

Association of *INOS*, *TRAIL*, *TGF- β 2*, *TGF- β 3*, and *IgL* genes with response to *Salmonella enteritidis* in poultry

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(Accepted 26 February 2003)

Abstract – Several candidate genes were selected, based on their critical roles in the host's response to intracellular bacteria, to study the genetic control of the chicken response to *Salmonella enteritidis* (SE). The candidate genes were: inducible nitric oxide synthase (*INOS*), tumor necrosis factor related apoptosis inducing ligand (*TRAIL*), transforming growth factor β 2 (*TGF- β 2*), transforming growth factor β 3 (*TGF- β 3*), and immunoglobulin G light chain (*IgL*). Responses to pathogenic SE colonization or to SE vaccination were measured in the Iowa *Salmonella* response resource population (ISRRP). Outbred broiler sires and three diverse, highly inbred dam lines produced 508 F₁ progeny, which were evaluated as young chicks for either bacterial load isolated from spleen or cecum contents after pathogenic SE inoculation, or the circulating antibody level after SE vaccination. Fragments of each gene were sequenced from the founder lines of the resource population to identify genomic sequence variation. Single nucleotide polymorphisms (SNP) were identified, then PCR-RFLP techniques were developed to genotype the F₁ resource population. Linear mixed models were used for statistical analyses. Because the inbred dam lines always contributed one copy of the same allele, the heterozygous sire allele effects could be assessed in the F₁ generation. Association analyses revealed significant effects of the sire allele of *TRAIL-StyI* on the spleen ($P < 0.07$) and cecum ($P < 0.0002$) SE bacterial load. Significant effects ($P < 0.04$) were found on the cecum bacterial load for *TGF- β 3-BsrI*. Varied and moderate association was found for SE vaccine antibody response for all genes. This is the first reported study on the association of SNP in *INOS*, *TRAIL*, *TGF- β 2*, *TGF- β 3*, and *IgL* with the chicken response to SE. Identification of candidate genes to improve the immune response may be useful for marker-assisted selection to enhance disease resistance.

Salmonella / genetic / candidate gene / PCR-RFLP / poultry

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1. INTRODUCTION

Salmonella contamination of poultry meat and eggs continues to be a global threat to public health [1]. *Salmonella enteritidis* (SE) was recently identified as the single most common cause of food poisoning in the United States [32]. Chronic subtherapeutic use of antimicrobial agents in domestic livestock may control bacterial diseases as well as promote growth. However, this approach may be a factor in transmitting antibiotic-resistant *Salmonella* to humans through the food chain [37]. Genetic approaches to improve the chicken's innate resistance to *Salmonella*, therefore, provide useful alternative solutions to controlling *Salmonella* infection in poultry and reducing potential microbial contamination of human food.

Detailed knowledge of molecular biology and genomics has facilitated the dissection of the genetics of complex traits. The candidate gene approach is one major strategy to identify the genes that influence traits of interest [33]. The hypothesis used in this approach is that candidate genes represent a large proportion of the quantitative trait loci (QTL) that determine a particular trait. Over the past 20 years, several genes have been found to affect the immune response to SE in poultry [5, 10, 21, 26]. The main objective of this study was to analyze new candidate genes for *Salmonella* response in poultry.

Nitric oxide (NO) is a messenger molecule with diverse functions throughout the body. Studies to investigate the biological role of *INOS* found that mice with mutant homozygous *INOS* alleles have significantly stronger Th1 type of immune response and are resistant to lipopolysaccharide-induced mortality [36]. Lui *et al.* [24] concluded that NO is required for cytokine-induced Heat shock protein (hsp) 70 mRNA expression.

Apoptosis serves an important role in programmed cell death [18]. The *TRAIL* (*TNF*-related apoptosis-inducing ligand), or the APO-2 ligand (Apo-2L) is capable of inducing apoptosis in some tumor necrosis factor (*TNF*)-resistant cancer cells [8, 35]. The *TNF* cytokine has a wide variety of functions, including apoptosis [31], and *TRAIL* is a member of the *TNF* cytokine family.

