DEVELOPMENT OF A MULTIPLEX FLUORESCENT MICROSPHERE IMMUNOASSAY FOR DIAGNOSIS OF THE PORCINE DISEASE COMPLEX

by

RUSSELL RANSBURGH

B.S., South Dakota State University, 2012

A THESIS

submitted in partial fulfillment of the requirements for the degree

MASTER OF SCIENCE

Department of Diagnostic Medicine and Pathobiology
College of Veterinary Medicine

KANSAS STATE UNIVERSITY
Manhattan, Kansas

2015

Approved by:

Major Professor
Ying Fang

Copyright

RUSSELL RANSBURGH

2015

Abstract

The Porcine Disease Complex (PDC) results in major economic problems for swine producers. PDC outbreaks result in increased mortality, decreased feed efficiency, higher cull rates, prolonged days to market and increased treatment costs. This disease involves the interaction and participation of many multifactorial etiologies including both bacterial and viral organisms playing a role in disease initiation and progression. The most common viral pathogens associated with the PDC include porcine reproductive and respiratory syndrome virus (PRRSV), porcine circovirus (PCV2) and swine influenza virus (swIV). The recent outbreak of porcine epidemic diarrhea virus (PEDV) in the US swine herd has made the PDC even more complicated. In aid of the prevention and control of the PDC, veterinarians and producers require fast and efficient diagnostic tests for controlling the disease. In this study, we have generated recombinant nucleocapsid antigens to these viruses for use in a Luminex™ technology-based fluorescent microsphere immunoassay (FMIA). Utilizing these recombinant nucleocapsid antigens, the FMIA was developed to simultaneously detect antibodies in serum from animals infected with PEDV, PRRSV, SwIV and PCV2. The FMIA was developed based on testing experimentally derived standard positive and negative control sera, and the diagnostic specificity and sensitivity were compared to that generated from the classical enzyme-linked immunosorbent assay (ELISA) or hemagglutination inhibition (HI) test. Based on an evaluation of 4147 serum samples with known serostatus, the multiplex FMIAs reached greater than 97.5% sensitivity and 92.3 % specificity. Results showed that multiplexing did not affect the diagnostic sensitivity or specificity of each individual assay. This work provides a platform for the development of multiplex assays for detecting various swine pathogens simultaneously and aids in preventing and controlling the PDC.

Table of Contents

List of Figures ... vi
List of Tables ... vii
Acknowledgements ... viii
Chapter 1 - Literature Review ... 1
Porcine Disease Complex ... 1
Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) ... 1
Porcine Circovirus Type 2 (PCV2) ... 3
Swine Influenza Virus (swIV) ... 5
Porcine Epidemic Diarrhea Virus (PEDV) ... 6
Fluorescent Microsphere Immunoassay ... 7
Figures and Tables ... 10
Chapter 2 - Development of a 5-Plex FMIA ... 13
Introduction ... 13
Materials and Methods ... 13
Expression and purification of recombinant viral nucleocapsid proteins: ... 13
Covalent coupling of recombinant proteins to fluorescent microspheres: ... 15
Fluorescent Microsphere Immunoassay (FMIA): ... 16
Data Analysis: ... 17
Assay validation: ... 17
Measurement of Repeatability: ... 18
Results ... 18
Expression of recombinant nucleocapsid proteins ... 18
Fluorescent microsphere immunoassay development ... 19
Establishment of control standard ... 19
Assay Optimization ... 19
Diagnostic Sensitivity and Specificity ... 20
Assessment of assay repeatability ... 20
Development of 5-plex multiplex assay ... 21

Figures and Tables 22
Chapter 3 - Discussion and Conclusions 29
References 31

List of Figures

Figure 1-1 Schematic representation of the porcine disease complex (PDC) viral nucleocapsid particles 10
Figure 1-2 Luminex xMAP Technology 11
Figure 1-3 Schematic of Luminex 200 System 12
Figure 2-1 SDS-PAGE gel for recombinant N proteins 22
Figure 2-2 Coupling efficiency of antigen coated beads using mAbs 23
Figure 2-3 Serum dilution optimization 24
Figure 2-4 Determination of Diagnostic Sensitivity and Specificity by ROC 26
Figure 2-5 Comparison of singleplex vs. multiplex 27

List of Tables

Table 2-1 Summary of ROC analysis of serum based FMIA results 25

Table 2-2 Assay repeatability of serum-based FMIAs 28

Acknowledgements

I would first like to express my sincere gratitude to my mentor, Dr. Ying Fang, for accepting me into her laboratory and allowing me opportunities to grow as a researcher and student during the past 5 years. She pushed me past the boundaries I thought myself capable when I first arrived as a “naïve” undergraduate and I feel better capable to pursue my future career goals.

Secondly, I would like to thank my graduate committee members, Dr. Bob Rowland and Dr. Elizabeth Davis, for their support and suggestions for my project.

I want to thank all of the great lab members I have had the pleasure of working with from South Dakota State University and Kansas State University. This includes Dr. Zhi Sun, Dr. Steve Lawson, Dr. Yanhua Li, Rob Langenhorst, Brianna Sandager, Zhiyong Jiang, Longchao Zhu, Rui Guo, Pengcheng Shang, Zhenhai Chen, Fangfeng Yuan and Yin Wang. I want to especially thank Dr. Zhi Sun and Dr. Steve Lawson for spending a great amount of time training me in the lab.

I would also like to thank my girlfriend, Jinhwa Lee, for all of her help and motivation. Finally, I owe everything I have achieved to my mom, Cindy Ransburgh, for constantly encouraging me and supporting my dreams.

Chapter 1 - Literature Review

Porcine Disease Complex

The Porcine Disease Complex (PDC) is a multifactorial disease complex in pig populations which causes severe economic losses for the swine industry. The pathogens involved in forming the PDC include common viruses linked to the Porcine Respiratory Disease Complex (PRDC): porcine reproductive and respiratory syndrome virus (PRRSV), porcine circovirus type 2 (PCV2), and swine influenza virus (swIV). Porcine epidemic diarrhea virus (PEDV) was recently introduced to the United States and has caused severe outbreaks of fatal diarrhea in young pigs and is included in the PDC.

Porcine Reproductive and Respiratory Syndrome Virus (PRRSV)

Porcine reproductive and respiratory syndrome (PRRS) is the most economically significant disease of swine. It costs US swine producers $644 million dollars a year (Miller *et al.,* 2011). The disease often manifests as reproductive failures in sow operations and severe pneumonia in young pigs. It causes late term abortions, stillbirths and mummified fetuses in breeding gilts or sows. Increased mortality and decreased growth performance to respiratory illness is common in young pigs with PRRS (Corzo et al., 2010). PRRS has been causing clinical outbreaks in the US since the late 1980s and Europe since the early 1990s but the responsible agent was still unknown (Wensvoort et al., 1991). Early names given to the disease included "mystery swine disease" and "blue-ear pig disease".

The etiologic agent responsible for PRRS was discovered to be porcine reproductive and respiratory syndrome virus (PRRSV) after it was isolated separately in the Netherlands and the USA in 1991 (Collins et al., 1992; Wensvoort et al., 1991). The prototypic North American strain was characterized as American Type Tissue Culture (ATCC) VR2332 while the European

isolate was identified as Lelystad virus (LV) (Benfield et al., 1992; Wensvoort et al., 1991). PRRSV is a single stranded positive sense RNA virus in the family *Arterivirdae* of the order *Nidovirales*. The other members of the family include lactate-dehydrogenase elevating virus (LDV), simian hemorrhagic fever virus (SHFV) and equine arteritis virus (EAV) (Cavanagh, 1997; Snijder & Meulenberg, 1998; Snijder, Kikkert, & Fang, 2013). PRRS viruses belong to two distinct genotypes including Type I (European) and Type II (North American) based upon their respective prototypic strain phylogeny. These genotypes only share around 63% nucleotide identity (Allende et al., 1999; Nelsen, Murtaugh, & Faaberg, 1999). The genotypes also differ in their antigenic properties, highlighting their separate evolutionary divergence (Drew, Meulenberg, Sands, & Paton, 1995; Wensvoort et al., 1992). Porcine alveolar macrophages (PAMs) are the primary cell targets for PRRSV infection (Duan, Nauwynck, & Pensaert, 1997; Nauwynck, Duan, Favoreel, Van Oostveldt, & Pensaert, 1999). Cell lines known to be permissive to PRRSV include African green monkey kidney cell line MA-104 and its derivatives which include MARC-145 (Kim, Kwang, Yoon, Joo, & Frey, 1993). The major cellular receptor for virus entry was determined to be CD163 which belongs to the scavenger receptor cysteine rich family (Calvert et al., 2007).

PRRSV can be transmitted to swine in a number of ways which makes control and prevention difficult in swine facilities. The virus has been isolated from many swine bodily fluids including serum, semen, saliva, urine, nasal swabs, oropharyngeal swabs as well as fecal samples. The primary method of transmission is through direct physical contact but aerosol transmission has also been reported (Rossow, 1998). Other methods of transmission include fomite transmission (Pitkin, Deen, & Dee, 2009) and semen from both vasectomized and non-vasectomized boars (Christopher-Hennings et al., 1998).

The PRRSV genome is roughly 15.4kb in length and encodes at least 10 open reading frames (ORFs). The replicase ORF1a and ORF1b genes are located at the 5' proximal end of the genome and consist of 75% of the total genome. Once released into the cytoplasm, two polyproteins pp1a and pp1ab are translated which are subsequently cleaved by proteases to yield 14 non-structural proteins (nsps). Translation of pp1ab relies on a -1 ribosomal frameshift signal in the ORF1a and ORF1b overlap region. The 3' terminal end of the genome encodes for structural proteins which include GP2a, GP2b, GP3, GP4, GP5, ORF5a, matrix (M) and nucleocapsid (N) proteins. The N protein is highly antigenic, and induces early antibody responses in pigs (Brown et al., 2009; Wootton, Yoo, & Rogan, 2000). It has also been used extensively in the development of commercial enzyme-linked immunosorbent assays (ELISAs) for PRRSV diagnosis.

Porcine Circovirus Type 2 (PCV2)

Porcine circovirus type 2 (PCV2) is a small closed circular single stranded DNA virus which is involved in a number of polymicrobial disease complexes including the porcine respiratory disease complex (PRDC), post weaning multisystemic wasting syndrome (PMWS), and porcine dermatitis and nephropathy syndrome (PDNS). Porcine circovirus type 2 associated disease (PCVAD) is a term which encompasses all pathologies resulting from PCV2 infection. Porcine circovirus (PCV) was first reported in 1974 as a cell culture contaminant in a porcine kidney cell line (PK-15) (Tischer, Gelderblom, Vettermann, & Koch, 1982). It was characterized as a small non-enveloped single-stranded DNA virus with a genome of 1.7kb in length (Tischer et al., 1982). Through experimental infection studies, it was found incapable of producing disease in pigs (Allan et al., 1995; Tischer, Mields, Wolff, Vagt, & Griem, 1986) .

In the late 1990s, a new PCV variant emerged in Europe and North America associated with pigs developing PMWS. This variant displayed many genetic differences to the original PCV isolate identified in the PK-15 cell line. The newly discovered pathogenic virus was termed PCV2 and the nonpathogenic virus PCV1 for differentiation. PCV1 and PCV2 have similar genomic organization. Both consist of two major open reading frames (ORFs) including ORF1 which encodes a replicase protein and ORF2 which encodes the nucleocapsid protein (Hamel, Lin, & Nayar, 1998; Meehan, Creelan, McNulty, & Todd, 1997; Meehan et al., 1998; Morozov et al., 1998). The 702 nucleotide ORF2 sequence of the PCV2 genome shares only 66% amino acid identity with that of the PCV1 ORF2 sequence, while the ORF1 sequences are 85% identical (Hamel et al., 1998). Among global PCV2 isolates, the ORF2 nucleotide sequence is highly conserved with 95-100% identities (Meehan et al., 1997; Meehan et al., 1998; Morozov et al., 1998; Nawagitgul et al., 2000). Previous studies have shown that the ORF2 encoded recombinant nucleocapsid protein is immunogenic and reacts strongly with serum from PCV2 infected swine (Nawagitgul et al., 2000; Nawagitgul et al., 2002).

Current diagnostic methods for PCV2 detection include serological tests such as the indirect immunofluorescence assays (IFA), indirect immunoperoxidase monolayer assay (IPMA), serum-virus neutralization assays (SVN) and enzyme linked immunosorbent assays (ELISA). The IFA, IPMA and SVN are highly technical and time-consuming assays which require previously infected porcine cell cultures. Because of the differences in staining interpretation, results may be varied depending on the technician. Virus cross-contamination is also a concern with these assays in cell culture. The ELISA is an attractive alternative to these methods in terms of being a higher throughput assay with lower risks of biases between results.

