TLR4 Single Nucleotide Polymorphisms (SNPs) Associated with Salmonella Shedding in Pigs

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Abstract

Toll-like receptor 4 (TLR4) is a key factor in the innate immune recognition of lipopolysaccharide (LPS) from Gram-negative bacteria. Previous studies from our group identified differences in the expression profile of TLR4 and genes affected by the TLR4 signaling pathway among pigs that shed varying levels of Salmonella, a Gram-negative bacterium. Therefore, genetic variation in this gene may be involved with the host’s immune response to bacterial infections. The current study screened for single nucleotide polymorphisms (SNPs) in the TLR4 gene and tested their association with Salmonella fecal shedding. Pigs (n=117) were intranasally challenged at 7 weeks of age with 1 x 10⁹ CFU of S. Typhimurium χ4232 and were classified as low or persistent Salmonella shedders based on the levels of Salmonella being excreted in fecal material. Salmonella fecal shedding was determined by quantitative bacteriology on days 2, 7, 14, and 20/21 post exposure, and the cumulative levels of Salmonella were calculated to identify the low (n=20) and persistent (n=20) Salmonella shedder pigs. From those 40 animals, the TLR4 region was sequenced, and 18 single nucleotide polymorphisms (SNPs) in TLR4 were identified. Twelve SNPs have been previously described and six are novel SNPs of which five are in the 5’ untranslated region and one is in intron 2. Single marker association test identified 13 SNPs associated with the qualitative trait of Salmonella fecal shedding, and seven of those SNPs were also associated with a quantitative measurement of fecal shedding (P < 0.05). Using a stepwise regression process, a haplotype composed of SNPs rs80787918 and rs80907449 (P ≤ 4.0 x 10⁻⁵) spanning a region of 4.9Kb was identified, thereby providing additional information of the influence of those SNPs on Salmonella fecal shedding in pigs.

Keywords: TLR4, single nucleotide polymorphisms (SNPs), Salmonella, swine

Introduction

Salmonella is a widespread foodborne pathogen with the ability to adapt to different environments. Swine (Sus scrofa) are an important reservoir of Salmonella because colonization and shedding of this bacterium, imposing elevated risks to public and animal health. Thus, diverse intervention strategies are needed to control the transmission of Salmonella from pork to humans and to the environment.

In bacterial infections, the severity of infection is impacted by the pathogenicity of the microorganism and its interaction with the host immune defense system (Zanella et al., 2011). Toll-like receptor 4 (TLR4) is a well-characterized gram-negative bacterial lipopolysaccharide (LPS) recognition receptor and a host inflammatory response activator well conserved among animal species (Noreen et al., 2012, Yang et al. 2012). Schröder and Schumann (2005) suggested that mutations in the TLR4 regions involved with pathogen recognition and transduction signaling may affect host susceptibility to infection. Polymorphisms in the TLR4 gene have been associated with different infectious diseases in humans, cattle, chicken and pigs (Noreen et al., 2012, Kataria et al. 2011, Leveque et al. 2003, Yang et al., 2012).

In swine, TLR4 is located on Sus scrofa 1 (SSC1) V10.2 (289,776,058 bp to 289,785,087 bp). Thomas et al. (2006) identified the genomic structure of porcine TLR4, and Shinkai et al. (2006) described the distribution of SNPs for five TLRs in pigs. Specifically for TLR4, 13 SNPs were widely distributed in 11 pig breeds, and of those, seven were non-synonymous. Thirty four SNPs were identified in TLR4 using pigs representing European commercial breeds and some traditional breeds (n=259), and of these, 17 SNPs were located in the non-coding region and 17 SNPs were found in the coding region (Palermo et al., 2009). Furthermore, polymorphisms in the TLR4 gene have been identified as potential genetic markers for disease susceptibility in pigs (Uenishi & Shinkay, 2009). Our group had reported up-regulation of TLR4 and its target genes in pigs challenged with Salmonella enterica serovar Typhimurium (Huang et al., 2011). Therefore, to determine if TLR4 is a possible candidate gene associated with Salmonella shedding, we first sequenced the TLR4 gene for SNPs identification and tested their associations with Salmonella shedding status.

Material and Methods
Briefly, 117 pigs were intranasally challenged at 7 weeks of age with 1 x 10^9 CFU of S. Typhimurium as previously described (Huang et al., 2011, Uthe et al., 2009). At days 2, 7, 14 and 20/21 post-inoculation (pi), Salmonella fecal shedding was quantified using a standard bacteriological test previously described (Uthe et al., 2009). Forty pigs were chosen based on their fecal culture status; quantitative classification of the phenotype was scored based on cumulative Salmonella fecal shedding. Genomic DNA was extracted from blood samples and purified as previously described (Uthe et al., 2009). Nine sets of primers were designed to cover all exons (n=3) of TLR4 (SSC1: 289,775,345bp - 289,786,312bp V. 10.2). PCR products were sequenced and polymorphisms were identified using Phred/Phrap/Consed/PolyPhred software. A single and multiple marker association tests were conducted within PLINK (V1.07) and R statistical programs (Purcell et al., 2007).

