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Association of toll-like receptor 4 (TLR 4) gene exon 3 variants with serostatus of the ovine Johne's disease (paratuberculosis) in Turkish sheep

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Abstract: In the present study, the effect of exon 3 of the toll-like receptor 4 (TLR4) gene on ovine Johne's disease (OJD) serostatus was tested in Turkish sheep breeds. Initially, a retrospective cohort was performed using indirect ELISA on a commingled flock consisting of 3 native and 4 composite breeds (n = 1750). Case-control matched pairs were constructed according to the breed type and age (n = 188). Subsequently, exon 3 of the TLR4 gene was sequenced and coding variants were investigated. A total of 15 single nucleotide polymorphisms (SNPs) were detected and 12 of those SNPs were nonsynonymous. Next, 3 haplotype blocks consisting of these SNPs were constructed. The McNemar's test was conducted on both the SNPs and haplotypes. As a consequence, within the detection limits (power, 0.95; odds ratio > 3.3), no association was detected between the OJD serostatus and TLR4 exon 3 coding variants in Turkish sheep.

Key words: Genetic association, genetic resistance to diseases, selective breeding

1. Introduction

Johne's disease, also known as paratuberculosis, is an incurable, chronic, contagious, and zoonotic disease that causes granulomatous changes in the intestines. The causative agent is *Mycobacterium avium* subsp. *paratuberculosis* (MAP). Numerous species, including farm animals, wild ruminants, birds, and humans have been reported as susceptible to MAP infection [1–3]. Due to its ability to survive in environmental sources such as water, soil, and foods of animal origin, MAP infection can be acquired from these sources by farm animals and humans [4]. Furthermore, evidence exists that MAP might be the causative agent of the human Crohn's disease [2].

There are 2 major strains of MAP in farm animals. Type S (Type 1) causes ovine Johne's disease (OJD). OJD is spread around the globe and causes significant economic losses in the sheep industry, and it is estimated that production losses caused by OJD range from 1% to 7.8% [5]. Eradication strategies for OJD mostly rely on serological screening, culling infected sheep, and preventing infected sheep from joining the flock. However, such control measures have been found to be ineffective [6,7]. Inactivated vaccines against OJD are available in some countries. Nevertheless, there is evidence that vaccination does not guarantee that MAP will not spread from infected sheep [8].

Several efforts have indicated the host genetic factor underlying resistance/susceptibility to OJD. Some genes, such as major histocompatibility complex [9], toll-like receptor (TLR) gene family (TLR 1, 2, and 4) [10], and PCP4, CD109 [11] have been proposed as candidate genes. Among these, TLR genes play a critical role in innate immunity by recognizing pathogen-associated molecular patterns (PAMPs) produced by the pathogens, such as bacteria, viruses, and fungi, etc., and by initiating inflammatory events [12,13]. There have been several reports that TLR genes are involved in natural genetic resistance against various diseases [14–17] by recognizing molecular patterns, such as lipopolysaccharides, envelope glycoproteins, glycoinositolphospholipids, or mannans of the pathogens [18]. Accordingly, Bhide et al. [10] reported that various mutations in the TLR1, TLR2, and TLR4 genes may alter immune response against MAP infection in sheep.

The ovar TLR4 gene is located on chromosome 2 and consists of 3 exons and 3 introns. The first 2 exons are relatively short in length, and harbor 93 and 167 nucleotides, respectively. The third exon is the longest coding region (2266bp) and contains dozens of polymorphisms (https://www.ensembl.org), and the biological functions of most of them have not been identified until now.

In the present study, the aim was to investigate the possible effects of TLR4 variants on OJD serostatus. For

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this purpose, a serosurvey was conducted to determine OJD serostatus in a commingled flock consisting of 3 native and 4 composite breeds. A case-control matched pair study was designed using surveyed ewes. Next, the ovine TLR4 exon 3 region of the matched pairs was sequenced, and the association between the TLR4 variants and OJD serostatus was investigated.

