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Study of Toll-Like Receptor 9 Gene Polymorphism and its Association with Mastitis Disease in the Holstein Cows

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ABSTRACT

Mastitis is causes considerable economic losses due to decrease in the quality and quantity of milk production, increases of the cost of treatment and veterinary services, and animal waste (increases of waste product). Inflammation of the udder caused by a traumatic event, toxic agent, or invasion of microorganisms may be indicated by an increase in numbers of somatic cells in milk. Bovine TLR9 is located on BTA22. The TLR9 mRNA consists of two exons and is 3255 bp including 5 and 3 UTRs, The genomic size of TLR9 is 4264 bp and the protein is 1029 aa. Blood samples were collected from 150 dairy cattle from six herds. DNA extraction was performed by salting out method. A fragment of 245 bp from intron 1 was amplified by the polymerase chain reaction and analyzed by single-strand conformation polymorphism to get the patterns of single-stranded DNA separated by native polyacrylamide gel electrophoresis and visualized by silver staining. Nine genotypes were revealed with the frequencies of 0.8150 (AA), 0.1481 (AB), 0.2666 (AC), 0.1704 (AD), 0.8880 (BB), 0.1480 (BC), 0.5920 (BD), 0.2960 (CC) and 0.1407 (CD). The allele frequencies for A, B, C and D were 0.3741, 0.2000, 0.2407 and 0.1852; respectively.Chi-square test didn't confirm Hardy-Weinberg (H-W) equilibrium for this locus. Associations between polymorphisms and the trait studied were evaluated using the MIXED procedure of the SAS 9.1 software. Results showed that the somatic cell score not have a significant association with genotypes of TLR9 gene.

Keywords: Holstein cow, polymorphism, TLR9 gene, somatic cell score.

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INTRODUCTION

Bovine mastitis

Bovine mastitis is a disease with high incidence worldwide, even in herds with mastitis control programs. It causes considerable economic losses due to decreases in the quality and

quantity of milk production, increases in the cost of treatment and veterinary services, and animal waste (Crist *et al*., 1997; Dego *et al*., 2002).There are many different contagious and environmental bacteria that cause mastitis (Hogan and Smith., 1998).Inflammation of the udder caused by a traumatic event, toxic agent, or invasion of microorganisms may be indicated by an increase in numbers of somatic cells in milk. Although SCC from an uninfected quarter average about100 x 10^3 cells/ml, the *SCC* in infected mammary quarters is generally much higher (Mattila, 1985; Sheldrake *et al*., 1983 a,b).
The conserved molecules that are unique to some classes of potential pathogens known as pathogen associated molecular patterns (PAMPs) are primarily identified by an array of specialized pattern recognition receptors (PRR) of innate immune system, which includes the TLR family (Toll-like receptors) (Medzhitov *et al*., 1997; Akira, 2001; Janeway and Medzhitov, 2002).

Molecular structure of TLRs

TLRs are evolutionarily conserved innate immune receptors that belong to a family of type Itransmembrane proteins with an extracellular amino terminus. The ectodomain of TLR molecules consists of 16-28 leucine rich repeat (LRR) domain (Matsushima *et al*., 2007), which is mandatory for identification of PAMPs (Fujita *et al*., 2003). The central part of the LRRs possesses more irregular or longer motifs and varies among different TLRs, implying the functional importance of the central parts in ligand recognition (Matsushima *et al*., 2007). The cytoplasmic regions of TLR molecules possess a conserved domain Toll/IL-1 receptor (TIR) domain involved in downstream signal transduction. Based on their localization in the cell and the ligands they recognize, TLR molecules are divided into two groups. TLRs of the first group (TLR1, TLR2, TLR4, TLR5, TLR6 and TLR10) are expressed on cell surface and recognize compounds derived mainly from microbes. TLRs belonging to the second group (TLR3, TLR7 and TLR9) are expressed on the membranes of intracellular organelles such as endosomes (Heil *et al*., 2003; Matsumoto *et al*., 2003) and recognize nucleic acids or derivatives of nucleotides.

TLR9 structure

TLR9 has been grouped in a subfamily with TLR7 and TLR8, receptors that are also expressed within the endosome and sense pathogen-derived RNA and DNA. ClustalW multiple sequence alignments (www.ebi.ac.uk/clustalw) of these mammalian TLR9 sequences revealed that the bovine TLR9 shared 79% homology with human TLR9 and 73% homology with murine TLR9.Bovine TLR9 is located on BTA22. The TLR9 mRNA consists of two exons and is 3255 bp including 5 and 3 UTRs, according to the NCBI reference sequence (Accession No. NC_007320). The genomic size of TLR9 is 4264 bp and the protein is 1029 aa (Cargill and Womack, 2007).

