Title: PRRSV Identification by Virochip - **NPB #11-109**

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Industry Summary:

Current identification and surveillance practices for PRRSV involves a few selected U.S. veterinary diagnostic laboratories performing quantitative RT-PCR and ORF5 nucleotide sequencing followed by restriction fragment length polymorphism, a process that typically takes two weeks or more. Therefore there is a substantial need for faster surveillance programs coupled with next generation detection methods to rapidly identify and track genomic changes in PRRSV for animal health preparedness for future outbreaks. For this project we constructed a novel microarray platform that is designed to rapidly and specifically identify and differentiate all known strains of PRRSV, including novel or emerging isolates encoding a high degree of genetic variability compared to known isolates. Because this microarray platform emphasizes multiple key regions encompassing the entire PRRSV genome it has increased sensitivity and the ability to track genetic variability. Our results indicate that this platform successfully identified and distinguished the genetic variability of four genetically divergent PRRSV isolates in cell culture samples. Additionally, our data demonstrates that the PRRSV microarray has a 10 to 1000 times improved sensitivity or increased limit of detection compared to currently deployed quantitative RT-PCR. We have also demonstrated this array platform successfully identified and differentiated PRRSV using antemortem clinical samples (nasal swab and serum) collected from pigs infected with a Chinese highly pathogenic PRRSV isolate and bacterial cocktail consisting of *Streptococcus suis*, *Haemophilus parasuis*, and *Actinobacillus suis*. This data demonstrates that the PRRSV microarray is a sensitive and specific tool that is able to quickly identify novel or emerging strains of PRRSV in clinical samples containing multiple swine pathogens.

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Keywords: PRRSV, microarray, detection, diagnostic, surveillance

Scientific Abstract:

Respiratory disease in pigs is the most important health concern for swine producers today and porcine reproductive and respiratory syndrome virus (PRRSV) is the most economically significant disease affecting the pork industry worldwide. Current identification and surveillance practices for PRRSV involves a few selected U.S. veterinary diagnostic laboratories performing quantitative RT-PCR and ORF5 nucleotide sequencing followed by restriction fragment length polymorphism, a process that typically takes two weeks or more. Therefore there is a substantial need for faster surveillance programs coupled with next generation detection methods to rapidly identify and track genomic changes in PRRSV for animal health preparedness for future outbreaks. In this report we describe the construction of a novel microarray platform designed to rapidly and specifically identify and differentiate all known strains of PRRSV, including novel or emerging isolates encoding a high degree of genetic variability compared to known isolates. Because this microarray platform emphasizes multiple key regions encompassing the entire PRRSV genome it has an increased sensitivity and the ability to track genetic variability. Our results indicate that this platform successfully identified and distinguished the genetic variability of four genetically divergent PRRSV isolates in cell culture samples. Additionally, the limit of detection by quantitative RT-PCR for these samples is approximately the 10^-6 dilution and the limit of detection for our novel PRRSV microarray is approximately 10^-7 to 10^-9 dilution. This data demonstrates that the PRRSV microarray (PRRSV Chip) has a 10 to 1000 times improved sensitivity compared to currently deployed quantitative RT-PCR. We have also demonstrated this array platform successfully identified and differentiated PRRSV using antemortem clinical samples (nasal swab and serum) collected from a pig infected with a Chinese highly pathogenic PRRSV isolate and a bacterial cocktail consisting of *Streptococcus suis*, *Haemophilus parasuis*, and *Actinobacillus suis.* This data demonstrates that the PRRSV Chip is a sensitive and specific tool that is able to quickly identify novel or emerging strains of PRRSV in clinical samples containing multiple swine pathogens.

Introduction:

