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Genes Expressed in Response to PRRSV

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Summary and Implications

Gene maps of livestock are rapidly being developed and have led to an explosion of knowledge in recent years about genes affecting economic traits. One potential application of this information that would have major economic value is in selection of livestock for resistance to disease. Even though much has been learned about Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) since it was first identified, PRRSV continues to cause significant economic losses in many herds. Traditional approaches to manage PRRSV can be effective, but may be costly and have not always resulted in permanent control. This is a disease for which application of molecular genetic knowledge to select for resistance would have significant economic advantages. An experiment was initiated at Nebraska to investigate possible genetic variation among pigs in response to PRRSV. Pigs from two populations were infected with PRRSV, responses over 14 days were recorded, and tissues were collected at necropsy for gene expression studies. Phenotypic data, including body weights and rectal temperatures, viremia, and lung lesion scores, provided substantial evidence that genetic variation in response to PRRSV exists. With that knowledge, we developed an index of high (H) and low (L) responders, indicating susceptible and resistant phenotypes, and measured expression differences in lung and bronchial lymph node of 11 immune function genes between H and L pigs. Ten of these genes, involving both innate and acquired immune function, were expressed differently in lung and/or lymph tissue. They tended to be up-regulated (expressed at greater levels) in H pigs. We demonstrated that genetic variation in response to PRRSV exists and that both innate and acquired genes are involved. We have not yet determined whether selection for the immune function genes involved or levels of the proteins they produce will be effective in selecting for PRRSV resistance. Results will be helpful in additional investigations aimed at developing methods to select for resistance to PRRSV.

Introduction

The National Pork Board has estimated that disease due to the Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) causes annual losses of approximately $560 million to U.S. pork producers. To combat PRRSV, the Coordinated Agricultural Program (CAP) grant awarded to researchers in the industry by USDA National Research Initiative (NRI), a major, multi-disciplinary initiative to eradicate PRRSV, was implemented.

One objective of the NRI CAP grant is to investigate the possibility of genetically modifying the pig as a means to control PRRSV. The hypothesis is that genetic variation in response to PRRSV exists, that the genes responsible for this variation can be identified, and that selection on these genes will be effective in developing lines of pigs resistant to PRRSV. An experiment was initiated at the University of Nebraska to test this hypothesis. The objectives were 1) to determine whether genetic variation exists, 2) to identify traits that differentiate animals that respond differently to PRRSV, 3) to build the phenotypic and genotypic records to quantify genetic variation between animals and 4) to identify the genes involved in the response.

Nebraska PRRSV Infection Experiment

The description of the experiment and biological responses relating to objectives 1 to 3 were reported in the *2004 Nebraska Swine Report.* An overview is included here.

Pigs of each of two populations were infected with PRRSV and their phenotypic responses over a 14-day period were recorded. A total of 200 pigs from the Nebraska Index line (I), a Large White-Landrace composite population that has been selected for increased litter size for 20 generations, and 200 pigs from a cross of Hampshire and Duroc lines (HD) that have been selected for rate and efficiency of lean growth were used. Line I pigs were born in the University of Nebraska swine research herd, whereas HD pigs were obtained from a commercial farm. Neither farm had experienced disease from PRRSV and pigs from both herds had tested negative for the presence of PRRSV by PCR methods and for PRRSV serum antibodies by the ELISA test. Pigs represented a total of 83 sires and 163 dams.

Pigs were transported at an average age of 23 days to the University of Nebraska Veterinary and Biomedical Sciences (VBS) Animal Research Facility and placed in environmentally controlled rooms, two pens per room, and 12 to 13 pigs per pen. One room was randomly assigned for treatment and the pigs were inoculated intranasally with 2 ml (1 ml per nostril) of 10^5 $CCID_{50}$ (cell culture infectious dose 50% per ml) of PRRSV strain 97-7985 supplied by Dr. Fernando Osorio. Pigs in the other isolated room were littermates to the infected pigs and served as controls. Data were collected on days 0, 4, 7 and 14 post-inoculation. Pigs

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were killed at day14, necropsy was performed, and samples of several tissues were colleted for gene expression experiments.

Phenotypic Traits Measured

Body temperature by rectal probe, body weight, and blood draws were collected and recorded just before inoculation (day 0) and 4, 7, and 14 days after inoculation. At necropsy, samples of lung, bronchial lymph node, and spleen were collected and stored at -80ºC. Viremia ($CCID_{50}$/ml), a measure of each pig's ability to replicate the virus, was measured in serum collected at days 4, 7, and 14, and in lung and bronchial lymph node collected at necropsy. An ELISA test was conducted on serum samples collected at d14 to determine the level of PPRSV antibody in infected pigs and to test for possible cross contamination in uninfected pigs.

