Natural Resistance-Associated Macrophage Protein 1 Gene Polymorphisms and Response to Vaccine Against or Challenge with *Salmonella enteritidis* in Young Chicks[1]

W. Liu, M. G. Kaiser, and S. J. Lamont[2]

Department of Animal Science, Iowa State University, Ames, Iowa 50011-3150

ABSTRACT *Salmonella enteritidis* (SE) contamination of poultry products is of global food-safety concern. The natural resistance-associated macrophage protein 1 (NRAMP1) affects host innate immunity to intracellular bacteria because of its ability to transport divalent cations in late endosome/lysosomes. Studying the association of the NRAMP1 gene and chicken innate immune response to SE can, therefore, aid understanding and enhancement of chicken genetic resistance to SE. The chicken NRAMP1 gene was investigated as a candidate gene for SE response in a unique resource population. Outbred broiler sires and three diverse, highly inbred dam lines (two major histocompatibility complex-congenic Leghorn and one Fayoumi line) produced F_1 progeny that were evaluated as young chicks for either bacterial load in spleen and cecum after pathogenic SE inoculation or antibody level after SE vaccination. Thirty-seven single nucleotide polymorphisms (SNP) were identified in 3.1 kb of genomic DNA of the NRAMP1 gene. A PCR-RFLP assay was developed to identify a SNP in a conserved transport motif. The sire NRAMP1 gene SNP was associated ($P < 0.02$) with antibody level to SE vaccine for Sire 8170 offspring in the two Leghorn crosses. In Sire 8296 offspring, NRAMP1 was associated ($P < 0.02$) with spleen bacterial load in the combined dam-line crosses. This study demonstrated the association of a SNP polymorphism in a highly conserved region of NRAMP1 with SE vaccine and pathogen challenge response in young chicks, indicating that either NRAMP1 or a linked gene controls these SE-response traits.

(*Key words*: natural resistance-associated macrophage protein 1, *Salmonella enteritidis*, polymerase chain reaction-restriction fragment length polymorphism, bacterial load, antibody level)

2003 Poultry Science 82:259–266

INTRODUCTION

Salmonella contamination of poultry products is of global concern because of the potential threat to public health (Barrow, 1997). The most common causes of human *Salmonella* foodborne disease are *S. enteritidis* and *S. typhimurium* (O'Brien, 1988; Angulo and Swerdlow, 1998; Hafez, 1999). Because SE infects the reproductive tract, as well as the gastrointestinal tract, SE can be deposited into unfertilized eggs that are intended for human consumption (Keller et al., 1995). SE can be vertically transmitted to progeny by infecting the developing embryo, as well as being horizontally transmitted to hatch-mates to cause a widespread pathogenic impact (Gast and Beard, 1990). There are increasing concerns regarding antibiotic usage to control zoonotic pathogens in livestock, because of the potential for evolution of antibiotic-resistant pathogens (Tollefson et al., 1999). Enhancement of natural genetic resistance to *Salmonella* in chickens is, therefore, an important alternative approach to maintain both animal welfare and public health.

One approach to enhance the genetics of immune response and disease resistance is to utilize candidate genes. 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, markers near or within the gene sequence can be developed for marker-assisted selection to improve immunity. A small number of potential candidate genes have already been identified for *Salmonella* response in the chicken and mouse. The natural resistance-associated macrophage protein 1 (NRAMP1) gene is linked to mortality induced by *S. typhimurium* in the mouse (Vidal et al., 1993) and chicken (Hu et al., 1997). The

Received for publication January 7, 2002.

Accepted for publication September 27, 2002.

[1]Journal paper No. J-19133 of the Iowa Agriculture and Home Economics Experiment Station, Ames, IA 50011; Projects 2237 and 3521.

[2]To whom correspondence should be addressed: sjlamont@iastate.edu.

Abbreviation Key: BPDTS = binding protein-dependent transport motif; ISRRP = Iowa *Salmonella* response resource population; NRAMP1 = natural resistance-associated macrophage protein 1; SNP = single nucleotide polymorphism.

NRAMP1 gene belongs to a large gene family encoding divalent cation transporters that are localized to late endosomes/lysosomes and are proposed to affect intraphagosomal microbial replication by modulating divalent cation content in this organelle. The many cellular functions that depend on metal ions as cofactors may explain the pleiotropic effects of NRAMP1 and its complex role in infectious diseases (Blackwell et al., 2000; Gruenheid and Gros, 2000). The SAL1 locus on chicken chromosome 5 was identified as a QTL for *Salmonella* response (Mariani et al., 2001). Chicken MHC haplotypes are associated with mortality to SE challenge (Cotter et al., 1997). The role of the Toll-like receptor 4 (TLR4) gene in chicken susceptibility to *Salmonella* infection is under active study (Beaumont et al., 2000; Leveque and Malo, 2000).

Investigation of antibody response to SE vaccination, as well as bacterial colonization with pathogenic SE, provides important information to characterize the genetic control of multiple levels of response to *Salmonella*. In the current study, F_1 chicks from outbred broiler breeder sires crossed with dams of three highly inbred lines [G-B1, G-B2, and Fayoumi (M15.2)] were used. The unique cross between genetically distant parental lines generated a high level of genetic variation, which has the advantage of improving the detection of linkage between markers and QTL (Hillel, 1997). The use of multiple dam lines allows detection of interaction of sire alleles with the genes of different dam lines. Because the founder lines were genetically distinct and the dam lines are highly inbred, the effect of heterozygous sire alleles could be studied in the F_1 generation (Lamont et al., 2002). Also, use of an F_1 generation with half of the genetics derived from inbred lines minimizes the genetic noise and helps to detect contribution of sire variation.

