	Pipette 100 µL per well of antibody	3
Incubation	30	Incubation	60
Wash step	10	Wash step	5
Prepare streptavidin-PE	2	Prepare substrate solution	2
Pipette 50 µL Strep-PE per well	3	Pipette 100 µL substrate per well	3
Incubation	30	Incubation	20
Washing step	10	Pipette 100 µL stop per well	3
Assay read-out	70	Assay read-out	10
Total	270min	**Total**	290min

Chapter 2 - Development of multiplex fluorescent immunoassay to multiple swine pathogens

Introduction

PRRSV, SIV and PCV2 are economically important and are widely distributed in both domestic and feral pigs. To date, few multiplex tests are available for antibody detection, especially for veterinary use. The FMIA has been developed for simultaneous detection of antibodies to PCV2 and PRRSV (Lin et al., 2011). In this study, we have applied the FMIA to develop a multiple serological detection test for PCV2, SIV and PRRSV as an efficient tool for diagnosis and surveillance of swine diseases. To develop the assay, we chose suitable antigens for each pathogen. The ORF7 gene, which encodes the N protein, of PRRSV, is highly conserved and immunogenic. The N protein is the earliest detected immunogenic protein, which was observed in diagnostic tests as early as 5-7 DPI. Hence, the N protein is the most suitable antigen to use for the surveillance of PRRS disease. The capsid protein (CP) of PCV2 is the only structural protein, which constitutes the outer protein of virion. For surveillance of PCV2-associated disease, two different antigens were used: CP (43-233) and CP (160-233). We chose CP (43-233), which includes all the known immunogenic epitopes, as antigen to monitor PCV2-associate disease. Antibodies targeted to the CP (160-233) region were detected only in PCVAD affected pigs associated with relatively low neutralizing antibody (NA) response. Therefore, we chose CP (160-233) as antigen to distinguish unprotected animals after PCV2 infection. The nucleoprotein (NP) of SIV is a group specific structural protein and is the major internal component of the virion. The highly conserved NP elicits immunity against all subtypes of SIV. Therefore, NP was selected as target antigen for surveillance of swine influenza disease.

The approach used for this study was as follows. Specific antigens of each pathogen were coupled to different color bead sets which were used to develop a multiplex assay. The assay was optimized by testing various parameters of positive and negative samples, such as sensitivity, specificity, reproducibility between different coupling batches and cross reactivity among

different bead sets. The results of the multiplex assay were compared with commercial ELISA. We applied this multiplex assay to identify early stage infection by detection of IgM antibody.

Methods

Expression and purification of recombinant antigen proteins

SIV NP, PRRSV NA N, PRRSV EU N, PCV2 CP (43-233), and PCV2 CP (160-233) antigens proteins were expressed as fusion proteins (His-tag and ubiquitin) in the pHUE vector using an *E. coli* expression system. The pHUE vectors containing the target protein cDNA were transformed into the BL-21(DE:3) *E. coli* cell line. *E. coli* were cultured in LB with ampicillin at 37°C until they reached an OD600 of 0.4-0.6. For the purpose of induction of protein expression, Isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to *E. coli* cultures to a final concentration of 1.0ug/ml. After addition of IPTG, bacteria were grown for an additional 4 hours and then harvested by centrifugation at 4,000g for 10 minutes.

Proteins were purified using a PrepEase His-Tagged Protein Purification High yield Kit (USB). The bacterial pellet was subjected to three freeze thaw cycles at -80°C and RT respectively. Then the pellet was suspended in 5mL of 1X lysis, equilibration, and wash (LEW) buffer (USB) and 50uL of EDTA free protease inhibitor cocktail (THERMO scientific). After adding lysozyme to a final concentration of 1mg/mL, the mixture was incubated on ice for 30 minutes. Sonication was then performed six times for 10 seconds each time, with 30 seconds incubations on ice between bursts. The bacterial lysate was then ultra-centrifuged at 20,000g for 30 minutes. The supernatant was then filtered through a 0.45um PVDF filter (Fisher) to get soluble proteins. In order to capture the 6X-His tagged target proteins, the filtered lysate was added to a Ni-TED mini column (USB). After washing with 3 column volumes of 1X LEW, the target proteins were eluted in four 1mL aliquots using 1X elution buffer (USB).

Purified protein concentrations were measured using Protein Assay (Bio-Rad). Briefly, purified polypeptides were mixed with 200uL of 1X dye reagent in a 96 well plate (flat bottom

Fisher). Absorbance values of each well were determined by reading at 595nm on a precision microplate reader (Molecular Devices). Protein concentrations were then determined by comparison to BSA standard protein concentrations.

Bead coupling protocol

The purified antigen proteins were covalently coupled to Luminex MagPlex® polystyrene, carboxylated beads with appropriate beads address. Briefly, 0.5mL of commercial beads (1.25×10^7 beads/mL) were taken from 4 °C and warmed to room temperature in a USA Scientific microcentrifuge tube. Beads should be protected from prolonged exposure to light throughout this procedure. The tube was placed into a magnetic separator for 1 minute to remove the supernatant. Beads were washed once with 100 µL of dd-H_2O and tube was sonicated for approximately 20 seconds. Beads were resuspended in 80 µL of sodium dihydrogenphosphate (NaH_2PO_4, 0.1M, pH 6.2) after removing supernatant, vortexed gently, and sonicated for 20 seconds. Ten micoliters of 50 mg/mL Sulfo-NHS (N-hydroxysulfocuccinimide, Thermo Scientific, Rockford, USA) and 10 µL of 50 mg/mL EDC (N-(3-dimethylaminopropyl)-N-ethylcarbodiimide Thermo Scientific, Rockford, USA) were added successively to the beads and vortexed gently. For the purpose of activation, beads were incubated for 20 minutes at room temperature with gentle mixing. The beads were washed twice with 250 µL phosphate buffered saline (PBS), pH 7.4 by vortexing and sonication for approximately 20 seconds. The activated and washed beads were resuspended in 100 µL PBS. Recombinant protein (25 uL) was added and total volume was brought to 500 µL in PBS. The tube was gently rotated on a shaker Rotisserie (Thermo Scientific) for two hours at RT in the dark for the coupling reaction to occur. Coupled beads were washed three times with 500 µL of PBS plus 4% goat serum (PBS-GS). Lastly, 1000uL PBS-GS was added to the washed beads to prepare bead stocks, which were stored at 2-8°C in the dark until use. Antibody detection protocol

For reaction with samples, bead stocks were diluted in PBS-GS to a working concentration of 2500 coupled beads per 50 µL working solution. Due to the light sensitive nature of the fluorescent assay, all procedures were performed in the dark by sealing the plate with foil, except while adding and removing of components. Fifty microliters of a 1:400 diluted serum sample was added to each test well of a 96-well plate (Costar polystyrene white, round bottom 96-well plate, Corning, NY) along with 50 µL of the bead working solution. The plate

was incubated at room temperature for 40 minutes on a plate shaker (Thermo Scientific), the supernatant was dumped with firm shakes by holding the assay plate onto a magnetic plate, and the assay plate was washed three times with 190 µL of PBS-GS. The bead-antibody complex was incubated with 50 µL of biotin-SP-conjugated affinity purified goat anti-swine secondary antibody (IgG, 2ug/mL,Jackson ImmunoResearch) or biotin-labeled affinity purified goat anti-swine IgM (KPL, 2ug/mL,Gaithersburg, MD) in the dark at RT on a plate shaker for 30 minutes. After incubation, the plate was washed three times with PBS-GS, and 50 µL of PBS-GS containing 2 µg/mL streptavidin-conjugated phycoerythrin (SAPE, Moss, Inc., Pasadena, Maryland) were added to each test well and incubated for 30 minutes at room temperature with shaking. The supernatant was dumped and the plate was washed three times with PBS-GS. The beads were resuspended in 100 µL of PBS-GS per well, and the assay plate was read on the MAGPIX instrument.