Swine Influenza Virus (swIV)

Swine influenza virus (swIV) causes acute respiratory illnesses in swine of all ages around the world. The etiologic agents are *Influenza A* viruses (IAVs) of the *Orthomyxoviridae* family. These are negative sense, single stranded and segmented RNA viruses. SwIV infection in pigs results in an acute illness with symptoms including fever, lethargy, decreased growth performance, coughing, sneezing and difficulty breathing. SwIV was first recognized during the Spanish influenza pandemic in 1918 as it coincided with human cases (Koen 1919). The classical swIV H1N1 strain genetically associated with this lineage was first isolated in 1930 (Shope, 1931). H1N1 is the most frequently isolated influenza strain in Asia, North America and most parts of Europe. H1N1 was the only subtype circulating in North America until 1998 when it re-assorted with both human H3N2 and an unknown avian influenza strain resulting in a triple re-assortment H3N2 SwIV. Because pigs contain alpha 2-3-linked as well as alpha 2-6-linked sialic acid receptors in their respiratory tract, they can be infected with human, avian or swine influenza viruses. For this reason, pigs are often considered the "mixing vessels" for IAVs (Ma et al., 2009; Zhou et al., 1999). H1N1, H3N2 and H1N2 are the current subtypes which are endemic globally.

The SwIV particle consists of an outer lipid envelope composed of the hemaglutinin (HA), neuraminidase (NA) and M1 structural proteins along with an inner core composed of matrix proteins surrounding the viral genome. The genome contains 8 RNA segments which encode for 4 structural proteins, 3 subunits of the RNA-dependent RNA polymerase (Rdrp) and 2 non-structural proteins. These are incorporated into the virion as ribonucleoprotein (RNP) complexes which are composed of the viral RNA (vRNA) as well as the Rdrp and the nucleoprotein (NP). The NP is a major structural protein of the viral RNP and plays many important roles for the virus replication cycle (Li et al., 2009). The NP is 498 amino acids in

length and contains highly conserved regions among Influenza A, B and C viruses (Mena et al., 1999; Portela & Digard, 2002). Antibody responses to the NP are robust and long-lasting (Amanna, Carlson, & Slifka, 2007).

The most commonly used serological tests for SwIV include the hemagluttination inhibition (HI) assay, virus neutralization (VN) and the indirect fluorescence antibody test (IFA) with the HI being the most common. The HI assay is designed to detect the highly variable, hemaglutinin surface protein (HA) so each test needs to be developed with a corresponding reference strain to each H subtype. The HI is also very labor intensive which makes high-throughput sample diagnostics problematic (Yoon, Janke, Swalla, & Erickson, 2004).

Porcine Epidemic Diarrhea Virus (PEDV)

Porcine epidemic diarrhea virus (PEDV) is a swine virus which belongs to the Coronaviridae family within the *Alphacoronavirus* genus. PEDV was first reported among grower and feeder pigs in the UK in 1971 (Oldham, 1971). The virus was subsequently isolated from Belgium in 1978 and the prototypic strain termed CV777 (Pensaert & de Bouck, 1978). PEDV is closely related to transmissible gastroenteritis virus (TGEV) which is another *Alphacoronavirus*. PEDV infection in pigs results in a severe watery diarrhea, dehydration, vomiting and decreased body weight (Song & Park, 2012; Stevenson et al., 2013). It is indistinguishable to TGEV in regard to clinical signs and pathology (Saif et al. 2012).

PEDV has been endemic in many countries of Europe and Asia over the last few decades (Song & Park, 2012). However, it wasn't until 2010 when substantial PEDV outbreaks occurred in China resulting in high mortalities among suckling pigs (Sun et al., 2012). Previously, North America had been free of the virus until a major outbreak in April 2013 when a highly virulent

strain of PEDV emerged and began spreading across the US (Chen et al., 2014). The virus has since spread to over 30 states and Canada. A recent phylogenetic study determined the PEDV strain circulating in the US to be almost identical to the AH2012 and CH/ZMDZY/11 PEDV strains, suggesting a Chinese origin (Huang et al., 2013). The virus circulating in North America has resulted in mortalities as high as 95% in young pigs (Chen et al., 2014; Stevenson et al., 2013). PEDV is a single-stranded positive sense RNA virus with a genome approximately 28kb in length and encodes 7 ORFs. The 5' two-thirds of the viral genome contains two large open reading frames (ORFs), 1a and 1b, which encode two nonstructural polyproteins, pp1a and pp1ab, that direct genome replication and transcription. The ORF1b is expressed by a -1 ribosomal frameshift mechanism. The pp1a and pp1ab are processed into at least 16 functional nonstructural proteins (nsps) by a virus-encoded complex proteolytic cascade. The 3' end of the viral genome encodes four structural proteins, including three membrane anchored proteins spike (S), membrane (M) and envelope (E) proteins, and the nucleocapsid (N) protein which encapsidates the genomic RNA (Spaan, Cavanagh, & Horzinek, 1988). Both the N and M antigens have been used in the development of previous serological assays (Song & Park, 2012). Figure 1.1 is a schematic depicting the nucleocapsid protein for each of the viruses included in the PDC.

Fluorescent Microsphere Immunoassay

The fluorescent microsphere immunoassay (FMIA) is an antigen or antibody detection assay which utilizes carboxylated microspheres or beads to serve as coupling surfaces for antigen-antibody binding reactions. Similar to ELISA capture assays, a capture antigen is immobilized on the bead surface and reacts with a target antibody within the sample. The FMIA is based upon xMAP technology which allows for efficient multiplexing within samples. A

detailed schematic of the xMAP technology is depicted in Figure 1.2. The technology relies on the use of different sets of internally dyed microspheres. Each 5.6 micron sized polystyrene microsphere contains a unique ratio of red and infrared dyes in order to distinguish one from another within the FMIA. Currently there are 500 available microsphere sets. Early xMAP technology was initially developed by Luminex Corporation in 1997 with the release of the FlowMetrix platform which consisted of 64 unique fluorescent microsphere sets (Fulton, McDade, Smith, Kienker, & Kettman, 1997; Gordon & McDade, 1997). The system utilized the principles of flow cytometry and a signal processor to analyze up to 64 coupled reactions in real-time. Newer platforms have since been developed including the Luminex 100/200, FLEXMAP 3D and the MAGPIX. While the Luminex 100/200 and FLEXMAP 3D systems utilizes the same principles of flow cytometry, the MAGPIX platform is based on fluorescent imaging which uses light emitting diodes (LEDs) instead of lasers for microsphere excitation and a CCD camera for detection. When performing the FMIA using the Luminex 200 platform, antigen or antibody coupled microspheres are transported through a fluidics system until they reach a narrow channel where they are excited by a dual-laser system. A red laser (635nm) will excite the internal dyes of the microsphere to distinguish it from other microsphere sets and a green laser (532nm) will excite the fluorescent dye on the fluorescent reporter. Four detectors will calculate the fluorescent signals, determine the specific bead set and differentiate between single or aggregate beads. The machine's software will generate mean fluorescent intensities (MFIs) for each sample by counting a minimum of 100 microspheres from each set tested. Figure 1.3 depicts a schematic of the Luminex 200 instrument and its dual laser system. The xMAP based FMIA has many distinct advantages compared to other methods for antigen/antibody detection including reduced cost and labor through multiplexing within samples, overall less sample required and faster

turnaround time. Multiplex FMIA panels would be well suitable in large-scale field application for disease surveillance and epidemiology studies.

Figures and Tables

Figure 1-1 Schematic representation of the porcine disease complex (PDC) viral nucleocapsid particles

(Figure adapted from http://viralzone.expasy.org)

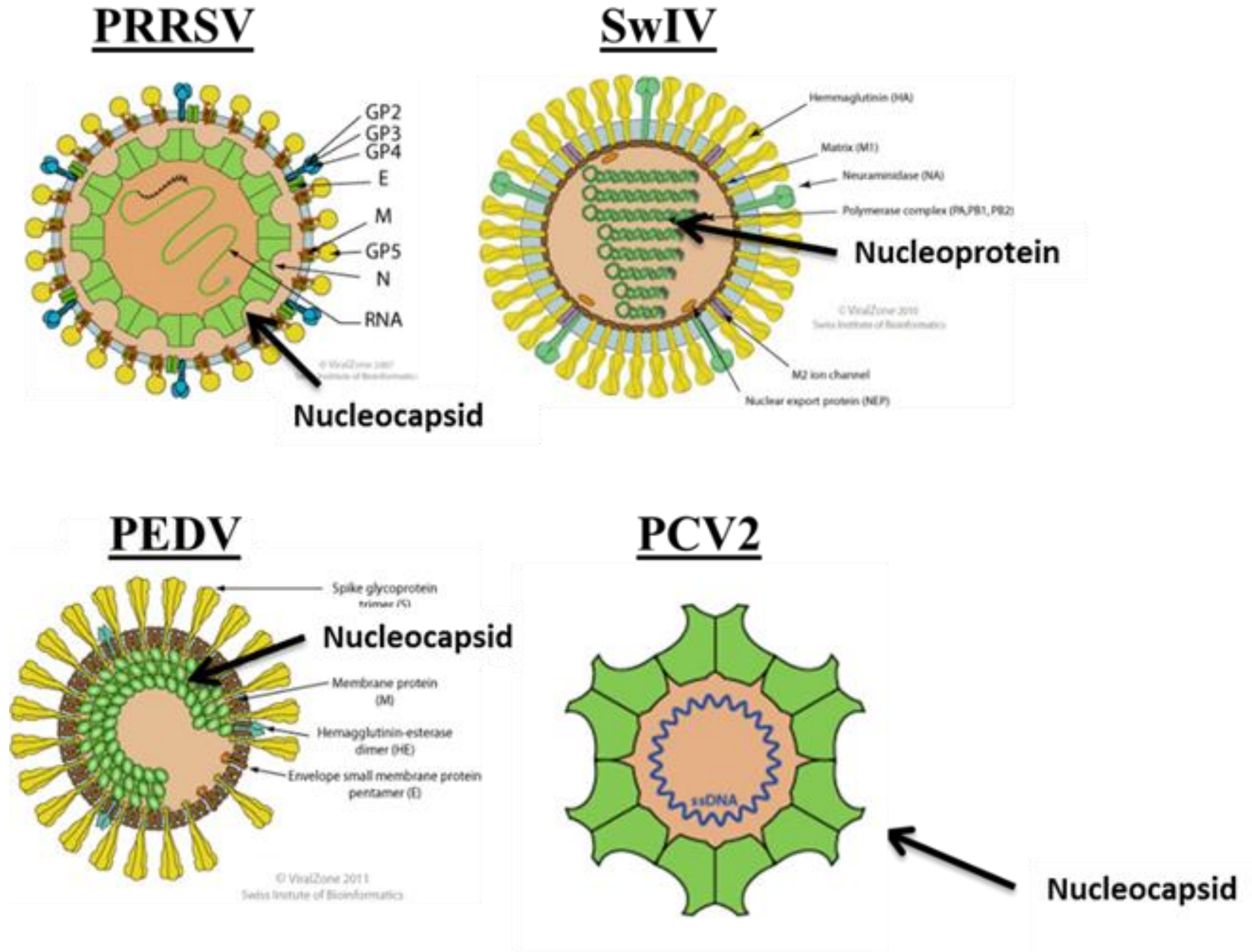
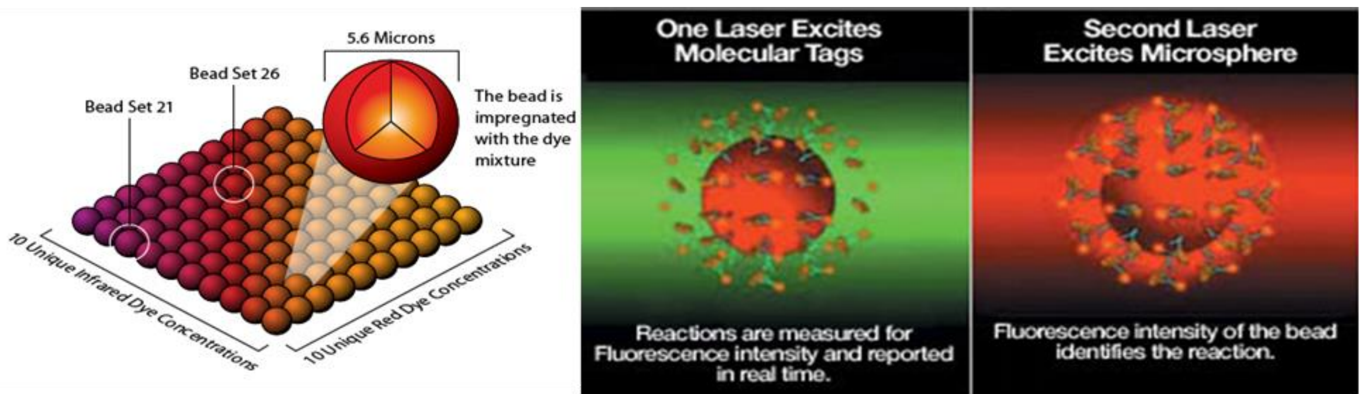


Figure 1-2 Luminex xMAP Technology
(Figure adapted from www.luminexcorp.com)



