



Short Communication

Correlating blood immune parameters and a CCT7 genetic variant with the shedding of *Salmonella enterica* serovar Typhimurium in swine[☆]J.J. Uthe^{a,b}, Y. Wang^b, L. Qu^{b,d}, D. Nettleton^{c,d}, C.K. Tuggle^{b,c}, S.M.D. Bearson^{a,*}^a USDA, ARS, National Animal Disease Center, Ames, IA 50010, USA^b Department of Animal Science, Iowa State University, Ames, IA 50011, USA^c Center for Integrated Animal Genomics, Iowa State University, Ames, IA 50011, USA^d Department of Statistics, Iowa State University, Ames, IA 50011, USA

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ABSTRACT

The porcine response to *Salmonella* infection is critical for control of *Salmonella* fecal shedding and the establishment of *Salmonella* carrier status. In this study, 40 crossbred pigs were intranasally inoculated with *Salmonella enterica* serovar Typhimurium (*Salmonella* 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* Typhimurium shedding levels at 2 and 7 dpi and serum interferon-gamma (IFN γ) levels at 2 dpi ($p < 0.05$), with *Salmonella* being shed in greater numbers from animals with higher IFN γ levels. A positive correlation was also observed between IFN γ levels and the number of banded neutrophils (2 dpi), circulating neutrophils (7 and 14 dpi), monocytes (7 dpi), and white blood cells (WBCs) (7, 14 and 20 dpi). We have further performed association studies on these immune response parameters as well as shedding status of the *Salmonella*-infected pigs with a single nucleotide polymorphism (SNP) in the porcine gene CCT7, previously shown by our group to be transcriptionally up-regulated in swine experimentally inoculated with *Salmonella* Typhimurium. Our analyses with the 40 pigs suggest a positive association ($p = 0.0012$) of SNP genotype A/G at position AK240296.c1153G > A of the CCT7 gene with *Salmonella* shedding at 7 dpi compared to the G/G homozygote genotype. Linking specific genes and genetic polymorphisms with the porcine immune response to *Salmonella* infection and shedding may identify potential markers for carrier pigs as well as targets for disease diagnosis, intervention and prevention.

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1. Introduction

A major problem in pre-harvest food safety is contamination on the farm or at the slaughter plant by animals shedding pathogenic bacteria such as *Salmonella enterica* (Ebner and Mathew, 2000; Hurd et al., 2001). An additional challenge is the difficulty in identifying which animals are carriers (and potential shedders) due to the often sub-clinical status of the *Salmonella* infection. As efficacy and restricted regulations influence the effectiveness of current antibiotic and vaccine treatments, the identification and

use of animals with enhanced disease resistance may serve as an alternative approach for disease control. If specific immune parameters and/or genes were identified from animals which are naturally more resistant to pathogens (e.g. *Salmonella*), direct improvement in food safety and animal health can be achieved by selecting favorable animals to breed disease-resistant offspring.

Immune cells, such as macrophages, monocytes and neutrophils, play a critical role in the innate immune response to *Salmonella enterica* infections by producing and releasing chemoattractants that recruit additional immune cells to the site of invasion and initiate a T helper 1 (Th1) response (Riber and Lind, 1999; Wick, 2004). The pro-inflammatory cytokine interferon- γ (IFN γ), has been shown by numerous investigations to play a pivotal role in the cell-mediated, Th1-dependent immune response during the early stages of *Salmonella* infection (Hyland et al., 2006; Splichal et al., 2002; Trebichavsky et al., 2003; Uthe et al., 2007; Zhao et al., 2006). The goal of this study was to investigate specific molecular and cellular parameters of the host's response to *Salmonella* Typhimurium infection, including porcine serum levels of IFN γ , the number of circulating blood immune cells and the specific genotype of a *Salmonella*-infection responsive gene (CCT7, chaperonin subunit) for their association with each other as well as to *Salmonella* shedding in swine. This information may assist in improving current and developing novel diagnostic assays to recognize animals harboring *Salmonella*, as well as support the identification of *Salmonella*-resistant lines of pigs.

