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Original scientific paper

GENETIC POLYMORPHISMS AND EXPRESSION PATTERNS OF *TLR4* GENE IN COMMERCIAL BROILER BREEDS

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Genetic polymorphisms and expression pattern of *TLR4* gene were studied in 300 one-day-old chicks of three breeds (Cobb, Avian and Ross) using PCR-RFLP and real time PCR. Blood samples were collected from chicks of each breed for DNA extraction. Spleens from randomly selected ten female chicks from each breed were collected for RNA extraction. PCR-*Taq*I digestion of 596-bp of a fragment of exon 1 of *TLR4* gene revealed two fragments (460 and 136-bp) for the genotype BB in all breeds and three fragments (596, 460 and 136-bp) for the genotype AB in Ross and Avian breeds. The incidence of *TLR4* genotypes and the frequencies of alleles indicated that B allele was more frequent than A allele in the population of all breeds. Gene expression pattern of *TLR4* revealed a significant up-regulation in Avian breed than Cobb and Ross as shown by the lower value of ΔCT. It could be concluded that genetic *TLR4*/*Taq*I locus polymorphism could be used as a marker assisted selection (MAS) for immune traits in commercial breeds of broilers. Also, changes in the expression patterns of *TLR4* gene could be a biomarker to follow up immune status of chicks to predict the most susceptible risk time for disease incidence and to build up an effective management regimen.

Keywords: TLR4 gene polymorphisms, broiler, PCR-RFLP, real time PCR

INTRODUCTION

Poultry industry has achieved a great popularity and rapid growth in the world as it provides human with a good quality protein including egg and meat which could overcome the problem of protein gap and malnutrition around the world. In recent decades, a tremendous development of broiler industry has occurred as body weight gain and feed utilization have been

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improved in broiler chicken. This development has been achieved due to the use of new technologies in poultry nutrition and genetics (BOGDANOV, 1990; HAVENSTEIN *et al*., 1994; SIEGEL, 2014; TALLENTIRE *et al*., 2016; ADEDOKUN and OLOJEDE, 2019). Genetic improvement or the response of individuals to selection is the major cause of most phenotypic changes in poultry. In the same respect, genetic variations among breeds hastened poultry production improvement through the development of new criteria in response to diseases, environmental changes and market conditions (ÇILEK and TEKIN, 2005). Therefore, the principle concern of poultry breeders is the selection of breed that could have a good growth rate, feed conversion, meat yield and immune response with high economic return (SEYEDABADI *et al.*, 2010).

In poultry, modulating the immune response has been greatly affected by genetics as it can improve innate immune system and thus minimize the use of vaccines or antibodies. Therefore, prevention and control of poultry infections becomes available without the emergence of antibody-resistant bacteria or drug residue for humans (MALEK *et al.*, 2004). The selection process relied on the fact that molecular genetic marker hastens genetic response of preferred traits to develop desirable performance in domestic animals (ANH *et al.*, 2015; ATEYA *et al.*, 2016). Molecular markers detect different criteria in DNA molecule and so can be used to distinguish between individuals in a population. The most common genetic markers used are RFLP (Restriction Fragment Length Polymorphism), AFLP (Amplified Fragment length Polymorphism), SNP (Single Nucleotide Polymorphism), SSR (Simple Sequence Repeat, or Microsatellite), STS (Sequence Tagged Site), RAPD (Randomly Amplified Polymorphic DNA), CAPS (Cleaved Amplified Polymorphic Sequence) and dCAPS (derived Cleaved Amplified Polymorphic Sequence) (PEREIRA *et al*., 2008).

The principle of restriction fragment length polymorphisms (RFLPs) is the utilization of restriction enzymes that recognize and cut DNA molecule at definite sites (restriction sites). A set of fragments with different length is originated that could be separated according to their molecular size by conventional gel electrophoresis (PEREIRA *et al.*, 2008; SARASWATHY and RAMALINGAM, 2011). In the same respect, quantitative nucleic acid analysis has been used to measure the quantity of a specific gene in the genome (SLAMON *et al.*, 1987). Differences in gene expression between tested samples can be evaluated using real-time quantitative PCR by comparing the amount of transcript gene in a cell (the target gene) to the amount of transcript reference gene (calibrator gene) (WONG and MEDRANO 2005; STEPHENSON, 2016). The use of real-time quantitative PCR provides few quantities of nucleic acid (one cell equivalent) analysis for several experiments that could not have been carried out using conventional methods (TICHOPAD *et al*., 2003).