Respiratory disease in pigs is the most important health concern for swine producers today and porcine reproductive and respiratory syndrome virus (PRRSV) is the most economically significant disease affecting the pork industry worldwide. With a mutation rate approximately thirty times higher than human immunodeficiency virus, PRRSV has the highest mutation rate of any known virus. Due to its high mutation rate and degree of genetic variability, there is a substantial need for improved diagnostic and surveillance tools to rapidly identify different and multiple PRRSV strains in swine samples and track genomic changes in field isolates of PRRSV. PCR-based assays are currently the gold standard for specific diagnosis of porcine respiratory disease complex (PRDC) pathogens, including PRRSV, due to the rapid and sensitive results they can provide. Despite high levels of sensitivity, major limitations exist in using PCR-based assays for pathogen identification. For example, PCR-based assays often fail to successfully detect more than one pathogen due to high failure rates associated with multiplex assays. PCR-based assays fail to provide complete subtype information, which may provide important information for vaccine and surveillance efforts. False negatives can occur when using PCR-based assays due to multiple pathogens being present in a single sample and, more notably, to mutations at primer binding sites, which is an issue for PRDC pathogens such as swine influenza virus and PRRSV that evolve rapidly and undergo reassortment/recombination at high frequency. An additional limitation in current surveillance strategies is time. Current identification and surveillance practices for PRRSV involves a selected few U.S. veterinary diagnostic laboratories performing quantitative RT-PCR and ORF5 nucleotide sequencing, followed by restriction fragment length polymorphism prediction, a process that may take two or more weeks. This extensive turnaround time highlights the substantial need for faster surveillance techniques coupled with next generation detection methods to rapidly identify, differentiate and monitor genomic changes in PRRSV for animal health preparedness for future outbreaks. Additionally, recent work on Asian highly pathogenic PRRSV (1) has shown that these viral strains are indeed a threat to our nations pork industry, and existing diagnostics are inadequate for prompt identification of these Asian strains.

Panviral DNA microarrays represent the best approach for massively parallel viral surveillance and discovery. A panviral or Virochip platform has proven to be extremely successful in both human clinical settings and veterinary clinical settings (2-4, 6, 8, 9). Additionally, we have successfully demonstrated that a Virochip platform can readily detect PRRSV in both cell culture and spiked serum samples, as well as antemortem clinical samples, such as serum, collected from PRRSV infected pigs (5). The major concerns with regard to using a microarray or Virochip platform are the cost, sensitivity and the inaccurate assumption of a long turnaround time. The idea that a microarray or Virochip platform has a long turnaround time is completely false given that it is only 4 days. Specifically, a typical parallel sample processing timeline for a Virochip platform is: (day 1) generate cDNA from isolated viral RNA, (day 2) amplify and label cDNA, (day 3) hybridize (combine complementary subunits) labeled cDNA to microarray, (day 4) scan microarray slide and visualize or read results. In addition, standardized techniques, kits with which to perform these steps, and the equipment to scan the completed assays, are all available. To identify genetic diversity including single nucleotide polymorphisms (SNPs) among large collections or numbers of PRRSV isolates some research labs are choosing to utilize next generation sequencing platforms, the cost of which far exceed that associated a microarray or Virochip platform. More significantly, for routine identification and surveillance purposes veterinary diagnostic laboratories are currently performing approximately 50 ORF5 sequencing reactions per

week. Given that the minimum cost of a single reaction is $130, this adds up to $26,000 per month for a result that provides no genome scale sequence information or variability among isolates. Collectively, after comparing resources invested, such as time and cost, to the quantity and quality of information gained, a microarray platform is a far superior choice to current diagnostic practices.

In this report we describe the development of a microarray platform to rapidly and specifically genotype PRRSV isolates, including novel or emerging isolates, especially those with the highly pathogenic Asian genotypes, encoding a high degree of genetic variability compared to known prototypes. As previously stated, we have demonstrated that a Virochip platform is able to successfully identify PRRSV in a variety of different clinical samples collected from PRRSV infected pigs (5). Building on our knowledge and expertise of microarray methodologies, we constructed a microarray platform (PRRSV Chip) to specifically detect PRRSV in clinical samples, such as serum, with a high degree of sensitivity to allow for rapid identification and concurrent tracking of genetic variability along the whole viral genome. To accomplish this, special emphasis was placed on targeting regions of the PRRSV genomic RNA that are known to be present in greater quantities during infection to increase sensitivity, and genomic regions that are to contain a high degree of genetic variability for epidemiology and surveillance purposes.

Objectives:

Objective 1. Design oligonucleotide probes corresponding to PRRSV genome with special emphasis placed on targeting regions of the PRRSV genomic DNA that are known to be present in greater quantities during infection to increase sensitivity and genomic regions that are known to contain a high degree of genetic variability for epidemiology and surveillance purposes .

Objective 2. Amplify and label cDNA generated from RNA isolated from clinical swine samples.

