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Bates, J. S.; Petry, D. B.; Eudy, J.; Bough, L.; and Johnson, Rodger K., "Differential expression in lung and bronchial lymph node of pigs with high and low responses to infection with porcine reproductive and respiratory syndrome virus" (2008). *Faculty Papers and Publications in Animal Science*. 454.

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Differential expression in lung and bronchial lymph node of pigs with high and low responses to infection with porcine reproductive and respiratory syndrome virus^{1,2}

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ABSTRACT: One hundred Hampshire × Duroc crossbred pigs and 100 Nebraska Index line pigs were infected with porcine reproductive and respiratory syndrome virus (PRRSV) and evaluated for resistance and susceptibility. Controls (100/line) were uninfected littermates to infected pigs. Viremia (V), BW change (WTΔ), and rectal temperature at 0, 4, 7, and 14 d postinfection were recorded. Lung, bronchial lymph node (BLN), and blood tissue were collected at necropsy (14 d postinfection). Infected pigs were classified as low or high responders to PRRSV based on the first principal component from principal component analyses of all variables. Low responders to PRRSV (low PRRSV burden) and their uninfected littermates were assigned to the low (L) class. High responders to PRRSV (high PRRSV burden) and their uninfected littermates were assigned to the high (H) class. Infected pigs in the L class had large WTΔ, low V, and few lung lesions; H-class pigs had small WTΔ, high V, and many lung lesions. Ribonucleic acid was extracted from lung and BLN tissue of the 7 highest and 7 lowest responders per line and from each of their control littermates. A control reference design was used, and cDNA from each reference sample

tissue was prepared from pooled RNA extracted from 2 control pigs from each line whose infected littermates had a principal component value of 0. Design variables in data analyses were line (Index vs. Hampshire × Duroc), class (H vs. L), treatment (infected vs. uninfected controls), and slide/pig as error. Oligo differential expression was based on $P < 0.01$ occurring in both lung and BLN. Line and treatment effects were significant for 38 and 541 oligos, respectively, in both lung and BLN. Line × class interaction existed for expression of thymosin β-4, DEAD box RNA helicase 3, acetylcholinesterase, and *Homo sapiens* X (inactive)-specific transcript in both tissues. Treatment × class existed for expression of CCAAT/enhancer-binding δ protein, nuclear factor of κ light polypeptide gene enhancer in B cells inhibitor α, thioredoxin-interacting protein, major facilitator superfamily domain containing 1, and unknown sequences SS00012040 and SS00012343. Line × treatment and line × treatment × class interactions were not significant. Possible important genetic associations for fine-mapping candidate genes related to response to PRRSV and determining causative alleles were revealed.

Key words: gene expression, microarray, pig, porcine reproductive and respiratory syndrome virus resistance

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J. Anim. Sci. 2008. 86:3279–3289
doi:10.2527/jas.2007-0685

INTRODUCTION

Porcine reproductive and respiratory syndrome virus (PRRSV) is one of the most economically important diseases of swine and causes reproductive failure, interstitial pneumonia, and weak piglets (Neumann et al., 2005). The virus targets alveolar macrophages (Murtaugh et al., 2002; Osorio, 2002) and induces apoptosis. Infected pigs are ineffective in eliminating the virus, and it may persist for several weeks to months postinfection (Labarque et al., 2003; Rowland et al., 2003; Chang et al., 2005).

Porcine reproductive and respiratory syndrome virus, a single-stranded 15-kb RNA-enveloped virus of positive polarity, contains 7 open reading frames (Allende et al., 1999). Its genome is highly variable, spe-

¹A contribution of the University of Nebraska Agricultural Research Division, supported in part by funds provided through the Hatch Act. Additional support was provided by USDA NRI PRRS CAP Grant 2004-35605-14197, the Nebraska Pork Producers Association, and USDA ARS project funds.

²The University of Nebraska Medical Center DNA Microarray Core Facility receives partial support from NIH grant number P20 RR016469 from the INBRE Program of the National Center for Research Resources.

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Received October 26, 2007.

Accepted July 15, 2008.

cifically between the North American and European isolates, due in part to many years of antigenic drift (Plagemann, 2003). Because of this variability, no effective vaccine against all PRRSV isolates currently exists. Therefore, alternative methods such as the use of genetic markers to select animals resistant or tolerant to PRRSV would be useful.

Studies with microarrays, which allow simultaneous assessment of mRNA transcription patterns of thousands of genes, have been used to identify genes and pathways involved in immune response to pathogens (Hernandez et al., 2003; de Koning et al., 2005). Microarray technology can determine changes of gene expression, host and virus interactions, and potential pathways for viral pathogenesis.

Breeds and lines of pigs respond differently to infection with PRRSV (Halbur et al., 1998; Lowe et al., 2005; Petry et al., 2005; Vincent et al., 2006), indicating that genetic variation in pigs in response to PRRSV exists. It is not practical to infect pigs in commercial populations with PRRSV, measure their response, and then select for resistance using quantitative methods. Therefore, selection for resistance using genetic markers or traits that can be measured in uninfected pigs seems more practical. The purpose of this experiment was to identify genes expressed differently in lung and bronchial lymph node (BLN) in pigs infected with PRRSV and then classified as susceptible or tolerant/resistant and in their uninfected littermates.

MATERIALS AND METHODS

This research was approved by the University of Nebraska Institutional Animal Care and Use Committee.

