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Association of *toll-like receptors 2* and *6* polymorphism with clinical mastitis and production traits in Holstein cattle

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Summary

Mastitis is a costly disease of dairy cattle as it causes a loss in milk yield and milk quality in affected cows. *Toll-like receptor* (*TLR*) genes play a role in the host response to a variety of organisms including those inducing mastitis. In the present study, we investigated the polymorphism of *TLR2*, *4*, *6* and *9* genes in Holstein cattle and their possible association with clinical mastitis (CM), milk somatic cell scores (SCS) and milk production traits. From a large commercial Holstein herd, thirty-eight blood samples were collected; 19 from cows without a previous lifetime history of mastitis (non-susceptible), and 19 from Holstein cows with at least three previous episodes of mastitis (susceptible). Genotyping of the four *TLRs* was done using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLPs) with *Rsa1*, *MSP1*, *Hha1*, *HaeIII*, and *Taq1* enzymes guided with DNA sequencing. Seven novel non-synonymous single-nucleotide polymorphisms (SNPs) were identified among *TLR2*, *4*, and *6* in susceptible animals. Association was found in *Taq1-TLR2* gene polymorphism with CM, fat percentage and peak yield (PY). The association of *Taq1-TLR6* and PY and lactation persistency was also shown. Mutations in *TLRs* that were repeatedly reported in susceptible cows provide potential genetic marker assisted selection (MAS) for mastitis resistance in dairy cattle.

Key words: Cattle, Genotypes, Mastitis, PCR-RFLPs, *TLRs*

Introduction

Mastitis is an inflammatory disease of the mammary gland and it is the most frequently occurring disease in dairy farms, worldwide. The global incidence of clinical mastitis (CM) ranged from 25-60 cases per 100 cows (Ruegg, 2003), and subclinical mastitis (SCM) is 20-80% (Contreras *et al*., 2011). It is considered a major source of economic loss in dairy farms, costs the dairy industry billions of dollars annually (NMC, 2005); mainly due to discarded milk from infected cows, replacement of culling cows, antibiotic therapies, extra labor and veterinary costs. However, the greatest loss is attributed to decreased milk production in affected cows (Akers and Nickerson, 2011). Besides the financial implications of mastitis, its importance in relation to public health should not be overlooked (Ibrahim, 2017). Mastitis is a highly complex disease due to the diversity as well as the variation in prevalence and abundance of mastitis-causing organisms as well as the variation in host responses (Rashad *et al*., 2016).

Using some identified genetic markers associated with mastitis resistance, marker assisted selection (MAS) would be an efficient way to improve the mammary health (Zhang, 2009). Identification of single-nucleotide polymorphism (SNP) in genes involved with the mammary innate immune response can be a useful marker in early detection of resistant/susceptible animals (He *et al*., 2011). The innate immune system gives the mammary gland the ability to combat a variety of invading pathogens due to the rapid response even upon first exposure and also non-pathogen-specific methods of recognition (Oviedo-Boyso *et al*., 2007). In the mammary gland, epithelial cells together with cells from the immune system are responsible for recognizing the invading pathogens via *toll-like receptors* (*TLRs*) (Alva-Murillo *et al*., 2017). These receptors recognize permanent structures of the microorganisms called pathogen-associated molecular patterns (PAMPs) and initiate the development of full immunological response. The detection of PAMPs by *TLRs* and subsequent initiation of signaling pathways to induce cytokine production can promote chemotactic migration of cells, including neutrophils and macrophages, from surrounding blood vessels to the site of infection (Cates *et al*., 2009). Ten different *TLRs* (*TLR1* to *TLR10*) have been identified in cattle. Among them, *TLR2*, *TLR4*, *TLR6* and *TLR9* genes have been found associated with mastitis resistance in cattle and play a role in innate immunity. *TLR2* and *TLR4* recognize bacterial cell components and are critical in the immune response against Gram-positive and Gram-negative bacteria. Bovine *TLR2* can properly transduce signals from *Staphylococcus aureus* and *Escherichia coli* (Yang *et al*., 2008). *TLR6* in association with *TLR2* recognizes a wide variety of bacterial cell wall components including lipopolysaccharides, lipoproteins and teichoic acid (Buwitt-Beckmann *et al*., 2006). *TLR9* recognizes unmethylated CpG dinucleotides of bacterial DNA (Kant *et al*., 2014). So these *TLR* genes are considered suitable

