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Investigation of *TLR2 -EcoRV*, *NOD2- BsaH*I ve *IFNγ- Hph*I allele frequency distribution among Turkish Native Cattle Breeds

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ARTICLE INFO	ABSTRACT
Research Article Received : 19/09/2021 Accepted : 25/05/2022 *Keywords:* Cattle Polymorphism Disease-related gene Native breed Marker-assisted selection	Changing climatic conditions and growing population size lead take precautions against potential risks. Researchers who study in the agricultural field, including animal production, focus on finding out variants and breeds resistant to diseases and environmental stress. Detecting gene regions that affect resistance to diseases and environmental stress might increase the performance of the selection in favor of these traits. Due to these reasons in this study, five Turkish native cattle breeds were investigated for SNPs belonging to TLR2, NOD2, and IFNγ (using *EcoRV*, *BsaH*I ve *Hph*I enzymes, respectively) reported to be associated with paratuberculosis previously. In total, 169 animals were analyzed from Native Southern Yellow (NSY= 29), East Anatolian Red (EAR=35), Anatolian Grey (AG=36), South Anatolian Red (SAR=34), and Native Black (NB=35). The genotype and allele frequencies were calculated with the PopGene32 program, and Khi square test was performed to determine whether the populations were in Hardy-Weinberg equilibrium (HWE). While at *TLR2-EcoRV* and NOD2- *BsaH*I loci, two alleles (A and C, C and T, respectively), *IFNγ- Hph*I locus was found to be monomorphic for the G allele. For *TLR2- EcoRV* locus, the C allele was found as predominant except NB breed, and the C allele was predominant for all breeds at *NOD2- BsaH*I locus. However, in all breeds, *NOD2- BsaH*I locus was at HWE, for *TLR2- EcoRV* NSY and SAR breeds showed derivation from HWE.

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Yerli Sığır Irklarında TLR2 *-EcoRV*, *NOD2- BsaH*I ve *IFNγ- Hph*I Allelerinin Frekans Dağılımının İncelenmesi

MAKALE BİLGİSİ	ÖZ
Araştırma Makalesi Geliş : 19/09/2021 Kabul : 25/05/2022 *Anahtar Kelimeler:* Sığır Polimorfizm Hastalık ilişkili gen Yerli ırk Marker destekli seleksiyon	Değişen iklim koşulları ve artan nüfus olası risklere karşı önlem alınması ihtiyacına yol açmıştır. Hayvansal üretimi de içine alan tarımsal alanda çalışan araştırıcılar hastalıklara ve çevresel strese dayanıklı çeşit ve türler bulma ve bunları geliştirme üzerine yoğunlaşmışlardır. Hastalık ve çevresel strese dayanıklılığı etkileyen gen bölgelerinin tespit edilmesi ile bu özellikler bakımından seleksiyon yapılması sağlanabilecektir. Bu nedenle gerçekleştirilen çalışmada beş yerli sığır ırkında genotipik yapı daha önce paratuberculosis ile ilişkili olduğu bildirilmiş *TLR2*, *NOD2* ve *IFNγ* genlerine ait SNP'ler (sırasıyla *EcoRV*, *BsaH*I ve *Hph*I enzimleri kullanılarak) bakımından PCR-RFLP yöntemi ile incelenmiştir. Yerli Güney Sarısı (NSY= 29), Güneydoğu Anadolu Kırmızısı (EAR=35), Boz Irk (AG=36), Güneydoğu Anadolu Kırmızısı (SAR=34), Yerli Kara (NB=35) ırklarından toplam 169 hayvan analiz edilmiştir. Allel ve genotip frekansları PopGene32 programı ile hesaplanmış, populasyonların Hardy-Weinberg dengesinde olup olmadıkları Khi-kare testi ile kontrol edilmiştir. *TLR2- EcoRV* ve *NOD2- BsaH*I lokuslarında iki allel (sırasıyla C ve A, C ve T) ve üç genotip belirlenirken, *IFNγ- Hph*I lokusu G alleli bakımından monomorfik bulunmuştur. *TLR2- EcoRV* lokusunda C alleli NB dışında tüm ırklarda, *NOD2- BsaH*I lokusunda tüm ırklarda C alleli predominanttı. *NOD2- BsaH*I lokusu bakımından tüm ırklar HW dengesinde bulunmuş ancak *TLR2- EcoRV* lokusu bakımından NSY ve SAR ırkları HW'den sapma göstermiştir.