2. Materials and methods

2.1. Animal study

Forty conventionally raised, mixed sex and breed piglets from sows identified as fecal-negative for *Salmonella* spp. were weaned at 10 days of age, shipped to the USDA-ARS-National Animal Disease Center (NADC) facility located in Ames, IA and raised in climate-controlled, fully enclosed isolation facilities. At 7 weeks of age, the *Salmonella*-free pigs were intranasally challenged with 1×10^9 colony forming units (cfu) of nalidixic acid resistant *Salmonella enterica* serovar Typhimurium χ 4232 (Fedorka-Cray et al., 1995) grown stationary in Luria Bertani (LB) broth at 37 °C. At 2, 7, 14, and 20 days post-inoculation (dpi), rectal temperatures as well as fecal and blood samples were collected from each animal. Blood samples were collected for the following procedures: DNA extraction using Promega's Wizard SV genomic DNA purification kit (Madison, WI), complete blood count (CBC) analysis to determine the number of cells per microliter of blood (performed by the Iowa State University, Veterinary Diagnostic Laboratory), and serum preparation for cytokine assays (see below). All procedures involving animals were lawful and approved by the USDA-ARS-NADC Animal Care and Use Committee.

2.2. Bacteriology

For quantitative bacteriology, 1 g of pig feces was combined with 5 ml phosphate buffered saline (PBS),

vortexed and 100 μ l directly plated to brilliant green agar with sulfadiazine (BGS, Difco, Detroit, MI) containing 30 mg/L nalidixic acid (10- and 100-fold dilutions were also performed for samples with >300 colonies per plate). Following 24 h 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 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 of each fecal sample was inoculated in 10 ml of GN-Hajna (GN, Difco, Detroit, MI) broth and tetrathionate (TET, VWR, Rutherford, NJ) broth for 24 and 48 h of growth at 37 °C, respectively. Following incubation, 100 μ l of each culture was transferred to 10 ml Rappaport-Vassiliadis medium (RV, Difco) and incubated at 37 °C for 18 h. The cultures were streaked on brilliant green agar plates with sulfadiazine (BGS) containing nalidixic acid. Colonies identified presumptively as *Salmonella* were streaked to triple sugar iron agar and lysine iron agar and further confirmed by serogroup antiserum agglutination.

2.3. ELISA assay for interferon- γ (IFN γ)

To determine the concentration of circulating IFN γ at 2 dpi, sera from the 40 experimental pigs were analyzed by ELISA using the porcine IFN γ (Pierce, Rockford, IL) ELISA kit according to the manufacturer's instructions.

2.4. Single nucleotide polymorphism (SNP) in porcine CCT7

The Institute for Genomic Research (TIGR) pig gene index search tool (<http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/gimain.pl?gudb=pig>) reported a potential synonymous SNP in the porcine CCT7 gene at position AK240296.c1153G > A. To confirm the predicted SNP, DNA from each of the 40 pigs in our population was isolated, and DNA sequence analysis (DNA Sequencing and Synthesis facility, Iowa State University, Ames, IA) was performed on four separate pools, each containing four DNA samples from the four breeds represented in the study (Landrace, Hampshire, Duroc and Yorkshire). Once the SNP was verified by sequence analysis, DNA from each individual pig was used to amplify the SNP region of the CCT7 gene using oligonucleotides SNP#2F (5'-TCCAGAC-CAGTGTGAATGC) and SNP#2R (5'-CCACCACGGAGGATGATAG). The PCR conditions were as follows: 5 min at 95 °C followed by 35 cycles of 1 min at 94 °C, 30 s at 55 °C, 1 min at 72 °C and final extension for 10 min at 72 °C. The SNP in the 533 bp PCR product was detected using restriction fragment length polymorphism (RFLP) analysis, with Sau96I producing fragments of 413, 85 and 35 bp (allele 1 = G) or 413 and 120 bp (allele 2 = A).