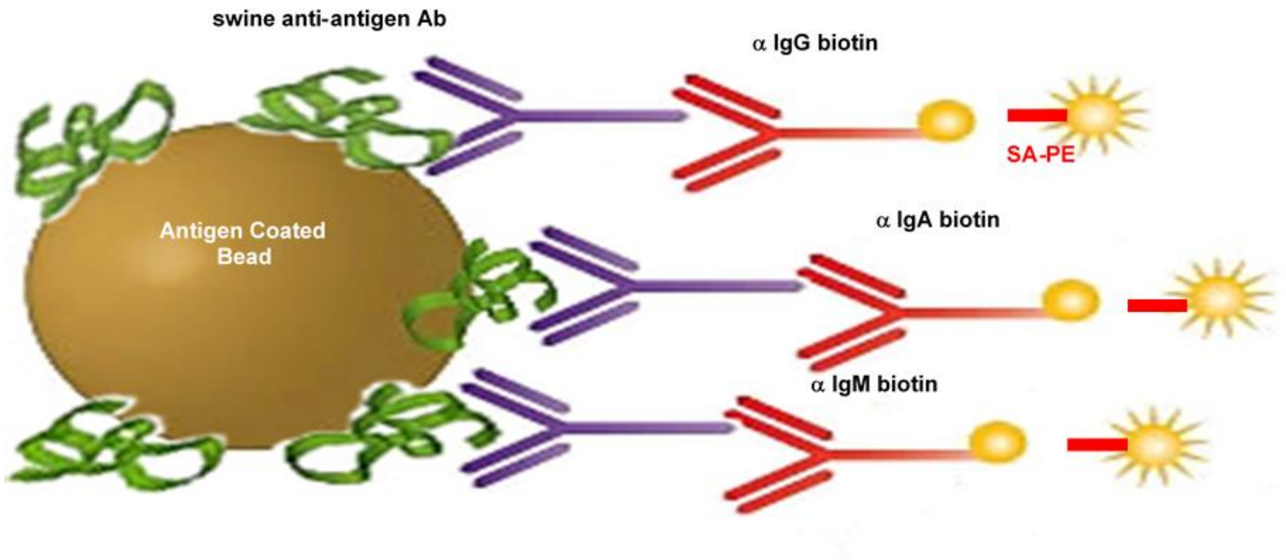
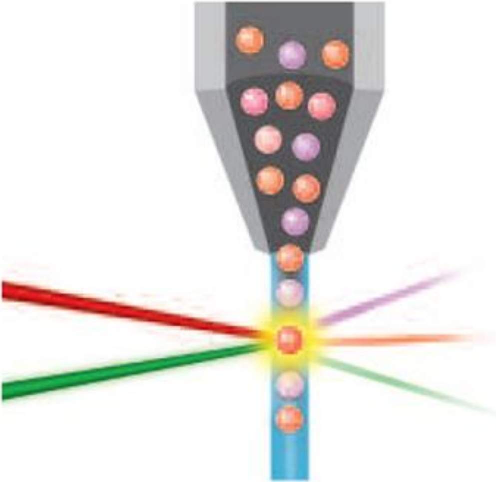
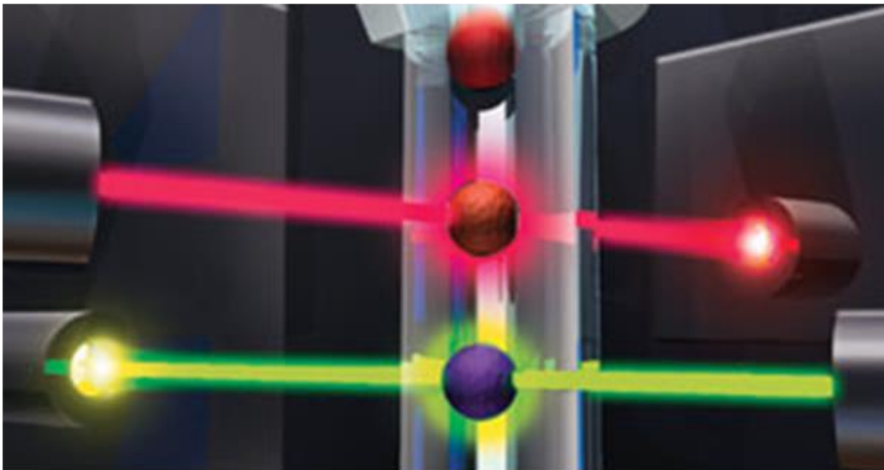


Figure 1-3 Schematic of Luminex 200 System

(Figure adapted from www.luminexcorp.com)





Chapter 2 - Development of a 5-Plex FMIA

Introduction

The PDC causes severe economic losses for the US swine industry and because of the complicated disease interactions and multifaceted nature, diagnosis and treatment is extremely difficult. Currently, there are very few effective multiplex assays available to test all pathogens in one sample. Due to biosecurity concerns, diagnostic reagents and materials which have already been developed for PEDV in other countries are difficult to import into the US. In this study, we have generated recombinant nucleocapsid antigens to these viruses for use in a Luminex™ technology-based fluorescent microsphere immunoassay (FMIA). Utilizing these recombinant nucleocapsid antigens, the FMIA was developed to serve as a serological diagnostic test for PEDV, PRRSV, SwIV and PCV2.

The nucleocapsid protein was chosen as the appropriate antigen as it is a highly conserved region, has proven immunogenicity and is the standard antigen used in many currently used diagnostic assays. In a previous study in our lab, the FMIA could detect the PRRSV N antibody as early as 7 days post infection (dpi) in experimentally infected animals (Langenhorst et al., 2012).

The multiplex FMIA was validated using a standard set of known negative and positive serological sample populations. Diagnostic sensitivity and specificity as well as assay repeatability were assessed and optimized utilizing these samples.

Materials and Methods

Expression and purification of recombinant viral nucleocapsid proteins:

Full length DNA fragments to PRRSV NA, PRRSV EU, SwIV N, PEDV N and truncated PCV2 (43-233) were amplified by reverse-transcription PCR and expressed as His-tag fusion

proteins in the pET28a prokaryotic expression vector. Once the fragments were successfully cloned into the pET vector, they were transformed into the BL-21 competent E.coli cell line for protein expression.

Initially, 5ml of Lysogeny broth- Lennox (Fisher Scientific) containing the antibiotic kanamycin at a concentration of 50μg/ml was inoculated with each respective protein and shaker incubated overnight at 37°C. The overnight cultures were then used to inoculate a 500ml culture of 2XYT *E. coli* growth medium containing Kanamycin at 50μg/ml and incubated shaking at 37°C for 2-3 hours until the OD600 was between 0.4-0.6. Once the cultures were at the appropriate OD, 250mM of isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to induce protein expression and further shaker incubated at 37°C for 4 hours. For optimal PCV2 protein expression, induction was alternatively performed at 30°C for 4 hours. Proteins were subsequently pelleted in 250ml bottles by centrifugation at 12,000 x g for 15 minutes.

Protein extraction from inclusion bodies was then performed using bacterial extraction reagents (B-PER, Pierce Thermo-Fisher) with all work performed on ice. Pellets were re-suspended in 10-20ml of B-PER containing 200μg/ml lysozyme and 1μg/ml PMSF protease inhibitor. The solution was made homogenous by pipetting up and down and by vortexing. The mixture was passed through a syringe and needle 20 times to disrupt the bacterial cell walls followed by centrifugation at 12,000 x g for 10 minutes. Pellets were then dissolved in a denaturation buffer containing 8M urea and incubated with Ni-NTA agarose (Qiagen) rotating overnight at 4°C.

Proteins were purified by nickel affinity chromatography as described previously (Brown et al., 2009). Denatured proteins were subsequently subjected to a further refolding process using a protein refolding kit (Novagen). Two beakers, each containing 2 litres of 1X phosphate

buffered saline (PBS) were prepared and cooled at 4°C for 2 hours. Pre-wetted dialysis membrane with a 12-14,000 kDa molecular weight cutoff (Spectrum Laboratories Inc.) was cut into an approximate 5 inch strip and capped on one end. All previously His-tag purified protein was carefully added into the dialysis membrane by pipette. A 1X solubilization buffer supplemented with 0.3% N-lauroylsarcosine was added to the protein within the membrane until protein particulate was no longer visible. The dialysis membrane was then completely capped and placed within the first beaker of 2L PBS. This PBS was supplemented with 0.1M dithiothreitol (DTT). The membrane was stir incubated at 4°C for 4 hours and then transferred to the second beaker of PBS and stir incubated for another 4 hours. Refolded protein was then carefully collected from the membrane and aliquoted into 1.7ml screw cap tubes. Protein purity was then analyzed by SDS-PAGE and protein concentration was determined by Lowry Assay.

Covalent coupling of recombinant proteins to fluorescent microspheres:

Proteins were coupled to magnetic Bio-Plex microspheres (Bio-Rad Laboratories, Inc.) utilizing a magnetic tube separator. Initially, 3.125×10^6 microspheres were washed with 250µl of activation buffer 0.1M NaH^2PO^4 followed by a second wash with 500µl activation buffer. Beads were resuspended by vortex and sonication for 30 seconds following each wash. They were allowed to separate by placing them back into the magnetic separator for 5 minutes. Microspheres were subsequently activated through the addition of 500 µl activation buffer containing 2.5 mg N-hydroxysulfosuccinimide (sulfo-NHS) and 2.5 mg N-(3-dimethylaminopropyl)-N- ethylcarbodiimide (EDC) (Pierce Chemical Company, Thermo-Fisher) and rotated at room temperature for 20 minutes. Activated microspheres were washed twice with phosphate-buffered saline (PBS) and sonicated. Coupling was performed by adding a volume containing 100µg of each protein to the activated microspheres and the volume was brought up

to 500µl in PBS. The samples were then incubated by rotation for 3 hours in the dark at room temperature. Coupled microspheres were washed by 1 ml of PBS plus 0.05% NaN3 and 1.0% bovine serum albumin (PBS-BN, Sigma-Aldrich) and blocked with an additional 1 ml of PBS-BN for 30 min to reduce any nonspecific binding. Microspheres were then washed twice, counted by hemocytometer and resuspended in PBS-BN to achieve a final concentration of 2.0x106 antigen-coupled microspheres/ml. Beads were stored in amber tubes at 4°C.

Fluorescent Microsphere Immunoassay (FMIA):

A working bead mixture was prepared in IDEXX buffer diluent so that there were 50 microspheres per microliter or 2500 beads per well (50µl of 5×10^4 beads/ml stock). Serum samples were prepared by diluting them 1/50 in PBS-BN. 50µl of the bead stocks were added to each appropriate well in a 96-well black clear bottom assay plate (Corning Inc.). 50µl of the diluted serum samples were then added to their respective wells. The plates were covered and incubated shaking for 1 hour at room temperature. Since the reactions are light sensitive, black plastic lids or aluminum foil covers were used. The plates were then secured in a magnetic plate separator (Luminex Corp.) for 1 minute to allow the beads to secure to the bottom of the plate and the supernatant was dumped by rapid forceful inversion 2-3 times in quick succession. The plates were washed 3 times with PBST (PBS plus 0.05% Tween 20) following this dumping procedure. Biotinylated goat anti-swine IgG (Jackson ImmunoResearch Laboratories) was diluted 1:10,000 in IDEXX buffer diluent and 50µl was added to each well. The plates were covered and incubated shaking for 1 hour at room temperature. The plates were washed by the same method previously described. The fluorescent reporter, Streptavidin-R-Phycoerythrin (Prozyme) was diluted to 2.5µg/ml in PBS-BN and 50µl was added to each well. The plates were incubated by shaking for 30 minutes at room temperature. Following a wash, the microspheres

were resuspended in 125µl of PBS-BN. The plate was shook for 5 minutes at room temperature and then analyzed on the Bio-Plex 200 instrument (Bio-Rad Laboratories, Inc.).

Data Analysis:

Data was analyzed through Biomanager software version 6.1 (Bio-Rad Laboratories, Inc.) which compiles the raw mean fluorescent intensity (MFI) for each sample. The MFI for 100 coupled microspheres for each individual target was calculated. Background well containing coupled beads with PBS-BN were present on every plate and were automatically subtracted from the samples as background. Serum positive or negative standard samples were added to each plate in duplicate or triplicate. The sample to positive (S/P) ratios were then calculated for each sample to determine the presence of antibody to each swine pathogen. The S/P ratios were calculated through the formula (MFI of sample – MFI of buffer) / (MFI of positive control – MFI of buffer).

Assay validation:

For assessing the diagnostic sensitivity and specificity for each assay, they were validated using samples taken from two distinct animal populations. The negative-testing populations of serum validation samples for NA PRRSV, EU PRRSV, SwIV, PCV2 and PEDV were composed of 371, 379, 629, 564 and 91 samples respectively. The positive-testing populations of serum validation samples for NA PRRSV, EU PRRSV, SwIV, PCV2 and PEDV were 716, 176, 566, 570 and 85 samples respectively. Receiver operating characteristic (ROC) analysis was conducted for each assay to determine assay cutoffs and diagnostic performance, using MedCalc®, version 10.4.0.0 (MedCalc® Software, Mariarke, Belgium)

Measurement of Repeatability:

The repeatability of the FMIA was assessed by running the same set of internal control serum standards multiple times on different plates. For all nucleocapsid assays, the intra- and inter-assay repeatability was calculated for 36 replicates on a single plate and repeated over a 3 day period to assess interassay repeatability. Each of the assays was run in a 5-plex format, and mean fluorescence intensity values are expressed as means, standard deviations, and percent coefficients of variation (% CV) for replicates. The % CV was calculated as described previously (Brown et al., 2009).

