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Association Analysis of SNPs in the 3'-UTR of the *MyD88* Gene with Resistance to *Salmonella* Pullorum Infection in Chickens

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Authors' Contribution
PR designed the study and wrote the article. XQL involved in the data analysis. CWY and HRD helped in sample collection. XSJ helped in preparation of the manuscript. YPL helped in conceiving and designing the study.

Key words
MyD88 gene, 3` Untranslated region, Salmonella Pullorum, SNPs, Polymorphisms

ABSTRACT

Pullorum disease is caused by *Salmonella* Pullorum and does a great loss to the poultry industry. As a universal innate immune gene, *Myeloid differentiation primary response gene 88* (*MyD88*) can activate the nuclear factor-κB (NF-κB) pathway and regulate downstream gene expression. Single nucleotide polymorphisms (SNPs) in the coding regions (CDS) of the *MyD88* gene have also been reported to be associated with inter-subject differences in responses to *Salmonella* Pullorum infection in chickens. However, whether the 3'-untranslated region (3'-UTR) of the *MyD88* gene is associated with resistance to *Salmonella* Pullorum infection still remains unknown. In this study, a total of eight SNPs, including three novel mutations [SNP4 (A4812316G), SNP6 (C4813363A) and SNP7 (C4813618T)] and five known loci, were found within 3292 bp sequenced fragments. The allele frequency and genotype frequency of SNP4 (A4812316G) were found to be significantly different ($P<0.05$) between the case and control groups. However, no significant differences were found in the haplotypes of SNP1 and SNP2 ($P>0.05$). These results suggest that SNP4 (A4812316G) in the 3'-UTR of the *MyD88* gene is linked to genetic resistance to *Salmonella* Pullorum infection and may provide an important reference for the marker-assisted selection of chickens during disease-resistance breeding.

INTRODUCTION

Salmonella Pullorum is one of the common bacterial diseases that limited the rapid development of the poultry industry (Barrow *et al*., 2003). Though many studies related to genetic resistance have been carried out during the past few decades (Severens, 1944; Li *et al*., 2010; Li *et al*., 2018), the identification of resistance genes and the determination of their underlying mechanisms against *Salmonella* Pullorum infection remain to be studied (Wigley, 2004). However, in recent years, accumulating evidence has shown that innate immune genes may play important roles in *Salmonella* Pullorum infection (Peng *et al*., 2010; Ramasamy *et al*., 2014; Qiu *et al*., 2017). Some evidence showed the importance of the *MyD88* gene in *Salmonella* Pullorum infection (Arques *et al*., 2009; Li *et al*., 2010; Liu *et al*., 2015). Other research also showed that deletion of the *MyD88* gene could substantially delay the innate immune response (Aderem and Ulevitch, 2000). The multiprotein complex of the MyD88-IRAK (Interleukin-1 receptor-associated kinase) family is used by receptors of Interleukin-1 (IL-1), Interleukin-18 (IL-18) and Interleukin-33 (IL-33), which are important for inflammation and host defenses (Netea *et al*., 2012).

During infection, the host inflammatory response is initiated by pattern-recognition receptors (PRRs), which recognize pathogen-associated molecular patterns (PAMPs), conserved structures of the pathogenic microorganisms. The toll-like receptor (TLR) family is a major class of PRRs that leads to nuclear factor-κB (NF-κB) translocation and transactivation (Moynagh, 2005). Most TLR signaling involves the adaptor molecule MyD88 (Myeloid differentiation factor 88), and it plays a key role in maintaining the normal response of innate immunity that has been frequently reported during pathogen infections (Naiki *et al*., 2005). The function of *MyD88* gene in the innate immune response against bacterial infection is largely known (Tauszigdelamasure *et al*., 2002; Serbina *et*

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al., 2003; Woods *et al*., 2008). Adenine- and uridine-rich elements (AREs) have been found in the 3'-UTR of certain messenger RNAs (mRNAs), and due to the function of AREs in the 3'-UTR (Kuersten and Goodwin, 2003; Barreau *et al*., 2006), it has been speculated that innate immune genes may be involved in regulating transcription and translation in the innate immune system (Sun and Ding, 2006; West *et al*., 2011). Despite being studied broadly among different species (Stockhammer *et al*., 2009; Issac *et al*., 2018), the role of the highly conserved *MyD88* gene in the response to *Salmonella* Pullorum infection remains elusive. Identification of SNPs located outside the typical structure of the *MyD88* gene, including the N-terminal death domain, C-terminal toll-interleukin 1 receptor (TIR) domain, and intermediate domain, and further studies focusing on the *MyD88* untranslated region are necessary (Netea *et al*., 2012).

