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Polymorphisms of *TLR1*, *TLR4* and *SLC11A1* Genes in Some Cattle Breeds Reared in Turkey

Korhan ARSLAN[a], Bilal AKYÜZ[a], Esma Gamze AKSEL[a], Fadime ÖZDEMİR[a], Mehmet Ulaş ÇINAR[b]

[a]*Erciyes University, Faculty of Veterinary Medicine, Department of Genetic, 38039, KAYSERİ*

[b]*Erciyes University, Faculty of Agricultural, Department of Animal Science, 38090, KAYSERİ*

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Corresponding Author: Korhan ARSLAN, E-mail: korhanars@gmail.com, Tel: +90 (352) 207 66 66
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ABSTRACT

The objective of this study was to examine the allelic and genotypic profiles of four SNPs of bovine Toll-Like receptor 1 (TLR1), Toll-Like Receptor 4 (TLR4) and Solute Linkete Carier 11A1 (SLC11A1) genes in Holstein, two Turkish native breeds and their crossbreds in Turkey. For this purpose, a total of 1023 cattle from Holstein (HL, n= 410), Anatolian Black (AB, n= 106), East Anatolian Red (EAR, n= 84), Anatolian Black Crossbreed (ABC, n= 124) and East Anatolian Red crossbreed (EARC, n= 299) breeds were examined for four SNPs. Samples were genotyped using by the PCR-RFLP method. According to the TLR1 (+1380) SNP, EAR was in Hardy-Weinberg equilibrium (HWE) while the AB, HL and native crossbreeds were deviated from HWE. All cattle breeds were in HWE for the TLR1 (+1596) SNP. In terms of TLR4 (+10) SNP, the CC genotype had the highest frequency in HL and native crossbreeds whereas the AB and EAR breeds were monomorphic and only CC genotype was found these breeds. According to the SLC11A1 (+1066) SNP, the CC genotype had the highest while the GG genotype frequency the lowest in all breeds. In addition EAR breed was in HWE while the AB, HL and native crossbreeds deviated from HWE.

Keywords: Polymorphism; Turkish native cattle; Toll-Like Receptor; *SLC11A1*; Marker-assisted selection

1. Introduction

The immunity is divided into two namely the innate and adaptive immunity, in mammals. Innate immunity is present both in vertebrates and invertebrates, whereas adaptive immunity is only present in vertebrates (Takeda & Akira 2001). Host genetic resistance is mainly sustained by innate immunity, providing protection against pathogens without being vaccinated or exposed to diseases (Prakash et al 2014). Deciphering host genotypes for disease resistance can help us to control livestock diseases and develop strategies for decreased economic losses in farm animal breeding.

TLR1 is associated with immune responses against many bacterial pathogens. It includes a TLR subfamily which creates heterodimers with *TLR2*. The resulting TLR1/TLR2 complex recognises different bacterial cell wall ingredients such as lipoproteins and lipopolysaccharides, thus mediating a natural immune response against Gram-positive and Gram-negative bacteria species (Buwitt-Beckmann et al 2006; Russell et al 2012). In this way,

TLR1/TLR2 heterodimers and also *TLR4* recognise mycobacterium Pathogen-Associated Molecular Patterns (PAMPs), then macrophages and dendritic cells are activated for immune response (Brightbill et al 1999; Chang et al 2006; Hawn et al 2007). It was reported that some mutations in the *TLR1* and *TLR4* genes decrease immune response against lipopeptide and lipopolysaccharide bacterial cell wall components (Hawn et al 2007). *TLR4* plays on important role in immune response against both Gram negative and positive bacteria (Underhill et al 1999). Additionally, the *TLR4* gene was reported to be a strong candidate gene for disease resistancy, such as against mastitis (Ogorevc et al 2009), paratuberculosis (Mucha et al 2009), and brucellosis (Prakash et al 2014).

Solute Linkete Carier 11A1 (*SLC11A1*) is a transmembrane protein and was reported to be one of the best known potential candidate genes that promote innate immunity against different intracellular pathogens (Kumar et al 2011). Therefore, the *SCL11A1* gene variants may be used to nature resistance or susceptibility to some important infections such as tuberculosis in water buffalo (Le Roex et al 2013) and brucellosis in cattle (Kumar et al 2011; Prakash et al 2014).

The present study was aimed at determining the allele and genotype frequency of four SNPs of bovine *TLR1, TLR4* and *SLC11A1* genes in Holstein, Anatolian Black (AB), East Anatolian Red (EAR) cattle breeds and their crossbreeds in Turkey.

