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RESEARCH ARTICLE

Investigating the Polymorphism of TLR2 Gene and its Association with Somatic Cell Count in the Milk of Aleppo Goats Raised in Sanliurfa Province

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Abstract

Mastitis is an important problem in the dairy industry which reduces milk yield and quality as well as causes economic loses. Somatic cell count (SCC) in milk is strongly associated to intra-mammary infections, thus represents an indicator of mammary health and milk quality. Toll-like receptor 2 (TLR2) is a member of of the Toll-like receptor family which is responsible for recognition of pathogens causing mastitis. Studies have shown that a 739G>A polymorphism in TLR2 gene is associated with SCC in milk in goat. This study aimed to determine the in the *TLR2* gene (Toll-Like-Receptor 2) in Aleppo goats in Sanliurfa and to investigate whether there is a relationship between this polymorphism and somatic cell count (SCC) in milk. Milk samples (50 ml) were collected twice at one-month intervals from 100 goats in five farms raising Aleppo goats in Sanliurfa. The milk samples were used for DNA isolation and determination of SCC. The 442 bp region of the target *TLR2* gene was amplified by PCR, and the PCR products were digested with the VspI enzyme and subjected to 2% agarose gel electrophoresis. Three genotypes, GG, GA, and AA, were observed in the entire sample. Of the 100 genotyped samples, 95 were GG, four were GA, and one was AA. The frequency of allele G was calculated as 0.97, and the frequency of allele A was calculated as 0.03. Mean SCC determined in milk samples from individuals carrying the A allele was found to be significantly lower. In conclusion, the G/A polymorphism of the *TLR2* gene has a significant effect on SCC and this polymorphism can be used as a marker for selection purposes in breeding programs aimed at improving SCC and mastitis resistance in Aleppo goats.

Key Words: Goat, PCR, polymorphism, somatic cell count, *TLR2*

Şanlıurfa İlinde Yetiştirilen Halep Keçilerinde TLR2 Genindeki Polimorfizm ve Sütteki Somatik Hücre Sayısı Arasındaki İlişkinin Araştırılması

Öz

Mastitis, süt endüstrisinde süt verimini ve kalitesini düşüren ve ekonomik kayıplara neden olan önemli bir sorundur. Sütteki somatik hücre sayısı (SCC), meme içi enfeksiyonlarla güçlü bir şekilde ilişkilidir ve bu nedenle meme sağlığı ve süt kalitesinin bir göstergesidir. Toll-like reseptör 2 (TLR2), mastitise neden olan patojenlerin tanınmasından sorumlu olan Toll-like reseptör ailesinin bir üyesidir. Araştırmalar, TLR2 genindeki 739G>A polimorfizminin keçi sütünde SCC ile ilişkili olduğunu göstermiştir. Sunulan bu çalışmada Şanlıurfa'daki Halep keçilerinde *TLR2* genindeki (Toll-Like-Receptor 2) 739G>A polimorfizminin belirlenmesi ve bu polimorfizm ile sütteki somatik hücre sayısı (SHS) arasında ilişki olup olmadığının araştırılması amaçlanmıştır. Şanlıurfa'da Halep keçisi yetiştiren 5 işletmedeki 100 keçiden birer aylık arayla iki kez 50 ml süt örneği alındı. Süt örnekleri DNA izolasyonu ve SHS'nin belirlenmesi amacıyla kullanıldı. Hedef *TLR2* geninin 442 bç'lik kısmı PCR işlemi ile çoğaltıldı ve *VspI* enzimi ile kesilen PCR ürünleri %2'lik agaroz jel elektroforezine tabi tutuldu. Tüm örneklem içerisinde GG, GA ve AA şeklinde üç genotip gözlendi. Genotiplenen 100 örnekten 95'inin GG, dördünün GA ve birinin ise AA genotipinde olduğu gözlendi. Allel G'nin frekansı 0.97, allel'nın A frekansı ise 0.03 hesaplandı. A allelini taşıyan bireylerin süt örneklerinde belirlenen ortalama SHS'nin önemli ölçüde daha düşük olduğu bulunmuştur. Sonuç olarak, TLR2 geninin G/A polimorfizmi SHS üzerinde önemli bir etkiye sahiptir ve bu polimorfizm Halep keçilerinde SCC ve mastitis direncini iyileştirmeyi amaçlayan ıslah programlarında seçim amaçlı bir belirteç olarak kullanılabilir.

