RESEARCH REPORT
PORK SAFETY

Title: Identifying the Genetic Profile of the *Salmonella*-carrier Pig to Improve Food Safety and Decrease Pre-Harvest Disease **– NPB #05-176**

Investigator: Shawn Michele Dunkin Bearson

Institution: USDA, Agricultural Research Service, National Animal Disease Center

Co-Investigators: Uthe, Jolita J., Wang, Yanfang, Qu, Long, Dekkers, Jack, Nettleton, Dan, Tuggle, Christopher

Date Submitted: February 1, 2007

Abstract

Salmonella shed from colonized swine can contaminate: slaughter plants and pork products during meat processing, edible crops when swine manure is used as a fertilizer, water supplies if manure used as crop fertilizer runs off into streams and waterways, and neighboring pigs resulting in a continuous food safety problem and animal health issue. Therefore, pre-harvest control of *Salmonella* in swine is an essential step in controlling animal disease, protecting the environment and preventing foodborne illness with *Salmonella*. A major focus of our collaborative research program is to investigate the porcine response to infection with *Salmonella* to 1) identify porcine genes differentially regulated during infection and 2) identify and associate genetic polymorphisms within these genes with infection status across swine populations. The goal of the research program is to provide molecular insight into the host gene expression responses that lead to the undesirable carrier state of *Salmonella* in pigs in order to identify approaches to control the re-emergence of *Salmonella* during transportation and marketing stress.

In the current study, 40 crossbred pigs were intranasally inoculated with *Salmonella enterica* serovar Typhimurium and monitored for *Salmonella* fecal shedding and blood immune parameters at 2, 7, 14 and 20 days post-inoculation (dpi). Using a multivariate permutation test, a positive correlation was observed between *Salmonella* shedding and interferon-gamma (IFNG) levels at 2 and 7 dpi ($p<0.05$), with a greater number of *Salmonella* shedding in the animals with higher IFNG levels. In addition, a positive correlation was observed of the IFNG levels with the number of circulating neutrophils at 7 and 14 dpi, mature banded neutrophils at 2 dpi, monocytes at 7 dpi and white blood cells (WBCs) at 7, 14 and 20 dpi. We have further performed association studies of immune response parameters or shedding status of the *Salmonella*-infected pigs with single nucleotide polymorphisms (SNPs) in 11 genes: VCP, CCT7, LCP1, CD47, SCARB2, SDCBP, CD163, CCR1, NCF2, IL8 and TYROBP. Expression of these genes was identified by our group as differentially-regulated during *Salmonella* infection, and assays for these SNPs have been developed in our laboratories. A

positive association of SNP genotype A/G at nucleotide 1026 (relative to start codon) of the CCT7 gene was observed with circulating neutrophils and WBCs ($p<0.05$) as well as *Salmonella* shedding ($p=0.0012$) at 7 dpi compared to the G/G heterozygote genotype. CCT7 encodes a molecular chaperone involved in tubulin folding and protection, and our work is the first report of its response to *Salmonella* infection. Thus, our analyses are linking the porcine immune response to *Salmonella* infection with specific genes and genetic polymorphisms, thereby providing potential markers for carrier pigs as well as targets for disease diagnosis, intervention and prevention.

Introduction

Currently, the most frequently applied methods for disease control in livestock involve the use of antibiotic drugs and/or vaccines. However, these approaches are not always effective; furthermore, antibiotic use is being regulated more strictly and may be banned from food products in the future. This creates a real need for alternative approaches to disease control to protect the food supply. One such approach is the identification and use of animals with enhanced disease resistance. If genes can be identified from animals which are naturally more resistant to microorganisms (such as *Salmonella),* direct improvement in food safety as well as animal disease can be achieved by selecting favorable animals to breed disease-resistant offspring.

The ability of animals to alter their gene expression in response to pathogenic bacteria is an important mechanism to fight infection. Studies in pigs have indicated that the host immune response is under genetic control. Differences in animal breeds for both disease-resistance and immune responsiveness have been documented and significant genetic control of disease resistance has been reported in pigs. With help from molecular biology, individual genes for control of disease can be identified by finding animals with high disease resistance.

One of the major problems in pre-harvest food safety is contamination on the farm or slaughter plant environment by animals shedding pathogenic bacteria such as *Salmonella enterica*. Shedding of *Salmonella* from infected pigs varies from non-shedding to persistent shedding. An additional challenge is the difficulty in identifying which animals are carriers and therefore, will in turn be shedders. Since the current technology is not entirely efficient in detecting *Salmonella*-infected pigs, the goal of this study was to identify differences in gene expression between non-shedders and persistent shedders of *Salmonella* so that differentially expressed genes can be tested for their contribution to the control of shedding. The transfer of this information may assist in developing diagnostic assays for carrier animals, as well as help develop *Salmonella*-resistant lines of pigs.

Objectives

Objective I. Characterize the transcriptional profile of the carrier state of *Salmonella*-infected pigs

Biological investigations by our research group have revealed that the *Salmonella*-shedding status of experimentally infected pigs varies greatly across the infected population from non-shedding to persistent shedding of the pathogen. The goal of Objective I is to investigate the carrier state of *S.* Typhimurium-infected pigs at the molecular level by examining the gene expression patterns of carrier pigs and determining how the carrier state of the animal relates to its shedding status.

Objective II. **Identify those differentially expressed genes that have sequence variants in pig populations to develop future diagnostic tests for resistance.**

Using molecular tests to screen for superior gene variants is now an established method to select for specific characteristics in animal breeding. We propose to use PCR tests to look for such gene variants from within the genes differentially responsive to infection. We believe such genes are excellent candidates for controlling the immune system's response to infection, and will lead to diagnostic PCR tests for selecting superior animals.

