RESEARCH REPORT

SWINE HEALTH

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Title: Novel multiplex diagnostic assays development for diagnosis of porcine respiratory disease complex – **NPB #11-037**

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Industry Summary:

Porcine Respiratory Disease Complex (PRDC) is a significant economic problem for swine producers. PRDC outbreaks can cause elevated mortality, decreased feed efficiency, higher cull rates, increased days to market, and increased treatment costs. This syndrome is caused by the interaction of multifactorial etiologies, including the participation of porcine reproductive and respiratory syndrome virus (PRRSV), swine influenza virus (SIV), and porcine circovirus (PCV2) in the disease progression. In aid of PRDC prevention and control, producers and veterinarians desire multiplex tests that can readily detect multiple pathogens at the same time, in the same sample, and at a reasonable cost. In this study, we generated a panel of recombinant viral antigens and monoclonal antibodies (mAbs) for the diagnosis of PRRSV, SIV and PCV2 infections. Using these reagents, we developed a multiplex fluorescent immunomicrosphere assay (FMIA) for detection of PRRSV, SIV and PCV2 simultaneously using serum or oral fluid samples. To meet the demanding of on-site field test, we also antibodies in the field. At the meanwhile, standard reagents and protocols have established through this study. They have been sharing with major diagnostic laboratories and swine disease researchers. Development of rapid, multiplex, cost effective diagnostic tests will be important in population-based epidemiological studies, which provide important data on the early identification of susceptible groups in the population and evaluation of vaccination and herd management strategies. These assays present advantages of simplicity, rapidity, cost-effectiveness, and potentially increase the sample number of representative individual animals in a large population. The dipstick test is user-friendly format, and can be performed on-site in a swine farm by untrained personnel. It is expected that the dipstick test can be used as an on-site initial screening test to determine the disease status of a swine population in the field, and the multiplex FMIA will be used as a laboratory confirmation test for the accurate identification of various pathogens in PRDC. Importantly, these tests are more suitable in the PRDC surveillance program at a population level. Technologies developed in this study could apply to other swine pathogens, such as porcine respiratory corona virus (PRCV), *Mycoplasma hyopneumoniae* (M. hyo.), Swine Brucellosis. Our ultimate goal is to develop a rapid test to detect various swine pathogens simultaneously to help preventing and controlling of PRDC. For more information, please contact Dr. Ying Fang, South Dakota State University, Phone: 605-688-6647, E-mail: ying.fang@sdstate.edu.

Keywords: porcine respiratory disease complex, PRRSV, swine influenza virus, PCV2, fluorescent microsphere immunoassay, diagnostics.

Scientific Abstract:

Development of diagnostic tests that are able to rapid, simultaneously detect multiple pathogens offers an important tool for disease surveillance and control measurements. In this study, we have developed a multiplexed fluorescent microsphere immunoassay for simultaneously detection of specific antibodies in oral fluid and serum samples from animals infected with porcine reproductive and respiratory syndrome virus (PRRSV), swine influenza virus (SIV), and porcine circovirus (PCV2). Recombinant nucleocapsid proteins of PRRSV, SwIV, PCV2 were generated and used as antigen and covalently coupled to Luminex fluorescent microspheres. Based on an evaluation of 1444 oral fluid samples with known serostatus, the oral fluid-based multiplex FMIA achieved greater than 92.0% sensitivity and 80.4% specificity. In serum samples (n = 2484), the multiplex FMIAs reached greater than 98.2% sensitivity and 98.3% specificity. Time course studies showed that the assay can detect antibody responses to PRRSV, SIV and PCV2 as early as 14 days post infection and for greater than 90 days post infection in oral fluid and serum. For detection of acute infection, a monoclonal antibody-based PRRSV antigen capture multiplex FMIA has been explored. Using 113 nasal swab samples from experimentally infected pigs with SIV, the assay generated 98.6% sensitivity and 92.7% specificity for capturing SIV. However, the assay showed low sensitivity and specificity for PRRSV and PCV2. To meet the demanding of on-site field test, a sandwich immuno-chromatographic dipstick assay was developed to detect antibodies from animals infected with PRRSV, SIV and PCV2. The sandwich immunochromatographic dipstick assay was developed based on the high binding capacity of a specific pathogen antigen to swine antibodies, and the conjugation of swine immunoglobulin with colloidal gold nanoparticles as a color probe. Using nucleocapsid proteins (SIV, PCV2) and nsp7 (PRRSV) as antigens, dipstick assays were developed based on testing standard positive and negative control sera as well as a panel of field serum samples, and the diagnostic specificity and sensitivity were compared to the classical enzyme-linked immunosorbent assay (ELISA) or hemagglutination inhibition (HI) test. Based on the evaluation of 456 serum samples of known serostatus from pigs experimentally infected with either type I or type II PRRSV, the PRRSV dipstick assay showed an overall sensitivity and specificity of 95% and 96% respectively, in comparison with the IDEXX HerdCheck X3 ELISA. The inter-rater agreement (kappa value) between dipstick test and ELSA was 0.965. The SIV dipstick test achieved a sensitivity of 96% and a specificity of 99% using 177 field serum samples previously tested in HI assay, and the kappa value between dipstick and HI was 0.953. For PCV2 antibody detection, results from nucleocapsid protein-based dipstick test and ELIA were compared using 135 samples from experimentally infected animals. The sensitivity and specificity of PCV2 dipstick test were 88% and 92%, respectively. These multiplex assays are more suitable for large-scale field application on porcine respiratory disease surveillance and epidemiology studies. They present advantages of simplicity, rapidity and cost-effectiveness. The dipstick test is field deployable with a user-friendly format, which can be performed on-site in a swine farm by untrained personnel, while the multiplex FMIA will be used as a laboratory confirmation test for the accurate identification of various pathogens in PRDC.

Introduction:

As modern swine production has evolved, the U.S. has been instrumental in developing technologies to increase productivity and efficiency. However, even with these advances, swine producers and veterinarians are suffering loses as a result of poor performance caused by infectious disease. Porcine Respiratory Disease Complex (PRDC) seems to have evolved with modern swine production. It is caused by the interaction of multifactorial etiologies, with both viral and bacterial organisms playing a major role in disease initiation and progression. The complex interaction between several infectious agents makes control difficult if not possible. The most common viral pathogens associated with the PRDC are porcine reproductive and respiratory syndrome virus (PRRSV), swine influenza (SIV), porcine circovirus (PCV2), and porcine respiratory corona virus (PRCV). In combination with bacterial organisms, these pathogens can sufficiently compromise the pig's respiratory immune defense system, resulting in severe respiratory disease. PRDC outbreaks can cause elevated mortality, decreased feed efficiency, higher cull rates, increased days to market, and increased treatment costs. PRCD has been reported to have resulted in morbidity as high as 70% with mortality averaging between 4 to 6%. According to a survey by the National Animal Health Monitoring Systems (NAHMS) on swine health and management, it is reported that the costs of PRDC can range from $0.21/pig in feeder pigs to $1.34/pig in a farrow to finish operation.