TLR4 belongs to evolutionarily conserved pattern recognition receptors group that counteract several pathogens by enhancing immune system and innate defense (LEVEQUE *et al.*, 2003). Chickens have ten *TLR* genes, six of them have orthologs in fish and mammals, one is shared by fish, and three are unique to birds (LYNN *et al.*, 2003; SMITH *et al.*, 2004; IQBAL *et al.*, 2005; PHILBIN *et al.*, 2005; ROACH *et al.*, 2005; YILMAZ *et al.*, 2005; HIGGS *et al.*, 2006; KEESTRA *et al.*, 2007; TEMPERLEY *et al.*, 2008)**.** Chicken *TLR4* is mapped to chromosome 17 and encodes 843 amino acid protein that has an extracellular transmembrane leucine-rich repeat (LRR) domain and Toll/interleukin I resistance (TIR) signaling domain as other TLR proteins (TEMPERLEY *et al.*, 2008)**.** Previous studies reported a 61% similarity between human and

chicken *TLR4* proteins however, 41 % only compared with other *TLR* family members was identified (LEVEQUE *et al.*, 2003).

Although numerous studies reported genetic polymorphisms and expression patterns of *TLR4* gene and its association with, and susceptibility and resistance to infection (LEVEQUE *et al.*, 2003; MALEK *et al.*, 2004; ABASHT *et al.*, 2009; LIU *et al.*, 2011; TOHIDI *et al.*, 2012; LI *et al.*, 2013; KHATAB *et al.*, 2017)**,** few of them reported genetic polymorphisms in *TLR4* gene in commercial broiler line breeds (RUAN *et al.*, 2012; SAIKHOM *et al.*, 2018).

Consequently, the objectives of the present study were to reveal the *Taq*I polymorphism in the exon1 of *TLR4* gene using PCR-RFLP and to elucidate the expression pattern of *TLR4* gene using real time PCR in three commercial broiler breeds.

MATERIALS AND METHODS

Experimental birds

A total of 300 one-day-old broiler chicks of both sexes of three breeds; Cobb, Avian and Ross (100 birds of each breed) was used. Chicks were purchased from a commercial hatchery. This study protocol was approved by Research Ethics Committee, Faculty of Veterinary Medicine, Mansoura University.

Experimental samples

Blood samples were collected from chicks into tubes containing anticoagulant disodium EDTA for DNA extraction**.** Ten female chicks from each breed were randomly selected and sacrificed for collection of spleen samples. Spleen samples were sterile collected, washed in phosphate buffer saline (PBS), snap frozen in liquid nitrogen and stored at -80°C for quantification of gene expression.

PCR-RFLP for TLR4 gene

PCR was done for amplification of a fragment of exon1 of *TLR4* gene with expected amplicon size of 596-bp using the primers as described previously (LEVEQUE *et al.*, 2003).

Forward: 5‘-GAAACGTTGTCAGAGGTTCCTATG-3‘

Reverse: 5‘-ACTTTGGTCCACCCATACTAATTT-3‘

Polymerase chain reaction mixture was done in a 25 µl consisted of: 3µl DNA, 8.5 µl H_2o (d.d water), 12.5 µl PCR master mix (Jena Bioscience, Germany), 0.5 µl of each primer. The final reaction mixture was achieved in a thermal cycler and the PCR temperature schedule program was carried out by an initial denaturation at 94 ^{0}C for 4 min followed by 34 cycles of 94°C for 1 min for denaturation, primer hybridization temperature at 58°C for 1 min, primer extension at 72°C for 1 min and the final elongation at 72°C lasted for 10 min.

Amplified DNA fragments of *TLR4* gene were digested with *Taq*I (Promega, USA) at 65°C for 3 hrs. The reaction volume was done in 20 µl consisted of: 7µl PCR product, 1µl 10x fast digest green buffer, 1µl restriction enzyme and 11µl H_2o (d.d water). The cleaved fragments were detected by agarose gel electrophoresis then the fragment patterns were visualized under U.V using gel documentation system (Gel Doc, Alpha-Chem, Umager, USA).