Materials and Methods

Animal study. Forty conventionally raised male and female piglets from sows identified as fecal-negative for *Salmonella* spp. were weaned at 10 days (d) of age, shipped to the National Animal Disease Center, Ames, IA and raised in climate-controlled, fully enclosed isolation facilities. To confirm that all piglets were fecal-negative for *Salmonella* spp. prior to challenge, bacteriological cultures were performed on rectal swabs at 2 and 5 weeks of age. At seven weeks of age, the pigs were intranasally challenged with 1×10^9 cfu of serovar Typhimurium χ4232 grown in Luria Bertani (LB) broth at 37ºC. At 2, 7, 14, and 20 days post-inoculation (dpi), rectal temperatures and clinical signs of infection (lethargy, loss of appetite and diarrhea) were recorded and fecal and blood samples were taken for each animal. At 21 dpi, tissue samples from the mesenteric and ileocecal lymph nodes were aseptically collected. Mesenteric and ileocecal lymph node samples were used in quantitative bacteriology and were immediately frozen in liquid nitrogen for RNA isolation, respectively. Blood samples were collected for the following: DNA extraction (see below), CBC analysis (ISU Veterinary Diagnostic Laboratory), serum preparation for cytokine assays (see below) and RNA extraction using the PAXgene Blood RNA tubes (PreAnalytiX, Valencia, CA). All procedures involving animals were lawful and approved by the USDA, ARS, NADC Animal Care and Use Committee.

Bacteriology. For quantitative bacteriology, one gram of pig feces was combined with 5 ml PBS, vortexed and 100 µl directly plated to brilliant green agar with sulfadiazine (BGS, Difco, Detroit, MI) containing nalidixic acid. For tissue samples, one gram of each tissue was combined with 2 ml of PBS in a whirlpak bag, pounded with a mallet and homogenized in a Stomacher (Seward, Westbury, NY) for 1 minute. One hundred microliters of the resulting solution was aliquoted onto brilliant green agar plates with sulfadiazine (BGS) containing nalidixic acid. One hundred microliters of a ten-fold dilution of each fecal and tissue sample were also plated, and additional dilutions were performed when colony forming units (cfu) reached >300/plate. Following 24 hours of incubation at 37ºC, colonies indicative of *Salmonella* were enumerated and a single colony from each plate was confirmed to be *Salmonella* by serogroup antiserum agglutination (Beckton, Dickinson and Co.,

Sparks, MD). The total number of cfu for each quantitative tissue or fecal sample was calculated per gram of sample by obtaining the number of *Salmonella* per plate and multiplying by the dilution factor.

Qualitative bacteriology of *Salmonella* was performed as follows: 1 gram (fecal) or 100 µl (homogenized tissue) samples were inoculated in 10 ml of GN-Hajna (GN, Difco, Detroit, MI) broth and tetrathionate (TET, VWR, Rutherford, NJ) broth for 24 and 48 hours of growth at 37ºC, respectively. Following incubation, 100 µl of each culture was transferred to 10 ml Rappaport-Vassiliadis medium (RV, Difco, Detroit, MI) and incubated at 37ºC for 18 h. The cultures were streaked on brilliant green agar plates with sulfadiazine (BGS) containing nalidixic acid. Colonies suspicious for *Salmonella* were stabbed/streaked to triple sugar iron agar and lysine iron agar and further confirmed by serogroup antiserum agglutination.

RNA extraction. Total RNA was isolated from pig mesenteric lymph node. Briefly, frozen tissues were disrupted in liquid nitrogen using a mortar and pestle and homogenized by a rotor-stator homogenizer (PRO Scientific, Oxford, CT). Total RNA was extracted from ~200 mg of tissue using an RNeasy Midi kit and DNase I-treated with the RNase-free DNase set (Qiagen, Valencia, CA). RNA from the blood samples was isolated from the PAXgene Blood RNA tubes using the PAXgene Blood RNA kit according to the manufacturer's instructions (Qiagen). Total RNA integrity, quality and quantity were assessed using the Agilent Bioanalyser 2100 and RNA Nano 6000 Labchip kit (Agilent Technologies, Palo Alto, CA).

Transcriptional analysis by DNA microarrays. Five µg total RNA was used for first and second strand cDNA synthesis according to manufacturer instructions (Affymetrix, Inc. Santa Clara, CA). The double stranded cDNA was purified, tested on an Agilent Bioanalyser 2100, and served as a template for the subsequent *in vitro* transcription (IVT) reaction for cRNA amplification. Labeling with cRNA biotin was performed by the GeneChip® One-Cycle target labeling kit (Affymetrix; Expression Analysis Technical Manual). Quality of the labeled cRNA was tested on an Agilent Bioanalyser 2100. Subsequently, labeled cRNA was fractionated and hybridized with the GeneChip® Porcine Genome Array according to the standard procedures provided by the manufacturer. Chips were washed and stained with a GeneChip Fluidics Station 450

(Affymetrix, Inc. Santa Clara, CA) using the standard fluidics protocol. Chips were then scanned with an Affymetrix GeneChip Scanner 3000 (Affymetrix, Inc. Santa Clara, CA).

MAS 5.0 (microarray analysis system 5.0, Affymetrix, Inc. Santa Clara, CA) default normalization methods were used to obtain the expression measure for each probeset. Logarithms were then taken on these expression measures. The median of the log expression measures for each chip was then subtracted from all the log expression measures on the same chip. Differentially expressed genes were identified by analyzing these normalized data using a general linear model analysis in SAS (SAS Insititue, Cary, NC) on a gene by gene basis.

Real-time PCR. Total RNA from the mesenteric lymph nodes and whole blood of persistent shedders (n=6) and non-shedders (n=4) pigs at 0, 2 and 20 d pi (60 samples total) was reverse transcribed to cDNA using Superscript reverse transcriptase (Invitrogen Corporation, Carlsbad, CA) and oligo-dT primer as previously described. For gene expression analysis, real-time PCR was performed in duplicate for each individual pig using 10 ng of cDNA (RNA equivalent)/25 µl reaction/well using the SYBR Green PCR master mix (Applied Biosystems Inc., Foster City, CA) and an BioRad Chromo4 Detector (BioRad, Hercules, CA). Thermal cycling parameters were as follows: 95°C for 10 min, 40 cycles at 95°C for 15 sec and 60°C for 1 min. At the 60°C step, fluorescent data acquisition was performed. Following PCR cycling, disassociation curve analysis was performed from 90°C to 55°C with 2°C increments and a hold time of 1 sec. Analysis of the disassociation curves (as well as agarose gel electrophoresis) confirmed that fluorescent signal was generated only from specific cDNA transcripts.