2. Material and Methods

A total of 1023 cattle made up of the Holstein (n= 410, from Kayseri, Kahramanmaraş, Balıkesir, Burdur, İzmir and Çanakkale), AB (n= 106, from Kayseri, Sivas, Ankara, Çankırı, Niğde and Yozgat), EAR (n= 84, from Erzurum, Kars and Ardahan), Anatolian Black crossbreed (ABC, n= 124 from Kayseri, Sivas, Çorum, Niğde and Yozgat) and East Anatolian Red crossbreed (EARC, n= 299, from Erzurum, Kars, Ardahan, Kayseri, Sivas, Çorum, Niğde and Yozgat) cattle were examined for four SNPs in the *TLR1* (*+1380, +1596*), *TLR4* (*+10*) and *SLC11A1* (*+1066*) genes. Genomic DNA was isolated from whole blood samples using the phenol-chloroform method (Sambrook et al 1989). Genotyping of *TLR1*, *TLR4*, *TLR9* and *SLC11A1* gene polymorphisms was performed by PCR-RFLP. Detailed information about the primers, amplification product lengths and enzymes are used shown in Table 1.

The PCR for all SNPs was performed in 20 µL reaction mixture, which included 1.5 mM $MgCl_2$, 200 µM dNTPs, 200 µM primer, 1×PCR buffer, 1U Taq polymerase and 50-10 ng genomic DNA. The thermal cycling condition consisted of pre-denaturation (94 °C for 5 min) followed by 35 cycles of 30 s at 94 °C, 30 s at the annealing temperature (Table 1), 30 s at 72 °C, and a final extension of 1 min at 72 °C. For RFLP, each of the PCR products was digested with the appropriate enzyme (Table 1)

Table 1- Primer sequences, amplification conditions, product sizes and restriction enzymes

SNP	*Primer sequence*	*AT (°C)*	*PS (bp)*	*RE*	*RE Heat, inactivation temparatrures and times*	*Reference*
TLR1 (+1380)	F: 5'-TTTAGCAGCCTTTCCATACT-3' R: 5'-TCTACCACGTCACTGGATACT-3'	55	179	*Bs1*I	Activation: 55 °C for 4 hours Inactivation: 80 °C for 20 minutes	Prakash et al 2014
TLR1 (+1596)	F: 5'-TTTAGCAGCCTTTCCATACT-3' R: 5'-CAGATCCAGGTAGATACAGAG-3'	64	354	*Bcl*I	Activation: 55 °C for 4 hours Inactivation: 80 °C for 20 minutes	Sun et al 2012
TLR4 (+10)	5'-CGTAACCCAGCACTGCTTTG-3' R: 5'-GCCTGTTAATGCCCTGTAACC-3	59.2	405	*BstU*I	Activation: 37 °C for 4 hours Inactivation: 65 °C for 20 minutes	Prakash et al 2014
SLC11A1 (+1066)	F: 5'-ATCTCCTTCCTACTGCCCG-3' R: 5'-CACAAACTGTCCCGCGTAG-3'	54	374	*Pst*I	Activation: 37 °C for 15 minutes Inactivation: 80 °C for 20 minutes	Prakash et al 2014

AT, annealing temperature; PS, product size; RE, restriction enzyme

in a reaction including 3 µL of the PCR product, 5 U of the restriction enzyme (MBI Fermentas) and 1 µL of the buffer. The reaction was incubated at the appropriate temperature for each enzyme (Table 1) for 4 h. Genotyping for each SNP was performed by 3% agarose gel electrophoresis.

The genotype and allele frequencies of the examined SNPs in each breed were calculated. The Hardy-Weinberg equilibriums (HWE) of the examined breeds for five SNPs in the *TLR1*, *TLR4* and *SLC11A1* genes were analysed using the Chi-square test. All statistical analyses were made by using FSTAT v.2.9.3.2 software.

3. Results and Discussion

After digestion with the *Bs1*I restriction enzyme digestion, the AA (179 bp), AG (179, 93 and 86 bp) and GG (93 and 86 bp) genotypes were observed for the *TLR1* (*+1380*) SNP (Figure 1). The 93 and 86 bp bands were not separated because of their close proximity to each other. However, genotypes could be detected by observing one or two fragments of 179 and around 100 bp. The AG genotype had the highest frequency while the AA genotype frequency had the lowest in all examined breeds. According to the *TLR1* (*+1380*) SNP, EAR breed was in HWE while the AB, HL and native crossbreeds deviated from HWE (Table 2).