As part of a comprehensive program to identify genetic markers of host response to SE, the objectives of this study were to identify sequence polymorphisms of the NRAMP1 gene in broiler and diverse inbred lines, to develop a method to characterize single nucleotide polymorphisms (SNP) in the resource population, and to investigate associations between an NRAMP1 sequence polymorphism and response to challenge or vaccination with SE.

MATERIALS AND METHODS

Genetic Material

The F_1 generation of the Iowa *Salmonella* Response Resource Population (ISRRP) of chickens was used. Four outbred broiler breeder male line sires (Kaiser et al., 1998), each mated to three to six dams of each of three highly inbred, diverse lines with inbreeding coefficients of 99% (Zhou and Lamont, 1999), produced an F_1 population in multiple hatches. The G-B1 and G-B2 inbred lines are two MHC-congenic Leghorn sublines that have been widely used for studies of MHC-restricted immune response and disease resistance (MacCubbin and Schierman, 1986; DiFronzo and Schierman, 1989). The Fayoumi (M15.2) line, which originated in Egypt, was imported to the United States because of anecdotal high resistance to leukosis. The Fayoumi line is genetically distant from the Leghorn and broiler lines and is unique in disease resistance characteristics and in specific alleles, compared with inbred Leghorn lines (Lamont et al., 1992; Lakshmanan et al., 1996; Zhou and Lamont, 1999).

Salmonella Pathogenic Challenge and Quantification of Bacterial Load

The F_1 chicks were raised in floor pens and given access to water and feed ad libitum meeting or exceeding National Research Council (1994) requirements. The F_1 chicks (n = 194) from three hatches were intraesophageally inoculated by intubation with 10^4 cfu/bird of virulent SE Phage type 13a at 1 d of age. The health status was monitored twice daily. Half of the birds each were euthanized at 6 or 7 d of age by cervical dislocation. Spleen and cecal content were aseptically harvested for bacterial quantification. The SE culture and quantification procedures were as described by Kaiser and Lamont (2001).

Salmonella Vaccination and Antibody Measurement

Chicks (n = 314) from two hatches were evaluated for antibody response to SE vaccination. Birds were administered 0.2 mL commercial killed SE vaccine[3] subcutaneously in the neck at 10 d of age. Blood samples (1.0 mL) were drawn from the wing vein of all chicks at 21 d of age. Prevaccination samples obtained from several chicks established that prevaccination SE antibody levels were not detectable. The blood was allowed to clot and was centrifuged (16,000 × *g*, 5 min); sera were collected and stored frozen until assayed. The ELISA assay was conducted using a commercial SE antibody kit,[4] with modifications as described by Kaiser et al. (1998). The antibody level was represented as (1-S/N), where S = sample optical density at 630 nm (OD_{630}), N = the triplicate means of the OD_{630} for the negative controls.

DNA Isolation, Primer Development, and Amplification

Genomic DNA was prepared by using a standard phenol/chloroform isolation procedure on erythrocytes collected from chicks challenged with SE at 5 d of age and chicks of SE vaccination at 21 d of age (Dunnington et al., 1990). Primers were designed using Oligo 5[5] from database sequence of GenBank accession S82465 and U40598 to PCR amplify the NRAMP1 genomic region and

[3]Biommune, Lenexa, KS.
[4]*Salmonella enteritidis* (antibody test), IDEXX Laboratories, Inc., Westbrook, ME.
[5]Applied Biosystems, Perkin-Elmer, Foster City, CA.

were synthesized at the DNA Sequencing and Synthesis Facility.[6] Primer set NR4F/NR4R (5′ GCACGATGCCCACCCTTG 3′; 5′ GGGACATTGCTGGCGTCAGT 3′) was used to amplify a 642-bp fragment from –220 bp to 202 bp, including the promoter region, exon 1, regulation factor in intron 1, and partial exon 2. Primer set NR5F/NR5R (5′ GGGACTGCTGTGCCAGAG 3′; 5′ TGCTAGCCAAAGGTCAGAGC 3′) was used to amplify an 884-bp fragment from exons 4 to 7. Primer set NR6F/NR6R (5′ CCGGCGCAGACGGAGGTGCT 3′; 5′ CCCGCGTAGGTGCCTGTCAT 3′) was used to amplify an 872-bp fragment from exon 8 to partial exon 11. Primer set NR7F/NR7R (5′ GGCGTCATCCTGGGCTGCTAT 3′; 5′ AGACCGTTGGCGAAGTCATGC 3′) was used to amplify an 801-bp fragment from exons 11 to 13. The PCR amplifications were carried out in a 25-μL reaction containing 25 ng genomic DNA, 0.8 μM of each primer, 200 μM of each dNTP, 1 U of *Taq* DNA polymerase,[7] 2.5 μL of 10 × PCR reaction buffer, and 1.5 mM $MgCl_2$. PCR was performed for 35 three-step cycles at 94 C for 1 min, 64 C for 90 s (66 C for NR5F and NR5R), and 72 C for 1 min. Amplified products were purified using a MICROCON[8] centrifugal filter. Nucleotide sequencing was performed by the DNA Sequencing and Synthesis Facility.[6] DNA samples from all four broiler sires and two individuals from each inbred dam line (total n = 10) were sequenced using both direction primers (total n = 20 sequences). Sequences were analyzed using Sequencher3.1.[9]