ELISA assay for interferon-γ (IFNG). To determine concentration of circulating IFNG, serum of 40 experimental pigs at day 2 p.i. was analyzed by ELISA using the porcine IFNG (Pierce, Rockford, IL) ELISA kit according to the manufacturer's instructions.

DNA extraction. To 1 ml of blood, 4 ml of solution A containing 0.32M sucrose, 1mM Tris HCl pH 7.5, 5mM $MgCl_2$ and 1% Trition X -100 was added. Following 10 minutes of centrifugation at 3000 rpm at 4°C, the pellet was resuspended in 8 ml of solution B (10mM Tris pH 8.0, 0.4 M NaCl, 2mM EDTA pH 8.0). Centrifugation was repeated and the pellet was resuspended in 4.5 ml of solution B. The addition of 0.5 ml of solution C (5% SDS, 2mg/ml Protease K in 2 ml H_2O) was followed by incubation at 45-55°C. Following overnight incubation in a shaking water bath, 1.3 ml 6M NaCl was added, the solution was vortexed for 15 seconds and the DNA was pelleted by centrifugation at 7500 rpm, 4°C for 15 minutes. The DNA was precipitated in 15 ml 95% ethanol, pelleted by centrifugation and washed the pellet in 1ml 70% ethanol. The DNA was air dried and resuspended in 0.1 ml 1X TE.

SNP identification. Several sequence analysis tools were employed to investigate potential SNPs in selected genes, including analysis of available bioinformatics data in web databases such as TIGR pig gene index search tool (http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/gimain.pl?gudb=pig) and NCBI SNP database (http://www.ncbi.nlm.nih.gov/projects/SNP/) as well as the software program Sequencher, to compare published sequence data of the selected genes (gene sequences available at http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=unigene). SNPs identified by computational means were further verified using our experimentally, *Salmonella*-infected pig population. To confirm potential SNPs and to reveal polymorphic allele frequencies in the population, target sequence regions were amplified by PCR followed by sequencing of 4 DNA pools, each representing a different pig breed. After the target SNP was confirmed by DNA sequencing, the entire experimental population was genotyped using restriction fragment length polymorphism (RFLP) assay for correlation analysis.

Correlation and association analyses. Correlation of serum levels of IFNG with *Salmonella* shedding as well as different blood cell counts was statistically analyzed using multivariate permutation test for Goodman and Kruskal's Gamma correlation with family-wise error rate controlled at 0.05.

Results

Objective I

Non-shedder and persistent shedder pigs. Forty 7 week old pigs were intranasally inoculated with *Salmonella enterica* serovar Typhimurium and monitored for fecal shedding over a 3 week period (**Table 1**). Four pigs were identified as non-shedders based on their initial *Salmonella* fecal positive status at 2 days post-inoculation (dpi) but lack of *Salmonella* shedding at 2 out of the 3 samplings at 7, 14, and 20 dpi, including 20 dpi. Six pigs were classified as persistent shedders based on their high numbers of cfu of *Salmonella* per gram of feces at all four sample time points.

Table 1. Colony forming units of *Salmonella* per gram of feces (cfu/g) following experimental inoculation. Qualitatively positive (+) or negative (-) data is given if the number of *Salmonella* was below the level of detection for the quantitative method. Qualitative data is also given for the ileocecal lymph nodes (ICLN).

Pig #	Classification	2 dpi (cfu/g feces)	7 dpi (cfu/g feces)	14 dpi (cfu/g feces)	20 dpi (cfu/g feces)	21 dpi (+ or -)
101	Non-shedder	100	112	-	-	-
102		200	128	+	112	-
103		120000	+	783	+	+
104		-	3178	385	-	-
105	Persistent shedder	120000	2526	373	194	+
106	Non-shedder	+	76	-	-	-
108	Persistent shedder	13800	3077	94	313	-
109		2500	+	+	-	-
110		3900	732	83	94	+
111		10200	1683	+	+	-
112		1000	5571	+	+	+
113		+	857	108	+	+
114		+	286	+	455	+
115	Persistent shedder	3500	11782	4330	413	+
116		100	-	500	+	+
117		22000	380	-	+	-
118	Persistent shedder	13000	421	1778	175	+
119		+	+	1509	+	-
121		400	345	1667	+	-
122		100	116279	+	+	-
123		4900	81	189	+	-
124		1100	531	+	+	+
125		300000	15968	+	+	-
126		95800	1101	119	+	+
128		200	+	85	+	-
129		+	+	+	+	-
130		400	388	88	322	-
131		+	+	+	+	-
132		43600	5149	1282	+	-
133		600	242	+	+	+
134	Persistent shedder	30000	4928	21667	54054	+
135		700	+	+	-	-
136		82400	23659	1197	+	+
137		+	2258	+	+	+
138		-	+	-	+	+
140	Non-shedder	+	-	-	-	-
141		26600	49429	2177	+	-
142	Persistent	320000	151515	1393	238	+

	shedder					
143	Non-shedder	+	-	+	-	-
144		200000	74646	+	+	+

Blood analyses of *Salmonella* infected pigs. As shown by our research group and others, the level interferon-γ (IFNG), a potent T helper 1 cytokine important in the host's immune response to infection, is elevated during infection with *Salmonella*. We have recently shown that the level of IFNG during serovar Typhimurium infections increases during the first 48 hours post-inoculation, then drops to the level of non-infected pigs by 7 dpi. Using an ELISA assay, the levels of IFNG in the blood were determined for the 40 pigs at 2 dpi (**Figure 1**).