candidate for mastitis resistance in dairy cattle. The present study aimed to screen for polymorphism in *TLR2*, *4*, *6* and *9* genes in Holstein cows, and its possible association with CM, milk somatic cell score (SCS), and production variables.

Materials and Methods

Study population, data and samples

The data and samples were collected from cows and performance records of a commercial Holstein herd (n=1875) located about 80 km on Cairo-Alexandria desert road, Egypt. The animals were housed free in open yards with corrugated metal sheets. Lactating cows were grouped according to their milk production, and concentrate feeding was offered accordingly. Cows were fed twice daily a total mixed ration (TMR) throughout the year. The TMR consisted of concentrates, corn silage, alfalfa hay, wheat bran, vitamins and minerals supplements, and calcium bicarbonate. Cows were machine milked three times daily at 8 h intervals starting at 06:00 am, and daily milk yield (DMY) was recorded for individual cows via computerized milking units.

Inspection of health records (n=1875) between 2013 and 2016, show 647 cows had contracted at least one episode of CM throughout their entire lactation. Thirty-eight blood samples were collected; 19 from Holstein cows without a previous lifetime history of mastitis (non-susceptible "NS") and 19 from Holstein cows with at least three previous episodes of mastitis (susceptible "S"). Details like age, parity, calving dates, stage of lactation, 305-day milk yield (305-DMY), peak yield (PY), average DMY, milk somatic cell count (SCC), milk composition and previous history of mastitis were collected from the electronic herd records. Cows in both groups were similar in parity (3.45 ± 0.51; vs. 3.30 ± 0.67; P>0.05), and days in milk (175 ± 44, vs. 176 ± 62; P>0.05) for non-susceptible and susceptible groups, respectively. Lactation means of SCC and milk composition were used. Lactation persistency was calculated according to Gajbhiye and Tripathi (1992) as a ratio of 305-DMY to peak yield. Blood samples were collected by jugular venipuncture into vacationer tubes containing EDTA as an anticoagulant. The samples were stored at -20°C till further processing for DNA isolation.

DNA extraction

DNA was extracted from blood samples using G-spin™ Total DNA Extraction Kit (Intron Biotechnology, Korea), it is carried out according to the manufacturer's instruction. The quality of extracted DNA was checked on 2% agarose. The presence of intact bands near wells with high molecular size indicated successful isolation of the genomic DNA.

Polymerase chain reaction (PCR) and sequencing

PCR was done for amplification of fragments in the transcribed exon of *TLR2*, *TLR4*, *TLR6* and *TLR9*. Primer sequences and their corresponding amplified fragment sizes along with the annealing temperatures are given in Table 1. The fragment of the *TLR4* gene was designed by primer 3 software (www.ncbi.nlm.nih.gov/tools/primer-blast/primertool.cgi) based on GenBank Accession No. NM_174198.6).

Amplification was performed in 25 µL reaction volume, containing 3 µL genomic DNA, 5 µL 10x buffer, 0.5 µL of dNTPs mix, 0.5 µL of each primer (10 Pmol), 0.3 µL Taq DNA polymerase (5 units/µL) and 15.2 µL d H_2O which was finally added. The final reaction mixture was placed in a thermal cycler (Techne, TC-3000, USA) and the PCR program was carried out by initial denaturation at 95°C for 5 min followed by 35 cycles of 94°C for 1 min for DNA denaturation, annealing temperature as seen in (Table 1) and extension at 72°C for 1 min and final extension at 72°C for 10 min. PCR products were stored at -20°C until check. The amplified DNA fragments were separated on 2-3% agarose gel, stained with ethidium bromide, visualized on a UV Transilluminator and photographed by Gel Documentation System (Alpha Imager M1220, Documentation and Analysis, System, Canada).