Results

Expression of recombinant nucleocapsid proteins

To develop an FMIA multi-plex assay, we initially expressed His-tagged fusion recombinant proteins of PRRSV NA-N from the Type II prototypic strain VR2332, PRRSV EU-N from Type I strain SD 01-08, SwIV-N from H3N2, PCV2-N from PCV2 genotype B and PEDV-N from a Nebraska PEDV field culture isolate. These proteins were expressed as inclusion bodies, so further protein purification, electro-elution and a protein refolding step was performed. The purity of the proteins was determined using SDS-PAGE followed by Coomassie brilliant blue staining. As shown in Fig. 2.1, all of the His-tagged recombinant proteins migrated according to their predicted sizes. Recombinant PRRSV NA and EU N proteins both displayed 17 kDa bands with >99% purity. The protein concentrations were found to be 2.19mg/ml and 3.16mg/ml respectively. Recombinant PCV2-N displayed a band which migrated to approximately 30 kDa with >99% purity. The protein concentration was determined to be 2.26mg/ml. Recombinant SwIV-N and PEDV-N both displayed a band at 50 kDa with purities >99% purity. Their protein concentrations were 1.42 and 2.86mg/ml respectively. The identity of

each protein was further confirmed by Western blot analysis using anti-His antibody (data not shown).

Fluorescent microsphere immunoassay development

Establishment of control standard

A set of internal control standards were established using serum collected from experimental animals for PRRSV, PCV2, and SwIV. For PEDV, a set of internal controls were established using pooled field sample serum. The serum standards were established as 'high positive', 'medium positive', 'low positive 'and 'negative' standard. For NA and EU PRRSV, SwIV, PEDV, and PCV2, the 'high positive' standard generates an MFI of 25,000- 29,000. The NA and EU PRRSV, PEDV, and PCV2 'medium positive' generates an MFI of 15,000-17,000 while the SwIV 'medium positive' generates an MFI of 7,000-9,000. The NA and EU PRRSV, PEDV, and PCV2 'low postive' generates an MFI of 7,000-9,000 while the SwIV 'low positive' generates an MFI of 1,500-2,000. The MFI for the 'negative' standards range from 10-200 MFI.

Assay Optimization

To determine the optimal concentrations of antigen to microsphere coupling, a series of couplings were performed using different concentration of antigen coated beads and analyzed against control standards. Five sets of beads each containing 3.125×10^6 beads were incubated with 3 different concentrations (500µg, 250µg and 100µg) of purified PRRSV NA and EU, SwIV, PCV2 and PEDV recombinant nucleocapsid proteins. Based on the highest signal to noise ratio for detection for these specific antibodies in serum, we determined 250µg per coupling reaction or 80µg per 1×10^6 microspheres was the optimal concentration for coupling these proteins. The coupling efficiency of the antigen coated beads was determined using antigen

specific monoclonal antibodies (mAbs) in a log_{10} dilution series. As shown in Fig. 2.2, relative coupling efficiency curves were generated and an average correlation coefficient (R^2) of 0.994 was calculated for both PRRSV SR-30 and PCV2 R. Magar regression analytes within the linear portion of the curve while the R^2 calculated for both SwIV mAbs 42-100 and HB-65 was 0.997. PEDV coupling efficiency is not shown as mAb to the nucleocapsid antibody was not available. The optimal serum dilutions were determined by diluting samples in a log_2 titration. Figure 2.3 shows a concentration dependent MFI signal for PRRSV NA antibody detection. It was determined that a 1:50 dilution of serum samples provided an optimal signal to noise ratio. A 1:50 dilution of serum sample showed optimal signal to noise ratio for all other virus infected control serum including EU PRRSV, SwIV, PCV2 and PEDV (not shown).

Diagnostic Sensitivity and Specificity

MedCalc statistical software was used for ROC analysis for each FMIA to determine an optimized cutoff value which maximizes both diagnostic sensitivity and specificity. Serum samples from known positive populations for each virus (2034 samples total) and serum samples from a known negative population for each virus (2113 samples total) were analyzed. These samples were obtained from experimental animals as described in the Materials and Methods section. The optimal cutoff value, diagnostic sensitivity and specificity of each individual test are presented in Figure 2.4. Each of the serum based FMIAs displayed >92% diagnostic sensitivity and specificity. Table 2.1 summarizes the results of the ROC analysis.

Assessment of assay repeatability

The precision of each individual FMIA was determined using internal control standards. Table 2.2 shows the intra and inter assay repeatability of each test. Both the intra and inter assay

repeatability values were <10% CV for all tests, suggesting these FMIAs are highly repeatable in diagnostic applications.

Development of 5-plex multiplex assay

Once the validation was completed for each of the individual nucleocapsid based FMIAs in singleplex format, we combined them into a 5-plex assay. The 5-plex assay was compared with each singleplex to determine whether there was any cross reactivity among the coupled bead sets. Each serum internal control standard along with the individual corresponding bead set was first tested in a single-plex format and then combined for testing in the 5-plex format. Correlation coefficients were determined for comparison between each individual nucleocapsid based FMIA and the 5-plex assay. As shown in Figure 2.5, there was no statistical difference between multiplex and singleplex analytes for the nucleocapsid based assays.

Figures and Tables

Figure 2-1 SDS-PAGE gel for recombinant N proteins

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of *E.coli* expressed recombinant protein preparations, followed by Coomassie brilliant blue staining. The left lane displays the protein molecular weight (MW) marker (lane 1); the remaining lanes represent nucleocapsid proteins of PRRSV NA (lane 2) and EU (lane 3), PEDV (lane 4), SwIV (lane 5) and PCV2 (lane 6).

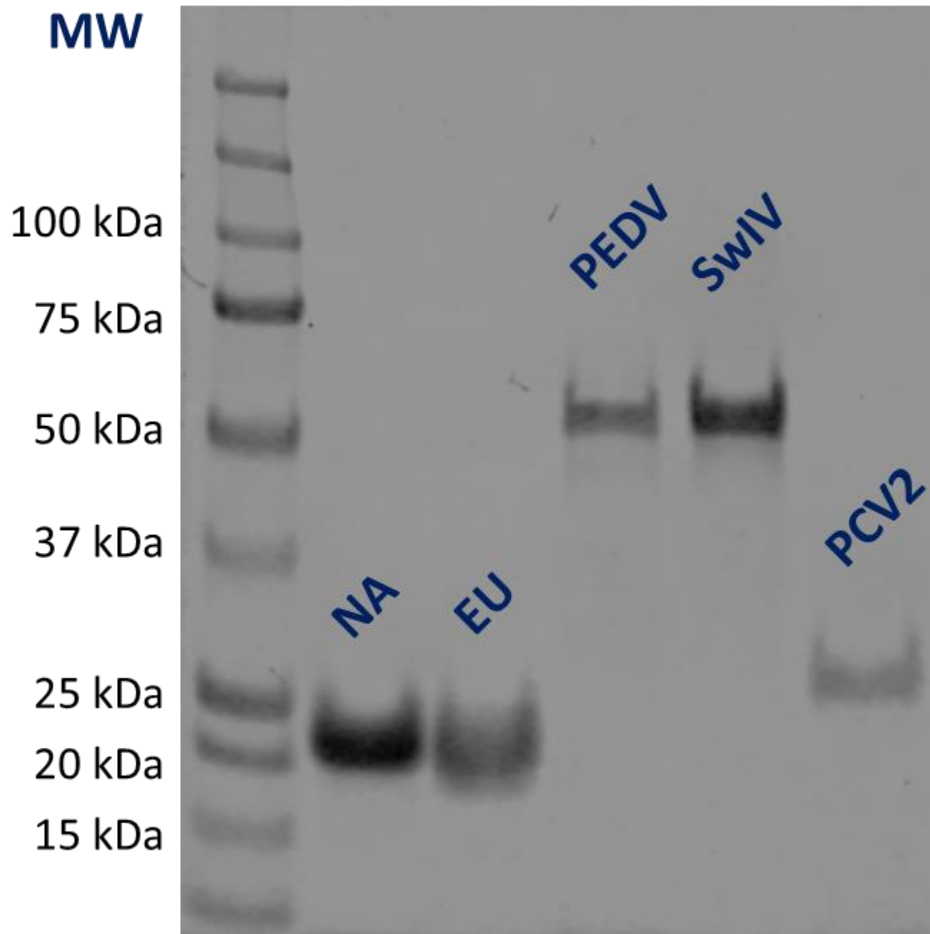


Figure 2-2 Coupling efficiency of antigen coated beads using mAbs

Coupling efficiency of the antigen coated beads determined using antigen-specific monoclonal antibody (mAb). Pairwise, PRRSV SR-30 & PCV2 R. Magar have similar coupling efficiencies (r=.994) as determined by the slope within the linear portion of the curves as do both SIV antibodies (r= .997).

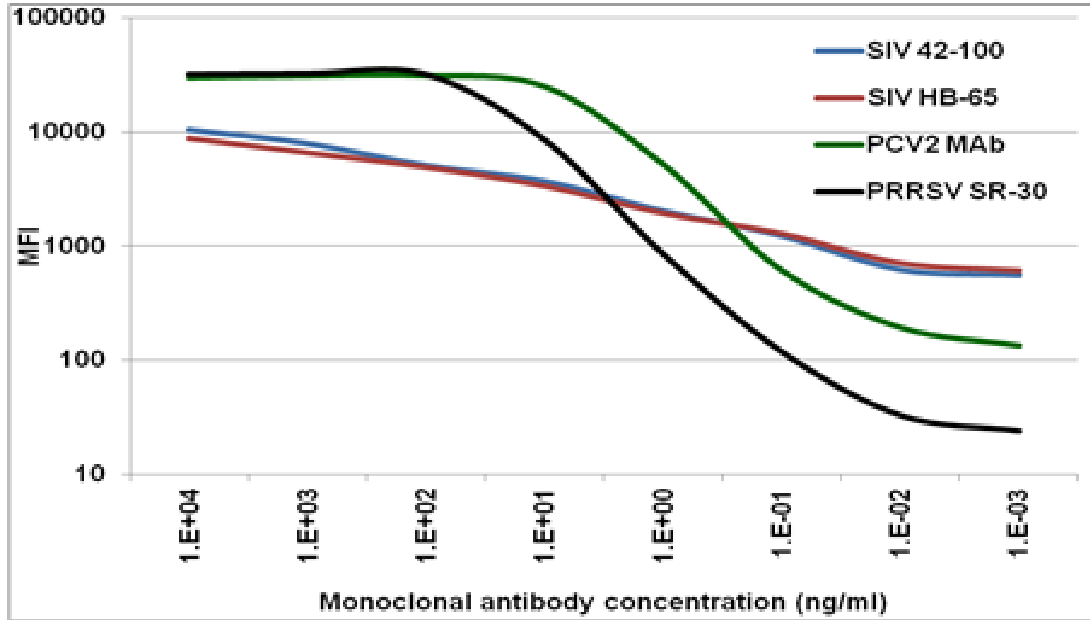


Figure 2-3 Serum dilution optimization

Optimization of the amount of infected control serum for the fluorescent microsphere immunoassay. The volume of internal control serum sample was 2-fold serial diluted against a fixed number of antigen coupled microspheres and then tested in the FMIA to generate a maximum signal to noise ratio of mean fluorescence intensity (MFI).