PCR-RFLP Genotyping

The restriction enzyme sites in these sequences were detected by a sequence analysis web server.[10] The *Sac*I restriction enzyme was selected to genotype F_1 chicks based on a single nucleotide sequence polymorphism at Ser^{379} among the parents. For PCR-RFLP, 8 μL of amplified PCR product was digested (4 h at 37 C) by adding 0.5 μL *Sac*I restriction enzyme[7] (10 unit/μL), 0.2 μL acetylated BSA (10 μg/μL), 2 μL 10 × Buffer J, and 9.3 μL H_2O to make final reaction volumes of 20 μL. Separation was by electrophoresis through 2% agarose gels. Two of the four sires were heterozygous for this SNP, and, therefore, only their F_1 offspring were genotyped using PCR-RFLP. The number of F_1 phenotypic observations for spleen bacterial count, cecal content bacterial count, and antibody level, were 57, 54, and 163, respectively, for offspring of these two sires.

Statistical Analysis

General linear models were used to estimate the association between the NRAMP1 genotype of the F_1 chicks of two heterozygous sires and the SE bacteria count using SAS software (SAS Institute, 1999). Model 1 was used for the combined two sire families. Sire allele (allele), sire, dam line, interaction between sire allele and sire, and interaction between sire allele and dam line were used as fixed effects in Model 1. The ranges for spleen SE (1.04×10^7 to 3.55×10^9 cfu/mL) and cecum count SE (1.06×10^7 to 5.5×10^8 cfu/mL) were large, and the distributions for both variables skewed from normality. Both the spleen and cecum SE counts were transformed to their natural logarithms as response variables, therefore, to achieve normality or homogeneous variance in model construction.

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

where Y_{ijkl} = response variables from each F_1 bird, natural logarithms of spleen bacteria count, or cecal bacterial count.

To calculate the percentage of phenotypic variation of spleen or cecum bacterial load accounted for by the sire NRAMP1 allele, Model 1 was taken as a full model and used to build a reduced model with only sire and dam-line fixed effect as explanatory variables. Let SSE_{full} and SSE_{red} be the residual sum of squares for the full and reduced models, and SST_{full} be the total sum of squares for the full model, then $100\% \times (SSE_{red}\text{-}SSE_{full})/SSE_{red}$ is the percentage phenotypic variation accounted by sire NRAMP1 allele effect after adjustment for sire and dam-line effect, and $100\% \times (SSE_{red}\text{-}SSE_{full})/SST_{full}$ is the percentage phenotypic variation accounted by sire NRAMP1 allele effect.

A linear mixed model was used to estimate the association between the NRAMP1 genotype of the F_1 chicks of the two heterozygous sire families (n = 175) and the SE vaccine antibody level. Model 2 was used for the combined two 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.

$$\text{Model 2: } Y_{ijklmn} = \mu + \text{allele}_i + \text{sire}_j + \text{dam line}_k + \text{allele} \times \text{sire}_{ij} + \text{allele} \times \text{dam line}_{ik} + \text{sex}_l + \text{plate}_m \text{ (random)} + e_{ijklmn}$$

where Y_{ijklmn} = response variables from each F_1 bird, (1-S/N) for antibody level.

To calculate the percentage of the phenotypic variation in antibody level accounted for by the sire NRAMP1 allele, set Model 3 as a full model, then build a reduced model with sire, dam line, and sex as fixed effects and plate as a random effect. Then the calculation is same as for spleen and cecum bacterial load.

RESULTS

NRAMP1 Allelic Diversity

The NRAMP1 genomic fragments were sequenced and characterized in four outbred broiler sires and two dams

[6]Iowa State University, Ames, IA.
[7]Promega Co., Madison, WI.
[8]Millipore Corporation, Bedford, MA.
[9]Gene Codes Corporation, Ann Arbor, MI.
[10]http://darwin.bio.genseo.edu./~yin/webGen e/RE.html.

TABLE 1. Distribution of nucleotide sequence polymorphisms in exons 1 to 13 coding regions of NRAMP[1] among parental chicken lines and GenBank sequence

Line	Thr^{122}	Leu^{167}	Gln^{196}	Ser^{267}	Tyr^{288}	Leu^{297}	Phe^{298}	Phe^{311}	Asn^{325}	Ile^{356}	Ser^{379}	Tyr^{386}
GenBankU40598	ACA	TTG	CGG	TCA	TAT	CTC	TTT	TTC	AAT	ATC	AGC	TAC
Broiler Sire 8170	••G	C••	•G•	••C	••C	••G	••C	••T	••C	••C	••C/T	••C/T
Broiler Sire 8291	••G	C••	•G•	••C	••C	••G	••C	••T	••C	••C	••T	••C
Broiler Sire 8296	••G	C••	•G•	••C	••C	••G	••C	••T	••C	••C	••C/T	••C/T
Broiler Sire 8338	••G	C••	•G•	••C	••C	••G	••C	••T	••C	••C	••C	••T
G-B1	••G	C••	•G•	••C	••C	••G	••C	••T	••C	••C	••T	••C
G-B2	••G	C••	•G•	••C	••C	••G	••C	••T	••C	••C	••T	••C
M15.2	••G	C••	•A•	••C	••C	••C	••C	••C	••C	••T	••C	••C

[1]Natural resistance-associated marcophage protein 1.

from each of three inbred lines, for a total of 10 individuals. Sequences are available in the GenBank database (GenBank accession AF447073, AY072001 to AY072007). The sequenced NRAMP1 genomic regions had 100% within-inbred line nucleotide sequence identity. A total of 37 polymorphisms were identified in the 3.1-kb region among Leghorn, Fayoumi, and broiler lines. Six polymorphisms were identified in the exon regions (Table 1) among the lines that developed the ISRRP, and others were in the intron or 5′ regulatory regions (data not shown). Most sequence polymorphisms (24 of 37) came from comparison of the Fayoumi line with the other lines.