PRRSV Infection Experiment

A gene expression study was conducted with 56 pigs from a PRRSV infection experiment involving a total of 400 pigs. Design of the experiment and details of the infection and tissue collection procedures are in Petry et al. (2005). An overview is presented here.

Two hundred pigs from the Nebraska Index line (**I**), selected 20 generations for increased litter size, and 200 pigs from a commercial Hampshire \times Duroc (**HD**) cross-selected for lean growth, were used. Johnson et al. (1999) described the selection history of line I. Responses in reproductive traits through generation 19 are reported in Petry and Johnson (2004). Two pigs of the same sex from each available litter, representing 83 sires and 163 dams, were selected at random for the experiment. A total of 200 pigs were infected with PRRSV, and 200 uninfected littermates served as controls. The experiment was conducted in 2 replicates within each of 2 seasons with 50 pigs per breed in each year-season-replicate.

Pigs were housed in 2 isolation rooms at the University of Nebraska Animal Research Facility of the Veteri-

nary and Biomedical Sciences Department. Each room contained 2 pens of 12 to 13 pigs per pen, with line I pigs in one pen and line HD pigs in the other pen. One room was randomly assigned for treatment, and pigs in it were inoculated intranasally with 10^5 cell culture infectious dose 50% (of PRRSV strain 97-7985; Osorio et al., 2002). Application rate was 1 cc per nostril. Littermates, serving as controls to the infected pigs, were placed in the second room.

Blood (3 to 5 mL per pig) was collected from the jugular vein of pigs using 6.0-mL SS tube with silica clot activator, polymer gel, silicone-coated interior BD Vacutainer Systems preanalytical solutions tube No. 367974 (BD Biosciences, San Jose, CA). Blood samples were placed on ice for approximately 2 h and then serum was isolated by centrifugation at $1,464 \times g$ for 10 min at 10°C. Phenotypic data included viremia from serum samples collected at 4, 7, and 14 d postinfection (**dpi**), changes in BW, and rectal temperature from 0 to 4, 4 to 7, and 7 to 14 dpi; lung and BLN viremia from tissue collected at necropsy at d 14; and severity of lung lesions. Blood serum at d 0 before infection was collected and stored. Shin and Molitor (2002) reported that more than 80% of infected pigs showed the peak concentration of viral RNA concentrations in serum at 5 dpi and began to clear the virus thereafter. Work by Osorio (2002) also indicated that early clinical signs of PRRSV are evident in the early postinoculation period and that viremic titers are at maximum by d 14. Based on these results, in addition to sampling at d 0, blood samples were collected at 4, 7, and 14 dpi to monitor changes during and shortly after the acute phase of viral infection.

Line I and line HD pigs responded differently to infection with PRRSV (Petry et al., 2005), indicating genetic variation in response to infection. Uninfected HD pigs gained more BW and had greater rectal temperature from 0 to 14 dpi than uninfected I pigs, whereas infected I pigs gained more BW and had lower temperature than infected HD pigs. Viremia (cell culture infectious dose 50%/mL) was also greater in HD than I pigs at 4, 7, and 14 dpi. Genetic line differences in lung and lymph viremia concentrations were not significant, but tended to be greater in HD than I pigs. Based on these results, the current microarray experiment was designed to determine whether expression of genes differed between pigs in the tails of the PRRSV response distribution.

Phenotypic data for infected pigs were subjected to principal component (**PC**) multivariate procedures to identify 28 pigs, 7 pigs within each line in the outermost tails of the distributions of viral response variables. The first PC eigenvector, which accounted for 27% of the variation, was used to rank pigs. The 28 control littermates to each of these pigs were also selected, resulting in 56 pigs used in the gene expression experiment. Infected pigs in the right tail of the PC distribution were considered to be susceptible to PRRSV. Those pigs and

their control littermates formed the high PRRSV burden (**H**) class. Pigs in the left tail were considered to be tolerant or resistant. They and their control littermates formed the low PRRSV burden (**L**) class. Phenotypes of H- and L-class pigs, and expression differences of 11 specific immune function genes in these pigs, are reported by Petry et al. (2007).

A $2 \times 2 \times 2$ factorial treatment design was utilized for the gene expression experiment. Design effects included class (H and L), line (I and HD), and treatment (infected and uninfected), with 7 pigs in each of the 8 categories. Petry et al. (2007) provided evidence that characterization into H and L classes was not likely confounded with other types of infection.

Tissue Storage and RNA Preparation

Lung and BLN tissue were collected at necropsy, placed in Optimal Cutting Temperature Compound (Sakura Finetec USA Inc., Torrance, CA), snap frozen in liquid nitrogen, and stored at -80°C . The RNA from the tissues of the 56 pigs was extracted with Trizol as described by Royae et al. (2004) and Dawson et al. (2005). Integrity, quantity, and quality of RNA were assessed using the Agilent Bioanalyzer 2100 and RNA 6000 Labchip Kit (Agilent Technologies, Palo Alto, CA) described by Mueller et al. (2004). In a review, Schroeder et al. (2006) discuss application of this instrument for assigning integrity values to RNA measurements. The RNA was treated with DNase before cDNA synthesis using the Superscript Reverse Transcriptase (Invitrogen, Carlsbad, CA) and oligo-dT. The reverse transcription PCR primers and TaqMan probes were designed with Primer Express and manufactured by BioSource (Invitrogen; Dawson et al., 2005). Normalization of the samples was performed by using a standard amount of RNA ($10\ \mu\text{g}$) for every sample for the cDNA production and confirmed by the cycle threshold value for the housekeeping gene RPL32. The reverse transcription PCR analyses were conducted in duplicate on $100\ \text{ng}$ of cDNA/ $25\ \mu\text{L}$ reaction per well utilizing the Stratagene Brilliant Kit (La Jolla, CA) and an Applied Biosystems PRISM 7700 Sequence Detector System (Foster City, CA). Conditions used for amplification were as follows: 50°C for 2 min, 95°C for 10 min, 40 cycles of 95°C for 15 s, 60°C for 1 min, and then remaining at 4°C .