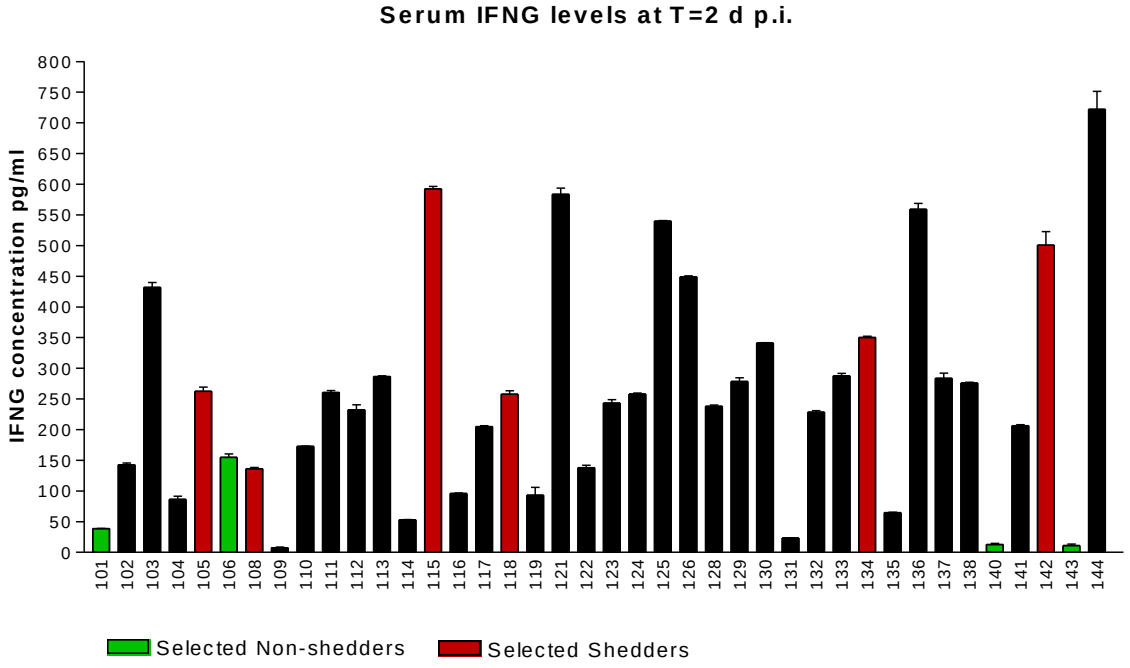

Figure 1. Serum IFNG levels at 2 dpi with serovar Typhimurium.

To determine if a connection exists between the levels of IFNG at 2 dpi and the shedding of *Salmonella* from the infected pigs, correlation analysis was performed. A significant and positive correlation was determined for IFNG levels at 2 dpi with bacterial shedding at 2 d and 7 d pi (**Figure 2**). In other words, the higher the IFNG level in the pigs at 2 dpi, the greater the bacterial shedding of *Salmonella* at 2 and 7 dpi.

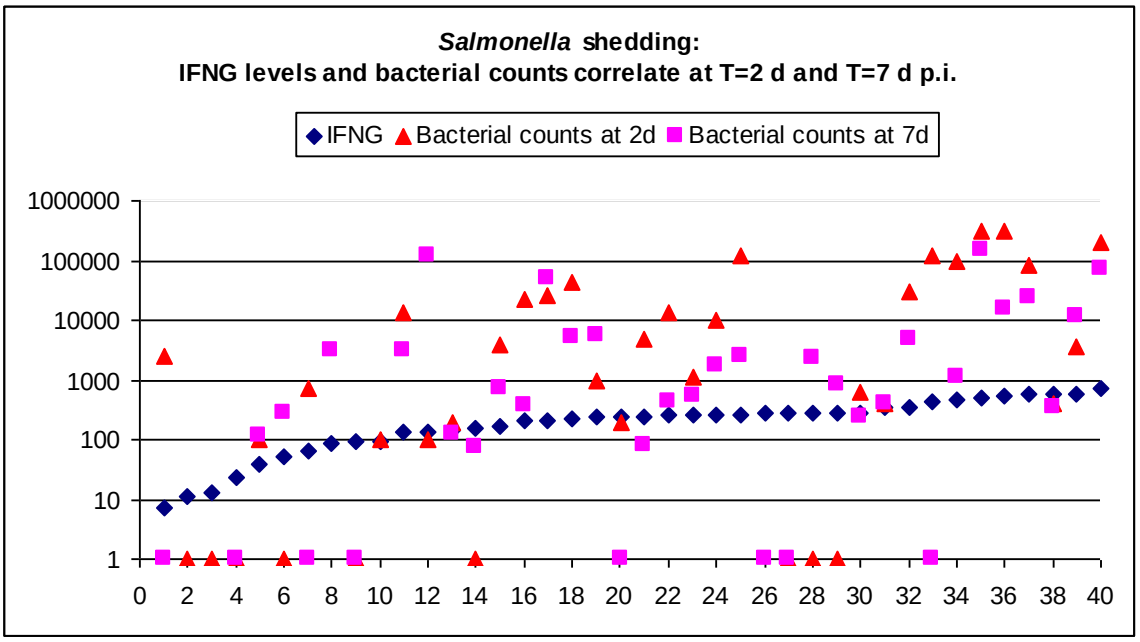

Figure 2. Serum IFNG levels at 2 dpi correlates with *Salmonella* shedding at 2 and 7 dpi. IFNG concentrations (pg/ml) and *Salmonella* cfu/g feces are plotted on theY-axis (log scale). The X-axis represents the pig population (n=40) sorted according their IFNG levels.

