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Development of confirmatory testing strategies for suspect false PRRS-positive reactors in diagnostic serology

by

Siyuan Liu

A thesis submitted to the graduate faculty

in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Major: Genetics

Program of Study Committee:
Kyoung-Jin Yoon, Co-Major Professor
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Iowa State University

Ames, Iowa

2010

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LIST OF ABBREVIATES

PRRSV – Porcine Reproductive and Respiratory Syndrome Virus
PRRS – Porcine Reproductive and Respiratory Syndrome
LV – Lelystad virus
EAV – Equine Arteritis Virus
LDV – Lactate-Dehydrogenase elevating Virus
SHFV – Simian Hemorrhagic Fever Virus
ORFs – Open Reading Frames
Nps – Nonstructural Proteins
dpi – Days Post Infection
IFA – Indirect Fluorescent Antibody
ELISA – Enzyme-Linked Immunosorbent Assay
NAs – Neutralizing Antibodies
ADE – Antibody-Dependent Enhancement
VI – Virus Isolation
IHC – Immunohistochemistry
PCR – Polymerase Chain Reaction
PAM – Pulmonary Alveolar Macrophages
RFLP – Restriction Fragment Length Polymorphism
SVN – Serum-Virus Neutralization
bELISA – Blocking ELISA
IPMA – Immunoperoxidase Monolayer Assay
SFP – Suspect False Positive
MIA – Microsphere Immunoassay
WIB – Western Immunoblotting Assay
FBS – Fetal Bovine Serum
Sf9 – *Spodoptera Frugiperda*
SDS-PAGE – Sodium Dodecyl Sulfate-polyacrylamide Gel Electrophoresis
CPE – Cytopathic Effect
S/P ratio – Sample-to-Positive Ratio
MAb – Monoclonal Antibody
ROC – Receiver Operation Characteristic
MFI – Mediam Fluorescence Intensity

CHAPTER 1: GENERAL INTRODUCTION

Thesis Organization

This thesis composed of three chapters. Chapter one presents a general introduction and review of different diagnostic methods for the detection of porcine reproductive and respiratory syndrome virus (PRRSV) or its infection in swine. This general introduction provides a broad overview of current knowledge of diagnosis perspective and background for research work presented in chapters two. Chapter two describes development of western immunoblotting and luminex® technology-based tests with recombinant PRRSV nucleocapsid protein prepared by baculovirus expression system and establishment of an epitope-based blocking ELISA using the existing commercial ELISA kit (2XR) for PRRSV (IDEXX) in comparison to a newer version of the commercial ELISA kit (X3), leading to developing A testing algorithm for confirmation of suspect false positives results occurred in the commercial ELISA. The final chapter includes general conclusions of the research studies and suggests possible direction of future studies.

Literature Review: PRRS virus diagnostics and diagnosis

Introduction

Historical aspect of PRRS

Porcine reproductive and respiratory syndrome (PRRS) was first observed in 1987 in the United States [42,48] and rapidly spread through North America. The first outbreak in Europe was recognized in late 1990 in Germany [55], and then became widely spread throughout the European continent. By 1994, PRRS was identified in most of major pig producing areas throughout the world [66].

PRRS was initially named 'Mystery Swine Disease' because no known etiologic agent could be established to be responsible for the disease (39). In Europe the disease was occasionally named "Blue ear pig disease" because of distinctive appearance of gross skin lesion (48). The causative virus was first isolated in The Netherlands in 1990 and designated as Lelystad virus (LV) [106] and shortly after in the US it was also isolated and named as the Swine Infertility and Respiratory Syndrome (SIRS) virus [17].

Although several names were applied to the disease since its emergence, International Office of Epizootics (O.I.E) designated the name 'porcine reproductive and respiratory syndrome' to the disease and resulted in the causal agent called 'Porcine Reproductive and Respiratory Syndrome Virus (PRRSV).'

Clinical Manifestations

In general, a PRRS outbreak is characterized by episodes of respiratory distress as well as decreased farrowing rate and elevated mortality due to an increased number of stillborn

pigliets [48,96]. However, polymorphic clinical presentations are one of the most common features of PRRS [108]. PRRSV infections in adult animals, particularly boars, can undergo unnoticed from a clinical point of view [68,86]. For example, only three out of 29 experimentally infected boars showed transient inappetence and depression for only one or two days [84,85]. Other research group also reported similar observation in experimentally infected boars [14,95].

The prevalence of PRRS varies depending upon animal age, pig density, health status, air quality, and exposure dose of virus [22,86]. Yet, the severity of the disease can also be affected by the strain of virus. Research done on pigs experimentally infected with nine different isolates of PRRSV showed differences in clinical signs, rectal temperatures, gross and histological lung lesions and mortality rate [38,39]. Furthermore, higher level of viremia and higher viral concentration in tissues may be related to the severity of PRRS. Highly virulent strains of PRRSV tend to replicate more efficiently in the host to a higher level [43]. In addition, a whole range of bacterial agents like *Streptococcus suis, Salmonella choleraesuis* and *Mycoplasma hyopneumoniae* appeared to enhance the severity of clinical manifestations following PRRS [27,97,109].

Economic impact of PRRS

For the past two decades, PRRS has become one of the most threatening diseases to swine health and production globally since it was first reported in the United States in the late 1980s [48,96]. Porcine reproductive and respiratory syndrome is currently considered to be the most economically important infectious disease that is faced by swine industry in the United States. Estimated annual economic losses due to PRRS in the US swine industry are

approximately $560 to $762 million [45,73]. The economic burden by PRRS is due to increased respiratory disease and pre-weaning mortality, decreased farrowing rates, higher chances for secondary infection by other diseases, decreased growth rate, and additional diagnostic, therapeutic and vaccine costs. The most significant economic loss due to PRRSV infection is generally observed in nursery and finishing phases of production [73]. Compared with other historical nightmare of swine producers, the annual cost of PRRS exceeds the costs of pseudorabies and classical swine fever [31,40]. In order to control and prevent the disease, a great deal of efforts has been mounted to develop efficacious vaccines for PRRSV [98]. Nonetheless, the effective control and eradiation strategy is still based on accurate identification of infected animals [4]. Therefore, rapid, convenient and reliable laboratory confirmatory tests are needed for accurate diagnosis.

Porcine reproductive and respiratory syndrome virus

Porcine reproductive and respiratory syndrome virus is a small enveloped, single-stranded positive-sense RNA virus with approximately 50-65nm in diameter. The PRRSV belongs to the family *Arteriviridae* with equine arteritis virus (EAV), lactate-dehydrogenase elevating virus (LDV) and simian hemorrhagic fever virus (SHFV) within the order *Nidovirales* [11,18,82]. Members of this family share many biological and molecular properties, including virion morphology, genomic organization, and gene expression strategies. They also have the ability to: a) induce prolonged viremia; b) establish persistent infections; and c) grow in macrophages [82]. Overall, PRRSV is more closely related to LDV and EAV than SHFV [63].

Two different major genotypes have been identified among PRRS viruses: the European

(type 1) and North American (type 2) types. They share less than 70% sequence homology in their entire genome. Comparative sequence analysis revealed certain degrees of amino acid identity between Lelystad virus and VR2332, ranging from 55% (ORF5), 79% (ORF6) and 64% (ORF7) [44,61,70,71,94].

The viral genome (approximately 15kb in size) encodes at least nine open reading frames (ORFs) that express a set of co-3' end nested subgenomic mRNAs with the same leader sequence at the 5' end [3,20]. The ORF1a and ORF1b compose almost 12kb of 5'end of the genome and encode nonstructural proteins (Nps) including RNA-dependent-RNA polymerases that was required for virus replication [23,62,63]. Three N-glycosylated minor envelope proteins (GP2, GP3, and GP4) were translated from ORF2, ORF3, and ORF4 respectively and form a heterotrimer, playing a role in the attachment of PRRSV to target cells [110]. ORF2b, which is completely embedded in ORF2a, encodes non-glycosylated minor envelope protein (2b) [112]. The major envelope glycoprotein of 25 kDa (GP5 or E) expressed by ORF5 is the main receptor molecule of PRRSV, forms a heterodimmer with 18-19 kDa unglycosylated matrix (M) protein that is expressed by ORF6 [59]. The 15-kDa nucleocapsid (N) protein, which is highly immunogenic and antigenically conserved among PRRS viruses, is expressed by ORF7 [65].

Immunology

Previously infected sows neither transmitted virus to their fetuses nor suffered from reproductive failure when subsequently challenged with a virulent strain during late gestation [34,51], indicating that solid protective immunity can be conferred by the prior infection. PRRSV-specific immunoglobulin M (IgM) and IgG responses rapidly appear in animals after

exposure to the virus [56,102]. Detectable antibody against PRRSV can be found in some pigs as early as 5-7 days post infection (PI) and by day 14 PI most, if not all, of the infected animals seroconvert to the virus [115,120] as determined by indirect fluorescent antibody (IFA) test or enzyme-linked immunosorbent assay (ELISA). The ontogeny of antibody response of pigs to PRRSV was studied with a North American PRRSV [72]. The antibody against the N protein was detected earliest, followed by the M protein, and then the GP5.

Despite of the fact that pigs rapidly mount virus-specific antibody after exposure, this rapid reaction does not correspond to the development of neutralizing antibodies (NAs) [116]. PRRSV is known to induce delayed production of virus-specific NA. In general, NA is not detected during the first 4 weeks after infection [57]. However, a critical role of NA in protection against PRRSV has been demonstrated [77]. Virus clearance from tissue and circulation of experimentally infected pigs was shown to coincide with the induction of NA [50]. GP5 is believed to be most important in inducing the protective antibody since most of the NAs are generated against GP5 and contains one of the major neutralization epitopes based on *in vitro* assessment [33,72,79,80]. In addition, GP4 and M protein were reported to contain neutralization epitopes [9,33,64,104,113]. Some reports also suggested that viral epitopes capable of inducing NAs reside on the GP3 protein [9,47]. Therefore, various membrane-associated proteins besides GP5 must be taken into consideration to achieve the full capacity of cross neutralization between different PRRS viruses [47].

The non-NAs developed at early stage may have a significant role in the pathogenesis of PRRSV. Yoon et al. reported an immunopathological condition known as antibody-dependent enhancement (ADE) of PRRSV in which virus-specific antibodies mediate and enhance virus infection in Fc receptor-bearing immune cells such as macrophages, resulting in higher

viremia for a longer period [118,119]. The epitopes that may induce ADE-mediating antibody for PRRSV were associated with the GP5 and N proteins [9,118]. Although exacerbation of disease severity due to ADE of virus infection has not been demonstrated under experimental conditions, enhanced severity of PRRS potentially due to ADE has been reported in the field [91,119].

Diagnostics

Diagnosis of PRRS based on clinical signs is unreliable considering that clinical disease is often not overt or of limited duration of clinical signs which can be similar to ones by other diseases. Furthermore, no pathogonomonic gross lesions specific for PRRSV infections have been demonstrated in infected pigs, and the only consistent microscopic lesion is interstitial pneumonia [19,83]. In addition, secondary or concurrent infection by other pathogens often occurs with PRRSV infection, which complicates the recognition of the disease. Therefore, laboratory diagnostic testing is necessary to aid accurate diagnosis of PRRS. Various laboratory tests have been developed for the detection of specific antibodies and for virus since the discovery of PRRSV.

Detection of PRRSV or viral components

Virus isolation (VI), immunohistochemistry (IHC) and polymerase chain reaction (PCR)-based assays are commonly employed to detect PRRSV directly.

1 Detection of infectious PRRSV – VI

Virus isolation test is considered as “gold standard” for PRRSV diagnosis. The VI test is done using porcine pulmonary alveolar macrophages (PAM), natural host cell, or African

monkey kidney cell line or its derivatives [17,46,106]. Differences of individual strains in the ability to grow in PAM or cell lines have been reported in a previous study [5]. For type 1 PRRSV, PAM have been reported to be most sensitive and in many cases is the only cell supporting PRRSV virus growth *in vitro*.

This technique, however, has some drawbacks. As mentioned above, PRRS viruses differ in their ability to grow in PAM and the cell lines. Hence, both cells should be utilized for better coverage of various PRRS viruses in VI. Use of PAM requires multiple batches since different batches may be not equally susceptible to the virus. The PAM need to be harvested from pigs under 6 to 8 weeks of age, preferably specific-pathogen-free pigs [114], which is cost-prohibited in some laboratories. Many times VI requires more than one passage [105]. Therefore, all these disadvantages make VI a rather laborious and expensive method which may not be suitable for large scale testing. In addition, longer turnaround time (7-14 days) of test result is another downside of using VI as routine diagnostic test when rapid diagnosis is critical for disease control.