The *TGF- β* are cytokines of the *TGF- β* superfamily. *TGF- β* family members are multifunctional cell-cell signaling proteins that play pivotal roles in cell differentiation, proliferation, and growth, extracellular matrix formation and immune function [22, 30]. The effects of *TGF- β* cytokines are likely because of their crucial roles in signal transduction [27, 29].

Immunoglobulin (Ig) molecules are composed of the basic structure of two heavy and two light chains joined by interchain disulfide bonds. The Ig light chain is a necessary component of all complete Ig molecules. The *IgL* gene is important in the immune response in chickens and other species [2, 12].

The objective of this study was to characterize associations between genomic regions bearing candidate genes and *Salmonella* response in poultry.

The overall goal was to identify DNA markers for phenotypic variation in response to bacteria.

2. MATERIALS AND METHODS

2.1. Experimental animals

The first filial (F_1) generation of the Iowa *Salmonella* response resource population (ISRRP) of chickens was used. The F_1 population was produced in five hatches by crossing four outbred males of a broiler breeder male line [15] with dams of three highly inbred dam lines: one Fayoumi and two MHC-congenic Leghorn lines (G-B1 and G-B2) with inbreeding coefficients of 99% [38]. These inbred lines have been used in many studies of immune response and disease resistance [6,19,20,25]. The genetic distance between the parental lines maximized the feasibility of finding molecular genetic polymorphisms [38].

2.2. *Salmonella* pathogenic challenge and quantification of bacterial load

F_1 chicks ($n = 194$) from three hatches were intraesophageally inoculated at 1 d of age with pathogenic SE as described by [16]. Half of the birds each were euthanized at 6 and 7 d of age. The SE culture and quantification procedures were previously described [13].

2.3. *Salmonella* vaccination and antibody measurement

Chicks ($n = 314$) from two hatches were injected at 10 d of age with 0.2 mL commercial bacterin SE vaccine (Biomune, Lenexa, KS 62215) for the evaluation of antibody response at 21 d of age. Vaccination and ELISA procedures to quantify SE vaccine antibody levels were previously described [15,14,17].

2.4. DNA isolation, PCR and sequencing

Genomic DNA was prepared from erythrocytes by using a standard phenol/chloroform isolation procedure [7]. To characterize each gene, a pair of primers was developed using Oligo 5 (National Bioscience, Inc., Plymouth, MN, USA) based on the published cDNA sequence of GenBank (Tab. I).

The PCR reactions were performed in 25- μ L reaction volumes containing 25 ng of chicken genomic DNA, an optimum concentration of $MgCl_2$ (Tab. I), 0.8 μ M of each primer, 200 μ M of each dNTP, 1 unit of *Taq* DNA polymerase, 2.5 μ L of 10 \times PCR reaction buffer, and 1.5 mM $MgCl_2$. The cycling conditions

Table I. Primer design for polymorphism identification of *INOS*, *TRAIL*, *TGF- β 2*, *TGF- β 3*, and *IgL* genes.

Gene	GenBank accession No.	Primer	Sequence	PCR product (bp)	Annealing temp./time
<i>INOS</i>	AF537190	Forward	5'-CCAATAAAAGTAGAAGCGA-3'	495 bp	50 °C/1 min
		Reverse	5'-CTCTTCCAGGACCTCCA-3'	genomic	
<i>TRAIL</i>	AF537189	Forward	5'-GTAAATTAGAGCCTCATCA-3'	786 bp	54 °C/45 S
		Reverse	5'-CACCTCAGTTCCTCCGA-3'	genomic	
<i>TGF-β2</i>	X58071	Forward	5'-GCC ATA GGT TCA GTG CAA G-3'	284 bp	52 °C/1 min
		Reverse	5'-TGA CAG AAG CTC TCA AGC C-3'	genomic	
<i>TGF-β3</i>	X60091	Forward	5'-CGG CCT GGA AAT CAG CAT AC-3'	1078 bp	56 °C/1 min
		Reverse	5'-GAA GCA GTA GTT GGT ATC CAG-3'	genomic	
<i>IgL</i>	M24403	Forward	5'-TTT ATA CCC GCG TCC TTC-3'	354 bp	57–61 °C/1 min
		Reverse	5'-GGG AAA TAC TGG TGA TAG GTG-3'	genomic	