Total RNA extraction, reverse transcription and quantitative real time PCR

Total RNA was extracted from spleen tissues in the one-day-old broiler chicks using Trizol reagent following the manufacturer instructions (RNeasy Mini Ki, Catalogue no.74104). The amount of extracted RNA was quantified and qualified using NanoDrop® ND-1000 Spectrophotometer. The cDNA of each sample was synthesized following the manufacture protocol (Thermo Fisher, Catalog no, EP0441). Absolute quantification of mRNA level of *TLR4* was performed by real-time PCR using SYBR Green PCR Master Mix (Quantitect SYBR green PCR kit, Catalog no, 204141). Primer sequences are shown in Table 1. The housekeeping gene *ß-actin* was used as a constitutive control for normalization. The reaction mixture was carried out in a total volume 25 µl consisted of total RNA 3 µl, 4 µl 5x Trans Amp buffer, 0.25 µl reverse transcriptase, 0.5 µl of each primer, 12.5 µl 2x Quantitect SYBR green PCR master mix and 8.25 µl RNase free-water. The final reaction mixture was placed in a thermal cycler and the following program was carried out: reverse transcription at 50°C for 30 min, primary denaturation at 94°C for 10 min followed by 40 cycles of 94°C for 15 s, annealing temperatures at 56°C for 30 s, and 72°C for 30 s. At the end of the amplification phase, a melting curve analysis was performed to confirm the specificity of the PCR product. ΔCT of each sample was calculated for *TLR4* gene using threshold cycle (CT) values that was normalized to those of the *β- actin* housekeeping. Lower ΔCT indicates increased expression (LIVAK and SCHMITTGEN, 2001; PFAFFL, 2001)**.**

Table 1. Oligonucleotide primers sequence of TLR-4 and ß. Actin genes in real time PCR

Gene	Oligonucleotide sequence	GenBank accession number	Reference
TLR-4	f 5·- GTTCCTGCTGAAATCCCAAA-3· r 5·- TATGGATGTGGCACCTTGAA-3·	NM_001030693	LU *et al.*, (2014)
ß-actin	f 5·- CCACCGCAAATGCTTCTAAAC-3· r 5·- AAGACTGCTGCTGACACCTTC-3·	NM_204305.1	YUAN *et al.*, (2007)

Gene and genotypic frequencies in TLR4-TaqI locus

Gene and genotypic frequencies were estimated by simple counting of the alleles based on the electrophoresis results using the equations described by FALCONER and MACKAY, (1996).

Statistical analysis

Gene expression pattern values in the broiler breeds were analyzed using SPSS16.0 (SPSS Inc., Chicago, IL) for one-way ANOVA analysis. The least significant difference was used for comparing differences among means of the groups. Comparisons were statistically significant at $P \leq 0.05$.

RESULTS

Effect of TLR4 gene polymorphism

Genomic DNA of chicks from each breed was used to amplify specific DNA fragments 596-bp of exon1 of *TLR4* gene (Figure 1). Restriction analysis of 596-bp PCR products digested

with *Taq*I revealed two fragments (460 and 136-bp) for the genotype BB in all breeds and three fragments (596, 460 and 136-bp) for the genotype AB in Ross and Aves breeds (Figure 2).

Incidences of *TLR4* genotypes and frequencies of alleles were calculated using PCR-RFLP method. B allele was more frequent than A allele in all breeds (Table 2).

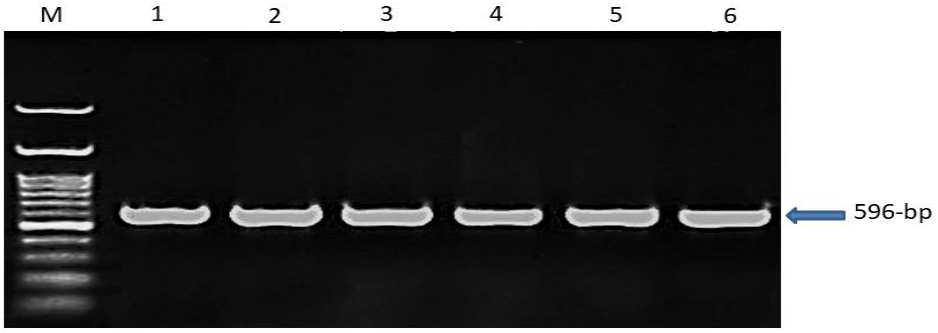

Fig. 1. Representative PCR results of TLR4 gene, lane M: DNA marker and lanes 1-6: 596-bp amplified fragment of TLR4 gene