Since the PRCD presents a substantial challenge to both veterinarians and producers, in order to combat this problem, a rapid diagnostic detection method is essential for controlling of the disease. With the previous support from NPB (grant #09-234), we developed a fluorescent immunomicrosphere assay (FMIA) to detect the PRRSV infection in serum and oral fluid samples. In current study, we explored the feasibility of expending this PRRSV FMIA techology into a multiplex assay to simultaneously detect antigens and antibodies from PRRSV, SIV and PCV2 infected animals. The natural characteristics of the FMIA system are ideal for multiplex assay development. The FMIA uses Luminex technology with the BioRad Bioplex instrumentation (BioRad Laboratories, Austin, TX). Recent studies from our laboratory showed that FMIA is about 10 times more sensitive than traditional ELISA formats (Langenhorst et al., 2012). FMIA requires minimal amount of coating antigens or antibodies (50-100 ug recombinant protein or monoclonal antibody per 3.125×10^6 microspheres for ten 96-well plates), which can detect about 100 pg/ml of host antibodies or less than 100 copies of viral antigens in a diagnostic sample. Another important characteristic of the technology is that FMIA allows uniform detection of multiple antigens and antibodies simultaneously within a small volume of sample. Therefore, the assay is well suitable for diagnosis of PRDC.

In addition to complex assays, such as Luminex, producers and veterinarians need a rapid and simple test that can be directly used in the field. Therefore, the second approach is to develop sandwich immunochromatographic assays. Immunochromatographic assay, also called lateral flow assay or simply dipstick assays. This technology is based on an immuno-chromatographic procedure that utilizes the property of high affinity monoclonal antibodies that recognize pathogen antigens (antigen capture) or the binding capacity of specific pathogen antigens to host antibodies (antibody detection). It has been widely used in the clinical medicine for human disease diagnosis, such as leptospirosis, *Salmonella typhi,* cholera, influenza, and West Nile (Keitel et al., 2010; Wang et al., 2006; Ryan et al., 2003; Hatta et al., 2002; Saengjaruk et al., 2002). The advantages of this assay include: user-friendly format, very short time to obtain test results, long-term stability of the dipstick (can store over a wide range of climates), and relatively inexpensive to make the strip and conduct the assay. These characteristics render it well suited for on-site testing by untrained personnel. In this study, we have developed sandwich immunochromatographic dipstick assays to detect antibodies from animals infected with PRRSV, PCV2 and SIV. The sandwich immunochromatographic dipstick assay was developed based on the high binding capacity of a specific pathogen antigen to swine antibodies, and the conjugation of swine immunoglobulin with colloidal gold nanoparticles as a color probe. Using nucleocapsid proteins (SIV, PCV2) and nsp7 (PRRSV) as antigens, dipstick assays were developed based on testing standard positive and negative control sera as well as a panel of field serum samples, and the diagnostic specificity and sensitivity were compared to the classical enzyme-linked immunosorbent assay (ELISA) or hemagglutination inhibition (HI) test.

Objectives:

Objective 1). To develop a FMIA multiplex test to simultaneously detect PRRSV, SIV and PCV2 antigens in serum and oral fluid samples.
Objective 2). To develop immunochromatographic dipstick tests for the rapid field diagnosis of porcine respiratory disease complex.

Materials & Methods:

Objective 1. To develop a FMIA multiplex test to simultaneously detect PRRSV, SIV and PCV2 antigens in serum and oral fluid samples.
Oral fluid and serum samples: For initial test development, a panel of 1100 field serum and oral fluid samples were used. They were collected from 10 barns, which are already run by RT-PCR for PRRSV, SIV, and PCV2. For the test validation, 180 oral fluid and 1195 serum samples with known SIV infection status was used. In addition, the set of 432 paired oral fluid and serum samples with known PRRSV infection status were characterized in from a previous study (Langenhorst et al., 2012), and a panel of oral fluid and serum samples (~1000 samples) with known PRRSV, PCV2 and SIV infection status were used.

Covalent Carbodiimide coupling of recombinant protein/mAb to fluorescent microspheres:
Fluorescent microsphere coupling was performed using a previously described method (Lawson et al., 2010). Briefly, 3.125×10^6 microspheres corresponding to a discrete spectral address were washed twice with 250ul activation buffer ($0.1MNaH_2PO4$, pH6.2) and sonicated for 60 s after each wash. For surface activation, 500ul of activation buffer containing 5 mg N-hydroxysulfosuccinimide (sulfo-NHS) and 5 mg N-(3-dimethylaminopropyl)-N-ethylcarbodiimide (EDC) (Pierce Chemical,Rockford, IL) was added to the microspheres and allowed to incubate 20 minutes at at room temperature. Activated microspheres were washed twice with phosphate-buffered saline (PBS) and sonicated after each wash. Coupling was initiated by the addition of 100 ug of the purified, recombinant nucleoprotein and brought to a final volume of 500 ul PBS. The coupling reaction was allowed to incubate in the dark for 3 h at room temperature with constant mixing. Coupled microspheres were washed once with 1 ml of PBS containing 0.05% NaN3 plus 1.0% bovine serum albumin (PBS-NB) and blocked with an additional 1 ml of PBS-NB for 30 min to reduce nonspecific binding. Microspheres were then washed twice and resuspended in PBS-NB to a final concentration of 2.0×10^6 antigen-coupled microspheres/ml. The amount of recombinant protein coupled to a microsphere was optimized by performing multiple coupling reactions with varying amounts of antigen (between 500 ug & 25 ug per reaction). The amount of antigen was titrated against a fixed number of microspheres (3.125×10^6) and then tested to generate a maximal signal-to-noise ratio using standard oral fluid or serum containing SIV antibodies.