included an initial denaturation at 94 °C for 3 min, followed by 36 cycles at 93 °C for 45 s, an optimum annealing temperature and optimal annealing time (Tab. I), 72 °C for 1 min and final extension at 72 °C for 10 min.

The PCR products were purified using a MICROCON® centrifugal filter (Millipore Corporation, Bedford, MA, USA). An ABI 377 sequencer (Perkin Elmer, Foster City, CA, USA) was used for direct sequencing using dye terminators. Each PCR product was sequenced at the Iowa State University DNA Sequencing and Synthesis Facility (Ames, IA, USA).

2.5. Polymorphisms and restriction fragment length polymorphism (RFLP) assays

For polymorphism characterization of each gene, one genomic DNA sample of broiler, Leghorn G-B1 and G-B2, and Fayoumi were sequenced using both direction primers (total n = 8 sequences). Sequencher software (Gene Codes Corporation, version 4.0.5, Ann Arbor, MI, USA) was used to assemble the sequences and to identify the polymorphisms.

The restriction enzyme sites for each gene were detected using a sequence analysis web server (<http://mbcr.bcm.tmc.edu/>). Table II describes the reaction conditions for each digestion; a total final reaction volume of 20 µL was used. The PCR products were digested overnight at 37 °C. The digested fragments

Table II. PCR-RFLP assay for genotyping SNP of *INOS-AluI*, *TRAIL-StyI*, *TGF-β2-RsaI*, *TGF-β3-BsrI*, and *IgL-Sau96I*.

Gene	SNP location	Dam Line genotype		Heterozygous sire ID	Restriction enzyme	Buffer (New England)
<i>INOS</i>	Pos. 173	Leghorn	C/C	8170	<i>AluI</i> 5 unit	Buffer 2
	# AF537190 (T → C)	Fayoumi	C/C	8296		
<i>TRAIL</i>	Pos. 82	Leghorn	A/A	8170	<i>StyI</i> 5 unit	Buffer 3
	# AF537189 (G → A)	Fayoumi	A/A			
<i>TGF-β2</i>	Pos. 640	Leghorn	T/T	8170	<i>RsaI</i> 6 unit	Buffer 3
	# X58071 (T → C)	Fayoumi	C/C	8338		
<i>TGF-β3</i>	Pos. 2833	Leghorn	A/A	8170	<i>BsrI</i> 10 unit	Buffer 1
	# X60091 (C → A)	Fayoumi	C/C	8291 8338		
<i>IgL</i>	Pos	Leghorn	T/T	8170	<i>Sau96I</i> 5 unit	Buffer 4
	# M24403 (T → C)	Fayoumi	C/C	8338		

were separated by electrophoresis through 2.5% agarose. Ethidium bromide staining was used for DNA visualization.

2.6. Statistical analysis

A linear mixed model was used to estimate the association between the sire allele of the F₁ chicks of the heterozygous sire families and the SE bacterial count using the JMP® program [34]. Model 1 was used for the combined heterozygous sire families for each candidate gene. Sire and dam line were taken as fixed effects. Both the spleen SE count (1.04×10^7 to 3.55×10^9 cfu · mL⁻¹) and cecum SE count (1.06×10^7 to 5.5×10^8 cfu · mL⁻¹) were transformed to their natural logarithms as response variables to achieve a normal distribution of the dependent variables in model construction.