Innate immune-mediated disease resistance is closely related to genetic factors. As a key factor in innate immunity, the relevance of the *MyD88* gene to *Salmonella* Pullorum infection remains unclear. To further explore the potential association between the *MyD88* gene and genetic resistance to *Salmonella* Pullorum infection, 81 case and 90 control samples (infected or uninfected with *Salmonella* Pullorum) from local Qing Jiao Ma hens were collected to conduct an association study between the SNPs in the 3'-UTR of the *MyD88* gene and the resistance to *Salmonella* Pullorum infection in chickens.

MATERIALS AND METHODS

Salmonella *Pullorum detection and sample collection*

Experimental hens at the age of 300 days are a pure line of local Qing Jiao Ma chickens from the Poultry Breeding Farm, Sichuan Agricultural University. Based on case-control design, the whole blood glass plate agglutination method (SN/T 1222-2003, AQSIQ) was used to test for *Salmonella* Pullorum infection (Liu *et al*., 2015), which is quick and less costly, exerting the greatest value on a flock basis. Following common laying hen immunization program, chickens were vaccinated timely of immunization. In this study, 2,200 Qing Jiao Ma hens have been tested at the time of 300 days of age, among which 81 infected subjects were collected as the cases and 90 uninfected subjects were collected as controls. The blood samples were collected from a vein under the wings of the chickens and stored at -20 °C for further use. All procedures carried out in this experiment were reviewed and approved by the Institutional Animal Care and Use Committee of Sichuan Agricultural University, China.

DNA extraction and pool construction

Genomic DNA from the blood samples was extracted by using the standard phenol/chloroform method. After the extracted DNA was tested by Nano Drop (ND-2000, Thermo Scientific) as previously described (Liu *et al*., 2019), the DNA samples were diluted to the level of (100 ± 3) ng/μL. A case DNA pool and a control one were composed of 30 samples that each contains 2μL DNA selected at random, respectively.

Primer design

A primer designing tool in NCBI (http://www.ncbi.nlm.nih.gov/ tools/primer-blast) was used for primer designing, and five primer pairs were designed to cover the 3'-UTR of the *MyD88* gene according to the genomic sequence of *Gallus gallus* (GenBank accession number: NM_001030962). Primer pairs were designed after analysis by Oligo 7 (http://www.oligo.net/downloads.html). The primer sequences are shown in Table I.

PCR amplification and sequencing

Polymerase chain reaction (PCR) amplification was conducted using a 25 μL reaction mixture containing 50-100 ng of DNA, 0.3 μL of each forward and reverse primer, and 15 μL of 2× Taq PCR Master Mix (Tiangen Biotech Co., China). The procedure was carried out with 1 cycle of denaturalization at 96 °C for 4 minutes; 36 cycles of 98 °C for 40 seconds, 50-60 °C (optimal annealing temperature of each primer pair, listed in Table I) for 30 seconds, and 72 °C for 1 minute; and a final cycle of 72 °C for 8 minutes. All PCR products of the DNA pools and individuals were directly sequenced by the Shanghai Sangon Biotechnology Company (Shanghai, China).

Statistical analysis

Sequence variations, compositions and variable sites were identified using Chromas software and DNASTAR software (DNASTAR Inc., Madison, WI, USA). Hardy-Weinberg equilibrium, pairwise linkage disequilibrium (*D'*) and association analyses were conducted with Haploview software (version 3.32; http://www.broad.mit.edu/mpg/haploview/). The determination of allele frequency differences in the *MyD88* gene between the case and control groups were performed by chi-squared test using R software (version 3.0.2, The R Foundation for Statistical Computing). In previous study, odds ratio (OR) and 95% confidence interval calculations were performed to determine the resistance or susceptibility to *Salmonella* Pullorum infection (Liu *et al*., 2015).

Table I. The primer sequences of the 3'-UTR used for amplification.

Name	Amplicon size (bp)	Sequence (5'-3')	Target region	Production (bp)	Annealing temperature (°C)
P1	23	F: CTACTTAATTCAGCGAGCAATAG	Start - 1978	894	59.0
	20	R: ACAAACTGGACCCACTTAGG			
P2	25	F: ACCCTTTAATAGAAACTCAGTCTTG	1869 - 2616	768	54.6
	21	R: CGAGTTTGTGAGCCTACCCTA			
P3	20	F: GGTGCTGTTGCTGCTTCCTC	2531 - 3521	1012	54.0
	22	R: CACATCTCAAGTGCCAAACCAC			
P4	19	F: CATACCCAACTTGTGCGTT	3479 - 3978	521	52.5
	22	R: ACTCCATTTTGTCATTCAGAGA			
P5	20	F: GTAAAATCCAGCTTATGCAC	3907 - End	499	59.6
	20	R: ATTCCTCACTAACACTTCCT			

Table II. Change in alleles in the 3'-UTR.