A PCR-RFLP method was developed to identify a *Sac*I polymorphism for Ser^{379} (Figure 1). Sires 8170 and 8296 were Ser^{379} heterozygous genotype C/T, sire 8291 and the Leghorn lines (G-B1 and G-B2) were homozygous T/T, and sire 8338 and the Fayoumi line (M15.2) were homozygous C/C. Thus, F_1 offspring from only the two heterozygous sires were genotyped to identify the sire allele effect on each SE response trait.

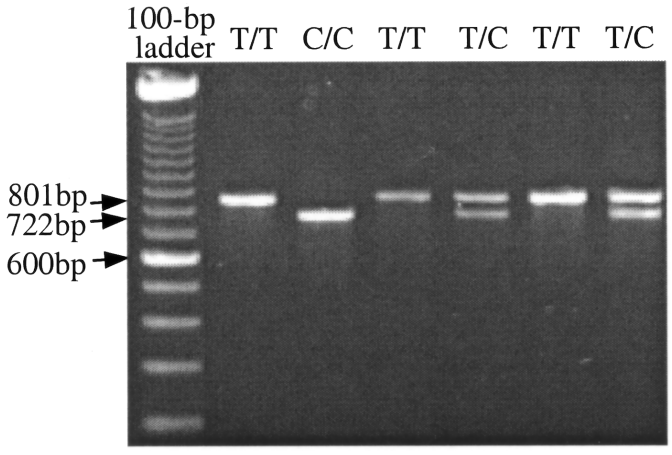

FIGURE 1. Ethidium bromide-stained 2% agarose gel electrophoresis of endonuclease *Sac*I-digested PCR product from natural resistance-associated macrophage protein 1 (NRAMP 1) gene showing characteristic polymorphic bands for the Ser^{379} alleles in the gene in the F_1 population. T/T = homozygotes of NRAMP Ser^{379} 801-bp allele lacking the *Sac*I site; C/C = homozygotes of NRAMP I Ser^{379} 722-bp allele, exhibiting only the digested fragment; C/T = heterozygotes, showing the presence of digested (722 bp) and undigested (801 bp) bands. The small *Sac*I digested 80-bp fragment is not present in the resolving range of a 2% agarose gel.

NRAMP1 Associations with Response to SE

The unique outbred-by-inbred F_1 population design was such that only the heterozygous NRAMP1 sire contributed genetic variation (C or T allele) in the NRAMP1 Ser^{379} alleles to the analysis within each dam-line cross. Thus, the analysis was of the effect of the sire allele only. Although one of the two SNP nucleotides (C or T) used to identify the two different sire alleles was identical with the SNP nucleotide of each dam line used in the cross, the NRAMP1 alleles are unique among the lines, based upon the sequence data (Table 1). For example, the sire NRAMP "T" allele is unique from that of the "T" allele inherited from the Leghorn dam lines. The analysis of both sire families combined for three *Salmonella* response phenotypes indicated a sire effect ($P < 0.05$ for antibody level, Table 2), a sire allele-by-dam-line interaction ($P < 0.03$ for antibody level, Table 2), and a sire allele-by-sire interaction ($P < 0.01$ for spleen SE count, Table 2). The NRAMP1 allele effect accounted for 19.1% of the phenotypic variation of spleen bacterial load and 3.1% of the phenotypic variation of cecum bacterial load, after adjustment for sire and dam-line effect (or 17.9% and 2.8% of the total phenotypic variation of spleen and cecum bacterial load, respectively). The NRAMP1 allele effect determined 5.9% of the phenotypic variation of antibody response to SE after adjustment for sire, dam-line, sex, and plate effects (or 3.6% of the total phenotypic variation of antibody response to SE). Sire NRAMP1 polymorphism at the Ser^{379} position was associated ($P < 0.02$) with spleen SE bacteria burden in Sire 8296 offspring in the combined three dam-line crosses, where the C allele was associated with lower spleen bacterial load (Table 3). In the Sire 8170 family, NRAMP1 polymorphism was associated ($P < 0.02$) with antibody levels in crosses with both Leghorn inbred lines. The C allele was associated ($P < 0.02$) with higher antibody levels in the G-B1 cross but with lower antibody levels in the G-B2 cross ($P < 0.01$) (Table 3). There was a difference ($P < 0.001$) in antibody level between the two sexes in the two sire families combined (data not shown). Females had a lower mean antibody level (0.14 ± 0.03) than males (0.22 ± 0.03). The lower antibody response of females was consistent in each sire family.

TABLE 2. Effect of sire NRAMP[1] allele, sire, dam line, sex, and two-way interactions with allele on three *Salmonella* response phenotypes (ANOVA, *P* values)

Source of variation	Bacterial load		Antibody level[4]
	Cecum[2]	Spleen[3]	
Allele	0.58	0.88	0.32
Sire	0.11	0.39	0.05
Dam line	0.91	0.47	0.07
Sex	—	—	0.001
Allele × sire	0.80	0.01	0.25
Allele × dam line	0.62	0.30	0.03
Plate (antibody level, random)	—	—	<0.001
Number of observations	54	57	153

[1]Natural resistance-associated macrophage protein.

