I. PROJECT TITLE: *"PRDC Pathogen Identification by Genome Microarray"*

NPB #01-069

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II. Abstract

Porcine respiratory disease complex involves several pathogenic agents. The diagnostic tests for many of these reagents often take several weeks to complete and often are of low sensitivity. This report describes our efforts to improve the differential diagnostic capability by utilization of a recently described technique, genomic microarray hybridization.

III. Introduction

Porcine respiratory disease complex, a major concern for pork producers, is characterized by slow growth, anorexia, cough, and dyspnea and results in decreased swine productivity. The disease complex most commonly consists of three pathogenic agents - porcine reproductive and respiratory disease syndrome virus (PRRSV), swine influenza virus, and Mycoplasma hyopneumoniae. However, other agents, such as Actinobacillus pleuropneumoniae, Actinobacillus suis, Pasteurella multocida, Streptococcus suis, Bordetella bronchiseptica and Haemophilus parasuis, as well as circovirus, may add to the complexity of this disease syndrome. The diagnostic tests for many of these agents are inadequate, insensitive, have high background, or are too costly or difficult for use in a diagnostic setting. A key component to understanding this complex is to develop better diagnostic tools with which to identify agents involved with specific respiratory disease states. A recent advancement in biotechnology, termed microarray analysis, has allowed nucleic acid hybridization to be completed using extremely small amounts of target DNA (pathogen) and amplified clinical sample RNA. We proposed to complete the first PRDC microarray and analyze its effective use in a diagnostic setting.

IV. Objectives

1. To produce target DNA fragments corresponding to all major swine respiratory pathogens and produce microarray hybridization slides that contain a pattern of all of the amplified targets.
2. RT-PCR amplify RNA and DNA in serum, lung fluid and nasal turbinate samples using biotinylated random primers.
3. Hybridize the amplified sample DNA to the fixed target DNA, wash with a strepavidin-labelled fluorometric probe and quantify resulting signal.
4. Determine specificity and sensitivity of the results and compare to our current PCR diagnostic tests.

V. Procedures

1. To produce target DNA fragments corresponding to all major swine respiratory pathogens and produce microarray hybridization slides that contain a pattern of all of the amplified targets.

As detailed in the 6 month progress report, all pathogen specific genome sequences were isolated, amplified, verified by agarose electrophoresis and arrayed on GAPS II coated slides (Corning Life Sciences) using a Biorobotic arrayer. All products are listed in the results section (Table I). The use of GAPS II coated slides circumvented variable and time consuming slide preparation.

2. RT-PCR amplify RNA and DNA in serum, lung fluid and nasal turbinate samples using biotinylated random primers.

The extraction and isolation of genomic material from lung tissue, nasal turbinate and serum was completed for both known clinical samples and for controls. We utilized standard methods. We deviated from the use of biotinylated random primers, and opted instead for a more advanced protocol of downstream hybridization.

Clinical samples that had been previously diagnosed by routine procedures were identified by Dr. Marie Gramer and frozen at -70˚C in phosphate buffered saline (PBS) or RNA later. Negative controls included PBS only or negative lung tissue from swine. Positive controls were the cloned DNA templates, purified PRRSV, FluA, and bacterial isolates acquired from several sources. For isolation of viral RNA from PRRSV and samples, we utilized the QIAamp Viral RNA Purification Kit from Qiagen. DNA was extracted from another aliquot of sample by Applied Biosystems' PrepMan Ultra Sample Preparation Reagent. After the DNA of several pathogens were isolated, multiplex PCR was used to incorporate a T7 promoter sequence into agent specific genomic regions. *In vitro* transcription then was completed on the amplified DNA to generate pathogen specific RNA. RNA from both procedures were combined and fluorometrically labeled using the Amino Allyl cDNA Labeling Kit (Ambion) in the presence of Cy3-dNTP vs. Cy5-dNTP, processed and utilized for hybridization.

3. *Hybridize the amplified sample DNA to the fixed target DNA, wash with a labeled fluorometric probe and quantify resulting signal.*

GAPS II slides were hybridized according to the manufacturer's protocol. The arrays were pre-hybridized by first incubating for 60 minutes at 42˚C in 50% formamide, 5X SSC, 0.1% SDS, 0.1 mg/mL BSA in a Coplin jar. After incubation, arrays were washed in water and rinsed in isopropanol until the SDS was completely removed. The arrays were then dried with compressed air. To hybridize the fluorescent-labeled cDNA probe to the DNA templates on the microarray, the probes were prepared in fresh hybridization solution consisting of 50% formamide, 5X SSC, 0.1% SDS, and 0.1 mg/mL salmon sperm DNA as a nucleic acid blocker. The probe solution was incubated at 95˚C for 5 min. After incubation, the probe was centrifuged for 2 min to collect condensation and the sample was allowed to cool to room temperature. The microarray slide and

coverslips were inspected for cleanliness, then placed into a hybridization chamber.The prepared, cooled probe was pipetted onto the surface of the printed side of the slide and under the coverslip under the coverslip. The hybridization chamber was sealed and submerged in a 42˚C water bath overnight.After hybridization, the coverslips were removed from the slide by immersing in 2X SSC, 0.1% SDS (at 42˚C) until the coverslip moved freely away from the slide. The arrays were washed in 2X SSC, 0.1% SDS for 5 min at 42˚C. This was followed by soaking in 0.1X SSC, 0.1% SDS for 10 min at room temperature, then in 0.1X SSC for 1 min at room temperature, repeating these soaking steps 4 times. Finally, the array was rinsed in 0.01X SSC for up to 10 seconds and dried with compressed air. The completed microarray was analyzed using Scanarray 3000 and provided software.