The PCR products were purified using MEGA quick-spin total fragment DNA purification kit (Intron Biotechnology). The purified PCR products were sent to LGC Company (Germany) for sequencing of 24 PCR products, six products for each gene (three non-susceptible cows and three susceptible). Sequence data were analyzed using Chromas 1.45 (http://www.technelysium.com.au). Sequence comparisons were performed using the BLASTN program from the National Center for Biotechnology information website http://www.ncbi.nlm.nih.gov/. Sequences were aligned using CLUSTAL-W version 1.8 (Thompson *et al*., 1994). Differences between individual sequences of non-susceptible and susceptible animals for each gene were classified as SNPs. The amino acid translation was done using MEGA version 6. Nucleotide sequences were

Table 1: Primer sequences, length of PCR product and their annealing temperatures

Primer	Primer sequence Forward (5´-3´) and Reverse (5´-3´)	Annealing temperature (C)	Product size (bp)	Reference
TLR2	Forward: GCTCCTGTGACTTCCTGTCC Reverse: CCGAAAGCACAAAGATGGTT	60	501	Menzies and Ingham (2006)
TLR4	Forward: GCCTAAACCACCTCTCCAC Reverse: AGAAGGGCTTGTAGACTTCT	59	682	Primer 3 software
TLR6	Forward: AAAGAATCTCCCATCAGAAT Reverse: GAAGGATACAACTTAGGTGA	63	515	Mariotti *et al*. (2009)
TLR9	Forward: CTGGAGGAGCTGAACCTGG Reverse: TGGTTGTAGGACAGCAGCAG	58	320	Menzies and Ingham (2006)

compared with that of the *Bos taurus* accession No. KT601038.1, DQ839567.1, XM_015471578.1, and AY859726.1 for *TLR2*, *TLR4*, *TLR6* and *TLR9*, respectively and the amino acids with acc. No. ACH92789.1, AAQ62700.1, ACH92795.1, and AAX56987.1 for *TLR2*, *TLR4*, *TLR6* and *TLR9*, respectively. Our sequence data were submitted to the GenBank and received the following accession number KY626164, KY626165, KY626166, KY626167.

Restriction fragment length polymorphism (RFLP)

Based on the sequence data of the *TLR* genes, a restriction map for each gene was constructed using Neb cutter software (http://tools.neb.com/NEBcutter2). The amplified DNA fragment was digested with selected restriction enzyme; *Rsa1*, *MSP1*, and *Taq1* for *TLR2* gene; *MSP1*, and *Rsa1* for *TLR4* gene; *HaeIII* and *Taq1* for *TLR6* gene; *Rsa1*, *Hha1*, *HaeIII*, and *MSP1* for *TLR9* gene.

RFLP in the case of *MSP1* (Thermo Scientific) was carried out in a reaction volume of 15 µL consist of 5 µL PCR product, 1 µL green buffer, 8 µL d H_2O and 1 µL of fast digest *MSP1* restriction enzyme. The reaction was incubated at 37°C for 5 min using a thermal cycler (Techne, TC-3000, USA). RFLP in the case of *Rsa1*, *HaeIII*, *Taq1* and *Hha1* restriction enzymes (Promega-USA) were carried out in a reaction volume of 20 µL consisting of 5 µL PCR product, 2 µL 10x buffer, 12.3 µL d H_2O, 0.2 µL acetylated BSA 10 mg and 0.5 µL from restriction enzyme. The reaction was incubated at 37°C for 2-4 h. The cleaved fragments were detected by 3% agarose gel electrophoresis and then the fragment patterns were visualized under UV in gel documentation system. Genotype was generated for each restriction enzyme cut.