Blood from the 40 pigs was also quantitatively analyzed for cells important in the immune response to infection: neutrophils, lymphocytes, monocytes and white blood cells (WBC). Correlation analyses identified the following positive correlations to IFNG levels at 2 dpi:

- WBC counts at 7, 14 and 21 dpi (**Figure 3**)
- Circulating neutrophils at 7 and 14 dpi and mature banded neutrophils at 2 dpi (**Figure 4**)
- monocytes at 7 dpi (**Figure 5**)

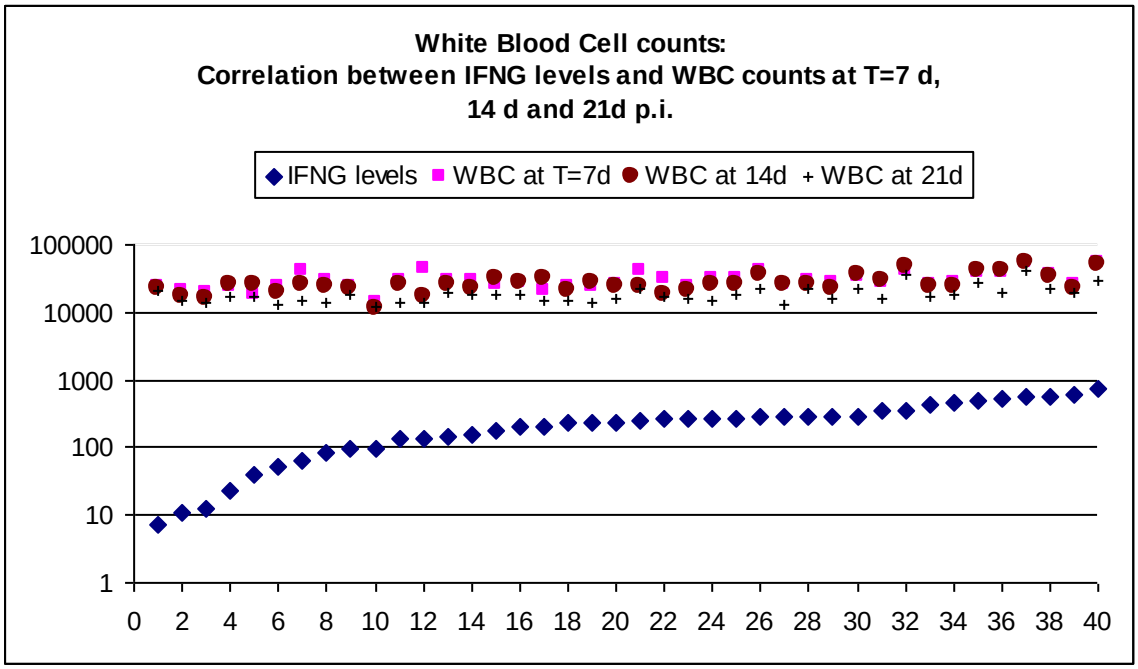

Figure 3. Correlation analysis between serum IFNG levels at 2 dpi and WBC counts. IFNG concentrations (pg/ml) and WBC counts/ml blood at 7, 14 and 21 dpi are plotted on the Y-axis (log scale). The X-axis represents the pig population (n=40) sorted according to IFNG levels.

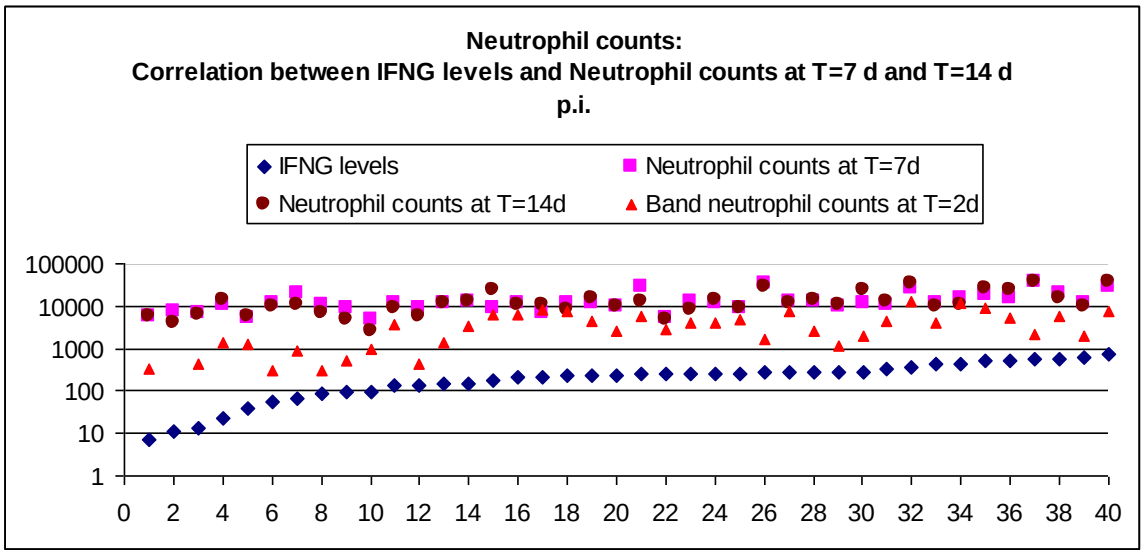

Figure 4. Correlation analysis between serum IFNG levels at 2 dpi and blood neutrophil counts. IFNG concentrations (pg/ml) and total neutrophil counts/ml blood at 7 and 14 dpi and mature band neutrophil counts at 2 dpi are plotted on the Y-axis (log scale). The X-axis represents the pig population (n=40) sorted according to IFNG levels.