2. Material and methods

2.1. Animals

Animal material used in the study was from the Sheep Breeding and Research Institute (SRI). Since the 1940s, a variety of crossbreeding studies have been employed to improve the meat yield of native sheep at the SRI. Moreover, some native sheep breeds have been conserved ex situ in vivo at the SRI. Serological tests were performed on 1750 ewes from 3 native (Karacabey merinosu, Kıvırcık, and İmroz) and 4 composite (Bandırma, Ramlıç, Black head mutton merino crossbred-SBA, and Hampshire crossbred) sheep. All of the sampled ewes were unvaccinated against OJD and had been reared in the same environmental and management conditions for 20 years. Therefore, the exposure intensity to the causative agent of OJD was estimated to be similar. For the genetic and serological tests, 2 tubes of peripheral whole blood, with and without EDTA, were collected under aseptic conditions from the ewes. To ensure adequate seroconversion for OJD, only sheep aged 2 years or over were sampled.

2.2. Serological tests

Serums were separated from fresh whole blood using a centrifuge, and kept in a deep freezer until laboratory examination. In order to determine OJD serostatus in the sheep, an indirect enzyme-linked immunosorbent assay (ELISA) was performed using commercial test kits (Idexx Laboratories Inc., Westbrook, USA). ELISA plates were read at 450 nm using a spectrophotometer (BioTek Instruments, Winooski, USA). Samples with suspected results were tested a second time, and samples with suspected results in the second test were excluded from the analysis.

2.3. Genetic analysis

Commercial kits were used for DNA extraction from whole blood. A partial amplification containing 1450 to 2266 bp per region of TLR4 exon 3 was performed via 2 separate polymerase chain reactions (PCRs). Primers used for the PCR were as follows: TLR4_1F: CCGGCTGGTTTTGGGAGAAT, TLR4_1R: GCCAGTAAAGATGCCGTGGA, TLR4_2F: TCCACGGCATCTTTACTGGC, and TLR4_2R: CTGGGACACCACGACAATCA. Primers were designed using the online tool of the National Center for Biotechnology Information (https://www.ncbi.nlm.nih.gov/tools/primer-blast). Sequence analysis was performed by following the standard protocol (prepurification, chain termination reaction, final purification, and capillary electrophoresis) using a BigDye Terminator v.3. 1 cycle sequencing kit (Thermo Fisher Scientific Inc., USA) on an Applied Biosystems ABI 3500 genetic analyzer (Thermo Fisher).

2.4. Statistical analysis

Calculation of the minor allele frequencies (MAFs), Hardy-Weinberg equation, and linkage disequilibrium, and construction of the haplotypes was performed using Haploview, v.4.2 [19]. Genetic association analyses of case-control studies require standardization of the duration and intensity of MAP exposure. Another major consideration causing false discovery is population stratification (or population structure), which results in systematic divergence in allele or haplotype frequencies between families or breeds. Therefore, in order to reduce the effect of population stratification and standardize MAP exposure conditions, 94 matched pairs (case and control, n = 188) were constructed according to their breed type, sex, and age. Briefly, a seropositive ewe was matched randomly with a seronegative ewe from the same age group and breed type. McNemar's analysis [20] for correlated proportions was performed for the recessive and dominant models. Both alleles and haplotypes were analyzed separately. Finally, power analysis was conducted using G*Power v.3.1 [21] software to evaluate the detection limits of the statistical analysis.

3. Results

The indirect ELISA test revealed that 114 of the 1750 tested ewes were seropositive for OJD. All of the native and composite breeds were infected with OJD and seroprevalances according to the breeds ranged from 2.3% to 8.2% (mean 6.5%). In the sequence analysis, a total of 15 single nucleotide polymorphisms (SNPs) were detected and 12 of those were nonsynonymous. The amino acid substitution and codon positions of the nonsynonymous SNPs were G230R, E286G, S294N, K295E, W298R, K344N, R351H, F356L, D363G, V364A, T366S, and D395Y, respectively, whereas the codon positions of the synonymous SNPs were A291, N299, and L378. All of the SNPs were in the Hardy-Weinberg equation within breeds/crossbreeds, except for G230R. A summary of the detected SNPs are presented in Table 1. A total of 3 haplotype blocks consisting of these SNPs were constructed (Figure 1). All of the SNPs were in strong linkage disequilibrium (LD) within the haplotype blocks and the r-square values ranged from 0.113 to 1.0. The haplotype block consisting of SNPs E286G, A291, S294N, K295E, W298R, N299 (LD r-square, 0.81 to 1.0) was selected as a candidate for association analysis and a specific haplotype $G_{286}A_{291}A_{294}G_{295}C_{298}T_{299}$ was tested against 3 common haplotypes.

