Association of Interleukin-10 Cluster Genes and Salmonella Response in the Chicken

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INTRODUCTION

Salmonella contamination of poultry meat and eggs continues to be a global threat to public health (Barrow and Duchet-Suchaux, 1997). Salmonella enteritidis is an enteric bacterium that is a zoonotic intracellular pathogen of poultry and humans as well as other species (McIlroy et al., 1989). Salmonella enteritidis is a major cause of human foodborne illness and is the most frequent serovar detected in outbreaks of human salmonellosis (Lu et al., 1999). In the United States, an estimated 500,000 cases of human illness are annually attributed to S. enteritidis due to contaminated food products (Schlosser and Ebel, 1998). In the advent of S. enteritidis infection, some animals die, but others can host the bacteria for several weeks or months without presenting any particular symptoms. These healthy carriers are therefore an obstacle to the eradication of S. enteritidis and are responsible for the transmission to humans. Different prophylactic measures to control S. enteritidis infection in poultry have been studied, including competitive exclusion and vaccination. The use of antibiotics in domestic livestock has been questioned due to the possible creation of antibiotic-resistant bacteria and the possibility of antibiotic residues being consumed by humans (White et al., 2001).

Host genetic factors clearly influence the epidemiology of S. enteritidis infection in chickens. Newer strategies to increase the overall level of resistance at flock and population levels that use selective breeding programs to enhance natural resistance are expected to contribute significantly in this regard (van der Zjipp, 1983; Warner et al., 1987; Womack, 1988). In recent years, much progress on the identification and characterization of candidate genes, microsatellite markers, and comparative gene mapping has been made. Identification of individual candidate genes that control natural resistance and the actions of these genes will greatly expand our knowledge of genetic resistance to bacterial diseases and the possibilities for practical application. A candidate gene is usually chosen, based on its biological or physiological functions, to study the association between its genetic polymorphisms and traits of interest (Rothschild and Soller, 1997). Once a candidate gene is identified and proven, causative mutations (functional markers) or polymorphic markers (direct markers) near or within the gene sequence can be developed for marker-assisted selection to improve immunity.
The ability to respond to *S. enteritidis* infections is age-dependent in poultry. In young chickens, salmonellosis is a major disease characterized by severe clinical signs of diarrhea and dehydration with high mortality rates. In adult chickens, it does not cause significant disease or mortality, and birds can carry the bacteria for several weeks without presenting any clinical signs, which constitutes a risk for public health (Wigley et al., 2002). The heritability estimates of *S. enteritidis* range from 0.13 to 0.53 for frequency in internal organs of challenged adult layers (Beaumont et al., 1999), 0.20 in chickens inoculated at 1 wk of age, 0.38 in hens inoculated at the peak of lay (Berthelot et al., 1998; Beaumont et al., 1999), and 0.10 for spleen infection using 13-d-old chicks (Girard-Santosuosso et al., 2002). These estimated heritabilities suggest that resistant populations could be developed through selection for a lower level of contamination of spleen and cecum. Over the past 10 yr numerous candidate gene studies have found allelic variation to affect the immune response to *S. enteritidis* in poultry (Hu et al., 1997; Cotter et al., 1998; Mariani et al., 2001; Lamont et al., 2002).

*Salmonella enteritidis* has lipopolysaccharide (LPS) as a major component of the outer membrane. Lipopolysaccharide is a major trigger of inflammation and pathology caused by a variety of neuroendocrine and immunological changes that activate different hematopoietic and nonhematopoietic cells (MacKay and Lester, 1992; Nakamura et al., 1998). In recent years, many studies on the effects of LPS on chickens have been reported using different in vivo and in vitro parameters. Those studies suggest that birds show patterns of response to endotoxins (such as LPS) similar to mammals (Miller and Qureshi, 1992; Zhang et al., 1995; Koh et al., 1996). A study by Xie et al. (2000) on *S. typhimurium* LPS showed that the LPS produced inflammatory responses in chickens characterized by an elevation in cloacal temperature, feed avoidance, loss of body weight, an increase in liver weight, the elevation of heterophil counts, an increase in plasma levels of interleukin 6, and selective changes in the concentrations of certain plasma proteins, notably a 65-kDa protein band. Xie et al. (2000) also demonstrated that LPS stimulated interleukin 10 (IL10) gene expression in chickens. In the present study, genes in the IL10 cluster [polymeric immunoglobulin receptor (*PIGR*), IL10, map kinase-activated protein kinase 2 (*MAPKAPK2*), and ligatin (*LGTN*)] and dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 1A (*DYRK1A*) were used to identify single nucleotide polymorphisms (SNP) associated with response to *S. enteritidis* challenge in *F*$_8$ advanced intercross lines (AIL) of chickens.