2 Detection of viral antigen(s) – IHC

Detection of PRRS viral antigen(s) in frozen sections of tissues, ethanol-fixed or formalin-fixed tissues by using immunoperoxidase or immunogold silver staining (IGSS) has been demonstrated as an effective diagnostic tool for PRRSV in nursery and grower/finishing pigs [37,58,83]. The method has been reported to be highly specific but with only moderately sensitivity [38]. The results from IGSS on different ethanol-fixed tissues are comparable to those of virus isolation [58]. However, it should be noted that this method, depending upon test type, can be time-consuming and may have a better application for research purpose than diagnosis. Besides, the uneven distribution of PRRSV antigen in tissues (particularly lungs)

of infected pigs often limits the detection of virus infected animals with this method, although other reports have indicated that IHC most consistently identified PRRSV in the lung [52,54,87].

3 Detection of viral genome – PCR

Assays based on PCR technology have been the focus of recent assay development for detecting PRRS viral nucleic acids in clinical specimens. Reverse transcription-polymerase chain reaction (RT-PCR) in various formats or platforms have been used to detect the viral RNA of PRRSV [15,49,53,54,60,101], [16]. In North American veterinary diagnostic laboratories, real-time RT-PCR is commonly used and commercial real-time RT-PCR reagents/kits are available to the diagnostic community [30,103].

It has been demonstrated that multiplex RT-PCRs can efficiently differentiate North American (type 2) strains from European (type 1) strains of PRRSV by means of specific oligonucleotide primers or probes that are designed according to the sequence of the nucleocapsid protein (N) gene [60]. Restriction fragment length polymorphism (RFLP) analysis of PRRSV's ORF5 which is coupled with PCR amplification of the target gene was devised to rapidly differentiate the PRRS vaccine virus from North American field strains of PRRSV [107]. Such a differential utility of the assay has diminished since the vaccine virus was reported to spread from vaccinated pigs to non-vaccinated animals causing the disease and continue to circulate in a population [74]. In addition, RFLP appears not to be able to accurately determine the relatedness among PRRS viruses since restriction enzyme sites frequently undergo genetic mutation [117].

PCR technology possesses a great advantage over virus isolation in detection of PRRSV in samples with reduced infectivity or cytotoxicity, such as autolyzed tissues, semen or

environmental samples [15]. In recent years, a number of PCR-based methods including one-step RT-PCRs and nested RT-PCRs have been published for the detection of PRRSV in tissue and body fluids of infected animals [32,35,49,60,76,92]. RT-PCRs for the detection and differentiation of PRRS viruses are used as sensitive and highly specific diagnostic tools to control PRRS or to monitor the negative status of herds. Although PCR provides promising sensitivity and rapid diagnosis, the approach is costly, as it requires specialized laboratory equipment and experienced technicians. In addition, increased false positive results were reported from herds negative for PRRSV infection [29].

Serological tests

In addition to direct methods of agent detection, antibody detection has been commonly used for serodiagnosis of PRRSV infection. Serologic tests for PRRSV include the indirect fluorescent antibody (IFA) test, serum-virus neutralization (SVN) test, immunoperoxidase monolayer assay (IPMA) and enzyme-linked immunosorbent assays (ELISA). Currently, commercialized ELISA kit (IDEXX Laboratories, Inc., ME) is most commonly used in North American veterinary diagnostic laboratories.

1 Immunoperoxidase monolayer assay and indirect fluorescent antibody test

The IPMA was the first serological test reported for the serodiagnosis of PRRS [106]. Subsequently, the IFA test, which is equivalent to the IPMA, was developed in the United States [115]. Both tests were initially developed using PAM cell, but later adapted to cell lines such as MA104, CL2621 or MARC-145 [5,46,106,120]. The IPMA was extensively used in European laboratories whereas the IFA test has traditionally been the preferred approach for serological screening of pigs for PRRSV infection in the United States. The IPMA and IFA

test are thought to be highly specific and sensitive [106,115]. Under experimental conditions, these tests can detect antibodies to PRRSV between seven to fourteen days after inoculation. Peak antibody titers tend to be reached after four to six weeks after infection and then decline slowly until approaching the lower detection limit of the assay around six to twelve months after initial exposure [1,120]. However, these tests need to be done in laboratories with special facilities, and test results, particularly endpoint titer, can vary due to subjective reading of test result by laboratory personnel. The existence of antigenic variation among PRRS viruses can lead to false negative in these tests if the virus used in the assay is antigenically distinct from the virus causing disease in a herd or farm. Furthermore, these tests need to use cell culture, which is laborious and cannot be automated for large-scale use.

2 Enzyme-linked immunosorbent assay

The advantage of the ELISA compared with IPMA and IFA is that it can be automated and performed economically for large-scale examination. Development of an indirect ELISA for detection of PRRSV-specific antibodies was first reported at 1992 [2], using cell culture supernatant of infected alveolar macrophage culture as antigen. This method was reported to be more sensitive than IPMA, particularly for early antibodies detection. However, unacceptable high background signals were also observed in an in-house ELISA for some seronegative sows [26]. Since then, several in-house indirect ELISAs have been reported by others to eliminate the high background issue [7,12,21,24,25,28,69,81,90,93,111]. Nevertheless, preparation of antigens and ELISA plates in a testing laboratory was time-consuming and required skill and rigid quality assurance to prepare those in a consistent manner with consistency and quality. The availability of a commercial ELISA kit (e.g., IDEXX HerdCheck PRRS 2XR antibody ELISA) to the diagnostic community has eliminated such

difficulties and concerns.

Development of a blocking ELISA (bELISA) with antigen derived from infectious PRRSV using polyclonal antibodies as the detection system, has been reported [41,93]. The test was reported to be specific and more sensitive than the IPMA when applied to field sera and to sera which were collected early after an experimental infection with PRRSV. High background which was observed in IPMA or indirect ELISA was not encountered with bELISA. Higher specificity of the assay made it more suitable as confirmatory testing when suspect false positive result occurred in other serologic assay including the commercial ELISA kit.

3 Serum-virus neutralization test

Virus-specific NAs are produced in animals after infection with PRRSV although antibody level varies among virus strains and animals. The antibodies can be measured in an SVN test using cell lines [67]. The SVN test is also considered to be a specific test, but with lower sensitivity as compared to IPMA, IFA or ELISA for detection of antibody early after infection [6,13,67]. However, the sensitivity of this method could be enhanced by adding fresh normal swine serum as source of complement to the serum being assayed [116]. This modification increased the sensitivity of the test and made it possible to detect neutralization titers as early as 11 days post infection. Those early detectable NAs were of IgM isotype whose neutralizing activity was dependent upon the presence of complements. The most significant drawback of SVN test is that test results can be severely compromised by antigenic variability which is known to exist among PRRS viruses [8]. Considering its laborious nature, SVN test is less suitable for routine diagnostic use even though it is only assay measuring functional antibody against PRRSV

Diagnosis

In the field, suspicion of PRRS in breeding animals and farrowing houses is based on clinical signs of reproductive failure (increased mummified fetuses and stillborn piglets) particularly at late gestation and a high level of neonatal/pre-weaning mortality [48,96]. Boars may show loss of appetite and libido. For growing-finishing animals, lethargy and respiratory distress (clear nasal secretion, dyspnea) is the main clinical presentation due to PRRS[100]. Therefore, similar respiratory and/or reproductive symptoms should be differentiated from PRRS through laboratory testing. PRRSV-induced reproductive disease need to be differentiated from leptospirosis, porcine parvovirus infection, and porcine enterovirus infection (SEMID), haemagglutinating encephalomyelitis virus infection causing Encephalomyelitis, Aujeszky's disease, African swine fever and classical swine fever. For the respiratory and post-weaning form of PRRS, differential diagnosis should include: swine influenza, Mycoplasma enzootic pneumonia, proliferative and necrotizing pneumonia (caused by porcine circovirus type 2), Haemophilus parasuis infection (caused by Haemophilus parasuis), haemagglutinating encephalomyelitis virus infection, porcine respiratory coronavirus infection, syncitial pneumonia and myocarditis (caused by respiratory *syncytial* virus), postweaning multisystemic wasting syndrome and Nipah virus infection [75].

Laboratory investigation consists of pathological examination, virological testing and/or serological testing depending upon disease stage. No pathognomic gross lesions specific for PRRS have been identified although abnormal changes are present in many internal organs (e.g., lung, regional lymph nodes, spleen) of affected animals. For examples, lungs from affected pigs are generally swollen, firm, tan-colored, and marble looking. Lymph nodes are enlarged [36]. Microscopically, type 2 interstitial pneumonia is the most common lesion in

lungs of affected pigs along with lymphadenopathy and inflammation in various secondary lymphoid tissues [88].

For virological testing, immunohistochemistry (IHC) and RT-PCR assays are commonly used although virus isolation test is considered to be “gold standard”. PRRSV and/or viral antigens have been detected in almost all internal organs (lung, spleen, tonsil, lymph nodes, thymus, heart, liver, kidney, brain), bone marrow and bodily fluid (serum, saliva, nasal secretion, fetal thoracic fluid, semen) [25,88]. Nonetheless, serum is often a preferred specimen for PRRSV testing because it can be used for both virological and serological tests [99]. Serum samples are particularly useful for the diagnosis of PRRS when PRRSV is suspected to cause reproductive disorder in breeding animals including boars [89]. Since PRRSV can easily lose infectivity at room temperature or higher, it is important to keep cold chain when fresh tissue or bodily fluid samples including serum are submitted to a diagnostic lab particularly for virus isolation [75]. Care must be also taken when interpreting virus testing results (particularly PCR) since PRRSV is known to induce prolong viremia and to persist in animals after infection without clinical disease[10]. It is recommended to do both histopathological examination and viral testing along with clinical assessment to get accurate diagnosis of PRRS.

For serology, ELISA is a preferred laboratory method for testing although IFA or SVN tests have been used. Yoon et al [120] studied the humoral immune response of pigs to PRRSV after experimental infection. Virus-specific antibodies were first detected by the IFA, IPMA, ELISA, and the SVN test 9-to-11, 5-to-9, 9-to-13, and 9-to-28 days post inoculation and reached their maximum values by 4-to-5, 5-to-6, 4-to-6, and 10-to-11 week’s dpi, respectively. It was estimated by regression analysis that the ELISA, IFA, IPMA, and the

SVN antibodies would approach the lower limits of detection by approximately day 137, 158, 324, and 356 dpi, respectively. Colostrum-derived passive antibody is believed to be weaned out by 6 to 10 weeks after birth. Since interpretation of serology results can be influenced by the presence of antibody incurred by prior infection, antibody of vaccination origin or material antibody, paired serology is highly recommended for accurate serodiagnosis. Considering that 65-70% of swine in the US can have a serological evidence of PRRSV infection, diagnostic value of serology can be enhanced if IgM-based assays are applied. Park et al. demonstrated a proof-of-concept using IFA test [78].

Conclusion

Generally speaking, virological diagnosis is difficult since cell culture or special equipment is needed, whereas serological diagnosis is easier to perform with good specificity and sensitivity on a herd level. Continuous genetic and antigenic variation among PRRS viruses has been identified as a strong impediment to the accuracy of diagnostic testing. Different kinetics of antibody persistence in individual animals and uncertainty over significance of seropositive results with respect to PRRS status may also be problematic. In addition, no serological test can be recognized as “gold standard” due to the fact that the sensitivity and specificity of tests can hardly reach to 100%. Therefore, an accurate and cost effective diagnostic method still needs to be developed.

Statement of Problem

Several serologic methods have been established to monitor the PRRS status of swine

herds. Among these tests, ELISA has been most commonly accepted by the diagnostic community because of its good repeatability, perceived good quality control, specificity, fast turnaround time and relatively low cost. A commercial PRRS ELISA kit manufactured by IDEXX Laboratories is in worldwide use for its sensitivity, reliability and ability to meet the high demand for both population-based testing and individual animal testing. It can detect antibody against both European and North American PRRSV simultaneously but not in a differential manner. Few, if any, serological tests reach 100% accuracy, and the PRRS ELISA is no exception to this rule. The occurrence of false positives in this commercial ELISA kit was claimed to be approximately 0.5-0.6% based on estimated specificity; however, field experience and research data suggested that a suspect false positive (SFP) rate in the kit can range from 0.5% to 15%. Because of our reliance on serology for effective PRRS prevention and control, it is necessary to develop assays with improved performance or testing algorithms for verifying test results when SFP samples are presented.

