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Fold changes in relative mRNA expression of immune response genes in lymphoid tissues of Rhode Island Red chicken

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Immune response in chicken is very complex phenomenon controlled by many genetic factors and plays important role in protection against diseases. Modern intensive poultry production system lowers immunity and exposes chicken to variety of potentially pathogenic organisms. Genetic selection for immune traits in chickens can increase general and specific resistance to pathogens (Swaggerty *et al*. 2008). Different lines and breeds of chickens have been reported with measureable differences in expression of genes responsible to provide immunity against pathogens (Redmond *et al*. 2009, Cheeseman *et al*. 2007). Cytokines are involved in regulation of both innate and adaptive immune responses. IL-1β, belonging to IL–1 super-family, is reported as one of the first chicken cytokines. It plays an important role in inflammatory response and increases antibody production similar to that of its mammalian counterpart (Sterneck *et al.* 1992, Leutz *et al*. 1989). Kim *et al*. (2008) reported IL-1β gene expression differed after parasitic infestation between chicken inbred lines disparate for the MHC. Nitric oxide is an important mediator of immune and inflammatory responses, produced by macrophages through activation of inducible enzyme nitric oxide synthase (iNOS) (Djeraba *et al.* 2000), and plays a powerful role in immune responses because of its antimicrobial and antitumor activity (Blanchette *et al.* 2003). Toll-like receptors (TLRs) are critical component of innate immune responses and play pivotal role in early recognition of pathogen as well as in the initiation of robust and specific adaptive immune response. Ten TLRs were identified in chickens. TLR15, a novel avian-specific TLR, with no homologue in other species, plays a central role in innate immunity. While most mammalian TLRs are well characterized, the chicken-specific TLR15 has not been extensively studied. Basal expression levels of immune response genes provide information about general immune status or response against pathogens in birds and vary among different breeds of chickens. Rhode Island Red (RIR) chicken is useful for backyard poultry production and believed to be more diseases resistant than other exotic breeds. The basal mRNA expression profiles in bird's different tissues are suggestive of its preparedness and ability to respond to pathogen assault. Understanding the genetics of basal expression levels of immunity related genes is mandatory for effective genetic selection for immune response (Kumar *et al.* 2011). Present investigation was carried out to determine the fold changes in the relative mRNA expression of three immune response genes, viz. IL1–β, iNOS and TLR15 in three lymphoid tissues, viz. bursa, spleen and thymus of selected pure strain of RIR chicken.

RIR birds (12: 6 of either sex) were randomly chosen at 6–8 weeks of age, maintained at experimental layer farm of this institute in deep litter system and provided *ad lib.* feed and water, and standard management and vaccination practices (Rahim *et al*. 2016). Three lymphoid tissues, viz. bursa, spleen and thymus, weighing approximately 50–100 mg were aseptically collected from each of the experimental birds in 1ml RNAlater® (Ambion, USA). Tissues were cut into small pieces to ensure proper infusion of RNAlater® into it and stored at –80°C until use. Total RNA was isolated by TRIzol® reagent (Invitrogen, USA) (Hongbao *et al.* 2008) and finally dissolved in 50 µl of nuclease-free water. Any possible contamination of genomic DNA was removed by treating 5 µl of each RNA sample with 5U of RNase-free DNase (Biogene, USA) at 37°C for 1 h. The DNase was subsequently inactivated by incubation at 65°C for 10 min. RNA purity and quantity were determined by NanoDrop Spectrophotometer (NanoDrop Technologies Inc., USA), and samples showing absorbance ratio (260/

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280) of ~2.0 were stored at –20°C till further use. Each DNase-treated total RNA sample was reverse transcribed using Verso cDNA synthesis kit® (Thermo Fisher Scientific Inc., USA). Concentration of cDNA of each sample was equalized to 25 ng/µl and stored at –20°C until further use. Expression of immune response genes, viz. IL1–β, iNOS and TLR15 was quantified by quantitative reverse transcription PCR (qRT-PCR) in CFX 96® in Real Time PCR detection system (BioRad Laboratories Inc., USA). Two primer pairs for IL1–β and β-actin were selected from the published reports (Higgs *et al*. 2006) and 2 primer pairs for iNOS and TLR15 were designed using PrimerQuest tool of integrated DNA technologies (IDT). The specificity of the primers was checked by NCBI blast program. All the primers were got synthesized from M/s Xcelris Genomics Labs Ltd., Ahmedabad (India) and details are given below:

All PCR reactions were performed in triplicate in 0.2 ml clear thin walled nuclease-free 8-tube strips with optically clear flat caps (Axygen Scientific Inc., USA). A negative control (NTC, no template control) in triplicate containing all the ingredients except cDNA was also set up to check any contamination. β-actin gene was used as housekeeping gene. The amplification was carried out in 20 µl reaction mixture using DyNAmo ColorFlash SYBR Green qPCR Kit® (Thermo Fisher Scientific Inc., USA). Real-time PCR cycling conditions were as follows: initial denaturation at 95°C for 7 min 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 by the real time detection system to generate amplification curve. Each sample was subjected to 60–95°C @ + 0.5°C increment for 10 sec to generate dissociation curve or melt curve to ensure specific amplification. Then threshold cycle (C_t) values and melting point temperature of each tube was retrieved and reviewed for its corresponding amplification and dissociation curve. The data from qRT-PCR experiment was imported in excel. The tissue showing highest ΔC_t value was chosen as the calibrator tissue. The fold expression was determined using the formula $2^{(-\Delta\Delta Ct)}$ as per Livak and Schmittgen (2001). The standard errors of ΔC_t values were calculated and the ΔC_t values among three tissues were compared (Yuan *et al*. 2006). The standard errors and significant differences (P<0.05) were plotted on the fold change bars in the graph.