Software and statistical analysis

Data were analyzed with the software Luminex$^{®}$ xPONENT 4.2, and the mean fluorescence intensity (MFI) for each sample was determined. The MFI is directly proportional to the amount of antibody bound to the microspheres. The MFI for at least 100 microspheres corresponding to each individual antigen was recorded for each well. All reported MFI measurements were background corrected.

The presence or absence of antibodies to each swine pathogen was evaluated by calculating the sample to positive ratio (S/P ratio). Positive and negative controls were performed for each plate.

S/P ratio = (Sample mean – negative control mean)/(positive control mean – negative control mean)

IDEXX ELISA also employs S/P ratio to analyze data. S/P ratio threshold was fixed at 0.4, for positive and negative controls which were provided by this commercial ELISA kit.

The determination of P values, t-test, or F-test between groups were performed by Excel data analysis function.

Establishment of control standards

For the positive and negative controls, serum samples were collected from infected experimental animals and uninfected animals. The sera corresponding to the five bead sets were serially titrated with 2-fold dilutions from 1:100 to 1:6400. Fifty microliters of serially diluted serum samples were added to corresponding wells containing multiple coupled bead sets. Standard protocol for detection was performed. The results were visualized as regression curves (Figure 2.1). For optimum discrimination between positive and negative samples the baseline negative sample should be at least 10 times lower than a positive sample. Based on this criterion, 1:400 was chosen as sample dilution for reaction. Samples that had large quantities were selected as controls for long-term usage. The mean fluorescence intensity (MFI) of positive controls were in the range of 15,000 to 30,000 and that of negative controls were from 300 to 1,000. Five positive controls for each antigen were chosen. Three negative controls were follows: SIV and PRRSV NA shared one negative control, PCV2(43-233) used the negative control which also used as SIV positive control, and PRRSV EU and PCV2 (160-233) had separate negative controls.

Results

Diagnostic sensitivity and specificity for detection of antibodies against PRRS NP

Sensitivity is defined as a test's ability to identify positive results, and a measure of the proportion of actual positives which are correctly identified. Specificity measures the proportion of negatives which are correctly identified, and relates to the test's ability to identify negative results. To measure the diagnostic sensitivity and specificity, two experimental groups of pigs were tested. The first group included 198 three weeks old healthy PRRSV antibody-negative uninfected pigs. The second group included 491 experimentally PRRSV infected pigs for which samples were collected 42 days post infection. The pigs were euthanized on day 42. The cutoff value was determined by optimal balance of diagnostic sensitivity and specificity as 0.2 (Figure 2.2). Greater than 99% diagnostic sensitivity and specificity were achieved in PRRSV NA N antigen protein-based FMIA. Validate this cutoff S/P ratio (0.2) in 711 PRRSV negative field pigs, indicates good specificity (Figure 2.3)

Coupling reproducibility

To assess reproducibility of different coupling batches of each bead set, samples of each pathogen and controls were tested by FMIA. Each sample was run 18 times with coupling batch1 and 21 times with coupling batch2. Comparisons were performed between the two coupling batches by t-test (Table 2-2). The results show that 57.5% samples have statistical differences between two coupling batches according to MFI values. However, only 25% samples show statistical differences after S/P calculation.

Comparison of PRRSV FMIA with ELISA

In order to compare the PRRSV FMIA with the standard IDEXX ELISA test, 183 three week-old pigs were infected with PRRSV and bled at 4, 7, and 11 DPI. Serum samples were used to perform ELISA and FMIA. Serum samples were diluted to 1:400 in FMIA followed by standard protocol and data analysis to obtain results. The ELISA was performed by the KSVDL. Samples were diluted to 1:40 and 100ul of diluted samples was dispended into a 96-well antigen-coated plate (provided in IDEXX PRRSV Antibody Test Kit). The plate was incubated at room temperature for 30 minutes, the supernatant was dumped into a waste reservoir, and the assay plate was washed three times with 300 μL of wash solution. Anti-swine secondary antibody labeled with HRP (100ul) was dispensed into each well followed by incubation for 30 min, then the supernatant was dumped and the plate was washed three times with 300ul wash solution. TMB substrate at 100 μL was added to each test well and incubated for 15 min at room temperature, followed by100ul Stop solution to stop the reaction. The absorption values of samples and controls were measured at A_{650}. The presence or absence of antibody to PRRSV is determined by calculating the S/P ratio. If the S/P ratio is greater than or equal to 0.40, then the sample is classified as positive for PRRS antibodies, otherwise, this sample is classified as negative.

The results of FMIA were compared to the commercially available ELISA (IDEXX Laboratories, Inc.) (Figure2.4). At 4 days post infection (PI), FMIA indicated that 4% of samples were positive compared to 0% in ELISA. At 7 days PI, the percentage of positive samples was 93% in FMIA compared to 41% in ELISA. The two assays showed the same 100% positive

results in samples 11 days PI. FMIA showed higher sensitivity in day 4 and day 7 PI in comparison with ELISA (Table 2-1).

*Detection of PRRSV **pan-*Ig* and *IgM* antibodies***

Seven 3 week old pigs were infected with PRRSV. Serum samples were collected at 4, 7, 11, 14, 21, and 28 days post infection (DPI). Two FMIA assay were run using different secondary antibodies to detect swine pan-Ig (IgG and IgM) and IgM antibodies against PRRSV NA N protein. Goat anti-swine IgG (H+L) which can react with whole molecule swine IgG and the light chains of other swine immunoglobulins was used to detect pan-Ig. Goat anti-swine IgM which reacts specifically with swine IgM was used to detect IgM.   The results are presented in Figure 2.5. Anti-PRRSV IgM antibodies appeared in serum by 7 DPI and reached a peak by 11 DPI, and then declined rapidly to low levels after 2-3 weeks post infection. Anti-PRRSV pan-Ig antibodies were first detected 7 DPI, peak at 14 DPI, and remained constant until 28 DPI. The trend of antibodies confirmed FMIA can be used to detect Pan-Ig and IgM antibodies in serum samples.

Comparison of single assays with the multiplex format

In developing the FMIA, assays are developed as single assays and combined into a multiplex format. After validating each individual FMIA in singleplex format, a multiplex (5-plex) assay was developed by combining the singleplex assays together. The multiplex assay was compared with the singleplex assays to check whether there was any cross-reactivity among bead sets. Each serum sample was first tested in a singleplex format and then testing in a multiplex format with all five bead sets. As show in Figure 2.6, Comparisons were performed between singleplex and multiplex assay of each bead set by Anova $\mathcal{F}$-test. All the P-values of $\mathcal{F}$-test were greater than 0.05, which suggested there was no statistically significant difference between multiplex and singleplex assay for each bead set. Thus, there is no cross-reactivity in the presence of multiple protein-coupled microspheres.

Figures and Tables

Figure 2.1 Two-fold dilution of positive controls (A) and negative controls (B)