Model 1: $Y_{ijkl} = \mu + \text{sire allele}_i + \text{sire}_j + \text{dam line}_k + \text{allele} \times \text{dam line}_{ik} + e_{ijkl}$

where Y_{ijkl} is defined as the response variable from each F₁ bird (natural logarithms of the spleen or cecal bacterial count).

A linear mixed model was used to estimate the association between each candidate gene genotype of the F₁ chicks of the heterozygous sire families and the SE vaccine antibody level. Model 2 was used for the combined heterozygous sire families. Sex and ELISA plate factors were included in the statistical model, based on frequent significance in other antibody studies. Plate effect, which varied among ELISA assays, was considered a random effect.

Model 2:

$Y_{ijklmn} = \mu + \text{sire allele}_i + \text{sire}_j + \text{dam line}_k + \text{sex}_l + \text{plate}_m(\text{random}) + e_{ijklmn}$

where Y_{ijklmn} is defined as the response variable from each F₁ bird, (1 – S/N) for antibody level.

Each gene was analyzed separately, because the inheritance pattern of the alleles indicated that the five genes were not linked, although only one (*TGF-β3*) is currently mapped [4].

3. RESULTS

3.1. Sequence variation and PCR-RFLP

Single nucleotide polymorphisms (SNP) were identified within *INOS*, *TRAIL*, *TGF-β2*, *TGF-β3*, and *IgL* that affect the recognition sequence for *INOS*-*AluI*, *TRAIL*-*StyI*, *TGF-β2*-*RsaI*, *TGF-β3*-*BsrI*, and *IgL*-*Sua96I* restriction enzymes, respectively (Tab. II). These enzyme-gene combinations were then used to follow the inheritance of the heterozygous sire alleles in the F₁ chicks.

A primer set (Tab. I) was used to amplify the 495 bp fragment of the *INOS* gene. Sequence analysis revealed one SNP, a T/C substitution at position 173 bp in our sequence (AF537190), in the intronic region. Sires 8170 and 8296 were heterozygous genotype T/C at this position. Sires 8291 and 8338 and Leghorn G-B1, G-B2 and Fayoumi lines were homozygous genotype C/C. The T/C substitution of *INOS*-*AluI* digested polymorphisms was selected for PCR-RFLP genotyping of the F₁ offspring. The PCR-RFLP resulting from the *INOS*-*AluI* digestion of the PCR product produced either an undigested PCR product of 495 bp for one allele, or 322 bp and 173 bp fragments for the other allele.

For the *TRAIL* gene, sequence analysis revealed one SNP, a G/A substitution at position 82 bp in our sequence (AF537189). This SNP occurred in the exonic region, but did not change the predicted amino acid. This polymorphic site was detected using *TRAIL*-*StyI* (Tab. I). Sire 8170 was heterozygous genotype G/A at this position. Sires 8296, 8291, and 8338, and Leghorn G-B1, G-B2 and Fayoumi lines were homozygous genotype A/A. The PCR-RFLP resulting from the *TRAIL*-*StyI* digestion of the PCR product produced either an undigested PCR product of 560 bp, or 480 bp and 80 bp fragments.

For the *TGF-β2* gene promoter region, the amplified 284-bp product was sequenced for offspring of heterozygous sires 8170 and 8338. There was a T/C SNP at position 640 (accession No. X58071) [3]. Sires 8291, 8296 and Leghorn G-B1, G-B2 lines were homozygous genotype T/T and the Fayoumi line was homozygous C/C. The restriction enzyme *RsaI*-digested PCR products had fragment sizes of 184 bp and 100 bp for one allele or 284 bp for the other allele.