Table 1. Summary of the detected SNPs.

Nucleotide position[1]	Codon position	MAF[2]	Alleles	Amino acid substitution
688	230	0.245	G/A	Glycine/arginine
857	286	0.122	A/G	Glutamic acid /glycine
873	291	0.122	G/A	Alanine/-
881	294	0.122	G/A	serine/asparagine
883	295	0.12	A/G	Lysine/glutamic acid
892	298	0.122	T/C	Tryptophan/arginine
897	299	0.101	C/T	Asparagine/-
1032	344	0.021	G/C	lysine/asparagine
1052	351	0.024	G/A	Arginine/histidine
1066	356	0.447	T/C	Phenylalanine/leucine
1088	363	0.128	A/G	Aspartic acid/glycine
1091	364	0.122	T/C	Valine/alanine
1097	366	0.12	C/G	Threonine/serine
1132	378	0.122	C/T	Leucine/-
1183	395	0.122	G/T	Aspartic acid /tyrosine

[1]Nucleotide positions of the SNPs are consistent with Oar_v3.1 assembly of the ovine genome sequence. [2]Minor allele frequency

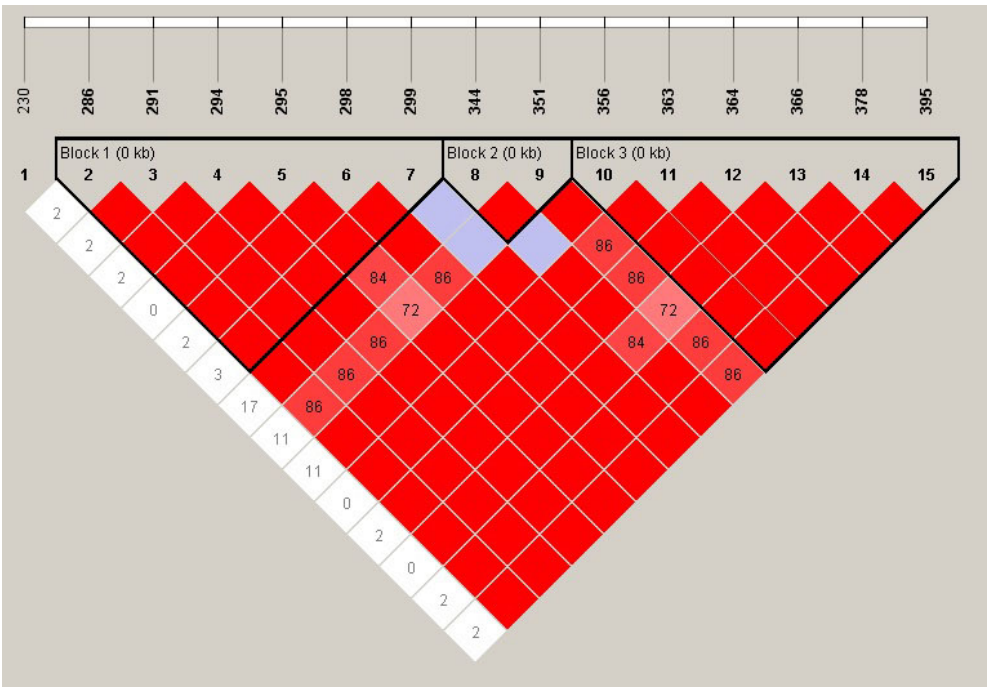

Figure 1. Constructed haplotype blocks on ovine *TLR4* exon 3.