Hypothesis, objective and specific aims

The hypothesis of the project presented here was that the suspect false positive (SFP) rate generated by IDEXX PRRS ELISA 2XR kit can be greatly reduced by following up alternative assays. The objective of the project was then to develop testing strategies to address the SFP issue when it occurs during primary testing with the following specific aims:

1. Establishing Western immunoblotting and microsphere immunoassay using recombinant PRRSV nucleocapsid proteins;
2. Applying a blocking ELISA format to the commercial ELISA 2XR kit and validate test

performance;

3. Assessing the diagnostic performance of IDEXX PRRS ELISA X3 kit which was recently released from IDEXX as new version of PRRS ELISA 2XR kit; and
4. Evaluating the diagnostic utility of all assays for verifying SFP results.

CHAPTER 2: TESTING ALGORITHM FOR CONFIRMATION OF SUSPECT FALSE POSITIVES IN A COMMERCIAL ELISA KIT FOR PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME VIRUS (PRRSV)

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(to be submitted to Journal of Veterinary Diagnostic Investigation)

Abstract

The commercial ELISA kit (IDEXX HerdCheck® PRRS) has been the main serodiagnostic tool for PRRS virus (PRRSV) in North America and many pig producing regions throughout the world. Although the kit has been mostly a reliable test for detecting PRRSV exposure at the herd level, the specificity of the kit has been frequently challenged by the occurrence of unexpected false positive results at varying rates. An indirect fluorescent antibody (IFA) test has been commonly employed to confirm or disprove the suspect false positive reactivity in the ELISA. However, the reliability of test results have been often questioned due to the subjectivity in determination of test result and perceived lower sensitivity. The current study was conducted first to develop immunoassays (Western

immunoblot and microsphere immunoassay) based on recombinant N protein of PRRSV as well as bELISA using the existing commercial ELISA kit (2XR). Then diagnostic performances of four different serologic assays (Western immunoblot, microsphere immunoassay, bELISA and new IDEXX's PRRS ELISA kit X3) was evaluated on the same set of experimental and field serum samples in comparison to ELISA 2XR kit to establish a confirmatory testing algorithm when suspect false positive (SFP) results occur in ELISA 2XR. All assays showed excellent performance on experimental samples, meaning that all tests are valid for specific and sensitive detection of the target antibody. Diagnostic sensitivity and specificity of the assays related to ELISA 2XR, however, varied on the field samples, although all of them greatly reduced the number of SFP samples. By combining two different assays (particularly ELISA X3 and Western immunoblotting), the SFP results could be reduced by as high as 73.5% which provide higher confidence in determining the PRRS status of animal when SFP results occur.

Key words: PRRSV, diagnostic confirmatory test performance, test algorithm

Introduction

Porcine respiratory and reproductive syndrome virus (PRRS) is caused by an arterivirus named PRRS virus (PRRSV) [34] has had a catastrophic economic influence on swine industry [45] since its emergence on three continents in the last two decades [2,3,11,28]. The disease was first observed in 1987 in the US and in 1990 in Europe [27] and is characterized by manifestation of late term abortion and stillbirths in sows and respiratory disease in pigs of all ages [29,40]. Since its appearance, two major genotypes have been identified, European (type 1) and North

American (type 2) [5,24,26,40], which develop similar clinical symptoms but shares only 50% nucleotide sequence homology [6]. Nowadays, both genotypes can be found in the United States and Europe [9,31].

PRRSV is an enveloped virus containing single-stranded positive-sense RNA genome of approximately 15Kb in length which encodes nine open reading frames (ORFs) [6,39,42]. ORFs 1a and 1b compose approximately 80% of the 5' end of genome and encode RNA replicase complex. ORFs 2a, 3 and 4 encode for minor envelope glycoproteins (GP2, GP3, GP4) of PRRSV [21]. ORF2b encodes a minor nonglycosylated protein (2b) associated with viral membrane [42]. The major structural proteins glycoprotein 5 (GP5 or E), nonglycosylated matrix (M) protein, and nucleocapsid (N) are encoded by ORF5, ORF6 and ORF7 respectively [21].

Among viral proteins, the N protein is expressed abundantly in infected cells and constitutes about 20-40% of the virion protein [1,18,26]. The N protein is highly basic and 15 kDa in size [19,21]. The N protein can multimerize to form icosahedral core structures around genomic RNA [18,21]. Most anti-PRRSV immunoglobulins in serum of infected animals are directed against the N protein [6]. Antibody against N protein can be detected within 7 days of infection [17,25,44]. The N proteins of European and North American PRRSV strains have both conserved and divergent epitopes that can be recognized by monoclonal antibodies [23]. Because of the advantages described above the N protein has been applied to several diagnostic tests as the target antigen [7,8,10,33,41].

Porcine reproductive and respiratory syndrome costs the US swine industry approximately $600 million annually [13] due to respiratory disease, increased mortality in piglets and reproductive disorder, weight loss and poor performance in grow-finishing pigs

[16,37]. In most of the cases, infected animals can undergo without overt clinical signs, which makes the accurate clinical diagnosis more difficult to perform [22,30]. Therefore, accurate and timely laboratory diagnosis of virus infection is necessary for effectively monitoring infection status and controlling the disease. Serology has been a useful tool to determine PRRS status of herds or individual animals and to monitor the PRRSV infection or vaccination. Several serological assays have been developed for PRRSV, such as indirect fluorescent antibody (IFA) test, serum-virus neutralization (SVN) test, immunoperoxidase monolayer assay (IPMA) and enzyme-linked immunosorbent assays (ELISA). Among those tests, the ELISA has been preferred because of its high sensitivity, relatively low cost, fast turnaround time and high throughput.

The commercial indirect ELISA kit (HerdCheck®) manufactured by IDEXX Laboratories is in use worldwide for its good sensitivity and specificity and built-in quality control. Additional advantage of IDEXX ELISA kit is that it can reliably detect antibodies to both genotypes of PRRSV. Even with many advantages, it has been observed that the IDEXX ELISA produced unexpected positive results in seronegative herds where the farm and herd history lead to the strong expectation of negative results [10,32]. The rate of unexpected false positive results (i.e., singleton reactors) has been as high as 5% or sometimes reached 15% at herd level based on data in a veterinary diagnostic laboratory (K-J Yoon, unpublished data). This is much higher than expected based on the specificity (99% or higher) claimed by the manufacturer. The occurrence of singleton reactors in the commercial ELISA kit have had significant impact on effective control of PRRS, which is based on an accurate identification of infected animals or accurate assessment of PRRS status in herd.

When false positive results are suspected, polymerase chain reaction (PCR) based assay,

virus isolation (VI), and/or indirect fluorescent antibody (IFA) tests have been commonly employed as follow-up methods to resolve the question of the unexpected positive samples. Although PCR and VI can detect viremic pigs within 24 hours post exposure, both methods have several drawbacks. The VI test has a long turnaround time of results. Not all PRRS viruses replicate equally well in vitro. The success of VI from serum decreases as time after infection goes on [4]. PCR test is a sensitive assay to detect the virus in various sample matrices. It can provide fast turnaround of result as compared to VI. However, due to the great sensitivity of the assay, false positive results could be a problem for PCR. False negative results can also occur in PCR due to continuous genetic mutation of PRRSV [38]. Both PCR and VI results can lack a correlation with the ELISA results due to timing of sampling (viremia versus antibody response) [10], not to mention that VI and PCR cost more than ELISA.

IFA test has been frequently used by many veterinary diagnostic laboratories as a confirmatory test when false positive results are suspected in the IDEXX ELISA. The IFA assay has shown the antibody detection kinetics similar to ELISA [15,44]. Limitations exist in IFA assay as well. Cell culture techniques are required to prepare antigens for assay, which can prolong turnaround time and may not be feasible to all labs. The PRRSV isolates used in the assay may be antigenically different from the virus strain causing the disease in the index herds, leading to false-negative results [43]. Besides, the subjectivity of reading the assay result can result in misinterpretation. Undesirable results can also be caused by nonspecific background staining.

Considering all drawbacks and limitations among the current diagnostic tests available for PRRSV, alternative serologic assays with better performance and reliability with potential

for automation and/or high throughput was desired. At the same time a testing algorithm for confirmation was needed when suspect false positive (SFP) results occurred. The following study was conducted to address these needs.

Material and Methods

Study Design

Four different binding assays were employed and evaluated on the same set of experimental and field serum samples in order to establish a confirmatory testing algorithm when SFP results occur in the current commercial PRRS ELISA kit (2XR). The assays employed were western immunoblotting assay (WIB), microsphere immunoassay (MIA), blocking ELISA (bELISA) and new IDEXX PRRS ELISA kit (X3) which had recently released to European market. WIB, MIA and bELISA were selected since these are known to have high specificity with sensitivity equivalent to indirect ELISA. The ELISA X3 was employed because the manufacturer claimed that the X3 kit has much higher specificity than the 2XR kit while it maintains the same sensitivity as the 2XR kit (IDEXX press release). All assays were virtually based on the nucleocapsid protein of PRRSV. WIB and MIA were developed using recombinant baculovirus-expressed nucleocapsid protein of PRRSV. The bELISA utilized the ELISA 2XR kit. For test validation, a total of 131 sera (27 known negative and 104 known positive) collected from experimental pigs with known PRRSV infection status were used. In addition, a total of 188 swine sera were collected from submissions to the Iowa State University Veterinary Diagnostic Laboratory (ISU-VDL) in 2009 and used to evaluated diagnostic performance of the selected tests in comparison to the

ELISA 2XR. Based on results of ELISA 2XR and IFA (and PCR if necessary) and historical PRRS monitoring data of farms, the field samples were categorized into 4 groups. Group 1 was composed of 44 samples that were positive by both ELISA and IFA and other herds or sites from the same farm were also tested positive for PRRSV. Group 2 had 68 SFP samples that were positive by ELISA with S/P ratio ≥0.4 but negative by IFA and PCR. These samples were submitted from farms expected to be PRRS negative based on historical monitoring data. Group 3 were consisted of 31 inconclusive (potentially false negative) samples with ELISA S/P ratios equal to greater than 0.2 but less than 0.4. Pigs in this category have been often considered to be positive by the swine industry and diagnostic community when samples were collected from historically PRRS negative or naïve herds. All these samples were tested negative by IFA. Group 4 were 45 true negative samples that were negative by ELISA with S/P ratio <0.2 and also tested negative by IFA (Appendix). Test results were compared to each other and statistically analyzed for the equivalency of performance among 4 diagnostic tests.

Cells and Viruses

The type 2 prototype PRRSV strain VR2332 and type 1 prototype PRRSV strain Lelystad virus were used as source of the ORF7 gene. Both strains were propagated in MARC-145 cells which is a highly permissive clone of African Monkey Kidney cell line MA104 [14] with Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA).

The Bac-to Bac baculovirus expression system was purchased from a commercial vendor (Invitrogen Inc.), as well as *Spodoptera frugiperda* (Sf9) insect cell line which was used as

the host for baculovirus transfer vector. Monolayer cultures of Sf9 cells and those infected with the recombinant baculovirus viruses were maintained in adherent and suspension culture with Sf-900 II serum free medium (GIBCO, Carlsbad, CA).

Biological samples

Three different independent sets of serum samples were used for the study. First, 32 sera with known PRRS status were obtained from an animal study conducted in our laboratory. These sera were collected from 16 inoculated pigs and 16 shame control pigs after 65 days post inoculation (dpi). All pigs were from a PRRS naïve herd and at 4 weeks of age at the time of virus inoculation.

Second, a total of 88 PRRSV antibody-positive serum samples were obtained through sequential bleedings from a previous animal study in our laboratory [36]. The samples represented 11 pigs randomly selected from a total of 112 pigs used in that study. The 11 pigs were inoculated with a type 2 PRRSV (VR2332, JA142 or chimeric virus) at day 0 and challenged with VR2332 or JA142 at 44 dpi. Serum samples were collected weekly over a 72-day period. Sera (n=11) collected at 0 dpi served as negative control sera. All of these sera were tested by IFA and serum-virus neutralization (SVN) tests for presence or absence of antibody specific for PRRSV.

Third, 188 field swine sera samples were selected from submissions to the ISU-VDL which had more than 10 serum samples. The samples represented various age groups. No more than 2 serum samples were selected from the same case submission or same farm/herd. All samples were tested by ELISA 2XR and, if necessary, IFA as part of routine serology diagnostic service. PCR was also performed on some of these samples when desired to

address SFP concern as per clients. Based on test results and historical PRRS monitoring data of index herds, 44 positive, 76 negative and 68 SFP sera were identified. The 76 negative sera were further divided into 2 categories based on ELISA S/P ratio: a) inconclusive (n=31; $0.2 \leq S/P < 0.4$) and b) true negative (n=45; S/P<0.2).