Lungs were scored for the presence of pneumonia (yes (1) or no (0)). Sections of lung were examined by light microscopy and scored on a scale of 1 to 3. Lungs receiving a score of 1 had no lesions or had mild multifocal interstitial pneumonia. A score of 2 was given to lung sections that had moderate interstitial pneumonia involving less than 50% of the area of the section. A score of 3 was given to lung sections that had greater than 50% involvement of severe interstitial pneumonia. If present, lesions suggestive of *mycoplasma hyopneumonae* and infectious bacterial pneumonia were recorded.

Identifying resistant and susceptible pigs

Principal component (PC) analysis, a multivariate procedure, was used to identify pigs in the outermost tails of the distribution of response variables. This procedure combined all variables into an index in which traits were weighted according to their contribution to total variation. Traits included were viremia at 4, 7, and 14 days post-infection (dpi), weight change from 0 to 4, 4 to 7, and 7 to 14 dpi, rectal temperature change from 0 to 4, 4 to 7, and 7 to 14 dpi, lung and bronchial lymph node viremia, and severity of lung lesions. Pigs receiving high PC values had high viremia and high symptoms of PRRSV (H) and pigs with low PC values had low viremia and low symptoms (L). Based on the PC analysis, 7 H and 7 L pigs in each population and their uninfected littermates (total of 56 pigs) were used in a 2*2*2 factorial design that included class (pigs classed as H or L responders), line (I or HD), and treatment (infected or uninfected).

Gene expression analyses

RNA from the lung and bronchial lymph node (BLN) tissues of the 56 pigs was extracted and gene expression was evaluated with RT-PCR. Gene expression cycle thresholds (Ct), which are directly related to the initial amount of target DNA present, were recorded. A low Ct value means that the expression level of that gene was high because fewer PCR cycles were required to reach the threshold, and vice versa, a high Ct value means that the expression level was low because it took more cycles to reach the threshold. Thus, samples producing low Ct values had more cDNA, indicating greater expression of the gene, than those with high values.

Five genes with innate immune function, five genes with acquired immune function, one regulator gene, and a housekeeping gene to monitor experimental procedures were evaluated. Innate immunity is the basic resistance to disease that an animal possesses. It is the first line of defense against infection. Responses are immediate and broad-spectrum, without memory, so there is no lasting protective immunity. Acquired immunity on the other hand occurs in response to infection and is often called adaptive, as the immune system must adapt itself to previously unseen molecules. Responses are slower, but in the case of certain organisms, this type of immunity can be long-lasting as the individual may be immunized against certain organisms.

Genes involved in Innate Immunity

• Interferon alpha (IFNA) – induces an antiviral response or resistance to viral replication by binding to the interferon □/□ receptor. Once bound, IFNA helps activate the JAK-STAT pathway, which in turn induces the transcription of several genes. Genes activated by IFNA contribute to the inhibition of viral replication. The IFNA gene is on chromosome 4.

• Interleukin 1 beta (IL1B) - Produced by activated macrophages, IL-1 stimulates thymocyte proliferation by inducting IL-2 release, B-cell maturation and proliferation and fibroblast growth factor activity. The IL1B gene is on chromosome 3.

• Interleukin 6 (IL6) - is a cytokine with a wide variety of biological functions: it plays an essential role in the final differentiation of B-cells into IG-secreting cells; it induces myeloma and plasmacytoma growth, nerve cell differentiation in hepatocytes, and acute phase reactants. IL6 is located on chromosome 9.

• Interleukin 8 (IL8) - is a chemotactic factor that attracts neutrophils, basophils and T-cells but not monocytes. It is also involved in neutrophil activation and is released from several cell types in response to an inflammatory response. IL8 is located on chromosome 8.

• Colony stimulating factor 2 (CSF2) – named for their ability to induce the formation of distinct hematopoietic cell lines. CSF2 is located on chromosome 5.

Genes involved in Th1 (acquired) immune response

• Interferon gamma (IFNG) - produced by lymphocytes and activated by specific antigens or

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mitogens. IFN-gamma, in addition to having antiviral activity, has important immunoregulatory functions. It is a potent activator of macrophages, and has anti-proliferative effects on transformed cells and it can potentiate the antiviral and anti-tumor effects of the type I interferons. IFNG is located on chromosome 5.

• Interleukin 12 beta (IL12B) – functions to stimulate the synthesis of interferon-gamma by T-lymphocytes and NK cells; increases the killing activity of CTLs and NK cells; and stimulates the differentiation of naive T4-lymphocytes into interferon-gamma producing Th1 cells. It is produced mainly by macrophages and dendritic cells. IL12B is located on chromosome 5.

