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Investigating whether microsatellites could influence the relative expression level of TLR4 gene in bursa of Rhode Island Red chicken

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Toll-like receptor 4 (TLR4) gene, an immune response gene (Leveque *et al*. 2003), was mapped to micro-chromosome E41W17 linkage group, which is one of the 50 linkage groups in consensus linkage map of the chicken genome (Groenen *et al*. 2000). In E41W17 linkage group, four microsatellite loci (ADL0149, HUJ0002, ADL0293 and MCW0330), were mapped around TLR4 gene (Forgetta 2001). Being one of the vital immune response genes linked to resistance to infection with *Salmonella enteric* serovar *Typhimurium* in chickens (Leveque *et al*. 2003), TLR4 was chosen as the candidate gene for this study. Hence, it was envisaged to assess whether the allelic types of microsatellites (ADL0149, HUJ0002, ADL0293 and MCW0330) impact the relative expression of such proximally positioned candidate gene TLR4 in bursa of Rhode Island Red (RIR) chicken.

Twelve randomly selected RIR chicks from Experimental Layer Farm of Central Avian Research Institute (CARI), Izatnagar, Uttar Pradesh, India were selected for the study. The birds were reared in a deep litter system, and *ad lib.* water and feed was provided with standard ventilation and lighting arrangements (Rahim *et al*. 2016). Birds were immunized with routine vaccines as followed at the institute. Experimental birds were randomly selected at the age of 6-8 weeks and bursa tissue samples, weighing ~50-100 mg, were aseptically collected in 2 ml centrifuge tube containing ~1 ml RNAlater®. Tissues were cut into small pieces to ensure proper infusion of RNAlater® into it. Finally, tissue samples were labelled and cryopreserved at −80°C until they were used to isolate DNA and RNA.

Total RNA and genomic DNA were isolated from the tissue samples using TRIzol® reagent following the protocol of Hongbao *et al.* (2008). The concentration of both RNA and DNA was directly measured in nanogram per microlitre (ng/µl) using a NanoDrop Spectrophotometer at an absorbance ratio of 260 nm/280 nm, and samples with an absorbance ratio ($OD_{260/280nm}$) of 2.0 and 1.8 were considered satisfactory for RNA and DNA purity, respectively. All of the RNA samples were treated with RNase-free DNase I enzyme to eliminate any possible DNA contamination. DNase-treated RNA samples were then labelled properly and stored at −20°C until further use.

The PCR amplification of four microsatellite loci (ADL0149, HUJ0002, ADL0293 and MCW0330) was carried out using gene specific primers with a programmable thermal cycler at their accompanying optimized annealing temperatures (Table 1).

Reaction mixture (25 µl) was prepared by addition of 2 µl of template DNA (50 ng/µl), 5 µl of 5× Green GoTaq® Flexi buffer, 2.5 µl of 25 mM $MgCl_2$, 0.5 µl of 10 mM dNTP mix, 1 µl of 10 pM forward primer, 1 µl of 10 pM reverse primer, 0.15 µl (0.75U) of GoTaq® Flexi DNA polymerase (5U/µl) and 12.85 µl of nuclease free water. The PCR cycling conditions were optimized for ADL0149, ADL0293 and MCW0330 microsatellite loci as follows: Initial denaturation at 95°C for 5 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 55°C or 55.4°C for 30 sec and extension at 72°C for 30 sec. The final extension was carried out at 72°C for 5 min. Touch-down PCR was used to amplify the HUJ0002 microsatellite locus. Initial denaturation at 95°C for 5 min was followed by 13 cycles of denaturation at 94°C for 45 sec, annealing at 65-52°C for 30 sec with increments of −1°C per cycle, and extension at 72°C for 30 sec. The second phase of amplification included 39 cycles of denaturation at 94°C for 45 sec, annealing at 52°C for 30 sec and extension at 72°C for 30 sec. Finally, a 5-min extension was performed at 72°C. Having confirmed the successful amplification of all the four loci in 2% agarose gel, the PCR amplicons were run and analyzed on 3.4% MetaPhor™ agarose gel electrophoresis (MAGE) to resolve microsatellite alleles for genotyping purpose as per the published procedure (Asif *et al*. 2008). The MetaPhor gel was examined with the Gel-doc system and microsatellite allelic patterns were photographed for genotyping. The molecular sizes (bp)

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Table 1. Primer sequences of microsatellites, TLR4 and β-actin gene along with optimized annealing temperatures (T_a)