Statistical analysis

Genotypic and allelic frequencies were calculated using the POPGENE software (ver. 1.31). The Hardy-Weinberg (H-W) equilibrium was determined by $\chi 2$ test. Owing to suitability for small sample sizes, low genotype frequencies, and the provision of exact significance, we used Fisher's exact test to assess the association between *TLR* loci with CM. SCC was converted into the SCS (SCS = log [SCC/ (100,000) + 3] (Wiggans and Shook, 1987)). The association of *Taq1-TLR2* and *Taq1-TLR6* genotypes with SCS and production traits was assessed with the GLM procedure using SAS software (Statistical Analysis System version 9.13). The full model included the effects of *TLR* genotype and cow factors (age, parity, and calving date). Variations among *MSP1-TLR2*, *MSP1-TLR4*, and *Hha1-TLR9* were not carried out because of low frequencies of the mutant genotypes.

Results

Polymerase chain reaction

The amplified PCR products of *TLR* genes were recognized on 2% agarose. *TLR2* gene (501 bp), *TLR4* (682 bp), *TLR6* (515 bp) and *TLR9* (320 bp), as shown in (Fig. 1A).

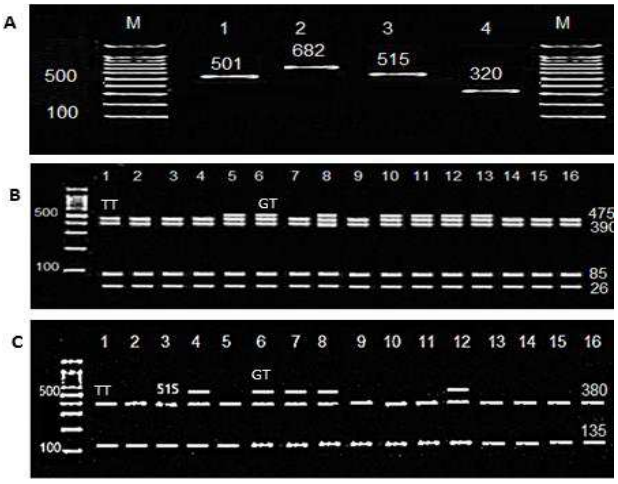

Fig. 1: Ethidium bromide stained agarose gel of PCR products. (**A**) Amplification of *TLRs*: *TLR2* (501), *TLR4* (682), *TLR6* (515), and *TLR9* (320) bp, (**B**) *TLR2-Taq1* (501 bp): GT (501 bp, 475 bp, 390 bp, 85 bp, and 26 bp) and TT (475 bp, 390 bp, 85 bp, and 26 bp), in susceptible cows, and (**C**) *TLR6-Taq1* (515 bp): GT (515 bp, 380 bp, and 135 bp) and TT (380 bp and 135 bp) in susceptible cows

Sequencing analysis and SNPs detection

The sequence analysis of *TLR2* gene revealed three non-synonymous SNPs; two transversions and one transition in susceptible cows compared with the non-susceptible, 2102 T>G, 2105 T>G, and 2107 C>T which altered amino acids 701 F>C, 702 V>G, and 703 P>S, respectively. Sequencing of *TLR4* gene revealed the presence of two non-synonymous SNPs 8731 A>G and 8732 G>A which changed the amino acid 322 S>D in susceptible cows. Analysis of *TLR6* sequences revealed the presence of two non-synonymous SNPs; transversion 979 T>G and 980 G>T in susceptible animals, leading to change of amino acid 174 C>V. No SNPs were detected in susceptible animals in *TLR9* gene. Phylogenetic analysis based on sequence data of *TLR9* revealed 100% similarities among susceptible animals and 100% similarities among the non-susceptible. All detected SNPs in *TLR2*, *4*, and *6* are illustrated in Figs. 2A-C.