4. ***Determine specificity and sensitivity of the results and compare to our current PCR diagnostic tests.***

Due to several unsuccessful attempts at sample labeling and downstream hybridization, we were unable to complete this aim. We provide evidence in the result section that the microarray will eventually prove beneficial.

VI. Results

1. To produce target DNA fragments corresponding to all major swine respiratory pathogens and produce microarray hybridization slides that contain a pattern of all of the amplified targets. Additional products will be added in later versions of the microarray.

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Table 1: PCR Products for Use As Targets for Genome Array

Pathogen	Target Gene	Nucleotide Length
PRRSV - EuroPRRSV	NSP2	204
PRRSV - EuroPRRSV	NSP2	204
PRRSV - EuroPRRSV	NSP9	202
PRRSV - EuroPRRSV	ORF5	220
PRRSV - JA142	NSP2	273
PRRSV - JA142	NSP2	273
PRRSV - JA142	NSP9	215
PRRSV - JA142	NSP9	215
PRRSV - JA142	ORF5	209
PRRSV - LV	NSP2	255
PRRSV - LV	NSP2	255

Table 1: PCR Products for Use As Targets for Genome Array

Pathogen	Target Gene	Nucleotide Length
PRRSV - LV	NSP9	202
PRRSV - LV	ORF5	220
PRRSV - PrimePac	NSP2	273
PRRSV - PrimePac	NSP2	273
PRRSV - PrimePac	NSP9	215
PRRSV - PrimePac	NSP9	215
PRRSV - PrimePac	ORF5	209
PRRSV - VR-2332	NSP2	273
PRRSV - VR-2332	NSP9	215
PRRSV - VR-2332	NSP9	215
PRRSV - VR-2332	ORF5	209
AV/Sw IV	HA4	115
AV/Sw IV	NA6	119
Swine IV, isolate 11	NP	73
Swine IV, isolate 12	NP	73
Swine IV, isolate 9	NP	73
Swine IV, H1N1	HA1	75
Swine IV, H3N2	HA3	68
Swine IV, H1N1	NA1	69
Swine IV, H3N2	NA2	74
Human IV, isolate 1	HA1	115
Human IV, isolate 1	NA1	111
Human IV, isolate 2	HA1	115
Human IV, isolate 2	NA1	111
Human IV, isolate 3	HA3	160
Human IV, isolate 4	HA3	160
Human IV, isolate 4	NA2	104

Table 1: PCR Products for Use As Targets for Genome Array

Pathogen	Target Gene	Nucleotide Length
Human IV, isolate 5	HA3	160
Human IV, isolate 5	NA2	104
Circovirus I + II, Universal	Replicase	150
Circovirus II, clone 1	Replicase	240
Circovirus II, clone 2	Replicase	240
Porcine Parvovirus	VP2	122
Actinobacillus pleuropneumoniae, Type 1	ApxIVA	377
Actinobacillus pleuropneumoniae, Type 5	ApxIVA	377
Actinobacillus pleuropneumoniae, Type 7	ApxIVA	377
Bordetella bronchiseptica, clone 1	5'UTR of flagellin	300
Bordetella bronchiseptica, clone 2	5'UTR of flagellin	300
Haemophilus parasuis	16S small subunit RNA	100
Leptospira species, clone 1		295
Leptospira species, clone 2		295
Mycoplasma hyopneumoniae, clone 1	MHYP	104
Mycoplasma hyopneumoniae, clone 2	MHYP	104
Pasteurella multocida, clone 1	ToxA	1-1
Pasteurella multocida, clone 2	ToxA	1-1
Streptococcus suis, types 1+14	cps	41
Streptococcus suis, type 2+1/2	cps	674
Streptococcus suis, type 2+1/2	cps	674
Streptococcus suis, type 7	cps	252
Streptococcus suis, type 9	cps	388

2. RT-PCR or PCR amplify RNA and DNA in serum, lung fluid and nasal turbinate samples.

A sample of DNA extraction and following PCR amplification is shown in Figure 1. For the assay itself, all pathogen-specific DNA primers were added to the purified sample DNA and subjected to PCR.