Determination of coupling efficiency: of the recombinant protein to the microspheres, 2.5×10^3 antigen-coupled microspheres were added to each well of a 96-well microtiter filter plate that was prewetted with 20 ul of PBS-NB. A solution of PBS-NB containing 1.0 mg/ml of each anti SIV-NP monoclonal antibody (mAbs 42-100 & HB-65), was serially titrated with 10-fold dilutions in PBS-NB. Fifty microliters of serially diluted antigen-specific mAb was added to corresponding wells containing coupled microspheres and allowed to incubate at room temperature for 1 h on a plate shaker. After washing of the microspheres with PBST (PBS plus 0.05% Tween 20) three times, 50 ul of goat anti-mouse IgG conjugated to streptavidin–R-phycoerythrin (SAPE, Invitrogen) was added to the antigen-antibody-microsphere mixture at a final concentration of 10 ug/ml in PBSNB. The microsphere/antibody-conjugate complex was incubated for 30 min at room temperature. Microspheres were subsequently washed with PBST three times and re-suspended in 125 ul of PBST. The microspheres were then transferred to a 96-well polystyrene optical plate. Uncoupled microspheres were included as a negative control. Microspheres were analyzed via the fluidics of a dual-laser Bio-Rad Bio-Plex 200 instrument. Data were analyzed with Bio-Plex Manager software (version 6.0). The mean fluorescence

intensity for 50 microspheres was recorded at each titration point, and a logarithmic regression curve was generated. Relative coupling efficiencies for each antigen-coated microsphere were determined by analyzing the MFI at each dilution point and interpolating the area under the linear portion of the curve.

Antigen-capture FMIA: Initially, a 96-well plate with 1.2 um filter was blocked with 150 ul of PBS-NB then aspirated via vacuum manifold and wetted with an additional 20 of PBS-NB buffer. Virus standards derived from cell culture supernatants were serially diluted ten-fold in serologically negative porcine serum or oral fluid. Next, porcine test serum or oral fluid was diluted 1/50 or 1/2 with in PBS-NB. After thorough mixing, oral fluid or serum standards were added to duplicated wells along with the testing samples. For the antigen capture, 2500 antibody-coupled microspheres were added to each in a volume of 50ul PBS-NB. All incubations were performed in the dark by sealing the plate with foil and incubated at 4^oC overnight on a plate shaker rotating at a speed of 500 rpm. The plate was then aspirated via vacuum manifold and washed three times with 200ul of PBST. Next, 50ul of a biotinylated, secondary anti-SIV, anti-PRRSV and anti-PCV2 monoclonal antibody was added at an optimized dilution to each well and incubated for 1 hour at room temperature. The plate was washed 3x with PBST and 50ul of conjugate solution containing 10mg/ml streptavidin r-phycoerythrin (SAPE) in PBS-NB was added to each well and incubated for 30 min at room temperature with shaking. The plate was then aspirated and washed 3x with PBST. Finally, the microspheres were resuspended in 125 ul of PBST per well and transferred to a clear 96-well polystyrene optical plate. Fluorescent signals were measured by dual laser detection system (Bio-Rad, Bio-Plex) as described previously. All reported mean fluorescence intensity (MFI) measurements were background corrected (normalized) and the final concentration of each virus was determined by interpolating the MFI of each analyte within the linear range of the regression curve generated by the standards.

Antibody detection FMIA: A 96-well hydrophilic membrane filter plate was blocked for 2 min with 150ul of PBS-NB, and then aspirated via a vacuum manifold and wetted with an additional 20ul of PBS-NB buffer. Fifty microliters of serum or oral fluid sample (diluted 1/50 or 1/2 in PBS-NB) was added to duplicate filter plate wells along with 50 ul of PBS-NB containing 2.5×10^3 of each antigen-coupled microspheres (1.0×10^4 microspheres total per well for the 4-plex assay). Since the microspheres and reporter moiety are light-sensitive, all incubations were performed in the dark by sealing the plate with foil. For the serum FMIA, the plate was incubated at room temperature for 1 h on a plate shaker rotating at 500 rpm. For the oral fluid FMIA, the plate was incubated at 4^0C overnight. The plate was washed three times with 150ul of PBST. For the serum FMIA, 50ul of biotinylated IgG (1:5000 dilution in PBS-NB, Jackson ImmunoResearch Laboratories) was added to the filter plate and incubated at room temperature for 1h. The oral fluid FMIA was treated the same as the serum FMIA except that biotinylated IgA and IgM (1:5000 dilution in PBS-NB, Bethyl laboratories) were added along with the biotinylated IgG as secondary antibodies. After incubation with secondary antibodies, 50ul of SAPE (2.5 ug/ml in PBS-NB) was added to each well and incubated for 30 min at room temperature with shaking. The supernatant was aspirated, and the plate was washed three times with PBST. Finally, the microspheres were re-suspended in 125ul of PBST per well and transferred to a clear 96-well polystyrene optical plate. Coupled microspheres were analyzed through the dual laser Bio-Rad, Bio-Plex 200® instrument. The MFI for 100 microspheres corresponding to each individual bead analyte was recorded for each well. All reported MFI measurements were normalized via F-Fo, where Fo was the background signal determined from the fluorescence measurement of a test sample in uncoated beads, and F was the MFI from a serological test sample in antigen-coated beads.

Objective 2. To develop an immunochromatographic dipstick tests for the rapid field diagnosis of porcine respiratory disease complex.
Dipstick test design: Two dipstick tests were explored: the "antigen capture" dipstick assay versus the "antibody detection" dipstick assay. For antigen capture dipstick assay, a pair of high-affinity anti-PRRSV, anti-SIV or anti-PCV2 mAbs was used for preparing the test strip. For antibody detection dipstick assay,