[2]Natural log(number of cecal content SE bacteria).

[3]Natural log(number of spleen content SE bacteria).

[4](1 – S/N) for SE vaccine antibody ELISA assay = sample OD_{630}/mean OD_{630} negative control. OD_{630} = optical density at 630 nm.

DISCUSSION

Unique NRAMP1 SNP were identified in the current study compared to those previously reported in other populations (Hu et al., 1997). The chicken NRAMP1 cDNA sequence and gene structure have been previously characterized (Hu et al., 1996; Girard-Santosuosso et al., 1997). In our study, 3.1 kb of the 5-kb NRAMP1 genomic region was characterized in 10 individuals from three breeds of chicken. The discovery of new SNP is likely because the current population includes outbred broiler, inbred Leghorn lines and inbred Fayoumi lines, which represents more diverse genetics than the inbred white Leghorn lines of the study of Hu et al. (1997). Most of the species-level SNP identified (24 of 37) came from comparison with the Fayoumi line (Table 1). This molecular divergence agrees with the distant and unique genetic background of the Fayoumi line and is in agreement with other molecular evaluations of this line (Chen and Lamont 1992; Plotsky et al., 1995; Zhou and Lamont 1999). Among all identified SNP, the *Sac*I polymorphism for Ser^{379} (Figure 1) is of particular interest because it is in the exon that codes for an evolutionarily conserved binding protein dependent transport motif (BPDTS) between TM8 and TM9 (Bairoch, 1991). Even though the Ser^{379} polymorphism of the NRAMP1 gene examined in the present study was a synonymous substitution (as were all exon polymorphisms identified in this study), its association with *Salmonella* response may be because of linkage to a functional polymorphism. The Ser^{379} polymorphism was reported, but not further examined, in the study of Hu et al. (1997), in which the association between the polymorphism G/A (Arg^{223}/Gln^{223}) in NRAMP1 and the *S. typhimurium*-induced survival curve was investigated.

The association between NRAMP1 polymorphism and SE responses identified in the current study indicated that the genomic region containing the NRAMP1 gene was linked to SE response. The linkage disequilibrium

TABLE 3. Least squares means, standard error (N) and *P*-value between Ser^{379} C and T sire alleles of the NRAMP1[1] gene for antibody response to SE vaccine, natural logarithms of spleen and cecum SE bacterial load in the F_1 population by dam line

Phenotype and dam line	Sire 8170			Sire 8296		
	C	T	*P*-value	C	T	*P*-value
Antibody level[2]						
All	0.21 ± 0.03 (41)	0.24 ± 0.03 (56)	0.38	0.18 ± 0.04 (32)	0.15 ± 0.04 (32)	0.50
G-B1	0.34 ± 0.05 (9)	0.19 ± 0.05 (11)	0.02	0.21 ± 0.06 (7)	0.14 ± 0.07 (5)	0.43
G-B2	0.14 ± 0.04 (20)	0.25 ± 0.04 (20)	0.01	0.15 ± 0.05 (19)	0.14 ± 0.04 (21)	0.93
M15.2	0.15 ± 0.05 (12)	0.16 ± 0.03 (25)	0.95	0.19 ± 0.07 (6)	0.18 ± 0.07 (6)	0.95
Spleen load[3]						
All	20.30 ± 0.18 (10)	20.02 ± 0.25 (6)	0.38	19.82 ± 0.11 (19)	20.20 ± 0.11 (22)	0.02
G-B1	20.32 ± 0.28 (4)	20.13 ± 0.55 (1)	0.76	19.70 ± 0.14 (10)	20.04 ± 0.14 (10)	0.09
G-B2	20.41 ± 0.28 (4)	19.77 ± 0.32 (3)	0.16	20.06 ± 0.22 (4)	20.20 ± 0.14 (9)	0.60
M15.2	20.17 ± 0.39 (2)	20.16 ± 0.39 (2)	0.99	19.69 ± 0.19 (5)	20.35 ± 0.25 (3)	0.04
Cecum load[3]						
All	18.52 ± 0.24 (9)	18.56 ± 0.32 (6)	0.92	18.95 ± 0.19 (18)	18.88 ± 0.19 (21)	0.79
G-B1	18.16 ± 0.32 (4)	19.52 ± 0.71 (1)	0.11	18.90 ± 0.27 (9)	18.80 ± 0.26 (10)	0.80
G-B2	18.50 ± 0.41 (3)	18.70 ± 0.41 (3)	0.74	19.04 ± 0.41 (4)	18.53 ± 0.26 (8)	0.29
M15.2	18.91 ± 0.50 (2)	17.48 ± 0.50 (2)	0.07	18.91 ± 0.36 (5)	19.05 ± 0.47 (3)	0.82

[1]Natural resistance-associated macrophage protein 1.

[2](1 – S/N) for SE antibody ELISA assay, where S = sample OD 630, and N = the triplicate mean negative control OD 630.

[3]Natural log(number) of SE bacteria load in site.

that exists in the F_1 population cannot, however, eliminate the alternate hypothesis that the association of the NRAMP1 SNP and SE responses was because of another gene syntenic to NRAMP1. But, considering the biological function of NRAMP1 identified in mouse (Vidal et al., 1993) and the association between NRAMP1 polymorphism and mortality in chicks after infection by *S. typhimurium* in an independent study (Hu et al., 1997), together with results from the current study, the NRAMP1 gene becomes a good candidate gene for response to the important pathogen, SE, based on these multiple independent studies.