The different color lines stand for different bead sets. The horizontal axis stands for dilutions of internal standard samples. MFI, mean fluorescence intensity
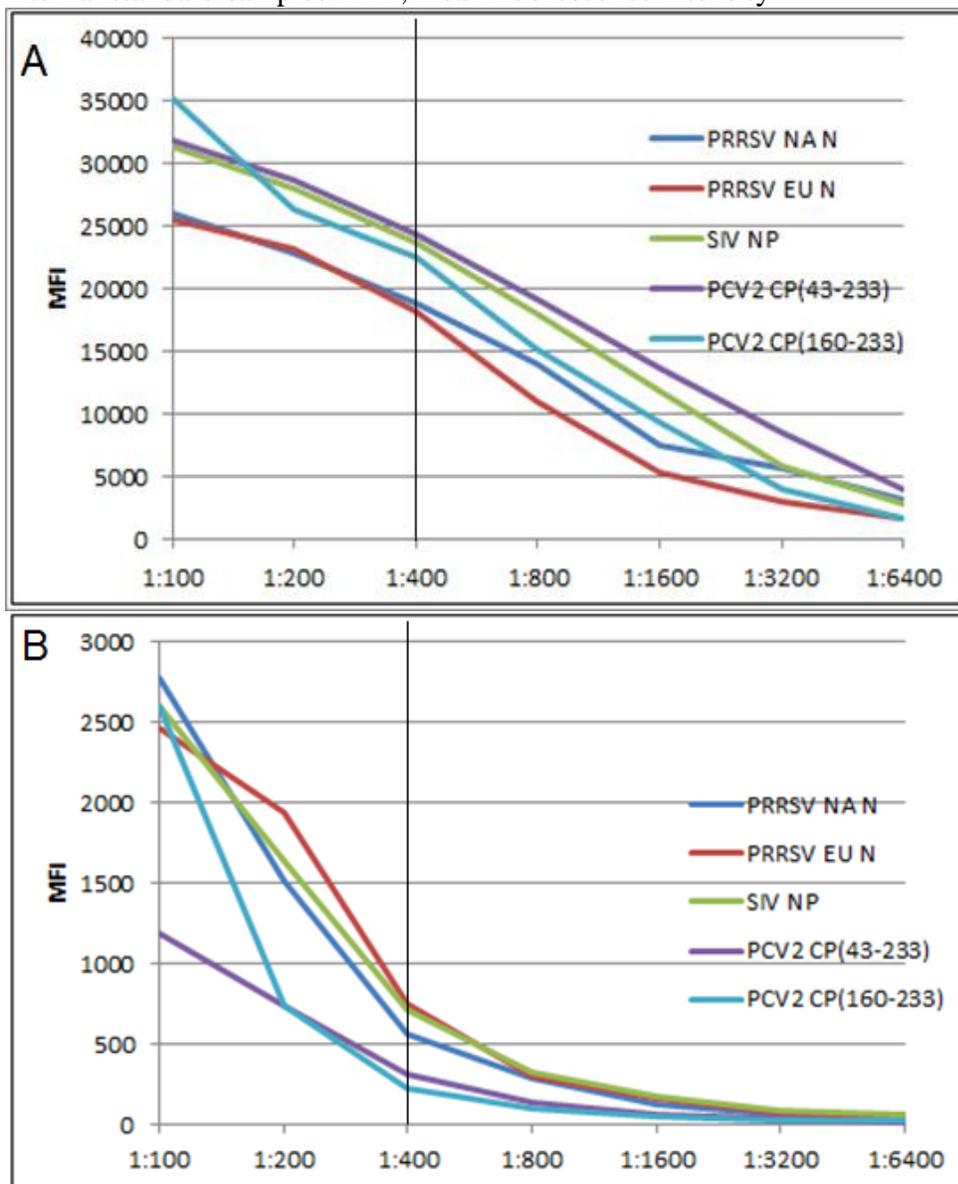
Figure 2.2 Diagnostic sensitivity and specificity of PRRSV FMIA.

The black x's are from pigs experimentally infected with PRRSV. The red x's were samples from a PRRSV negative herd. The horizontal dotted cutoff line between the positive and negative populations represents the cutoff value (99.6% diagnostic sensitivity and 100% specificity).The vertical axis is the S/P ratio
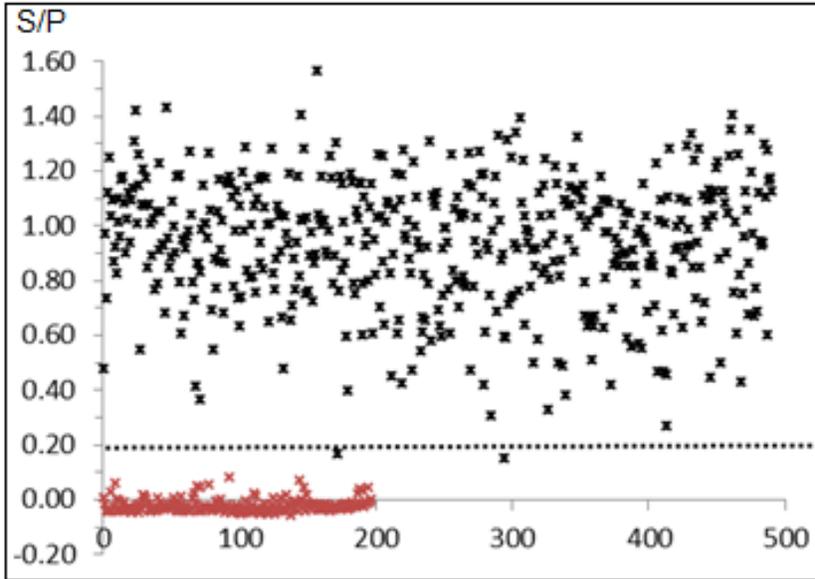
Figure 2.3 Validate cutoff value of S/P ration in PRRSV negative field pigs

The black x's are from field pigs without PRRSV infection.

21
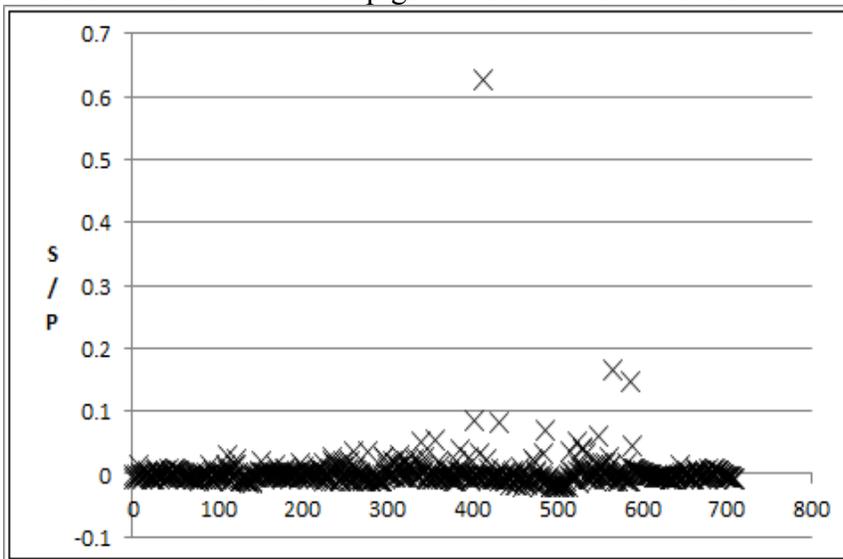
Figure 2.4 Comparison of PRRSV FMIA and ELISA
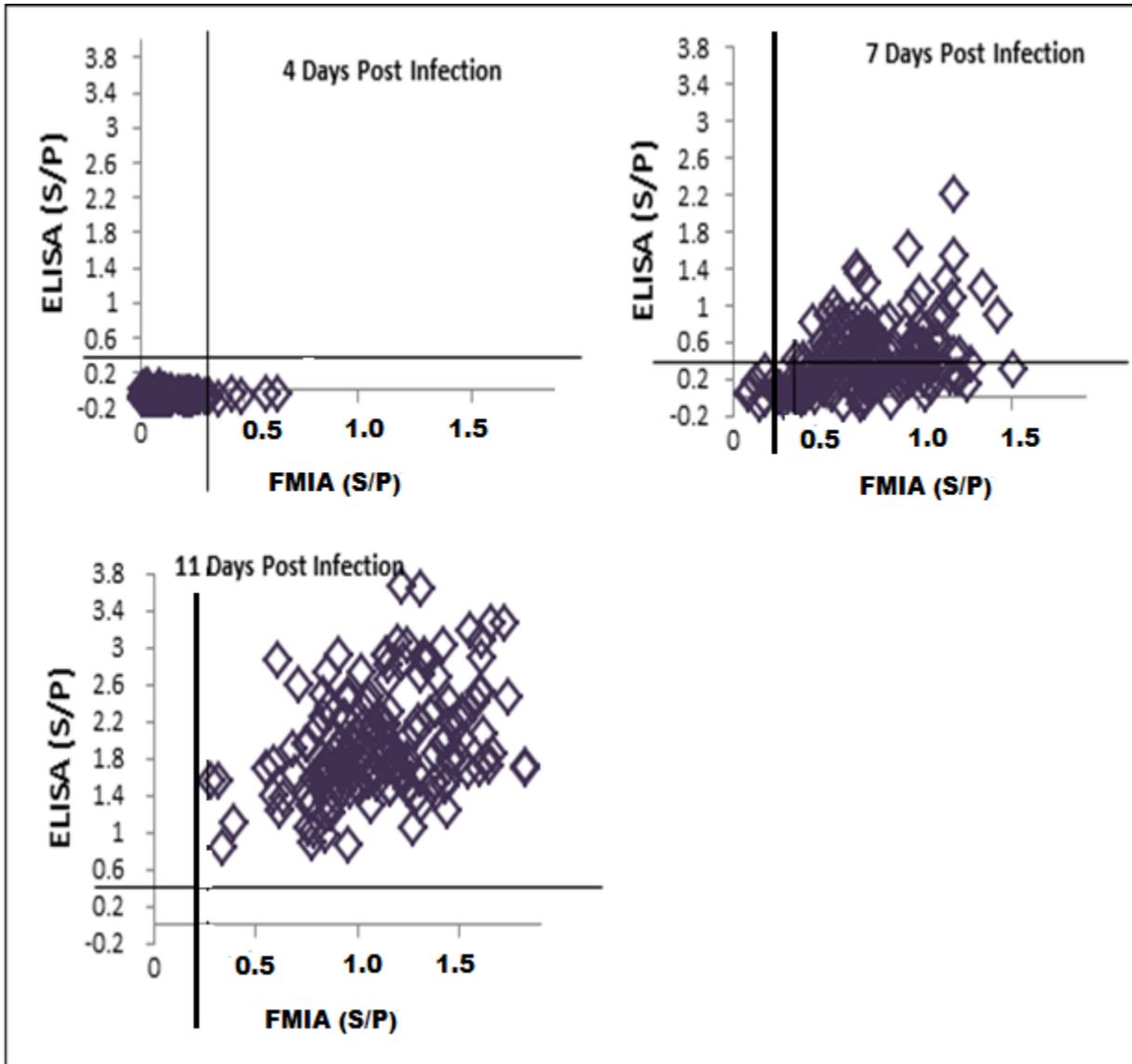
Figure 2.5 IgM and pan-Ig responses following PRRSV infection