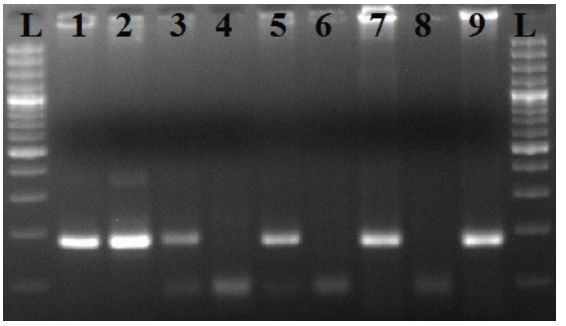

Figure 1- Picture of restriction fragments of *TLR1* (*+1380*) locus after digestion with *Bs1*I restrictiom enzyme Lane L, markers (100 bp); lanes 1, 2, 7 and 9, AA genotypes; lanes 3 and 5, AG genotypes; lanes 4, 6 and 8, GG genotypes

As a result of restriction enzyme digestion, three genotypes for the *TLR1* (*+1596*) polymorphism were detected: 261, 72 and 21 bp for the GG genotype; 333, 261, 72 and 21 bp for the GH genotype; 333 and 21 bp for the HH genotype (Figure 2). The GH genotype had the highest frequency in all breeds followed by HH and GG, respectively, in all examined breeds except for EAR in which HH was found as the most frequent genotype compared to GH. All cattle breeds were in HWE for this SNP (Table 2).

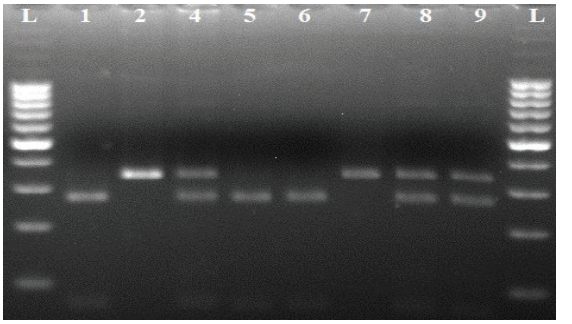

Figure 2- Picture of restriction fragments of *TLR1* (*+1596*) locus after digestion with *Bcl*I restriction enzyme Lane L, markers (100 bp); lanes 1, 5 and 6, GG genotypes; lanes 2 and 7, HH genotypes; lanes 4, 8 and 9, GH genotypes

Following digestion with *BstU*I enzyme for the *TLR4* (*+10*) SNP, in the CC genotype two bands with a length of 246 and 159 bp, and in the CT genotype three bands with a length of 405, 246 and 159 bp were observed (Figure 3). However, the TT genotype was not observed (Figure 3). The genotype CC had the highest frequency in HL and native crossbreeds whereas the monomorphic genotype was found in AB and EAR breeds (Table 2).

The polymorphism in the *SLC11A1* (*+1066*) mutation was identified by digestion of the PCR product with *Pst*I enzyme. In this study, three genotypes were obtained for the *SLC11A1* (*+1066*) SNP (CC, CG and GG) in the examined cattle breeds. After digestion with PstI, one fragment was observed for the CC genotype (348 and 95 bp), two fragments were observed for the GG genotype (293 and 81 bp) and three fragments were found expected

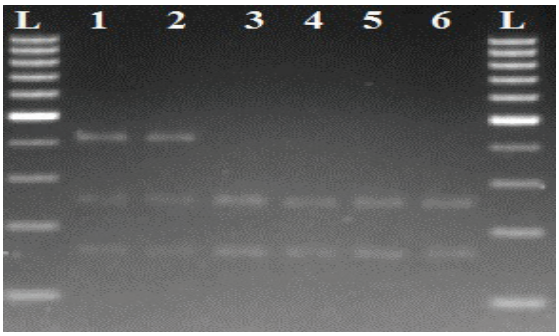

Figure 3- Picture of restriction fragments of *TLR4* (*+10*) locus after digestion with *BstU*I restriction enzyme Lane L, markers (100 bp); lanes 1 and 2, CT genotypes; lanes 3, 4, 5 and 6, CC genotypes

for the AB genotype (374, 293 and 81 bp) (Figure 4). The CC genotype had the highest frequency while the GG genotype frequency was the lowest in all breeds. Turkish native cattle breeds were in HWE while HL and crossbreeds deviated from HWE (Table 2).