Genotyping of TLR genes using RFLP technique

The cutting pattern of *Rsa1* in *TLR2*, *TLR4*, and *TLR9*; *HaeIII* in *TLR6* and *TLR9*; *Msp1* in *TLR9* are homozygous cutting pattern in both susceptible and non-susceptible cows (figures are not shown). Restriction analysis of PCR-RFLP-*MSP1* of *TLR2* (501 bp) showed two different genotypes: AB (370 bp, 131 bp, 98 bp, and 33 bp) and BB (370 bp and 131 bp), genotype AA is absent; both genotypes are recorded along both susceptible and non-susceptible cows (data are not shown). Restriction analysis of PCR-RFLP-*TLR2-Taq1* showed two different genotypes: GT (501 bp, 475 bp, 390 bp, 85 bp, and 26 bp) and TT (475 bp, 390 bp, 85 bp, and 26 bp), but genotype GG was absent (Fig. 1B). The TT was the only genotype found among non-susceptible

cows with a frequency of 1.00 compared to 0.6316 in susceptible cows (Table 2).

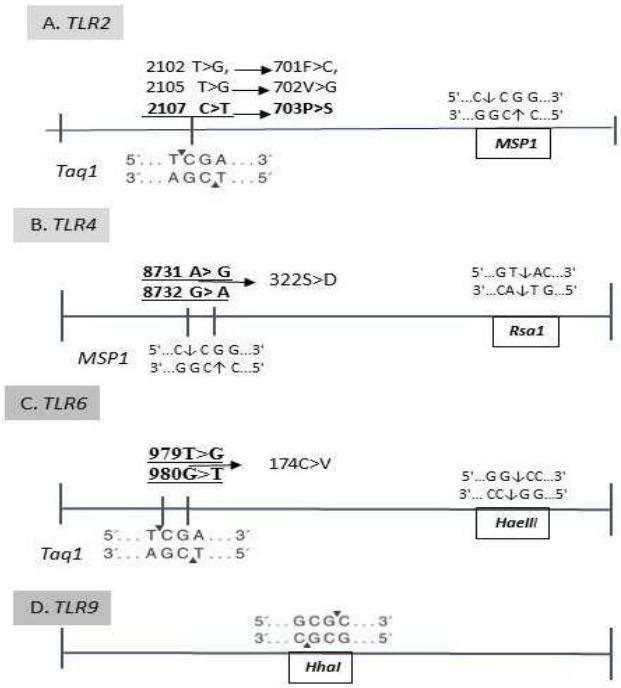

Fig. 2: Schematic representation of SNPs and amino acid change in relation to restriction sites in *TLR2* (**A**), *TLR4* (**B**), *TLR6* (**C**), and *TLR9* (**D**). Underline and bold litter indicates SNPs altered restriction sites and amino acid, surrounding restriction enzymes produce polymorphic cuts in susceptible and non-susceptible animals

The effect of *MSP1* restriction enzyme on *TLR4* gene region (682 bp) in 19 susceptible cows resulted in three fragments (490 bp, 110 bp, and 82 bp) for BB and four fragments (682 bp, 490 bp, 110 bp, and 82 bp) for AB genotype; both genotypes are recorded along with both susceptible and non-susceptible cows (data are not shown).

The effect of *Taq1* restriction enzyme on *TLR6* gene region (515 bp) resulted in two fragments (380 bp and 135 bp) for TT genotype and three fragments (515 bp, 380 bp, and 135 bp) for GT genotype (Fig. 1C).

The effect of *Hha1* restriction enzyme on *TLR9* gene region (320 bp) resulted in three fragments (155 bp, 115 bp, and 50 bp) for CC genotype and four fragments (320 bp, 155 bp, 115 bp, and 50 bp) for TC genotype (figures are not shown).

Susceptible animals have a heterozygous genotype associated with a cutting pattern for *Taq1-TLR2*, *Taq1-TLR6* and *Hha1-TLR9* which are repeated in four animals (Table 2).

Genotype and allele frequencies

Overall Chi-square test showed that all loci were in H-W equilibrium (P>0.05 not shown in the Tables). The results of Fisher's exact test of *Taq1-TLR2* revealed a significant (P=0.007) difference in genotypic frequencies between non-susceptible and susceptible animals (Table 2).