Porcine Microarray and Oligonucleotides

The 13,297 oligonucleotides (porcine AROS 1.0/1.0 Extension; <http://www.quiagen.com>; last accessed Oct. 2, 2008) used in this experiment were an accumulation of porcine cDNA and expressed sequence tags and were designed from TIGR Tentative Consensus (SsGI Release 5.0). Zhao et al. (2005) showed that of these oligonucleotides, 8,541 uniquely matched human or mouse reference sequences or pig annotated gene Na-

tional Center for Biotechnology Information accession numbers, indicating a high specificity of the array. The oligonucleotides were synthesized at the University of Minnesota microarray facility.

Microarray Design

A control reference design was used. The reference sample was developed from uninfected control full sibling littermates of infected pigs that had a PC value of 0 and that were not included in the sample of 28 infected pigs used in the microarray experiment. Two pigs from line I and 2 pigs from line HD made up the reference sample. One microarray assay per experimental pig, with the reference sample included in each one, was conducted. Thus, every sample of interest was compared indirectly through the connection with the reference sample.

Two micrograms of total RNA was reverse-transcribed using oligo-dT primer and reagents from the Ambion Amino Allyl MessageAmp aRNA Amplification Kit (Ambion, Austin, TX; Van Gelder et al., 1990). After cRNA generation, $5\ \mu\text{g}$ of cRNA was coupled to either CY5 or CY3 depending upon the sample. The samples were hybridized overnight using Ambion slide hybridization buffer at 42°C for 16 h. Slides were washed per the suggestions of the manufacturers and were scanned with an Axon 4000B scanner. In total, 112 oligo microarray slides were used: 56 for lung and 56 for BLN.

Microarray Analysis

Medians were normalized by adjusting out a common median point. Global median normalization, which shifts the center of the distribution of the log ratio of 635/532 to zero, but does not affect the spread within the slide, was performed to remove systematic variation across slides. Data points falling in the range of -150 to 150 in intensity value and those with ratios less than -15 and greater than 15 were removed. Also, data generated from microarray spots with problems (i.e., scratches, smears, etc.) were not used.

Variation is expected within slides; however, systematic bias within slides is not expected to be repeated across slides. Therefore, Lowess normalization, which is intended to remove systematic intensity dye bias and outliers, was not performed. Each slide represented 1 pig, and variation among the 7 experimental units within each line-treatment-class category includes slide and pig variation.

Background adjustments for the Cy3 and Cy5 intensity medians were made. Statistical analyses, which used a linear mixed model after normalization of the data, were performed on the ratio of medians. The ratio of medians is defined as: (median foreground intensity of Cy5 – median background intensity of Cy5) divided by (median foreground intensity of Cy3 – median background intensity of Cy3). The statistical model in-

Table 1. Overall line means (\bar{X}), SD, and means for the 7 high (H⁺) and low (L+) responders for Index (I) and Hampshire-Duroc (HD) cross pigs infected with porcine reproductive and respiratory syndrome virus

Trait ¹	Line I				Line HD			
	\bar{X}	SD	H ⁺	L+	\bar{X}	SD	H ⁺	L+
V4	4.17	0.60	4.39	4.11	4.54	0.82	5.11	3.10
V7	3.91	0.76	4.47	3.20	4.40	0.70	5.13	3.64
V14	3.00	1.21	4.49	0.50	3.59	0.99	5.29	2.51
WC ₀₋₄	0.32	0.39	0.08	0.58	0.29	0.34	-0.04	0.53
WC ₄₋₇	0.33	0.41	-0.01	0.92	0.06	0.33	-0.18	0.37
WC ₇₋₁₄	1.35	0.79	0.31	2.21	0.71	0.78	-0.57	1.80
TC ₀₋₄	0.92	1.56	1.09	0.33	1.76	1.46	0.79	1.94
TC ₄₋₇	0.58	1.25	1.00	0.66	0.82	1.70	1.36	-0.06
TC ₇₋₁₄	-0.16	1.17	0.36	-1.01	-0.36	1.63	-2.84	-0.26
LV	3.96	1.28	5.07	2.40	4.45	0.78	4.71	4.21
LNV	2.55	1.30	3.33	1.31	3.12	0.99	3.70	2.66
L	1.26	0.79	1.57	1.00	1.96	0.57	1.57	2.00

¹V4, V7, and V14 = viremia titer, expressed as log₁₀ cell culture infectious dose 50% (CCID₅₀)/mL, in serum collected at 4, 7, and 14 d, respectively (SEM = 0.07); WC₀₋₄, WC₄₋₇, and WC₇₋₁₄ = BW change (kg) from d 0 to d 4, d 4 to d 7, and d 7 to d 14, respectively (SEM = 0.05); TC₀₋₄, TC₄₋₇, and TC₇₋₁₄ = temperature change (°C) from d 0 to d 4, d 4 to d 7, and d 7 to d 14, respectively (SEM = 0.10); LV and LNV = viral titer (log₁₀ CCID₅₀/mL) in lung (SEM = 0.11) and bronchial lymph (SEM = 0.07), respectively, collected at necropsy on d 14; L = severity score of lung lesions (SEM = 0.12).

cluded line, treatment, class, and all possible interactions as fixed effects. Litter within class by line was included as a random effect. Age of pig was fitted as a covariate.