The observed heterozygosity varied from 0.284 to 0.568 for *TLR1 +1380*, from 0.401 to 0.502 for *TLR1 +1596*, from 0.000 to 0.018 for *TLR4 +10*, and from to 0.168 to 0.370 for the *SLC11A1 +1066* polymorphism in the five examined cattle populations. The expected heterozygosity, the observed heterozygosity, the allele and genotype

Table 2- Heterozygosity, allele and genotype frequencies of examined loci in cattle breed reared in Turkey

SNP	*Breed*	*n*	*Allele frequency*		*Genotype frequency*			*Heterozygosity*		X^2 *(df= 1)*
			A	*G*	*AA*	*AG*	*GG*	H_E	H_O	
TLR1 (*+1380*)	HL	410	0.270	0.730	0.003	0.539	0.458	0.395	0.537	53.500*
	AB	106	0.190	0.810	0.000	0.387	0.613	0.303	0.371	6.090*
	ABC	124	0.270	0.730	0.008	0.524	0.468	0.395	0.524	13.440*
	EAR	84	0.150	0.850	0.000	0.298	0.702	0.245	0.284	2.570 NS
	EARC	299	0.290	0.710	0.003	0.569	0.428	0.410	0.568	44.880*
			G	H	GG	GH	HH			
TLR1 (*+1596*)	HL	410	0.400	0.600	0.151	0.502	0.347	0.481	0.502	0.730 NS
	AB	106	0.440	0.560	0.217	0.453	0.330	0.493	0.474	0.720 NS
	ABC	124	0.420	0.580	0.210	0.427	0.363	0.490	0.427	1.930 NS
	EAR	84	0.310	0.690	0.095	0.429	0.476	0.429	0.419	0.000 NS
	EARC	299	0.460	0.540	0.258	0.401	0.341	0.497	0.401	10.980*
			C	T	CC	CT	TT			
TLR4 (*+10*)	HL	410	0.998	0.002	0.995	0.005	0.000	0.002	0.004	0.000
	AB	106	1.000	0.000	1.000	0.000	0.000	0.000	0.000	0.000
	ABC	124	0.996	0.002	0.992	0.008	0.000	0.004	0.019	0.000
	EAR	84	1.000	0.000	1.000	0.000	0.000	0.000	0.000	0.000
	EARC	299	0.990	0.010	0.980	0.020	0.000	0.010	0.008	0.030 NS
			C	G	CC	CG	GG			
SLC11A1 (+1066)	HL	410	0.850	0.150	0.763	0.171	0.066	0.254	0.168	45.990*
	AB	106	0.850	0.150	0.755	0.189	0.056	0.262	0.206	7.380*
	ABC	124	0.800	0.200	0.702	0.194	0.104	0.323	0.193	19.720*
	EAR	84	0.760	0.240	0.570	0.370	0.060	0.374	0.370	0.000 NS
	EARC	299	0.780	0.220	0.679	0.201	0.120	0.344	0.200	51.920*

$H_{E,}$ expected heterozygosity; H_O, observed heterozygosity; X^2, Chi-square; HL, Holstein; AB, Anatolian Black; EAR, East Anatolian Red; ABC, Anatolian Black Crossbreed; EARC, East Anatolian Red Crossbreed; df, freedom degree; *, statistical significance 0.05; NS, Non significant

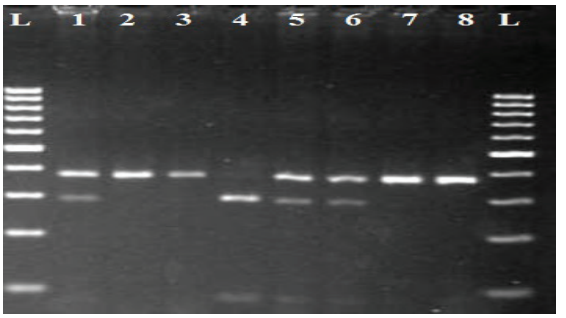

Figure 4- Picture of restriction fragments of *SLC11A1* (+*1066*) locus after digestion with *Pst*I restriction enzyme Lane L, markers (100 bp); lanes 1, 5 and 6, CG genotypes; lanes 2, 3, 7 and 8, CC genotypes; lane 4, GG genotype

frequencies for four polymorphism of the HL, AB, ABC, EAR, EARC populations are shown in Table 2.

Genomic and phenotypic selection for increasing milk yield have resulted in a decrease in the health traits of high yielding cattle breeds such as Holstein (Egger-Danner et al 2015). However, increasing milk production is no longer as important trait as it used to be, compared to health and longevity. In today's dairy cattle breeding, health and longevity are more important for selection than milk production (Egger-Danner et al 2015). The main goal of dairy cattle farming is to increase income by obtaining higher milk and meat yield and to lower the costs caused by lower fertility, high disease and culling rates (Strapáková et al 2016).