Association of TLRs genotypes with SCS and milk production traits

Least square means of *Taq1-TLR2* genotypes for SCS and milk production traits are presented in Table 3. Cows of GT genotype at the *Taq1-TLR2* locus tended (P<0.10) to have higher PY and fat percentage. As shown in Table 4, animals with the GT genotype of *Taq1-TLR6* locus peaked at a higher level (P=0.003) but were less persistent in lactation (P=0.03). On the other hand, genotypes at both loci do not vary in SCS (Tables 3 and 4).

Table 2: Genotype and allele frequencies of *TLR* loci in relation to susceptibility to CM

Loci	Group	Genotypes			Alleles		P-value
MSP1-TLR		**AA**	**AB**	**BB**	**A**	**B**	0.48
	NS	0.00 (0)	0.00 (0)	1.00 (19)	0.00	1.00	
	S	0.00 (0)	0.1053 (2)	0.8947 (17)	0.0526	0.9474	
Taq1-TLR2		**GG**	**GT**	**TT**	**G**	**T**	0.007
	NS	0.00 (0)	0.00 (0)	1.00 (19)	0.00	1.00	
	S	0.00 (0)	0.3684 (7)	0.6316 (12)	0.1842	0.8158	
MSP1-TLR4		**AA**	**AB**	**BB**	**A**	**B**	1.00
	NS	0.00 (0)	0.00 (0)	1.00 (19)	0.00	1.00	
	S	0.00 (0)	0.0526 (1)	0.9474 (18)	0.0263	0.9737	
Taq1-TLR6		**GG**	**GT**	**TT**	**G**	**T**	1.00
	NS	0.00 (0)	0.2105 (4)	0.7895 (15)	0.10525	0.89475	
	S	0.00 (0)	0.2632 (5)	0.7368 (14)	0.1316	0.8684	
Hha1-TLR9		**TT**	**TC**	**TT**	**T**	**C**	0.229
	NS	0.00 (0)	0.00 (0)	1.00 (19)	0.00	1.00	
	S	0.00 (0)	0.1579 (3)	0.8421 (16)	0.0789	0.9211	

Total number of cows was 19 in each group. NS (non-susceptible): Cows have never had clinical mastitis, and S (susceptible): Cows have been affected with clinical mastitis at least 3 times throughout lactations. Values in parentheses are numbers of each genotype

Table 3: Association of *Taq1-TLR2* genotypes with milk production variables in Holstein cows

Trait	*Taq1-TLR2* genotype		P-value
	GT	TT	
Somatic cell score	5.81±0.76	4.88±0.28	0.1959
305 d milk yield (kg)	9417±345	9031±243	0.5640
Peak yield (kg)	50.7±2.30	45.5±1.15	0.0903
Daily milk yield (kg)	29.4±2.45	28.7±0.85	0.7436
Persistency	191±8.25	200±5.73	0.5851
Fat (%)	3.82±0.29	2.99±0.10	0.0814
Protein (%)	3.15±0.26	3.66±0.08	0.1761
Lactose (%)	4.50±0.37	5.31±0.11	0.1383
Ash (%)	0.66± 0.06	0.78±0.02	0.1262

Values are least squares means and standard errors

Table 4: Association of *Taq1-TLR6* genotypes with milk production variables in Holstein cows

Trait	*Taq1-TLR6* genotype		P-value
	GT	TT	
Somatic cell score	5.54±0.55	5.13±0.33	0.4497
305 d milk yield (kg)	8999±316	9128±258	0.8081
Peak yield (kg)	52.7±1.50	45.6±1.19	0.0035
Daily milk yield (kg)	29.3±1.93	28.9±0.86	0.7726
Persistency	173±8.13	202±5.86	0.0321
Fat (%)	3.19±0.27	2.94±0.11	0.3825
Protein (%)	3.79±0.07	3.68±0.10	0.6463
Lactose (%)	5.49±0.10	5.34±0.15	0.6543
Ash (%)	0.81±0.02	0.78±0.02	0.6092

Values are least squares means and standard errors

Discussion

The marked differences in mastitis prevalence among or within breeds suggest the presence of resistance or susceptibility to mastitis, and therefore its prevalence is influenced by genetic factors (Rupp and Boichard, 2003). The present study was carried out to screen for polymorphism in *TLR2*, *TLR4*, *TLR6* and *TLR9* genes in Holstein cows, and its possible association with CM, milk SCS, and production variables.