### MATERIALS AND METHODS

#### Experimental Birds

The *F*$_8$ generation of 2 related AIL of the Iowa *Salmonella* Response Resource Population (ISRRP) was used (Hasenstein and Lamont, 2007). The *F*$_1$ generation of this population was generated by crossing outbred broilers (Kaiser and Lamont, 2002) with dams of 2 unrelated, highly inbred lines (Leghorn and Fayoumi; Zhou and Lamont, 1999). Further intercrossing of *F*$_1$ birds within the 2 granddam lines yielded the *F*$_8$ birds. The genetic distance between the parental lines maximized the feasibility of finding molecular genetic polymorphism, and the inbred nature of the founder dams meant that they consistently contributed the same allele to all offspring. To ensure that the maternal immune status of all hens producing the chicks was equivalent and would not interfere with testing their chicks for *Salmonella* response, all hens were kept under the same biosecure management conditions. Chicks were tested for *Salmonella* before challenge. The chicks were equally divided with regard to genetic line and sex into 3 biosafety-level-2 animal rooms.

#### Salmonella Pathogenic Challenge and Quantification of Bacterial Load

The *F*$_8$ chicks (n = 132) were intraesophageally inoculated at 1 d of age with *S. enteritidis* phage type 13a via syringe equipped with an infusion teat (Kaiser et al., 2002). Each chick received an inoculation dose of $1 \times 10^4$ cfu/chick in Luria broth (Qbiogene, Irvine, CA). Chicks were monitored twice daily for clinical expression of the disease. Birds that survived to d 7 or 8 were euthanized, and the spleen and cecal contents were cultured for quantification of *S. enteritidis* load. Spleens were cultured at a ratio of 1:10 (spleen:medium; wt/vol), and cecal contents were collected by wiping a 2.5-cm section of the lower cecum with a sterile swab and placing the swab into 10 mL of selenite enrichment broth (Difco, Detroit, MI). After 24 h of enrichment at 37°C, the cultures were held for 24 h at 4°C and then serially diluted on brilliant green agar plates (Fisher Scientific, Pittsburgh, PA). Colonies were counted after 24 h at 37°C (Kaiser and Lamont, 2001).

#### DNA Isolation, PCR, and Sequencing

Genomic DNA was isolated from blood using the Gentra Puregene Kit (Gentra System, Plymouth, MN). Sequence information of the 5 genes was retrieved from UCSC Chicken genome browser database (http://genome.ucsc.edu/cgi-bin/hgGateway). Primers were designed, 2 for each gene, using the Primer3 program (Rozen and Skalicky, 1998; Table 1). The PCR mixture contained 5 ng of genomic DNA, 1 unit of *Taq* polymerase (Promega, Madison, WI), 1.5 mM MgCl$_2$, 200 μM deoxyribonucleotide triphosphates (dNTP), and 50 pmol of each primer in a total volume of 25 μL. The amplification went through 30 cycles as follows: 94°C denaturation, 45°C annealing, touch-down annealing from 58.5 to 59.3°C for 30 s, and extension at 72°C for 35 s; repeated for 34 cycles. The PCR primers and dNTP were removed before sequencing: 10 μL of PCR product was incubated with 2 μL of ExoSAP-IT (Amersham Pharmacia, Cleveland, OH) for 45 min at 37°C, followed by 20 min at 80°C for enzyme inactivation and purification. Three birds per AIL (6 birds total, 12 sequences per gene) were analyzed to detect potential SNP. Sequencing of the purified PCR products was done on an...
Table 1. Primer pair and single base pair extension primers of the 5 candidate genes analyzed