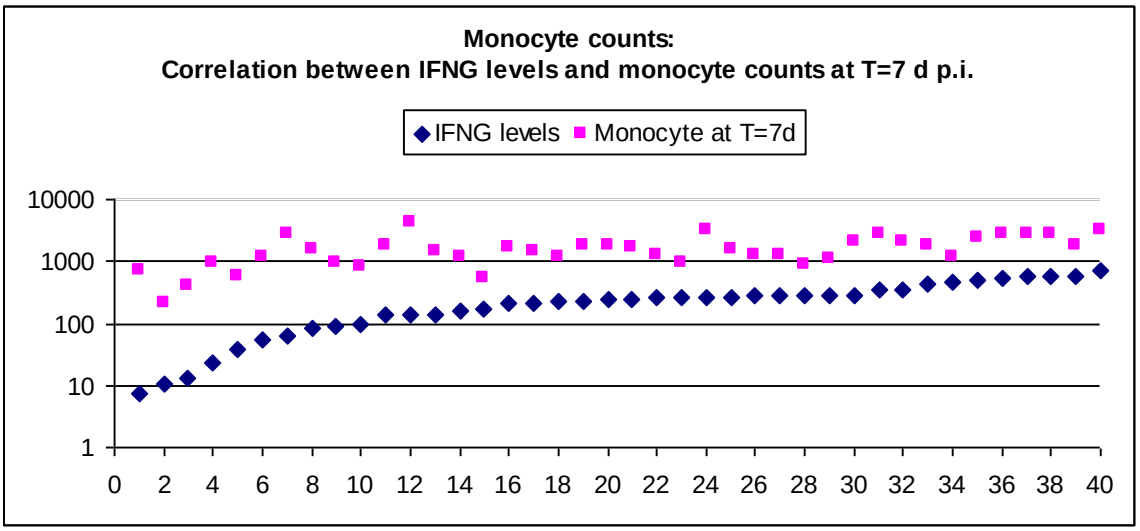

Figure 5. Correlation between serum IFNG levels at 2 dpi and monocyte counts. IFNG concentrations (pg/ml) and monocyte counts/ml blood at 7 dpi are plotted on the Y-axis (log scale). The X-axis represents the pig population (n=40) sorted according to IFNG levels.

Differential gene expression. To identify genes that may be responsible for the carrier status of *Salmonella* in swine, we searched for genes that were expressed differently between the non-shedder and persistent shedder groups at 21 dpi. Transcriptional profiling of the non-shedder (n=4) and persistent shedder (n=6) pigs was performed using the Affymetrix porcine DNA microarray representing over 22,000 swine genes. Analysis of the RNA isolated from the mesenteric lymph nodes of the 10 pigs at 21 dpi revealed only seven genes differentially expressed between the low and high shedder groups (fold change >1.5, $p<0.01$, $q<0.22$) (**Table 2**). The cellular functions of several of these genes have been identified in humans: apoptosis, cytoskeleton rearrangements, signal transduction, protein degradation and immunity. Genes with unknown functions were also identified as differentially-expressed between the low- and high-shedders.

Table 2. Genes differentially expressed in non-shedder versus persistent shedder pigs at 21 dpi.

Affy ID	Human NM number	Gene name and official gene symbol	Gene function	Fold change	p	q
Ssc.27354.1.S1_at	NM_139244	syntaxin binding protein 5 (STXBP5)	transport/cargo protein family; also involved in signal transduction and cell communication; plays a role in neurotransmitter release by stimulating SNARE complex formation.	5.29	0.00013	0.19
Ssc.6694.1.S1_at	NM_018947	cytochrome c; somatic (CYCS)	component of the electron transport chain in mitochondria and involved in initiation of apoptosis through activation of procaspase 9	2.42	0.00012	0.19
Ssc.19323.1.S1_at	NM_138389	hypothetical protein BC001096	Biological function unknown. The protein posseses a coiled coil domain.	2.17	0.00006	0.19
Ssc.9975.1.A1_at		gi:15178549, Ssc.9975, Transcribed locus, S. scrofa	Function unknown	2.10	0.00003	0.19
Ssc.14558.1.S1_at	NM_000442	plateletendothelial cell adhesion molecule (CD31 antigen) (PECAM1)	Role in modulation of integrin-mediated cell adhesion and migration, angiogenesis, apoptosis, signal transduction. Negative regulator of immune cell signalling, autoimmunity, macrophage phagocytosis, neutrophil transmigration. Sensor of oxidative stress, perhaps most importantly during the process of inflammation.	1.77	0.00010	0.19
Ssc.6524.2.S1_at	NM_015373	PKD2 interactor; golgi and endoplasmic reticulum associated 1 (PGEA1)	Interacts directly with beta-catenin, inhibiting oncogenic beta-catenin-mediated transcriptional activation.	1.76	0.00018	0.19
Ssc.29722.1.S1_at		gi:6745680, Ssc.29722, Transcribed locus, S. scrofa	Function unknown	1.51	0.00022	0.22

Additionally, RNA was extracted from blood collected from the low- and high-shedders during the acute (2 days post-inoculation) and chronic (20 days post-inoculation) stages of infection as well as from the pigs prior to inoculation with *Salmonella*. In our search for genes whose expression could possibly serve as markers for *Salmonella* infection, we performed real-time PCR tests to determine the gene expression profile in this group of pigs for the CXCL10 gene. CXCL10 is a gene identified in our previous work as up-regulated during the acute phase of infection. However, CXCL10 expression does not appear to differ distinctly between the low and high *Salmonella* shedders (**Figure 6).**