recombinant viral proteins were used as the test antigen for preparing the strip. A schematic description of the immunochromatographic dipstick test device is shown in Figure 1. The dipstick was prepared using a cellulose acetate supported strip of nitrocellulose membrane (HF180MC100, 0.5 x 25 mm), and the reagents were dotted on the membrane. In the detection zone, the control antibody and mAbs (or recombinant proteins) specific to PRRSV, SIV or PCV2 were separately spotted onto control region (C) and test region (T). For antigen capture assay, the virus specific mAb-coated colloidal gold particles were spotted near the other end (bottom) of the strip. For antibody detection assay, the detection reagent consists of a mAb that recognizes the FC region of the swine immunoglobulin, which was conjugated to colloidal gold. The control Ab consists of a anti-staphelococcus protein A mAb. . When a sample is placed on the sample application site, the combined solution of test sample and detection reagent rise up the membrane and colloidal gold was deposited at the site of the solid-phase antibody. The specific virus-binded mAb/gold particle complex (antigen capture) or viral antigen/swine antibody/gold partical complex (antibody detection) was trapped in the test region, while mAb-gold particle was reacted with control antibody and trapped in the control region.
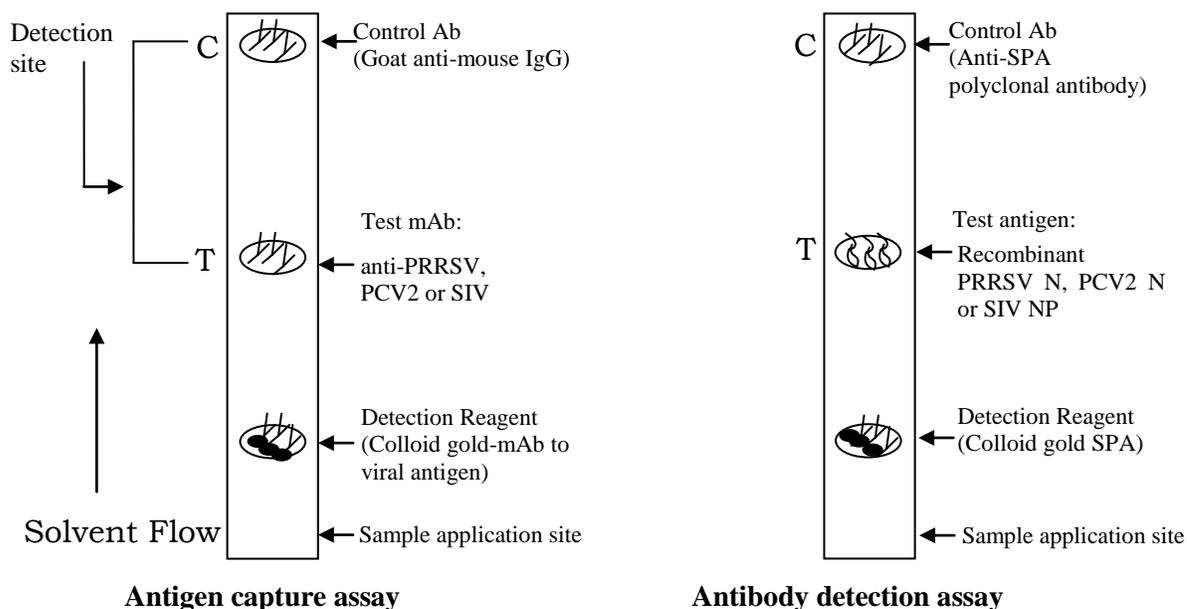
Figure1. The schematic presentation of the immunochromatographic test strips. T: test antibodies; C: control antibodies.

Colloidal gold-mAb conjugates preparation: Three mAbs 126-107, 42-100, and 39-29 were raised against PRRSV, SIV and PCV2, respectively. The mAb against Fc constant region of the swine Ig was purchased from an antibody company (Jackson ImmunoResearch Laboratories, Inc.). These mAbs were purified using a caprylic acid and ammonium sulfate method. Each antibody was conjugated to gold nanoparticles as previously described (Wang et al., 2007).

Immunochromatographic dipstick test development: The dipstick was prepared according to previously described methods (Hatta et al., 2002; Shyu et al., 2002). Briefly, pathogen specific mAbs or recombinant proteins were applied on the nitrocellulose membrane, and then air dried at room temperature. The membrane was blocked by incubating with 1% (w/v) polyvinyl alcohol dissolved in 20 mM Tris/HCl, pH 7.4 for 30 min at room temperature. The strip was washed with water and dried. To assist free mobility of the labeled reagent, the membrane was soaked to sucrose solution (5% w/v in water) and dried (We expect the dipstick can be stored at this stage, which can be carried to the field for testing). The assay was carried out by applying a sample to the bottom of the dipstick. After sample application, the test strip was washed with PBS Tween, and soaked into a silver enhancer for 5 min and fixed with sodium thiosulfate solution for 3 min at room
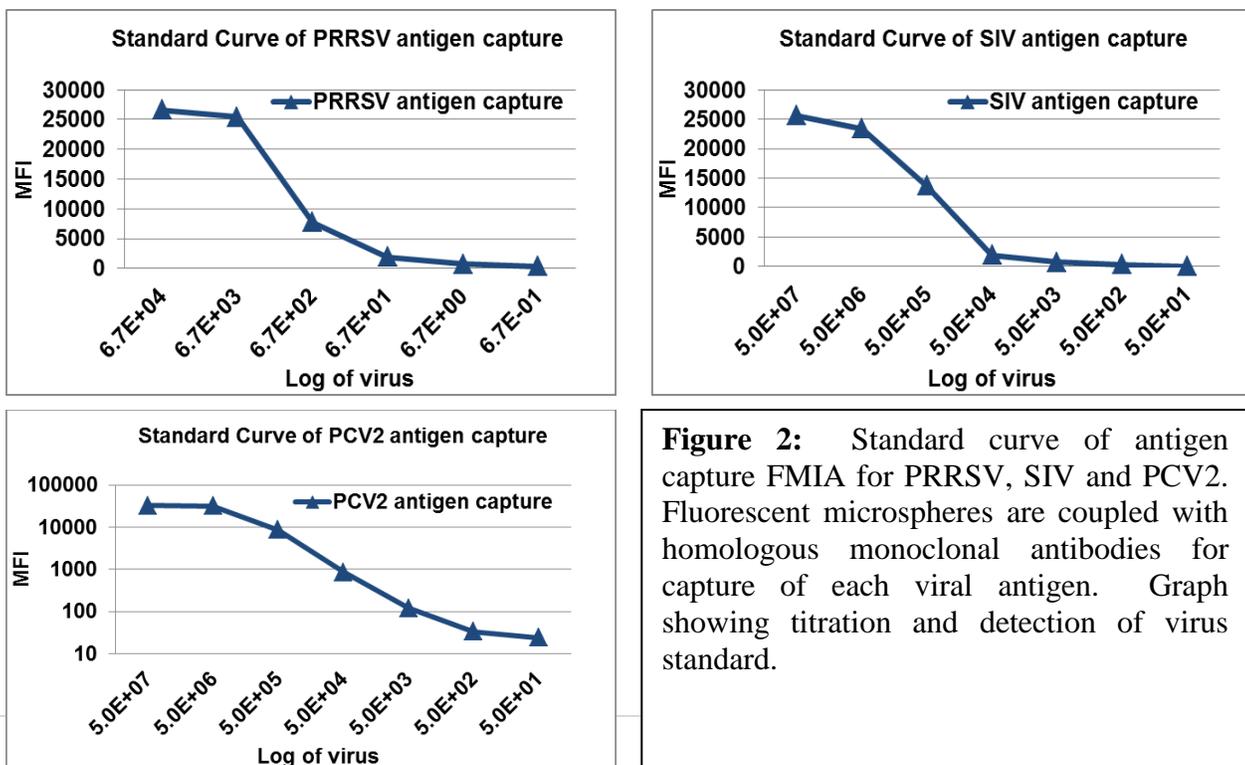
temperature. The optimal concentration of detection/testing antibody (antigen) was determined by testing the serial dilution of mAb (recombinant protein) (range from 0.1ug to 1ug) for each virus. The detection limit of the test was determined by spiking various concentration of virus or antibody in oral fluid or serum (10 fold serial dilution of $1x10^5$ $TCID_{50}$ virus stock; 0.1-1ug of antibody), and then assayed by the dipstick.