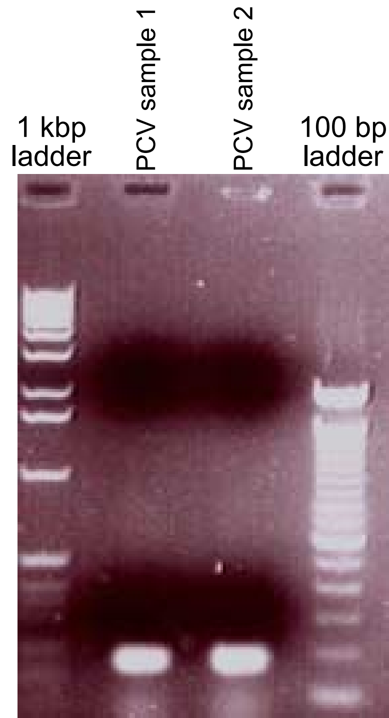
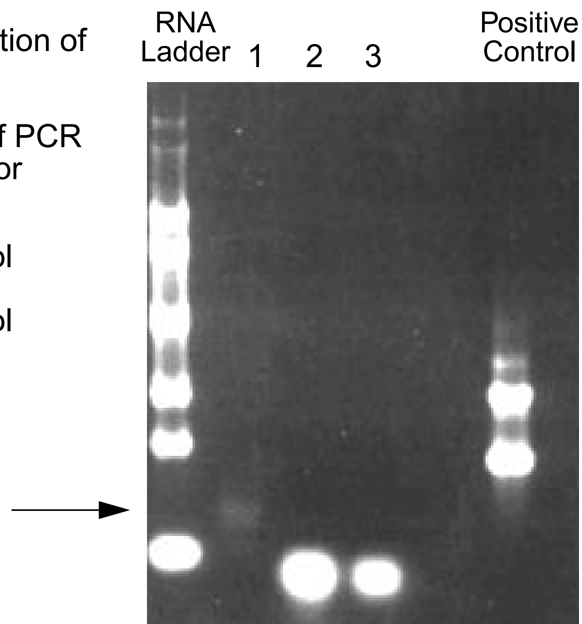


Figure 1. Purification and amplification of Porcine Circovirus Type II

Following modest PCR amplification, the products were *in vitro* transcribed, as shown in Figure 2.

Figure 2. *In vitro* transcription of multiplexed PCR.

1. Multiplex transcription of PCR amplified sample DNA for P. multocida
2. P. multocida DNA control
3. P. multocida DNA control
4. *In vitro* transcription of Kit supplied control



3. Hybridize the amplified sample DNA to the fixed target DNA, wash with a labeled fluorometric probe and quantify resulting signal.

We encountered many problems with adequately amplifying target pathogens from the samples, and in optimally labeling the RNA products. Preliminary results suggest we are making headway, as a PRRSV-NA yielded a fluorescent positive spot to PRRSV VR2332.

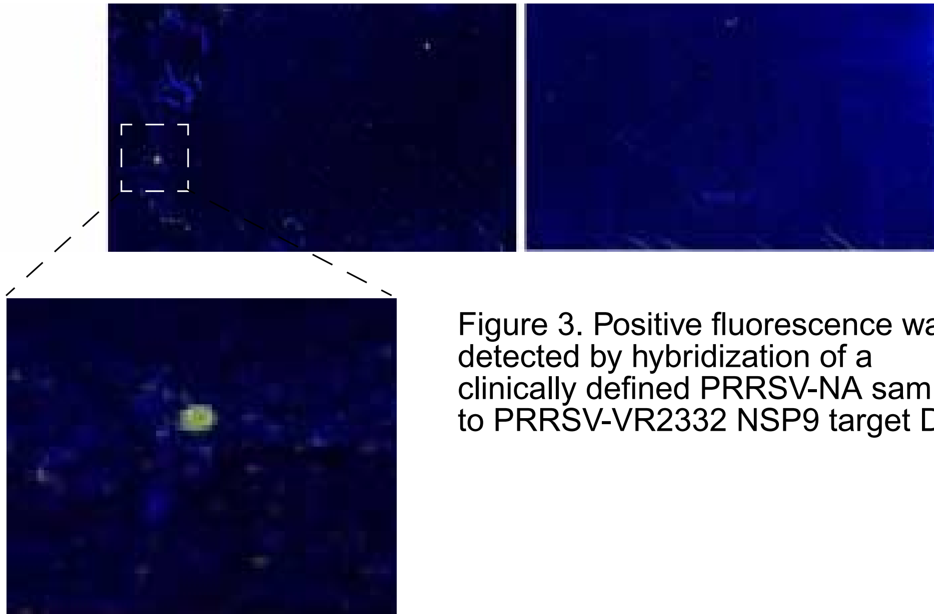


Figure 3. Positive fluorescence was detected by hybridization of a clinically defined PRRSV-NA sample to PRRSV-VR2332 NSP9 target DNA

Summary: The work presented describes the development to date of a novel procedure for detection of all PRDC pathogens. When completed, with BRDC funding, the PRDC microarray should be able to simultaneously detect all agents of this syndrome, and will aid in studies of PRRSV recombination and evolution, and FluA transmission between and within herds.

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