Objective 3. Hybridize the amplified samples and analyze the microarray data for determining the specificity and sensitivity of the PRRSV -specific microarray compared to current PCR diagnostic tests.

Materials & Methods:

Virus strains and clinical specimens

PRRSV strains VR2332, MN184, SDSU73, Lelystad were used in this study. Virus stocks were propagated on MARC-145 cells and were cultured and maintained in medium composed of MEM supplemented with 10% FBS and 50 mg/liter of gentamicin (Gibco-Invitrogen) at 37°C, 5% CO_2. Clinical PRRSV samples were obtained from serum and nasal swab collected at 10 days post infection (dpi) from one pig infected with Chinese highly pathogenic PRRSV (HP-PRRSV) strain rJXwn06 and a bacterial cocktail consisting of *Streptococcus suis*, *Haemophilus parasuis*, and *Actinobacillus suis*.

RNA extraction and specimen processing

Viral RNA was purified from the different samples with the MagMAX-96 Viral RNA Isolation Kit (Ambion), following the manufacturer's instructions. Once RNA was isolated, quantification of total RNA was achieved by using a Nanodrop spectrophotometer (Eppendorf). Ten-fold serial dilution of all samples were then prepared.

Pan viral DNA microarray analysis

Nine microliters of each RNA sample was randomly amplified and labeled and was then hybridized to the PRRSV Virochip microarray as described elsewhere (protocol S1 in Wang et al.[(9)]). Microarrays were scanned using the Axon 4000B scanner and GenePix software (version 3; Axon Instruments). E-predict, a computational tool developed for the analyzing Virochip array hybridization signature, was used to determine all Virochip results, with a P-value cutoff positivity of 0.05, as described elsewhere (7).

qRT-PCR and virus quantification

Samples were titered by serial dilution on MARC-145 cells to determine the TCID50/ml to verify diagnostic capabilities. PRRSV was quantitated using a Tetracore PRRS Real-Time RT-PCR kit (Tetracore, Rockville, USA), according to the manufacturer's instructions.

Results:

Objective 1:

Worldwide, there are 3-4 known genotypes of Type 1, and 4 major genotypes of Type 2 PRRSV that have undergone full genome sequencing. Oligonucleotide probes were designed using bioinformatic approaches to specifically target several regions of the PRRSV genome. To specifically address sensitivity issues in detecting PRRSV, probes targeting the genotype-specific (Type 1 and 2) nucleocapsid protein (ORF 7) were designed. The rational for specifically targeting this region of the PRRSV genome is that it is known to be present in greater quantities during infection, especially in easily acquired clinical samples such as serum, and is the region where most quantitative RT-PCR test are detecting. Genotype differential probes targeting the 5' and 3' untranslated regions were also developed, as these two regions have been shown to have the highest degree of conservation and are expressed in equal abundance to ORF7. ORF5, coding for the viral attachment protein and most often sequenced, was mined to identify 60mer regions that have the capacity to discriminate between different PRRSV strains. Additionally, several probes were designed targeting the region near the 5' terminus (nsp2), which has been shown to vary in length and sequence between many strains. Lastly, the highly conserved polymerase region (nsp9) was targeted. A final total of 1,500 60mer oligonucleotide probes were chosen, synthesized, and used for further analysis.

Objective 2:

To begin testing the usefulness of the PRRSV Chip to positively detect PRRSV, RNA isolated from tissue culture cells infected with four distinct PRRSV isolates (VR2332, MN184, SDSU73, or Lelystad) and quantitated by qRT-PCR analysis to quantitate the number of viral copies per microliter present in each sample and titered to determine the TCID50 per microliter (Table 1). To assess the performance of the PRRSV Chip in a clinical veterinary setting, RNA isolated from antemortem clinical samples (nasal swab and serum) collected from a pig infected with a Chinese highly pathogenic PRRSV isolate and bacterial cocktail consisting of *Streptococcus suis*, *Haemophilus parasuis*, and *Actinobacillus suis* (Table 1).