Microsatellite Loci	FP/RP	Primer sequence (5'-3')[#]	T_a (°C)
ADL0149	FP	5′ATA GCA TAC ACC CAG CCA CC3′	55.4
	RP	5′GAA TAA GAA TGT TNC CCT GC3′	
HUJ0002	FP	5′CAT CTC ACA GAG CCA GCA GTG3′	65 and 52*
	RP	5′GAA TCC TGG ATG TCA AAG CC3′	
ADL0293	FP	5′ACA TAC CGC AGT CTT TGT TC3′	55
	RP	5′GTA ATC TAG AAA CCC CAT CT3′	
MCW0330	FP	5′TGG ACC TCA TCA GTC TGA CAG3′	55
	RP	5′AAT GTT CTC ATA GAG TTC CTG C3′	
Gene of target and housekeeping for expression study			
TLR4 gene	FP	5′CCACCCTGGACTTGGACCTCAG3′	58
	RP	5′AAGGCTGCTAGACCCAGGTGCT3′	
β-actin gene	FP	5′GGAAGTTACTCGCCTCTG3′	
	RP	5′AAAGACACTTGTTGGGTTAC3′	

FP/RP, Forward/Reverse primer. [#]Available at http://poultry.mph.msu.edu. *Touch down PCR performed @ −1°C per cycle for 13 cycles.

of all the alleles at the microsatellites investigated were determined using Quantity One® software. The presence of microsatellite alleles and band patterns in the MetaPhor gel were used to determine the genotypes of all the birds.

First strand cDNA was synthesized from DNase-treated total RNA using Thermo Scientific RevertAid first strand cDNA synthesis kit®. The tube containing resultant first stand cDNA was labelled carefully and stored at −20°C till further use. At this stage, the concentration of cDNA of each sample was equalized to 25 ng/µl for subsequent usage in quantitative reverse transcriptase PCR (qRT-PCR).

The relative quantification of TLR4 gene-mRNA expression in the bursa of RIR chicken was performed by qRT-PCR method using CFX 96®-Real Time PCR detection system. All reactions were carried out in 0.2 ml clear, thin walled nuclease-free 8-tube strips with optically clear flat caps. The qRT-PCR was carried out using DyNAmo ColorFlash SYBR Green qPCR Kit® in 20 µl reaction mixture. To perform it, initial denaturation at 95°C for 7 min was followed by 40 cycles of denaturation at 95°C for 10 sec, annealing at 58°C for 20 sec, and extension at 72°C for 20 sec, followed by detection of fluorescent signal using the real-time detection system to build an amplification curve. After 40 cycles, each sample was heated to 60-95°C for 10 sec at +0.5°C increments to construct a dissociation or melt curve to determine whether the amplification was particular or non-specific. Each tube's threshold cycle (C_t) value and melting point temperature were carefully retrieved and inspected for their associated amplification and dissociation curves after RT-PCR was completed. The ΔC_t value for each sample was calculated after subtracting average C_t value of housekeeping gene from average C_t value of the target gene. For each sample, the ΔC_t value was subtracted from 40 (total cycle number) so as to obtain '$40-\Delta C_t$' whose greater value indicates higher expression (Mackinnon *et al.* 2009). The '$40-\Delta C_t$' value data was analyzed by least squares analysis of variance using JMP 9.0.0 statistical program package (SAS 9.22, 2010) taking microsatellite genotype as a fixed effect in the linear model:

$$Y_{ij} = \mu + Gi + e_{ij}$$

where Y_{ij}, '$40-\Delta C_t$' value of mRNA expression of TLR4 gene in i^{th} genotype of j^{th} individual at a particular locus; µ, overall mean; G_i, fixed effect of i^{th} genotype (i = 1 to total number of genotypes); e_{ij}, random error of i^{th} genotype of j^{th} individual associated with mean zero and variance σ^2. Because the microsatellites were independent, a microsatellite-by-microsatellite analysis was performed.

All the twelve birds were genotyped for four microsatellite loci (ADL0149, HUJ0002, ADL0293 and MCW0330) after resolving the accompanying PCR amplified alleles on 3.4% MetaPhor™ agarose gel. Representative allelic patterns are presented in Fig. 1.