**Results**

For the quantitative measurement of Salmonella shedding cumulative measurements were taken within days 2, 7, 14 and 20/21 pi (Figure 1). Sequencing analysis of those forty (n=40) animals identified 18 SNPs; twelve were previously described in the literature and/or annotated in GenBank and six are novel SNPs (Table 1).

Of the 18 SNPs, thirteen (n=13) SNPs were associated (P ≤ 0.05) with Salmonella shedding as a qualitative phenotype using a Chi-squared test; of those 13 SNPs, seven were also associated with Salmonella shedding as a quantitative phenotype using a Wald Statistical test (Table 1).

![Figure 1: Area under the log curve illustrating the log of cumulative colony forming units (CFU). Quantitative bacteriology of Salmonella shedding in swine fecal samples was performed at day 2, 7, 14 and 20/21 days post-challenge with S. Typhimurium, and CFU were determined.](image)

**Table 1.** Identified SNPs and position in the TLR4 gene of Salmonella low and persistent shedder pigs.

| SNP  | Location in Sus Scrofa genome (bp) | GenBank accession number | Single Marker Association (P-value) |
|------|----------------------------------|--------------------------|-----------------------------------|
|      |                                  |                          | qualitative | quantitative |
| SNP1# | SSC1:289,774,983 No                | 0.033                    | 0.064    |
| SNP2# | SSC1:289,775,046 No                | 0.033                    | 0.064    |
| SNP3# | SSC1:289,775,081 No                | 0.033                    | 0.064    |
| SNP4# | SSC1:289,775,543 No                | 0.133                    | 0.244    |
| SNP5  | SSC1:289,775,665 rs80830544        | 0.363                    | 0.550    |
| SNP6# | SSC1:289,775,979 No                | 0.025                    | 0.056    |
| SNP7  | SSC1:289,780,226 rs80881287        | 0.004                    | 0.029    |
| SNP8**| SSC1:289,780,292 rs80787918        | 0.002                    | 0.013    |
| SNP9# | SSC1:289,782,761 No                | 0.001                    | 0.025    |
| SNP10 | SSC1:289,782,834 rs80923358        | 0.003                    | 0.037    |
| SNP11 | SSC1:289,782,933 rs80951861        | 0.003                    | 0.037    |
| SNP12*| SSC1:289,783,127 rs80811682        | 0.007                    | 0.054    |
| SNP13*| SSC1:289,783,342 (Shinkai et al., 2006) | 0.285 | 0.514 |
| SNP14 | SSC1:289,783,476 rs80981701        | 0.064                    | 0.105    |
| SNP15*| SSC1:289,783,478 rs80955017        | 0.034                    | 0.046    |
| SNP16*| SSC1:289,783,543 rs80894552        | 0.176                    | 0.231    |
| SNP17 | SSC1:289,784,913 rs80834103        | 0.025                    | 0.056    |
| SNP18**| SSC1:289,785,250 rs80907449        | 0.002                    | 0.011    |

*non-synonymous SNPs; **haplotype components; # novel SNPs
Using a haplotype construction and the backward-elimination process, the most significant haplotype for both measurements of *Salmonella* shedding, qualitative ($P \leq 7.9 \times 10^{-4}$) and quantitative ($P \leq 4.0 \times 10^{-3}$) comprised a region of 4.9Kb composed of SNPS, rs80787918 (SNP8) and rs80907449 (SNP18) ($r^2=0.902$) located at SSC1:289,780,292 bp and SSC1:289,785,250 bp, respectively (Table 2).

**Discussion**

Four SNPs, SNP12, SNP13, SNP15 and SNP16, located on exon three of TLR4 gene are non-synonymous mutations and they are positioned between SNP8 and SNP18. When the additive effects of those markers were tested within the haplotype constructed with markers SNP8 and SNP18, it was not observed any improvement in the association test. The significance of those results was possibly penalized by the addition of markers into the association test, due to the limited number of samples.

The haplotype CC of SNP8 and SNP18 was identified in higher frequency in persistent shedding pigs (67.5%) compared to low shedding pigs (30%); furthermore, the frequency of haplotype TT in low shedding pigs (65%) was greater when compared to persistent shedding pigs (32.5%). No animals from the persistent shedding group were identified with the haplotype TC or CT, while it was observed in low frequency in the low shedding group (2.5%). Together, these results suggest that the region located between SNP8 and SNP18, more specifically on exon 3, is possibly harboring the causative mutation for *Salmonella* colonization and shedding variation in swine.

**Conclusion**

The results from this study support the concept that TLR4 is an important modulator associated with the porcine response to *Salmonella* infection in swine.

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**Table 2.** Haplotypes frequency (SNPs rs80787918 and rs80907449) and associations with qualitative and quantitative phenotypes of *Salmonella* shedding.

| Haplotype | Persistent Shedders | Low Shedders | Qualitative (P Val.) | Quantitative (P Val.) |
|-----------|---------------------|-------------|---------------------|----------------------|
| CC        | 0.675               | 0.3         | 0.00079             | 0.004201             |
| TC        | 0                   | 0.025       | 0.3143              | 0.1054               |
| CT        | 0                   | 0.025       | 0.3143              | 0.1445               |
| TT        | 0.325               | 0.65        | 0.00334             | 0.02912              |
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