There were important procedural differences between the current study and the study by Hu et al. (1997). Hu et al. (1997) reported NRAMP1 to be linked with mortality in chicks after infection by *S. typhimurium*. In their study, pathogen-challenged chicks were intramuscularly inoculated with highly virulent *S. typhimurium* strain on the day of hatch. In the current study, chicks were administered a different species of *Salmonella*, *S. enteritidis*, through the gastrointestinal tract, to mimic the usual oral exposure route of infection. The response to *S. enteritidis* was characterized by quantification of SE bacterial numbers that colonized two sites (spleen and cecal contents). Thus, the phenotypes measured in the current study incorporate the important effects of mucosal defenses (McGhee et al., 1992) and also characterize defense properties against bacterial colonization of internal organs in the host animal. This model of the natural route of exposure and detailed quantification of gastrointestinal and internal organ bacterial burden may yield information on candidate genes of relevance to field conditions and, therefore, be of practical importance for applied marker-assisted selection for enhanced genetic resistance to SE.

Gene interaction and linkage disequilibrum are important issues in candidate gene studies (Rothschild and Soller, 1997). The unique population design of crossing outbred broiler sires with multiple, diverse inbred dam lines allowed detection of gene interaction between the sire NRAMP1 allele and sire and between sire NRAMP1 allele and dam line genetics on the F_1 offspring response to SE. This result could help to determine whether the associations between genetic markers and traits of interest are detectable only in a specific cross, or if they are robust over a wide range of genetic combinations and therefore expected to be more effectively applied in marker-assisted selection across populations. Also, the interaction between sire NRAMP1 allele and dam lines on F_1 response to SE could be more clearly detected, because of the homogeneity within each dam line and the genetic distance between the dam lines. There was a significant interaction effect between sire NRAMP1 allele and sire on the F_1 offspring spleen bacterial load and between sire NRAMP1 allele and dam line on F_1 offspring antibody response to SE vaccine. NRAMP1 can influence antigen presentation directly or indirectly. The NRAMP1 allele directly affects antigen presentation by regulating MHC II expression, which affects antigen processing and presentation (Lang et al. 1997). The indirect effects may arise from the regulatory function of NRAMP1 on genes related to antigen processing and presentation, such as tumor necrosis factor-α (TNF-α) and interleukin 1β (IL-1β) (Lang et al., 1997). The regulatory effects may utilize complex pathways. The detected gene interactions may arise from pleiotropic effects of the NRAMP1 gene on macrophage activation pathways and macrophage function (Soo et al., 1998; Blackwell et al., 2000).

The NRAMP1 allele was significantly associated with chick systemic response to *S. enteritidis* challenge. Lumenal *Salmonella* can penetrate the epithelial barrier through M cells in the gastrointestinal tract via the type III secretory system (Collazo and Galan, 1997). Internalized *Salmonella* can be engulfed by macrophages beneath epithelial cells via phagocytosis and carried to the spleen. *Salmonella* may survive and replicate within vacuoles inside macrophages (Finlay and Brumell, 2000). Dendritic cells can also sample lumenal *Salmonella* and transport bacteria to the spleen (Rescigno et al., 2001). Therefore, the spleen SE bacterial load may represent the overall status of host systemic immune response to SE. In mice, the bacterial load of the spleen was significantly lower in Nramp1(r) (resistant) mice than Nramp1(s) (susceptible) mice. (Lalmanach et al., 2001).

The NRAMP1 allele was significantly associated with antibody response to SE specifically in the MHC-congenic Leghorn crosses. The effect of *Nramp1* on immune response to *Salmonella* vaccine was also shown in mice (Gautier et al., 1998; Soo et al., 1998). In a study by Gautier et al (1998), congenic BALB/c-susceptible (*Ity/Nramp1*^{s}) mice showed a significantly higher antibody level to *S. abortusovis* vaccine strain Rv6 than C.D2-resistant mice (*Ity/Nramp1*^{r}). The influence of *Nramp1* on response to recombinant salmonella vaccines was shown by Soo et al. (1998), who used tetanus toxoid antigen and leishmainial gp63 carried by live attenuated *S. typhimurium aroA aroD* mutants.

The interaction between NRAMP1 and MHC on antibody response to *S. enteritidis* in the current study may be related to NRAMP1 regulation of MHC class II expression as reported in other studies. The *Nramp1*^{r} allele upregulates MHC class II expression in mice (Lang et al., 1997; Wojciechowski et al., 1999). The G-B1 and G-B2 dam lines used in the current study are MHC congenic lines (Chen and Lamont, 1992). Single-strand conformation polymorphism assay of the MHC class IIβ genomic region showed different patterns between G-B1 and G-B2, confirming MHC class IIβ gene polymorphisms between the two lines (Liu and Lamont, unpublished data). Thus, the interaction between NRAMP1 and dam line (of the two MHC-congenic lines) may reflect the NRAMP1 affecting antibody response to SE vaccine through differential regulation of MHC class II expression of the two MHC alleles.

The NRAMP1 gene showed no association with SE cecum bacterial load in the present study, even though the NRAMP1 gene is important for control of spleen bacterial load. Bacteria load in cecal content was not correlated with spleen bacterial load in a study of young chick survival and pathogen load to after SE challenge (Kaiser and

Lamont, 2001). This finding suggests that independent host genetic mechanisms are responsible for control of pathogen load at the two sites. Bacterial load in cecal content is an estimator of fecal bacterial shedding; therefore, results of the current study suggest that NRAMP1 may not affect the level of shedding of SE bacteria.