Production of recombinant nucleocapsid protein of PRRSV

Construction of recombinant baculovirus

To construct baculovirus expression vectors containing ORF7 gene and 6Xhistidine tag, the specific cDNA of type 2 and type 1 PRRSV ORF7 was first amplified from the genomic RNA of VR2332 and Lelystad Virus, respectively, by Reverse transcription- PCR (RT-PCR). Amplification primers contained *EcoRI* and *XbaI* sites at the upstream and downstream of ORF7, respectively for further manipulation of PCR products. The sequences of the PCR primers were as follows:

NAEcoF 5'-GAATTCGAATTCCAAATATGCCAAATAACAACG-3'

NAXbaR 5'-TCTAGATCTAGAAACACTGAGATGCCTCAAGAA-3'

EUEcoF 5'-GAATTCGAATTCATGGCCGGTAAAAACCAGAG-3'

EUXbaR 5'-TCTAGATCTAGATTAACTTGCACCCTGACTGG-3'

Each PCR product and the baculovirus protein expression plasmid vector pFastBac HT (Invitrogen) were digested by *EcoRI* and *XbaI* (New England BioLabs, Beverly, MA, USA), purified after being electrophoresed, and ligated to each other by utilizing the restriction enzyme sites. After transformation and selection, pORF7NA, and pORF7EU were obtained, in which VR2332 and LV ORF7 cDNA were confirmed by sequencing in the correct orientation with respect to the polyhedron promoter. The recombinant pFastBac HT donor

plasmid, containing ORF7 gene, was then transformed into competent DH10Bac™ *E. coli* cells for transposition into the bacmid according to the manufacturer's instructions ("BAC-TO-BAC™" manual, Invitrogen). Correct bacmid construction was confirmed by nested PCR using M13 and ORF7 gene specific primers as well as sequencing.

Transfection of Sf9 cells with recombinant bacmid DNA

Sf9 insect cells (Invitrogen) were transfected with bacmid DNA bac-6×His-ORF7EU or bac-6×His-ORF7NA and incubated at 30°C for 72h or until start to see signs of cytopathic effect. Rescued recombinant baculoviruses bac-6×His-ORF7EU and bac-6×His-ORF7NA in the supernatant were harvested and confirmed by sequencing as P1 viral stock and then used to infect fresh insect cells to amplify baculoviral stock until P3 which generated a suitable titer (e.g. 1×10^8 pfu/ml). Cells infected with such viruses were then used as crude antigen for western immunoblotting to confirm the presence of each recombinant protein of 17.5 kDa and 17.3 kDa in size, respectively. Uninfected cells and the cells infected with wild type baculovirus were used as controls for assay.

Antigen purification

The expressed bac-6×His-ORF7EU and bac-6×His-ORF7NA recombinant proteins are tagged with 6×His. ProBond™ purification system (Invitrogen) was used to purify the recombinant fusion proteins under denaturing condition following manufacturer's recommended procedure.

SDS-PAGE and Western immunoblotting

Sample preparation

Sf9 cells infected with baculovirus-expressed recombinant N protein of PRRSV or wild

baculovirus (virus control) and uninfected cells (mock control) were pelleted by centrigufation for 10 min at 2000 × *g*. Each of the resulting cell pellets was lysed by three cycle of freeze-and-thaw and mixed with 5X sample buffer containing 0.6M Tris-HCl (pH6.8), 25% glycerol, 2% SDS, 10% β-mercaptoethanol and 0.1% bromophenol blue, and boiled at 100°C for 5 minutes. Samples were cooling down by running water for 30 sec and centrifuge for 1 min at 10000 *x g*.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Twenty five microliters of each sample were loaded on each well of a 12% polyacrylamide precast gel (Bio-Rad Laboratories., Hercules, CA) and electrophoresed for 90 min at a constant voltage of 100 along with prestained molecular weight markers (Bio-Rad). After electrophoresis, recombinant proteins and the markers separated on each gel were electrotransferred in transfer buffer (Bio-Rad) onto a 0.45μm nitrocellulose membrane (Bio-Rad) for 1 h at a constant voltage of 100 with ice pack. After transfer, membranes were blocked with 2% skim milk (Nestlé S.A., Vevey, Switzerland) solution diluted with PBST by immerging and gently rocking for 30 minutes at ambient temperature.

Western immunoblotting (WIB)

Each serum sample was diluted 1:40 in 20mM Tris-buffered saline solution (pH7.5) with 0.05% Tween 20 (TBST) and incubated with the membrane contains polypeptides and markers for 1 hour at ambient temperature with slow and gentle rocking, and then rinsed three times for 5 minutes each using TBST. The membrane was then incubated with optimally diluted goat-anti-swine IgG labeled with horseradish peroxidase (Kirkegaard-Perry Laboratories, Gaithersburg, MD, USA) for 1 h at ambient temperature with slow and gentle rocking. The membrane was rinsed three times for five minutes each using TBST. Then,

antigen-antibody reactions were visualized by adding 3,3', 5,5''-tetramethylbenzidine (TMB) substrate (Kirkegaard-Perry Laboratories) to the membrane and incubating for up to 5 minutes at ambient temperature. Distilled water was added to stop colorimetric reaction. The presence of antibodies specific for PRRS viral protein in each serum was confirmed by existence of specific band with molecular weight as 17.5kDa and 17.3kDa respectively for bac-6×His-ORF7EU and bac-6×His-ORF7NA recombinant proteins.

Indirect fluorescent antibody (IFA) Test

MARC-145 cells were prepared in 96-well plates (Corning Inc., Lowell, MA) and incubated at 37°C with supply of 5% CO_2 in a humid environment for 48-60 hours. Cell monolayers were infected with 100 µl of PRRSV (JA142) at a rate of 10^4 $TCID_{50}$/ml for each well. After incubation for 20 hours, cells were rinsed three times by 1× phosphate-buffered saline (PBS, pH 7.4) and fixed by cold 80% acetone aqueous solution. Uninfected cell monolayers were prepared in the same manner and used as cell control antigens. All the plates were dried and stored at -20°C until use. Before testing, plates were brought to ambient temperature and each well was washed three times with 300 µl of PBS containing 0.1% Tween 20 (PBS-T). One hundred µl of each serum sample (1:20 diluted) were added to duplicate wells, after incubation for 30 minutes at 37°C, serum samples were removed from each plate and each well was washed three times by submerging in PBS-T and standing for 5 min at ambient temperature. Fluorescein isothiocyanate (FITC) -conjugated goat anti-swine antibody (KPL) diluted 1:100 in PBS was then added to each well and incubated for 30 minutes at 37°C. After washing three times with PBS-T, plates were observed under a fluorescence microscope. Samples were considered positive for PRRSV antibody in which

specific cytoplasmic fluorescence with some intranuclear staining can be observed.

Serum-virus neutralization (SVN) test

Sera were heat-inactivated for 45 minutes at 56°C prior to being tested. Two-fold serial dilutions was made with each serum in RPMI-1640 (Sigma, St. Louis, MO) supplemented with 10% FCS (Sigma), 20mM L-glutamine (Sigma), and an antibiotic-antimycotic mixture (Sigma) which contained 100 IU/ml penicillin, 100µg/ml streptomycin, 50µg/ml gentamicin and 0.25mg/ml amphotericin B (hereafter, RPMI growth medium). Each diluted serum sample was then mixed with an equal volume of PRRSV VR2332 at a rate of 10^3 $TCID_{50}$/ml. After incubating at 37°C for 1 h, serum-virus mixtures were then transferred onto MARC-145 cell monolayers prepared in 96-well plates and incubated for another 1 h at 37°C. Then all inoculums were removed and cell monolayers were replenished with 200 µl of fresh RPMI growth medium. Cells were incubated at 37°C for up to 5 days and monitored daily for cytopathic effect (CPE). The presence of virus-specific CPE in each well was recorded. The presence of virus in wells without visible CPE was further determined by an immunofluorescence microscopy using monoclonal antibody SDOW17 conjugated with FITC (Rural Technologies, Brookings, SD). Neutralizing antibody titer of each serum was determined as the reciprocal of the highest dilution in which no evidence of virus growth was detected.

Microsphere immunoassay (MIA)

Coupling of recombinant PRRSV nucleocapsid antigen to carboxylated microspheres

Microspheres (1.25×10^{6}) with a carboxylated surface were coated with recombinant N protein using two-step carbodiimide procedure as recommended by Luminex Corporation (Austin, TX). In brief, selected COOH beads for the protein coupling reaction were activated by first mixing with 10 µl of freshly made 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (Pierce Biotechnology, Rockford, IL) at a concentration of 50 mg/ml followed by adding 10 µl of freshly prepared *N*-hydroxysulfosuccinimide (sodium salt) solution (Pierce Biotechnology, Rockford, IL) at a concentration of 50 mg/ml and then agitating the beads while being protected from direct light on a rotator at ambient temperature for 20 minutes. The beads were then washed twice in 150 µl 1× PBS (pH 7.4) and pelleted by centrifugation at 14,000 × g for 4 min. The activated beads were resuspended in 100 µl of PBS and sonicated (Bransonic, Danbury, CT) for 15 sec. 12 µg of protein sample was added to the activated beads, and the final volume was adjusted to 500µl with PBS. Cover the microcentrifuge tube with aluminum foil and the beads were agitated with a rotator at ambient room for 2 h. The coupled microspheres were then washed with 500 µl wash buffer provided in the Bio-Plex™ amine coupling kit (Bio-Rad Laboratories, Hercules, CA), centrifuged down, resuspended with 250 µl blocking buffer which was included in kit, and then rotated at ambient temperature for 30 min while being protected from light. After washing once with 500 µl storage buffer in kit and centrifuge at 16,000 *x g* for 6 min, the resulting coupled bead pellet was stored in 150 µl of the storage buffer in 4°C. The concentrations of coupled microspheres in each batch were determined by counting on a hemocytometer so that equivalent quantities of each bead set could be applied into the assay.

Microsphere immunoassay

To perform a two-step suspension MIA, multiscreen 96-well filter plates (Millipore,

Bedford, MA) and a multiscreen vacuum manifold (Millipore, Bedford, MA) were employed to facilitate microsphere washing. Briefly, filter plate was blocked with 100µl PBN buffer consisted of 1× PBS (pH 7.4), 1% BSA (Sigma) and 0.05% NaAzide (Sigma) for 2 minutes at what temperature. Plate was washed once with 190 µl PBS with 0.05% Tween20 (PBST), and keep wells moist by adding 20 µl of PBN buffer. Fifty µl of each serum sample which was diluted 1:100 in PBN buffer were added to test wells and approximately 2500 antigen coated beads in a 50 µl volume of PBN buffer were also added, making final sample dilution 1:200. The filter plate was sealed with aluminum foil covers and allowed to be incubated on a shaker (VWR LabShop., Batavia, IL) at 37°C for 30 minutes and then washed three times with PBST by using the vacuum manifold. Optimally diluted affinity-purified biotin-labeled anti-swine IgG (1:2500 dilution in 50 µl of PBN buffer) was added to each well. After incubation in dark with shaking for 30 minutes at 37°C, plates were washed three times with PBST by using the vacuum manifold. After adding steptavidin conjugate (1:100 dilution in 50 µl of PBN) to each well, incubate the plate in the dark on a shaker at 37°C for 30 minutes. After washing twice with PBST by using the vacuum manifold, microspheres were then resuspended in 125 µl of PBN buffer per well. Seventy five µl aliquot of each suspension was transferred to a clear polystyrene 96-well plate (Costar, Corning, NY) and evaluated for the microsphere fluorescence intensity with a Luminex^{TM} 100 instrument (Luminex Corp., Austin, TX). The instrument was calibrated with CL1 and CL2 calibration microspheres (Bio-Rad Laboratories,) by following manufacturer's directions. The median fluorescence intensity (MFI) of fluorochrome-conjugated secondary antibody bound to individual microsphere was derived from a flow analysis of 100 microspheres per well for each sample.

Enzyme-linked immunosorbent assay (ELISA)

Two IDEXX PRRS ELISA kits (HerdChek® PRRS 2XR and HerdCheck® PRRS X3, Westbrook, ME) were employed and used as directed by the manufacturer. Samples with sample-to-positive (S/P) ratios ≥0.4 were considered positive for antibody against PRRSV.