<table>
<thead>
<tr>
<th>Gene</th>
<th>Accession</th>
<th>Product size (bp)</th>
<th>Primer sequence 1</th>
<th>Single base extension primers 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>PIGR</td>
<td>AF303371</td>
<td>467</td>
<td>5′ GTGCAAACACTTCCCCCTAA 3′</td>
<td>5′ TTTTGTGCAAATGGAGAACGA 3′</td>
</tr>
<tr>
<td>IL10</td>
<td>AJ621254</td>
<td>416</td>
<td>5′ TGGAGCCTTAAATCCCACTG 3′</td>
<td>5′ TAATGCAAGCCTCATTGTGC 3′</td>
</tr>
<tr>
<td>DYRK1A</td>
<td>AJ459381</td>
<td>491</td>
<td>5′ GGACAGATGGTGTTGCCTTT 3′</td>
<td>5′ ACAGGGGTTGAAAGGGACTT 3′</td>
</tr>
<tr>
<td>MAPKAPK2</td>
<td>NM_032960</td>
<td>412</td>
<td>5′ CAGTGATGGGAGTGTTGGTG 3′</td>
<td>5′ CACTGTGGCCTCCCAGTATT 3′</td>
</tr>
<tr>
<td>LGTN</td>
<td>AJ719434</td>
<td>410</td>
<td>5′ CCAGCAGGGCTATAATGGAA 3′</td>
<td>5′ GTCTTTGGGGTTGCAAGAAA 3′</td>
</tr>
</tbody>
</table>

1 PIGR = polymeric immunoglobulin receptor; IL10 = interleukin 10; MAPKAPK2 = map kinase-activated protein kinase 2; LGTN = ligatin; DYRK1A = dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 1A.

Table 2. Associations (P-values) between single nucleotide polymorphism and *Salmonella enteritidis* count in spleen and cecal content of F8 advanced intercross lines (broiler × Fayoumi and broiler × Leghorn combined)

<table>
<thead>
<tr>
<th>Gene 1</th>
<th>Tissue measured for bacterial load</th>
</tr>
</thead>
<tbody>
<tr>
<td>PIGR</td>
<td>Spleen 0.0265 Cecum 0.0377</td>
</tr>
<tr>
<td>IL10</td>
<td>Spleen 0.0006 Cecum 0.0004</td>
</tr>
<tr>
<td>MAPKAPK2</td>
<td>Spleen 0.0002 Cecum 0.0004</td>
</tr>
<tr>
<td>LGTN</td>
<td>Spleen 0.344 Cecum 0.314</td>
</tr>
<tr>
<td>DYRK1A</td>
<td>Spleen 0.23 Cecum 0.279</td>
</tr>
</tbody>
</table>

1 PIGR = polymeric immunoglobulin receptor; IL10 = interleukin 10; MAPKAPK2 = map kinase-activated protein kinase 2; LGTN = ligatin; DYRK1A = dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 1A.

Genotyping

The F8 population was genotyped for 1 SNP per gene by using the Multiplexed SNaPshot assay (Applied Biosystems, Foster City, CA). For multiplexing of PCR, 2.5 μL of each PCR product of the 5 genes was pooled and purified by treating with 2 μL of ExoSAP-IT (USB Corporation, Cleveland, OH). The SNaPshot reaction was carried out with 1.5 μL of the purified PCR product, 0.5 μL of the 5 pooled SBP extension primers, 0.5 μL of H2O, and 2.5 μL of SNaPshot Multiplex Ready Reaction Mix (Applied Biosystems) in a total reaction volume of 5 μL. The SNaPshot reaction was run on the thermal cycler for 10 s at 90°C, followed by 5 s at 50°C, and 30 s at 60°C, and was repeated 25 times. To purify the unincorporated ddNTP, the post-SNaPshot reactions were incubated with 0.5 μL of shrimp alkaline phosphatase (Promega) for 1 h at 37°C and for 15 min at 72°C. The postextension samples were analyzed on an ABI Prism 3100 sequencer at the Nucleic Acid Facility, Iowa State University, Ames, and were genotyped using Genescan software (Perkin Elmer Biosystems, Foster City, CA).