Twenty-four oligos were repeated either 12 or 17 times within each slide. Data for these oligos were fitted to the same mixed model with spot within slide included to account for repeated measures.

Correlations in gene expression patterns for 11 immune function genes between lung and BLN were weak (Petry et al., 2007), indicating that patterns for one tissue cannot be extrapolated to other tissues. Therefore, expression data for lung and BLN tissues were analyzed separately using the SAS software (SAS Inst. Inc., Cary, NC). However, considering all genes on the array used in this experiment, it is expected that some expression patterns may be highly correlated between lung and BLN.

To minimize the number of false positives, a threshold of $P < 0.01$ was used for each oligo in BLN and in lung. To control false positive rate, the P -values were subcategorized by tissue for each effect in the model (line \times treatment \times class, treatment \times class, line \times class, line \times treatment, line, treatment, and class). If, for any oligo, true expression differences for effects in the model (treatments, classes, lines, or interaction effects) do not exist, then P -values for these effects across tissues are expected to be independent. For these oligos, false positive rates in lung are uncorrelated with those in BLN. Therefore, the effects in the model for an oligo were considered to be significant if the P -value was less than 0.01 in both BLN and lung. With that assumption, the probability of a false positive occurring in both tissues is less than or equal to 0.0001.

RESULTS

Means for the 7 H and 7 L pigs within each line and means and SD for all infected pigs were reported by Petry et al. (2007) and are in Table 1 to illustrate phenotypic differences among pigs for which gene expressions were evaluated. All pigs challenged with PRRSV became infected as indicated by the 4 dpi viremia values, but L-class pigs in both populations began to clear the virus more quickly and thus had less viremia at 7 and 14 dpi, whereas H-class pigs had a continual increase in viremia from d 4 to 14. Pigs in the L class also had increasing BW change after infection with PRRSV, whereas pigs in the H class had minimal or negative BW change.

Gene Expression

The experiment was designed to identify genes that are expressed differently between pigs infected with PRRSV and uninfected littermates, genes expressed differently between high and low responders, and to determine whether these expression patterns are consistent across 2 distinctly different populations. To address these objectives, genes for which main effects significantly affected expression patterns are presented first, followed by those genes for which interactions among main effects in expression patterns were significant.

Line, Treatment, and Class Effects. Line was significant ($P < 0.01$) for 76 oligos in lung, 358 in BLN, and 38 in both tissues. Treatment was significant for 1,027 oligos in lung, 1,398 in BLN, and 541 in both tissues. Class was significant for 75 oligos in lung, 50 in BLN, but none in both lung and BLN.

Table 2. Genes and oligonucleotide identification number (ID) for which line × class and treatment × class were significant ($P < 0.01$) in both lung and bronchial lymph node (BLN)

Gene ¹	Oligonucleotide ID	P-values	
		Lung	BLN
Line × class			
<i>Homo sapiens</i> DEAD box RNA helicase 3 (DDX3)	SS00006751	0.0014	0.00007
Thymosin β-4 (Tβ ₄)	SS00008272	0.0002	0.0029
Acetylcholinesterase (ACHE)	SS00011278	0.0043	0.0014
<i>Homo sapiens</i> X (inactive)-specific transcript (XIST)	SS00012135	0.0015	0.0065
Treatment × class			
Nuclear factor of κ light polypeptide gene enhancer in B cells inhibitor α (NF-κBIα)	SS00000892	0.0013	0.0086
Thioredoxin-interacting protein (TXNIP)	SS00008321	0.0017	0.0026
Major facilitator superfamily domain containing 1 (MFS1)	SS00009186	0.0078	0.0069
CCAAT/enhancer-binding δ protein (CEBPδ)	SS00010698	0.000007	0.0081
Unknown	SS00012040	0.0004	0.0016
Unknown	SS00012343	0.0073	0.0027

¹Oligonucleotide sequence, TIGR Tentative Consensus accession, and reference sequence ID can be found in the supplementary table of the online version of Zhao et al., 2005 (<http://jas.fass.org/content/vol86/issue12>).

Although line, treatment, and class effects were not significant in both tissues for some oligos, oligos for which these effects were significant in at least 1 tissue may be of interest to others studying genetic resistance to PRRSV. Therefore, nucleotide number and results for these oligos are reported for line, treatment, and class effects in supplementary Tables 1, 2, and 3, respectively (<http://jas.fass.org/content/vol86/issue12>).

The supplementary table of the online version of Zhao et al. (2005) contains additional information about genes listed in the supplementary tables of this manuscript, including the oligonucleotide sequence and the gene that the sequence represents. Main effects of line, treatment, and class will not be discussed further.