2.5. Correlation and association analyses

The correlation of serum levels of IFN γ with *Salmonella* shedding as well as different blood cell counts were

statistically analyzed using a multivariate permutation test (Yoder et al., 2004) for Goodman and Kruskal's Gamma correlation (Goodman and Kruskal, 1954) based on 50,000 random permutations of IFN γ levels while preserving the dependency among bacterial counts and blood cell counts. This procedure controls family-wise error rate (FWER) for all the 29 tests (5 counts for blood cells at each of day 0, 2, 7, 14 and 20; and 1 *Salmonella* shedding count at each of day 2, 7, 14, 20) according to Westfall and Young (1993). Association analysis of the CCT7 SNP to *Salmonella* shedding and blood cell counts was performed by permutation testing with 100,000 permutations. The Bonferroni method was used to control the FWER at the 0.05 level.

3. Results

3.1. Correlation of serum interferon- γ (IFN γ) levels at 2 dpi with *Salmonella* shedding status and blood immune cells in *Salmonella*-infected pigs

As shown by our research group and others (Hyland et al., 2006; Splichal et al., 2002; Trebichavsky et al., 2003; Uthe et al., 2007; Zhao et al., 2006), the level of interferon- γ (RNA and/or protein), a potent T helper 1 cytokine important in the host's immune response to intracellular pathogens, is elevated during infection with *Salmonella*. We have recently shown that the level of serum IFN γ during a *Salmonella* Typhimurium infection of swine increases during the first 48 h post-inoculation, then drops to the level observed in non-infected pigs by 7 dpi (Uthe et al., 2007). Using an ELISA assay, the levels of serum IFN γ were determined at 2 dpi for the 40 pigs intranasally inoculated with *Salmonella* Typhimurium (Fig. 1). To determine if a correlation exists between the levels of IFN γ at 2 dpi and fecal shedding of *Salmonella* from the infected pigs at 2, 7, 14, and 20 dpi, correlation analysis was performed. A significant positive correlation (FWER < 0.05) was found for IFN γ levels at 2 dpi with bacterial shedding at 2 and 7 dpi ($p < 0.05$). In other words, the higher the IFN γ level in pigs at 2 dpi, the greater the *Salmonella* shedding at 2 and 7 dpi (Fig. 1).

In addition, at 2, 7, 14 and 20 dpi, blood samples from the 40 pigs were analyzed to determine the numbers of cells involved in the immune response to *Salmonella* Typhimurium infection. Correlation analyses indicated a positive correlation (FWER < 0.05) between IFN γ levels at 2 dpi with white blood cell counts at 7, 14 and 20 dpi ($p < 0.05$), monocytes at 7 dpi ($p < 0.01$), circulating neutrophils at 7 and 14 dpi ($p < 0.01$) and banded neutrophils at 2 dpi ($p < 0.01$) (data not shown).

3.2. Association of the CCT7 porcine gene with *Salmonella* fecal shedding and blood immune cells at 7 dpi

A presumptive SNP identified by The TIGR pig gene index search tool in the CCT7 gene at position AK240296.c1153G > A was confirmed in this study using PCR-RFLP. Analyses were performed to determine if an association exists between the CCT7 sequence variant and *Salmonella*-shedding levels, serum levels of IFN γ , or counts of various white blood cell types in the population of the 40 pigs. Statistical analysis indicated an association of the CCT7 SNP with *Salmonella* shedding ($p = 0.0012$) at 7 dpi. As shown in Fig. 2, pigs shedding *Salmonella* at higher levels were more likely to have genotype G/A than genotype G/G. When using the Bonferroni method to adjust for multiple testing across 30 traits (serum level of IFN γ , *Salmonella*-shedding levels at four time points, and five blood cell counts at each of five time points), the p -value for CCT7 association with shedding became 0.036. No other associations with CCT7 were significant when controlling FWER at the 0.05 level. However, unadjusted p -values for CCT7 association with IFN γ at 2 dpi, circulating WBCs, neutrophils, and monocytes at 7 dpi, and circulating WBCs at 20 dpi were all less than 0.05 and suggestive of increased levels for pigs with the G/A genotype.