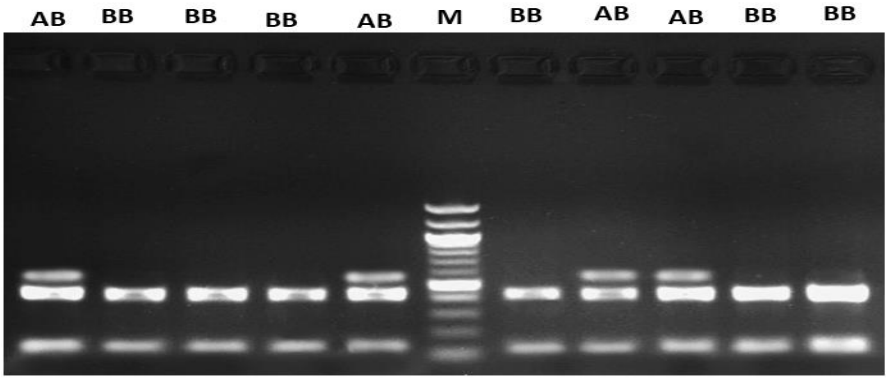

Fig. 2. Representative TaqI restriction fragment pattern of TLR4 gene (596-bp). BB: Restriction fragment of 460 and 136-bp, AB: Restriction fragment of 596, 460 and 136-bp and M: DNA ladder

Table 2. Frequency of genotypes and alleles in TLR4 locus

Breed	No. of birds	Number/frequency of genotypes		Allele frequency	
		AB	BB	A	B
Cobb	100	-	100/1	-	1
Ross	100	20/0.2	80/0.8	0.1	0.9
Avian	100	35/0.35	65/0.65	0.175	0.825

TLR4 gene expression pattern

Levels of *TLR4* gene expression in spleen of different breeds were higher in Avian than in either Cobb or Ross breed, as indicated by the lower values of ΔCT (Figure 3).

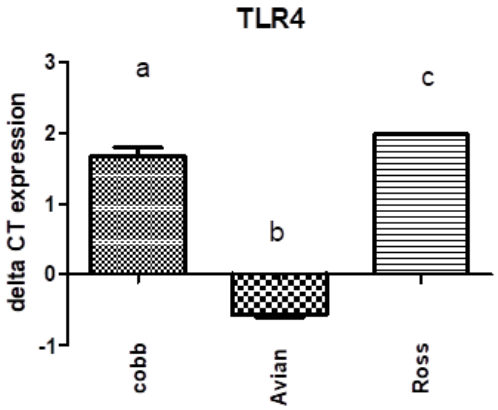

Figure 3. Relative TLR4 gene expression in spleen of different breeds. β-actin gene was used as a reference gene to normalize the data and shown as ΔCT ± SE. Lower ΔCT values indicate increased expressions.

DISCUSSION

Improving economic traits in chicken has become of the major hurdle that can be achieved by identification and utilization of QTLs that provide the potential for genetic improvement in selection programs without slaughtering. Recent advances in molecular genetics and development of molecular techniques have led to the discovery of genes, or markers associated with genes involved in economic traits (GAO *et al.*, 2007; GHANEM *et al.*, 2016). When a polymorphic locus is associated with the economic traits of animals, that polymorphic locus can therefore be used as a genetic marker for the trait and become useful as a selection criterion for genetic improvement of this trait (marker-assisted selection) (EL-SHAFAEY *et al.*, 2017; ELDOMANY *et al.*, 2019).

In this context, *TLR4* gene polymorphism in the three chicken breeds investigated revealed a specific PCR product of a desirable size (596-bp), involving exon 1 of *TLR4* gene.

The following DNA restriction fragments were obtained for *TLR4-Taq*I digestion: two fragments (460 and 136-bp) for genotype BB in Cobb, Avian and Ross breeds and three fragments (596, 460 and 136-bp) for genotype AB in Ross and Avian breeds. The incidence of *TLR4* genotypes and the frequency of alleles were calculated. B allele was more frequent than A allele in the three breeds. DNA fragments reported in the current study were similar to those obtained by KHATAB *et al.*, (2017); however with different gene and genotypic frequencies. The authors reported the association between *TLR4* gene and genetic resistance/susceptibility to Salmonella enteritidis in Fayoumi and Hy-line strain in Egypt. The differences in gene and genotype frequencies between current and previous studies may be attributed to the difference in genetic backgrounds of the breeds and to the number of chickens (RUAN *et al.*, 2012; SAIKHOM *et al.*, 2018). Few studies reported genetic polymorphisms in *TLR4* gene in commercial broiler line breeds. RUAN *et al.*, (2012) reported chicken *TLR4* gene polymorphisms in different breeds, and reported that amino acid sequence analysis indicated there were 9 sites of amino acid sequence polymorphism; eight of them were located in the extracellular domain, while 1 polymorphic site was located in the cytoplasmic domain. SAIKHOM *et al.*, (2018) also investigated *TLR4* gene exon II (248-bp) polymorphism on Haringhata Black Chicken using polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) and reported two (EF and FF) genotypes. Genotype frequencies were 0.634 for EF and 0.366 for FF with allele frequency 0.317 and 0.683 for E and F respectively.