For the fourth intron region of *TGF-β3*, a 294-bp product was amplified from genomic DNA and sequenced. A C/A SNP occurred at base 2833 (accession No. X60091). Sire 8170, 8291, and 8338 were heterozygous genotype C/A at this position. Sire 8296 and Leghorn G-B1 and G-B2 lines were homozygous genotype A/A and the Fayoumi line was homozygous C/C. The restriction enzyme *BsII*-digested PCR products produced fragments of 124 bp, 75 bp, 74 bp, and 20 bp for one allele, and fragments of 145 bp, 75 bp, and 74 bp for the other allele.

Primers (Tab. I) were used to amplify the promoter region of the *IgL* gene (GenBank accession No. M24403). The initial PCR conditions were developed by Heltemes *et al.* [9]. The primers were then redesigned, based on the specific sequence of the lines used in this study, to amplify a 354 bp fragment. The PCR conditions were the same, except for the annealing temperature (57 to 61 °C). The sequence analysis revealed one SNP at 60 bp upstream of the octamer sequence. Sires 8170 and 8338 were heterozygous genotype T/C at this position. Sires 8291 and 8296 and Leghorn lines G-B1, G-B2 were homozygous genotype T/T; and the Fayoumi line was homozygous C/C. This polymorphic

site was detected using *IgL-Sau96I* which resulted in 161 bp fragments and two predicted 10 bp fragments for the Leghorn and Fayoumi lines. The Leghorn line had an additional line-specific 173 bp fragment. The Fayoumi lines had two additional fragments of 103 and 70 bp.

3.2. Association of candidate genes with SE response

The F₁ progeny of sires that were heterozygous for each specific gene were genotyped for that gene. The associations are summarized in Table III.

The F₁ progeny analysis suggested an association between the *INOS-AluI* sire allele and spleen SE bacterial load ($P < 0.15$). There was an association of $P < 0.21$ of the *INOS-AluI* sire allele and antibody level to the SE vaccine.

The *TRAIL-StyI* sire allele was associated with both spleen ($P < 0.07$) and cecal contents ($P < 0.0002$) bacterial load (Tab. III). The allele effect, however, was in opposite directions in the two organ sites, in that the sire allele "A" was associated with a higher spleen, but a lower cecal, bacterial load. For the SE vaccinated group, there was a moderate association between sire allele and antibody response to the SE vaccine ($P < 0.20$).

The sire allele of *TGF-β2-RsaI* did not have a major effect on bacterial load. There was, however, an association of $P < 0.07$ between the *TGF-β2* sire allele and antibody level to the SE vaccine.

Although there was no significant association with the spleen bacterial load, the *TGF-β3-BsrI* sire allele was significantly associated ($P < 0.04$) with the

Table III. Associations between *INOS*, *TRAIL*, *TGF-β2*, *TGF-β3* and *IgL* gene polymorphisms and SE response.

Gene	<i>P</i> -value ¹		
	Bacterial Load		Vaccine Antibody (N)
	Spleen (N ²)	Cecum (N)	
<i>INOS</i>	0.15 ¹ (51)	0.57 (51)	0.21 (134)
<i>TRAIL</i>	0.07 (12)	0.0002 (12)	0.20 (68)
<i>IgL</i>	0.90 (36)	0.24 (36)	0.05 (77)
<i>TGF-β2</i>	0.90 (45)	0.90 (45)	0.07 (119)
<i>TGF-β3</i>	0.92 (40)	0.04 (40)	0.17 (34)

¹ $P = P$ -value (main effects); ² N = number of phenotyped F₁ progeny from sires heterozygous for single nucleotide polymorphism evaluated for this gene.

cecum content bacterial load. A moderate association ($P < 0.17$) was found between the *TGF-β3-BsrI* sire allele and antibody response to the SE vaccine.

For the *IgL* gene, there was no association between *IgL-Sau96I* sire allele and bacterial load. The *IgL-Sau96I* sire allele was significantly associated ($P < 0.05$) with antibody response to SE vaccine.