Data Analysis: Descriptive statistics for each assay was generated using JMP 7.0.2 (SAS Institute, Inc). Data was analyzed by multivariate analysis of variance (MANOVA) using time as the repeated measure. To assess the diagnostic sensitivity and diagnostic specificity, results were validated using the samples from two distinct animal populations. The negative-testing (non-infected) validation population was composed of samples from individual animals of negative control groups. The positive-testing (infected) validation population was composed of samples from experimentally infected animals. Receiver Operating Characteristic (ROC) was conducted for each assay to determine assay cutoffs and diagnostic performance using MedCalc® Version 10.4.0.0 (MedCalc® Software, Mariakerke, Belgium).

Results

**Objective 1.** To develop a FMIA multiplex test to simultaneously detect PRRSV, SIV and PCV2 antigens in serum and oral fluid samples.

Antigen-captured FMIA development: Initially, we generated standard virus stocks for antigen-captured FMIA development. Fluorescent microspheres were coupled with homologous mAbs for capture of a specific viral antigen in virus-infected cells. As shown in Fig. 2, PRRSV capture FMIA can detect greater than 6.7 x 10 $TCID_{50}$/ml of virus; SIV capture FMIA can detect greater than 5 x 10^4 $TCID_{50}$/ml of virus; while PCV2 capture FMIA can detect 1 x 10^4 $TCID_{50}$/ml virus. Although we were successfully developed a standard curve for PRRSV and PCV2 antigen capture in cell culture supernatant, we were not able to effectively capture native PRRSV and PCV2 in serum and oral fluid from field or experimental samples at any time point post infection. In contrast, using 113 nasal swab samples from experimentally infected pigs with SwIV, we obtained 98.6% sensitivity and 92.7% specificity for the SIV capture FMIA. We further tested 472 field samples, including 217 nasal swab, 120 field virus isolates and 135 lung tissues. Of these samples, 344 tested positive by PCR test, the FMIA showed 92.5% agreement with PCR result. However, when 128 field samples tested as negative by PCR test, the FMIA only showed 61.8% agreement with PCR result.

Figure 2: Standard curve of antigen capture FMIA for PRRSV, SIV and PCV2. Fluorescent microspheres are coupled with homologous monoclonal antibodies for capture of each viral antigen. Graph showing titration and detection of virus standard.

Antibody detection FMIA development

Expression of recombinant proteins: Recombinant nucleocapsid proteins from SwIV, PCV2, and PRRSV were expressed as His-tagged fusion proteins in *E. coli*. PCV2 was expressed at high levels, and was purified in soluble form. In contrast, recombinant PRRSV and SIV nucleocapsid proteins formed inclusion bodies, and a protein refolding step was performed. The purity of the recombinant proteins was evaluated using SDS-PAGE followed by Coomassie blue staining. As shown in Fig.3, all of the His-tagged recombinant proteins migrated according to their predicted sizes. These proteins showed greater than 95% purity. The protein concentrations were determined to be approximately 1.2-1.8 mg/ml. The identity of each protein was further confirmed by Western blot analysis with anti-His antibody. Each of these antigens were prepared in large amount as a stock, aliquoted and stored in -80^0C.
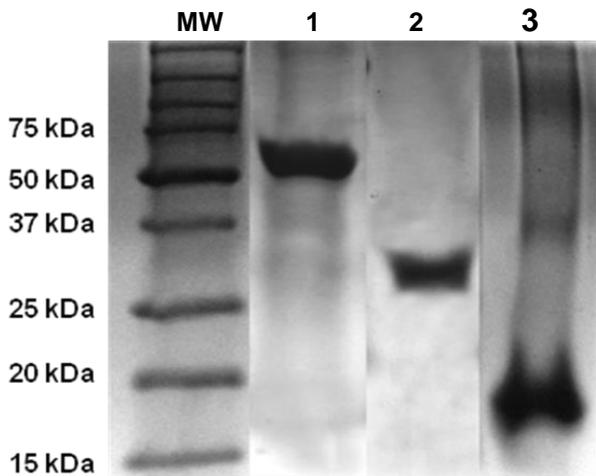
Figure 3. SDS-PAGE analysis of recombinant proteins expressed in *E. coli*. Purified recombinant proteins were separated on 12% polyacrylamide gel and stained with Coomassie Brilliant Blue G-250. The position of molecular weight (MW) marker is indicated at the left side of the panel. The remaining lanes represent SIV-NP protein (lane 1), PCV2 nucleocapsid (N) protein (lane 2), and PRRSV nucleocapsid (N) (lane 3).

Establishment of control standards: Two sets of internal control standards were established for each pathogen 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 PRRSV, SwIV and PCV2 nucleocapsid protein based-FMIA

Sample type	Standard (MFI*)	Antigen		
		PRRSV N	**SIV NP**	**PCV2 N**
Serum	**High**	25,000-29,000	25,000-30.000	25,000-30,000
	Low	12,000-15,000	4000-6000	12,000-15,000
	Negative	800-1,200	200-400	100-500
Oral fluid	**High**	25,000-29,000	7,000-10,000	15,000-19,000
	Low	12,000-15,000	2,000-4000	5,000-8,000
	Negative	800-1,200	100-400	150-700

*MFI: mean fluorescence intensity.

Test optimization: To determine the optimal concentrations for coupling each antigen, a series of couplings were performed using various concentrations of the antigen and analyzed against control standards in order to determine the optimal amount of antigen per microsphere. Four sets of beads, each containing 3.125 x 10^6 beads, were incubated with various concentrations (500ug, 250ug and 100ug) of purified PRRSV EU-N, NA-N, SIV N, or PCV2 N recombinant protein. Based on the highest signal-to-noise ratio for detection of virus-specific antibodies in standard oral fluid or serum, we determined that 250ug per coupling reaction (80ug