Of the 114 seropositive ewes, 94 were available for matched pairs. A breed and age composition of the ewes included in the analysis is given in Table 2. Matched pairs were coded as (0:1), (1:0), (0:0), and (1:1) ,which meant that one member of the pair was a carrier of the risk allele/haplotype but the other member was not (0:1

Table 2. Distribution of the case-control matched pairs according to age and breed.

Age (year)	Karacabey Merinosu	Kıvırcık	İmroz	Bandırma	Ramlıç	SBA	Hamph. Cross	Total
2	7	2	1	2	-	-	-	12
3	17	1	-	-	-	1	-	19
4	14	2	-	4	-	-	1	21
5	17	4	-	2	-	-	-	23
6	5	2	-	1	1	-	-	9
7	5	-	1	2	1	-	-	9
8	-	-	-	1	-	-	-	1
Total	65	11	2	12	2	1	1	94

or 1:0), or both members were carriers of the risk allele/haplotype (1:1) or none of the members were not (0:0). Typically, discordant pairs (0:1 and 1:0) were informative in the McNemar's correlated proportion analysis and the number of discordant pairs determined the strength of the statistics. In the current study, the number of discordant pairs ranged from 32 to 36 for the SNP alleles and 30 for the candidate haplotype.

Power analysis was performed using the actual percentage of discordant pairs and size of the matched pairs (n = 94) and P = 0.05. Within the statistical limits (power of detection of 0.95; odds ratio threshold of >3.3) no association was detected between the TLR4 exon 3 variants and OJD serostatus in Turkish native and composite sheep.

4. Discussion

Distribution of OJD in Turkish flocks/breeds has been reported with various seroprevalence ranging from 8.3% to 55.8% [22,23]. There are no reports regarding the production losses caused by OJD in Turkey; however, as an example, it has been estimated that the cost of OJD is more than USD10,000 for each flock in Australia [5]. There is no treatment for OJD, and vaccination is very limited in Turkey and many parts of the world. Selective breeding for genetically less OJD-susceptible sheep might be an opportunity for disease control. Molecular markers can be a powerful tool to achieve this goal, and the TLR gene family might comprise candidate genes due to PAMP recognition ability, to investigate the genetic background of OJD resistance/susceptibility.

In the present study, the scientific question was whether any nucleotide change in TLR4 coding variants affects OJD susceptibility in Turkish sheep. For this purpose, the serostatus of 1750 ewes were determined using indirect ELISA. Among the SRI breeds, statistically significant differences regarding OJD seroprevalence was not observed.

In order to determine a TLR4 variant profile, it was necessary to genotype the polymorphic region (exon 3) of the TLR4 and perform association analysis over case-control matched pairs. A total of 12 missense SNPs were identified in this study, all of which were reported previously by Zhou et al. (2007) [24], except for G230R.

Recent studies have revealed that the TLR gene family could play a crucial role in innate immunity. It was reported that the variations at 5′ upstream of the bovine TLR4 gene altered the expression profile and could affect immune response [25]. Additionally, it has been demonstrated that expression of the TLR gene family in the course of OJD significantly differed when compared to healthy sheep [26].

Bhide et al. [10] reported 15 missense SNPs in ovine TLR4 and found that the SNPs caused P356L amino acid substitution associated with the occurrence of OJD at an odds ratio of 1.64 in Tsigai sheep. The P356L variant was also detected in this study at a frequency of 0.447. The McNemar's test, according to either the dominant or recessive model for this variant, did not indicate any significant association (P > 0.05).

In conclusion, the ELISA results indicated that OJD is prevalent in Turkey. Sequence analysis revealed that the exon 3 region of the ovine TLR4 gene was highly polymorphic in the sampled breeds; however, no association was detected between the TLR4 exon 3 variants and OJD serostatus in Turkish sheep. Considered together, it could be suggested that other genes belonging to the TLR family could still be candidate genes for genetic resistance/susceptibility to OJD; however, further research is required to explore their effects on the immunity of the TLR gene family.

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Conflict of Interest
The author declares that there is no conflict of interest.

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