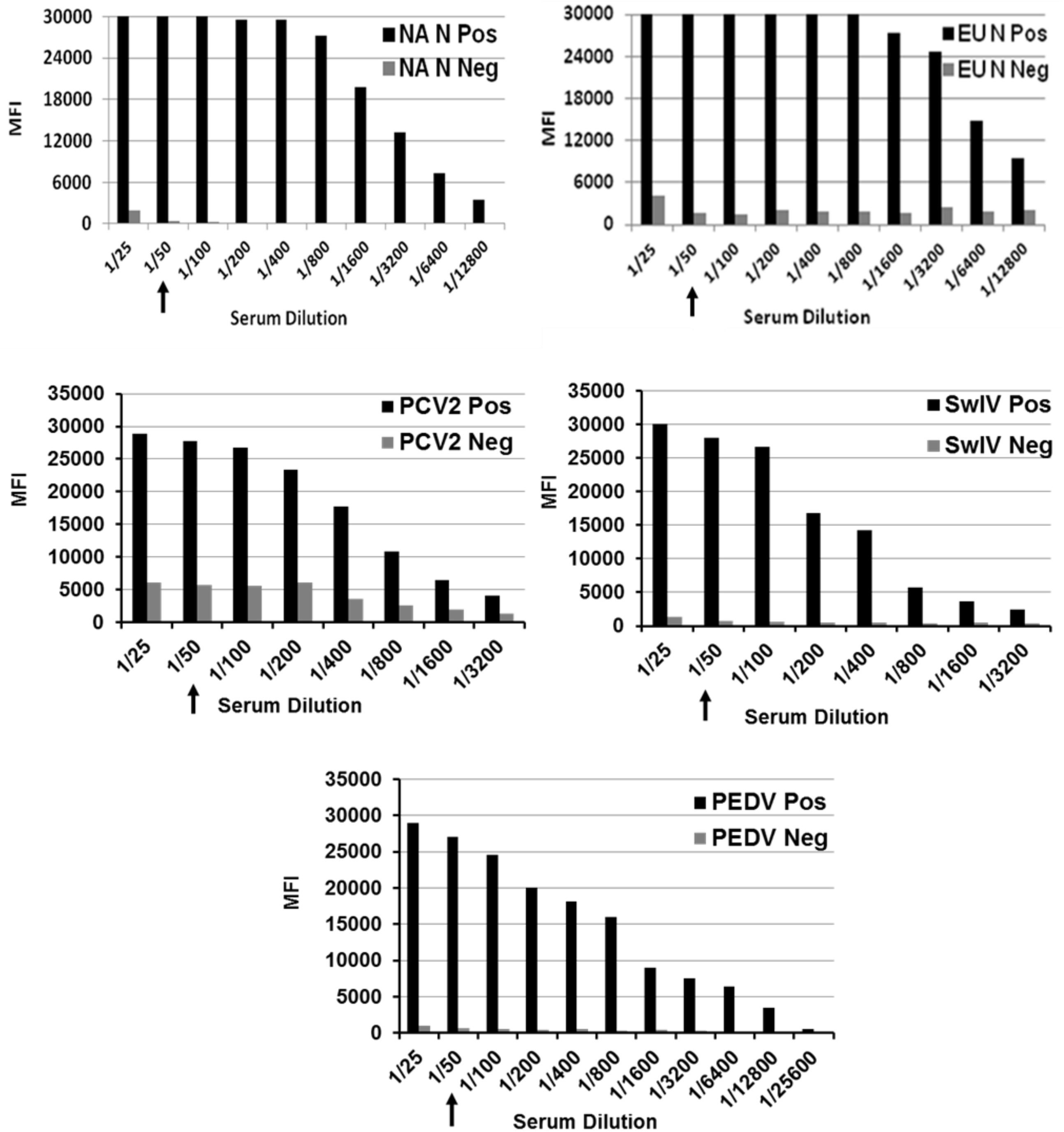


Table 2-1 Summary of ROC analysis of serum based FMIA results

	Serum		
Antigen	**Diagnostic cut off**	**Sensitivity**	**Specificity**
PRRSV Eu-N (N = 555)	0.54	100%	99.7%
PRRSV NA-N (N= 1087)	0.34	99.3%	98.9%
SwIV NP (N= 1195)	0.19	98.2%	98.3%
PCV2 N (N = 1134)	0.57	96.4%	90.1%
PEDV N (N= 176)	0.40	98.6%	96.3%

Figure 2-4 Determination of Diagnostic Sensitivity and Specificity by ROC

Determination of diagnostic sensitivity and specificity by receiver operating characterstic (ROC) analysis for serum based FMIA. Diagnostic sensitivity and specificity were calculated using samples from known PCV2, SwIV, PRRSV and PEDV infected swine populations (4147 serum samples total). ROC analysis was performed using MedCalc® Version 10.4.0.0 (Medcalc® Software, Mariakerke, Belgium). In each panel, the interactive dot plot on the left side indicates the negative population, and the dot plot on the right side indicates the positive population. The horizontal line between the positive and negative population represents the cut off value which provides optimal diagnostic sensitivity and specificity.

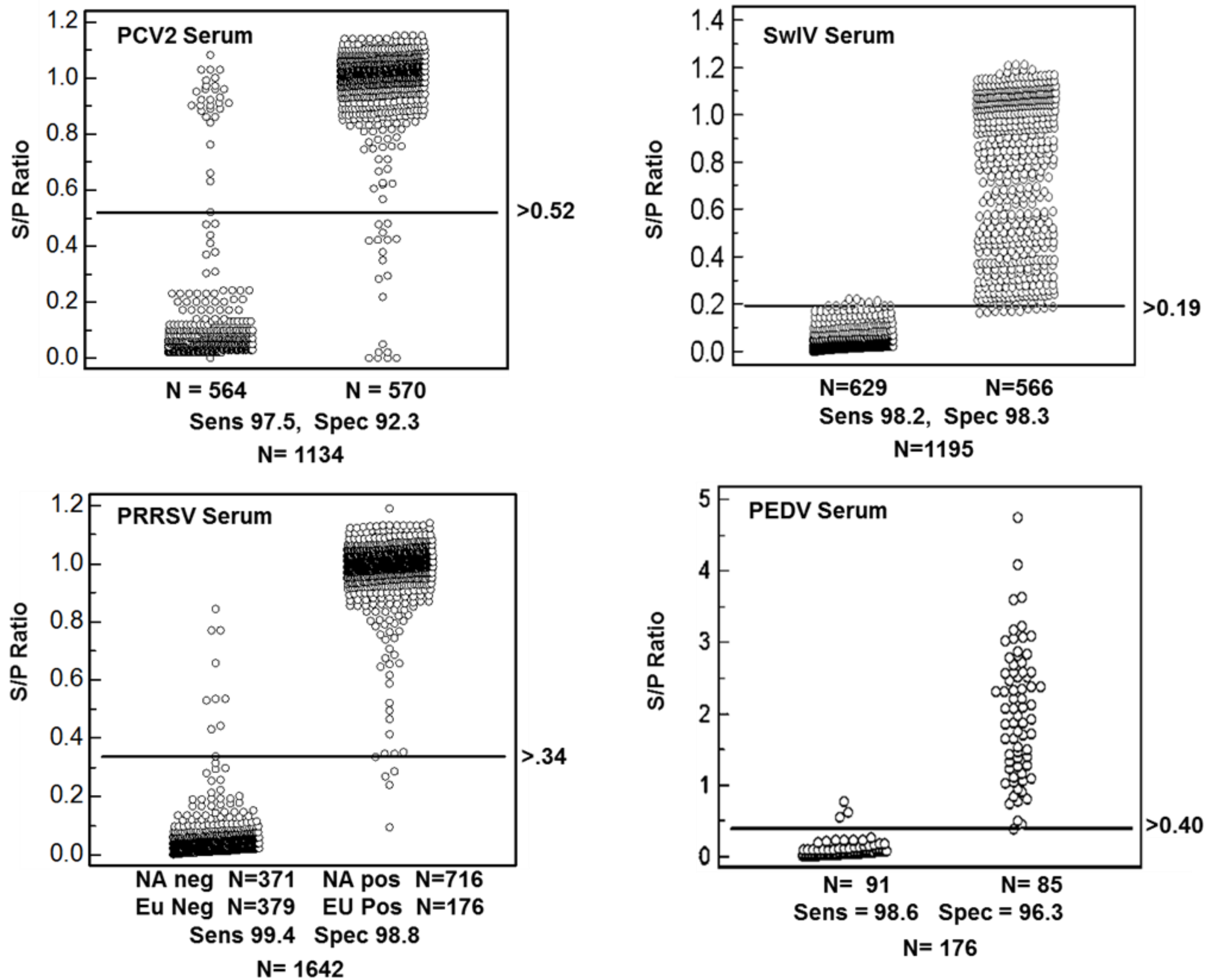


Figure 2-5 Comparison of singleplex vs. multiplex

Development of a multiplex fluorescent microsphere immunoassay for the simultaneous detection of PRRSV (A), PCV2 (B), SIV (C) and PEDV (D) using standard serum samples. Each individual bead set was first tested in a singleplex format and then combined to test all five antigens for a multiplex format. Samples were from pigs co-infected with PRRSV, PCV2, SIV and PEDV. SP: singleplex; MP: multiplex.

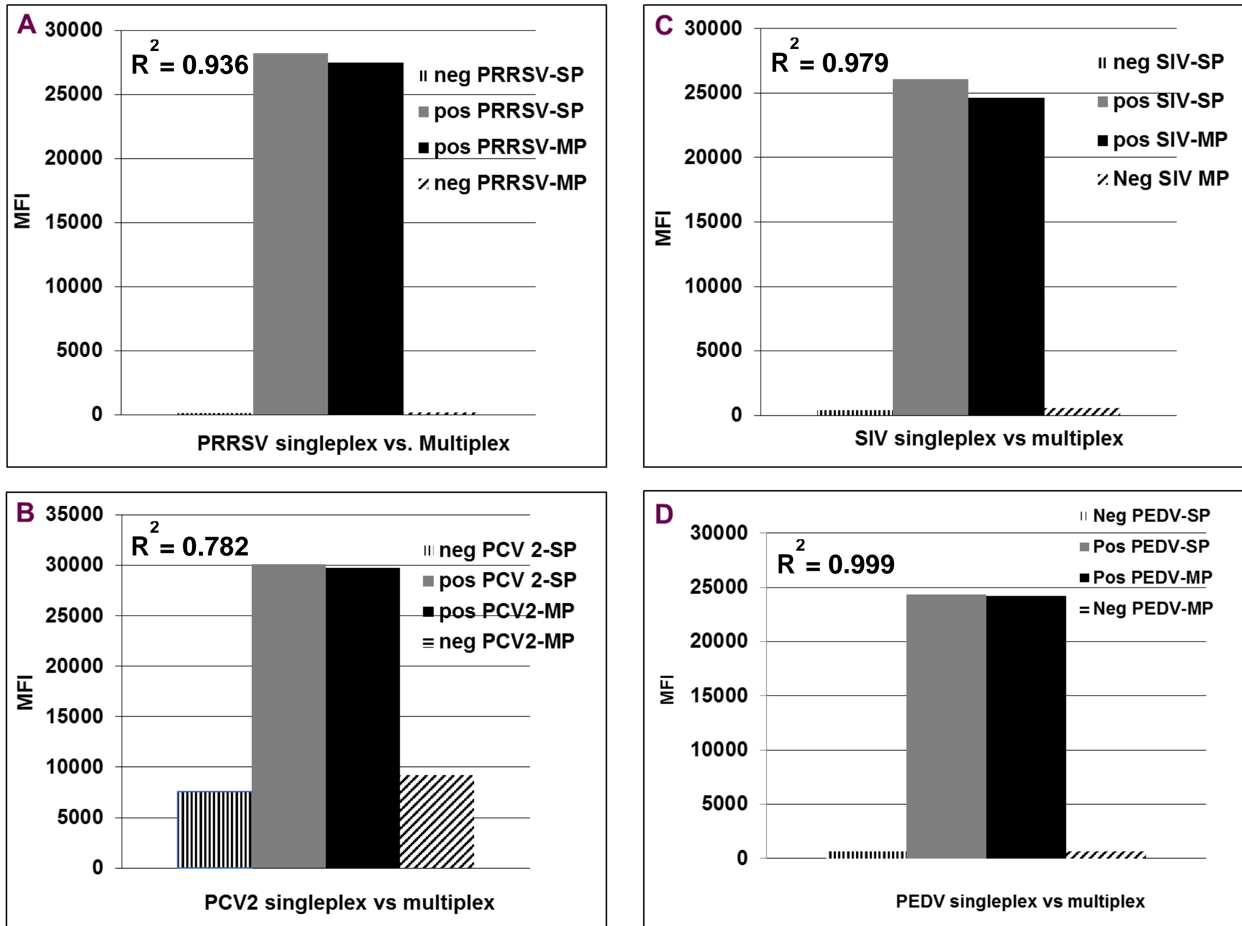


Table 2-2 Assay repeatability of serum-based FMIAs

Repeatability	PRRSV NA-N	PRRSV EU-N	SIV NP	PCV2 N	PEDV N
Serum Intra-assay repeatability (%CV)	1.9	1.7	5.6	2.0	1.4
Serum Inter-assay repeatability (%CV)	4.7	3.4	8.6	2.2	1.6

Chapter 3 - Discussion and Conclusions

Recently, many high-health status herds have fallen victim to severe porcine disease complex (PDC) outbreaks. The PDC 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 quickly and simultaneously detect multiple pathogens in the PDC offers an important tool for disease surveillance and control measurements.

In this study, we developed a 5-plex FMIA for the simultaneous detection of antibodies against the PDC in serum. The nucleocapsid protein was chosen because it is highly conserved among the strains of the respective viruses in this study, it has highly immunogenicity and because many of the current commercially available serological tests are based on this protein.

All of the antigens were expressed as recombinant proteins in an *E. coli* expression system. The generation of highly purified recombinant proteins while maintaining their native conformations is required for these assays. High levels of expression for these nucleocapsid proteins resulted in the formation of inclusion bodies and required further purification and refolding steps to return them to native forms. To prevent degradation of the proteins they were kept at cold temperatures, multiple freeze-thaw cycles were avoided by creating multiple aliquots and using them only once and a protease inhibitor (PMSF) was utilized during the protein extraction and purification protocols.

The diagnostic sensitivity and specificity was greater than 92% for each of the serum based FMIAs and multiplexing did not show any significant differences in MFI. In two previous studies performed in our lab, the PRRSV antibody response to the N protein could be detected as early as 7dpi in a standard panel of serum samples by the FMIA whereas the same panel was

tested previously by IDEXX ELISA could only detect antibody at 14dpi (Brown et al., 2009; Langenhorst et al., 2012). This highlights the increased sensitivity of the FMIA as compared to the ELISA.

