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Association between ovine Toll-like receptor 4 (*TLR4*) gene coding variants and presence of *Eimeria* spp. in naturally infected adult Turkish native sheep

Yalçın Yaman^a , Ramazan Aymaz^a, Murat Keleş^a, Veysel Bay^a, Mehmet Özüoğlu^b, and Bayram Şenlik^b

^aDepartment of Biometry and Genetics, Sheep Breeding and Research Institute, Bandırma, Turkey; ^bVeterinary Faculty, Department of Parasitology, Uludağ University, Bursa, Turkey

ABSTRACT

Coccidiosis caused by *Eimeria* spp. is a protozoan disease prevalent in farm animals, and it is responsible for serious economic losses especially in young animals. It has been popular to breed disease-resistant animals due to the concern about food safety, animal welfare, and public health. Toll-like receptor (*TLR*) gene family plays a key role in the innate immune system participating in host-antigen interaction, therefore, they are candidate genes for breeding disease-resistant animals. In the present study, possible genetic associations between *TLR4* gene coding variants and the presence of *Eimeria* spp. in adult Turkish sheep were investigated. For this purpose, the presence of *Eimeria* spp. in fecal samples from six native Turkish sheep were determined, and approximately 1450 bp region in the 3rd exon of the ovine *TLR4* gene was sequenced. Ten nonsynonymous and four synonymous single nucleotide polymorphisms (SNPs) were detected in the targeted region. Statistical analyses revealed that the SNP at the codon at 356th position encoding Leucine instead of Phenylalanine (F356L) was significantly associated with the presence of *Eimeria* spp. It was found that the individuals carrying at least one Leucine amino acid sequence at this position have 2.3-fold more risk for the presence of *Eimeria* spp.

KEYWORDS

Sheep; Coccidiosis; genetic resistance/susceptibility; *TLR4* gene

Introduction

The innate immune system of vertebrate animals is the first defense system genetically programmed to detect conserved structures on the pathogens cell surface via dendritic cells, macrophages, and neutrophils. These conserved structures are called pathogen-associated molecular patterns (PAMPs) and recognized by host-encoded pattern recognizing receptors (PPRs) of the innate immune system.^{1–3} Toll-like receptors (TLRs) are the first discovered PPRs and their ability to recognize a wide variety of PAMPs was demonstrated in earlier studies. TLRs are membrane-bound proteins and encoded by the Toll-like receptor gene family (*TLR1–TLR13*). While the extracellular leucine-rich repeat (LRR) segment is involved in PAMPs recognition process, the intracellular domain of the TLRs, which have homology with IL-1R and that is called Toll/IL-1R (TIR), initiate a downstream signaling pathway for anti-inflammatory events against pathogens.⁴

Many studies have demonstrated that TLRs have a key role in host-pathogen interaction such as in tuberculosis,⁵ paratuberculosis,⁶ salmonellosis,⁷ and gastrointestinal

nematode infections,⁸ as a part of the innate immunity. Recently, *TLR4* from the *TLR* gene family has been shown to be involved in the host response to some protozoal infections such as leishmaniasis,⁹ trypanosomiasis,¹⁰ malaria¹¹ and chicken coccidiosis,¹² etc.

Eimeria spp. infection (coccidiosis) is a prevalent protozoan disease in farm animals causing serious economic losses. Coccidiosis is a contagious enteric disease that is characterized by diarrhea, weight loss and being lethal in some cases.¹³ There are eleven known *Eimeria* species in sheep and two of them are known to be highly pathogenic.¹⁴ Although young animals are more sensitive to disease, sheep of all ages can be affected. In the intensive breeding systems with high animal density, coccidiosis infections can cause much more serious outcomes.¹⁵ There are few studies on the role of *TLR4* gene in *Eimeria* spp. infection in chicken, however, to the best of our knowledge there is no genetic association study in sheep.

The objectives of the present study were to investigate the possible association between the coding variants in the ovine *TLR4* gene (on chromosome 5,

consisting of three exons, and encoding 841 amino acids) and the existence of the *Eimeria* spp. in naturally infected native Turkish sheep, and evaluate selective breeding opportunities against coccidiosis.