Markers	ID	Position	Obs HET	Expt HET	Allele change	HWE (P)	MAF
SNP1	rs317890917	4810191	0.457	0.412	A>G	0.4784	0.330
SNP2	rs14131328	4810253	0.258	0.349	A>G	0.5507	0.327
SNP3	rs14131329	4810257	0.405	0.419	C>T	0.4578	0.354
SNP4	A4812316G	4812316	0.481	0.481	A>G	0.8340	0.342
SNP5	rs14131331	4810276	0.436	0.483	C>T	0.8480	0.368
SNP6	C4813363A	4813363	0.471	0.477	C>A	0.8152	0.409
SNP7	C4813618T	4813618	0.474	0.483	C>T	0.6792	0.111
SNP8	rs312369633	4813635	0.072	0.082	C>T	0.9977	0.146

Obs HET, observed heterozygosity; Expt HET, expected heterozygosity; HWE (*P*), *P* value of the Hardy-Weinberg equilibrium test; MAF, minimum allele frequency.

RESULTS

Sequencing the 3'-UTR of the MyD88 gene

In total, eight SNPs were detected in the 3'-UTR of the *MyD88* gene: three novel mutations located in the chicken genome, named A4812316G (SNP4), C4813363A (SNP6) and C4813618T (SNP7), and five known SNPs (http://www.ncbi.nlm.nih.gov/projects/SNP), named SNP1, SNP2, SNP3, SNP5, and SNP8.

The Hardy-Weinberg equilibrium

Eight SNPs were analyzed with the Hardy-Weinberg equilibrium (HWE) test, and the results were shown in Table II. The observed heterozygosity of all SNPs was at a general level as expected. All the eight SNPs fit the assumption of the Hardy-Weinberg equilibrium (P>0.05). The minor allele frequency (MAF) of the SNPs was greater than 0.01.

Allele and genotype frequency of the mutated loci

The results of the allele and genotype frequency analyses of the 8 SNPs in the case and control groups were shown in Tables III and IV. The allelic distributions did not significantly differ between cases and controls in the 3'-UTR except SNP4 (A4812316G, P=0.0315, χ^2=4.625). Table III shows that all of the OR values were less than 1, including the data for SNP4. In addition, the genotype distribution of SNP4 was significantly different between the case and control groups in the analysis of resistance to *Salmonella* Pullorum infection (P<0.05).

Association between haplotypes and susceptibility to Salmonella *Pullorum*

There were no significant differences in the linkage disequilibrium (LD) structures of the 8 SNPs between the cases and controls (Fig. 1). There was only one block which was in strong linkage disequilibrium state (D>0.8). The analysis of the haplotypes showed that the haplotype

Table III. Allele frequency of mutations in the 3'-UTR.

Markers	Alleles		χ2, P value	OR	95% CI
SNP1 (rs317890917)	A	G	χ^2=0.115 *P*=0.7344	0.9249	0.8392-1.1316
Cases	55 (0.340)	107 (0.660)			
Controls	58 (0.322)	122 (0.678)			
SNP2 (rs14131328)	A	G	χ^2=0.048 *P*=0.8269	0.9508	0.8480-1.1409
Cases	54 (0.333)	108 (0.667)			
Controls	58 (0.322)	122 (0.678)			
SNP3 (rs14131329)	T	C		0.6739	0.7408-1.0198
Cases	65 (0.401)	97 (0.599)	χ^2=3.029 *P*=0.0818		
Controls	56 (0.311)	124 (0.689)			
SNP4 (A4812316G)	G	A	χ^2=4.625 *P*=0.0315	0.6088	0.531-0.9759
Cases	116 (0.716)	46 (0.284)			
Controls	109 (0.606)	71 (0.394)			
SNP5 (rs14131331)	C	T	χ^2=1.424 *P*=0.2327	0.765	0.7689-1.0669
Cases	65 (0.401)	97 (0.599)			
Controls	61 (0.339)	119 (0.661)			
SNP6 (C4813363A)	C	A	χ^2=0.074 *P*=0.786	0.925	0.5882-1.4942
Cases	135 (0.833)	27 (0.167)			
Controls	148 (0.822)	32 (0.178)			
SNP7 (C4813618T)	C	T	χ^2=1.9 *P*=0.1681	0.6149	0.3473-1.2097
Cases	148 (0.914)	14 (0.086)			
Controls	156 (0.867)	24 (0.133)			
SNP8 (rs312369633)	C	T	χ^2=2.655 *P*=0.1032	0.6057	0.8499-1.0163
Cases	29 (0.179)	133 (0.821)			
Controls	21 (0.117)	159 (0.883)			

