Associations of the porcine immune response and genetic polymorphisms with the shedding of *Salmonella enterica* serovar Typhimurium

Bearson, S.(1) , Utte, J.(1,2) , Wang, Y.(2) , Qu, L.(2) , Dekkers, J.(2,3) , Nettleton, D.(3,4) , Bearson, B.(3) , Tuggle, C.(2,3)

(1) USDA, ARS, National Animal Disease Center, Ames, IA, 50010, USA  
(2) Department of Animal Science, Iowa State University, Ames, IA, 50011, USA  
(3) Center for Integrated Animal Genomics, Iowa State University, Ames, IA, 50011, USA  
(4) Department of Statistics, Iowa State University, Ames, IA, 50011, USA  
(5) USDA, ARS, National Soil Tilth Lab, Ames, IA, 50011 USA

Shawn M.D. Bearson: sbearson@nadc.ars.usda.gov

**Abstract**

A major focus of our collaborative research 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. 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 between IFNG levels and 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 9 genes: VCP, CCT7, LCP1, CD47, SCARB2, CD163, CCR1, TLR4 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. Specifically, preliminary analysis suggests a positive association ($p<0.05$) of SNP genotype A/G at nucleotide 1026 (relative to start codon) of the CCT7 gene with circulating neutrophils and WBCs and with *Salmonella* shedding at 7 dpi compared to the G/G heterozygote genotype. CCT7 encodes a molecular chaperone involved in tubulin folding and protection. 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 (1); 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.

A major problem in pre-harvest food safety is contamination on the farm or slaughter plant environment by animals shedding pathogenic bacteria such as *Salmonella enterica* (2,3). Another 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 investigate specific molecular parameters for their...
contribution to Salmonella 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.

Materials and methods

Animal study. Forty conventionally raised, mixed sex piglets from sows identified as fecal-negative for Salmonella spp. were weaned at 10 days of age, shipped to the NADC, Ames, IA and raised in climate-controlled, fully enclosed isolation facilities. At seven weeks of age, the Salmonella-free pigs were intranasally challenged with 1 x 10^9 cfu of serovar Typhimurium χ4232 grown in Luria Bertani (LB) broth at 37°C. 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 ileocecal lymph nodes (ICLN) were aseptically collected for quantitative bacteriology. Blood samples were collected for the following procedures: DNA extraction, CBC analysis (ISU Veterinary Diagnostic Laboratory), and serum preparation for cytokine assays.

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 ICLN 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. Following 24 hours of incubation at 37°C, 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. For qualitative bacteriology of Salmonella, the following was performed: 1 gram (fetal) 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 37°C, respectively. Following incubation, 100 μl of each culture was transferred to 10 ml Rappaport-Vassiliadis medium (RV, Difco, Detroit, MI) and incubated at 37°C 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.

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.

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/tgi-bin/tgi/index.pl?gdb=pig) and NCBI SNP database (http://www.ncbi.nlm.nih.gov/projects/SNP/) as well as the software program Sequencher (Genecodes, Ann Arbor, MI), 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, created by sampling litters from sows with similar breed backgrounds. After the target SNP was confirmed by DNA sequencing, the entire experimental population was genotyped using restriction fragment length polymorphism (RFLP).