Anahtar Kelimeler: Keçi, PCR, polimorfizm, somatik hücre sayısı, *TLR2*

INTRODUCTION

Goat milk and its products, such as yoghurt, cheese and powdered milk, are an important source of nutrition for humans. In developing countries, more people are fed with goat milk than with cow milk. Goat milk provides an alternative source of protein for people who cannot consume cow's milk due to gastrointestinal disorders or an allergy to cow's milk. In developed countries, goat's milk is also in demand to cater for different tastes. (1).

Mastitis, which is the inflammation of udder tissue, is an important problem in the dairy industry due to its negative impact on economic performance, animal welfare, and milk yield and quality (2). Inappropriate environmental conditions, contaminated milking equipment and poor udder cleaning before and after milking can all lead to mastitis. In addition, selection procedures applied to increase milk yield can increase susceptibility to mastitis. As there is a close relationship between mastitis and somatic cell count (SCC), SCC is considered an indicator of milk quality and general udder health (3–5). The heritability of SCC has been reported to be 0.24 ± 0.01 and 0.20 ± 0.01 in Saanen and Alpine goats, respectively (5). While there is no definitive upper limit for SCC in goat's milk, some researchers have suggested that milk with a count exceeding 3 500 000 cells/ml is not suitable for sale (6), and that the maximum acceptable level for Class A goat's milk is 840 000 cells/ml (7).

Proteins defined as pattern recognition receptors (PRRs) found on cell surface, endosomes and cytoplasm recognize the molecular structures of pathogens play a crucial role in innate immunity. Toll-like receptors (TLRs) represent an important group among PRRs (8). The main function of TLRs in vertebrates is the recognition of pathogen-associated molecular structures originating from pathogenic microorganisms such as viruses, bacteria and fungi. Among them *TLR1*, *TLR5*, *TLR6* and TLR10 recognize structures of bacterial origin, while *TLR3*, *TLR7, TLR8* and *TLR9* recognize structures specific to viruses. *TLR2* and *TLR4* recognize structures originating from both bacteria and viruses. TLR proteins can also be classified according to the molecular structures they bind, i.e. their ligand structure. These structures are lipids, proteins and nucleic acids (9). *TLR1* and *TLR2* play a role in the response to lipopeptides in mycobacteria as heterodimers (10).

The goat *TLR2* gene is approximately 26.21 kb in size and consists of two exons. This gene encodes a protein consisting of 794 amino acids and weighing 91.4 kD. In goats, the *TLR2* gene is located on chromosome 17. TLR genes are candidate genes for susceptibility or resistance to various diseases due to their role in the recognition of pathogen-associated molecular structures. Therefore, several studies have been conducted on the relationship between polymorphisms in TLR genes and mastitis in sheep and cattle (11-14).

The association between mastitis in goats and polymorphism in *TLR2* gene was first investigated by Ruiz-Rodriguez et al. (15) who identified three polymorphic nucleotides in *TLR2* gene as 39G>A, 840G>A, and 1083A>G (GenBank accession no: JQ911706). The 739G>A polymorphism results in a substitution of isoleucine amino acid for valine. The other two polymorphisms do not cause amino acid substitution. In the study in which a total of 39 animals were examined, the mean SCC in the milk of 8 heterozygous (GA) individuals in terms of 739G>A polymorphism was found to be significantly lower (146.222) than that of homozygous (GG) individuals (537.367) (P<0.004).

Ogorevc et al. (2) developed a method to detect 739G>A polymorphism by PCR-RFLP method (*VspI*) and investigated the association between this polymorphism and SCC in milk of 61 goats. In the study in which a total of 863 records were used, the mean SCC in the milk of goats with GG genotype was significantly higher than that of GA and AA genotypes. The difference was found to be statistically significant.

