RESEARCH REPORT
SWINE HEALTH

Title: Serum Markers of PRRSV Infection **#04-123**

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Abstract

Detection of PRRS by serological screening for antibodies is simple, reliable, and of benefit to the swine industry. However, there is a delay of one to two weeks between onset of infection and ELISA antibody response. During this time transmission can occur, leading to outbreaks. ELISA tests also are ineffective in differentiating acute from persistent infection and infected from vaccinated animals. The response of animals to infectious challenge includes changes in the composition of proteins in blood and serum. We hypothesized that PRRSV infection will produce a characteristic profile of protein differences in serum that is an early diagnostic signature of infection detectable by mass spectrometry (MS). Early identification of serum protein changes that indicate PRRS infection status, would have great value for monitoring the health of individual pigs and herds. We have identified a protein of 9244 molecular weight that is associated with PRRSV infection and is present as early as one day after infection.

Monitoring PRRS infection in herds or individual pigs by presence of antibodies in serum misses the first critical 1-2 weeks of acute infection. Early detection would facilitate more rapid interventions to limit the spread of infection, thus improving herd health and productivity.

We hypothesize that the PRRS infection status of pigs can be determined by examination of protein profiles in serum because the response to infection involves an immediate local and global mobilization of the immune system and major organ systems. Proteins involved in alarm responses, host defense, and intracellular signaling travel through the blood and can be analyzed in samples of serum. The composition of proteins in blood and serum change rapidly and are specific for individual disease conditions.

We expect that PRRSV will produce a characteristic signature profile of protein changes in serum that may differentiate acute and persistent infection, vaccination and field infection, and level of virulence. The proposed research will establish the feasibility of serum profiling for PRRS, and identify candidate proteins for which specific diagnostic and monitoring tests can be developed. The proposed studies would also establish the value of serum profiling for health and disease assessment for other infectious and noninfectious conditions, including environmental stress, that impact on animal health and productivity.

Objectives

Objective 1. To determine the serum protein profile of PRRSV noninfected, acutely infected and persistently infected pigs.

Research Question 1a. What is the total protein profile of swine serum?

Research Question 1b. What is the effect of PRRSV infection on the protein profile?

Objective 2. To determine the effect of removal of the abundant protein fraction on differentiation of infected and noninfected serum.

Research Question 2a. What is the effect of albumin removal on PRRS-positive and PRRS-negative serum profiles?

Research Question 2b. What is the effect of high-molecular weight protein removal on PRRS-positive and PRRS-negative serum profiles?

Objective 3. To identify specific proteins in signature profiles that characterize the PRRS status of a pig.

Research Question 3a. What are the amino acid sequence identities of peaks in a protein profile that are diagnostic for PRRS infection?

Research Question 3b. Do antibodies raised to diagnostic proteins identify PRRS-positive pigs in an ELISA format?

Materials & Methods

Serum samples were obtained from pigs belonging to different age groups that were infected with different isolates of PRRSV as shown in Table 1.

Table 1. Sources of serum used in the study.

Isolates	Pig Age	Day after infection	No. of Pigs	
			Infected	Controls
VR2332	2-3 weeks	0, 2, 7	4	2
MN30100	16 weeks	1, 3	4	1
MN184	6 weeks	0, 1, 5	5	1
JA142	3, 15 weeks	0, 1, 3	5	5
ATP	3, 15 weeks	0, 1, 3	4	4

Serum samples were also collected from pigs infected or inoculated with *Salmonella sp* (n=5), *Lawsonia intracellularis* (n=5), porcine circovirus 2 (n=3), swine influenza virus (5), *Mycoplasma hyopneumoniae* (2) and keyhole limpet hemocyanin (n= 4). Serum samples (10µl) were diluted 5-fold with normal saline. Acetonitrile was added to the final concentration of 66.7%. Then samples were incubated at the room temperature for 30 minutes and spun at 13,200 rpm for 10 minutes to pellet the precipitate. Supernatants were collected and dried in Speed-Vac concentrator. Dried protein was rehydrated with 10ul of autoclaved water.

Acetonitrile-treated samples were desalted by C4 resin-loaded Zip-Tip column. Ten µl of the rehydrated sample was drawn and expelled through the Zip-Tip repeatedly for a minute (at least 10 times). The Zip-Tip was washed by 10ul of wash solution (0.1% TFA) for seven times. Absorbed proteins were eluted in 1.6 µl of 75% acetonitrile containing 0.1% trifluoroacetic acid. The elution was spotted on the MALDI target and mixed with sinapinic acid (matrix) in 1:1 ratio.