Material and methods

Animals

Six native Turkish sheep breeds; Karacabey merino ($n = 67$), Kivircik ($n = 18$), Imroz (Gokceada) ($n = 38$), Chios (Sakiz) ($n = 18$), Cine capari ($n = 43$) and Karakacan ($n = 41$) were used in the study and they were apparently healthy. Flock compositions were; flock 1 (Karacabey merino), flock 2 (Kivircik and Imroz), flock 3 (Chios), flock 4 (Cine capari), and flock 5 (Karakacan). The ages of examined sheep were ranked from two to eight. Sampling of feces was performed in 2017.

All breeds except Karacabey merino have been conserved under the 'Conservation and Sustainable Use of Animal Genetic Resources' project by the Republic of Turkey Ministry of Agriculture and Forestry. Cine capari and Karakacan breeds are in danger of extinction. The last two Karakacan flocks were scattered due to the death of the owners, and a few hundred Cine capari adults remained. Karacabey merino, Kivircik, Imroz and Chios breeds were from the research flocks of the Sheep Breeding and Research Institute (SRI), and have been using the same pasture, others were from different regions of Turkey.

Parasitological analysis

None of the studied flocks was treated for coccidiosis at least for six months before sampling. Approximately 20-30 g fecal samples were obtained from the rectum of each individual and kept in the deep freezer until the laboratory examination. A modified McMaster method¹⁶ was used to determine *Eimeria* spp. status. Briefly, 3 g fecal sample was homogenized in 42 ml water, filtered, and centrifuged at 300 g. Supernatant was discarded and 15 ml saturated saltwater was added onto the sediment. Finally, McMaster slide chambers were filled with this mixture by Pasteur pipette and under a light microscope at 40× magnification, gastrointestinal nematode eggs were separated and the presence of *Eimeria* spp. oocysts were investigated in a minimum of two ruled grids.

Genetic analysis

A tube of whole blood with EDTA was taken from V. jugularis in aseptic condition. Genomic DNA was

isolated using commercial extraction kits (GeneAll Exgene, GeneAll Biotechnology, Co., Ltd. Korea) following the manufacturer's instructions. Two sets of primers were designed using an online primer blast tool (<https://www.ncbi.nlm.nih.gov/tools/primer-blast>). About 1450 bp of exon 3 of the ovine *TLR4* gene (accession number: NM_001135930.1) was amplified with two separate polymerase chain reactions (PCR). The primers; TLR4_1F: CCGGCTGGTTTGGGAGAAT, TLR4_1R: GCCAGTAAAGATGCCGTGGA, TLR4_2F: TCCACGGCATCTTTACTGGC and TLR4_2R: CTGGGACACCACGACAATCA were used for the PCR. The reaction mixture was composed of 50–100 ng of template DNA, 1.25 U Taq DNA polymerase, 0.2 mM of each dNTP, 10X PCR buffer, 2.5 mM MgCl₂, 10 pmol of each primer and ddH₂O up to 25 μL final volume. PCR conditions were as follows; 94 °C for 4 min; 35 cycles of 94 °C for 1 min, 60 °C for 1 min, and 72 °C for 1 min; and a final extension at 72 °C for 10 min. Sequencing was performed in two separate runs with standard chain termination protocol. Briefly, PCR products subjected to pre-purification using Exo-Sap incubation, cycle sequencing using BigDye™ Terminator v3. 1 Cycle Sequencing Kit, final purification with ethanol precipitation, and capillary electrophoresis on ABI 3500 platform.

Statistical analysis

Chromatograms were visualized using FinchTV v1.4.0 (Geospiza, Inc) and aligned by MEGA v6.0 software.¹⁷ Minor Allele Frequency (MAF) and Hardy-Weinberg (HW) equation calculated by Plink v1.07,¹⁸ haplotype construction was performed using Haploview v4.2.20.¹⁹ Allelic association analysis was performed using Mixed Linear Model (MLM) using GEMMA software²⁰ whereas haplotypic association analysis performed using the same model in Plink 1.07 software. The Association formula was as follows:

$$y(\text{phenotype}) = u (\text{Mean}) \\ + W\alpha (\text{fixed effects; breed} + \text{pasture}) \\ + x\beta + Zu + \varepsilon$$

where 'y' is the vector of phenotypes, 'W' is covariate matrix, 'α' is the vector of associated fixed effects (Breed and pasture), 'x', represents the vector of genotypes (coded as 0/1/2), 'β' is the regression coefficient of the phenotype on the genotypes, 'Z' is the design matrix for the vector 'u' of random polygenic effects, and 'ε' represents the vector of residual errors.