• Interleukin 15 (IL15) – stimulates NK cell proliferation and proliferation of T-lymphocytes. IL-15 is produced by various cells including macrophages. IL15 is located on chromosome 8.

• Signal transducer and activator of transcription 1-alpha (STAT1) – binds to phosphorylated tyrosine residues playing an essential role in the signaling pathways of a variety of cytokines. STAT1 is located on chromosome 15.

• Tumor necrosis factor (TNF) – it is a cytokine that binds to TNFRSF1A/TNFR1 and TNFRSF1B/TNFBR. It is mainly secreted by macrophages and can induce cell death of certain tumor cell lines. It is a potent pyrogen causing fever by direct action or by stimulation of interleukin 1 secretion and is implicated in the induction of cachexia. Under certain conditions it can stimulate cell proliferation and induce cell differentiation. TNF is located on chromosome 7.

Genes involved in the T-regulatory immune function

• Interleukin 10 (IL10) – stimulates proliferation of B cells, thymocytes, and mast cells. It also antagonizes generation of the Th1 subset of helper T cells so it is an inhibitor of activated macrophages and dendritic cells. As such, it regulates innate immunity and cell-mediated immunity. IL10 is located on chromosome 1.

Housekeeping gene

• Ribosomal protein L32 (RPL32) – ubiquitous protein found throughout the body. This gene is used to determine if protocols were working properly because all pigs should have approximately the same level of the protein, however levels may differ across tissues. RPL32 is found on several chromosomes.

Results

There was substantial evidence for genetic variation as pigs of the two populations had distinctly different responses to PRRSV (see the 2004 Nebraska Swine Report). With that evidence in hand, we developed the Principal Component Index to describe susceptible and resistant pigs. The seven highest and lowest responders in each population and their littermates were used to determine whether specific immune function genes were expressed differently in tissues of these pigs.

Table 1 contains the overall line means for individual traits and the means of the seven pigs in each line classified as susceptible (H, high viremia and high symptoms of PRRSV) or resistant (L, low viremia and low symptoms). All pigs replicated PRRSV as evidenced by elevated serum viremia values at day 4. Serum viremia of L class pigs in Line I dropped sharply at day 7 and declined to values near zero by day 14, whereas serum viremia of H pigs remained high throughout. In both lines, L pigs showed immediate symptoms of PRRSV with slightly decreased weight gain and increased temperature, but they recovered quickly and gained weight at near normal rates and had near normal temperatures from day 7 to 14, whereas H pigs had high temperatures, with the exception of temperature of HD pigs, from day 7-14, and low or negative weight gain during each period. The general nature of the response was similar in both lines, but the difference between H and L pigs was greater in I than in HD, suggesting resistant I pigs (the L class) had a stronger innate immune response to PRRSV than HD pigs. This difference in response between lines was supported by the gene expression data (see below).

Differences in gene expression levels in lung and bronchial lymph nodes between infected and uninfected pigs, between susceptible (H) and resistant (L) pigs that had been infected with PRRSV, and between uninfected littermates of H and L pigs are in Table 2. Gene expression is measured by the number of cycles to produce a predefined threshold level of cDNA, the complementary DNA of the RNA harvested from the tissue. Each cycle doubles the amount from the previous cycle. Therefore, differences of 1 Ct represent a two-fold difference in cDNA, differences of 2 represent a four-fold difference, etc.

The first contrast (I – UI) contrasts gene expression between infected (+) and uninfected (-) pigs. It shows which immune function genes were stimulated to produce RNA in response to PRRSV. Seven of the 11 genes studied responded in lung tissue ($P < 0.05$) and all were up-regulated as it took fewer Ct cycles to produce the threshold level of cDNA. Three genes (IL1B, IL8, and CSF2) are innate immunity genes, and four (IFNG, IL12B, STAT1, and TNF) are part of the acquired immune response. Each of these genes except IL1B also was up-regulated in bronchial lymph nodes. Three additional genes also were up-regulated in lymph tissue (INFA, IL10, and IL6). Therefore, every gene studied except IL15, including the regulator gene IL10, was expressed in greater amounts in either or both lung and lymph

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tissue in response to PRRSV.

The next contrasts (H - L) are between pigs classed as susceptible (H) and resistant (L). It was done both in infected (+) and in uninfected littermates (-) to determine whether resistance occurred because pigs had a greater capacity to respond to the virus (measured in infected pigs), or whether there was naturally greater expression of the gene even in the absence of virus (measured in uninfected littermates).