protein/ $1x\ 10^6$ microspheres) was the optimal concentration for the coupling of these recombinant proteins. The optimal serum and oral fluid dilution was determined by diluting serum and oral fluid samples in a $\log_2$ titration. In oral fluid FMIA, it was determined that a 1:3 dilution of oral fluid samples gave an optimal signal-to-noise ratio, while in serum FMIA, a 1:50 dilution of serum samples gave an optimal signal-to-noise ratio. The coupling efficiency of the antigen-coated beads was determined using antigen-specific monoclonal antibody in a $\log_{10}$ dilution. As shown in Fig. 4, relative coupling efficiency curves were generated, and an average correlation coefficient (R^2) for each antigen was calculated for all regression analytes within the linear portion of the curve. PRRSV and PCV2 antigens had similar coupling efficiency of $R^2 = 0.994$, while SIV had coupling efficiency of $R^2 = 0.997$.
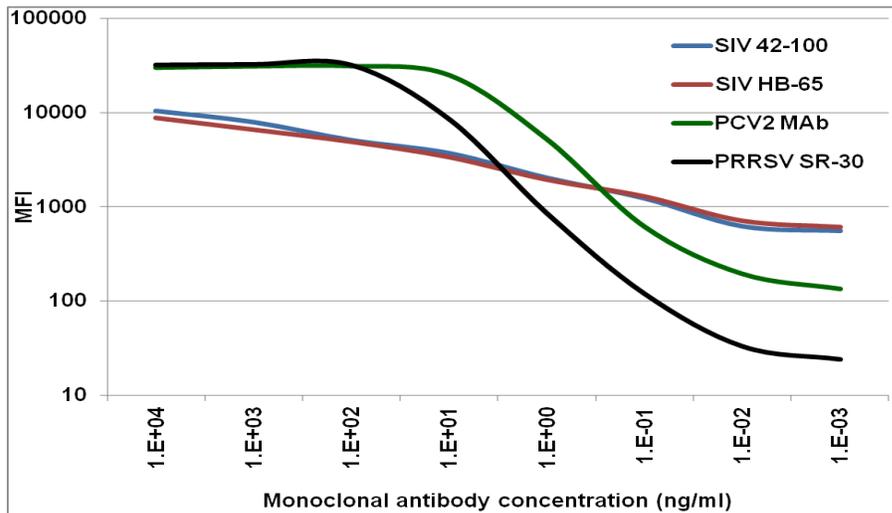

Fig. 4. Coupling efficiency for nucleocapsid proteins of PRRSV, SIV and PCV2. Coupling efficiencies of antigen-coated beads were determined using anti-nucleoprotein monoclonal antibodies (mAbs) developed in the laboratory. The commercial available mAb HB-65 was used as a control. Note that antigen-specific mAbs were detected at approximately 10pg/ml.

Cutoff determination, diagnostic sensitivity, and diagnostic specificity: GRAPH ROC software was used for ROC analysis of each FMIA to determine an optimized cutoff that maximizes both the diagnostic specificity and diagnostic sensitivity of each 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. These samples were obtained from experimental animals as described in the Materials and Methods section. Each test showed greater than 90% diagnostic sensitivity and specificity in oral fluid-based FMIAs, while greater than 95% diagnostic sensitivity and specificity were achieved in serum-based FMIAs. Table 2 summarizes the results of ROC analysis. Diagnostic sensitivity and specificity was generally lower for the oral fluid-based assays. This result is expected, since the antibody concentration is substantially higher in serum than in oral fluid (Langenhorst et al., 2012).

Table 2. Summary of ROC analysis of serum and oral fluid- based FMIA results

Antigen	Serum			Oral fluid		
	Diag cut off	Sensitivity	Specificity	Diag cut off	Sensitivity	Specificity
PRRSV Eu-N	0.54	100%	99.7%	0.31	95%	95.7%
PRRSV NA-N	0.34	99.3%	98.9%	0.18	100%	97.5%
SIV NP	0.19	98.2%	98.3%	0.16	90.0%	80.4%
PCV2 N	0.20	98.4%	98.5%	0.37	95.8%	90.0%

Assessment of test repeatability: The precision of each individual FMIA was determined using internal control standards. Table 3 shows the intra-assay or inter-assay repeatability for each test. Both intra-assay and inter-assay repeatability are less than 10% coefficient of variability for all the tests, which suggests that these FMIAs are highly repeatable in diagnostic applications.

Table 3. Assay repeatability of oral fluid and serum-based FMIAs

Repeatability	PRRSV NA-N	PRRSV EU-N	SIV NP	PCV2 N
Serum Intra-assay repeatability (%CV)	1.9	1.7	5.6	2.0
Serum Inter-assay repeatability (%CV)	4.7	3.4	8.6	2.2
Oral fluid Intra-assay repeatability (%CV)	3.2	1.5	5.7	4.2
Oral fluid Inter-assay repeatability (%CV)	3.0	1.4	8.7	6.2

Development of a multiplex assay: Once we validated each of individual FMIA in single-plex format, we combined the single-plex format into a 3-plex assay for each type of samples (oral fluid or serum). The 3-plex assay was compared with single-plex to determine whether there was any cross-reactivity among bead sets. Each oral fluid and serum internal control standard along with their individual corresponding bead set was first tested in a single-plex format and then combined to test in a 3-plex format. Correlation coefficients were determined for comparison between each individual FMIA and the 4-plex assay. As shown in Figure 5, there is no statistical difference between multi-plex and single-plex analytes at any time point for both oral fluid and serum-based assay.

Objective 2. To develop immunochromato -graphic dipstick tests for the rapid field diagnosis of porcine respiratory disease complex.

Expression of recombinant proteins: Recombinant proteins of nucleocapsid proteins from SIV, PCV2, and both genotypes of PRRSV nsp7 were expressed as His-tagged fusion proteins in *E. coli*. PCV2 NP and PRRSV nsp7 were expressed at high levels, and was purified in soluble form. In contrast, recombinant SIV N proteins formed inclusion bodies, and a protein refolding step was performed. The purity of the recombinant proteins was evaluated using SDS-PAGE followed by Coomassie blue staining. As shown in Fig.6, all of the His-tagged recombinant proteins migrated according to their predicted sizes. The protein concentration was determined to be approximately 1-3 mg/ml. The identity of each protein was further confirmed by Western blot analysis with anti-His antibody. Each of these antigens were prepared in large amount as a stock, aliquoted and stored in -80^0C.
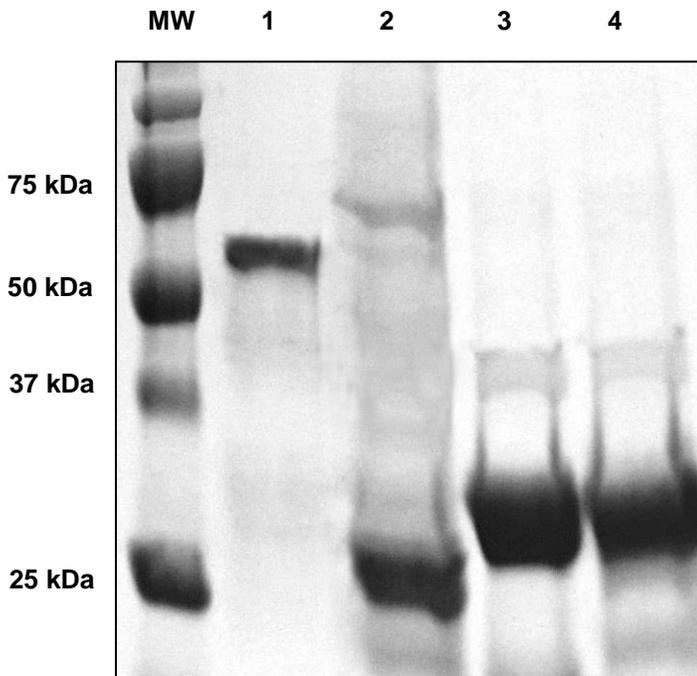
Figure 6. SDS-PAGE analysis of recombinant protein expressed in *E. coli*. Purified recombinant proteins were separated on 12% polyacrylamide gel and stained with Coomassie Brilliant Blue G-250. The position of molecular weight (MW) marker is indicated at the left side of the panel. The remaining lanes represent SIV-NP protein (lane 1), PCV2 nucleocapsid protein (lane 2), type II PRRSV nsp7 (lane 3) and type I PRRSV nsp7 (lane 4).