Figure 2.6 Comparison of assay run as a "singleplex" (blue) versus multiplex format (red)

Table 2-1 Sensitivity comparison of PRRSV FMIA of ELISA

Test	Percent of samples showing a positive result		
	DAY 4	DAY 7	DAY 11
FMIA	4%	93%	100%
ELISA	0%	41%	100%

Table 2-2 Comparing between different coupling batches to estimate reproducibility

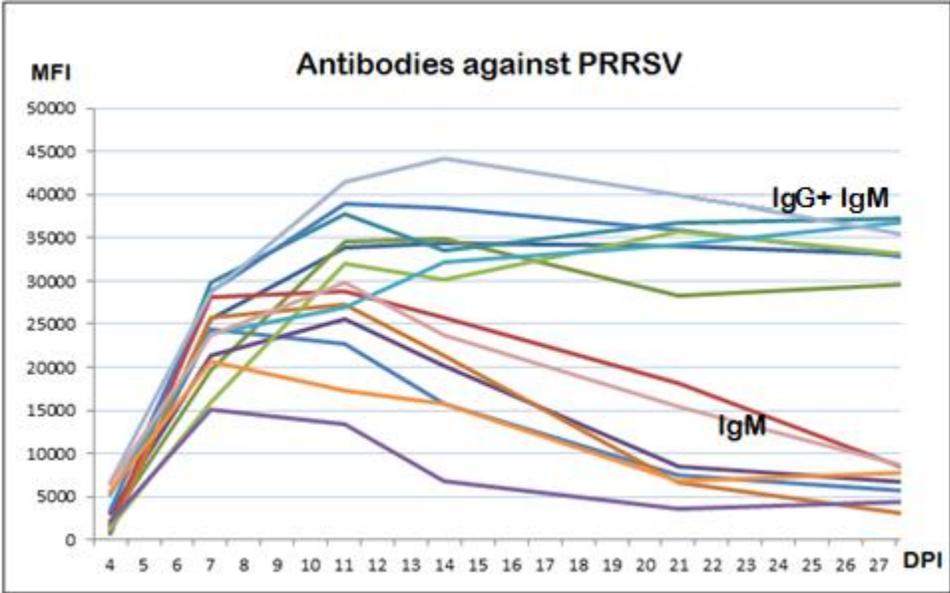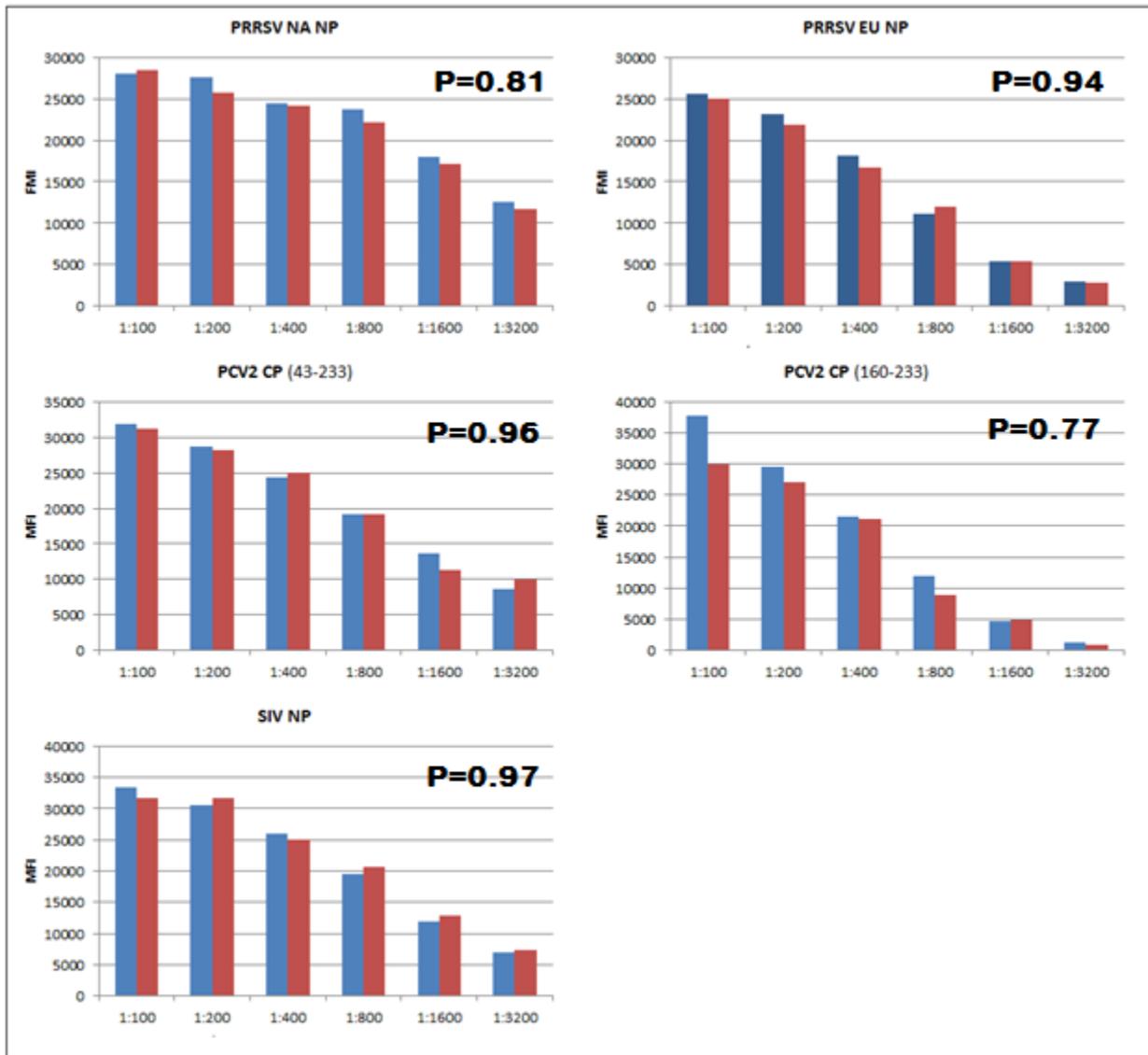| | | MFI | | | | | S/P | | | | |
| | | coupling1 | | coupling 2 | | P value | coupling1 | | coupling 2 | | P value |
	samples	mean	SD	mean	SD		mean	SD	mean	SD	NA
SIV	1	2282.2	373.8	3228.5	869.4	0.0001	0.071	0.013	0.094	0.022	0.0002
	2	1814.5	311.3	2133.9	572.2	0.034	0.052	0.008	0.054	0.014	0.49
	3	5150.8	1517.9	6401.9	1395.4	0.011	0.189	0.058	0.212	0.036	0.16
	4(positive)	24792.5	2516.2	27645.2	3120.9	0.003	1.000	0.000	1.000	0.000	NA
	5	12030.9	1589.3	14679.7	2200.1	0.0001	0.471	0.047	0.520	0.057	0.03
	6	467.3	131.9	479.7	123.6	0.76	-0.004	0.005	-0.006	0.004	0.08
	7	760.4	165.1	902.7	199.1	0.019	0.008	0.005	0.009	0.004	0.5
	8(negative)	554.8	109.7	648.2	131.6	0.02	0.000	0.000	0.000	0.000	NA
PCV2 (43-233)	1	23867.5	3039.5	24467.3	2169.8	0.49	1.243	0.202	1.283	0.172	0.51
	2(positive)	19620.2	3319.6	19531.3	3468.4	0.93	1.000	0.000	1.000	0.000	NA
	3	986.2	365.8	847.7	282.8	0.2	0.033	0.010	0.026	0.007	0.01
	4(negative)	330.7	123.0	328.5	95.4	0.95	0.000	0.000	0.000	0.000	NA
	5	31774.0	7200.9	30821.4	4399.3	0.65	1.656	0.122	1.604	0.141	0.24
	6	7948.4	2354.3	7010.1	1728.4	0.17	0.391	0.073	0.346	0.046	0.03
	7	30379.1	3375.3	29758.7	3077.9	0.55	1.575	0.131	1.560	0.189	0.76
	8	36200.7	10539.4	32653.4	4706.3	0.19	1.835	0.232	1.706	0.219	0.08
PRRSV NA	1	24496.2	3860.9	29255.7	3266.3	0.0002	0.801	0.101	0.914	0.105	0.001
	2	211.3	140.0	143.6	35.9	0.06	-0.013	0.007	-0.014	0.007	0.58
	3	13407.2	2396.9	19812.1	3874.9	4E-07	0.428	0.052	0.610	0.107	1E-07
	4	14510.4	2893.8	22824.4	4136.9	1E-08	0.463	0.056	0.706	0.109	5E-10
	5	7364.3	1243.5	13039.1	3257.5	8E-08	0.216	0.043	0.395	0.090	7E-09
	6(positive)	30697.2	5411.4	32018.7	2210.8	0.34	1.000	0.000	1.000	0.000	NA
	7	5803.2	1516.6	13200.1	4643.2	3E-07	0.173	0.034	0.399	0.136	2E-07
	8(negative)	602.0	335.5	596.8	238.0	0.95	0.000	0.000	0.000	0.000	NA
PRRSV EU	1(positive)	20534.5	2906.5	24662.4	2676.4	6E-05	1.000	0.000	1.000	0.000	NA
	2	8244.3	2519.1	10418.5	2372.5	0.009	0.389	0.127	0.409	0.071	0.56
	3(negative)	584.8	214.2	470.4	139.4	0.06	0.000	0.000	0.000	0.000	NA
	4	17487.3	3592.0	21481.1	3293.3	0.001	0.863	0.212	0.866	0.070	0.94
	5	6434.8	1419.0	8660.2	1911.3	0.0003	0.282	0.120	0.337	0.057	0.1
	6	12687.4	2829.5	14895.4	3170.6	0.02	0.625	0.192	0.594	0.102	0.54
	7	14832.2	1531.7	17624.9	2523.0	0.0002	0.732	0.153	0.708	0.053	0.54
	8	1334.4	570.2	1105.0	280.3	0.13	0.040	0.025	0.027	0.012	0.053