Table 1: Samples used to test the PRRSV Chip

Sample	TCID50/ mL	Viral copy number
VR2332	3.9 x 10^4	3.14 x 10^7
MN184	6.3 x 10^3	3.14 x 10^7
SDSU73	6.4 x 10^3	8.07 x 10^7
Lelystad	1.0 x 10^4	9.67 x 10^6
clinical		
Nasal swab	1.67x10^-2	ND
Serum	1.47x10^-1	ND

ND=not determined

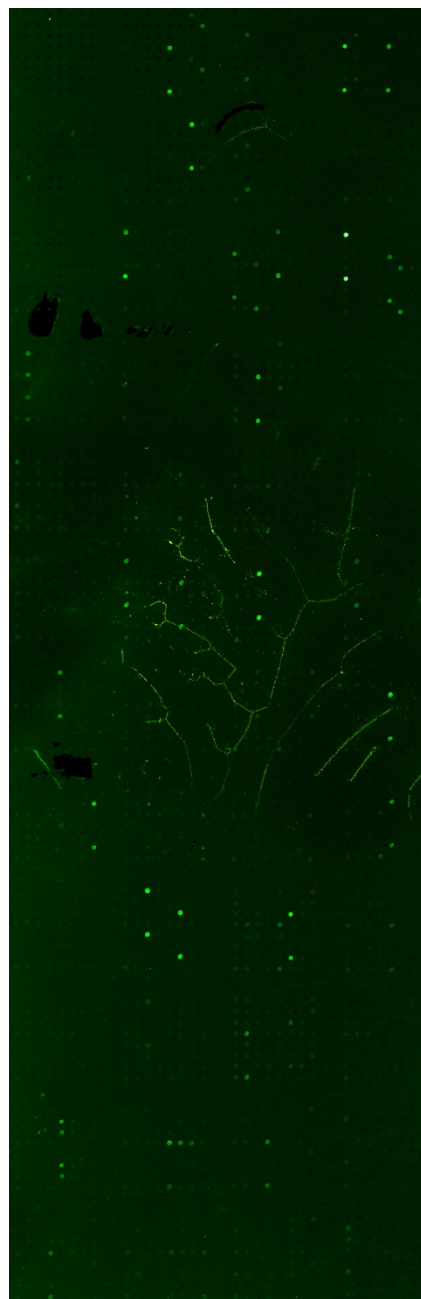
Objective 3:

Ten-fold serial dilutions were prepared for sample and nine micoliters was subsequently used to generate cDNA from all of the RNA samples. The cDNA was randomly amplified, labeled, and hybridized to the PRRSV Chip to begin evaluating the ability of the PRRSV Chip to positively detect PRRSV. We found the PRRSV Chip could successfully identify and distinguished the genetic variability of the four genetically divergent cell-cultured passaged PRRSV isolates that we evaluated (Figure 1A-H). The limit of detection by quantitative RT-PCR for these samples is approximately the 10^-6 dilution and the limit of detection for our novel PRRSV microarray is approximately 10^-7 to 10^-9 dilution. This data demonstrates that the PRRSV microarray has a 10 to 1000 times improved sensitivity compared to currently deployed quantitative RT-PCR.

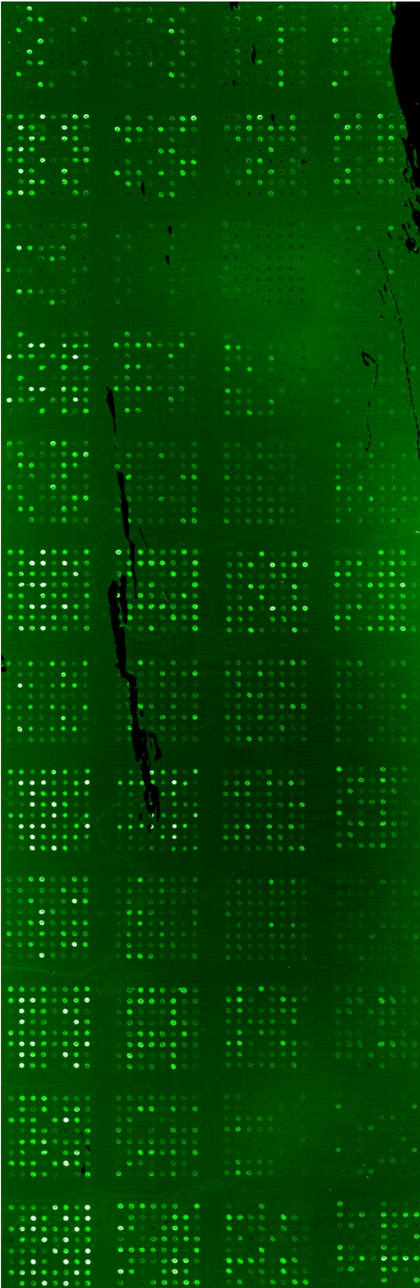
Figure 1. Detection of cell-cultured passaged PRRSV isolates by DNA microarray hybridization using the PRRSV Chip. Each green spot indicates a positive hybridization or match between a designed 60mer oligonucleotide probes and PRRSV nucleic acid present in sample.