Line × Class Interactions. Line × class interaction existed (Table 2) for 4 oligos in both lung and BLN: DEAD box RNA helicase 3 (**DDX3**), thymosin β-4 (**Tβ₄**), acetylcholinesterase (**ACHE**), and *Homo sapiens* X (inactive)-specific transcript (**XIST**). Mean

fold differences in expression of these genes are presented in Table 3.

Significant differences in expression of all 4 of these genes occurred between L-class and H-class pigs in line I, but not in line HD. Expression of DDX3 in line I was 2.72 times greater in lung of L-class pigs (1.96 vs. 0.72) and 3.25 times greater in BLN (1.95 vs. 0.60) than in H-class pigs, compared with ratios of 0.99 (lung) and 1.24 (BLN) of line HD, L-class to H-class pigs.

Expression of Tβ₄ was 3.8 times greater (1.79 vs. 0.47) in the lung and 3.1 times greater (1.83 vs. 0.59) in the BLN in line I L-class than H-class pigs. Greater expression in HD, L-class than H-class pigs also occurred, but the relative differences were much less (1.26-fold increase in lung and 1.74-fold increase in BLN).

Conversely, expression of ACHE and XIST was significantly less in line I, L-class than in H-class pigs. The fold differences in expression of ACHE were 0.33 in lung (0.42 vs. 1.28) and 0.38 (0.44 vs. 1.16) in BLN. These

Table 3. Fold differences, relative to common control, in expression of genes in lung and bronchial lymph node (BLN) for which line [line Index (I) vs. line Hampshire-Duroc (HD)] × class (high vs. low viremia) was significant

Gene ¹	Oligonucleotide ID	Lung						BLN					
		High viremia			Low viremia			High viremia			Low viremia		
		I	HD	SE	I	HD	SE	I	HD	SE	I	HD	SE
DDX3 ²	SS00006751	0.72	0.69	0.19	1.96	0.68	0.24	0.60	0.51	0.12	1.95	0.63	0.15
Tβ ₄ ³	SS00008272	0.47	0.42	0.16	1.79	0.53	0.17	0.59	0.31	0.18	1.83	0.54	0.19
ACHE ⁴	SS00011278	1.28	1.46	0.13	0.42	1.43	0.16	1.16	1.34	0.11	0.44	1.33	0.13
XIST ⁵	SS00012135	1.18	1.32	0.12	0.32	1.24	0.07	1.11	1.33	0.13	0.43	1.34	0.14

¹Oligonucleotide sequence, TIGR Tentative Consensus accession, and reference sequence identification numbers (ID) can be found in the supplementary table of the online version of Zhao et al., 2005 (<http://jas.fass.org/content/vol86/issue12>).

²DDX3 = DEAD box RNA helicase 3.

³Tβ₄ = thymosin β-4.

⁴ACHE = acetylcholinesterase.

⁵XIST = *Homo sapiens* X (inactive)-specific transcript.

Table 4. Fold differences, relative to common control, in expression of genes in lung and bronchial lymph node (BLN) for which treatment [infected (In) vs. uninfected (Un)] × class (high vs. low viremia) was significant

Gene ¹	Oligonucleotide ID	Lung						BLN					
		High viremia			Low viremia			High viremia			Low viremia		
		In	Un	SEM	In	Un	SEM	In	Un	SEM	In	Un	SEM
NF- κ BI α ²	SS00000892	0.94	0.53	0.07	0.80	0.69	0.07	0.66	0.52	0.05	0.59	0.68	0.05
TXNIP ³	SS00008321	0.74	0.52	0.08	0.56	0.73	0.08	1.51	0.90	0.19	1.22	1.58	0.19
MFSD1 ⁴	SS00009186	1.28	0.80	0.06	1.14	0.94	0.06	1.21	0.87	0.08	0.82	0.85	0.08
CEBP δ ⁵	SS00010698	1.21	0.75	0.07	0.92	0.93	0.07	1.10	0.84	0.09	0.92	1.03	0.10
Unknown	SS00012040	1.47	0.76	0.07	0.87	0.60	0.07	1.29	0.90	0.11	0.78	0.95	0.10
Unknown	SS00012343	1.15	0.86	0.06	0.95	0.91	0.06	0.93	0.63	0.06	0.90	0.92	0.06

¹Oligonucleotide sequence, TIGR Tentative Consensus accession, and reference sequence identification numbers (ID) can be found in the supplementary table of the online version of Zhao et al., 2005 (<http://jas.fass.org/content/vol86/issue12>).

²NF- κ BI α = nuclear factor of κ light polypeptide gene enhancer in B cells inhibitor α .

³TXNIP = thioredoxin-interacting protein.

⁴MFSD1 = major facilitator superfamily domain containing 1.

⁵CEBP δ = CCAAT/enhancer-binding δ protein.

same ratios for expression of XIST were 0.27 (0.32 vs. 1.18) in lung and 0.39 (0.43 vs. 1.11) in BLN. Again, differences in line HD in expression of these genes between L-class and H-class pigs were small.

Treatment × Class. Treatment × class interaction existed (Table 2) for expression of 6 oligos in both lung and BLN: nuclear factor of κ light polypeptide gene enhancer in B cells inhibitor α (NF- κ BI α), thioredoxin-interacting protein (TXNIP), major facilitator superfamily domain containing 1 (MFSD1), CCAAT/enhancer-binding δ protein (CEBP δ), and 2 unknown oligos, identification number (ID) = SS00012040 and ID = SS00012343. Mean fold differences in expression of these genes are presented in Table 4.