PCV2(160-233)	1	1038.2	250.6	1576.9	546.1	0.0003	0.015	0.014	0.034	0.022	0.003
	2	2738.4	858.9	2700.9	868.8	0.89	0.098	0.034	0.088	0.025	0.3
	3	259.3	81.3	348.9	126.8	0.01	-0.024	0.012	-0.026	0.007	0.61
	4	284.8	110.5	412.1	127.2	0.001	-0.022	0.013	-0.023	0.009	0.87
	5(negative)	727.3	237.4	876.4	273.0	0.08	0.000	0.000	0.000	0.000	NA
	6	8389.7	2716.2	8556.8	1857.1	0.84	0.309	0.183	0.376	0.063	0.15
	7(positive)	21004.8	2517.6	21291.2	2793.0	0.74	1.000	0.000	1.000	0.000	NA
	8	36394.2	11562.7	32596.9	4713.6	0.2	1.756	0.549	1.559	0.181	0.16

*NA, not applicable   SD, standard deviation

Chapter 3 - Application of multiple FMIA--Simultaneous detection of antibodies against swine pathogens in feral swine

Introduction

Feral swine are free ranging swine which were introduced into the USA by early European settlers over 300 years ago. USDA Wildlife Services recently estimated that there are 3-6 million feral swine in the United States compared with 1-2 million in 2004. Their distributions appear to be expanding from 39 states (2004) to 45 states (SCWDS, 2012).   Recent studies have indicated that feral swine act as reservoirs of important viral and bacterial pathogens that affect commercial swine (Wyckoff *et al.*, 2009) (Corn *et al.*, 2009) (Corner, 2006). Disease agents may be transmitted by various routes, including direct contact (Hahn *et al.*, 1997) or indirect contact via contamination of food, water, or fomites (Pritchard *et al.*, 2005).

Feral swine might play an important role in the epidemiology of swine pathogens, such as swine influenza (SIV), porcine circovirus 2 (PCV-2), and porcine respiratory and reproductive syndrome (PRRSV), which affect commercial swine in the United States. The objectives of this study were to determine the prevalence of antibodies against SIV, PRRSV, and PCV2 in selected regions of Hawaii State and Texas State.

Materials and methods

Hawaii sample collection

Serum samples from 345 feral swine were collected from Oahu and Hawai'i islands in the state of Hawaii (Figure 3.1). Serum sample were collected by field biologists of wildlife service during 2007-2010 as part of the USDA disease surveillance program.

Texas sample collection

Serum samples of 230 feral swine were collected from Aransas, Calhoun, Coke, Leon, Webb and Zapata counties in the state of Texas (Figure 3.2). Serum sample collections were done by wildlife service's field biologists during 2007-2010 as part of the USDA disease surveillance program.

Luminex assay

Feral serum samples were diluted 1:400 in PBS-GS buffer and tested following the antibodies detection protocol as described previously. A panel of five selected positive serum samples and three negative serum samples were used as controls in all assays (Table 3.1).