A) VR2332 (undiluted)

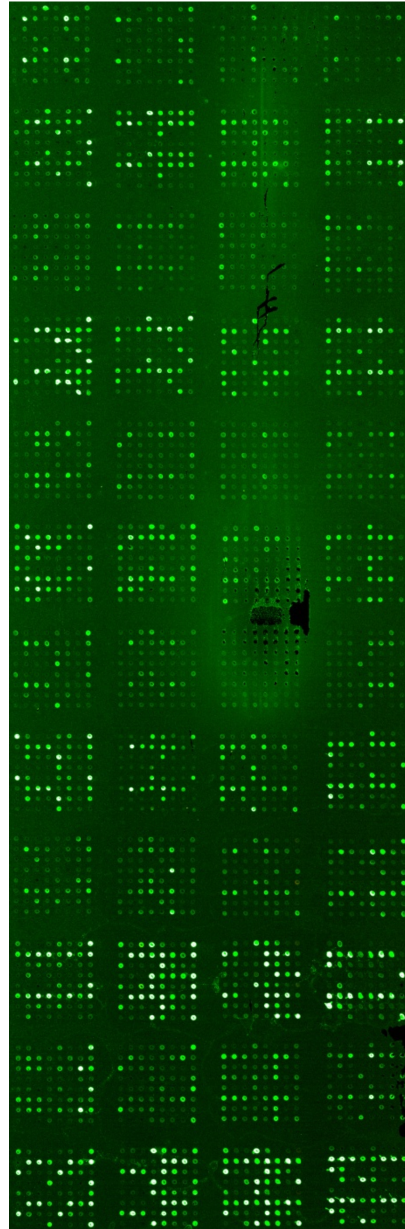
B) VR2332 (10^-6 dilution)



C) MN184 (undiluted)

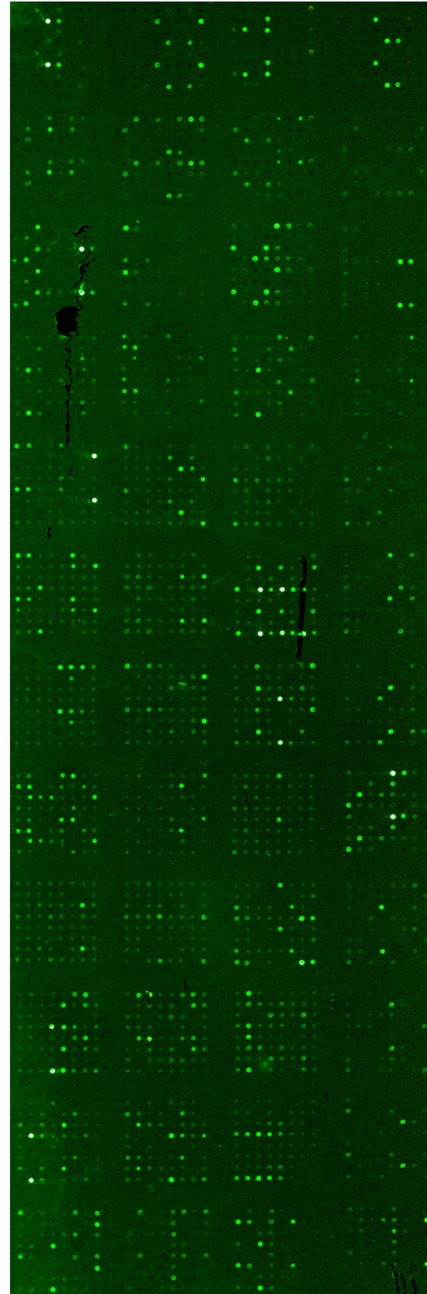


D) MN184 (10^-6 dilution)