In summary, the Ser^{379} position SNP of the NRAMP1 gene was associated with spleen bacterial load after exposure to pathogenic SE and also with antibody production to SE vaccination. It may, therefore, be useful for marker-assisted selection to improve resistance to SE in populations in which potential gene interactions are defined. This work supports a previous report of the effect of NRAMP1 on chicken innate immune response to *Salmonella* that evaluated different genetic host populations, *Salmonella* challenge species, and phenotypic traits. The current study confirms and expands previous research on the associations of NRAMP1 and *Salmonella* responses and clearly demonstrates the importance of gene interaction on NRAMP1 effects.

ACKNOWLEDGMENTS

We thank William Larson and Huaijun Zhou for assistance with phenotypic data collection, Kenneth J. Koehler for very helpful advice on statistical analysis, and Nader Deeb for helpful discussion. We thank IDEXX Laboratories., Inc., for donation of some of the ELISA kits used in this study.

REFERENCES

Angulo, F. J., and D. L. Swerdlow. 1998. Salmonella enteritidis infections in the United States. J. Am. Vet. Med. Assoc. 213:1729–1731.

Bairoch, A. 1991. PROSITE: a dictionary of sites and patterns in proteins. Nucleic Acids Res. 19(Suppl.):2241–2245.

Barrow, P. A. 1997. Novel approaches to control of bacterial infections in animals. Acta Vet. Hung. 45:317–329.

Beaumont, C., J. Protais, P. Colin, F. Pitel, F. Plisson-Petit, A. Vignal, M. Protais, and D. Malo. 2000. Testing the effect of two candidate genes on the resistance to *Salmonella* carrier stat in poultry. Proceedings of the World's Poultry Congress XXI. WPSA, Montreal, Quebec, Canada.

Blackwell, J. M., S. Searle, T. Goswami, and E. N. Miller. 2000. Understanding the multiple functions of Nramp1. Microbes Infect. 2:317–321.

Chen, Y., and S. J Lamont. 1992. Major histocompatibility complex class I restriction fragment length polymorphism analysis in highly inbred chicken lines and lines selected for major histocompatibility complex and immunoglobulin production. Poult. Sci. 71:999–1006.

Collazo, C. M., and J. E. Galan. 1997. The invasion-associated type-III protein secretion system in Salmonella—A review. Gene 192:51–59.

Cotter P. F., R. L. Taylor Jr., and H. Abplanalp. 1998. B-complex associated immunity to Salmonella enteritidis challenge in congenic chickens. Poult. Sci. 77:1846–1851.

DiFronzo, N. L., and L. W. Schierman. 1989. Transplantable Marek's disease lymphomas. III. Induction of MHC-restricted tumor immunity by lymphoblastoid cells in F1 hosts. Int. J. Cancer 44:474–476.

Dunnington, E. A., O. Gal, Y. Plotsky, A. Haberfeld, T. Kirk, A. Goldberg, U. Lavi, A. Cahaner, P. B. Siegel, and J. Hillel. 1990. DNA fingerprints of chickens selected for high and low body weight for 31 generations. Anim. Genet. 21:247–257.

Finlay, B. B., and J. H. Brumell. 2000. Salmonella interactions with host cells: in vitro to in vivo. Philos. Trans. R. Soc. Lond. B Biol. Sci. 355:623–631.

Gast, R. K., and C. W. Beard. 1990. Isolation of Salmonella enteritidis from internal organs of experimentally infected hens. Avian Dis. 34:991–993.

Gautier A. V., I. Lantier, and F. Lantier. 1998. Mouse susceptibility to infection by the Salmonella abortusovis vaccine strain Rv6 is controlled by the Ity/Nramp 1 gene and influences the antibody but not the complement responses. Microb. Pathog. 24:47–55.

Girard-Santosuosso, O., N. Bumstead, I. Lantier, J. Protais, P. Colin, J. F. Guillot, C. Beaumont, D. Malo, and F. Lantier. 1997. Partial conservation of the mammalian NRAMP1 syntenic group on chicken chromosome 7. Mammal. Genome 8:614–616.

Gruenheid, S., and P. Gros. 2000. Genetic susceptibility to intracellular infections: Nramp1, macrophage function and divalent. Curr. Opin. Microbiol. 3:43–48.

Hafez, H. M. 1999. Poultry meat and food safety: Pre- and post-harvest approaches to reduce foodborne pathogens. World's Poult. Sci. J. 55:269–280.

Hillel, J. 1997. Map-based quantitative trait locus identification. Poult. Sci. 76:1115–1120.

Hu, J., N. Bumstead, P. Barrow, G. Sebastiani, L. Olien, K. Morgan, and D. Malo. 1997. Resistance to salmonellosis in the chicken is linked to NRAMP1 and TNC. Genome Res. 7:693–704.

Hu, J., N. Bumstead, E. Skamene, P. Gros, and D. Malo. 1996. Structural organization, sequence, and expression of the chicken NRAMP1 gene encoding the natural resistance-associated macrophage protein 1. DNA Cell Biol. 15:113–123.

Kaiser, M. G., and S. J. Lamont. 2001. Genetic line differences in survival and pathogen load in young layer chicks after Salmonella enterica serovar enteritidis exposure. Poult. Sci. 80:1105–1108.