Susceptible pigs (H class) had greater expression of four genes in both lung and lymph. Two of these genes are involved in innate immunity (IL1B and IL8), INFG is involved with acquired immunity, and IL10 is a regulator gene. An additional acquired immunity gene, STAT1, had greater expression in lung. In all cases, resistant pigs (L class) had less expression of these genes. For two of these genes (INFG and IL10), uninfected littermates of resistant pigs also had lower expression levels than uninfected susceptible pigs. This suggests that the expression of these genes may be an inherent difference between H and L pigs that exists without the stimulus of PRRSV. There were no similar cases in lymph tissue. Therefore, most of the differences in gene expression between susceptible and resistant pigs probably occurred as a response to PRRSV.

Table 1. Overall line means and means for the seven high (H) and low (L) responders within each line based on Principal Component Index.

	Line I			Line HD		
Item	Overall[a]	H[b]	L[c]	Overall[a]	H[b]	L[c]
	Viremia, $\log_{10}$ $CCID_{50}$/ml					
Serum, day 4	4.17	4.39	4.11	4.54	5.11	3.10
Serum, day 7	3.91	4.47	3.20	4.40	5.13	3.64
Serum, day 14	3.00	4.49	0.50	3.59	5.29	2.51
Lung	3.96	5.07	2.40	4.45	4.71	4.21
Lymph	2.55	3.33	1.31	3.12	3.70	2.66
	Body weight change, lb					
Day 0 to 4	0.71	0.18	1.28	0.64	-0.09	1.17
Day 4 to 7	0.73	-0.02	2.03	0.13	-0.40	0.82
Day 7 to 14	2.98	0.68	4.87	1.57	-1.26	3.97
	Rectal temperature change, °F					
Day 0 to 4	1.66	1.96	.59	3.17	1.42	3.49
Day 4 to 7	1.04	1.80	1.19	1.48	2.45	-0.11
Day 7 to 14	-0.30	0.65	-1.81	-0.65	-5.11	-0.47
	Lung lesion score					
	1.26	1.57	1.00	1.96	1.57	2.00

[a]Overall line mean (n = 100).
[b]Mean of 7 pigs classed as high responders based on principal component index.
[c]Mean of 7 pigs classed as low responders based on principal component index.

Table 2. Differences in gene expression levels (Ct values) between infected (I) and uninfected (UI) pigs, between infected susceptible (H^+, high responders) and infected resistant (L^+, low responders), and between uninfected littermates (-) of H and L class pigs.

	Lung			Bronchial lymph node		
Gene	I - UI	H^+ - L^+	H^- - L^-	I - UI	H^+ - L^+	H^-- L^-
IFNA	-0.03	-0.88	-0.71	-0.53**	-0.60	-0.48
IFNG	-2.15**	-2.40**	-1.36**	-2.18**	-1.43**	0.25
IL15	-0.12	-1.51	-0.76	-0.15	-1.73	-0.82
STAT1	-0.82**	-1.59*	-0.88	-0.82**	-0.56	0.00
IL1B	-2.03**	-1.38*	0.21	-0.38	-1.66**	0.89
IL12B	-1.22**	-0.31	1.02	-0.69*	-0.16	0.66
CSF2	-1.10**	-0.71	0.28	-0.73**	0.23	0.48
IL8	-2.26**	-1.80*	-0.24	-2.02**	-2.17*	0.98
IL10	-0.43	-2.53**	-1.75**	-0.68*	-1.76*	-0.42
IL6	-0.06	-0.81	-0.50	-0.56*	-0.60	0.14
TNF	-0.68*	-1.36	-0.44	-0.67**	-0.08	0.64

Interactions

Only a few interactions of class (resistant vs susceptible) with genetic line existed, indicating that the general nature of expression differences between H and L pigs was consistent across lines. However, several significant interactions of genetic line with treatment (infected vs uninfected) existed. Interaction existed ($P < 0.05$) for expression of IFNG, IL12B, Il8, and TNF in lung, and for IFNA in lymph node. The general nature of these suggested that Line I had a greater innate response to PRRSV whereas Line HD had a greater acquired response.

As an example of these interactions, expression of the innate gene IFNA in lymph was nearly identical in infected and uninfected I pigs (30.3 vs 30.4 Ct), whereas the difference was more than two-fold between infected and uninfected HD pigs (30.5 vs 29.3). Infection reduced expression of INFA in HD, but had no effect in I pigs.

Expression of the IFNG gene involved in acquired immunity is another example. The Ct values for uninfected and infected I pigs were 27.3 and 26.2, respectively, whereas values for uninfected and infected HD pigs were 28.1 and 24.9, respectively. Infection stimulated a significantly greater expression of IFNG in HD pigs.

In general these interactions supported the suggestion from the phenotypic data that I-pigs had a stronger innate response to PRRSV, whereas HD pigs had a stronger acquired response. All pigs exhibited symptoms of PRRSV, but due to greater innate immunity, I pigs recovered more quickly and had greater weight gain and lower rectal temperatures, especially from day 7 to 14, than HD pigs.

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