The Aleppo goat breed is resistant to high temperatures in hot climates and has a high milk yield. They successfully utilise poor and barren pastures and are therefore widely raised in the southeastern Anatolia region, where vegetation cover is short. Breeding goats is also an excellent way to make good use of stubble areas after harvest (16).

The association of the 739G>A polymorphism in *TLR2* gene with SCC in goat has been investigated by Ogorevc et al. (2) and Ruiz-Rodriguez et al. (15) on 39 and 61 goats. However in Türkiye, there is no research investigating the relationship between SCC in goat milk and polymorphism in TLR genes. Goat raising and especially Aleppo goats are crucial part of animal production in the region. Defining the genetic structure of the goats in this region would help improve production capacity and quality of this breed. Assessing the relationship between genetic polymorphism and SCC can be used for marker assisted selection. The aim of this study was to investigate the relationship between 739G>A polymorphism and SCC in milk by and PCR-RFLP method using *VspI* enzyme in Aleppo goats raised in Sanliurfa province of Türkiye.

MATERIAL AND METHODS

The animal material of this study consisted of 100 Aleppo goats from 5 goat breeding facilities in Haliliye and Eyyübiye districts of Şanlıurfa province. The study was approved by Harran University Animal Experiments Local Ethics Committee (Decision No: 2022/001/03).

After the onset of parturitions, 50 ml milk samples from each animal were collected twice at one month intervals. After each animal was completely milked in a separate clean container, 50 ml of milk sample was taken into the sample collection tube. Of the collected milk samples, 25 ml was used for DNA isolation and the other 25 ml was used for assessing somatic cell count. In order to assess SCC raw milk samples were heated in a water bath to +40°C and then loaded into an automatic sampling device (Combi 150 milk analyzer, Bentley-United States of America).

Isolation of DNA from the collected milk samples was performed as reported by Liu et al. (17) with some modifications. Briefly, 15 ml of milk sample was centrifuged at 3000xg for 15 minutes and the upper fat layer and watery fraction were discarded. The cell pellet was re-suspended with 600 µl TE buffer and transferred into a 1.5 milliliter microcentrifuge. After centrifugation at 10 000xg for 10 minutes. the pellet was suspended in 600 µl lysis solution (0.1% Sodium dodecyl sulfate, 10 mMTris and 1 mM EDTA) containing 10 µl proteinase K (10 mg/ml). The tubes were kept at 56°C until the pellet was completely dissolved. DNA isolation from cell lysate was performed by phenol chloroform extraction method as reported by Sambrook et al. (18). Concentrations of

DNA the solutions were measured with a micro volume spectrophotometer at 260 nm wavelength and then diluted with TE-buffer to 100 ng/µl.

The target region in exon 2 of *TLR2* gene was amplified using the primers

F:5'-ATCTGCGGACCCTGAAAGTA-3'andR:5'-GCTGTAAA-ATCGCCAATTCC-3' and PCR conditions reported by Ogorevc et al. (2 2019). The reaction mixture was prepared in a final volume of 12.5 µl by adding 1 µl genomic DNA, 1,25 µl of 10X PCR Buffer, 2 mM MgCl2, 200 µM dNTP mix and 1.25 U of Taq polymerase (5 U/µl). Temperature treatments for amplification were applied for the first denaturation step at 95°C for 5 minutes, followed by 35 cycles of 95°C for 30 seconds, 58°C for 30 seconds and 72°C for 30 seconds, followed by a final extension step at 72°C for 3 minutes.

The PCR products obtained were treated with *VspI* enzyme. The reaction solution was prepared in a volume of 30 µl containing 10 µl PCR product, 3 µl 10X reaction buffer solution (Buffer O) and 2.5 U *VspI* enzyme. The samples were incubated in an oven at 37°C for 16 hours and then kept at 65°C for 20 minutes to inactivate *VspI* enzyme. The restriction products were separated by electrophoresis were on 2% agarose gel containing ethidium bromide and the results were visualized by UV light.