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Introduction

Infection diseases lead to economic losses and animal welfare problems in animal production field. Besides losing animals by death, time and money are loosed when treatment is performed on infected animals. For diagnosis, spending time and money are also inevitable. Using antibiotics is the other serious problem for both animal and consumer health. In addition, future environmental risks such as climatic changes enforce discovering genomic regions related to host genetic resistance to pathogens cause infection. However, that doesn't mean infection diseases will be able to prevent by genetic management; it has already been shown that genetic resistance and susceptibility of the animals vary between breeds and genotypes (Ameni et al., 2007). Difficulties in detecting carriers from phenotype determination of genomic regions related to host resistance may lead to the selection of animals in favor of the resistance for genetic improvement of the breeds. Bovine Tuberculosis (TB), an infectious disease of cattle caused by *Mycobacterium Bovis,* is one of the most common livestock diseases overall in the World. It leads vast amount of economic losses every year. Several genes have been reported to be related to the disease; among these genes, TRL2, NOD2, and IFNγ have been studied in different cattle breeds and seem to be potent candidate genes for infectious disease resistance.

Toll-like receptor 2 (*TRL2*) is one of the thirteen members of Toll-like receptors (TLRs), which belong to pathogen recognition receptors (PPR); as a member of the family, the *TRL2* gene is responsible for coding molecules that have functions in recognition of infection factors and initiation of the immune response (Akira et al., 2001; Saiga et al., 2011; Kawai et al., 2010). Studies were carried out to reveal new gene variants and associations between tuberculosis in different livestock species. Several authors reported numerous variants in cattle breeds, including SNPs and indels, and some relationships with bTB, paratuberculosis, and mastitis. The previous study focused on Turkish native cattle protein models, and amino acid analysis reflected *TLR2* as the most critical gene among the genes investigated (Bilgen et al., 2016).

The gene is also investigated in Turkish native cattle breeds using NGS methods and revealed quite important genetic diversity among native breeds compared with Holstein Friesian cattle used as the control group in the study (Bilgen et al., 2016).

Nucleotide-binding oligomerization domain containing 2 (NOD2) is also known as the caspase recruitment domain member 15 (CARD15) proteins that belong to pattern recognition receptors (PRR) like TLRs. They recognized that some components of the mycobacterial cell wall and activate the nuclear factor kappa-beta (NF-κB) proinflammatory signaling pathway (Abbot et al., 2004; Oh et al.,2005). Polymorphisms in different regions of the gene were found to be related to paratuberculosis in German (Higner, 2009, Küpper et al., 2013) and Spanish Holsteins (Ruiz-Larrañaga et al., 2010), Brahman X Angus cross (Pinedo et al., 2009), as well.

Interferons are a multiple gene group belonging to the inducible cytokine's family. Besides their other tasks, they make cells resistant to viruses; being a member of this family, IFNɣ initiates the host immune response to pathogens. This role makes it a strong candidate for disease-resistant livestock. In sheep, some IFNɣ mutations were found to be associated with paratuberculosis (Schmidt et al., 2002), while some of those were influential in response to the vaccine (Dukkipati et al., 2010).

This study aimed to investigate the frequency distribution of three SNPs located in the genes mentioned above among five native Turkish cattle breeds.

Material Methods

Sampling and DNA Isolation

The study was approved by the Ethics Committee of Uludag University (UUHADYEK) (approval date: UÜHADYEK: 04/09/2016; no:2016-11-05). Total 169 blood samples were collected from native cattle breeds such as Native Southern Yellow (NSY= 29), East Anatolian Red (EAR=35), Anatolian Grey (AG=36), South Anatolian Red (SAR=34), and Native Black (NB=35), where they breed originally. Genomic DNA was extracted with a commercial DNA extraction kit (NucleoSpin Blood, Macherey-Nagel GmbH & Co. KG) according to the instructions provided in the manual. Afterward, quantification and qualification of DNA were controlled using NanoDrop 2000 (Thermo Scientific, USA).

Genotyping and Statistical Analysis

Three SNPs named rs55617172, rs111009394, and rs110853435 located on *TLR2*, *NOD2*, and *IFNγ* genes genotyped according to PCR-RFLP methodology reported by Sadana et al. (2015). To determine polymorphisms on rs55617172, rs111009394, and rs110853435 *EcoRV*, *BsaH*I ve *Hph*I enzymes were used, respectively. Gene and genotype frequencies, expected and observed heterozygosity (He and Ho), and Chi-Square (χ2) values were calculated by POPGENE32 software program (Yeh 2000).