To account for the possible breed effect, a genetic relationship matrix (GRM) was constructed and added to the model, additionally, breed and pasture were also fitted to the MLM as fixed effects. Principal component analysis (PCA) was performed to see whether there is any population stratification according to breeds for the phenotype of interest (presence of *Eimeria* spp.). PC plots were visualized using R software.

Results

All individuals from Chios and Cine capari flocks were negative for *Eimeria* spp. presence. *Eimeria* spp. status of other breeds was ranked from 5.6 to 53.7% (Table 1). Since there was no *Eimeria* spp. positive individual in Chios and Cine capari, these breeds were excluded, and the other four breeds were subjected to the statistical analysis.

Sequence analysis revealed fourteen SNPs including ten nonsynonymous and four synonymous SNPs. The nonsynonymous SNPs were E286G, S294N, K295E, W298R, F356L, D363G, V364A, T366S, D395Y, and A514T (Table 2). Kivircik and Cine capari breeds were polymorphic only at codons 356 and 514, whereas other breeds were polymorphic for all the detected SNPs. All SNPs except the ones at 356th and 514th position have similar frequencies for all breeds. All of the SNPs were in Hardy-Weinberg (HW) equilibrium within breeds.

The haplotype construction using Haploview v4.2 showed that all detected SNPs were linked to each other with D' value ranked from 0.76 to 1.0 and r^2 value ranked from 0.05 to 1.0 (Fig. 1). From a single haplotype block, three possible haplotypes were predicted; A₂₈₆G₂₉₁G₂₉₄A₂₉₅T₂₉₈C₂₉₉T₃₅₆A₃₆₃T₃₆₄C₃₆₆C₃₇₈G₃₉₅G₅₁₄G₆₇₉, A₂₈₆G₂₉₁G₂₉₄A₂₉₅T₂₉₈C₂₉₉C₃₅₆A₃₆₃T₃₆₄C₃₆₆C₃₇₈G₃₉₅A₅₁₄G₆₇₉, and G₂₈₆A₂₉₁A₂₉₄G₂₉₅C₂₉₈T₂₉₉T₃₅₆G₃₆₃C₃₆₄G₃₆₆T₃₇₈T₃₉₅A₅₁₄A₆₇₉. Haplotype frequencies were 0.48, 0.44 and 0.07, respectively.

PC 1 versus PC 2 (first two PC) plots indicated no population stratification among breeds (Fig. 2). Statistical analysis showed that the SNP at 356th codon causing Phenylalanine to Leucine substitution (F356L) was detected to be associated with the presence of *Eimeria* spp. in adult native Turkish sheep (exact p -value = 0.049). A nonsignificant but potential association between *Eimeria* spp. presence and Alanine to Threonine substitution at codon 514 (A514T) was also observed (exact p -value = 0.056). Because all detected SNPs are in strong LD and acts like single SNP, no correction for multiple testing is needed. The sheep carrying at least one 'C' at 1066th nucleotide position (L at codon 356) was found to be 2.3 times more infected with *Eimeria* spp., thus, the presence of at least one Leucine at 356th codon was defined to be a risk factor for coccidiosis. When evaluated in a breed level, the number of 'C' nucleotide carrier *Eimeria* spp. positive sheep were consistently much more than the number of 'T' nucleotide carriers (F at codon 356) within each breed (Table 3).

Table 1. Presence of *Eimeria* spp. oocyst in feces according to Turkish native breeds.

Breeds	Karacabey Merino	Kivircik	Imroz	Chios	Cine capari	Karakacan	Total/Mean
<i>n</i>	67	18	38	18	43	41	225
Presence of <i>Eimeria</i> spp. oocyst in feces (%)	19.4	5.6	28.9			53.7	17.9

Table 2. A summary for detected SNPs and distribution of them according to breeds.