Increased expression of all 6 oligos occurred in both lung and BLN of pigs infected with PRRSV and that had a high PRRSV burden (H class) compared with their uninfected littermates (Table 4). Fold differences ranged from 1.27 (0.66 vs. 0.52) for expression of NF- κ BI α in BLN to 1.93 (1.47 vs. 0.76) for expression of unknown oligo (ID = SS00012040) in lung. However, in pigs with low PRRSV burden (L class), no consistent pattern existed in expression differences between infected and uninfected pigs. Fold differences in expression of these genes between L class infected and uninfected littermates ranged from 0.77 to 1.45.

DISCUSSION

Genetic variation for disease resistance is due to effects of genes that regulate innate and adaptive immune responses. Variation in gene expression among pigs and differences between lung and bronchial lymph tissue do not necessarily indicate genetic variation. However, interactions among line, treatment, and class found for several genes are evidence that genetic variation in the mechanisms involved in immune responses to PRRSV exist.

One objective was to determine whether response to infection was a general characteristic of H- and L-class pigs or whether expression differences could be measured only in the presence of virus. From a genetic selection standpoint, it would be desirable to select on a trait in uninfected pigs that is correlated with response in infected pigs. In this study, main effect of class was not significant for expression of any oligo for both tissues; however, interaction of line × class existed for 4 oligos, and interaction of treatment × class existed for 6 oligos. Although exact pathways in immune responses to infection with PRRSV are not fully understood, further study of the genes for which interaction existed could give rise to discoveries for genetic mechanisms involved in PRRSV immunity. Therefore, emphasis is placed on genes for which line × class and treatment × class interactions were significant in both lung and BLN (Table 5).

Expressions of DDX3 and T β ₄ were considerably greater in both lung and BLN of line I pigs with low PRRSV burden than in line I pigs with high PRRSV burden. Differences in expression of these oligos between L-class and H-class line HD pigs were relatively small. Different expression of 2 other oligos (ACHE and XIST) between L-class and H-class pigs also occurred in line I but not in line HD, but expression of each of these genes in line I was greater in pigs with high PRRSV burden than in those with low burden. Based on biological responses in pigs of this same experiment, Petry et al. (2005) concluded that line I pigs were more resistant to PRRSV than line HD pigs. Results reported herein suggest that DDX3, T β ₄, ACHE, and XIST may play a role, either directly or through genes in immune pathways, to genetic resistance to PRRSV.

The DEAD box proteins, RNA helicases, which contain the conserved amino acid motif Asp-Glu-Ala-Asp, are essential in RNA metabolism, which includes RNA transport, transcription, spliceosome function, ribosome assembly, initiation of translation, and RNA

Table 5. Genes and oligonucleotide identification number (ID) for significant differential expression ($P < 0.01$) in both lung and bronchial lymph node and molecular function

Gene ¹	Oligonucleotide ID	Molecular Function
DEAD box RNA helicase 3 (DDX3)	SS00006751	Controls RNA viral latency, RNA virus replication
Thymosin β -4 (T β ₄)	SS00008272	Apoptosis inhibitor
Acetylcholinesterase (ACHE)	SS00011278	Apoptosis regulator
<i>Homo sapiens</i> X (inactive)-specific transcript (XIST)	SS00012135	X-chromosome silencer
Nuclear factor of κ light polypeptide gene enhancer in B cells inhibitor α (NF- κ BI α)	SS00000892	Innate anti-RNA virus response, RNA virus enhancer, apoptosis
Thioredoxin-interacting protein (TXNIP)	SS00008321	Apoptosis enhancer, inhibits natural killer cell development, increases cell growth arrest (G ₀ phase)
Major facilitator superfamily domain containing 1 (MFSD1)	SS00009186	Unknown
CCAAT/enhancer-binding δ protein (CEBP δ)	SS00010698	Increases cell growth arrest (G ₀ phase)
Unknown	SS00012040	Unknown
Unknown	SS00012343	Unknown

¹Oligonucleotide sequence, TIGR Tentative Consensus accession, and reference sequence ID can be found in the supplementary table of the online version of Zhao et al., 2005 (<http://jas.fass.org/content/vol86/issue12>).

degradation (Krishnan and Zeichner, 2004). These authors also suggest that DDX3 may be important in human immunodeficiency virus (HIV) latency. The oligo DDX3 is found on the X chromosome of *H. sapiens*, *Bos taurus*, *Gallus gallus*, *Mus musculus*, *Canis lupus familiaris*, and *Rattus norvegicus* but is currently not mapped to a swine chromosome ([http://www.ncbi.nlm.nih.gov/sites/entrez?db = gene](http://www.ncbi.nlm.nih.gov/sites/entrez?db= gene)). Because of the location conservation across several species, it is plausible that DDX3 is also on the swine X chromosome.

It has been found that DDX3 is a crucial component in the pathogenesis of HIV and hepatitis C, and like PRRSV, HIV and hepatitis are also single-stranded positive RNA viruses. The precise biological function of DDX3 is not fully understood. However, DDX3 is known to harbor a cell growth-suppressive property, thus acting as a tumor suppressor (Chao et al., 2006), is required in viral replication of HIV and hepatitis C, and is involved in gene splicing (Kim et al., 2001; Krishnan and Zeichner, 2004; Ariumi et al, 2007).