Many aspects of the FMIA make it an attractive prospect for broad scale diagnostic, surveillance and epidemiological applications. The multiplex FMIA format presents the advantages of simplicity, rapidity, cost-effectiveness, and the potential to 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 the early identification of susceptible groups so that such animals can be quarantined or removed quickly to prevent transmission to naïve herd, the evaluation of vaccine efficacy and the use of these data in mathematical modeling in order to predict disease outbreaks and design better management strategies.

References

Allan, G. M., McNeilly, F., Cassidy, J. P., Reilly, G. A., Adair, B., Ellis, W. A., & McNulty, M. S. (1995). Pathogenesis of porcine circovirus; experimental infections of colostrum deprived piglets and examination of pig foetal material. *Veterinary Microbiology, 44*(1), 49-64. doi:037811359400136K [pii]

Allende, R., Lewis, T. L., Lu, Z., Rock, D. L., Kutish, G. F., Ali, A., Osorio, F. A. (1999). North American and European porcine reproductive and respiratory syndrome viruses differ in non-structural protein coding regions. *The Journal of General Virology, 80 (Pt 2)*(Pt 2), 307-315.

Anderson, S., Wakeley, P., Wibberley, G., Webster, K., & Sawyer, J. (2011). Development and evaluation of a luminex multiplex serology assay to detect antibodies to bovine herpes virus 1, parainfluenza 3 virus, bovine viral diarrhoea virus, and bovine respiratory syncytial virus, with comparison to existing ELISA detection methods. *Journal of Immunological Methods, 366*(1-2), 79-88. doi:10.1016/j.jim.2011.01.010 [doi]

Balasuriya, U. B., Shi, P. Y., Wong, S. J., Demarest, V. L., Gardner, I. A., Hullinger, P. J., MacLachlan, N. J. (2006). Detection of antibodies to west nile virus in equine sera using microsphere immunoassay. *Journal of Veterinary Diagnostic Investigation: Official Publication of the American Association of Veterinary Laboratory Diagnosticians, Inc, 18*(4), 392-395.

Barbe, F., Labarque, G., Pensaert, M., & Van Reeth, K. (2009). Performance of a commercial swine influenza virus H1N1 and H3N2 antibody enzyme-linked immunosorbent assay in pigs experimentally infected with european influenza viruses. *Journal of Veterinary Diagnostic Investigation: Official Publication of the American Association of Veterinary Laboratory Diagnosticians, Inc, 21*(1), 88-96. doi:21/1/88 [pii]

Batista, L., Pijoan, C., Dee, S., Olin, M., Molitor, T., Joo, H. S., Murtaugh, M. (2004). Virological and immunological responses to porcine reproductive and respiratory syndrome virus in a large population of gilts. *Canadian Journal of Veterinary Research, 68*(4), 267-273. doi:pg267 [pii]

Bautista, E., Meulenberg, J., Choi, C., Pol, J., & Molitor, T. (1996). PRRSV-specific T cell responses in infected and vaccinated pigs. Paper presented at the *Proc 14th IPVS, 63*

Bautista, E. M., & Molitor, T. W. (1997). Cell-mediated immunity to porcine reproductive and respiratory syndrome virus in swine. *Viral Immunology, 10*(2), 83-94.

Benfield, D. A., Nelson, E., Collins, J. E., Harris, L., Goyal, S. M., Robison, D., Chladek, D. (1992). Characterization of swine infertility and respiratory syndrome (SIRS) virus (isolate ATCC VR-2332). *Journal of Veterinary Diagnostic Investigation: Official Publication of the American Association of Veterinary Laboratory Diagnosticians, Inc, 4*(2), 127-133.

Bierk, M. D., Dee, S. A., Rossow, K. D., Otake, S., Collins, J. E., & Molitor, T. W. (2001). Transmission of porcine reproductive and respiratory syndrome virus from persistently infected sows to contact controls. *Canadian Journal of Veterinary Research = Revue Canadienne De Recherche Veterinaire, 65*(4), 261-266.

Brown, E., Lawson, S., Welbon, C., Gnanandarajah, J., Li, J., Murtaugh, M. P., Fang, Y. (2009). Antibody response to porcine reproductive and respiratory syndrome virus (PRRSV) nonstructural proteins and implications for diagnostic detection and differentiation of PRRSV types I and II. *Clinical and Vaccine Immunology: CVI, 16*(5), 628-635. doi:10.1128/CVI.00483-08 [doi]

Calvert, J. G., Slade, D. E., Shields, S. L., Jolie, R., Mannan, R. M., Ankenbauer, R. G., & Welch, S. K. (2007). CD163 expression confers susceptibility to porcine reproductive and respiratory syndrome viruses. *Journal of Virology, 81*(14), 7371-7379. doi:bJVI.00513-07 [pii]

Campitelli, L., Donatelli, I., Foni, E., Castrucci, M. R., Fabiani, C., Kawaoka, Y., Webster, R. G. (1997). Continued evolution of H1N1 and H3N2 influenza viruses in pigs in italy. *Virology, 232*(2), 310-318. doi:S0042-6822(97)98514-7 [pii]

Carson, R. T., & Vignali, D. A. (1999). Simultaneous quantitation of 15 cytokines using a multiplexed flow cytometric assay. *Journal of Immunological Methods, 227*(1-2), 41-52. doi:S0022-1759(99)00069-1 [pii]

Cavanagh, D. (1997). Nidovirales: A new order comprising coronaviridae and arteriviridae. *Archives of Virology, 142*(3), 629-633.

Chae, C. (2005). A review of porcine circovirus 2-associated syndromes and diseases. *Veterinary Journal (London, England: 1997), 169*(3), 326-336. doi:S1090023304000176 [pii]

Chand, R. J., Trible, B. R., & Rowland, R. R. (2012). Pathogenesis of porcine reproductive and respiratory syndrome virus. *Current Opinion in Virology, 2*(3), 256-263. doi:10.1016/j.coviro.2012.02.002 [doi]

Chen, Q., Li, G., Stasko, J., Thomas, J. T., Stensland, W. R., Pillatzki, A. E., Zhang, J. (2014). Isolation and characterization of porcine epidemic diarrhea viruses associated with the 2013 disease outbreak among swine in the united states. *Journal of Clinical Microbiology, 52*(1), 234-243. doi:10.1128/JCM.02820-13 [doi]

Chen, R., Yu, X., Gao, X., Xue, C., Song, C., Li, Y., & Cao, Y. (2015). Bead-based suspension array for simultaneous differential detection of five major swine viruses. *Applied Microbiology and Biotechnology,*doi:10.1007/s00253-014-6337-8 [doi]

Cho, J. G., & Dee, S. A. (2006). Porcine reproductive and respiratory syndrome virus. *Theriogenology, 66*(3), 655-662. doi:S0093-691X(06)00258-5 [pii]

Choi, C., & Chae, C. (2001). Colocalization of porcine reproductive and respiratory syndrome virus and porcine circovirus 2 in porcine dermatitis and nephrology syndrome by double-labeling technique. *Veterinary Pathology, 38*(4), 436-441.

Christopher-Hennings, J., Holler, L. D., Benfield, D. A., & Nelson, E. A. (2001). Detection and duration of porcine reproductive and respiratory syndrome virus in semen, serum, peripheral blood mononuclear cells, and tissues from yorkshire, hampshire, and landrace boars. *Journal of*

Veterinary Diagnostic Investigation: Official Publication of the American Association of Veterinary Laboratory Diagnosticians, Inc, 13(2), 133-142.

Christopher-Hennings, J., Nelson, E. A., Nelson, J. K., Rossow, K. D., Shivers, J. L., Yaeger, M. J., Benfield, D. A. (1998). Identification of porcine reproductive and respiratory syndrome virus in semen and tissues from vasectomized and nonvasectomized boars. *Veterinary Pathology, 35*(4), 260-267.

Claas, E. C., Burnham, C. A., Mazzulli, T., Templeton, K., & Topin, F. (2013). Performance of the xTAG(R) gastrointestinal pathogen panel, a multiplex molecular assay for simultaneous detection of bacterial, viral, and parasitic causes of infectious gastroenteritis. *Journal of Microbiology and Biotechnology, 23*(7), 1041-1045. doi:10.4014/jmb.1212.12042 [pii]

Collins, J. E., Benfield, D. A., Christianson, W. T., Harris, L., Hennings, J. C., Shaw, D. P., Joo, H. S. (1992). Isolation of swine infertility and respiratory syndrome virus (isolate ATCC VR-2332) in north america and experimental reproduction of the disease in gnotobiotic pigs. *Journal of Veterinary Diagnostic Investigation: Official Publication of the American Association of Veterinary Laboratory Diagnosticians, Inc, 4*(2), 117-126.

Corzo, C. A., Mondaca, E., Wayne, S., Torremorell, M., Dee, S., Davies, P., & Morrison, R. B. (2010). Control and elimination of porcine reproductive and respiratory syndrome virus. *Virus Research, 154*(1-2), 185-192. doi:10.1016/j.virusres.2010.08.016 [doi]

Croft, H., Malinowski, T., Krizbai, L., Mikec, I., Kajic, V., Reed, C., James, D. (2008). Use of luminex xMAP-derived bio-plex bead-based suspension array for specific detection of PPV W and characterization of epitopes on the coat protein of the virus. *Journal of Virological Methods, 153*(2), 203-213. doi:10.1016/j.jviromet.2008.07.016 [doi]

Dea, S., Wilson, L., Therrien, D., & Cornaglia, E. (2000). Competitive ELISA for detection of antibodies to porcine reproductive and respiratory syndrome virus using recombinant E. coli-expressed nucleocapsid protein as antigen. *Journal of Virological Methods, 87*(1-2), 109-122. doi:S0166093400001580 [pii]

Drew, T. W., Meulenberg, J. J., Sands, J. J., & Paton, D. J. (1995). Production, characterization and reactivity of monoclonal antibodies to porcine reproductive and respiratory syndrome virus. *The Journal of General Virology,76 (Pt 6)*(Pt 6), 1361-1369.

Duan, X., Nauwynck, H. J., & Pensaert, M. B. (1997). Effects of origin and state of differentiation and activation of monocytes/macrophages on their susceptibility to porcine reproductive and respiratory syndrome virus (PRRSV). *Archives of Virology, 142*(12), 2483-2497.

Duan, X., Nauwynck, H. J., & Pensaert, M. B. (1997). Virus quantification and identification of cellular targets in the lungs and lymphoid tissues of pigs at different time intervals after inoculation with porcine reproductive and respiratory syndrome virus (PRRSV). *Veterinary Microbiology, 56*(1-2), 9-19. doi:S0378-1135(96)01347-8 [pii]

Dunbar, S. A. (2006). Applications of luminex xMAP technology for rapid, high-throughput multiplexed nucleic acid detection. *Clinica Chimica Acta; International Journal of Clinical Chemistry, 363*(1-2), 71-82. doi:S0009-8981(05)00426-2 [pii]

Dunbar, S. A., & Jacobson, J. W. (2000). Application of the luminex LabMAP in rapid screening for mutations in the cystic fibrosis transmembrane conductance regulator gene: A pilot study. *Clinical Chemistry, 46*(9), 1498-1500.

Dunham, E. J., Dugan, V. G., Kaser, E. K., Perkins, S. E., Brown, I. H., Holmes, E. C., & Taubenberger, J. K. (2009). Different evolutionary trajectories of european avian-like and classical swine H1N1 influenza A viruses.*Journal of Virology, 83*(11), 5485-5494. doi:10.1128/JVI.02565-08 [doi]

Fachinger, V., Bischoff, R., Jedidia, S. B., Saalmuller, A., & Elbers, K. (2008). The effect of vaccination against porcine circovirus type 2 in pigs suffering from porcine respiratory disease complex. *Vaccine, 26*(11), 1488-1499. doi:10.1016/j.vaccine.2007.11.053 [doi]

Fenaux, M., Halbur, P. G., Gill, M., Toth, T. E., & Meng, X. J. (2000). Genetic characterization of type 2 porcine circovirus (PCV-2) from pigs with postweaning multisystemic wasting syndrome in

different geographic regions of north america and development of a differential PCR-restriction fragment length polymorphism assay to detect and differentiate between infections with PCV-1 and PCV-2. *Journal of Clinical Microbiology, 38*(7), 2494-2503. doi:0371 [pii]

Fenaux, M., Halbur, P. G., Gill, M., Toth, T. E., & Meng, X. J. (2000). Genetic characterization of type 2 porcine circovirus (PCV-2) from pigs with postweaning multisystemic wasting syndrome in different geographic regions of north america and development of a differential PCR-restriction fragment length polymorphism assay to detect and differentiate between infections with PCV-1 and PCV-2. *Journal of Clinical Microbiology, 38*(7), 2494-2503.

Fitzgerald, C., Collins, M., van Duyne, S., Mikoleit, M., Brown, T., & Fields, P. (2007). Multiplex, bead-based suspension array for molecular determination of common salmonella serogroups. *Journal of Clinical Microbiology,45*(10), 3323-3334. doi:JCM.00025-07 [pii]

Fulton, R. J., McDade, R. L., Smith, P. L., Kienker, L. J., & Kettman, J. R.,Jr. (1997). Advanced multiplexed analysis with the FlowMetrix system. *Clinical Chemistry, 43*(9), 1749-1756.