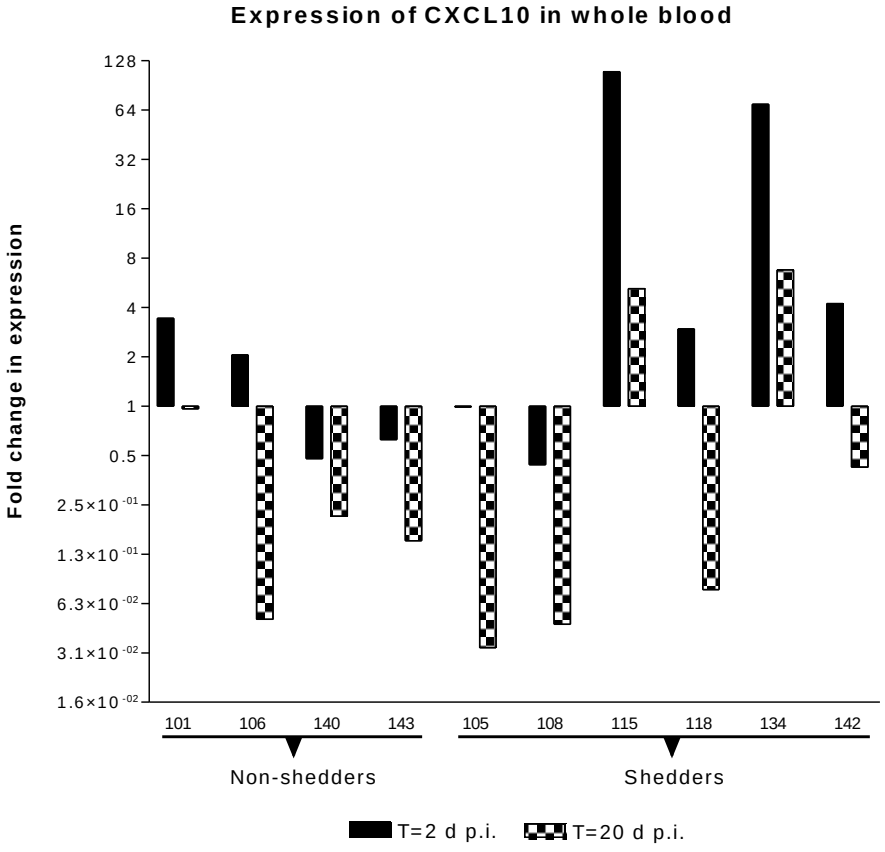

Figure 6. Real-time PCR of CXCL10 from porcine blood RNA at 2 and 20 dpi with *Salmonella* Typhimurium.

Objective II

Identifying Single Nucleotide Polymorphisms (SNPs) in porcine genes differentially expressed during *Salmonella* infection. Over the last few years, our research group has employed various molecular techniques to identify many genes that are differentially regulated in the pig during infection with *Salmonella* (Uthe et al, Vet Micro 2006; Zhao et al, Mammalian genome 2006; Uthe et al, Mol Immunol 2007; Wang et al, submitted to Genomics). As potential candidates for affecting the porcine response to *Salmonella* (and, thus, the outcome of disease), several of these genes were chosen for sequence analysis to search for sequence variants (SNPs): CXCL10, SDCBP, ARPC2, HSPH1, CXCL2, CCT7, LCP1, VCP, CD47/IAP, SCARB2, CD163, MARCO, CCR1, IL8, TYROBP and NCF2. Our analyses confirmed 12 SNPs in 11 genes that appear to respond to and/or manage the host's response to infection (**Table 3**).

Table 3. Single Nucleotide Polymorphisms (SNPs) identified in genes that are differentially regulated during infection with *Salmonella*.

Gene name	Identified SNPs	Confirmed SNPs	Polymorphic allele frequencies (%)	Amino acid	Location of confirmed SNPs	PCR primers (5'– 3')	Enzymes for RFLP
SCARB2	2	1	A 44, G 55	-	3'UTR	f: ATGGATGAGGGAACTGCAGACGAA r: TCAGGGACCACTGGCTTGAAGAAA	Tse I
SDCBP	1	1	A 21.3, G 78.8	V / I	Exon 3	f: GGAGCTCTCTCAGTACATG r: CCCCTGAACTGGTGCTC	Rsa I
VCP	3	1	A 30, G 70	E	Exon10	f: GTCGCTTTGACAGGGAGGTAG; r: TGTTCAAGGTCCACATCATCTG	BstY I
CD163	9	1	T 73.8, C 26.3	C	Exon 12	f: ATATGGCTCAATGAAGTGAAGTG r: GGGATTCTCGGCTCTTTGC	Afl III
CCT7	3	1	G 76.3, A 23.8	L	Exon 9	f: TCCAGACCAGTGTGAATGC r: CCACCACGGAGGATGATAG	Sau96 I
CCR1	5	1	A 45, G 55	-	3'UTR	f: CCCATCAGCAGAACCACAAC r: GATTTATTGTCTTGGGAAAGTGAT	Acu I or Tsp509 I
LCP1	2	1	T 85, C 15	D	Exon 2	f: CTGCCTGCTTGCCTCTG r: GATAAACTCGTCAAAGCTGATC	BtsC I
CD47/IAP	2	1	G 90, A 10	-	3'UTR	f: GTGCACCTGTGTAAGTTAGGCAC; r: CAGCAAACCACTTGGTCCCAGAAT	Tsp509 I
IL8	1	1	T 33.8, C 66.3	-	3'UTR	f: TCAGTAAAGATGCCAACACAAC r: ACAAAAAGCCAAAACAGGATTTC	Tsp509 I
TYROBP	3	2	T 66.3, A 33.8; C 58.8, T 41.3	R A	Exon 3	f: TGGTGCTGACCCTCCTC r: CTCAGCGATGTGTTGTTTCC	Afl III, Btg I
NCF2	6	1	C 80, T 20	?	Exon 6	f: ACCACAGAACCTCACCTAAAG r: ATGATGTCCCCTTCCAGAAAG	Acu I

The identified sequence variants (SNPs) were tested for their association to *Salmonella*-shedding levels, serum levels of IFNG and the various blood cell counts. To look for meaningful associations, one-way ANOVA analysis was employed (model: $y=\mu+genotype+\varepsilon$) followed by students t test. The analysis was further verified by multivariate permutation test with controlled family-wise error rate. Statistical analysis indicates a positive association of SNP genotype G/A of the CCT7 gene with circulating neutrophils and WBCs ($p<0.05$) as well as *Salmonella* shedding ($p=0.0012$) at 7 dpi compared to the G/G genotype in our 40 pig experiment. As shown in **Figure 7**, the pigs shedding *Salmonella* at higher levels were more likely to have the G/A genotype that the pigs with the DNA sequence G/G.