Results

Seroprevalence for Hawaii

Seroreactivity was determined for pan-Ig and IgM antibodies against SIV NP, PCV2 CP (43-233), PCV2 CP (160-233), PRRSV NA N, and PRRSV EU N proteins (Figure 3.3). PCV2 pan-Ig antibody was the highest among these four pathogens at 61% followed by SIV at 7% and PRRSV EU at 5%. Prevalence of the PRRSV NA pan-Ig antibody showed the lowest positive percentage at 3.5%. The percentage of multiple infections in Hawaiian feral pig population is 7.8% (Figure 3.6). The 10% pan-Ig against PCV2 CP (160-233), which also show pan-Ig against PCV2 CP (43-233), indicate this 10% feral pigs have a low protective response. Co-infections of PRRSV and SIV with PCV2 were more frequent than co-infection of SIV with PRRSV (Figure 3.6). Two feral pigs were detected by pan-Ig as having antibodies against all 3 pathogens. IgM antibodies were detected against the 5 tested antigens which indicated the presence of an early stage of infection for all 4 pathogens. Seroprevalence of IgM was less than or equal to 3% (Figure 3.3).

The seroprevalence of the antigens was also analyzed for the sample collection year (Figure 3.4). The percentage of seropositive responses to PCV2 was relatively stable and PRRSV seroprevalence over time remained low. However, the percentage of seropositive responses to SIV increased from 0% of 2007 to 19% of 2010.

To investigate geography-specific pan-Ig and IgM antibody seroreactivity, the serum samples from each of the two islands were compared. The overall animal level seroprevalence indicates that Oahu Island has a higher rate of disease than Hawai'i Island for both pan-Ig and IgM (Figure 3.5).

Seroprevalence for Texas

Texas swine pathogens seroreactivity was determined for the pan-Ig and IgM antibodies against SIV NP, PCV2 CP (43-233), PCV2 CP (160-233), PRRSV NA N, and PRRSV EU N antigen proteins (Figure 3.7). The positive percentage for each antigen was determined by using the calculated cutoff values. Seroprevalence of the PCV2 pan-Ig antibody was the highest among the pathogens at 54% followed by SIV at 3% and PRRSV EU at 1%. Antibodies to PRRSV EU were not detected in sera from any of the 230 samples collected in Texas. IgM antibodies were detected against all 5 antigens which indicate the presence of an early stage of infection for all 4 pathogens. Seroprevalence of IgM was less than or equal to 4% (Figure 3.7).

To investigate geography-specific antibody seroreactivity, the samples from each of the 6 counties were compared (Figure 3.7). Webb County possessed the highest prevalence of PCV2 antibodies (89.7%) followed by Aransas County(82.3%) and Zapata County(56.9%). The other three counties were less than 41%. Coke County possessed the highest prevalence of SIV antibodies (13.6%) followed by Aransas County (8.6%). Antibodies to SIV were not detected in Calhoun, Leon and Webb Counties. Three serum samples were antibody positive for PRRSV NA, one in Zapata County, two in Webb County.

The seroprevalence of the antigens was also analyzed for the sample collection year (Figure 3.8). It can be seen that 2010 had the highest percentage of seropositive responses to all antigens, in particular the PCV2 antigen. Antibodies to SIV first appeared in 2010. Three serum samples were antibody positive for PRRSV NA, one was detected in 2008, while two were detected in 2010.

Figures and Tables

Figure 3.1 Map of Hawaii showing sample collection sites.

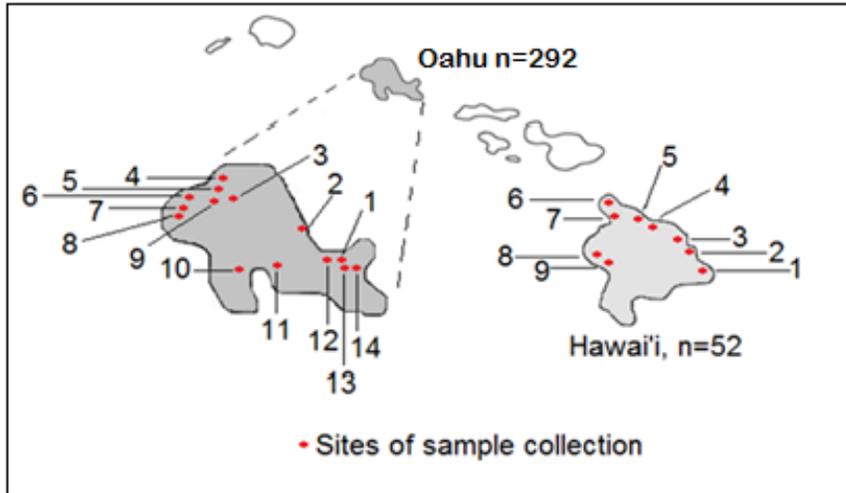Red dots represent the general locations of feral swine sample collection.

Figure 3.2 Map of Texas showing sites of sample collection.

Red colors show the location of the 6 counties where samples were collected.
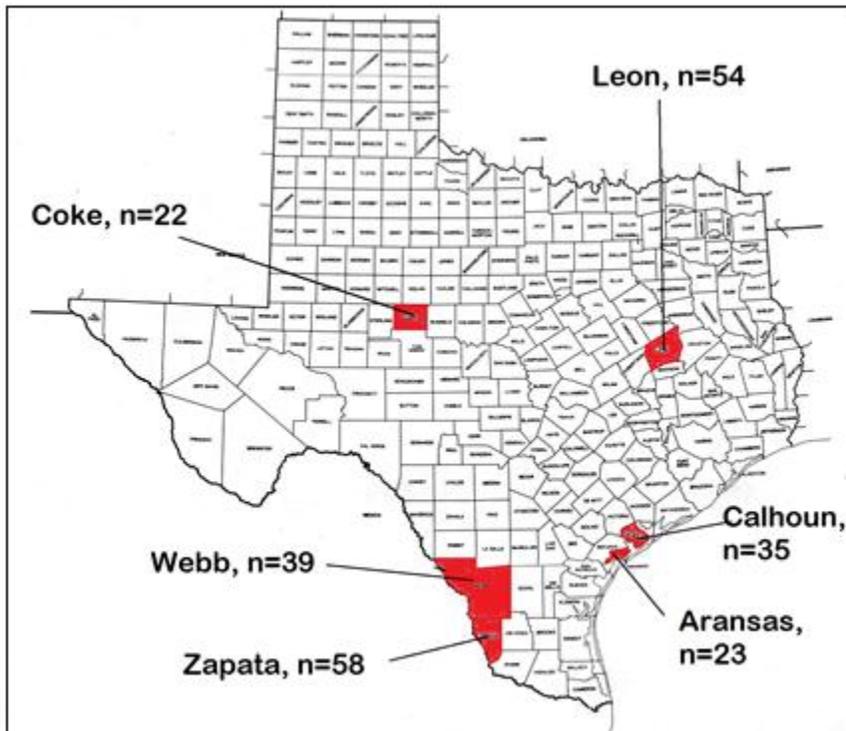
Figure 3.3 Seroresponses to PCV2 CP (43-233), PCV2 CP(160-233), PRRSV NA N, PRRSV EU N, and SIV NP antigen in feral swine populations in Hawaii.