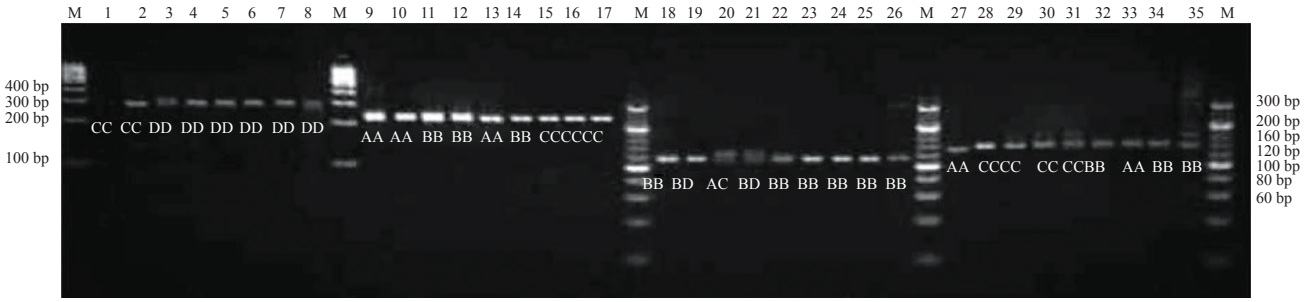

Fig. 1. PCR amplification of microsatellite alleles in 3.4% MetaPhor™ agarose gel. From left to right: Lane M, 100 bp ladder; Lane 1-8, MCW0330; Lane 9-17, ADL0149; Lane 18-26, ADL0293; Lane 27-35, HUJ0002.

Table 2. Least squares analysis of variance of '40−ΔC_t' values of mRNA expression level of TLR4 gene in tissue bursa of RIR chicken under different microsatellite genotypes

Source of variation	df	TLR4 gene-mRNA expression levels		Factors	N	'40−ΔC_t' mean values of TLR4 gene-mRNA expression levels
				Genotypes		
ADL0149		MSS	p value			
Genotype	3	50.858	0.4177	AA	3	35.9800±3.996
Remainder	8	47.907		BB	4	33.7350±3.460
				BC	1	22.9500±6.921
				CC	4	30.2800±3.460
HUJ0002						
Genotype	2	42.788	0.4570	AA	2	36.6750±5.001
Remainder	9	50.027		BB	5	29.3980±5.001
				CC	5	33.3220±5.001
ADL0293						
Genotype	2	32.191	0.5621	AC	1	31.140±7.237
Remainder	9	52.378		BB	9	31.217±2.412
				BD	2	37.425±5.117
MCW0330						
Genotype	4	81.629	0.1167	AA	1	25.120±5.468
Remainder	7	29.901		AC	1	22.950±5.468
				BD	1	21.840±5.468
				CC	2	34.580±3.866
				DD	7	35.411±2.066

Table 2 represents the results of least squares analysis of variance to demonstrate whether the microsatellite markers could impact the relative expression of the TLR4 gene in RIR chicken-bursa and p value indicates non-significant association of the markers with the relative expression levels of the TLR4 gene. A thorough study on a large number of samples could identify a marker microsatellite to accurately predict the TLR4 gene expression which plays central role in genetic resistance to Salmonellosis in chicken.

The results (Table 2) could only demonstrate variable expression levels of the TLR4 gene in tissue bursa of RIR chicken having different microsatellite genotypes. To mention, MCW0330 microsatellite genotypes potentially varied ($p \leq 0.12$) in the expression levels of the TLR4 gene-mRNA in bursa. The mean '40−ΔC_t' values for DD and CC genotypes with mean '40−ΔC_t' values were numerically higher than other genotypes. It was demonstrated that the microsatellites placed physically close to a gene may influence its expression. Leveque *et al*. (2003) mapped chTLR4 in microchromosome E41W17 with respect to the microsatellite markers ADL0293, MCW0330, and Tenascin C (TNC). The TLR4 had linkage with resistance and susceptibility of chicken to *Salmonella* serovar *Typhimurium* and alleles at microsatellite loci MCW0330 were found to co-inherit with the TLR4 gene. A thorough investigation taking a large sample size could confirm our hypothesis as tested by present study. Beaumont *et al*. (2003) studied association between microsatellites and associated immune response gene expression in brown egg hens. One of the immune genes targeted was TLR4 and the associated microsatellites were ADL0293, ADL0149, HUJ0002, MCW0330, and ADL0199. Sawaya *et al*. (2012) reviewed the effect of microsatellite motifs on expression of genes like *nos1, pax6, fli1* and *hmga2* and documented that microsatellite in and around genes modulate levels of gene expression in multiple organisms, ranging from bacteria to humans, due to variations in microsatellite lengths that modulate gene expression through change of B-DNA structure to Non-B DNA secondary structure.

SUMMARY

TLR4 gene, vitally linked to resistance to infection with *Salmonella enteric* serovar *Typhimurium* in chickens, was chosen as a candidate gene for this investigation to assess the probable impact of microsatellite genotypes on relative expression of TLR4 gene. Our study documented variable expression level of TLR4 gene in tissue bursa of RIR chicken encoded with different microsatellite genotypes. The DD and CC genotypes of MCW0330 microsatellite demonstrated numerically higher mRNA expression than its AA, AC and BD genotypes. Therefore, The MCW0330 genotyping may predict TLR4 gene expression if confirmed by a thorough investigation taking a large number of samples and then would play an important role in host-pathogen genetics with respect to Salmonellosis in poultry.

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