Gauger, P. C., Loving, C. L., & Vincent, A. L. (2014). Enzyme-linked immunosorbent assay for detection of serum or mucosal isotype-specific IgG and IgA whole-virus antibody to influenza A virus in swine. *Methods in Molecular Biology (Clifton, N.J.), 1161*, 303-312. doi:10.1007/978-1-4939-0758-8_25 [doi]

Gillespie, J., Opriessnig, T., Meng, X. J., Pelzer, K., & Buechner-Maxwell, V. (2009). Porcine circovirus type 2 and porcine circovirus-associated disease. *Journal of Veterinary Internal Medicine, 23*(6), 1151-1163. doi:10.1111/j.1939-1676.2009.0389.x

Gillespie, J., Opriessnig, T., Meng, X. J., Pelzer, K., & Buechner-Maxwell, V. (2009). Porcine circovirus type 2 and porcine circovirus-associated disease. *Journal of Veterinary Internal Medicine, 23*(6), 1151-1163. doi:10.1111/j.1939-1676.2009.0389.x

Goodell, C. K., Prickett, J., Kittawornrat, A., Johnson, J., Zhang, J., Wang, C., & Zimmerman, J. J. (2014). Evaluation of screening assays for the detection of influenza A virus serum antibodies in swine. *Transboundary and Emerging Diseases,* doi:10.1111/tbed.12214 [doi]

Gordon, R. F., & McDade, R. L. (1997). Multiplexed quantification of human IgG, IgA, and IgM with the FlowMetrixTM system, *Clinical Chemistry, 43*(9), 1799-1801.

Griffin, S. M., Chen, I. M., Fout, G. S., Wade, T. J., & Egorov, A. I. (2011). Development of a multiplex microsphere immunoassay for the quantitation of salivary antibody responses to selected waterborne pathogens. *Journal of Immunological Methods, 364*(1-2), 83-93. doi:10.1016/j.jim.2010.11.005 [doi]

Hamel, A. L., Lin, L. L., & Nayar, G. P. (1998). Nucleotide sequence of porcine circovirus associated with postweaning multisystemic wasting syndrome in pigs. *Journal of Virology, 72*(6), 5262-5267.

Harding, J. C. (2004). The clinical expression and emergence of porcine circovirus 2. *Veterinary Microbiology, 98*(2), 131-135. doi:S0378113503003286 [pii]

Hou, X. L., Yu, L. Y., & Liu, J. (2007). Development and evaluation of enzyme-linked immunosorbent assay based on recombinant nucleocapsid protein for detection of porcine epidemic diarrhea (PEDV) antibodies. *Veterinary Microbiology, 123*(1-3), 86-92. doi:S0378-1135(07)00077-6 [pii]

Huang, Y. W., Dickerman, A. W., Pineyro, P., Li, L., Fang, L., Kiehne, R., . . . Meng, X. J. (2013). Origin, evolution, and genotyping of emergent porcine epidemic diarrhea virus strains in the united states. *Mbio, 4*(5), e00737-13. doi:10.1128/mBio.00737-13 [doi]

Keij, J. F., & Steinkamp, J. A. (1998). Flow cytometric characterization and classification of multiple dual-color fluorescent microspheres using fluorescence lifetime. *Cytometry, 33*(3), 318-323. doi:10.1002/(SICI)1097-0320(19981101)33:3<318::AID-CYTO5>3.0.CO;2-C [pii]

Kellar, K. L., & Iannone, M. A. (2002). Multiplexed microsphere-based flow cytometric assays. *Experimental Hematology, 30*(11), 1227-1237. doi:S0301472X02009220 [pii]

Kennedy, S., Moffett, D., McNeilly, F., Meehan, B., Ellis, J., Krakowka, S., & Allan, G. M. (2000). Reproduction of lesions of postweaning multisystemic wasting syndrome by infection of conventional pigs with porcine circovirus type 2 alone or in combination with porcine parvovirus. *Journal of Comparative Pathology, 122*(1), 9-24. doi:10.1053/jcpa.1999.0337 [doi]

Kim, H. S., Kwang, J., Yoon, I. J., Joo, H. S., & Frey, M. L. (1993). Enhanced replication of porcine reproductive and respiratory syndrome (PRRS) virus in a homogeneous subpopulation of MA-104 cell line. *Archives of Virology, 133*(3-4), 477-483.

Kim, J., Chung, H. K., & Chae, C. (2003). Association of porcine circovirus 2 with porcine respiratory disease complex. *Veterinary Journal (London, England: 1997), 166*(3), 251-256. doi:S1090023302002575 [pii]

Kim, S. H., Kim, I. J., Pyo, H. M., Tark, D. S., Song, J. Y., & Hyun, B. H. (2007). Multiplex real-time RT-PCR for the simultaneous detection and quantification of transmissible gastroenteritis virus and porcine epidemic diarrhea virus. *Journal of Virological Methods, 146*(1-2), 172-177. doi:S0166-0934(07)00246-7 [pii]

Kim, W. I., Wu, W. H., Janke, B., & Yoon, K. J. (2006). Characterization of the humoral immune response of experimentally infected and vaccinated pigs to swine influenza viral proteins. *Archives of Virology, 151*(1), 23-36. doi:10.1007/s00705-005-0615-9 [doi]

Knuchel, M., Ackermann, M., Muller, H. K., & Kihm, U. (1992). An ELISA for detection of antibodies against porcine epidemic diarrhoea virus (PEDV) based on the specific solubility of the viral surface glycoprotein. *Veterinary Microbiology, 32*(2), 117-134. doi:0378-1135(92)90100-8 [pii]

Koen J. (1919). A practical method for field diagnosis of swine diseases. *American Journal of Veterinary Medicine*, 14:468–470.

Krakowka, S., Allan, G., Ellis, J., Hamberg, A., Charreyre, C., Kaufmann, E., Meehan, B. (2012). A nine-base nucleotide sequence in the porcine circovirus type 2 (PCV2) nucleocapsid gene

determines viral replication and virulence. *Virus Research, 164*(1-2), 90-99. doi:10.1016/j.virusres.2011.10.027 [doi]

Langenhorst, R. J., Lawson, S., Kittawornrat, A., Zimmerman, J. J., Sun, Z., Li, Y., Fang, Y. (2012). Development of a fluorescent microsphere immunoassay for detection of antibodies against porcine reproductive and respiratory syndrome virus using oral fluid samples as an alternative to serum-based assays. *Clinical and Vaccine Immunology: CVI, 19*(2), 180-189. doi:10.1128/CVI.05372-11 [doi]

Lawson, S., Lunney, J., Zuckermann, F., Osorio, F., Nelson, E., Welbon, C., Christopher-Hennings, J. (2010). Development of an 8-plex luminex assay to detect swine cytokines for vaccine development: Assessment of immunity after porcine reproductive and respiratory syndrome virus (PRRSV) vaccination. *Vaccine, 28*(32), 5356-5364. doi:10.1016/j.vaccine.2010.05.016 [doi]

Lee, B. W., Bey, R. F., Baarsch, M. J., & Emery, D. A. (1993). Subtype specific ELISA for the detection of antibodies against influenza A H1N1 and H3N2 in swine. *Journal of Virological Methods, 45*(2), 121-136. doi:0166-0934(93)90097-B [pii]

Lin, K., Wang, C., Murtaugh, M. P., & Ramamoorthy, S. (2011). Multiplex method for simultaneous serological detection of porcine reproductive and respiratory syndrome virus and porcine circovirus type 2. *Journal of Clinical Microbiology, 49*(9), 3184-3190. doi:10.1128/JCM.00557-11 [doi]

Lindau-Shepard, B. A., & Pass, K. A. (2010). Newborn screening for cystic fibrosis by use of a multiplex immunoassay. *Clinical Chemistry, 56*(3), 445-450. doi:10.1373/clinchem.2009.132480 [doi]

Liu, C., Ihara, T., Nunoya, T., & Ueda, S. (2004). Development of an ELISA based on the baculovirus-expressed capsid protein of porcine circovirus type 2 as antigen. *The Journal of Veterinary Medical Science / the Japanese Society of Veterinary Science, 66*(3), 237-242.

Liu, Q., Tikoo, S. K., & Babiuk, L. A. (2001). Nuclear localization of the ORF2 protein encoded by porcine circovirus type 2. *Virology, 285*(1), 91-99. doi:10.1006/viro.2001.0922 [doi]

Ma, W., Lager, K. M., Vincent, A. L., Janke, B. H., Gramer, M. R., & Richt, J. A. (2009). The role of swine in the generation of novel influenza viruses. *Zoonoses and Public Health, 56*(6-7), 326-337. doi:10.1111/j.1863-2378.2008.01217.x [doi]

Ma, W., Lager, K. M., Vincent, A. L., Janke, B. H., Gramer, M. R., & Richt, J. A. (2009). The role of swine in the generation of novel influenza viruses. *Zoonoses and Public Health, 56*(6-7), 326-337. doi:10.1111/j.1863-2378.2008.01217.x [doi]

Martins, T. B. (2002). Development of internal controls for the luminex instrument as part of a multiplex seven-analyte viral respiratory antibody profile. *Clinical and Diagnostic Laboratory Immunology, 9*(1), 41-45.

McNeilly, F., McNair, I., O'Connor, M., Brockbank, S., Gilpin, D., Lasagna, C., Allan, G. M. (2002). Evaluation of a porcine circovirus type 2-specific antigen-capture enzyme-linked immunosorbent assay for the diagnosis of postweaning multisystemic wasting syndrome in pigs: Comparison with virus isolation, immunohistochemistry, and the polymerase chain reaction. *Journal of Veterinary Diagnostic Investigation: Official Publication of the American Association of Veterinary Laboratory Diagnosticians, Inc, 14*(2), 106-112.

Meehan, B. M., Creelan, J. L., McNulty, M. S., & Todd, D. (1997). Sequence of porcine circovirus DNA: Affinities with plant circoviruses. *The Journal of General Virology, 78 (Pt 1)*(Pt 1), 221-227.

Meehan, B. M., McNeilly, F., Todd, D., Kennedy, S., Jewhurst, V. A., Ellis, J. A., Allan, G. M. (1998). Characterization of novel circovirus DNAs associated with wasting syndromes in pigs. *The Journal of General Virology, 79 (Pt 9)*(Pt 9), 2171-2179.

Memoli, M. J., Tumpey, T. M., Jagger, B. W., Dugan, V. G., Sheng, Z. M., Qi, L., Taubenberger, J. K. (2009). An early â€˜classicalâ€™ swine H1N1 influenza virus shows similar pathogenicity to the

1918 pandemic virus in ferrets and mice. *Virology, 393*(2), 338-345. doi:10.1016/j.virol.2009.08.021 [doi]

Miller, M. (2011). PRRS price tag, $664 million. Porknetwork http://wwwporknetworkcom/pork-news/127963843html.

Morozov, I., Sirinarumitr, T., Sorden, S. D., Halbur, P. G., Morgan, M. K., Yoon, K. J., & Paul, P. S. (1998). Detection of a novel strain of porcine circovirus in pigs with postweaning multisystemic wasting syndrome. *Journal of Clinical Microbiology, 36*(9), 2535-2541. doi:0089 [pii]

Nainys, J., Lasickiene, R., Petraityte-Burneikiene, R., Dabrisius, J., Lelesius, R., Sereika, V., Gedvilaite, A. (2014). Generation in yeast of recombinant virus-like particles of porcine circovirus type 2 capsid protein and their use for a serologic assay and development of monoclonal antibodies. *BMC Biotechnology, 14*(1), 100. doi:s12896-014-0100-1 [pii]

Nauwynck, H. J., Duan, X., Favoreel, H. W., Van Oostveldt, P., & Pensaert, M. B. (1999). Entry of porcine reproductive and respiratory syndrome virus into porcine alveolar macrophages via receptor-mediated endocytosis.*The Journal of General Virology, 80 (Pt 2)*(Pt 2), 297-305.

Nawagitgul, P., Harms, P. A., Morozov, I., Thacker, B. J., Sorden, S. D., Lekcharoensuk, C., & Paul, P. S. (2002). Modified indirect porcine circovirus (PCV) type 2-based and recombinant capsid protein (ORF2)-based enzyme-linked immunosorbent assays for detection of antibodies to PCV. *Clinical and Diagnostic Laboratory Immunology, 9*(1), 33-40.

Nawagitgul, P., Harms, P. A., Morozov, I., Thacker, B. J., Sorden, S. D., Lekcharoensuk, C., & Paul, P. S. (2002). Modified indirect porcine circovirus (PCV) type 2-based and recombinant capsid protein (ORF2)-based enzyme-linked immunosorbent assays for detection of antibodies to PCV. *Clinical and Diagnostic Laboratory Immunology, 9*(1), 33-40. doi:0113 [pii]

Nawagitgul, P., Morozov, I., Bolin, S. R., Harms, P. A., Sorden, S. D., & Paul, P. S. (2000). Open reading frame 2 of porcine circovirus type 2 encodes a major capsid protein. *The Journal of General Virology, 81*(Pt 9), 2281-2287.