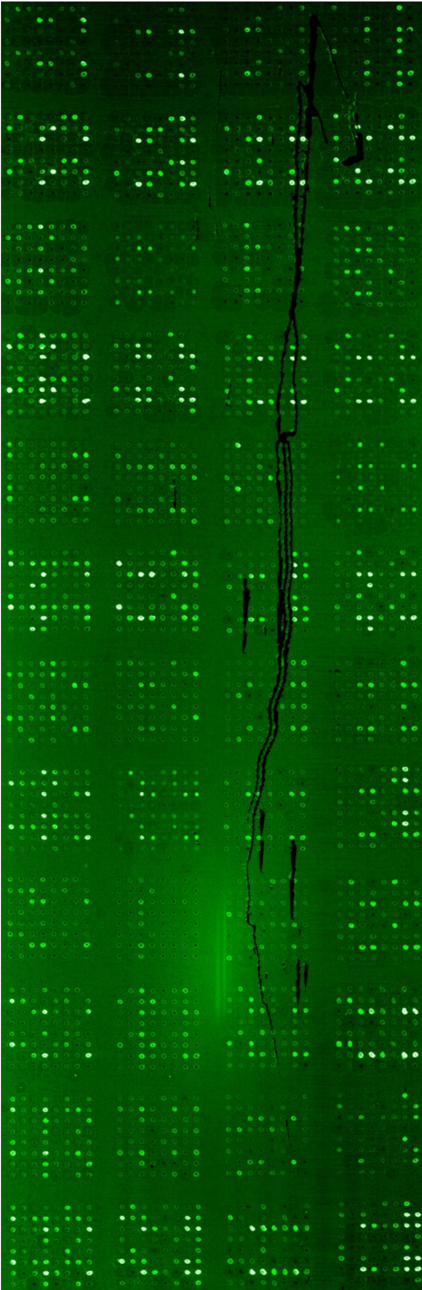


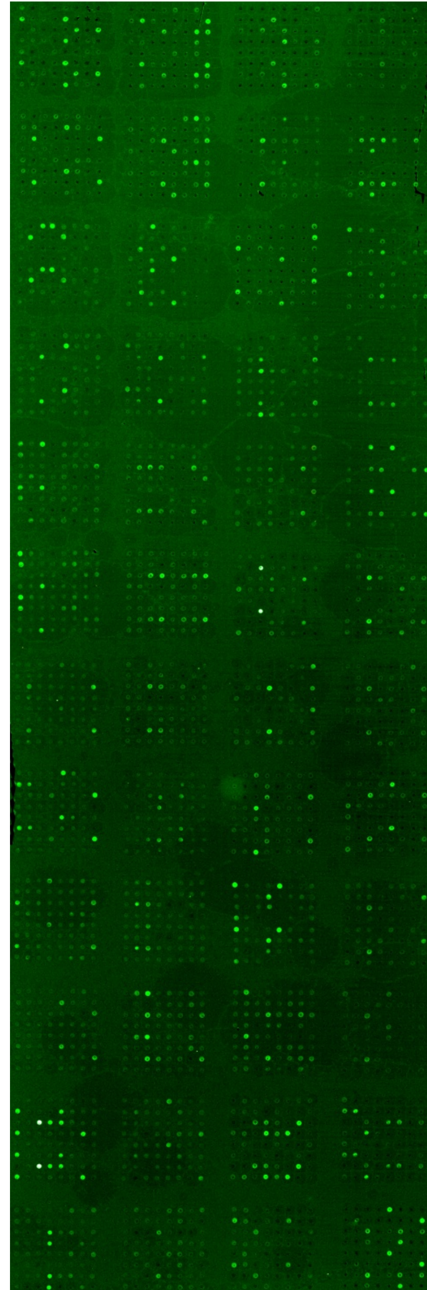
E) SDSU73 (undiluted)

F) SDSU73 (10^-6 dilution)



G) Lelystad (undiluted)