Correlation and association analyses. The correlation of serum levels of IFNG with Salmonella shedding as well as different blood cell counts was statistically analyzed using the multivariate permutation test for Goodman and Kruskal's Gamma correlation, with family-wise error rate (FWER) controlled at 0.05. Association analysis of each SNP to Salmonella shedding and blood cell counts was performed by one-way ANOVA (model: y=μ+genotype+ε) followed by Student t tests, with FWER controlled at 0.05 by multivariate permutation tests.
Identifying 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. Four pigs were identified as non-shedders based on their initial Salmonella fecal positive status at 2 days post-inoculation (dpi) and 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>
<thead>
<tr>
<th>Pig #</th>
<th>Classification</th>
<th>2 dpi (cfu/g feces)</th>
<th>7 dpi (cfu/g feces)</th>
<th>14 dpi (cfu/g feces)</th>
<th>20 dpi (cfu/g feces)</th>
<th>21 dpi (cfu/g feces)</th>
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</thead>
<tbody>
<tr>
<td>101</td>
<td>Non-shedder</td>
<td>100</td>
<td>112</td>
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<td>-</td>
<td>-</td>
</tr>
<tr>
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<td>Persistent shedder</td>
<td>120000</td>
<td>2526</td>
<td>373</td>
<td>194</td>
<td>+</td>
</tr>
<tr>
<td>106</td>
<td>Non-shedder</td>
<td>+</td>
<td>76</td>
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<td>-</td>
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</tr>
<tr>
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<td>94</td>
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<tr>
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<td>Persistent shedder</td>
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<td>11782</td>
<td>4330</td>
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</tr>
<tr>
<td>118</td>
<td>Persistent shedder</td>
<td>13000</td>
<td>421</td>
<td>1778</td>
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</tr>
<tr>
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<td>21667</td>
<td>54054</td>
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<tr>
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<td>+</td>
<td>-</td>
<td>-</td>
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<tr>
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<td>1393</td>
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<tr>
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<td>Non-shedder</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Blood analyses of Salmonella infected pigs. As shown by our research group and others (5), the level of 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 blood were determined for the 40 pigs at 2 dpi. To determine if a connection exists between the levels of IFNG at 2 dpi and shedding of Salmonella from the infected pigs, correlation analysis was performed. A significant positive correlation (FWER<0.05) was found for IFNG levels at 2 dpi with bacterial shedding at 2 and 7 dpi (p<0.05). In other words, the higher the IFNG level in pigs at 2 dpi, the greater the Salmonella shedding at 2 and 7 dpi.

Blood was also quantitatively analyzed for cells involved in immune response to infection. Correlation analyses identified positive correlations between IFNG levels at 2 dpi with: white blood cell counts at 7, 14 and 21 dpi (p<0.05); circulating neutrophils at 7 dpi and mature banded neutrophils at 2 dpi (p<0.01); monocytes at 7 dpi (p<0.01).
Identifying Single Nucleotide Polymorphisms 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. 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 were identified in the following genes: CXCL10, SDCBP, ARPC2, HSPH1, CXCL2, CCT7, LCP1, VCP, CD47/IAP, SCARB2, CD163, MARCO, CCR1, IL8, TYROBP and NCF2. The identified SNPs were tested for their association to Salmonella-shedding levels, serum levels of IFNG and the various blood cell counts. Statistical analysis indicated a positive association of SNP genotype G/A of the CCT7 gene with Salmonella shedding (p=0.0102) as well as circulating neutrophils (p=0.0102) and WBCs (p=0.0152) at 7 dpi compared to the G/G genotype in our 40 pig experiment. As shown in the graph, pigs shedding Salmonella at higher levels were more likely to have genotype G/A than genotype G/G.

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 pigs that are carriers and may become shedders, but it is also important to prevent carrier status and eliminate Salmonella shedding. The goals of this research project were to address these issues by 1) investigating porcine genes that may play a role in Salmonella shedding and 2) characterizing genetic variations in porcine response genes that may associate with Salmonella shedding in swine. Our data suggests an important role for IFNG, a cytokine involved in stimulating Th1 immunity, during Salmonella infections, since higher levels of IFNG correlated with greater numbers of circulating immune cells and with Salmonella shedding. Furthermore, genetic variation in the gene encoding CCT7, a molecular chaperone involved in protein folding (4) and identified by our group as up-regulated in Salmonella-infected pigs (5), was associated with Salmonella shedding. Since the pigs used in this study were of mixed breed composition, this association could represent breed differences and must be validated in other populations. Investigating factors in the pig that control the ability of the animal to combat disease will assist in developing diagnostic tools for classifying potential carrier pigs as well as identify genetic markers to select for Salmonella resistant pigs.

Conclusions

From this study, our preliminary conclusions are as follows:

1. During acute infection with Salmonella, pigs with higher IFNG levels are more likely to shed greater numbers of Salmonella.
2. During an acute Salmonella infection, pigs with higher IFNG levels have greater circulating white blood cells, neutrophils and monocytes.
3. Within the studied mixed breed population, pigs with the SNP genotype G/A in the CCT7 gene are more likely to shed Salmonella than pigs with the G/G genotype.

Our current research goal is to test these associations in a larger pig population.

References