In order to validate the genotyping results one sample of each genotype was sequenced by Sanger sequencing. Sequence analysis was outsourced to a private company. The reaction mixture of PCR for sequence analysis was prepared in a volume of 20 µl, containing 0.5 µM primer, 10 µl 2X sequence master mix (fluresan-labeled dNTP, MgCL2, DNA-Polymerase enzyme and reaction buffer) and 3 µl PCR product. For the PCR process used in sequence analysis, initial denaturation was performed at 95 °C for 120 seconds, followed by 35 cycles of 95 °C for 30 seconds, 52 °C for 30 seconds and 72 °C for 20 seconds. The product to be used for sequence analysis was prified from other reaction components by spin column method. The base sequences of PCR products were determined using an ABI PRISM 3130XL (Applied Biosystems) automated sequence analyzer.

Genotype and allele frequencies were assessed by counting. Chi-square test was used to analyze whether the observed genotype numbers were in Hardy-Weinberg equilibrium. Normality of the data was assessed by using Shapiro-Wilk test. The t-test was used to determine the effect of genotype on Somatic Cell count in milk. In order to obtain a normal distribution data were log-transformed. The mean SCC in milk samples collected from the same animal in two different periods were included in the analysis. Spss 17.0 package program was used for statistical analysis.

RESULTS

A 442 base pair fragment of the goat *TLR2* gene was amplified by PCR. Agarose gel electrophoresis image of PCR products of some samples is shown in Figure 1. Some of the restriction products of the PCR products obtained after treatment with *VspI* enzyme are shown in Figure 2. After treatment with VspI enzyme, three genotypes were observed as GG, GA and AA (Figure 2).

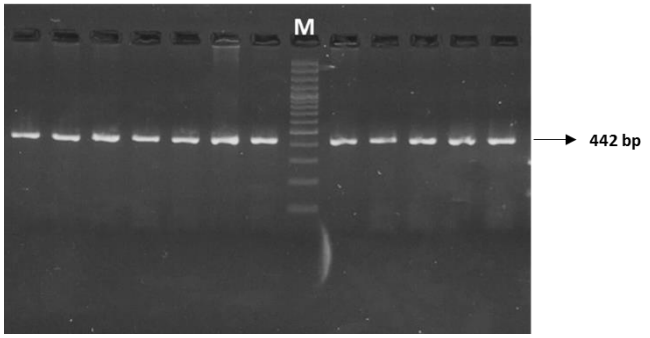

Figure 1. Agarose gel image of PCR products of some samples. M: 100 bp ladder

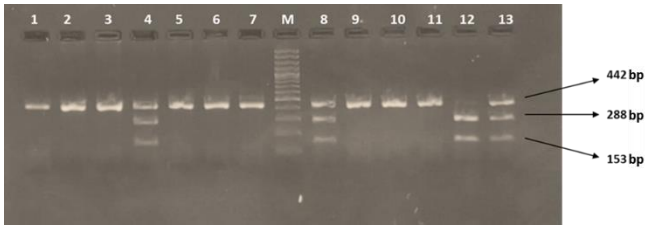

Figure 2. Restriction products of some samples treated with *VspI* enzyme. 1,2,3,5,6,7,9,10,11, GG; 4, 8, 13 GA; 12 AA. M: 100 bp ladder

Genotypes observed on agarose gel were confirmed on representative samples by sequence analysis. The electropherogram images of the genotypes identified by sequence analysis are shown in Figure 3.