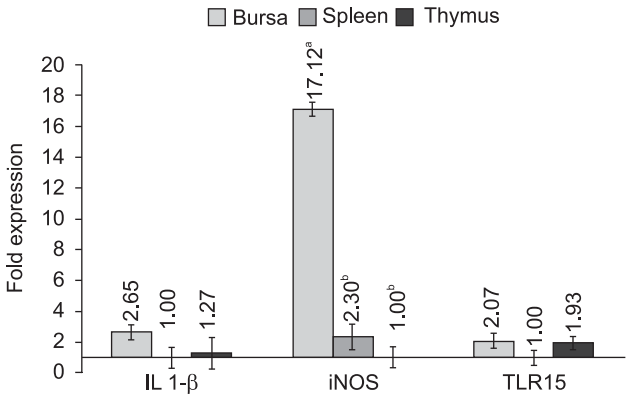

Fig. 1. Fold expression of IL1–β, iNOS and TLR15 genes in bursa, spleen and thymus of RIR chicken. Numerical values on the top of bars represent fold expressions. Similar superscripts represent non-significant differences in the ΔC_t values (P<0.05).

Gene		Primer sequences	T_m (°C)	Size (bp)	NCBI- Accession nos. / References
TLR15	F	5'-TGTGGTATGTGAGAATGGGC-3'	58	85	NM_001037835.1
	R	5'-GCATCGAAGGGCTTATTTTCTG-3'			
iNOS	F	5'-GGCATCTGTATGTCTGTGGAG-3'	59	147	NM_204961.1
	R	5'-CTTCATGGTATCGCTTTTGGC-3'			
IL1-β	F	5'-CGCTCACAGTCCTTCGACATC-3'	56	230	Higgs *et al*. (2006)
	R	5'-CCGCTCATCACACACGACATGT-3'			
β-actin	F	5'-GGAAGTTACTCGCCTCTG-3'	56/58/59*	114	Higgs *et al*. (2006)
	R	5'-AAAGACACTTGTTGGGTTAC-3'			

*Annealing temperature that was used for each of the 3 target genes in respective experiment.

Folds changes of relative mRNA expression of three immune response genes *viz.* IL1–β, iNOS and TLR-15 in three different lymphoid tissues (bursa, spleen and thymus) of RIR chicken are presented in Fig. 1.

Melting curve analysis demonstrated a single predominant peak with a distinct melting temperature for each of the primer pairs. Basal gene expression of the three genes was several folds higher in busra as compared to spleen and thymus. It was found that the relative expression of IL1–β gene in term of fold changes in bursa (2.65±0.47) and thymus (1.27±0.99) was higher in comparison to spleen (1.00±0.65), although the differences were statistically non-significant**.** The fold expression of iNOS gene was significantly (P<0.05) more (17.12±0.40 folds) in spleen bursa in comparison to (2.30±0.87 folds) and thymus (1.00±0.69 folds). The fold expression of TLR15 gene was 2.07±0.48 and 1.931.93±0.47 folds more in bursa and thymus, respectively, in comparison to spleen (1.00±0.43), but the differences were statistically non-significant. Generally higher basal expression of all three studied immune response genes in bursa than other tissues might be due to fact that bursa is the principal site for production of B-lymphocytes in chicken. Kumar *et al*. (2011) while analyzing basal expression levels of five immune response genes (IL-1β, IL–2, iNOS, TLR4, and TLR15) also observed

no significant differences in the assayed genes among 3 genotypes. There are not many reports available on the aspect to compare or contrast. Present findings suggested the need for further investigations into cytokine gene expression in chickens so as to generate a better understanding of the host defense process.

SUMMARY

Fold changes in relative mRNA expression of three immune response genes viz. IL1–β, iNOS and TLR15 were determined in bursa, spleen and thymus tissues of Rhode Island Red chicken. Total RNA was isolated from 12 birds, aged around 6–8 weeks. Relative quantification of mRNA expression was assessed by qRT-PCR. Fold expressions were determined using average threshold cycle (C_t) values employing $2^{(-\Delta\Delta Ct)}$ method. There was wide variation in basal expression levels of immune response genes among different tissues. Basal mRNA expression of IL1–β, iNOS and TLR15 genes was several folds higher in bursa than in spleen and thymus. This investigation has generated important findings related to immune response genes expression which could pave way to further investigation in host-pathogen genetics and finally to develop breeding strategies for improvement of diseases resistance so as to have better protection and production in chicken.

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