Results and Discussion

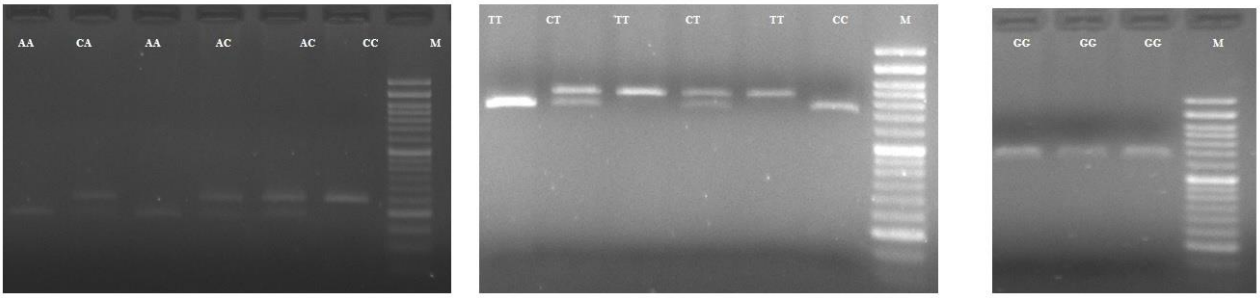
According to PCR-RFLP results, two alleles, A and C, and three genotypes have been found at rs55617172. These alleles created three genotypes named AA, AC, and CC (Figure 1). For *TLR2- EcoRV* among breeds investigated, the C allele was found as predominant, except the NB breed has a frequency of A higher than 50%. Although the distribution of genotypes also varies from breed to breed for the locus, the CC genotype is predominant in the majority of the breeds. The frequency of heterozygotes was the highest in AG and NB breeds (Table 1).

A critical study on diversity in the different TLR genes was carried out to investigate them in Turkish native cattle breeds using new generation sequencing (NGS) methods. The study revealed that *TLR2* had higher diversity than the other TLR genes had (Bilgen et al., 2016). The study showed that *TLR2* also exhibited an important level of haplotype diversity with 36 haplotypes, while this number was found as four in the Holstein population (Bilgen et al., 2016). A recent study on Czech cattle breeds has supported this finding (Novák et al., 2019).

Table 1. Genotypic structure and some basic population parameters for rs55617172 (*TLR2- EcoRV*), rs111009394 (*NOD2- BsaH*I), and rs110853435 (*IFNγ- Hph*I)loci

Locus	n	Allele frequencies (%)		Genotype frequencies (%)			He	Ho	χ2
		Native Southern Yellow (NSY)							
		A	C	AA	AC	CC			
rs55617172	29	31.03	68.97	20.69	20.69	58.62	0.4356	0.2069	7.741**
		C	T	CC	CT	TT			
rs111009394	29	82.76	17.24	68.97	27.59	3.44	0.2904	0.2759	0.032^{ns}
		Native Black (NB)							
		A	C	AA	AC	CC			
rs55617172	35	54.29	45.71	34.29	40	25.71	0.5004	0.4118	1.318^{ns}
		C	T	CC	CT	TT			
rs111009394	35	95.71	4.29	91.43	8.57	0.00	0.0856	0.0882	0.070^{ns}
		Anatolian Grey (AG)							
		A	C	AA	AC	CC			
rs55617172	36	48.61	51.39	25	47.22	27.78	0.5067	0.4722	0.108^{ns}
		C	T	CC	CT	TT			
rs111009394	36	80.56	19.44	61.11	38.89	0.00	0.3177	0.3889	2.098^{ns}
		East Anatolian Red (EAR)							
		A	C	AA	AC	CC			
rs55617172	35	32.86	67.14	14.29	37.14	48.57	0.3714	0.4476	0.876^{ns}
		C	T	CC	CT	TT			
rs111009394	35	82.86	17.14	68.57	28.57	2.86	0.2882	0.2857	0.001^{ns}
		South Anatolian Red (SAR)							
		A	C	AA	AC	CC			
rs55617172	34	33.82	66.18	20.59	26.47	52.94	0.4543	0.2647	5.679*
		C	T	CC	CT	TT			
rs111009394	34	88.23	11.77	76.47	23.53	0.00	0.2107	0.2353	0.664^{ns}

ns=not significant, * P<0.05, **P<0.01



a) *TLR2- EcoRV* b) *NOD2- BsaH*I c) *IFNγ- Hph*I

Figure 1. Electrophoretic illustration of loci analyzed (M: Marker, 50 bp)

Bilgen et al. (2016) reported that AG has the most heterogeneous breed for the *TLR2* gene. Although we analyzed only one SNP in the gene, our findings can confirm the previous study (Bilgen et al., 2016) regarding the highest heterozygote genotype frequency in the AG breed (Table 1). The *TLR2-EcoRV* locus is a nucleic acid substitution that causes an amino acid change from aspartic acid (Asp) to glutamic acid (G) and symbolized as D63E and is supposed to be influential on MAP infection. A recent study carried on in Pakistan to impact the prediction of some SNPs on the gene estimated benign and low impact for the SNP rs55617172 (Batool et al., 2020). Deferent authors have published similar results (Novák et al., 2019; Bjelka and Novák, 2020). Among Indian native cattle breeds, the rs55617172 SNP was found polymorphic with a similar allele and genotype distribution (Sadana et al., 2015). They also found the C allele and CC genotype as predominant.