The HerdCheck® PRRS X3 is a new PRRS antibody test kit from IDEXX which was made available to the European market in mid 2009. The kit was purchased from the vendor with United States Department of Agriculture's permission for import. The manufacturer claimed that the specificity and sensitivity of the kit was 99.9% and 98.8% respectively with the reduction of false-positive singleton reactors by 90%.

Epitope-based blocking ELISA (bELISA)

The blocking ELISA was conducted by utilizing PRRS ELISA 2XR kit and following the manufacturer's recommended procedure with a few modifications. Anti-PRRSV monoclonal antibody (MAb) SR30 (Rural Technologies Inc., Brookings, SD) which can recognize both type 1 and type 2 PRRS viruses was used as detecting antibody after its optimal concentration for the assay was determined via a checker board titration. All components (diluents, washing buffer, stop solution) provided in the ELISA 2XR kit except the secondary antibody were used for the blocking ELISA. Virtually the ELISA was done by following the manufacturer's recommended procedure for ELISA 2XR with a few modifications.

For testing, the plates were incubated with 100 µl each of 1:40 diluted test serum samples for 30 min at ambient temperature. For each sample additional set of viral and cell control antigen wells was assigned and left without incubating with the sample. Each well

was washed with approximately 300 µl of 1X wash buffer three times. 100 µl of optimally diluted SR30 MAb (Rural Technologies Inc., Brookings, SD) was added to all wells and incubate for 1 h at room temperature. After washing 3 times with wash buffer, 100 µl of optimally diluted peroxidase-labeled goat anti-mouse IgG (H+L) (KPL) was added, and plates were incubated further for 1h at ambient temperature. The plates were rinsed with 1X wash buffer three times and the substrate was added for 15 minutes. Color development was stopped by the addition of 100 µl of stop solution. Optical density (OD) of each well was measured at 650 nm wavelength in a microplate reader (BioTek, Winooski, VT). The blocking index (BI), which is the degree of test serum sample blocking the binding of the detecting antibody (i.e., SR30) to PRRSV antigen in well, was calculated using the following formula:

BI=1- (adjusted OD with serum) ÷ (adjusted OD without serum)

where adjusted OD is the net difference in OD between PRRS well and NHC well (i.e., OD of PRRS well - OD of NHC well).

Data analyses

JMP software (SAS Institute Inc., Cary, NC, USA) was used for receiver operation characteristic (ROC) curve analysis on results from experimental serum samples with known PRRS status in order to determine the cutoff of each assay.

For evaluation of clinical serum samples, the diagnostic sensitivity of each assay was calculated as the ratio of the number of clinical samples that tested positive to the total number of samples that positively identified by IDEXX ELISA 2XR and IFA test. The diagnostic specificity of the assay was calculated as the ratio of the number of samples that

tested negative to the total number of negative samples.

Cochran’s Q test (InStat version 2.04; GraphPad Software, San Diego, CA) was used for group comparison to assess the equivalency of performance among 4 diagnostic tests employed for the study (MIA, WIB, ELISA X3, bELISA) in comparison to the ELISA 2XR. Cochran's Q test provides a method for testing differences between three or more matched sets of frequencies or proportions. The Cochran test statistic Q was calculated as formulation below:

$$Q = \frac{c(c-1)\sum_{j=1}^{c} C_j^2 - (c-1)N^2}{cN - \sum_{i=1}^{r} R_i^2}, \qquad (1)$$

where c is the total number of treatments, r is the total number of samples, C_j is the outcome totals for *jth* treatment, R_i is the outcome totals for *ith* sample, and N is the total outcomes for all the samples for all the treatment. The analysis was conducted using the following hypothesis:

Among the four groups (i.e., true positive, suspect false positive, inconclusive ($0.2 \leq S/P < 0.4$), and true negative ($S/P < 0.2$)),

H_0: The four treatments are equally effective

H_1: At least two of the four treatments differ in effectiveness.

McNemar’s test (InStat version 2.04; GraphPad Software, San Diego, CA) (pairwise homogenous comparison) was applied to each pair of the employed assays (MIA, WIB, ELISA X3, and bELISA) for assessing if any two treatments were equally effective. The McNemar test is a non-parametric method used on nominal data to determine whether the row and column marginal frequencies are equal using the following formula:

$$\chi^2 \sim \frac{(b-c)^2}{b+c}, \qquad (2)$$

where b and c denote the discordants between two treatments.

The hypotheses were:

H_0: The two treatments are equally effective

H_1: The two treatments differ in effectiveness

Results

Analysis of recombinant bacmid

Traditional restriction endonuclease digestion analysis was difficult to perform with recombinant bacmid DNA due to its large size, which was greater than 135kb. Therefore, nested PCR analysis was used to verify the presence of PRRV ORF7 in correct position as it was unlikely that any of the unwanted PCR products contained binding sites for both M13 and PRRSV ORF7 specific primers. Each PCR yielded products with expected molecular size. Figure 1A and 1B show PCR products of recombinant bacmid transposed with type 1 or type 2 PRRSV ORF7 (2.7kb) from the first run with the M13 primers and type 1 or 2 PRRSV ORF7 (400bp) from the second run (nested PCR) with the PRRSV ORF7 specific primers, respectively, indicating that the recombinant bacmid containing the PRRSV ORF7 gene were constructed successfully.

Expression and analysis of recombinant PRRSV N protein

The expressed recombinant proteins of bac-6×His-ORF7EU and bac-6×His-ORF7NA

were found in the insoluble form. The target protein of type 1 and 2 PRRSV ORF7 with a molecular weight of 17.5 kDa and 17.3 kDa, respectively, were immunoprecipitated by porcine anti-PRRSV serum sample from lysates of Sf9 cells infected with bac-6×His -ORF7EU and bac-6×His -ORF7NA bacmid DNA as shown in Figure 2. The purified target recombinant protein of each genotype could be obtained using a metal–chelating resin under denaturing conditions, which was still specifically recognized by the PRRSV antiserum (Figure 3).

The antigenic properties of the recombinant N protein expressed by bac-ORF7 were further investigated in WIB with a panel of anti-N monoclonal antibodies (MAbs). The expressed recombinant protein of type 1 PRRSV ORF7 was recognized only by MAbs SDOW17 and SR30 (Fig. 4A) whereas the expressed recombinant protein of type 2 PRRSV ORF7 was reactive with all of the MAbs (Fig. 4B). In an immunofluorescence microscopy, MAbs SDOW17 and SR30 stained positively with cells infected with bac-6×His -ORF7EU or bac-6×His -ORF7NA as these MAbs were expected to recognize N protein of both genotypes of PRRSV. On the other hand, MAbs EP147, VO17 and JP28, which specifically recognize N protein of type 2 PRRSV isolates [26], did not react with cells infected with bac-6×His-ORF7EU (Fig. 4).

Development of recombinant PRRSV N protein-based Western immunoblotting assay

Two expressed recombinant proteins (bac-6×His-ORF7EU and bac-6×His-ORF7NA) were used to set up WIB assay. Total protein concentration of recombinant antigen bac-6×His-ORF7EU and bac-6×His-ORF7NA was 8.67 mg/ml and 7.96 mg/ml, respectively. Serial 10-fold dilutions from the original antigens were made to determine the optimal

working concentration. According to the signal recognition of antigens, 10^{-2} dilution from the original bac-6×His -ORF7EU antigen was chosen for WIB while the 10^{-1} dilution for the bac-6×His -ORF7NA antigens was chosen for the assay. At the given dilution, each antigen preparation had total protein concentration of approximately 0.8 mg/ml

When each of the optimally diluted antigens was tested against polyclonal porcine antisera to the VR2332 (type 2) and Lelystad virus (type 1) strains after being serially diluted in 2-fold from 1:20 to 1:2560, the antigen bac-6×His-ORF7NA showed the positive result with both type 1 and 2 PRRSV antibodies with the endpoint of 1:80 and 1:160 respectively. The antigen bac-6×His-ORF7EU had the same results as the bac-6×His-ORF7NA, suggesting that both recombinant ORF7 proteins have the same cross reactivity and either one can be used to detect antibody against both type 1 and type 2 PRRSV.

When 32 experimental serum samples from animal study were tested on WIB using the recombinant N protein, the test results were highly in accordance with the expected status of samples (i.e., no false positive and no false negative).

Establishment of the recombinant N protein-based MIA

A set of microsphere beads was selected to establish the assays to detect antibodies specific for PRRSV. When the 32 known positive and negative swine sera were tested on MIA in order to establish the cutoff for the assay, clear separation between positives and negatives was made by the assay (Fig. 5). The medium fluorescence intensity (MFI) value for positive and negative sera was 27349 and 1042, respectively. The cutoff of MFI value was set at 4436 as determined by ROC analysis. Results from repeated runs on the same set of the

samples demonstrated inter-operator reproducibility of the assay.

Validation and optimization of bELISA

Based on the checkerboard titration, the optimal concentration of MAb SR30 and serum samples dilution was set at 1 μg/ml and 1:40, respectively, which provided the highest BI between known positive and negative control sera with minimal background. The cut-off value was BI of 0.138 as determined by ROC analysis on 32 experimental serum samples with known PRRS status from an animal challenge study.

Comparison of test performance over time

Swine sera samples collected weekly from 11 pigs at 9 different dpi were tested by ELISA 2XR, WIB, MIA, bELISA, and ELISA X3. All of the tests performed on these samples similarly with respect to detecting PRRSV-specific antibody (Fig. 6). While all day 0 samples were negative for PRRSV antibody by all assays evaluated, seroconversion to PRRSV was detected in all pigs by all the assays as early as 14 dpi. Antibody reactive to the recombinant N protein remained detectable by the last sampling day (i.e., 72 dpi) in experimentally infected populations. Interestingly, MIA demonstrated seroconversion to the recombinant PRRSV N protein as early as 7 dpi. A total of 2 serum samples which were collected from 2 different pigs at two different days were negative on WIB after 14 dpi.

Diagnostic performance of assays in relation to ELISA 2XR

A total of 120 selected field samples, which consisted of 44 positive and 76 negative samples as determined by ELISA 2XR and IFA, were tested by the MIA, WIB, ELISA X3

and bELISA. All assays yielded varying numbers of false positive results. False negative results were also observed in all the assays except the ELISA X3 kit. In comparison to ELISA 2XR, MIA displayed the diagnostic sensitivity and diagnostic specificity of 95% and 92.4%, respectively, when using the cutoff of MFI 4464. The same diagnostic sensitivity (95%) and specificity (92.4%) were observed for bELISA with the cutoff at 0.138BI. The diagnostic sensitivity and specificity of WIB was estimated to be 96.7% and 97.8% respectively. ELISA X3 showed the diagnostic sensitivity and specificity of 100% and 84.8%, respectively.

WIB had the highest test agreement (97.4%) with ELISA 2XR, while ELISA X3 gave the lowest test agreement (90.8%) with ELISA 2XR. Results of MIA and bELISA showed 93.4% agreement with those of ELISA 2XR. All the test agreements were supported by kappa value (Table 1).

Diagnostic performance of assays on suspect false positive samples

When four diagnostic methods applied to the SFP samples (group 2), none of them was successful in giving all negative results. Frequency of negative result given by each method is summarized in Table 2. Individually, MIA and WIB performed best in reducing the rate of SFP, although both of them still had 12 positive samples out of the 68 SFP samples. ELISA X3 still detected 15 samples as positive although it was still better than bELISA which gave 24 positives in total. Among the 68 SFP samples, 3 were identified as positive by all assays.

The majority (86.7%=10.3%+ 27.9%+ 48.5%) of the tested SFP samples were identified as PRRS negative when multiple assays were applied (Table 3). The best outcome for reducing the SFP rate were obtained when combining results of WIB and ELISA X3 (73.5%)

if more than one method had been randomly combined. Combination of MIA and WIB (72.1%) or MIA and ELISA X3 (70.6%) showed comparable outcome. The bELISA, however, performed poorly in reducing the SFP rate when compared with other three methods individually or in combination

Statistical analysis of test performance

Cochran's Q Test and McNemar's Test were used to compare the effectiveness of MIA, WIB, ELISA X3 and bELISA when diagnosing cases of SFP, validation positive and validation negative samples. The results of analyses are summarized in Table 4.

The Cochran's Q Test showed that all four tests were equally effective when testing true positive and true negative samples (i.e., group 1 and 4), but they have different effectiveness when testing cases of SFP (group 2) or inconclusive (group 3). For the cases of SFP, McNemar's Test showed that any two methods among MIA, WIB and ELISA X3 were equally effective ($p>0.05$). In contrast, bELISA was determined not to have the same effectiveness ($p<0.05$) as MIA or WIB but appeared to have the same effectiveness as ELISA X3.