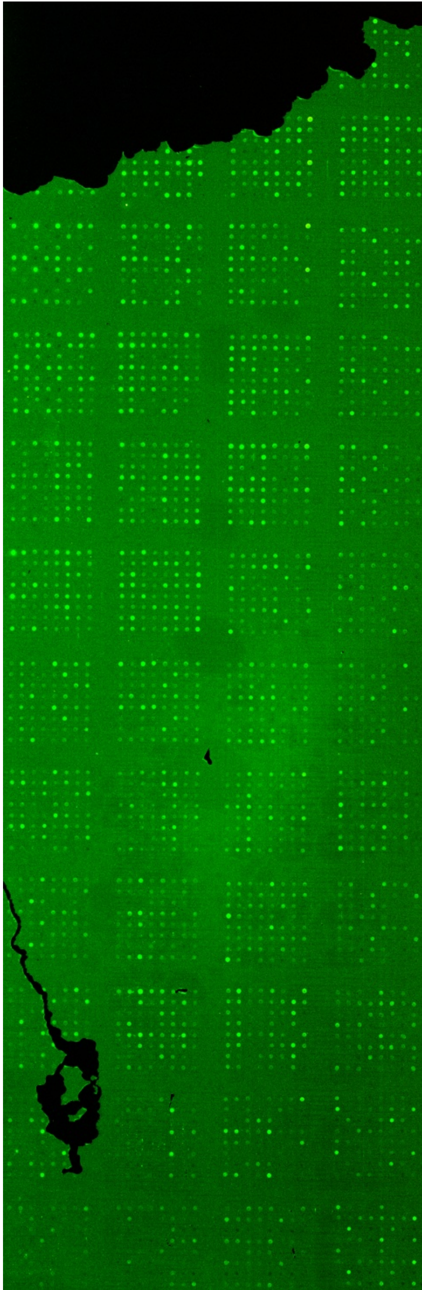


H) Lelystad (10^-6 dilution)

Figure 2: Detection of Chinese highly pathogenic PRRSV (HP-PRRSV) strain rJXwn06 by DNA microarray hybridization using the PRRSV Chip from samples isolated from pig coinfected with rJXwn06 and a bacterial

cocktail. Each green spot indicates a positive hybridization or match between a designed 60mer oligonucleotide probes and PRRSV nucleic acid present in sample.

A) Nasal swab (undiluted)



B) Serum (undiluted)

To assess the performance of the PRRSV Chip in a clinical veterinary setting, we examined serum and nasal swab samples collected from a pig infected with Chinese highly pathogenic PRRSV (HP-PRRSV) strain rJXwn06 and a bacterial cocktail consisting of *Streptococcus suis*, *Haemophilus parasuis*, and *Actinobacillus suis*. We specifically chose to evaluate nasal swab and serum samples because they are easy to collect in that they do

not require extensive training and can be collected antemortem, allowing multiple samples to be drawn from a single animal, making excellent choices for use in disease surveillance programs. cDNA generated from RNA isolated from serum and nasal swab samples collected from this pig was randomly amplified, labeled, and hybridized to the Virochip. The PRRSV Chip successfully detected PRRSV nucleic acid present in both samples (Figure 2A and B).

IX. Discussion:

Respiratory disease in pigs is the most important health concern for swine producers today and porcine reproductive and respiratory syndrome virus (PRRSV) is the most economically significant disease affecting the pork industry worldwide. With a mutation rate approximately thirty times higher than human immunodeficiency virus, PRRSV has the highest mutation rate of any known virus. Due to its high mutation rate and degree of genetic variability, there is a substantial need for improved diagnostic and surveillance tools to rapidly identify different and multiple PRRSV strains in swine samples and track genomic changes in field isolates of PRRSV. PCR-based assays are currently the gold standard for specific diagnosis of porcine respiratory disease complex (PRDC) pathogens, including PRRSV, due to the rapid and sensitive results they can provide. Despite high levels of sensitivity, major limitations exist in using PCR-based assays for pathogen identification. For example, PCR-based assays often fail to successfully detect more than one pathogen due to high failure rates associated with multiplex assays. PCR-based assays fail to provide complete subtype information, which may provide important information for vaccine and surveillance efforts. False negatives can occur when using PCR-based assays due to multiple pathogens being present in a single sample and, more notably, to mutations at primer binding sites, which is an issue for PRDC pathogens such as swine influenza virus and PRRSV that evolve rapidly and undergo reassortment/recombination at high frequency. An additional limitation in current surveillance strategies is time. Current identification and surveillance practices for PRRSV involves a selected few U.S. veterinary diagnostic laboratories performing quantitative RT-PCR and ORF5 nucleotide sequencing, followed by restriction fragment length polymorphism prediction, a process that may take two or more weeks. This extensive turnaround time highlights the substantial need for faster surveillance techniques coupled with next generation detection methods to rapidly identify, differentiate and monitor genomic changes in PRRSV for animal health preparedness for future outbreaks. Additionally, recent work on Asian highly pathogenic PRRSV (1) has shown that these viral strains are indeed a threat to our nations pork industry, and existing diagnostics are inadequate for prompt identification of these Asian strains.