Interestingly they reveal a positive association between the A allele and resistance to MAP infection, while the infection was more often among animals with heterozygote genotype. The authors explained this contradiction as a possible advantage of heterozygote AC for a different unknown related trait. Sadana et al. (2015) saw the derivation from HWE for this locus as a possible sign of a combined selection without taking it into account by the producer. The study also carried out on Indian native cattle for the same locus with different nomenclature (Bhaladhare et al., 2016) reported a similar distribution of the allele and genotypes and significant differences in distribution between the case and control group for infection without any heterozygote disadvantages. As can be seen from Table. 1, among Turkish native cattle breeds investigated, NSY and SAR breeds showed derivation from HWE for *TLR2- EcoRV* (Table 1). A similar study aimed to analyze the genetic structure of three Turkish native cattle breeds (AG, NB, and EAR) for disease-resistant genes, including *TLR2-EcoRV* locus, also found the C allele as predominant. At the same time, AG was exceptional due to the higher frequency of the A allele (Karayel and Karslı, 2022). In the three cattle breeds, heterozygote genotypes had the highest frequency, while in Holstein Friesian breed AA homozygote genotype was predominant in the same study (Karayel and Karslı, 2022). It was reported that the genotype had a positive effect on resistance to MAP infection (Sadana et al., 2015; Bhaladhare et al., 2016). Our finding consists of the results found by Karayel and Karslı (2022). We also found AC genotype predominant in AG and NB while in EAR breed, the CC genotype had a higher frequency than others (Table 1).

At rs110853435, we have detected only one allele (G) and a single type of genotype (GG) (Figure 1, Table 1). However, the G allele was found in Indian native cattle breeds at a high frequency as 82% (Sadana et al., 2015). On the other hand, Uganda native cattle breeds had a moderate frequency of almost 30%, while the A allele was widespread among these breeds (Okui et al., 2021). Nevertheless, neither Uganda breeds nor Indian breeds had any relationship with MAP infection and the alleles (Sadana et al., 2015; Okui et al., 2021).

At rs111009394 locus, two alleles (C and T) and three genotypes (CC, CT, and TT) were found (Figure1 and Table 1). The C allele and CC genotype were predominant in all breeds without exception (Table 1). The frequency of the T allele is as low as <5%; even there was no found T allele in some breeds (AG, SAR, and NB) (Table 1). *NOD2- BsaH*I locus was at HWE for all breeds. Allele and genotype frequencies found in this study were similar to those previously reported (Sadana et al., 2015; Okui et al., 2021). Although in the previous studies any relation was found between the infection existences and *NOD2- BsaH*I alleles (Sadana et al., 2015; Okui et al., 2021). On the other hand, some previous studies have reported some important relations between further SNPs and MAP-infected animals (Ruiz-Larrañaga et al., 2010; Küpper et al., 2013; Vázquez et al., 2014; Wang et al., 2015). Due to these fragmented results, an increased number of studies should be carried out on the effects of SNPs on this gene. Likewise, Okui et al. (2021) took attention to the possibility of important allele combination effects that need proof.

Conclusion

As well-known that infectious diseases cause so many risks for both animal and human health and welfare. They also lead to a lot of economic losses. Besides pathogens host immune system has an impact on the diseases. In this sense, the genetic structure of immune system-related genes became critical for achieving disease resistance herd by selected carrier animals for diseases resistant related genomic regions. The polymorphism level of the genes in target populations should be figured out to perform appropriate selection programs. According to our findings, it may be said that genetic diversity on rs55617172 would allow further research to investigate possible associations. At the same time, rs110853435 had a low level of polymorphism would be a limitation for association studies. The rs111009394 had no variation when considering previously reported low diversity for the SNP it may be said that the SNP is not appropriate for further association studies.

Additionally, it should be kept in mind that genetic characterization of native livestock populations for diseases and environmental stress-related genes has a key role in possible future environmental risks. Native breeds are also seen as a kind of guarantee for unknown future conditions due to their high adaptive skills. For this reason, the number and depth of studies carried out on native breeds for mentioned genes should be increased.

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Conflict of Interests

The authors decelerate no conflicts of interest.

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