Many studies reported the association between *TLR4* gene polymorphism and susceptibility and resistance to infection in chicken. LEVEQUE *et al.*, (2003) indicated that, there was a link between *TLR4* gene variation and susceptibility to Salmonella enterica serovar typhimurium infection in chickens. Analysis of chicken *TLR4* genes in a Salmonella enteritidis resource population of chickens has been investigated (MALEK *et al.*, 2004; TOHIDI *et al.*, 2012; KHATAB *et al.*, 2017). Similarly, LIU *et al.*, 2011 studied relatedness between genetic polymorphism at exon2 of *TLR4* gene and resistant traits in chicken. The association between *TLR4* gene polymorphism and Salmonella natural and artificial infection was also studied in Chinese native chicken breeds (LI *et al.*, 2013).

In the present study real time PCR was carried out to quantify mRNA level of *TLR4* gene in the spleen of three broiler chicken breeds. Our findings revealed that the expression pattern was higher in Avian breed compared to in Cobb and Ross breeds. This was shown by the lower values of ΔCT. Our study is the first approach to detect polymorphisms of *TLR4* mRNA level in commercial broiler line breeds using real time PCR. However, many studies reported the association between changes in the relative expression of *TLR4* gene and incidence of infection in chickens. ABASHT *et al.* (2009) demonstrated an effect to genetic line on *TLR4* gene expression in the spleen of Salmonella Enteritidis-infected birds which may partly explain genetic variability in immune response. KOGUT *et al.* (2012) also analyzed *TLR4* gene expression in heterophils from genetic chicken lines that differed in their susceptibility to Salmonella enteritidis. Changes in the *TLR4* gene expression in chicken embryo fibroblasts from chickens resistant and susceptible to Marek's disease was also reported (HAUNSHI and CHENG, 2014).

CONCLUSION

We elucidated that distinguished *TLR4* gene polymorphisms in commercial broiler breeds can be carried out by examining *TLR4*/*Taq*I locus. The polymorphism can potentially be

explained by the differences in *TLR4* gene and genotype frequencies; where B allele was the most frequent in the broiler breeds. Genetic *TLR4*/*Taq*I locus polymorphism could be used as a marker-assisted selection for immune traits which could help enhance disease resistance in broiler chicken. The variable expression pattern of *TLR4* gene in chicken broiler breeds can also be done using real time PCR analysis; where Aves breed had higher *TLR4* gene expression. Also, the expression patterns of *TLR4* gene could be a biomarker that can be used to follow up immune status of chickens not only to predict the most susceptible risk time for disease occurrence but to also build up an effective management protocol to improve chicken health through breeding and vaccination. Further studies should be carried out to investigate the association of various polymorphisms located in different regions of the *TLR4* genes. Other chicken breeds should be also considered.

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GENETIČKI POLIMORFIZMI I NAČIN EKSPRESIJE *TLR4* GENA KOD KOMERCIJALNIH BROJLERA

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Izvod

Genetski polimorfizmi i način ekspresije *TLR4* gena proučavani su kod 300 jednodnevnih pilića tri rase (Cobb, Avian i Ross) koristeći PCR-RFLP i RT-PCR. Uzorci krvi prikupljeni su od pilića svake rase radi ekstrakcije DNK. Slezine slučajno odabranih deset ženki pilića iz svake rase prikupljene su za ekstrakciju RNA. PCR-Taq digestija 596-bp fragmenta eksona 1 *TLR4* gena otkrila je dva fragmenta (460 i 136-bp) za genotip BB kod svih rasa i tri fragmenta (596, 460 i 136-bp) za genotip AB u rasama Ross i Avian. Učestalost *TLR4* genotipova i učestalost alela ukazivali su da je alel B češći od alela A u populaciji svih rasa. Način ekspresije gena *TLR4* pokazao je značajnu ekspresiju kod rase Avian u odnosu na Cobb i Ross, što pokazuje i niža vrednost ΔCT. Moglo bi se zaključiti da se genetski *TLR4*/*Taq* polimorfizam lokusa može koristiti za selekciju pomoću markera (MAS) za imunološka svojstva komercijalnih rase brojlera. Takođe, promene u načinu ekspresije *TLR4* gena mogu biti biomarker za praćenje imunološkog statusa pilića radi predviđanja najosetljivijeg vremena rizika za pojavu bolesti.

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