Nucleotide position ^a	Alles	Codon position	Imroz	Kivircik	Karakacan	Karacabey Merino	Cine capari	Chios	Overall MAF	Amino acid substitution
857	A/G	286	0.039		0.098	0.097		0.056	0.073	Glutamic acid /glycine
873	G/A	291	0.039		0.098	0.097		0.056	0.073	Alanine/-
881	G/A	294	0.039		0.098	0.097		0.056	0.073	Serine/asparagine
883	A/G	295	0.039		0.098	0.097		0.056	0.073	Lysine/glutamic acid
892	T/C	298	0.039		0.098	0.097		0.056	0.073	Tryptophan/arginine
897	C/T	299	0.039		0.098	0.097		0.056	0.073	Asparagine/-
1066	T/C	356	0.434	0.278	0.402	0.493	0.326	0.389	0.439	Phenylalanine/leucine
1088	A/G	363	0.039		0.122	0.104		0.056	0.082	Aspartic acid/glycine
1091	T/C	364	0.039		0.098	0.097		0.056	0.073	Valine/alanine
1097	C/G	366	0.039		0.098	0.097		0.056	0.073	Threonine/serine
1132	C/T	378	0.039		0.098	0.097		0.056	0.073	Leucine/-
1183	G/T	395	0.039		0.098	0.097		0.056	0.073	Aspartic acid/tyrosine
1540	G/A	514	0.459	0.278	0.5	0.4	0.314	0.333	0.494	Alanine/threonine
2037	G/A	679	0.027		0.098	0.1		0.028	0.071	Valine/-

^aNucleotide positions of the SNPs are consistent with Oar_v3.1 assembly of the ovine genome sequence. MAF: Minor allele frequency.

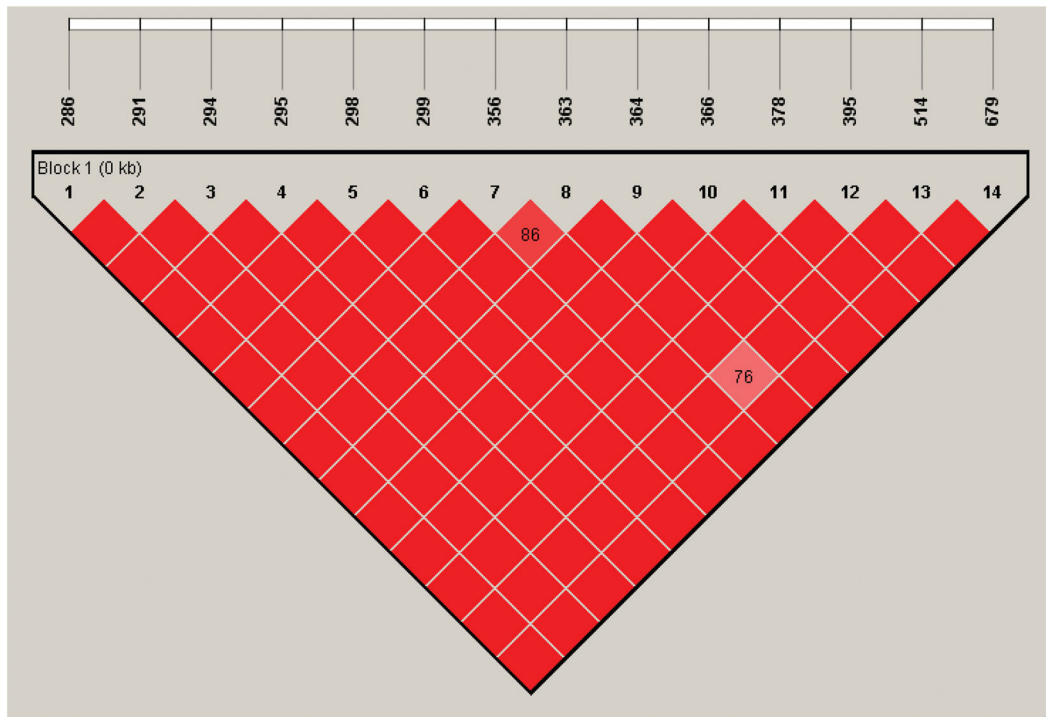


Figure 1. Haplotype block of detected 14 SNPs.