4. Discussion

Salmonella can establish a carrier state in pigs, thereby providing a reservoir for the pathogen. Once the *Salmo*-

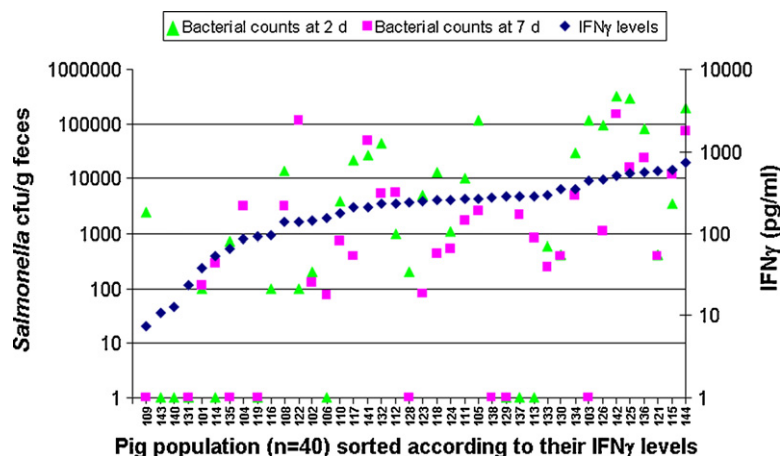


Fig. 1. Correlation between porcine IFN γ levels and *Salmonella* fecal shedding. Using a multivariate permutation test for Goodman and Kruskal's Gamma correlation, a positive correlation between the serum levels of IFN γ at 2 dpi (diamonds) and the fecal shedding of *Salmonella* at 2 dpi (triangles) and 7 dpi (squares) was observed in a group of 40 pigs inoculated with *Salmonella* Typhimurium.

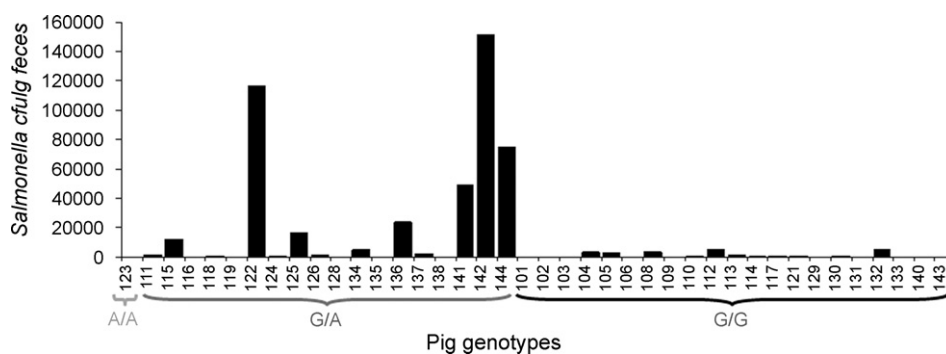


Fig. 2. Association of G/A SNP in the CCT7 porcine gene with elevated *Salmonella* fecal shedding. Pig genotypes are provided on the X-axis of the graph. The levels of fecal shedding of *Salmonella* for each pig at 7 dpi are represented on the Y-axis.

nella-carrier pig is placed under stressful conditions (i.e. transportation, mixing, etc.), the pathogen may re-emerge from the animal and be shed in the feces to contaminate and 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 goal of this research project was to investigate correlations between circulating immune blood cells, serum levels of IFN γ , shedding status of pigs colonized with *Salmonella* Typhimurium, and a sequence variant in the *Salmonella*-responsive, porcine CCT7 gene.

A precedent for investigating the importance of IFN γ and its relationship with *Salmonella* was established in patients unable to produce or respond to IFN γ due to mutations in the genes that encode major proteins of the type 1 cytokine axis; their enhanced disease susceptibility to intracellular pathogens (including *Salmonella*) illustrates not only a role for IFN γ in *Salmonella* infections but also the influence of genetic host factors in disease control (Ottenhoff et al., 2002; van de Vosse et al., 2004). Another investigation involving patients with previous *Salmonella* infections showed that a SNP in the IFNG gene was a risk factor for developing recurrent episodes of gastroenteritis (Doorduyn et al., 2008). Our data also suggests an important role for IFN γ during *Salmonella* infections since higher serum levels of IFN γ correlated both with greater numbers of circulating immune cells and with *Salmonella* shedding in swine.