OR, odds ratio; CI, confidence interval. When the *p*-value of the Chi-square test is less than 0.05, the maximum value of the 95% CI less than 1 means resistance effect.

groups GG and AA in block one of the 3'-UTR had no significant association with *Salmonella* Pullorum infection between the cases and controls (Table V).

DISCUSSION

Sequencing the 3'-UTR of the MyD88 gene

A previous study revealed that 2 SNPs in the coding region (CDS) of the *MyD88* gene were significantly associated with susceptibility to *Salmonella* Pullorum infection (Liu *et al*., 2015). In addition, further evidence confirmed that higher expression of TLR signaling activation via MyD88-dependent pathway is more beneficial to chicken mononuclear cells mediated innate immunity (Karnati *et al*., 2015). Therefore, the 8 SNPs located in the 3'-UTR of the *MyD88* gene were predicted to be potential alleles in the follow-up studies.

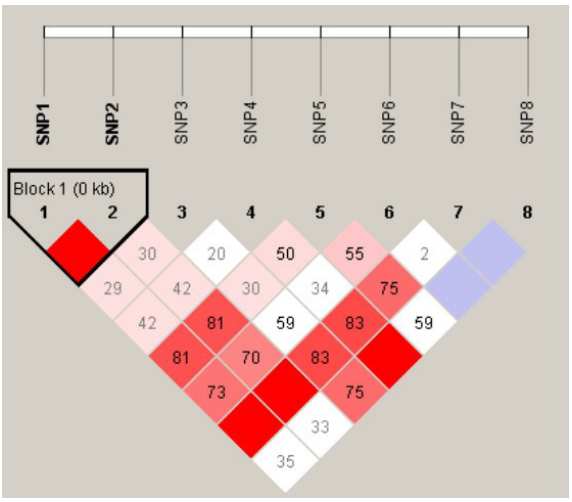

Fig. 1. Linkage disequilibrium analysis of the 8 SNPs.

Table IV. Genotype frequency of mutations in the 3'-UTR.

Markers	Genotypes			χ^2, P value
SNP1 (rs317890917)	AA	AG	GG	χ^2=0.12
Cases	8 (0.099)	39 (0.481)	34 (0.420)	*P*=0.9398
Controls	8 (0.089)	42 (0.467)	40 (0.444)	
SNP2 (rs14131328)	AA	AG	GG	χ^2=0.06
Cases	8 (0.099)	38 (0.469)	35 (0.432)	*P*=0.9705
Controls	8 (0.089)	42 (0.467)	40 (0.444)	
SNP3 (rs14131329)	TT	TC	CC	χ^2=3.50
Cases	13 (0.161)	39 (0.481)	29 (0.358)	*P*=0.1734
Controls	11 (0.122)	34 (0.378)	45 (0.500)	
SNP4 (A4812316G)	GG	GA	AA	χ^2=6.31
Cases	40 (0.494)	36 (0.444)	5 (0.062)	*P*=0.0426
Controls	36 (0.400)	37 (0.411)	17 (0.189)	
SNP5 (rs14131331)	TT	TC	CC	χ^2=1.52
Cases	28 (0.346)	41 (0.506)	12 (0.148)	*P*=0.4681
Controls	39 (0.433)	41 (0.456)	10 (0.111)	
SNP6 (C4813363A)	CC	CA	AA	χ^2=2.98
Cases	54 (0.667)	27 (0.333)	0 (0.000)	*P*=0.3118
Controls	61 (0.678)	26 (0.289)	3 (0.033)	
SNP7 (C4813618T)	TT	TC	CC	χ^2=1.86
Cases	1 (0.012)	12 (0.148)	68 (0.840)	*P*=0.4302
Controls	2 (0.022)	20 (0.222)	68 (0.756)	
SNP8 (rs312369633)	TT	TC	CC	χ^2=4.76
Cases	53 (0.654)	27 (0.333)	1 (0.013)	*P*=0.0599
Controls	71 (0.789)	17 (0.189)	2 (0.022)	

P, *P* value of the Chi-square test.