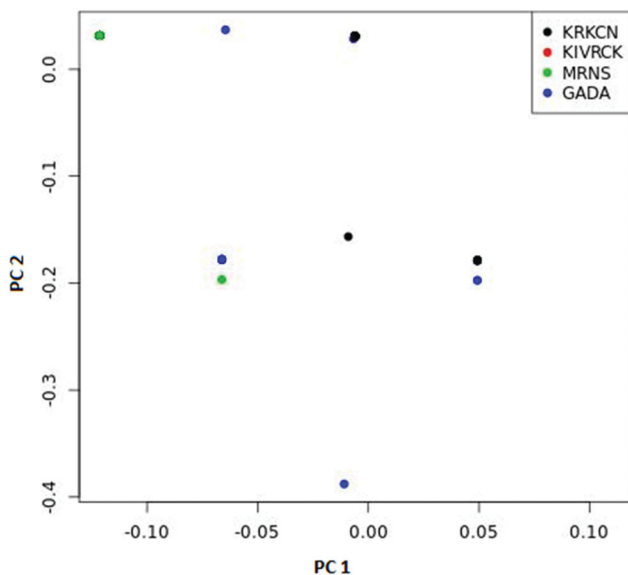


Figure 2. PC 1 versus PC 2 plot graphic for breeds and phenotype of interest. As there is only one *Eimeria* spp. positive in Kivircik breed, R software has ignored to visualize of the 'red dot' for this breed.

Discussion

Coccidiosis caused by *Eimeria* spp. is a highly prevalent protozoan disease of sheep worldwide including Turkey. Although young animals are more susceptible, adult sheep are also infected.^{21–23} It was found that indigenous goats in Zimbabwe are more resistant to coccidiosis compared to Boer breed,²⁴ and similar

observations were reported in chicken breeds²⁵ and rabbits.²⁶ Among studied breeds, Karacabey merino, Kivircik, Imroz, and Chios have been reared at SRI in the same management and environmental condition, and all of them have been using the same pasture. *Eimeria* spp. status of SRI breeds was ranked from 0 to 28.9% which indicates that breed-specific resistance/susceptibility against *Eimeria* spp. could also exist in sheep.

There are studies investigating the association between fecal eggs count (FEC) of gastrointestinal nematodes and host genetics in sheep^{27,28} and cattle.^{29,30} Some candidate genes or QTLs have been proposed to be potential genetic resistance/susceptibility factors against gastrointestinal nematodes. At the molecular basis, genetic resistance to *Eimeria* spp. infection is well studied in chickens^{31–33} compared to the other farm animals. Nevertheless, there is no study investigating the molecular mechanisms involved in coccidiosis resistance/susceptibility in sheep.

In the present study, we identified a Phenylalanine to Leucine substitution at codon 356 (F356L) in the 3rd exon of ovine *TLR4* gene as a risk factor for the presence of *Eimeria* spp. in Turkish adult sheep. Breed-wise distribution of the identified F356L variation for Imroz, Kivircik, Karakacan, Karacabey merino, Cine capari, and Chios breeds were 0.434, 0.278, 0.402, 0.493, 0.326, and 0.389, respectively. Within our detection limit, the presence of at least

Table 3. Distribution of at least one 'C' nucleotide carriers at codon 356 in *Eimeria* spp. positive sheep according to breeds.

Breeds	Total number of <i>Eimeria</i> spp. positive sheep	At least one copy of 'C' carriers in <i>Eimeria</i> spp. positives (%)	No copy of 'C' carriers in <i>Eimeria</i> spp. positives (%)
Karakaçan	22	63.6	36.4
Merino	13	76.9	23.1
Imroz	11	72.7	27.3
Kivircik	1	100.0	0.0
Total	47		

one Phenylalanine at the codon 356 (356 F) provides 2.3 times greater risk for the presence of *Eimeria* spp. with no clinical signs in adult sheep. In humans, the part of the *TLR4* gene from codon 24 to 631 encodes the extracellular LRR rich domain of the TLR4 protein (<https://www.uniprot.org/uniprot>; accession number: A8K1Y8), hence, it could be speculated that F356L amino acid substitution might be playing a role in cell surface antigen recognition process.

In conclusion, we characterized a part of the 3rd exon of the ovine *TLR4* gene and performed a genetic association study to seek any possible association between the *TLR4* coding variants and the presence of *Eimeria* spp. in adult native Turkish sheep. A significant association between the SNP F356L and *Eimeria* spp. status was detected (exact *p*-value = 0.049), and a non-significant but potential association for A514T amino acid substitution was observed (exact *p*-value = 0.056), as well. This is the first report to investigate the potential genetic resistance/susceptibility to *Eimeria* spp. in sheep, and further studies are required to elaborate the role of the *TLR* gene family in the innate immune response to coccidiosis.

Ethical approval

All animal procedures in the study were reviewed and approved by the local ethics committee of Sheep Breeding and Research Institute (Approval Number: 1282412).

Disclosure statement

No potential conflict of interest was reported by the author(s).

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ORCID

Yalçın Yaman  <http://orcid.org/0000-0003-2705-2831>

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