The gene that encodes T β ₄, TMSB4X, is also found on the X chromosomes of *H. sapiens*, *B. taurus*, *G. galus*, and *M. musculus* ([http://www.ncbi.nlm.nih.gov/sites/entrez?db =gene](http://www.ncbi.nlm.nih.gov/sites/entrez?db= gene)), and like DDX3, T β ₄ is also not mapped to a swine chromosome. Thus, it is probable that TMSB4X is also on the swine X chromosome. In humans, TMSB4X escapes X inactivation (entrez gene: TMSB4X T β ₄, X-linked) caused by XIST.

Thymosin β -4 has many functions, including cell proliferation, migration, and differentiation, and has also been shown to be involved in resistance to apoptosis (Muller and Hannappel, 2003; Hsiao et al., 2006). Cytotoxic T lymphocytes, natural killers, and lymphokine-activated killers utilize the Fas ligand to trigger apoptosis (Berke 1995; Liu et al., 1995; Eischen et al., 1996). Hsiao et al. (2006) showed that overexpression of T β ₄ increased the cellular apoptosis resistance of Fas ligand-bearing T cells. Upon induction of apoptosis, a reduc-

tion in mRNA of T β ₄ has been shown, further suggesting a role for T β ₄ in apoptosis (Muller and Hannappel, 2003). Findings here along with those cited above suggest that increased concentrations of T β ₄ in line I pigs with low PRRSV burden increased resistance to apoptosis, a primary effect of PRRSV infection.

Acetylcholinesterase is involved in protective immunity. Possible roles of ACHE in immune response to PRRSV are difficult to postulate, but it too may be involved in apoptosis. Inhibition of ACHE has been shown to increase cell counts and enhance cellular proliferation (Jiang et al., 2007). Phosphorylation of c-Jun N-terminal kinases (JNK) was enhanced during apoptosis and was coupled with increases in ACHE expression in apoptotic cells. The upregulation of ACHE was abolished after administering a JNK inhibitor and silencing JNK with small interfering RNA (Deng et al., 2006).

Homo sapiens X (inactive)-specific transcript has been mapped to the swine X chromosome and is responsible for X inactivation in females (Ng et al., 2007). It is unlikely that XIST itself has an effect on immune response to PRRSV. However, XIST may indirectly affect immune response to PRRSV through inactivation of the second X chromosome in females, affecting the expression of genes such as DDX3 or T β ₄. In males, however, a homolog of DDX3 is present on the Y chromosome (Kim et al., 2001). If DDX3 is an important gene in immune response to PRRSV, sex-linked responses could occur. This experiment was too small and not designed so as to test that hypothesis.

We found that expression of 6 genes was greater in pigs infected with PRRSV and that they had a greater PRRSV burden than their uninfected littermates. However, there was little difference in expression of these genes between infected pigs with low PRRSV burden and their uninfected littermates. Thus, it appears that these are genes that are activated to a greater degree in

pigs that respond most to PRRSV, but there was no innate difference detected between uninfected littermates of high and low responders.

The NF- κ B gene inhibits the NF- κ B pathway. Lee and Kleiboeker (2005) showed that upon PRRSV infection in MARC-145 cells, the NF- κ B pathway was activated as a result of NF- κ B α degradation, resulting in induction of reactive oxygen species (ROS). In that study, PRRSV infection induced oxidative stress in cells by generating ROS, and antioxidants inhibited NF- κ B DNA binding activity in PRRSV-infected cells, suggesting ROS as a mechanism where NF- κ B was activated by PRRSV infection. It has been found that NF- κ B plays a crucial role in apoptosis of virus-infected cells, and NF- κ B activation may maximize viral replication through delaying cell death or increase the rate of apoptosis in infected cells as a mechanism to increase rate of spread of virus (Mi et al., 2001; Bowie et al., 2004).

Infection in humans with viruses such as HIV (Israel and Gougerot-Pocidallo, 1997), influenza virus (Flory et al., 2000), and hepatitis C (Gong et al., 2001) activate the NF- κ B pathway. It is plausible that expression of NF- κ B α in response to PRRSV is increased in some pigs (those with high PRRSV burden), affecting the NF- κ B pathway, whereas this same level of activation did not occur in pigs with low PRRSV burden.

Thioredoxin-interacting protein, also known as vitamin D₃ upregulated protein 1, is important in cell stability, has numerous cellular functions, and can be induced by several stress stimuli (Kim et al., 2007). They found that overexpression of TXNIP inhibited cellular proliferation through cell cycle arrest in the generation 0 and generation 1 phases, whereas underexpression of TXNIP was strongly associated with tumorigenesis. A role for TXNIP in apoptosis has also been shown (Xiang et al., 2005). It has been found that TXNIP inhibited the interaction between thioredoxin and apoptosis signal-regulating kinase 1 (ASK-1), resulting in increases in ASK-1 concentrations. It has been found that ASK-1 is important in cell cycle and proliferation, and activation of ASK-1 induces apoptosis through the JNK and p38 mitogen-activated protein kinase pathway (Tobiume et al., 2001; Song and Lee, 2003).