Cellular responses to TLR9 signaling

A variety of cells have been shown to respond to CpG ODN stimulation but these responses may reflect either direct stimulation through TLR9 or indirect activation through CpG ODN-induced cytokine secretion. In humans, it appears that only B cells and plasmacytoid dendritic cell (pDC) express TLR9 and respond directly to CpG ODN stimulation (Hornung *et al*., 2002; Bauer *et al*., 2001). The production of IFN-a by pDC plays a key role in the activation of NK cells and other innate immune responses. A similar cellular pattern of TLR9 expression has been reported for mice with the exception that myeloid DC and macrophages also respond directly to CpG ODN stimulation (Sparwasser *et al*., 1997, 1998). There is evidence that CpG ODN can directly stimulate purified bovine B cells to proliferate and express IL-6 and stimulate purified bovine monocytes and macrophages to express IL-6 and IL-12 (Brown *et al*., 1998; Zhang *et al*., 2001). Cultured bovine macrophages and myeloid DC (derived from CD14+ monocytes) were also used to study CpG

ODN-induced responses and CpG-specific induction of IL-10, IL-12 and TNF secretion was observed (Werling *et al*., 2004). To date, there have no study which has been showed association between TLR9 and mastitis disease.
The objectives of the current study were to detect polymorphisms of TLR9 and determine association of such polymorphisms with Somatic Cell Score in Holstein cattle of RazaviKhorasan province.

MATERIAL AND METHODS

Blood samples collection and DNA processing

Blood samples (at 10 ml volume, the tubes containing of Na_2EDTA) of 150 Iranian Holstein cows (female, upper than 2 lactation period) were collected randomly from six Iranian Holstein cattle farms in RazaviKhorasan. The blood samples were kept to isolated in -20 °C. Genomic DNA was isolated from blood samples by the salting out method (Iranpur and Esmailzadeh, 2010) and the quality and quantity of DNA was investigated by Nanodrop set and loaded on a 1% agarose gel. Polymerase chain reactions (PCR) were carried out in 25 µl volume including 250 ng of genomic DNA, 10 pmol each primer (Figure1) and 12µl Master Mix (sinaclone company, Iran).

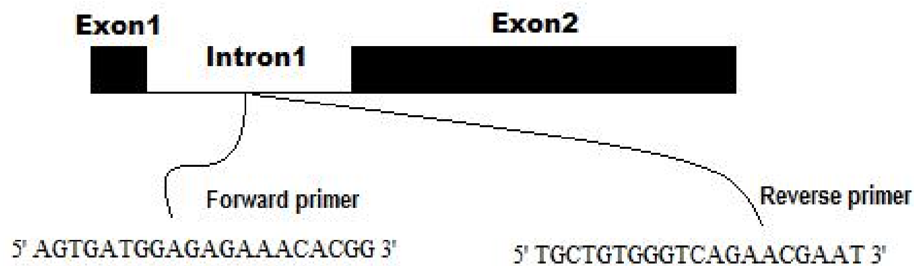

Figure 1: The selection subregion of primers and their sequencing

The PCR protocol was 95 °C for 5 min, followed by 35 cycles of 95° C for 30 s, annealing for 40 s and 56°C for 40 s, 72 °C for 45s with a final extension at 72°C for10 min. 1% agarose gel and Marker (500bp, Fermentaz company) was used to investigation quality of the PCR products (figure 2).

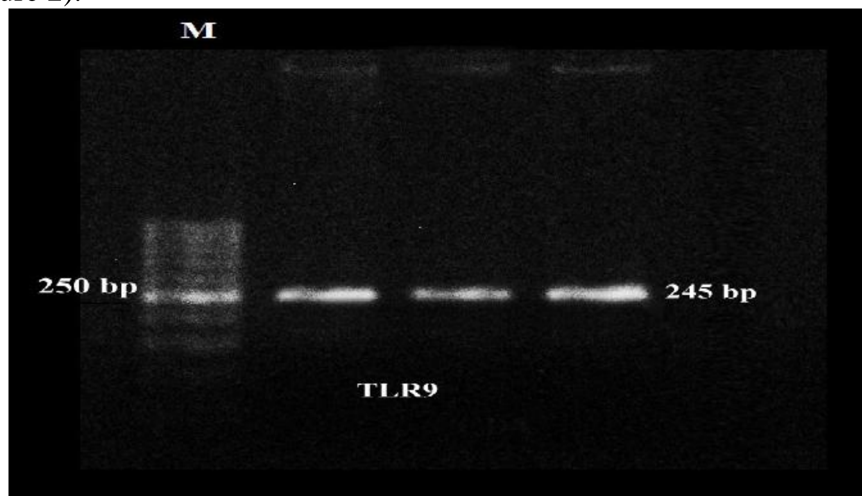

Figure 2: Quality of PCR products for TLR9 gene, identified by Marker (500bp from fermentaz Co).