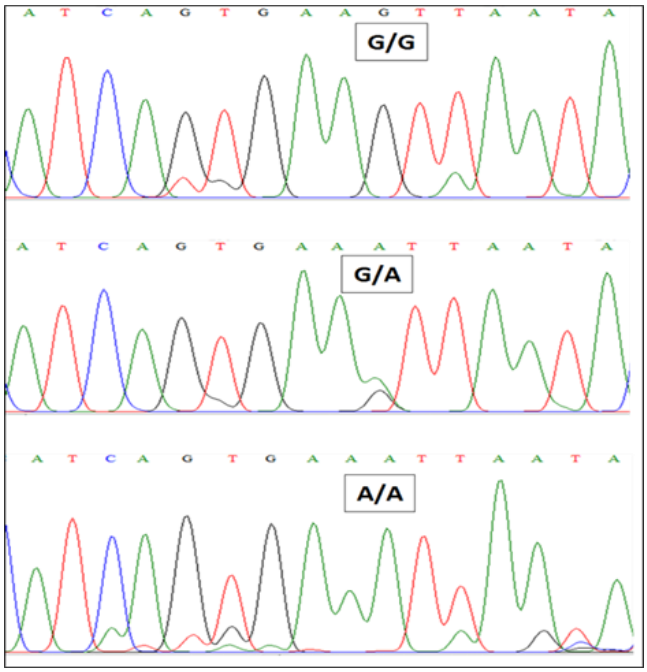

Figure 3. The electropherogram images of the genotypes.

Among 100 samples genotyped, 95 had GG, four had GA and one had AA genotype. Based on the genotype data, the frequency of the G allele was calculated as 0.97 and the frequency of the A allele as 0.03 (Table 1). The observed genotype frequencies were significantly different from Hardy-Weinberg equilibrium ($P<0.001$).

Table 1. Genotype and allele frequencies

Genotype frequencies			Total	Allele frequencies		χ^2	P
GG	GA	AA		G	A		
95 (0.95)	4 (0.04)	1 (0.01)	100	0.97	0.03	9.78	0.001

There was a moderate positive correlation between the first and the second measurements in terms of somatic cell count (r=0.439; P<0.001). While the difference between genotype averages in terms of actual somatic cell count in goat milk was statistically insignificant (P>0.05), the difference was found significant after logarithmic correction (P<0.05). It was determined that the genotype of *TLR2* gene had a significant effect on SCC (Table 2).

Table 2. Genotype means for SCC

Genotype	SCC (X1000)		LogSCC	
	Min.-Max.	Mean±S.E.	Min.-Max.	Mean±S.E.
GG	145.00 - 13 265.00	2 582.22±285.86	5.16 - 7.12	6.17±0.05
A-	114.50 – 1 523.50	725.60±303.51	5.06 - 6.18	5.66±0.22
Over all	114.50 – 13 265.00	2 467.61±272.99	5.06 - 7.12	6.14±0.05
p	0.098		0.010	

DISCUSSION

Somatic cells in milk consist of leukocytes and mammary epithelial cells and are an important indicator for determining both udder health and milk quality. Many researchers have reported a negative relationship between the increased somatic cell count (SCC) in milk as a result of mastitis and milk yield (19-21). On the other hand, Haenlein (3) has reported that SCC also varies depending on the stage of lactation. Rupp et al. (5) reported the heritability of somatic cell count in Saanen and Alpine goats as 0.24±0.01 and 0.20±0.01, respectively.

In the milk samples examined the mean SCC per milliliter ranged between 114 500 ($0.114x10^6$) and 13 265 000 ($13 265x10^6$) with a mean of 2 457 108 ($2.457x10^6$). In a study in which the same polymorphism was investigated on 39 goats of different breeds, by Ruiz-Rodriguez et al. (15) it was found that this value varied between 19 000 and 3 485 000 and the average (P<0.004) was 407 283. Ogorevc et al. (2) found that the mean SCC of individuals with GG, GA and AA (n=2) genotypes was 1 475 660, 1 055 380 and 577 110 (P<0.0007), respectively. Although there is no definite limit for the maximum SCC in goat milk, it is assumed that milk containing higher than 3 500 000 SCC/ml is not suitable for sale and and this number should not be higher than 840 000 SCC/ml for class A goat (6,7). The mean values determined in our study were significantly higher than these values. This may be due to the hygiene level of the environment where the goat herds included in the study were raised, the lactation period when milk samples were collected, the goat breed and the number of lactations (15,22,23).