Natural killer cells are important in innate immunity. One of their functions is the elimination of major histocompatibility complex class-I-deficient virus-infected cells (Colucci et al., 2002; Moretta et al., 2002). Lee et al. (2005) showed that in TXNIP knockout mice, a significant reduction in natural killer cells occurred and that TXNIP is a critical factor in the development and function of these cells. Increased expression of TXNIP in infected H-class pigs compared with uninfected littermates may have caused a reduction in natural killer, whereas this same event may not have occurred in pigs with low PRRSV burden.

Overexpression of TXNIP has also been shown to increase concentrations of ROS, which are often considered to be toxic stresses against cells. The regulation of

the redox system is important in cell proliferation, gene expression, cell cycle, and apoptosis (Armstrong et al., 2002; Liu and Chen, 2002; Vaculova et al., 2002; Rosato and Grant, 2003). Thioredoxin plays a protective role against oxidative stress and modulates DNA-binding activities of transcription factors of the NF- κ B pathway (Kim et al., 2007).

Greater expression of CEPB δ occurred in both tissues of infected than uninfected H-class pigs, whereas expression in infected L-class pigs and their uninfected littermates was very similar. Pathways by which CEPB δ are involved in immune response to PRRSV are not clear, but perhaps it is involved in cell growth and apoptosis. Cells with greater expression of CEPB δ exhibited a rapid decline of cyclin D₁ and phosphorylated retinoblastoma protein concentrations, a rapid increase in the cyclin-dependent kinase inhibitor 27, and accelerated generation 0 growth arrest and apoptosis (O'Rourke et al., 1999; Sivko et al., 2004). Upon restoration of normal CEPB δ concentrations, O'Rourke et al. (1999) showed that generation 0 growth arrest and apoptosis returned to normal, suggesting that CEPB δ plays a key role in regulating generation 0 growth arrest and apoptosis.

Expression of CEPB δ concentrations are generally low, but upon stimulation by interferon γ , IL1, IL6, lipopolysaccharide, and tumor necrosis factor α , expression concentrations increase (Thangaraju et al., 2005). The CEPB genes are regulators of early inflammatory mediators, including IL6, and tumor necrosis factor α (Chini et al., 1998; Poli, 1998; Fan et al., 2001). Using the same pigs as in this study, Petry et al. (2007) found that IL1B concentrations in lung were significantly greater in pigs infected with PRRSV than in their uninfected littermates. Interleukin 1 in the lung is known to activate both CEPB δ and the NF- κ B pathway (Seppanen et al., 2005). However, the NF- κ B pathway has been shown to be repressed by NF- κ B α . Thus, in pigs with high PRRSV burden, the effect IL-1 has on the NF- κ B pathway may also be repressed due to increases in NF- κ B α activity. Upon PRRSV infection, CEPB δ may be enhanced by IL-1, as well as other innate immunity genes involved in regulation of CEPB δ , in particular STAT3, which has been shown to regulate CEPB δ (Thangaraju et al., 2005).

Increases in oxidative stress have also been coupled with increases in CEPB δ expression, suggesting a relationship between CEPB δ and ROS (Alan and Papaconstantinou, 1992; Choi et al., 1995; Sugahara et al., 1999; Cassel and Nord, 2003). Henderson et al. (1995) demonstrated that upon HIV infection, CEPB proteins play an essential role in transcription of HIV in macrophage-monocytes, suggesting a possible role for CEPB δ in transcription of PRRSV. This could explain why only infected pigs with high PRRSV burden had elevated concentrations of CEPB δ .

Major facilitator superfamily domain containing 1, unknown oligo SS00012040, and unknown oligo SS00012343 were oligos for which differential expres-

sion existed within treatment \times class subclasses. The function of these 3 oligos is unknown, and thus potential pathways for their involvement in immune response to PRRSV cannot be postulated.

Fold differences in expression of oligos for which treatment \times class was significant were relatively small. No expression differences between pigs with high and low PRRSV burden, regardless of infection status, were greater than 2-fold, a difference often considered important in gene expression studies. However, because a microarray analysis was done for each pig, adequate replication occurred to detect interactions with smaller fold differences in expression. In addition, some mRNA have relatively short half-lives, in particular CEPB δ , which has a half-life of \sim 35 min (Dearth and DeWille, 2003). Relatively small changes in transcription or half-life of short-lived mRNA can result in significant (\sim 1,000-fold) differences in mRNA content over a short period of time (Ross, 1996; Guhaniyogi and Brewer, 2001). Therefore, the importance of oligos with smaller fold differences in expression should not be overlooked.

Results of this experiment provide insights into genes and possible mechanisms by which pigs are able to tolerate PRRSV infection and not show symptoms of porcine reproductive and respiratory syndrome. Many oligos found to be differentially expressed are known to interact with one another, indicating potential pathways involved in response to PRRSV infection.

Many genes were found to be involved in the immune response to PRRSV. Genes including T β ₄, DDX3X, ACHE, and XIST, CEPB δ , NF κ B1 α , TXNIP, MFS1, and unknown sequences SS00012040 and SS00012343 need additional evaluation to determine their role in response to PRRSV infection. They are candidates for genes that could be included in selection for greater immunity to PRRSV, either through selection for allelic variants that allow resistance-tolerance to PRRSV, or selection on the gene products themselves. Although these genes may not be the only genes associated in the immune response to PRRSV, they do provide insight into the genetic mechanisms associated with the host immune response to PRRSV.

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