Each circle represents one sample; horizontal lines represent cutoff values. Percentage values indicate seropositivity. MFI, median fluorescent intensity. S/P, sample over positive.
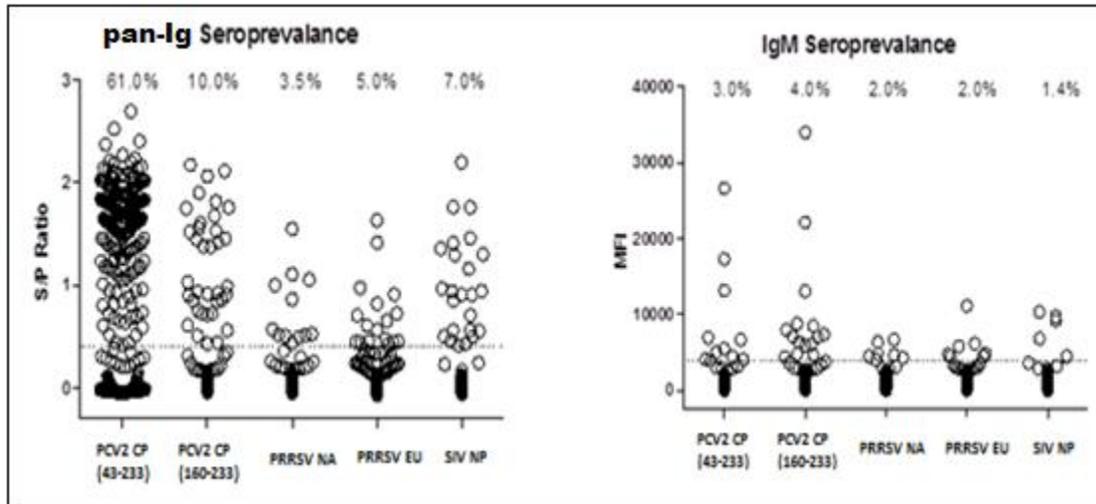

Figure 3.4 Sample collection year related seroprevalence of PCV2, PRRSV NA and SIV in Hawaiian feral swine populations.
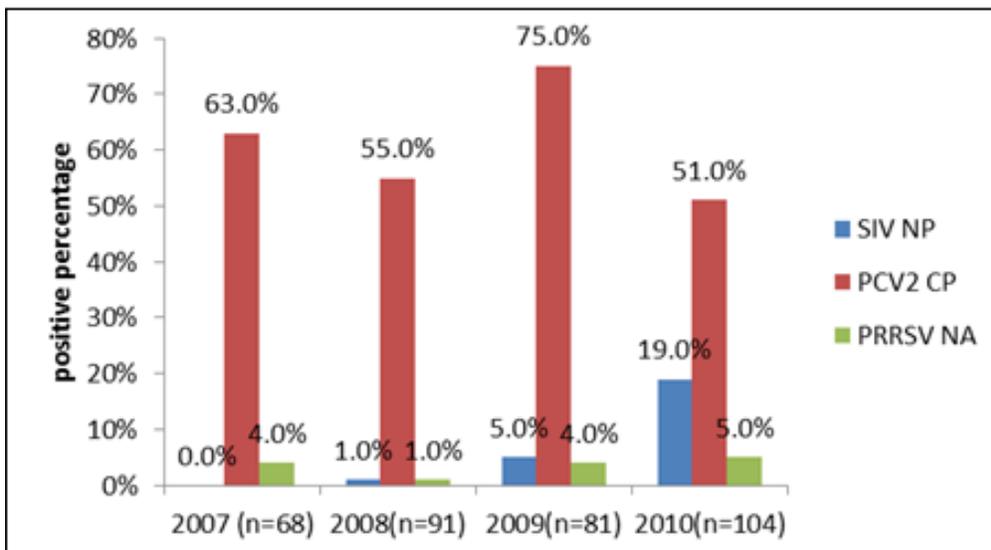
Figure 3.5 Geographic-related seroprevalence of PCV2, PRRSV NA and SIV NP pan-Ig (left) and IgM (right) antibodies in feral swine populations, state of Hawaii.

The population was divided into 2 islands: Hawai'i (n = 52) and Oahu (n = 293). Each circle represents one serum sample, and the horizontal lines represent cutoff values. MFI, median fluorescent intensity. S/P, sample over positive.
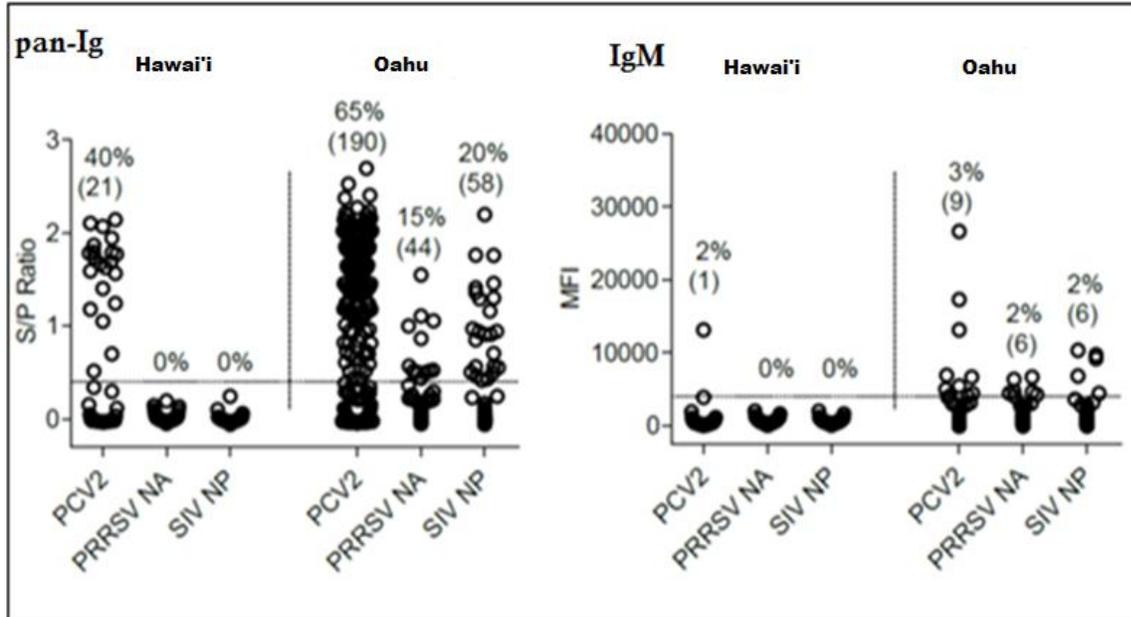
Figure 3.6 Pattern of pan-Ig antibody distribution in the Hawaiian feral pig population.

The numbers in the brackets indicate the total positive samples for the corresponding virus. The 20 PRRSV positive samples include PRRSV NA and EU.
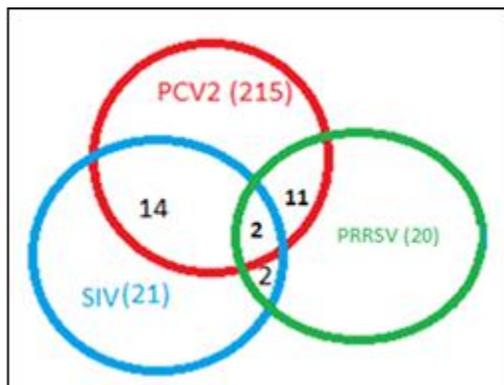
Figure 3.7 Seroresponses to PCV2 CP (43-233), PCV2 CP(160-233), PRRSV NA N, PRRSV EU N, and SIV NP in feral swine populations in Texas.
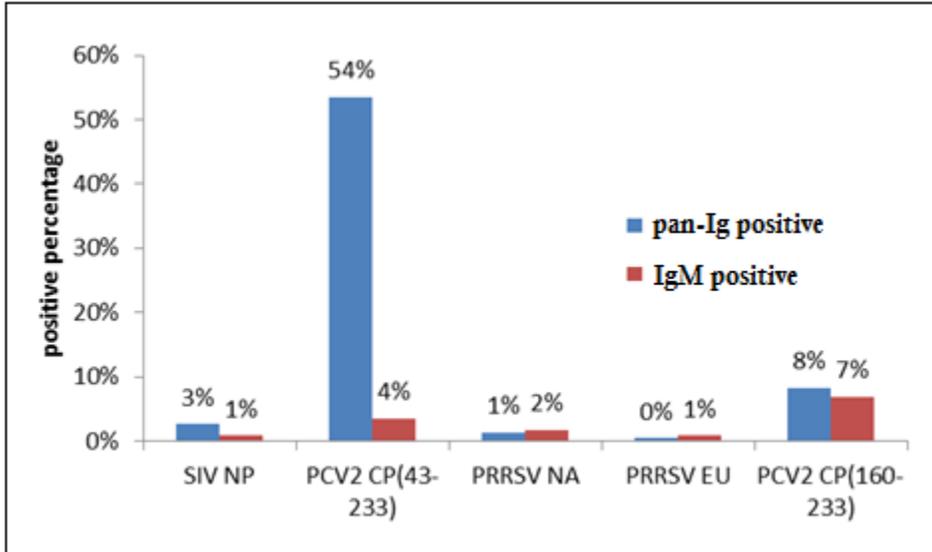