4. DISCUSSION

This is the first report, to our knowledge, of associations between the chromosomal regions marked by *INOS*, *TRAIL*, *TGF-β2*, *TGF-β3* and *IgL* gene polymorphisms and response to *Salmonella enteritidis* in chickens. The polymorphisms of each examined gene were not amino acid substitution sites, thus suggesting that the associations of each SNP gene with *Salmonella* response is because of linkage of the SNP to other functional polymorphisms in the same or nearby genes. The SNP, however, serve as useful markers for positional cloning and for marker-assisted genetic selection. The probability that the SNP are in causative genes is enhanced by the selection of the genes on the pre-existing knowledge (comparative genomics) of their function in similar pathways in other species.

The *TGF-β* genes have effects on spleen growth and development in the chicken [11, 23]. Zhou and colleagues [39] conducted an association study of *TGF-β* genes and antibody kinetics in a resource population that shared two founder lines with the present study. They found that *TGF-β* genes contribute to the genetic control of antibody response kinetics in the chicken. In the current study, both *TGF-β2* and *TGF-β3* showed moderate association with the SE vaccine antibody response. There was no association between the spleen bacterial load and sire allele of *TGF-β2* or *TGF-β3*; however, a significant association was found between the *TGF-β3-BsrI* sire allele and cecal bacterial contents. The different genetic effects on cecal contents and spleen bacterial load are consistent with a previous report [13] of independent host genetic mechanisms for the control of bacterial load in the spleen or cecum contents.

The detected association of the *INOS-AluI* sire allele with spleen bacterial load and circulating antibody response to the SE vaccine may be a result of the key role of *INOS* in the intracellular pathway for processing *Salmonella* antigens [28]. Further proof in a larger scale experiment is however needed to verify the results. The significant association between the *IgL* gene and antibody response to SE vaccine supports the finding of Zhou *et al.* [40] for the effect of *IgL* gene promoter polymorphisms on the primary antibody response to *Salmonella* in chickens.

The investigated SNP in the *TRAIL* gene showed associations with both spleen and cecal bacterial load. This finding, however, was based on a progeny

group of only 12 chicks from the single heterozygous sire and therefore must be interpreted with caution until supported by a larger number of observations.

The ISRRP F₁ resource population was generated from a cross between divergent breeds with outbred sires and highly inbred dams. The advantages of this population are the consistent contribution of the same alleles to all offspring by each highly inbred dam line, thereby reducing “genetic noise” and allowing a more refined estimate of the contribution of the sire alleles. A disadvantage is that large linkage disequilibrium is expected. Therefore, future studies should investigate and confirm the effects of the *INOS-AluI*, *TRAIL-StyI*, *TGF-β2-RsaI*, *TGF-β3-BsrI*, and *IgL-Sua96I* polymorphism to determine whether these genes are indeed directly causative or only linked to other loci controlling the observed variation in pathogen response to *Salmonella*. Currently, advanced intercross generations of the ISRRP are being produced to reduce the linkage disequilibrium interval for the purpose of fine-mapping the genes controlling *Salmonella* response.

Several new chromosomal regions with effects on response to *Salmonella* in chickens were identified in this study. Considering the associations observed with multiple response traits to *Salmonella*, this study suggests that these candidate gene polymorphisms could potentially be used as markers to discover the specific DNA sequence differences responsible for the phenotypic variation. After this confirmation, these genes can be used in marker-assisted selection to enhance response to *Salmonella*. Identification of genes in protective pathways of disease resistance will also open possibilities to design specific therapeutic strategies (e.g. vaccines) to match population genotypes for crucial genes.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge the members of the Poultry Research Center at Iowa State University for help in managing the birds; Michael Kaiser, Bill Larson, Wei Liu, and Huaijun Zhoun for technical support; and Animal Health, State of Iowa, and Multistate Research Funds and Research Grant IS-3021–98CR from BARD for financial support. Massoud Malek is a Cargill Research Excellence Fellow.

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