In this report we describe a novel microarray platform referred to as a PRRSV Chip. The PRRSV Chip is designed to rapidly and specifically identify and differentiate all known strains of PRRSV, including novel or emerging isolates encoding a high degree of genetic variability compared to known isolates. Because this microarray platform emphasizes multiple key regions encompassing the entire PRRSV genome it has increased sensitivity and the ability to track genetic variability. Our results indicate that this platform successfully identified and distinguished the genetic variability of four genetically divergent PRRSV isolates in cell culture samples. Additionally, the limit of detection by quantitative RT-PCR for these samples is approximately the 10^-6 dilution and the limit of detection for our novel PRRSV microarray is approximately 10^-7 to 10^-9 dilution. This data demonstrates that the PRRSV microarray has a 10 to 1000 times improved sensitivity compared to currently deployed quantitative RT-PCR. We have also demonstrated this array platform

successfully identified and differentiated PRRSV using antemortem clinical samples (nasal swab and serum) collected from a pig infected with a Chinese highly pathogenic PRRSV isolate and bacterial cocktail consisting of *Streptococcus suis*, *Haemophilus parasuis*, and *Actinobacillus suis*. This data demonstrates that the PRRSV microarray is a sensitive and specific tool that is able to quickly identify novel or emerging strains of PRRSV in clinical samples containing multiple swine pathogens.

The most successful DNA microarray used for pathogen surveillance and detection is the "Virochip", a panviral DNA microarray designed by DeRisi and colleagues. The Virochip has been used to indentify SARS, a xenotropic murine leukemia virus-related (a novel retrovirus) from patients with familial prostate cancer, and a novel clade of human rhinoviruses (2-4, 6, 8, 9). It has additionally proven to be successful in a clinical veterinary setting by successfully identifying a novel coronovirus from a Beluga Whale and by identifying foot-and-mouth disease virus (FMDV) in ticks collected from a livestock market in Nairobi Kenya (6). We recently tested the Virochip platform on a set of clinical veterinary specimens and found that it while could detect a number of swine viruses, it failed to detect known viruses in several clinical samples (5). This failure is mainly due to the intended use of the Virochip, which is designed for viral discovery not detection or diagnostics, or surveillance.

Our novel PRRSV microarray platform (PRRSV Chip) differs from the Virochip in two main aspects: sensitivity and genome coverage. The Virochip contains only five probes targeting a region of the PRRSV genome that is present in significantly less quantities during infection than other regions of the genome, such as the nucleocapsid gene. Our novel array platform contains 1500 probes targeting multiple regions throughout the PRRS virus genome and specifically emphasizes regions of the genome that are known to be present in greater quantities during infection, especially in easily acquired clinical samples such as serum. As a result, the PRRSV Chip has a sensitivity greater than quantitative RT-PCR. An even greater improvement afforded by our novel PRRSV microarray over the Virochip is the increased genome coverage. The 1500 probes present in our PRRSV microarray target multiple regions throughout the entire genome and specifically emphasize regions that are known to contain the highest degree of conservation as well as regions that are known to contain the most sequence diversity (thus simultaneously allowing considerable capacity for genetic characterization of the virus strain detected). This comprehensive coverage over the entire PRRSV genome allows our PRRSV Chip to not only verify the existence of PRRSV in a clinical sample, but more importantly, track genomic changes occurring in circulating field isolates for surveillance purposes. The only other existing method that can provide similar information is whole genome sequence analysis, which is highly complex, laborious, time consuming and too costly for routine veterinary diagnostic laboratory usage. Further work is continuing to clearly define the limit of detection for the PRRSV Chip and to determine the minimum number of oligonucleotide probes needed to successfully identify and differentiate PRRSV isolates.

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