Statistical Analysis

Statistical tests for associations of SNP with spleen and cecal content *S. enteritidis* burden were analyzed by AN-
OVA by using the JMP software (SAS Institute, 2000). Each of the 5 SNP was independently tested for association with the phenotype, fitting the GLM to the data. Main effects included SNP, line, sex, and BW as fixed effects, and sire, hatch day, and room as random effects.

The model was

$$Y_{ijklmnop} = \mu + \text{Genotype}_i + \text{Line}_j + \text{Sex}_k$$

$$+ (\beta(BW)_l + \text{Sire}[\text{Line}]_m + \text{Room}_n + \text{Hatch}_p$$

$$+ (\text{Room} \times \text{Hatch})_p + \epsilon_{ijklmnop}$$

where $$Y_{ijklmnop}$$ was the response variable (individual birds’ spleen and cecum bacterial count expressed as natural logarithms); $$\mu$$ was the mean; Genotype, was the fixed effect of the ith SNP genotype; Line was fixed effect of the jth line; Sex was the fixed effect of the kth sex; $$\beta(BW)_l$$ was the lth Bw as a fixed effect covariate; Sire[Line] was the random effect of the mth sire in the jth line; Room was the random effect of the nth room; Hatch was the random effect of the pth hatch; (Room × Hatch) was the interaction effect of the nth room and the pth hatch; and $$\epsilon_{ijklmnop}$$ was the residual error term.

RESULTS AND DISCUSSION

Unique SNP were analyzed in the current study for 3 of the genes studied (DYRK1A, LGTN, and PIGR), whereas the SNP analyzed in IL10 and MAPKAP2 were reported previously (Wong et al., 2004). The discovery of new SNP was possible because the current population utilized a different breed (Fayoumi) from those used to identify the SNP in the Beijing Genomic Institute study (Wong et al., 2004).

There was generally no line effect; therefore, results are presented (Table 2) for analysis of the combined data of the 2 AIL. Because 5 genes were tested, a P-value < 0.01 was considered significant, and $$P < 0.05$$ suggestive. The MAPKAP2 SNP showed a highly significant association with both spleen ($$P = 0.0002$$) and cecal content ($$P = 0.0004$$) of S. enteritidis. There was a highly significant association of the IL10 SNP with spleen tissue ($$P = 0.0006$$) and cecal content ($$P = 0.0004$$). The PIGR SNP showed a suggestive association with spleen tissue ($$P = 0.0265$$) and cecal content ($$P = 0.0377$$). Although the DYRK1A SNP was not associated with bacterial burden in the combined-line analysis, in the broiler × Fayoumi AIL it was suggestively associated with both spleen ($$P = 0.036$$) and cecal content ($$P = 0.027$$; data not shown). No association of LGTN and bacterial burden was observed.

The MAPKAP2 gene is involved in many cellular processes including stress, inflammatory responses, nuclear export, gene expression regulation, and cell proliferation (Beyaert et al., 1996; Kotlyarov et al., 1999; Meng et al., 2002; Schindler et al., 2002). Interleukin 10 is a cytokine with potent antiinflammatory and immunoregulatory activities and is produced primarily by monocytes and to a lesser extent by lymphocytes (Flynn and Chan, 2001). The DYRK1A gene plays a significant role in a signaling pathway that regulates cell proliferation (Shindoh et al., 1996).

An increasing amount of evidence indicates the important impact of genetics on modulating the immune response in poultry (Lamont, 1998). By using genetic approaches to improve the innate immune systems and minimize the need for use of vaccines or antibiotics, it might be possible to reduce S. enteritidis contamination in poultry without the hazards of antibody-resistant bacteria or residue consumption for humans. This study identifies associations of polymorphisms in genes in the IL10 region and response to S. enteritidis challenge in chickens. The polymorphism of each gene was in the noncoding sequence of the gene, suggesting that the associations were because of linkage of the SNP to other functional polymorphisms in the same gene or nearby genes. The SNP, however, serve as useful markers for positional cloning of causal genes and for marker-assisted selection.

In conclusion, the SNP in MAPKAP2 and IL10 genes were strongly associated with bacterial burden of spleen and cecal content after exposure to pathogenic S. enteritidis. Use of these SNP in marker-assisted selection may enhance disease resistance. The current study illustrated the efficacy of using genes of biological function defined in other species to identify candidate genes in chicken.

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