Host genetic factors are important in determining susceptibility to pathogens, including *Salmonella*. Investigations of an inbred strain of mice with increased susceptibility to intracellular pathogens identified the natural resistance-associated macrophage protein gene (Nramp1) (Gros et al., 1981; Skamene et al., 1982). Furthermore, a study by van Diemen et al. (2002) on breeding swine for resistance to *Salmonella* Choleraesuis revealed functional differences in circulating immune cells: the pigs with higher resistance to *Salmonella* Choleraesuis exhibited higher numbers of circulating neutrophils and enhanced polymorphonuclear neutrophil (PMNs) function. In the present study, a genetic variant (SNP) was identified in the gene encoding CCT7, a subunit of the CCT chaperonin. CCT (chaperonin containing TCP-1; also known as TRiC for TCP-1 ring complex) is a cytosolic

chaperonin made up of 8 subunits (CCT 1-8) and is essential for ATP-dependent protein folding of the cytoskeletal proteins actin and tubulin as well as an estimated 9–15% of all newly synthesized proteins (Spiess et al., 2004; Thulasiraman et al., 1999; Valpuesta et al., 2002). Recently published research by our group was the first to demonstrate transcriptional up-regulation of CCT7 in pigs experimentally infected with *Salmonella enterica* serovar Typhimurium and serovar Choleraesuis (Uthe et al., 2007). As a transcriptionally activated gene during *Salmonella* infection, CCT7 may serve as a potential candidate for affecting variation in the porcine response to *Salmonella* (and, thus, the outcome of disease); therefore, associations between the CCT7 SNP with *Salmonella* shedding and circulating immune cells were investigated. At 7 dpi, pigs with the G/A genotype shed significantly greater numbers of *Salmonella* in their feces and had higher numbers of circulating neutrophils, WBCs and monocytes. Since *Salmonella* alters the host's cytoskeleton during invasion via effector proteins of the Type III secretion system (reviewed by Patel and Galan, 2005) and the CCT chaperonin is essential for actin folding (reviewed by Dunn et al., 2001), transcriptional activation of CCT7 during *Salmonella* infection and the identification of a SNP in CCT7 that associates with *Salmonella* shedding is intriguing as a prospective genetic marker for *Salmonella* susceptibility.

In this study, we observed the greater the number of *Salmonella* present in swine feces, the higher the IFN γ levels in the serum. In addition, the higher the serum levels of IFN γ , the greater the number of circulating immune cells. Therefore, pigs with high IFN γ levels and elevated neutrophils and white blood cells in the blood during a *Salmonella* infection may shed *Salmonella* at higher levels and for longer periods of time. Although we are uncertain of its biological interpretation, others have also shown elevated levels of IFN γ correlating with a disease state. For example, in patients with celiac disease, higher IFNG gene expression correlated with the severity of tissue remodeling in duodenal biopsies (Wapenaar et al., 2004). Also, a SNP in the IFNG gene (+874T/A allele) showed a protective association with tuberculosis susceptibility (Pacheco et al., 2008). While the authors are unclear of the physiological mechanism involved, the SNP has also been shown to associate with a stronger IFN γ response to tuberculosis (Lopez-Maderuelo et al., 2003; Sallakci et al., 2007),

suggesting that increased levels of IFN γ during the early stages of infection may control the spread of *M. tuberculosis* and, thus, the disease outcome in the host.

In this study, we also propose a genetic variant (SNP) in the CCT7 porcine gene that associates with circulating neutrophils, white blood cell and monocytes as well as shedding of *Salmonella* in the feces of experimentally infected pigs. Thus, pigs with the CCT7 A/G genotype may be more prone to shedding greater numbers of *Salmonella* than swine with the G/G genotype. This observation requires further investigation using additional pig populations (in progress), but is interesting since a potentially powerful approach in dissecting *Salmonella* disease resistance versus susceptibility in domestic animals is (1) identifying genetic variations (SNPs) in genes that play a role in the host's immune response to *Salmonella* infection, and (2) associating the genetic variants with phenotypes observed in swine during *Salmonella* infections. Investigating factors in pigs 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.

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