The sequence analysis of *TLRs* loci revealed that *TLR* genes are highly polymorphic. Three novel non-synonymous SNPs were identified in *TLR2*; two SNPs resulted from transversions and one from a transition. Several SNPs have been identified in *TLRs* in various cattle breeds (Zhang *et al*., 2009; Huang *et al*., 2011; Bai *et al*., 2012; Girish *et al*., 2015). No SNPs in *TLR2* were found in an earlier study of Pant *et al*. (2007). We reported a significant association of the GT genotype at the *Taq1-TLR2* locus with CM based on their frequency. Similarly, Seabury *et al*. (2014) found an association between *TLR2* gene polymorphism with CM. Furthermore, the higher peak milk and fat percentage of GT genotype of *Taq1-TLR2* gene may be attributed to the well-known positive correlation between milk production and susceptibility to mastitis.

Mastitis cows tend to have a higher PY than non-mastitis cows before they develop CM (26, 27, 28, and 29) (Gröhn *et al*., 2004; Wilson *et al*., 2004; Gan *et al*., 2013; Zhang *et al*., 2016). In addition, milk fat content increases as a result of mastitis (Pyorala *et al*., 2003; Bruckmaier *et al*., 2004). Opsal *et al*. (2008) did not detect a significant association between the chromosomal regions surrounding *TLR2* and mastitis in Norwegian Red cattle. The current study revealed a numerical increase in SCS in GT individuals of *Taq1-TLR2* gene. A similar, but significant association between *TLR2* polymorphism, and SCS was reported by Zhang *et al*. (2009) and Prebavathy *et al*. (2015). The lack of statistical evidence in our study may be due to the small sample size.

We identified two novel non-synonymous SNPs resulted from a transition in *TLR4* gene. No SNPs were identified in *TLR9* sequence of susceptible animals, but polymorphism in the *TLR9* was revealed by RFLP (*TLR9-Hha1*). Also, studies which were conducted on the bovine *TLR9* gene demonstrated some genetic mutations in this gene (Griebel *et al*., 2005; Cargill and Womack, 2007; Sun *et al*., 2012). Due to the low frequencies of the mutant genotypes at *MSP1-TLR4* and *Hha1-TLR9* loci, differences among genotypes could not be statistically analyzed.

Two novel non-synonymous SNPs resulted from transversion were identified in *TLR6*. In a study of Mariotti *et al*. (2009), 855 G>A and 2315 T>C were identified in *TLR6*, and six polymorphic sites were found by Chu *et al*. (2009). The GT genotype of *Taq1-TLR6* locus was significantly associated with higher PY; however, the TT cows were more persistent in their lactation. This study is considered the first record revealing the association of *TLR6* genotypes with lactation persistency. Therefore, we suggest that TLR6 polymorphism and its RFLPs markers could be used in breeding for genetic improvement of Holstein cattle.

Mutations that were repeatedly reported in some susceptible animals in *TLRs* loci indicate that *TLR* genes could be used as a potential genetic MAS for culling of mastitis in Holstein cows.

Association was found in *Taq1-TLR2* gene polymorphism with CM, fat percentage and PY, and *Taq1-TLR6* with PY and lactation persistency. Repeated mutations that were reported in some mastitis animals in *TLRs* loci indicate that these genes could be used as a potential genetic MAS for mastitis resistance in dairy cattle. However, our results should be verified with larger sample size in a further study.

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