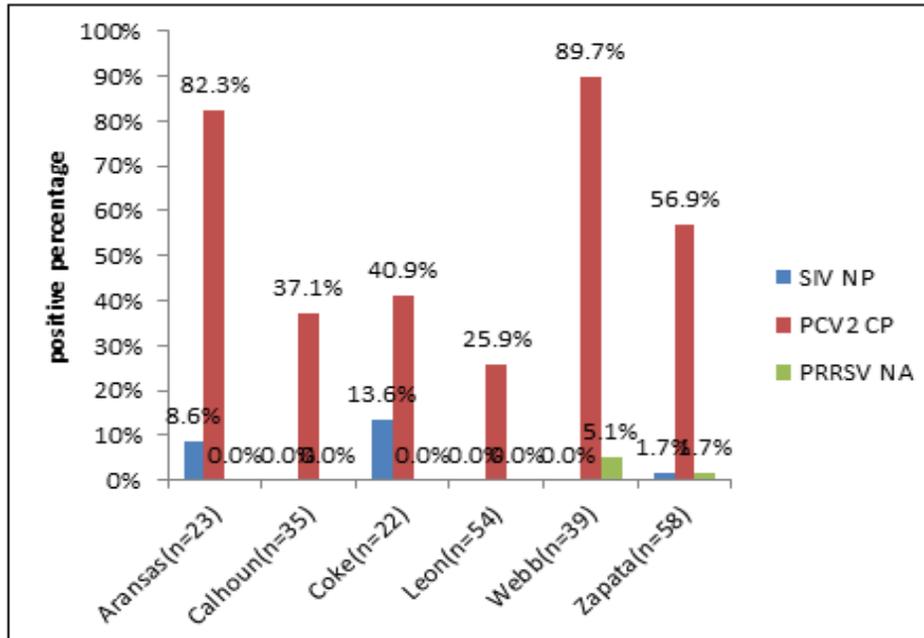**Figure 3.8 Seroprevalence in different Texan counties.**

Figure 3.9 Sample collection year related seroprevalence of PCV2, PRRSV NA and SIV pan-Ig antibodies in Texan feral swine populations
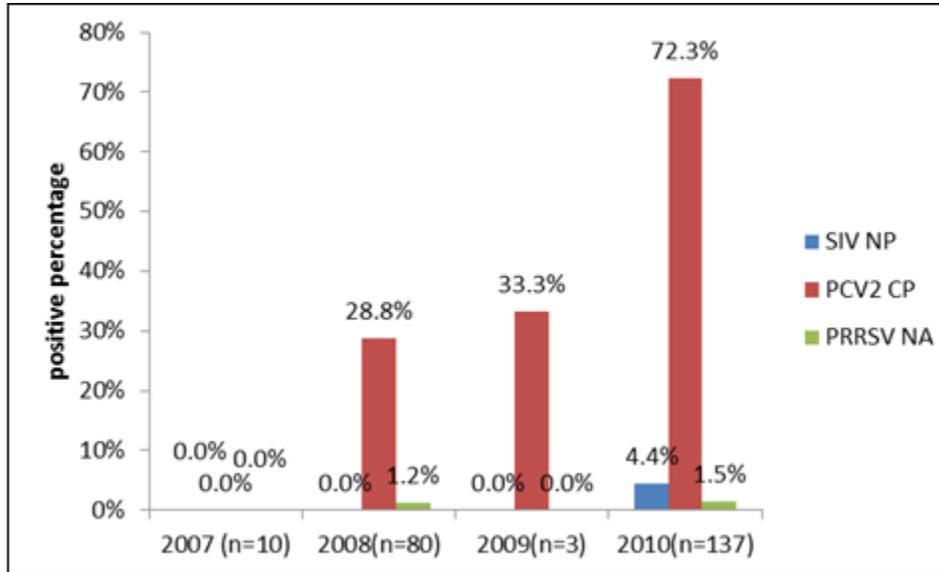

Table 3-1 Map of 96-well plate of FMIA.

Blank stands for blank control(PBS-GS buffer); P1 stands for PRRSV NA positive control; P2 stands for PRRSV EU positive control; P3 stands for PCV2 CP(43-233) and PCV2 CP(160-233) positive control; P4 stands for SIV positive control; P5 stands for PRRSV NA IgM positive control; N1 stands for PRRSV NA and SIV negative control; N2 stands for PCV2 negative control; N3 stands for PRRSV EU negative control; S1-S78 stand for feral serum samples 1-78.

Blank	P 4	N 3	S 7	S 15	S 23	S 31	S 39	S 47	S 55	S 63	S 71
Blank	P 4	N 3	S 8	S 16	S 24	S 32	S 40	S 48	S 56	S 64	S 72
P 1	P 5	S 1	S 9	S 17	S 25	S 33	S 41	S 49	S 57	S 65	S 73
P 1	P 5	S 2	S 10	S 18	S 26	S 34	S 42	S 50	S 58	S 66	S 74
P 2	N 1	S 3	S 11	S 19	S 27	S 35	S 43	S 51	S 59	S 67	S 75
P 2	N 1	S 4	S 12	S 20	S 28	S 36	S 44	S 52	S 60	S 68	S 76
P 3	N 2	S 5	S 13	S 21	S 29	S 37	S 45	S 53	S 61	S 69	S 77
P 3	N 2	S 6	S 14	S 22	S 30	S 38	S 46	S 54	S 62	S 70	S 78

Chapter 4 - Discussion

This study developed and validated a fluorescent microsphere immunoassay (FMIA) for simultaneous detection of antibody responses to three important swine pathogens: PRRSV, SIV and PCV2. This assay is the first immunoassay using antigen PCV2 CP (160-233) to detect infected animal.

The FMIA is a promising immunoassay for diagnosis and surveillance. It requires a smaller sample volume and less time and money compared with the traditional ELISA immunoassay. Moreover, it overcomes the limitation of the requirement for a single analyte per turn in ELISA. The performance of our FMIA was better in sensitivity compared to the commercial ELISA kit where PRRSV was detected in experimentally infected pigs.

In this study, we applied FMIA for epidemiological surveillance of feral swine populations in Hawaii and Texas. The seroprevalence of three economically important swine pathogens was evaluated. The antibodies against PCV2 show highest percentage of prevalence among these four pathogens in both Hawaiian and Texas feral swine populations. Hence we consider PCV2 as the most prevalent pathogen in feral pigs in Hawaii and Texas, and this pathogen poses the greatest threat to commercial pigs. SIV seroprevalence increased from 2007 to 2010 in Hawaii State, suggesting an increasing risk for commercial pigs. Moreover, yearly surveillance in Texas State shows an increase in seropositivity to all pathogens, particularly PCV2.

After investigation of geographic surveillance, we found antibodies against all three viruses were detected on Oahu, whereas, Hawai'i was only seropositive for PCV2. Moreover, antibodies to PCV2 indicate Oahu has a higher rate of disease compared with Hawai'i. There is also a difference in prevalence among counties in Texas State. Webb County and Aransas County possess higher prevalence of PCV2 antibodies (>80%); Calhoun County, Coke County and Zapata County show moderate prevalence of PCV2 antibodies (from 37% to 57%); Leon County possesses the lowest prevalence of PCV2 antibodies (26%).

The development of FMIA for detection of antibodies to multiple swine pathogens in serum samples offers an important alternative for swine disease surveillance in commercial and feral herds. Thus FMIA has the potential for detection of broad range of pathogens. Increasing the multiplexing capability of FMIA will result in a high-throughput and sensitive platform which could apply to diagnostic, epidemiological, and disease surveillance fields.

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