Antigen capture dipstick assay development: The antigen capture dipstick assay has been developed. For each viral antigen, we used a pair of high-affinity anti-PRRSV, anti-SIV or anti-PCV2 mAbs for preparing the test strip. A schematic description of the immunochromatographic dipstick test device is shown in Fig. 1. The dipstick is prepared using a cellulose acetate supported strip of nitrocellulose membrane (FF85, capillary rise 70-150s, 4 cm, 0.5 x 25 mm), and the reagents were dotted on the membrane. In the detection zone, the control antibody and secondary mAbs specific to PRRSV, SIV or PCV2 are separately spotted onto control region (C) and test region (T). The virus specific primary mAb-coated colloidal gold particles are spotted near the other end (bottom) of the strip. When a sample is placed on the sample application site, the combined solution of test sample and detection reagent rise up the membrane and colloidal gold is deposited at the site of the solid-phase antibody. The specific virus-binded mAb/gold particle complex (antigen capture) was trapped in the test region, while mAb-gold particle was reacted with control antibody and trapped in the control region.

Initially, viral antigen standards were established using PRRSV, SIV or PCV2 viruses propagated in cell cultures. The titer of each viral stock was determined by titrating on the cells, and each standard was diluted in negative serum, oral fluid, or nasal secretion (for SIV only) samples. The dipstick assay was performed to determine the detection limit for each antigen. Using the antigen standard prepared in serum, the dipstick assay can detect at least 1×10^3 TCID$_{50}$/ml of PRRSV, 1×10^3 TCID$_{50}$/ml of SIV and 1×10^2 TCID$_{50}$/ml of PCV2 (Table 4). However, the assay was not sensitive for the viral antigens in oral fluid or nasal secretion.

Table 4. Detection limit of the antigen capture immunochromatographic dipstick assays

| Assays	Detection limit
PRRSV dipstick	1×10^3 TCID$_{50}$/ml of virus standards diluted in serum
SIV dipstick	1×10^3 TCID$_{50}$/ml of virus standards diluted in serum
PCV2 dipstick	1×10^2 TCID$_{50}$/ml of virus standards diluted in serum

The diagnostic sensitivity and specificity of these tests were evaluated using a panel of serum samples with known infection status, and the results were compared with those generated by real-time quantitative qRT-PCR. The PCV2 and SIV antigen-capture dipstick assays were not sensitive enough to capture the viral particles in diagnostic samples. The PRRSV capture dipstick assay resulted in 100% specificity, but only 81.1% sensitivity (Table 5).

Table 5. Comparison of PRRSV antigen capture dipstick assay with real-time (q) RT-PCR on evaluation of serum samples

Sample group	qRT-PCR pos	qRT-PCR neg	dipstick pos	dipstick neg
qRT-PCR pos (n = 74)	74	0	60	14
qRT-PCR neg (n = 25)	0	25	0	25

Antibody detection dipstick assay development: Sandwich immunochromatographic dipstick assays to detect antibodies from animals infected with PRRSV, PCV2 and SIV were developed. The assay was performed based on the high binding capacity of a specific pathogen antigen to swine antibodies, and the conjugation of swine immunoglobulin with colloidal gold nanoparticles as a color probe. Using nucleocapsid proteins (SIV, PCV2) and nsp7 (PRRSV) as antigens, dipstick assays were initially developed based on testing standard positive and negative control sera. The assay setting is shown in Figure 1. The assay conditions were optimized. Figure 6A shows typical progression of increasing signal strength as serum antibody levels are increased. Serum dilution used for each antigen was also optimized. The effect of serum inhibition is evident on lower dilutions and while the dilution is increased, there is a rapid decrease in signal strength. Figure 6B shows an example of adjusting gold- SPA capture-detection reagents. Serum dilution for detection of antibody response to SIV and PCV2 was also optimized. The result showed that 1/30, 1/30 and 1/20 are the optimal dilution to detect antibodies to PRRSV, SIV and PCV2, respectively (Figure 7).
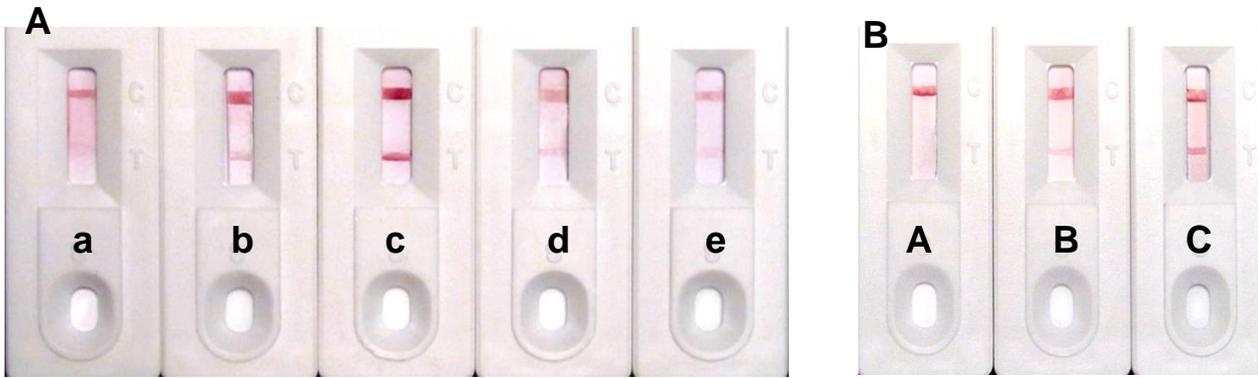
Figure 6. Immunochromatographic dipstick assay condition optimization. **A**. Optimization of serum concentration for SIV immunochromatographic dipstick assay. Serum dilutions of a): 1/10, b): 1/20, c): 1/30, d): 1/40, and e): 1/50 were performed respectively; **B**. Optimization of the immunochromatographic assay by adjusting gold- SPA capture-detection reagents. **a**). negative control serum; **b**). PRRSV weak positive; **c**). PRRSV high positive.
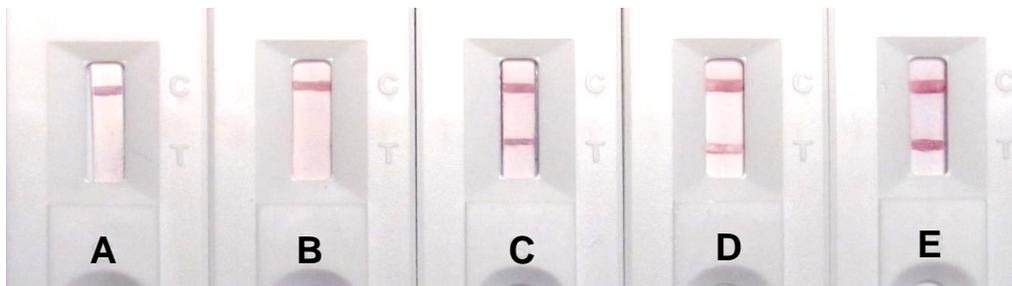
Figure 7. Typical immunochromatographic dipstick assay results. **A**). Assay run with transport buffer only (negative control); **B**). SIV, PCV2 or PRRSV negative swine serum; **C**). PRRSV positive serum; **D**). PCV2 positive serum; **E**). SIV positive serum sample.