Samples were run on a Biflex III (Bruker Daltonics, Billerica, MA) MALDI-TOF (matrix-assisted laser desorption/ionisation-time of flight) mass spectrometer (MS) operated in linear mode. The mass spectrometer was calibrated externally with the +1 and +2 charged states of Cytochrome C. Then the spectra were calibrated internally with consistent protein peaks of m/z values 8330 and 4165. The spectra were analyzed by Bruker XTOF processing software. Peaks within the mass range 4kD – 15kD were analyzed.

Processed sera were pooled and resolved by SDS-PAGE to fractionate the serum proteins based on their molecular mass. Essentially, the pooled processed samples were run on 16.5% Tris-Tricine pre-cast criterion gels (Biorad) and protein bands were visualized by staining the gel with deep purple stain (Amersham).

In-gel tryptic digestion was performed on resolved protein bands in the SDS-PAGE. Digested peptide mixtures were loaded and analyzed on LTQ LC-MS/MS instrument (Thermo Electron corporation). Initially, the peptides were fractionated on Liquid Chromatography (LC) using an acetonitrile gradient. Then each fraction was analyzed by MS scan to identify peptides. Three predominant peptides from each fraction were further fragmented into small peptide ions and mass will be calculated at the second step MS. The mass values obtained through the second MS were subjected to a BLAST search with theoretical masses of peptides available in the *Sus scrofa* Unigene data base (31,550 entries) by using the Bioworks software (Applied Biosystem).

Results

The low molecular weight protein profile in pig sera was detected by mass spectrometry. The comparative analysis of spectra from PRRSV infected and non infected pig sera were reproducible and revealed that a

protein with m/z ratio 9244±2 was found in most of the PRRSV-infected pigs This protein peak was rarely observed in the specific pathogen free animals. (Figures1 and 2)

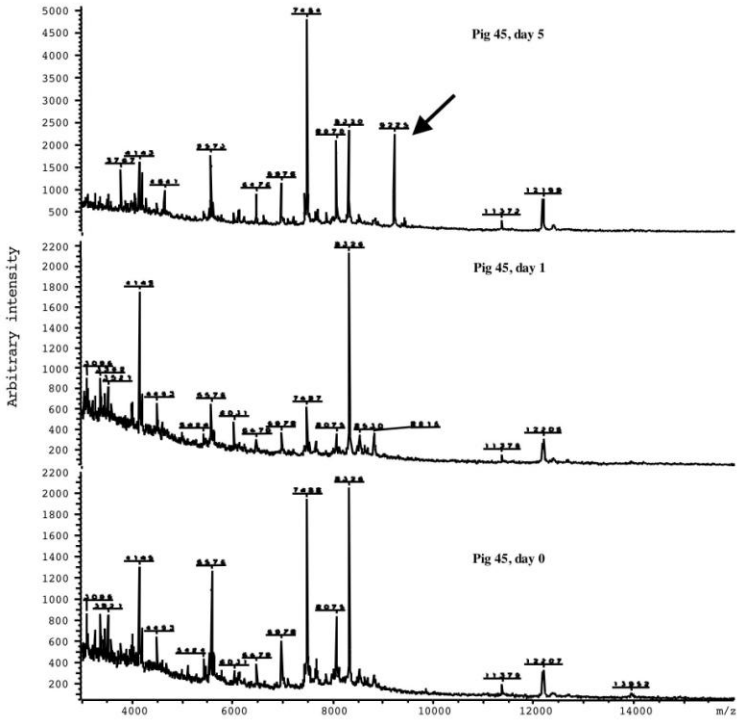
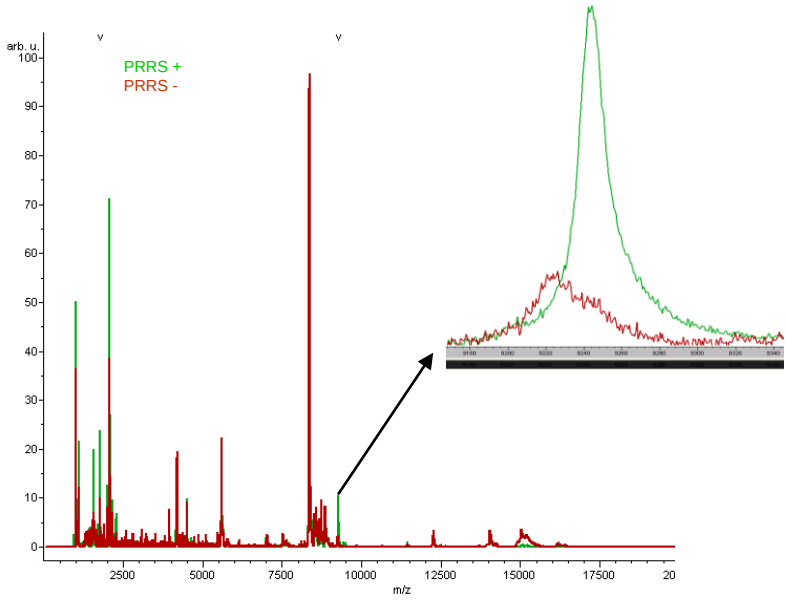