On group 3 samples with S/P ratios between 0.2 and 0.4 (i.e., inconclusive potentially false negative or marginally true negative), McNemar's Tests showed that all pairwise comparisons among the 4 assays had the same effectiveness, except comparing WIB and ELISA X3. WIB determined 30 of the 31 samples negative (97.4% specificity), but ELISA X3 determined only 20 of the 31 samples negative which gave a specificity of 81.6%.

Discussion

Monitoring the serologic status of swine herds for PRRSV is critical for disease prevention and control. Although there is no true "gold standard" for PRRSV serodiagnosis, ELISA has been most commonly used in veterinary diagnostic laboratories because the assay is relatively easy to perform and can provide high throughput while other serologic tests can be expensive and/or time-consuming [20]. Once a commercial ELISA kit (IDEXX) became a widely accepted tool in the diagnostic community, the occurrence of unexpected false positive results at a higher rate than expected has become a great concern to the swine industry with respect to disease control and prevention. Several competitive or bELISA have been applied to minimize the occurrence of SPF results [7,12,35]. However, lower-than-desired levels of specificity were obtained, leading to the desire for a more thoroughly validated serologic assay with high level of accuracy.

Products of baculovirus expressing system are generally considered to be immunogenic; therefore, they can be used to establish various immunoassays. In this study, recombinant baculoviruses containing the gene encoding for the putative N proteins of type 1 or 2 PRRSV were constructed and used to express the proteins in insect cells. The N protein was chosen since it is known to be antigenically conserved among PRRS viruses. Baculovirus-expressed fusion N proteins were shown to be antigenically similar to the native N protein as they were detected by porcine anti-PRRSV sera in WIB. Using the recombinant N protein, WIB and MIA were able to clear more than 80% of SFP sera as negative, demonstrating their usefulness as good confirmatory test. A disadvantage of WIB was that the assay could not detect antibody specific for the N protein until 14 dpi, whereas MIA was able to detect the

antibody at 7 dpi.

Microsphere immunoassay has shown many promising characteristics as future serologic assay but has not been applied to PRRSV diagnostics. This study demonstrated that the diagnostic sensitivity and specificity of the recombinant PRRSV N protein-based MIA were excellent in a small aliquot of serum (≤10μl). In comparison to IFA test which has been commonly used as confirmatory test for SFP samples in the veterinary diagnostic community, MIA is more convenient and can provide rapid turnaround, especially when screening a large numbers of sera. The MIA also provides a quantitative test result, whereas the IFA generally produces qualitative result unless endpoint titration is carried out. The significant advantage of MIA in comparison to WIB is its capacity for multiplexing with additional target antigen(s), therefore, the specificity of the test can be enhanced while maintaining the same level of sensitivity.

The epitope-based bELISA had the same sensitivity (95%) and specificity (92.4%) as MIA. However, the assay produced most false positive samples when it was conducted on the SFP samples. The fluctuation in test performance was also observed among different ELISA 2XR plates which were used in bELISA, which may account for poor performance of the bELISA as compared to the other three assays. The IDEXX PRRS ELISA X3 kit was able to reduce the SFP rate as claimed by the manufacturer. Interestingly, the kit produced more positive results when it was run on samples tested negative by ELISA 2XR, particularly ones with S/P ratio between 0.2 and 0.4. This could be due to the increased sensitivity of the assay or test specificity has been compromised. The comparative assessment in this study indicated that the bELISA and ELISA X3 may have lower specificity than ELISA 2XR. Therefore, these 2 ELISAs are not recommended as confirmatory testing. The assays, particularly

ELISA X3 would be rather more suitable for rapid screening tests which can substantially reduce a number of SFP reactors.

Although all the assays employed in the study displayed a relatively good diagnostic sensitivity and diagnostic specificity and were capable of reducing the SFP sample numbers, none of individual assay could reduce the rate by 100%. The maximum reduction of the SFP rate by individual testing was 82.4%. On the other hand, when more than one assay were combined, the frequency of SPF samples was further reduced by as high as 73.5%, implying that more than one test may be needed for confirmation of SFP samples. The study results suggest that WIB and ELISA X3 are the best pair with best performance for confirmatory testing. Considering that the ELISA X3 may have lower-than-desired specificity, a testing algorithm suggested for confirmation is then that samples with SFP result in the ELISA 2XR should be tested first by ELISA X3 and then by WIB.

Acknowledgement

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FIGURE LEGEND

Figure 1. Photographs of agarose gels showing the PCR products of recombinant virus bac-6xHis-ORF7NA or bac-6xHis-ORF7EU (A) and nested PCR products of type 1 or type 2 PRRSV ORF7 (B). Lanes 1-3 and 4-6 in the panel A are PCR product of bac-6xHis-ORF7NA and bac-6xHis-ORF7EU, respectively, with M13 primers. Lanes 1-3 and 4-6 in the panel B are PCR products of type 1 and 2 PRRSV ORF7, respectively which were amplified with gene specific primers from the corresponding PCR products shown in the panel A as template.

Figure 2. Western immunoblotting confirmation of expression of the recombinant PRRSV nucleocapsid proteins in sf9 insect cells transfected with bacmid DNA bac-6xHis-ORF7EU and bac-6xHis-ORF7EU using anti-PRRSV polyclonal antiserum raised against VR2332. WT represents crude antigen prepared from wild-type baculovirus-infected Sf9 cells. Molecular weight (MW) markers are given in kDa to the left of the figure.

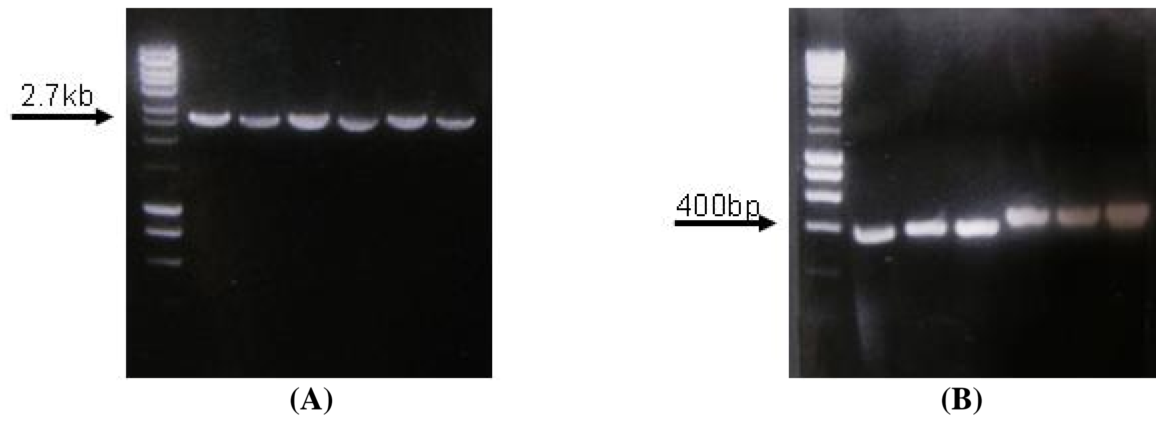
Figure 3. Western immunoblot analysis of bac-6xHis-ORF7EU and bac-6xHis-ORF7NA recombinant protein before and after purification. Lanes 1 and 2 on each nitrocellulose membrane is unpurified and purified proteins recognized by PRRSV-specific antiserum, respectively.

Figure 4. Western immunoblot analysis of the reactivity of purified recombinant type 1 PRRSV nucleocapsid (bac-6xHis-ORF7EU) protein (A) and recombinant type 2 PRRSV nucleocapsid (bac-6xHis-ORF7NA) protein (B) with PRRSV nucleocapsid protein-specific monoclonal antibodies (MAbs) SDOW 17, SR 30, VO17, JP24 and EP147, porcine anti-LV serum, and negative control serum. MAbs VO17, JP24 and EP147 are specific only for type 2 PRRS viruses whereas MAbs SDOW17 and SR30 detect both type 1 and type 2 PRRS viruses. Molecular weight (MW) markers are given in kDa to the left of the figure.

Figure 5. Readout of microsphere immunoassay using recombinant PRRSV nucleocapsid protein on serum samples collected from experimental pigs with known PRRSV infection status. The median fluorescence intensity (MFI) of each serum is indicated by red bar. X1 to X16 represent 16 known negative sera whereas X17 through X32 represent 16 known positive sera.

Figure 6. Comparison of test performance over time on serum sequentially bled from 11 pigs experimentally infected with PRRSV. The X axis represents days post infection; Y axis represents number of positive/negative results. Each method is represented by a different color bar as indicated on the figure.

Figure 1.



(A) **(B)**

Figure 2.

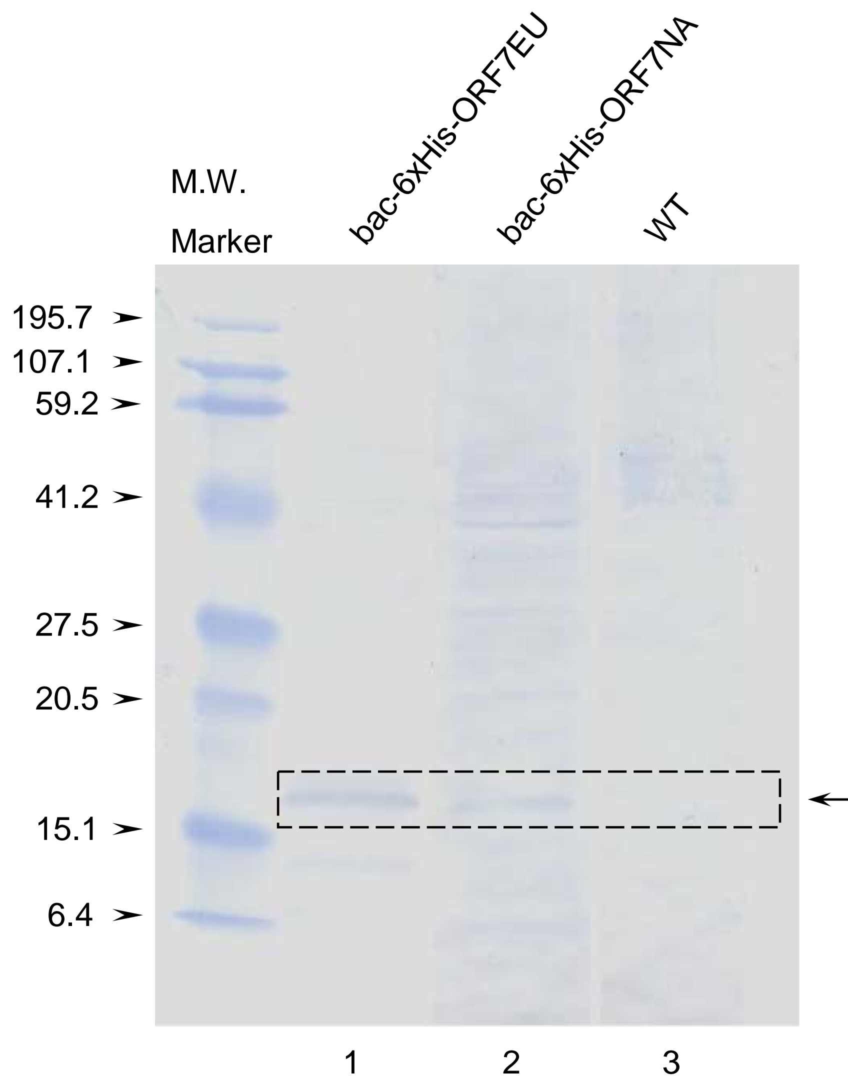


Figure 3.