Genotyping

The PCR products were genotyped by single-strand conformation polymorphism (SSCP) to screen the mutations within the amplified region. In total, 3µl of the PCR product of each sample was mixed with 7µl of denaturing buffer (98% formamide, 1% NaoH, 0.5% bromophenol blue and 0.5% glycerol) and then denatured at 95°C for 10 min, followed by a rapid chill on ice for 10min.

The denatured PCR products were electrophoresed on 10% polyacrylamide gels for 12 h at 8 V/cm and stained by 0.2% AgNO3 for 20min. Genotypes were recorded according to band patterns.

Statistical Analysis

The program POPGENE 32 (Francis *et al*., 1999) was used to test the number of alleles per locus (N), effected number of alleles (Ne), expected (He) and observed (Ho) heterozygosity, and departures from Hardy–Weinberg equilibrium (HWE).

The relationship between different genotypes and SCS trait, which was Log of Somatic Cell Count, were analyzed using mixed procedure of SAS9.1 package (SAS Inst. Inc., Cary, NC).

Tukey-Kramer test were used to compare the mean values of the attributes traits for the different genotypes. The statistical model is as follows:

$$ScSijkmno = \mu + Cowi + Gj + Hk + Ym + Sn + Dim + qDim + Po + Milkij + FPij + PPij + E_{ijkmno}$$

Where:
SCS_{ij}- somatic cell score of cow i and genotype j.
µ- means of population,
Cow_i- The fixed effect of cow i,
G_j- the fixed effect of genotype j,
H_k- the fixed effect of herd k, (k=1, 2, 3, 4, 5, 6).
Y_m- the fixed effect of calving year m.
S_n- the fixed effect of calve season n, (n=1, 2, 3, 4)
DIM- the number of lactation days.
qDIM- square of the number of lactation days.
P_o- parity O of cow, (O= 2,3,4,5,6,7)
$Milk_i$- monthly milk yields for each of cow i,
PP_i and FP_i- protein and fat percentage respectively.
E_{ijkmno}- the random errore.

RESULTS

Nine genotypes were revealed with the frequencies of 0.8150 (AA), 0.1481 (AB), 0.2666 (AC), 0.1704 (AD), 0.8880 (BB), 0.1480 (BC), 0.5920 (BD), 0.2960 (CC) and 0.1407 (CD). The allele frequencies for A, B, C and D were 0.3741, 0.2000, 0.2407and 0.1852, respectively.The chi-square test confirmed that this population is not in H-W equilibrium for the studied locus (Figure3 and Table1).

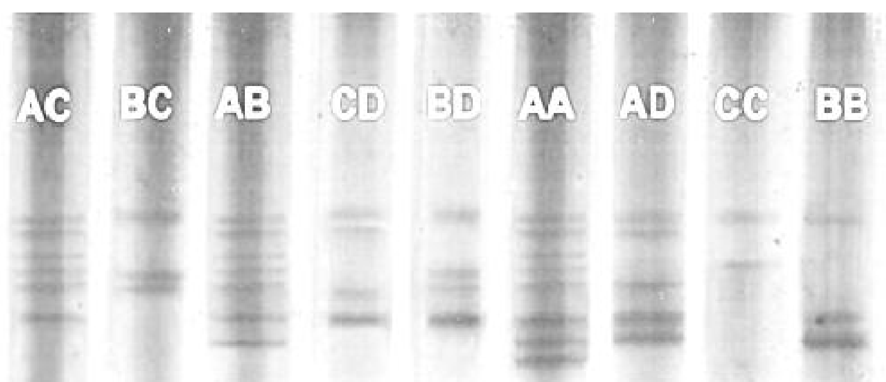

Figure3: genotypes derived from the electrophoresis of Polyacrylamide gel for TLR9 gene.

Table 1: the frequencies of Allele and genotypes for TLR9 gene

Allele frequency (%)				Genotype frequency (%)						
A	B	C	D	AA	AB	AC	AD	BB	BC	X^2
37.41	20.00	24.07	18.52	08.15	14.81	26.66	17.04	08.88	01.48	38.15
				BD	CC	CD				
				05.92	02.96	14.07				

X^2 =chi-square for HW equilibrium

Genetic diversity parameters such as observed and expected homozygosity and heterozygosity, Observed number of alleles (Na), Effective number of alleles (Ne), Shannon and Nei index, obtained from POPGENE are shown in table 2.