Kaiser, M. G., T. Wing, and S. J. Lamont. 1998. Effect of genetics, vaccine dosage, and postvaccination sampling interval on early antibody response to *Salmonella enteritidis* vaccine in broiler breeder chicks. Poult. Sci. 77:271–275.

Keller, L. H., C. E. Benson, K. Krotec, and R. J. Eckroade. 1995. *Salmonella enteritidis* colonization of the reproductive tract and forming and freshly laid eggs of chickens. Infect. Immun. 63:2443–2449.

Lalmanach, A. C., A. Montagne, P. Menanteau, and F. Lantier. 2001. Effect of the mouse Nramp1 genotype on the expression of IFN-gamma gene in early response to Salmonella infection. Microbes Infect. 3:639–644.

Lakshmanan, N., M. G. Kaiser, and S. J. Lamont. 1996. Marek's disease resistance in MHC congenic lines from Leghorn and Fayoumi breeds. Pages 57–62 in Current Research on Marek's Disease. R. F. Silva, H. H. Cheng, P. M. Coussens, L. F. Lee, and L. F. Velicer, ed. American Association of Avian Pathologists, Kennett Square, PA.

Lamont, S. J. 1998. The chicken major histocompatibility complex and disease. Rev. Sci. Technol. 17:128–142.

Lamont, S. J., Y. Chen, H. J. Aarts, M. C. van der Hulst-van Arkel, G. Beuving, F. R. Leenstra. 1992. Endogenous viral genes in thirteen highly inbred chicken lines and in lines selected for immune response traits. Poult. Sci. 71:530–538.

Lamont, S. J., M. G. Kaiser, and W. Liu. 2002. Candidate genes for resistance to Salmonella enteritidis colonization in chickens as detected in a novel genetic cross. Vet. Immunol. Immunopathol. 87:423–428.

Lang, T., E. Prina, D. Sibthorpe, J. M. Blackwell, 1997. Nramp1 transfection transfers Ity/Lsh/Bcg-related pleiotropic effects on macrophage activation: influence on antigenprocessing and presentation. Infect. Immun. 65:380–386.

Leveque, G., and D. Malo. 2000. Identification of the chicken Toll-like receptor 4 (TLR4) gene and its role in susceptibility to Salmonella infection. Proceedings of the World's Poultry Congress XXI. WPSA, Montreal, Quebec, Canada.

MacCubbin, D. L., and L. W. Schierman. 1986. MHC-restricted cytotoxic response of chicken T cells: expression, augmentation, and clonal characterization. J. Immunol. 136:12–16.

Mariani, P., P. A. Barrow, R. Negrini, H. Cheng, M. Groenen, R. Negrini, and N. Bumstead. 2001. Localisation of a locus detennining salmonellosis resistance to chicken chromosome 5. Immunogenetics 53:786–791.

McGhee J. R., J. Mestecky, M. T. Dertzbaugh, J. H. Eldridge, M. Hirasawa, and H. Kiyono. 1992. The mucosal immune system: From fundamental concepts to vaccine development. Vaccine 10:75–88.

National Research Council. 1994. Nutrient Requirements of Poultry. 9th rev. ed. National Academy Press, Washington, DC.

O'Brien, J. D. 1988. Salmonella enteritidis infection in broiler chicks. Vet. Rec. 122:214.

Plotsky, Y., M. G. Kaiser, and S. J. Lamont. 1995. Genetic characterization of highly inbred chicken lines by two DNA methods: DNA fingerprinting and polymerase chain reaction using arbitrary primers. Anim. Genet. 26:163–170.

Rescigno, M., M. Urbano, B. Valzasina, M. Francolini, G. Rotta, R. Bonasio, F. Granucci, J. P. Kraehenbuhl, and P. Ricciardi-Castagnoli. 2001. Dendritic cells express tight junction proteins and penetrate gut epithelial monolayers to sample bacteria. Nat. Immunol. 2:361–367.

Rothschild, M. F., and M. Soller. 1997. Candidate gene analysis to detect genes controlling traits of economic importace in domestic livestock. Probe Newslett. Agric. Genomics 8:13–20.

SAS Institute. 1999. SAS/STAT User's Guide. Version 8.1. SAS Institute, Inc., Cary, NC.

Soo, S. S., B. Villarreal-Ramos, C. M. Anjam Khan, C. E. Hormaeche, and J. M. Blackwell. 1998. Genetic control of immune response to recombinant antigens carried by an attenuated Salmonella typhimurium vaccine strain: Nramp1 influences T-helper subset responses and protection against leishmanial challenge. Infect. Immun. 66:1910–1917.

Tollefson, L., P. J. Fedorka-Cray, and F. J. Angulo. 1999. Public health aspects of antibiotic resistance monitoring in the USA. Acta Vet. Scand. 92(Suppl.):67–75.

Vidal, S. M., D. Malo, K. Vogan, E. Skamene and P. Gros. 1993. Natural resistance to infection with intracellular parasites: isolation of a candidate for Bcg. Cell 73:469–485.

Wojciechowski, W., J. DeSanctis, K. Skamene, and D. Radzioch. 1999. Attenuation of MHC class II expression in macrophages infected with Mycobacterium bovis bacillus Calmette-Guerin involves class II transactivator and depends on the Nramp1 gene. J. Immunol. 163:2688–2696.

Zhou, H., and S. J. Lamont. 1999. Genetic characterization of biodiversity in highly inbred chicken lines by microsatellite markers. Anim. Genet. 30:256–264.