Figure 1. The MALDI-TOF profile of acetonitrile-extracted peptides present in the serum of pig #45 at days 0, 1, and 5 after PRRSV infection. The arrow indicates a novel peak at 9236±13 m/z at 5 days of infection. M/z is mass to charge ratio. The charge is 1 since there is no other peak at 0.5 or 2-fold.

Figure 2. Average mass spectra of PRRSV-infected and non-PRRSV infected pig sera.



Based on these findings, a protein with a m/z value 9244±2 was a potential disease marker for PRRS. Twenty-two serum samples from PRRSV inoculated pigs and 27 PRRSV-negative control samples were analyzed to detect the presence of the potential marker (Table 2). In PRRSV infected animals 9244±2 D protein appeared within 7 days after infection.

Table 2. Presence or absence of the 9244±2 D (test +) protein in pig sera.

	Test positives		Test negatives		Total
Isolates	Infected	Non infected	Infected	Non infected	
VR 2332	3	1	1	5	10
MN 30100	4	0	0	1	5
MN 184	5	0	0	6	11
JA142	4	1	0	5	10
ATP	4	0	1	8	13
Total	20	2	2	25	49

Considering the presence of 9244±2 D protein as a disease diagnostic marker for PRRS data in

Table 1 was further summarized in a 2 x 2 table (Table 2). According to the 2 x 2 table use of 9244±2 D protein as a diagnostic marker for acute PRRSV infection gave a sensitivity of 0.91 and specificity of 0.92.

Table 3. Presence of the 9244±2 D protein in pig sera as PRRS diagnostic marker.

	Infected	Controls	Total
Test +	20	2	22
Test -	2	25	27
Total	22	27	49

To confirm the specificity of 9244±2 D protein for PRRS in pig additional 24 serum samples from PRRS free pigs infected or inoculate with other pathogens and keyhole limpet hemocyanin were tested. The aggregate results from all serum samples are shown in Table 4. The test sensitivity was 0.91 and the specificity was 0.80.

Table 4. Presence of the 9244±2 D protein in pig sera as a differential PRRS specific diagnostic marker.

	Infected	Controls	Total
Test +	20	10	30
Test -	2	41	43
Total	22	51	73

The preceding results were obtained from serum extracted with acetonitrile as described in Methods & Materials. The method was highly effective in removing high molecular weight proteins from the sample (Figure 3). Other methods of fractionating serum were proposed, but not pursued. Consultation with colleagues

and examination of the literature indicated that no other method gave reproducible, and quantitative results, or was as effective.

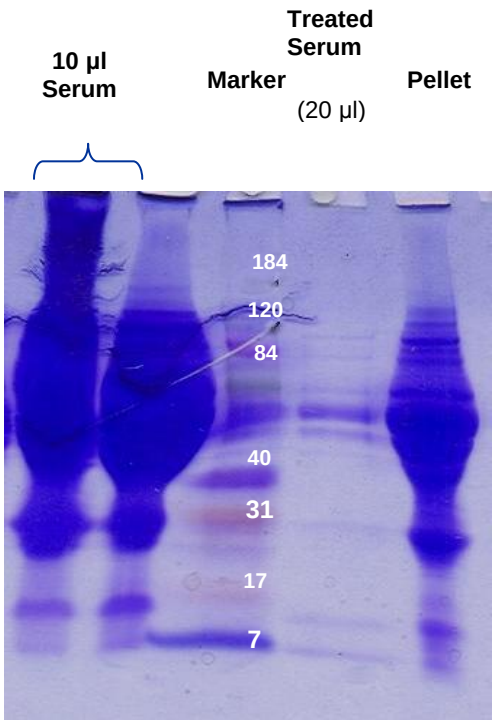


Figure. 3. Acetonitrile removes the highly abundant proteins from serum.