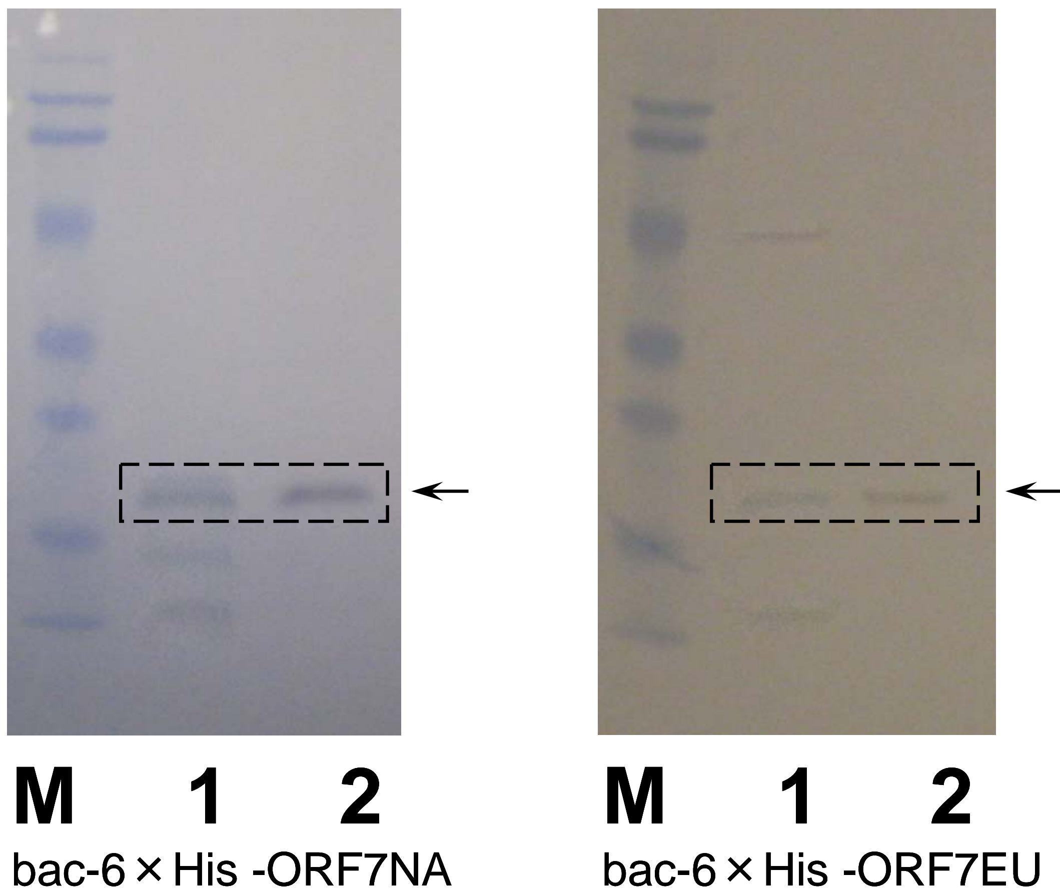


Figure 4.

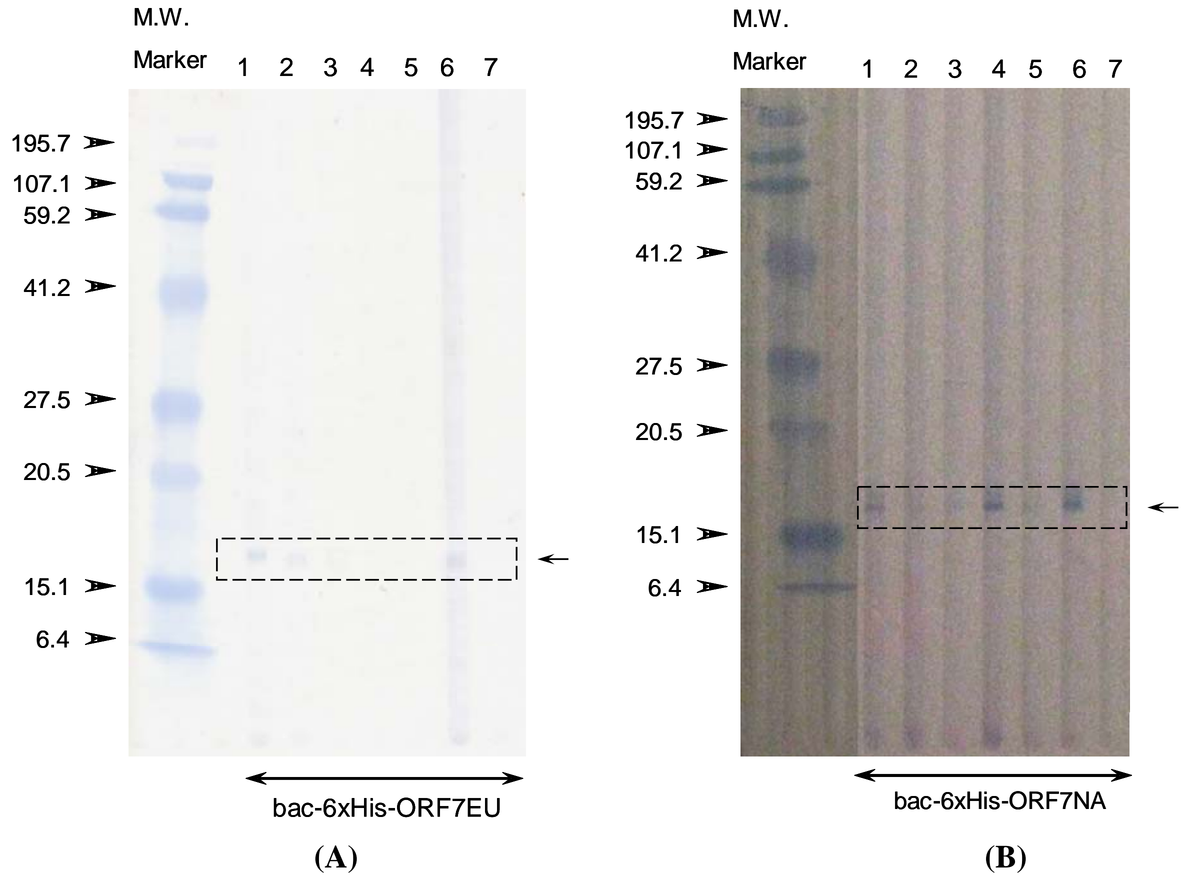


Figure 5.

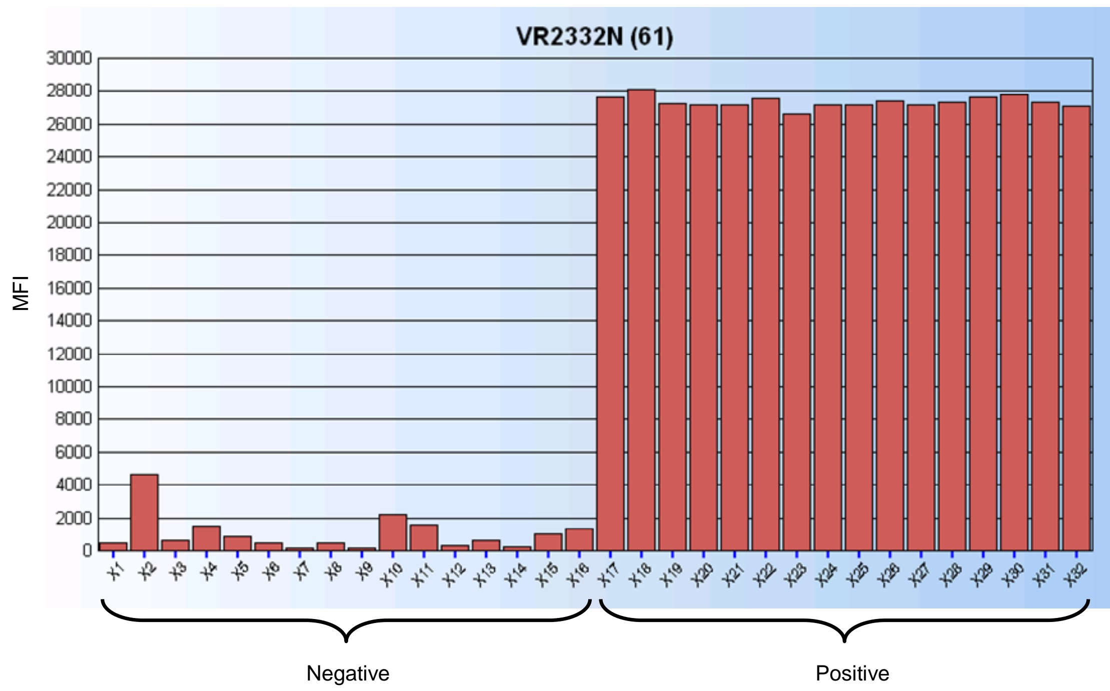


Figure 6.

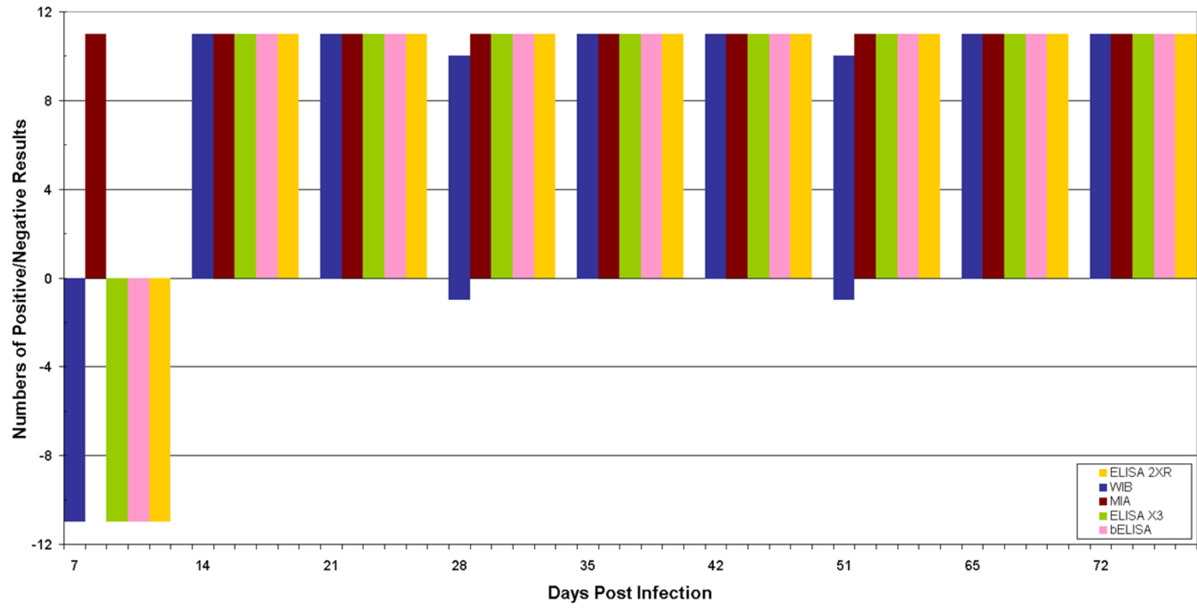


Table 1. Diagnostic performance of selected serologic assays in comparison to ELISA 2XR on swine sera selected from submissions to the Iowa State University Diagnostic Laboratory which had more than 10 serum samples per case. The status of each sample was first determined by ELISA 2XR and IFA/PCR along with herd history.

Category	ELISA 2XR	MIA	WIB	ELISA X3	bELISA
TP	60	57	58	60	57
TN	92	85	90	78	85
FP	0	7	2	14	7
FN	0	3	2	0	3
Sensitivity(%)	N/A	95.0	96.7	100.0	95.0
Specificity(%)	N/A	92.4	97.8	84.8	92.4
Test Agreement (%)		93.4	97.4	90.8	93.4
κ Value		0.864	0.945	0.815	0.864

TP: True Positive
TN: True Negative
FP: False Positive
FN: False Negative
Sensitivity%= 100*TP/(TP+FN)
Specificity%= 100*TN/(TN+FP)
Test agreement=100*number of samples with agreed test results/number of total samples tested
κ value: 0.81-1.00 almost perfect agreement

Table 2. Frequency of negative results given by each assay on suspect false positive samples which were determined by ESLIA 2XR and IFA/PCR along with herd history.

Method	Frequency	% reduction
MIA	56/68	82.4
WIB	56/68	82.4
ELISA X3	53/68	77.9
bELISA	44/68	64.7

Table 3. Frequency of negative results in suspected false positive samples based on different combinations of results from all four assays evaluated. Methods 1, 2, 3 and 4 are recombinant PRRSV nucleocapsid protein-based microsphere immunoassay, Western immunoblotting using recombinant PRRSV nucleocapsid protein, ELISA X3 (IDEXX) and epitope-based blocking ELISA utilizing ELISA 2XR (IDEXX), respectively.

PRRS Negative Samples, based on	Frequency	%
General Combinations		
None of the Methods	3/68	4.4
Any 1 Method	6/68	8.8
Any 2 Methods	7/68	10.3
Any 3 Methods	19/68	27.9
Any 4 Methods	33/68	48.5
Specific Combinations		
Methods 1 and 2	49/68	72.1
Methods 1 and 3	48/68	70.6
Methods 1 and 4	39/68	57.4
Methods 2 and 3	50/68	73.5
Methods 2 and 4	38/68	55.9
Methods 3 and 4	38/68	55.9

Table 4. Cochran's Q test and McNemar's Test results. P value<0.05 is indicative of significant difference (i.e., not equal between assays).