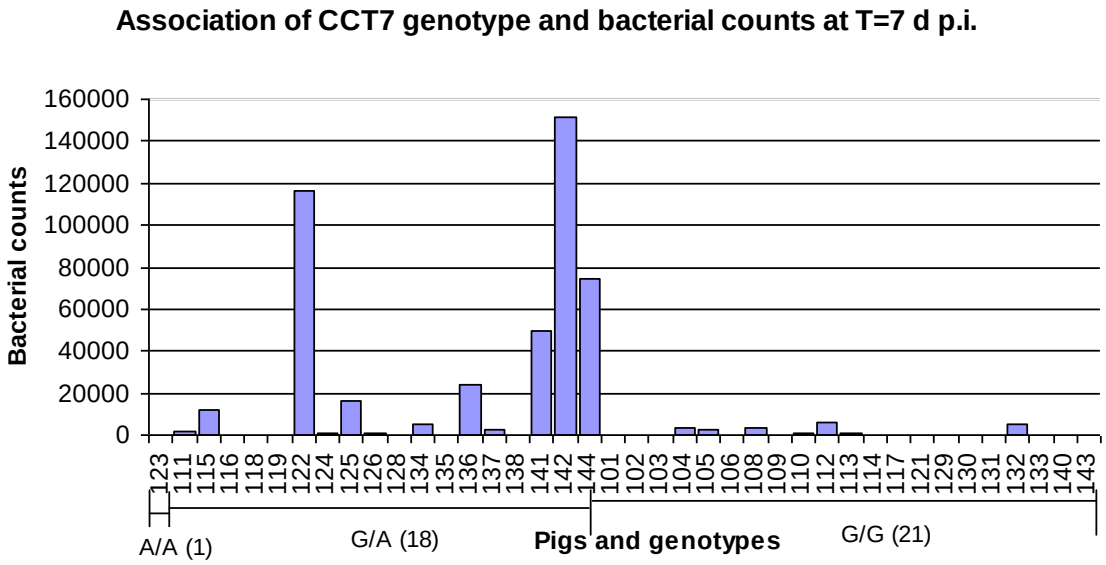

Figure 7. Association (p=0.0012) between CCT7 genotypes A/A, G/A and G/G with *Salmonella* shedding (cfu/g feces) at day 7 p.i. Number of pigs in each genotype is indicated in the brackets.

Discussion

Salmonella can establish a carrier state in pigs, thereby providing a reservoir for the pathogen. Once the *Salmonella*-carrier pig is placed under stressful conditions (i.e. transportation, mixing, etc.), the pathogen can re-emerge from the animal and be shed in the feces to contaminate/infect pen mates as well as the environment. Therefore, it is not only important to identify the pigs that are carriers and may become shedders, but it is also important to prevent the carrier status and eliminate *Salmonella* shedding. The goals of this research project were to address these issues by 1) identifying markers associated with *Salmonella* status, 2) investigating porcine genes that may play a role in *Salmonella* shedding and 3) characterizing genetic variations in porcine response genes that may associate with *Salmonella* shedding in swine.

Our data indicates an important role for IFNG, a cytokine involved in stimulating Th1 immunity, in the pig's response to *Salmonella* infection. As demonstrated by correlation analysis, the higher the IFNG levels in the pigs at 2 dpi, the more likely they were to shed higher numbers of *Salmonella* in their feces at 2 and 7 dpi. Furthermore, elevated levels of IFNG during the acute stage of infection (2 dpi) were also associated with greater numbers of immune cells circulating in the blood including white blood cells (7, 14 and 20 dpi), neutrophils (2, 7 and 14 dpi) and monocytes (7 dpi).

The association of single nucleotide polymorphisms (SNPs) with a selected phenotype can provide DNA markers for identification and selection for a desired trait. This project identified 12 SNPs in 11 genes that are associated with and potentially control *Salmonella* infections in swine. Furthermore, a positive association was identified for the CCT7 gene and bacterial shedding of *Salmonella* at 7 dpi: the pigs shedding higher levels of *Salmonella* at 7 dpi were more likely to contain the G/A genotype and the pigs shedding lower levels of *Salmonella* were more likely to contain the G/G genotype.

CCT7 is one of the 8 subunits of CCT a cytosolic chaperonin that belongs to group II chaperonin family. Classically, the CCT complex is known to assist in folding of actin and tubulin in ATP-dependent

manner. However, more resent studies indicate that CCT is involved in folding of about 9-15% of newly synthesized proteins including cytoskeleton-associated and cell cycle regulatory proteins. Furthermore, as an adaptor protein, CCT plays a role in inhibition of NO-stimulated sGC activity. The expression of CCT is controlled at the mRNA level and is closely associated with cell growth and cell cycling in mouse and human cells.

Reducing the incidence of pre-harvest *Salmonella* infections in pigs will provide a safer food supply, improve animal health, lower producer's expenditures, and provide competition for new international markets. To achieve disease control, multiple approaches should be examined and may need to be implemented; examples of such approaches may include biosecurity, vaccination, feed composition and additives, etc. Our research group is using a functional genomics approach - identifying genes that respond to infection by increasing (or decreasing) their expression level and use this expression information to develop markers associated with decreased disease transmission (bacterial shedding) to improve pig genetics. Due to the complexity of identifying and accurately measuring disease resistance in the field, we have first identified genes that correlate with disease in experimentally challenged pigs. These disease markers will then be tested in real world populations. Information about such genes may also be used to develop diagnostic tools to identify "carrier" animals, a major source of *Salmonella* contamination.

Lay Interpretation

Pigs that shed *Salmonella* in their feces are an animal health issue, a food safety problem and an environmental contamination risk. Our research program investigates the undesirable carrier state of *Salmonella* in pigs in order to identify approaches to control both clinical and sub-clinical (carrier) infections. Our research has identified pig genes that respond to infection with *Salmonella*. A DNA sequence variation was identified in one of the *Salmonella*-response genes (CCT7). When we compared the sequence of the CCT7 gene with the *Salmonella* shedding status of 40 infected pigs, we found that pigs with a specific DNA sequence were less likely to shed *Salmonella* in their feces than pigs with a different DNA sequence. Thus, pigs with this specific DNA sequence may be more resistant to infection with *Salmonella*. Furthermore, the amount of a specific immune compound (interferon-γ) in the blood of the infected pigs also correlated the *Salmonella* shedding status of the pigs as well as specific blood cells that fight infection. Identifying factors in the pig that control the ability of the animal to combat disease will uncover markers for classifying potential carrier pigs as well as targets for disease diagnosis, intervention and prevention.

Contact information:
Shawn Bearson
USDA, ARS, National Animal Disease Center
2300 Dayton Ave
Ames, IA 50010
sbearson@nadc.ars.usda.gov