Table 2: Genetic diversity values for TLR9 gene

Parameters of genetic diversity	TLR9 gene
Na	4
Ne	3.6740
Obs_Het	0.8000
Obs_Hom	0.2000
Exp_Het	0.7305
Exp_Hom	0.2695
Nei	0.7278
I	1.3448

Na = Observed number of alleles,Ne = Effective number of alleles (Hartl and Clark, 1989), I = Shannon's Information index (Shannon, 1948), Obs-Het= observatedheterozygosity, Obe-Hom= observatedhomozygosity, Exp-Het=Expected heterozygosity, Exp-Hom= Expected homozygosityand were computed using Levene (1949), Nei=Nei's index (1973).

Another criteria is used for loss of heterozygosity in the population is the fixation index that is referred to as the inbreeding coefficient (Wright, 1977). If the fixation index value is negative, indicating that the loci alleles have low correlation with each other and is high the heterozygosity in it locus, values of the fixation index was showed in table 3.

Table 3: The fixation index for TLR9 gene

Allele	Value[1]
A	-0.2496
B	0.3056
C	-0.1550
D	-0.2273
Total	-0.0992

[1] Values of negative, indicating that is heterozygosity in it locus

Association between TLR9 gene and SCS trait

The somatic cell score not showed a significant association with genotypes of TLR9 gene (Table 4).According to table 5, BD genotype have a lower SCS than other genotypes. Maybe, can be said that this genotype were considerable to breeding programs.

Table4: Statistical values of the somatic cell score trait and its association with TLR9 gene

Trait	TLR9 gene		
	Mean	**RMSE**	**P value**
SCS[1]	2.1428	0.5273	0.0955

SCS= Somatic Cell Score; RMSE= root mean square error
[1] The somatic cell score not showed a significant association with genotypes P>0.05.

Table5: Least Squares Means of genotypes for SCS trait

Trait	Genotypes (μ ± s.e.)								
	AA	AB	AC	AD	BB	BC	BD	CC	CD
SCS	2.10±0.063	2.15± 0.050	2.08± 0.046	2.19± 0.045	2.09± 0.0612	2.05±0.104	2.03±0.064	2.04±0.089	2.05±0.055

SCS= somatic cell score

DISCUSSION

Polymorphism was observed in 245bp fragment from intron 1 of TLR9 gene in Holstein cattle of RazaviKhorasan. Mastitis is the most frequent and costly disease in dairy production and the innate immune system is considered to be important as the first line defense against this disease. Mastitis infections have been correlated with over expression of TLR2 and TLR4 in mammary glands of cattle and TLR2 in pigs; this correlation is even more evident with increased severity of infection (Goldammer *et al*., 2004; Ibeagha-Awemu *et al*., 2008; Petzl *et al*., 2008; De Schepper *et al*., 2008; Yang *et al*., 2008a,b; Zhu *et al*., 2008). The limited role of TLR9 in pathogen recognition of mammary gland infection of cattle have been suggested both by *in vivo* and *ex vivo* studies (Goldammer *et al*., 2004; Mount *et al*., 2009). However, species difference in TLR9 expression during mastitis exists as CpG-ODN has been shown to promote the expression of its specific receptor (TLR9 mRNA) in goat mammary tissue (Zhu *et al*., 2007). In cattle significant association exists between TLR2 and TLR4 polymorphisms and mastitis indicated by somatic cell scores, an indirect index to measure the mastitis phenotype (Wang *et al*., 2007; Pant *et al*., 2008; Zhang *et al*., 2009). In contrast, Opsal *et al*. (2008) failed to detect any significant association between the chromosomal regions surrounding TLR2 and TLR4 and mastitis in Norwegian red cattle. TLR genes 2, 4 and 6 polymorphisms and their relationship with somatic cell count and natural bacterial infections of the mammary gland in sheep have also been reported (Swiderek *et al*., 2006). Moreover, TLR4 polymorphisms in cattle have also been shown to be associated with lactation persistency (Sharma *et al*., 2006). Based on established association between TLRs and somatic cell scores, selecting animals for breeding programs with specific TLR gene polymorphisms could help improve herd resistance to mastitis. However, the somatic cell score not showed a significant association with TLR9 gene polymorphisms.

CONCLUSION

Probability, one of the reasons that there was no significant association between genotypes and SCS trait, can be small sample size and large genotypic variation in this research.

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