	Group			
	Positive (s/p≥0.4)	False Positive	Inconclusive (s/p<0.2)	Negative (0.2≤s/p<0.4)
Overall Difference (Cochran's Q)	N/A	0.0103*	0.6659	0.0061
MIA vs. WIB	1.0000	1.0000	1.0000	0.1250
MIA vs. ELISA X3	0.25	0.5811	0.6250	0.0625
MIA vs. bELISA	1.0000	0.0169	1.0000	1.0000
WIB vs. ELISA X3	0.5	0.5078	0.6250	0.0020
WIB vs. bELISA	1.0000	0.0227	1.0000	0.2188
ELISA X3 vs. bELISA	0.25	0.0784	1.0000	0.1460

*P value for comparison of proportions between diagnostic tests.

Appendix

Group 1 samples test results by four diagnostic methods.

Sample #	MIA	WIB	ELISA X3	bELISA
1	pos	neg	pos	pos
2	pos	pos	pos	pos
3	pos	pos	pos	pos
4	pos	pos	pos	pos
5	pos	pos	pos	pos
6	pos	pos	pos	pos
7	pos	pos	pos	pos
8	pos	pos	pos	pos
9	pos	pos	pos	pos
10	pos	pos	pos	pos
11	pos	pos	pos	pos
12	pos	pos	pos	pos
13	pos	pos	pos	pos
14	pos	neg	pos	neg
15	pos	pos	pos	pos
16	pos	pos	pos	pos
17	pos	pos	pos	pos
18	pos	pos	pos	pos
19	pos	pos	pos	pos
20	pos	pos	pos	pos
21	pos	pos	pos	pos
22	neg	pos	pos	pos
23	pos	pos	pos	pos
24	pos	pos	pos	pos
25	neg	pos	pos	pos
26	neg	pos	pos	pos
27	pos	pos	pos	pos
28	pos	pos	pos	pos
29	pos	pos	pos	pos
30	pos	pos	pos	pos
31	pos	pos	pos	pos
32	pos	pos	pos	neg
33	pos	pos	pos	pos
34	pos	pos	pos	pos
35	pos	pos	pos	pos
36	pos	pos	pos	pos
37	pos	pos	pos	pos
38	pos	pos	pos	neg

Group 1 samples test results by four diagnostic methods continued.

39	pos	pos	pos	pos
40	pos	pos	pos	pos
41	pos	pos	pos	pos
42	pos	pos	pos	pos
43	pos	pos	pos	pos
44	pos	pos	pos	pos

Group 2 samples test results by four diagnostic methods.

Sample #	MIA	WIB	ELISA X3	bELISA	Sample #	MIA	WIB	ELISA X3	bELISA
1	neg	neg	neg	neg	35	neg	neg	neg	pos
2	neg	neg	neg	neg	36	neg	pos	neg	neg
3	neg	neg	neg	neg	37	pos	neg	neg	pos
4	neg	neg	neg	neg	38	neg	neg	neg	neg
5	neg	neg	neg	neg	39	neg	neg	neg	neg
6	neg	neg	neg	neg	40	neg	pos	neg	pos
7	neg	neg	neg	neg	41	neg	neg	neg	neg
8	neg	neg	neg	neg	42	neg	neg	neg	pos
9	neg	neg	neg	pos	43	neg	neg	neg	pos
10	neg	neg	neg	neg	44	neg	neg	neg	pos
11	neg	neg	neg	pos	45	neg	neg	neg	pos
12	neg	neg	neg	neg	46	neg	neg	neg	pos
13	neg	neg	neg	neg	47	neg	neg	neg	pos
14	pos	pos	pos	neg	48	neg	neg	neg	pos
15	neg	neg	neg	pos	49	neg	neg	neg	neg
16	neg	neg	neg	neg	50	neg	neg	neg	neg
17	neg	neg	neg	neg	51	neg	neg	neg	neg
18	pos	neg	pos	pos	52	pos	neg	pos	pos
19	neg	neg	neg	neg	53	pos	neg	neg	pos
20	pos	neg	neg	neg	54	pos	pos	pos	pos
21	neg	neg	neg	neg	55	neg	neg	neg	neg
22	neg	pos	pos	neg	56	neg	neg	pos	pos
23	neg	pos	pos	pos	57	neg	neg	neg	neg
24	neg	pos	neg	neg	58	neg	neg	neg	neg
25	neg	neg	neg	pos	59	neg	neg	neg	neg
26	neg	neg	neg	neg	60	neg	pos	pos	pos
27	neg	neg	neg	neg	61	pos	pos	pos	pos
28	pos	pos	pos	pos	62	neg	neg	neg	neg
29	neg	pos	pos	neg	63	neg	neg	neg	neg
30	pos	pos	pos	neg	64	neg	neg	neg	neg
31	neg	neg	pos	neg	65	pos	neg	neg	neg
32	neg	neg	pos	neg	66	neg	neg	neg	neg
33	pos	neg	neg	neg	67	neg	neg	neg	neg
34	neg	neg	pos	pos	68	neg	neg	neg	neg

Group 3 samples test results by four diagnostic methods.

Sample #	MIA	WIB	ELISA X3	bELISA
1	neg	neg	neg	neg
2	neg	neg	neg	neg
3	neg	neg	neg	pos
4	neg	neg	pos	neg
5	neg	neg	neg	neg
6	neg	neg	neg	neg
7	neg	neg	neg	neg
8	neg	neg	neg	neg
9	neg	neg	neg	neg
10	neg	neg	neg	neg
11	pos	neg	pos	neg
12	pos	neg	pos	neg
13	neg	neg	neg	neg
14	neg	neg	pos	pos
15	pos	neg	pos	pos
16	neg	neg	neg	neg
17	neg	pos	pos	neg
18	neg	neg	neg	neg
19	neg	neg	pos	neg
20	neg	neg	pos	neg
21	neg	neg	neg	neg
22	neg	neg	neg	neg
23	neg	neg	neg	neg
24	pos	neg	pos	neg
25	pos	neg	pos	neg
26	pos	neg	pos	neg
27	neg	neg	neg	neg
28	neg	neg	neg	neg
29	neg	neg	neg	pos
30	neg	neg	neg	neg
31	neg	neg	neg	pos

Group 4 samples test results by four diagnostic methods.

Sample #	MIA	WIB	ELISA X3	bELISA
1	neg	neg	neg	neg
2	neg	neg	neg	neg
3	neg	neg	neg	neg
4	neg	neg	neg	neg
5	neg	neg	neg	neg
6	neg	neg	neg	neg
7	neg	neg	neg	neg
8	neg	neg	neg	neg
9	neg	neg	neg	neg
10	neg	neg	neg	neg
11	neg	neg	neg	neg
12	neg	neg	neg	neg
13	neg	neg	neg	neg
14	neg	neg	neg	neg
15	neg	neg	neg	neg
16	neg	pos	neg	neg
17	neg	neg	neg	neg
18	neg	neg	neg	neg
19	neg	neg	neg	neg
20	neg	neg	neg	neg
21	neg	neg	neg	neg
22	neg	neg	neg	neg
23	neg	neg	neg	neg
24	neg	neg	neg	neg
25	neg	neg	neg	neg
26	neg	neg	neg	neg
27	neg	neg	neg	neg
28	neg	neg	neg	neg
29	neg	neg	pos	neg
30	neg	neg	neg	neg
31	neg	neg	neg	neg
32	neg	neg	neg	neg
33	neg	neg	neg	neg
34	neg	neg	pos	neg
35	neg	neg	neg	neg
36	neg	neg	neg	neg
37	neg	neg	neg	neg
38	neg	neg	neg	neg
39	pos	neg	neg	neg
40	neg	neg	pos	neg

Group 4 samples test results by four diagnostic methods continued.

41	neg	neg	neg	pos
42	neg	neg	neg	neg
43	neg	neg	neg	neg
44	neg	neg	neg	neg
45	neg	neg	neg	pos

CHAPTER 3: GENERAL CONCLUSION

Serology diagnostic tools aid in PRRS control strategies

Samples for serodiagnostics can be easily obtained from live pigs to determine the PRRS status of the animals. Several tests detect the pig's immune response to PRRSV and ELISA has been most commonly used for that purpose. Few, if any, serological tests reach 100% accuracy, no exception for PRRS diagnostics. All serological tests are expected to have some degree of error, and even widely used commercial IDEXX ELISA 2XR kit has is known to have the false-positive rate of 0.5-1%. In populations expected to be PRRS-negative, even 1% false positive rate can be a serious concern for PRRSV control and eradication. Follow-up tests commonly including PCR, VI, IFA and SVN are 'must' to confirm or disprove the unexpected test results. The inherent limitations of each of these tests, however, made them less desirable as follow-ups. In our study, three in-house developed serology methods (Western immunoblotting, microsphere immunoassay, epitope-based blocking ELISA) as well as newly released commercial ELISA X3 kits were evaluated for their performance as possible confirmatory tests.

Diagnostic sensitivity and specificity of all four methods were good and can be acceptable for use in diagnosis. As compared to the current IDEXX PRRS ELISA (2XR), all of them were able to reduce the number of suspected false positive samples substantially, ranging from 64.7% to 82.4%. Combining results of two different methods also reduced the SFP results by as high as 73.5%. Although this is not as good as individual method results, it does provide higher level of confidence when dealing a SFP sample. Although different

combination of tests can be chosen by laboratories and veterinarians depending upon their concerns and testing infrastructure, a recommendation for confirmatory testing based on observations of the current study would be a combination of WIB and ELISA 3X.

Obviously the more assays we apply to samples, the higher confidence we will get to confirm the true status of the samples. In reality, however, not all of the diagnostic methods can be employed by laboratories due to various practical constraints (e.g., infrastructure, expertise and personnel, etc.). For producers, the cost is an important factor to consider when diagnostic investigation or disease surveillance is necessary. Serology is not an exception to that even though it is much cheaper than other diagnostics such as PCR, virus isolation or tissue immunoassays. Our study suggested that a recombinant nucleocapsid protein-based WIB or MIA can be a rapid and reliable alternative to traditional IDEXX ELISAs when evaluating sera with suspected false positive results. Their high sensitivity and specificity also makes these tests useful in determining PRRSV infection status which can aid in the management of PRRS outbreaks. In case that proper instrumentation or skills to develop WIB or MIA is lacking, bELISA format of the ELISA 2XR may be a viable option since no special equipment is required for this assay and the original commercial ELISA kit can still be utilized without too many changes. If a single assay must be chosen among the assays evaluated, the new IDEXX PRRS ELISA X3 has promising future as it showed much better performance than the 2XR kit in reducing SFP rate while maintaining the level of sensitivity similar to that of the 2XR and high throughput capability. All these advantages make the X3 kit an excellent screening test with higher accuracy.

Accurate data interpretation

For all PRRS diagnostics, it is important to understand benefits and limitations of each assay to insure proper use and interpretation. Taking ELISA as a good example and comparing to other serology assays, it provides excellent quantitative information on PRRSV infection status of a herd and also provides a standardized universal platform for diagnostic testing whose results can be interchangeable between laboratories. However, care should be taken not to over-interpret the S/P ratio beyond determination of herd status for PRRSV infection. One of key elements that should be taken into consideration when interpreting result is the timing of the sampling in relation to time of exposure to the virus (i.e., disease kinetics). In our study, ELISA, bELISA, WIB and IFA test could not detect PRRSV-specific antibodies until 14 dpi whereas MIA detected the antibodies as early as 7 dpi.Another important element is the sample pool/size used for test evaluation. Because there is no universally accepted method to determine the true status of field samples, care should be taken in selecting samples, otherwise biased results can be obtained, leading to the incorrect conclusion. Thus, diagnostic tools are only as good as their valid and reliable use and the careful interpretation of their results.

Future work

Our present study demonstrated four assays can greatly reduce the SFP rate when subjected with unexpected positive samples. However, diagnostic sensitivity and specificity were not extremely high as hoped for. Although field samples used in this study were

carefully selected from submissions to ISU-VDL according to their ELISA and IFA or PCR results as well as the knowledge based on the farm history, it would have been impossible for us to know their absolutely true status for PRRSV. Therefore, biased results might be generated and affected the performance of the assays employed in the study. This suggests that the methods used may still have a room for improvement. In order to prove that, more samples with known infection status should be tested by all the assays in the near future to improve our understanding on their performance and feasibility in PRRSV diagnosis.

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