It has been shown that TLRs are associated with innate immune response in various livestock species (Le Roex et al 2013; Prakash et al 2014). Therefore, interest in breeding animals which are resistant to major infectious diseases by using TLRs genes has increased tremendously in recent years (Novák 2014).

The *TLR1* (*+1380*) SNP was firstly reported by Prakash et al (2014) in Indian native cattle breeds and their crossbreeds and they observed three genotypes for the *TLR1* (*+1380*) locus. The AG genotype frequency was higher than other genotypes, and AA genotype frequency was found to be lower in Indian native cattle (Prakash et al 2014). In a study conducted in Turkey, five different cattle breeds had been genotyped with this SNP. According to this study, although the genotype GG was the most common genotype in all breeds genotyped, the AA genotype was not found among examined animals. (Çınar et al 2016). Similarly, in this study AA genotype was not found in AB and EAR breeds. The AA genotype was the lowest in Holstein and crossbreeds in our examined population (Table 2). However, in Turkish native cattle breeds (AB and EAR) no AA genotype was observed (Table 2). In the present study the G allele was the most frequently found allele in all the examined breeds and the GG genotype was found to be higher in the investigated Turkish native cattle breeds (Table 2). In Holstein and crossbreeds, the frequencies of the genotype AG were found to be slightly higher compared to the GG genotype (Table 2). The *TLR1* (*+1596*) SNP was firstly reported in the Holstein breed (Sun et al 2012). The authors observed three genotypes (GG, GH and HH) and the frequency of the GH genotype was found to the highest in the Holstein breed (Sun et al 2012). Similarly, we found three genotypes in all examined breeds, and the GH genotype frequency was the highest in Holstein, AB and crossbreeds, whereas the HH genotype was found to be highest in the EAR breed (Table 2). The *TLR4 (+10)* SNP was monomorphic in Turkish native cattle breeds; additionally, this SNP was almost monomorphic in Holsteins and crossbreeds (Table 2). Similarly, Bilgen et al (2016) reported low variation in the AB, EAR and Holstein breeds in terms of *TLR4* SNP with. However, Prakash et al (2014) observed three genotypes (CC, CT and TT) for *TLR4* (*+10*) in Indian native cattle breeds. The *SLC11A1* (*+1066*) SNP was polymorphic in all examined breeds in the present study (Table 2). The frequency of the CC genotype was found to be the highest in the investigated animal populations. Prakash et al (2014) also observed three genotypes; however, in contrast to our study, the CC genotype was the least common in Indian native cattle.

Three of the SNPs genotyped [*TLR1* (*+1380*), *TLR4* (*+10*) and *SLC11A1* (*+1066*)] in this study were taken from a study that investigates *Bos indicus* cattle

derived by Prakash et al (2014). There they reported that samples, they examined were polymorphic and found in HWE. In contrast, in our study, *Bos taurus* origin cattle were used and in terms of these SNPs, deviation from HWE was observed in all breeds except EAR (Table 2). It is thought that this may have been due to the origins of examined cattle breeds (Lin et al 2010). In the light of those findings, it was observed that examined all breeds were in HWEs in terms of *TLR1* (*+1596*) SNP. HWE was not observed just in breed of EARC (Table 2).

This study is the first to investigate the genotype and allele frequencies of *TLR1* (*+1380, +1596*), *TLR4* (*+10*) and *SLC11A1* (*+1066*) SNPs in cattle breeds reared in Turkey. The present study indicated that among the investigated SNPs, heterozygosity was detected as <0.5, which shows low variation for these SNPs (Table 2). Prakash et al (2014) reported an association between the *TLR1* (*+1380*) AA, *TLR4* (*+10*) TT and *SLC11A1* (*+1066*) CC genotypes and resistance to bovine brucellosis. In addition, Sun et al (2012) found an association between the *TLR1* (*+1596*) GG genotype and resistance to bovine tuberculosis. According to the findings of Prakash et al (2014) and Sun et al (2012), we hypothesise that our investigated populations in Turkey had a greater relative risk of incidence to bovine brucellosis and tuberculosis. This is because, the genotypes given by Prakash et al (2014) as indicating susceptibility to bovine brucellosis and tuberculosis were found to have a higher frequency in our examined populations, except for *SLC11A1* (*+1066*).

Prakash et al (2014) reported that the SNPs used in this study might be used in genomic selection against bovine brucellosis and tuberculosis. In conclusion, increasing the frequencies of the *TLR1 (+1380-A*, *+1596-G*) and *TLR4* (*+10-T*) alleles may help to control bovine brucellosis and tuberculosis infections in Turkey. Further studies which study the association between reported SNPs and bovine brucellosis and tuberculosis infections in Turkey are needed.

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