To determine the diagnostic specificity and sensitivity, a panel of field serum samples was tested and the results were compared to the classical enzyme-linked immunosorbent assay (ELISA) or hemagglutination inhibition (HI) test (Table 6). Based on the evaluation of 456 serum samples of known serostatus from pigs experimentally infected with either type I or type II PRRSV, the PRRSV dipstick assay showed an overall sensitivity and specificity of 95% and 96% respectively, in comparison with the IDEXX HerdCheck X3 ELISA. The inter-rater agreement (kappa value) between dipstick test and ELSA was 0.965. The SIV dipstick test achieved a sensitivity of 96% and a specificity of 99% using 177 field serum samples previously tested in HI, and the kappa value between dipstick and HI was 0.953. For PCV2 antibody detection, results from nucleocapsid protein-based dipstick test and ELIA were compared using 135 samples from experimentally infected animals. The sensitivity and specificity of PCV2 dipstick test were 88% and 92%, respectively. We further tested a set of serum samples from a time course study. Both PRRSV and SIV dipstick assays detected antibody responses in more than half of all animals by 14 days post infection and greater than 95% of the animals by 28 days post infection. Checkerboard cross-reactivity experiments were performed and the result showed that there was no antibody cross-reactivity between PRRSV, SIV and PCV2 in each specific test.

Table 6. Comparison of PRRSV and PCV2 immunochromatographic assay with ELISA, and SIV immunochromatographic assay with HI as reference tests.

	Type I PRRSV		Type II PRRSV		Type I & II PRRSV		SIV		PCV2	
	ELISA (IDEXX X3)	LFD	ELISA (IDEXX X3)	LFD	ELISA (IDEXX X3)	LFD	HI	LFD	ELISA (SDSU)	LFD
Positive	151	142	149	142	300	284	74	71	123	116
Negative	63	61	93	88	156	149	103	102	106	96
Total # of samples	n=214		n=242		n=456		n=177		n=229	
Sensitivity	94.4%		95.5%		95%		96.1%		94.6%	
Specificity	96.9%		94.9%		96%		99%		91.4%	
Kappa	0.880		0.896		0.965		0.953		0.850	
95% CI	0.811 to 0.949		0.838 to 0.953		0.939 to 0.991		0.908 to 0.999		0.782 to 0.919	

Discussion

Recently, many high-health status herds have fallen victim to severe PRDC outbreaks. The PRDC has emerged as a significant economic problem for producers. Since this complex is caused by the interaction of multifactorial etiologies, development of diagnostic tests that are able to rapid, simultaneously detect multiple pathogens in the PRDC offers an important tool for disease surveillance and control measurements. In this study, we explore the possibility to develop multiplex FMIA and dipstick test to simultaneously detect multiple pathogens. The antigen capture assays (FMIA and dipstick) appeared to have low sensitivity and specificity, especially when comparing to currently available diagnostic assays for viral detection, such as real-time quantitative PCR. In contrast, antibody detection assays demonstrated acceptable diagnostic sensitivity and specificity, which has potential to further develop into standard assays for diagnosis of multiple pathogens simultaneously. Consistent with our previous findings (Langenhorst et al., 2012), the oral fluid-based assay has slightly lower diagnostic sensitivity and specificity in comparing to serum-based assay. This result is expected, since lower concentration of antibodies was present in oral fluid samples. The markedly lower levels of antibody in oral fluid may be due to physiological and anatomical differences in how antibody is secreted through gingival crevicular tissues. On the other hand, it may be due to environmental factors such as high levels of proteases present in the bucal mucosa and oral fluid. However, in comparison to oral fluid-based ELISA, the oral fluid-based FMIA had a signal-to-noise ratio significantly higher than that observed in ELISA (9.9 and 2.4 for N protein-based FMIA and ELISA, respectively). Although further optimization of oral fluid - based ELISA may be necessary to improve the test sensitivity, our results demonstrated the feasibility of using FMIA as an alternative to ELISA for detection of viral infection in oral fluid. The dipstick tests are user-friendly, and can be performed on-site in a swine farm by untrained personnel. The multiplex FMIA and dipstick test format present advantages of simplicity, rapidity, cost-effectiveness, and potentially increase the number of representative individual animals in a large population. Data on the proportion of a herd population that has been infected have many important epidemiologic applications, including 1) to early identify susceptible groups in the population, so that such animals can be quarantined or removed timely to prevent transmission to naïve herd; 2) to evaluate vaccine efficacy: it would be possible to detect whether there is pathogen circulating in vaccinated animals; 3) the use of these data in mathematical modeling to predict disease outbreaks and design better management strategies. The current study represents the "proof of concept" phase

for new diagnostic tests development. In the future, we will apply our technology to include other swine pathogens, such as porcine respiratory corona virus, *Mycoplasma hyopneumoniae*, Swine Brucellosis, *Streptococcus suis*, *Actinomyces pyogenes, Salmonella choleraesuis*. Our ultimate goal is to develop a rapid multiplex test to detect various swine pathogens simultaneously to help preventing and control of PRDC.