Nelsen, C. J., Murtaugh, M. P., & Faaberg, K. S. (1999). Porcine reproductive and respiratory syndrome virus comparison: Divergent evolution on two continents. *Journal of Virology, 73*(1), 270-280. doi:1126 [pii]

Oh, J. S., Song, D. S., Yang, J. S., Song, J. Y., Moon, H. J., Kim, T. Y., & Park, B. K. (2005). Comparison of an enzyme-linked immunosorbent assay with serum neutralization test for serodiagnosis of porcine epidemic diarrhea virus infection. *Journal of Veterinary Science, 6*(4), 349-352. doi:200512349 [pii]

Oldham, J., 1972. Letter to the editor. Pig Farming (Suppl.), 72–73.

Oliver, K. G., Kettman, J. R., & Fulton, R. J. (1998). Multiplexed analysis of human cytokines by use of the FlowMetrix system. *Clinical Chemistry, 44*(9), 2057-2060.

Opriessnig, T., McKeown, N. E., Harmon, K. L., Meng, X. J., & Halbur, P. G. (2006). Porcine circovirus type 2 infection decreases the efficacy of a modified live porcine reproductive and respiratory syndrome virus vaccine.*Clinical and Vaccine Immunology, 13*(8), 923-929. doi:0074-06 [pii]

Opriessnig, T., Meng, X. J., & Halbur, P. G. (2007). Porcine circovirus type 2 associated disease: Update on current terminology, clinical manifestations, pathogenesis, diagnosis, and intervention strategies. *Journal of Veterinary Diagnostic Investigation : Official Publication of the American Association of Veterinary Laboratory Diagnosticians, Inc, 19*(6), 591-615. doi:19/6/591 [pii]

Ouardani, M., Wilson, L., Jette, R., Montpetit, C., & Dea, S. (1999). Multiplex PCR for detection and typing of porcine circoviruses. *Journal of Clinical Microbiology, 37*(12), 3917-3924.

Owolodun, O. A., Gimenez-Lirola, L. G., Gerber, P. F., Sanford, B. J., Feagins, A. R., Meng, X. J., . . . Opriessnig, T. (2013). Development of a fluorescent microbead-based immunoassay for the detection of hepatitis E virus IgG antibodies in pigs and comparison to an enzyme-linked immunoassay. *Journal of Virological Methods, 193*(2), 278-283. doi:10.1016/j.jviromet.2013.06.010 [doi]

Pensaert, M. B., & de Bouck, P. (1978). A new coronavirus-like particle associated with diarrhea in swine. *Archives of Virology, 58*(3), 243-247.

Pitkin, A., Deen, J., & Dee, S. (2009). Further assessment of fomites and personnel as vehicles for the mechanical transport and transmission of porcine reproductive and respiratory syndrome virus. *Canadian Journal of Veterinary Research = Revue Canadienne De Recherche Veterinaire, 73*(4), 298-302.

Racine, S., Kheyar, A., Gagnon, C. A., Charbonneau, B., & Dea, S. (2004). Eucaryotic expression of the nucleocapsid protein gene of porcine circovirus type 2 and use of the protein in an indirect immunofluorescence assay for serological diagnosis of postweaning multisystemic wasting syndrome in pigs. *Clinical and Diagnostic Laboratory Immunology, 11*(4), 736-741. doi:10.1128/CDLI.11.4.736-741.2004 [doi]

Rossow, K. D. (1998). Porcine reproductive and respiratory syndrome. *Veterinary Pathology, 35*(1), 1-20.

Rovira, A., Balasch, M., Segales, J., Garcia, L., Plana-Duran, J., Rosell, C., Domingo, M. (2002). Experimental inoculation of conventional pigs with porcine reproductive and respiratory syndrome virus and porcine circovirus 2. *Journal of Virology, 76*(7), 3232-3239.

Ryan, J. T., & Rose, T. M. (2013). Development of whole-virus multiplex luminex-based serological assays for diagnosis of infections with kaposi's sarcoma-associated herpesvirus/human herpesvirus 8 homologs in macaques. *Clinical and Vaccine Immunology: CVI, 20*(3), 409-419. doi:10.1128/CVI.00673-12 [doi]

Saif LJ, Pensaert MB, Sestak K, Yeo SG, Jung K. (2012. Coronaviruses, p. 501-524. In Zimmerman J. J., Karriker L. A., Ramirez A., Schwartz K. J., Stevenson G. W. (ed.), Disease of swine, 10 th ed, vol. 35. John Wiley & Sons, Inc., NY, USA.

Sattler, T., Wodak, E., Revilla-Fernandez, S., & Schmoll, F. (2014). Comparison of different commercial ELISAs for detection of antibodies against porcine respiratory and reproductive syndrome virus in serum. *BMC Veterinary Research, 10*(1), 300. doi:s12917-014-0300-x [pii]

Schmitt, M., Bravo, I. G., Snijders, P. J. F., Gissmann, L., Pawlita, M., & Waterboer, T. (2006). Bead-based multiplex genotyping of human papillomaviruses. *Journal of Clinical Microbiology, 44*(2), 504-512. doi:1829-05 [pii]

Segales, J., & Domingo, M. (2002). Postweaning multisystemic wasting syndrome (PMWS) in pigs. A review. *The Veterinary Quarterly, 24*(3), 109-124. doi:10.1080/01652176.2002.9695132 [doi]

Segales, J., Rosell, C., & Domingo, M. (2004). Pathological findings associated with naturally acquired porcine circovirus type 2 associated disease. *Veterinary Microbiology, 98*(2), 137-149. doi:S0378113503003389 [pii]

Shi, M., Lam, T. T., Hon, C. C., Hui, R. K., Faaberg, K. S., Wennblom, T., . . . Leung, F. C. (2010). Molecular epidemiology of PRRSV: A phylogenetic perspective. *Virus Research, 154*(1-2), 7-17. doi:10.1016/j.virusres.2010.08.014 [doi]

Shoma, S., Verkaik, N. J., de Vogel, C. P., Hermans, P. W., van Selm, S., Mitchell, T. J., . . . van Belkum, A. (2011). Development of a multiplexed bead-based immunoassay for the simultaneous detection of antibodies to 17 pneumococcal proteins. *European Journal of Clinical Microbiology & Infectious Diseases: Official Publication of the European Society of Clinical Microbiology, 30*(4), 521-526. doi:10.1007/s10096-010-1113-x [doi]

Shope, R. E. (1931). Swine influenza: Iii. filtration experiments and etiology. *The Journal of Experimental Medicine, 54*(3), 373-385.

Song D, Park B. 2012. Porcine epidemic diarrhoea virus: a comprehensive review of molecular epidemiology, diagnosis, and vaccines. Virus genes. 696 44:167-175.

Smith, P. L., WalkerPeach, C. R., Fulton, R. J., & DuBois, D. B. (1998). A rapid, sensitive, multiplexed assay for detection of viral nucleic acids using the FlowMetrix system. *Clinical Chemistry, 44*(9), 2054-2056.

Snijder, E. J., Kikkert, M., & Fang, Y. (2013). Arterivirus molecular biology and pathogenesis. *The Journal of General Virology, 94*(Pt 10), 2141-2163. doi:10.1099/vir.0.056341-0 [doi]

Snijder, E. J., & Meulenberg, J. J. (1998). The molecular biology of arteriviruses. *The Journal of General Virology, 79 (Pt 5)*(Pt 5), 961-979.

Stevenson, G. W., Hoang, H., Schwartz, K. J., Burrough, E. R., Sun, D., Madson, D., . . . Yoon, K. J. (2013). Emergence of porcine epidemic diarrhea virus in the united states: Clinical signs, lesions, and viral genomic sequences. *Journal of Veterinary Diagnostic Investigation: Official Publication of the American Association of Veterinary Laboratory Diagnosticians, Inc, 25*(5), 649-654. doi:10.1177/1040638713501675 [doi]

Sun RQ, Cai RJ, Chen YQ, Liang PS, Chen DK, Song CX. 2012. Outbreak 775 of porcine epidemic diarrhea in suckling piglets, China. Emerg. Infect. Dis. 776 18:161-163

Thacker, E. L. (2001). Immunology of the porcine respiratory disease complex. *The Veterinary Clinics of North America.Food Animal Practice, 17*(3), 551-565.

Tischer, I., Gelderblom, H., Vettermann, W., & Koch, M. A. (1982). A very small porcine virus with circular single-stranded DNA. *Nature, 295*(5844), 64-66.

Tischer, I., Mields, W., Wolff, D., Vagt, M., & Griem, W. (1986). Studies on epidemiology and pathogenicity of porcine circovirus. *Archives of Virology, 91*(3-4), 271-276.

Tsai, Y. C., Chang, H. W., Jeng, C. R., Lin, T. L., Lin, C. M., Wan, C. H., & Pang, V. F. (2012). The effect of infection order of porcine circovirus type 2 and porcine reproductive and respiratory syndrome virus on dually infected swine alveolar macrophages. *BMC Veterinary Research, 8*, 174-6148-8-174. doi:1746-6148-8-174 [pii]

van Reeth, K., & Nauwynck, H. (2000). Proinflammatory cytokines and viral respiratory disease in pigs. *Veterinary Research, 31*(2), 187-213. doi:10.1051/vetres:2000113 [doi]

Van Reeth, K., Nauwynck, H., & Pensaert, M. (1996). Dual infections of feeder pigs with porcine reproductive and respiratory syndrome virus followed by porcine respiratory coronavirus or swine influenza virus: A clinical and virological study. *Veterinary Microbiology, 48*(3-4), 325-335.

Van Reeth, K., & Pensaert, M. (1994). Prevalence of infections with enzootic respiratory and enteric viruses in feeder pigs entering fattening herds. *The Veterinary Record, 135*(25), 594-597.

Vignali, D. A. (2000). Multiplexed particle-based flow cytometric assays. *Journal of Immunological Methods, 243*(1-2), 243-255. doi:S0022-1759(00)00238-6 [pii]

Voicu, I. L., Silim, A., Morin, M., & Elazhary, M. A. (1994). Interaction of porcine reproductive and respiratory syndrome virus with swine monocytes. *The Veterinary Record, 134*(16), 422-423.

Walker, I. W., Konoby, C. A., Jewhurst, V. A., McNair, I., McNeilly, F., Meehan, B. M., . . . Allan, G. M. (2000). Development and application of a competitive enzyme-linked immunosorbent assay for the detection of serum antibodies to porcine circovirus type 2. *Journal of Veterinary Diagnostic Investigation: Official Publication of the American Association of Veterinary Laboratory Diagnosticians, Inc, 12*(5), 400-405.

Waterboer, T., Sehr, P., Michael, K. M., Franceschi, S., Nieland, J. D., Joos, T. O., Pawlita, M. (2005). Multiplex human papillomavirus serology based on in situ-purified glutathione s-transferase fusion proteins. *Clinical Chemistry, 51*(10), 1845-1853. doi:clinchem.2005.052381 [pii]

Webby, R. J., Swenson, S. L., Krauss, S. L., Gerrish, P. J., Goyal, S. M., & Webster, R. G. (2000). Evolution of swine H3N2 influenza viruses in the united states. *Journal of Virology, 74*(18), 8243-8251.

Wensvoort, G., de Kluyver, E. P., Luijtze, E. A., den Besten, A., Harris, L., Collins, J. E., . . . Chladek, D. (1992). Antigenic comparison of lelystad virus and swine infertility and respiratory syndrome

(SIRS) virus. *Journal of Veterinary Diagnostic Investigation: Official Publication of the American Association of Veterinary Laboratory Diagnosticians, Inc, 4*(2), 134-138.

Wensvoort, G., Terpstra, C., Pol, J. M., ter Laak, E. A., Bloemraad, M., de Kluyver, E. P., Wagenaar, F. (1991). Mystery swine disease in the netherlands: The isolation of lelystad virus. *The Veterinary Quarterly, 13*(3), 121-130. doi:10.1080/01652176.1991.9694296 [doi]

Witte, S. B., Chard-Bergstrom, C., Loughin, T. A., & Kapil, S. (2000). Development of a recombinant nucleoprotein-based enzyme-linked immunosorbent assay for quantification of antibodies against porcine reproductive and respiratory syndrome virus. *Clinical and Diagnostic Laboratory Immunology, 7*(4), 700-702.

Wootton, S., Yoo, D., & Rogan, D. (2000). Full-length sequence of a canadian porcine reproductive and respiratory syndrome virus (PRRSV) isolate. *Archives of Virology, 145*(11), 2297-2323.

Yin, S. H., Yang, S. L., Tian, H., Wu, J. Y., Shang, Y. J., Cai, X. P., & Liu, X. T. (2010). An ELISA based on a truncated soluble ORF2 protein for the detection of PCV2 antibodies in domestic pigs. *Virologica Sinica, 25*(3), 191-198. doi:10.1007/s12250-010-3085-5 [doi]

Zhou, N. N., Senne, D. A., Landgraf, J. S., Swenson, S. L., Erickson, G., Rossow, K., . . . Webster, R. G. (1999). Genetic reassortment of avian, swine, and human influenza A viruses in american pigs. *Journal of Virology,73*(10), 8851-8856.

Zimmerman, J. J., Yoon, K. J., Wills, R. W., & Swenson, S. L. (1997). General overview of PRRSV: A perspective from the united states. *Veterinary Microbiology, 55*(1-4), 187-196.