Table V. Haplotype analysis of the 8 SNPs.

	Haplotype groups	Frequency (cases)	Frequency (control)	χ^2	P value
Block 1	GG	0.660	0.678	0.115	0.7344
	AA	0.333	0.322	0.048	0.8269

P, *P* value of the Chi-square test.

Highly conserved in its CDS, the *MyD88* gene plays a key role in the innate immune response. Though multiple previous studies have reported that the *MyD88* gene may be involved in the response to pathogenic bacteria infection, most of these studies were performed at the gene expression level (Peng *et al*., 2010; Li *et al*., 2017). To reveal the role of the *MyD88* gene in resistance to *Salmonella* Pullorum infection, we analyzed the 3'-UTR of the *MyD88* gene in local Qing Jiao Ma hens (infected or uninfected with *Salmonella* Pullorum). Meanwhile, a growing number of studies have shown that the 3'-UTR plays a considerable role in mRNA stability and the efficiency of translation (Cok and Morrison, 2001; Wang *et al*., 2006). The AREs of the 3'-UTR speed up the degradation of mRNA in *Cyclooxygenase-2* (*COX-2*) (Sureban *et al*., 2007). Collectively, SNPs of the 3'-UTR in the *MyD88* gene have been speculated to have an association with the regulation of the innate immune response to *Salmonella* Pullorum infection (Tsai *et al*., 2004).

The Hardy-Weinberg equilibrium

The Hardy–Weinberg principle, also known as the Hardy-Weinberg equilibrium, states that allele and genotype frequencies in a population will remain constant from generation to generation in the absence of other evolutionary influences, such as mate choice, mutations, selection, genetic drift, gene flow and meiotic drive (Salanti and Ioannidisb, 2008). Recently, Hardy-Weinberg tests performed in marker-disease association studies have been used for genotyping. The results showed that all these 8 SNPs fit the assumption of the HWE, and they can be used for further analysis. When analyzing the MAF of the 8 SNPs, the effects of selection and foreign blood were found to be eliminated from the chicken population. In other words, these SNPs are sporadic mutations and are vital in genetic breeding.

Allele and genotype frequency of the mutated loci

The odds ratio (OR) value is an important index to quantify how strongly the presence or absence of one property is associated with the presence or absence of another property in a given population (Cornfield, 1951). When the *p*-value of the Chi-square test is less than 0.05 (the OR value is meaningful), the maximum value of the 95% CI less than 1 means resistance effect (Liu *et al*., 2015). This suggested that the polymorphisms of SNP4 might have a significant correlation with resistance to *Salmonella* Pullorum infection. Besides the SNPs of *MyD88*, multiple genotypes of the innate immunity genes, including the *Avian β-defensins* and *Cytokines* SNPs, were previously reported to be associated with resistance to *Salmonella* infection (Sadeyen *et al*., 2006). It would be quite interesting to unravel the associations between resistance to disease and SNPs in other genes, particular those with the ability to fight against infections (Yu *et al*., 2018; Zhang *et al*., 2019).

Association between haplotypes and susceptibility to Salmonella Pullorum

It is known that the association between haplotype and disease is more effective than a single mutation analysis (Durrant *et al*., 2004). In past decades, haplotypes have repeatedly been used for analyses of the disease associations found in wild and domesticated populations of chickens (Hosomichi *et al*., 2008). Furthermore, studies have indicated linkage of the ability to respond to *Salmonella* infection with particular MHC class I or class II haplotypes in inbred lines of chickens (Liu *et al*., 2002; Eimes *et al*., 2010). Another study revealed the regulating effect of the 3'-UTR of chMAVS on mRNA by choosing two representative 3'-UTR haplotypes (Yu *et al*., 2014). It is notable that none of the haplotype groups showed significant associations with *Salmonella* Pullorum infection in our study. A possible explanation is that the effects of the SNPs in the haploid type groups canceled each other out due to potential interactions between genes, leading to no significant link between haplotypes and disease resistance.

CONCLUSION

In this study, we detected a significant novel mutation (SNP4) of the 3'-UTR in the *MyD88* gene between the cases and controls. This finding suggests that SNP4 (A4812316G) may have an effect on the individual immune. But further functional studies are necessary to evaluate the molecular mechanism caused by polymorphisms of the 3'-UTR of *MyD88* gene. Our results provide some clues to help better understand the potential role of genetic resistance to *Salmonella* Pullorum infection in poultry breeding.

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Statement of conflict of interest

The authors declare no conflict of interest.

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