There is no information on the age or lactation period of the goats included in the study. The breeding season of goats raised in Sanliurfa province is between February and April and their lactation period is approximately 5 months. The milk samples used were collected in May-June. Therefore, SCC may have been found to be high because the milk samples were collected in the later stages of the lactation period.

Three genotypes as GG, GA and AA were observed after treatment of the PCR-RFLP products with *VspI* enzyme. Out of the 100 genotyped samples, 95 had the GG, four had the GA and one had the AA genotype. Based on the genotype data, the frequency of the G allele was calculated as 0.97 and the frequency of the A allele as 0.03. Allele frequencies may vary from population to population. For example, Ruiz-Rodriguez et al. (15) found the frequencies of alleles G and A to be 0.898 and 0.102 respectively. Ogorevc et al. (2) found the frequencies of alleles G and A in Slovenian Alpine goats as 0.85 and 0.15, respectively. The frequency of G allele was higher and the frequency of A allele was lower than Ruiz-Rodriguez et al. (15) and Ogorevc et al. (2).

A statistically significant relationship between 739G>A polymorphism detected by *TLR2* gene *VspI* enzyme and somatic cell count in milk in Aleppo goats. Ruiz-Rodriguez et al (15) examined 39 animals from different breeds and found that 8 animals were heterozygous (GA) and the others were homozygous (GG) for 739G>A polymorphism. While the mean SCC in the milk of homozygous individuals was 537.367, this value was 146.222 in heterozygotes (P<0.004). Ogorevc et al. (2) investigated the relationship between this polymorphism and SCC in 61 goats. In the study in which a total of 863 records were used, the mean SCC per milliliter of goats with GG genotype was found to be 1 475 660 GA, 1 055 380 for the genotypes, and 577 110 for individuals with AA genotype (2 individuals). The difference was found to be statistically significant (P<0.0007).

Similar relationships between SCC and certain polymorphism in TLR4 gene have been reported in cattle, water buffaloe and camel (24,25,26). Ruiz-Rodriguez et al (15) have analyzed the effect of the amino acid change on the protein structure of *TLR2* and reported that both Val and Ile are nonpolar and hydrophobic so that a Val/Ile substitution does not represent a radical change. The authors attributed the effect to transcriptional changes through promoter or enhancer variants linked to the this polymorphism or synonymous mutations in this gene which potentially may have role for codon usage relying on a different tRNA for the mRNA to

be translated into protein. In accordance with this suggestion it has been reported that expression of TLR genes in mammary gland are significantly up-regulated in the case of exposure to *Staphylococcus aureus* and mastitis (27,28,29)

Among the samples included, only five animals carrying the A allele as homozygous or heterozygous were identified. All of these animals belonged to one farm. This can be attributed to a low frequency of A allel in this breed. On the other hand, no information on confounding factors such as lactation stage, parity or management conditions was available. Therefore, the genotypic effect detected is quite low for a strong conclusion. The moderate correlation between the samples collected in two different periods indicates that the estimation based on samples collected once may be misleading. Therefore, milk samples were taken twice from each animal and a total of 200 records from 100 animals were used. Although this number is lower than the 863 records of 61 animals reported by Ogorevc et al. (2), it is higher than the number of samples collected at one time from 39 goats of different breeds in Ruiz-Rodriguez et al. (15). However, an analysis with higher statistical power would be necessary by including more individuals carrying the A allele in future studies.

If the relationship between this polymorphism and somatic cell count is determined in this breed, this polymorphism can be used as a marker to be used in selection for breeder selection for breeding studies in terms of somatic cell count and mastitis resistance.

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CONFLICT OF INTEREST

There is no conflict of interest to be declared by the authors

AUTHOR CONTRIBUTIONS

SGE and FB contributed to study planning, sample collection, the writing of the study and final check, MB organized sample collection, MOA contributed to laboratory analyses and final check.

ETHICAL STATEMENT

The study was approved by the Harran University Animal Experiments Local Ethics Committee (HAYDEK) on February 9, 2022, with approval number 2022/001/03.

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