To identify of 9244 MW protein of interest, acetonitrile-extracted serum was fractionated by one dimensional electrophoresis. At least six protein bands within 7-17 D were resolved successfully (Figure 4A). Further to precisely identify proteins which gave signals in MS spectra one dimensional electrophoresis was performed on 20 pooled acetonitrile-treated and zip tipped sera (Figure 4B).

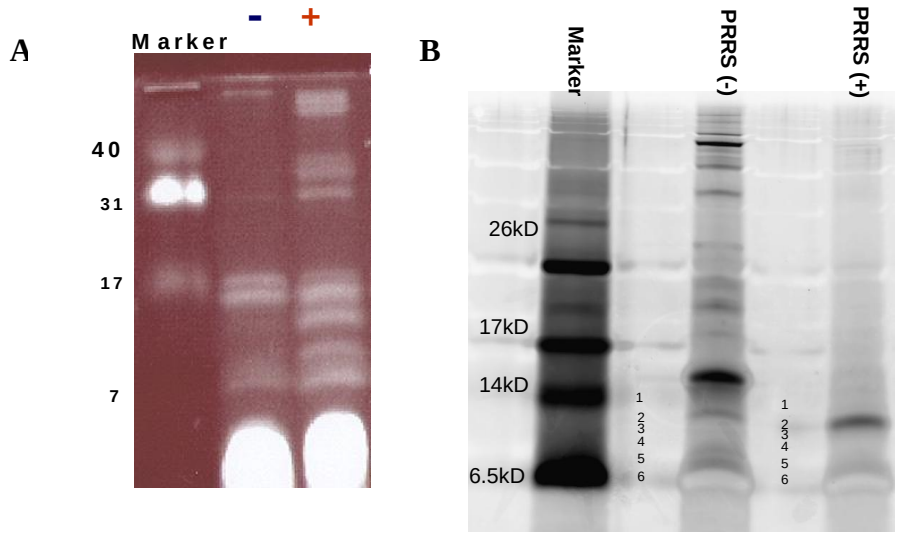


Figure 4. Electrophoretic fractionation of acetonitrile-extracted serum. (A) PRRS infection reveals two candidate bands in the range of 7-17 kD. (B) Acetonitrilettreated and zip-tipped pig serum.

The proteins resolved in one dimensional PAGE in Figure 4B (tiny numbers) were excised and subjected to tryptic digestion. The digested peptide mixture was analyzed by LC/MS/MS. Among the six bands analyzed, band number 2 was identified as the αS1 subunit of haptoglobin. The molecular weight of the αS1 subunit of haptoglobin is 9246 D. This finding strongly suggests that the protein with m/z 9244±2 is the αS1 subunit of haptoglobin. We are currently working on the confirmation of this protein by western blot with anti-porcine haptoglobin antibody and N terminal sequencing.

Discussion

Our preliminary results suggest that the identified protein with 9244±2 D molecular weight is associated with the acute PRRSV infection in pigs. The presence and kinetics of this protein in chronic and persist PRRSV infection is not yet resolved. But, the appearance of 9244±2 D protein as early as day one of PRRSV infection indicate that this protein could be used as a potential early diagnostic marker for PRRSV infection.

Further analysis of porcine serum proteins through one dimensional gel electrophoresis coupled with LC/MS/MS revealed that the 9244±2 D protein most likely is the αS1 subunit of haptoglobin. Haptoglobin is an acute phase protein previously examined with respect to health and growth in swine. However, fragments of

haptoglobin have not been described previously in swine. We are currently working on the confirmation of this protein by western blot with anti-porcine haptoglobin antibody and N terminal amino acid sequencing. Since the sequence of pig genome is not complete it is also possible that the 9244 D protein might be a novel protein.

Lay Interpretation

Early detection of infectious disease is crucial for preventing the spread of the infectious agent. In PRRS, current diagnostic methods mainly use the antibody response of individual pigs or herd, which requires at least 7-10 days. Some biological molecules appear in the serum at very early stages of infection and may be useful diagnostically. Our project explored the possibility of identifying such a biomarker for PRRSV infection. We obtained reproducible profiles of low molecular weigh proteins, including one associated with early PRRSV infection, that are present in the serum of pigs. Now we are in the process of fully characterizing the PRRS-associated protein.