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Evaluation of *CD109*, *PCP4* and *SEMA3D* genes for their association with Ovine Johne's disease in Turkish sheep

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ABSTRACT

Johne's disease is a chronic, contagious, zoonotic disease that affects numerous species including livestock and sometimes humans. The disease is globally distributed in sheep populations and caused by *Mycobacterium avium* Subsp. *paratuberculosis* (MAP). A previous genome-wide association study identified single nucleotide polymorphism (SNP) markers associated with OJD serostatus in *CD109*, *PCP4,* and *SEMA3D* genes. Our aim was to evaluate the same markers for association with OJD seroprevalence in Turkish sheep in a retrospective matched case-control study. The serological status for OJD in 1801 sheep was determined for four native and four composite breeds from three research flocks. One hundred eleven matched case-control pairs were constructed according to breed type and age from 1750 comingled ewes reared in the same environment. A Single Nucleotide Primer Extension (SNuPE) assay was designed to genotype *PCP4*-Intron 1, *PCP4*-3'UTR, *SEMA3D*, *CD109*-intron 2 and *CD109*-intron 8 markers and a McNemar's test was performed on the matched pairs. An association with these five markers was not detected with the OJD serostatus in Turkish sheep (power of detection, 0.95; odds ratio >3; McNemar's $p < .05$). Thus, a wider search may be needed to identify any major underlying genetic risk factors for OJD in Turkish sheep.

KEYWORDS
Paratuberculosis; Ovine Johne's disease; genetic association; disease susceptibility

Introduction

Paratuberculosis, also called as Johne's disease, is a chronic disease of the small intestine characterized with chronic granulomatous enteritis and progressive wasting. Paratuberculosis is contagious, zoonotic and affecting numerous species, including farm animals and humans.[1,2] The causative agent of paratuberculosis is *Mycobacterium avium* Subsp. *paratuberculosis* (MAP). MAP is facultative, intracellular bacteria and obligated to host macrophages to reproduce itself.[3–5] A wide range of host organisms has been reported for MAP infection, especially livestock, wildlife ruminants and birds. It is also reported that many insects and protozoa species can carry and transmit the MAP bacteria to susceptible hosts (reviewed by Ref.[6]) In addition to cross-species transmission from infected animals, MAP can survive in surface water, soil and manure for a long time owing to its spore-like state, thus, infection can be acquired from such environmental sources.[7] Furthermore, MAP cannot be inactivated by pasteurization procedure, therefore, raw and/or pasteurized milk and processed milk products (i.e., Baby formula, cheese, etc.) are potential risk factors, as well.[8,9] Although its role is not well defined, MAP has been suggested as a potential causative agent of Crohn's disease, a chronic inflammatory bowel disease in humans.[1] There are also concerns that MAP might be associated with other diseases such as sarcoidosis, Blau syndrome, Type 1 diabetes, Hashimoto's thyroiditis and multiple sclerosis. Additionally, MAP is thought to trigger autoimmune diabetes and autoimmune thyroiditis (reviewed by Ref.[10]) Regardless of controversy surrounding the evidence, the suggestion of MAP as an agent associated with human disease has significant implications for international marketing of affected livestock and increases its negative impact on livestock producers.

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Johne's disease is globally distributed in cattle and sheep. Two major strains been identified: are Type I (Type S), first cultured from sheep; and Type II (Type C), first cultured from cattle. Although the course of the infection is similar in cattle and sheep, chronic diarrhea is not a general feature in sheep in contrast to cattle.[11] It is estimated that production loss due to Ovine Johne's Disease (OJD) in sheep is 4–5% in the UK, 1–4% in New Zealand and up to 7.8% in Australia.[12] In sheep industry, classical control measures such as culling and restocking with uninfected sheep were found to be inadequate and/or not cost-effective for the eradication of the OJD.[13,14] Commercial vaccines against OJD are available in some countries. Vaccination with killed MAP 316 F strain reportedly reduced the mortality and achieved some success in reducing the prevalence of MAP shedding. However, it has been reported that multibacillary lesions found at necropsy of vaccinated sheep was shown to be shedding enormous numbers of MAP.[15]

Previous serological surveys and experimental infection studies indicated that there were breed-level differences in susceptibility to OJD. For example, fine-wool breeds (Merino and Corriedale) found to have a higher seroprevalence than others (Romney and composite breeds).[2] In an experimental infection study, clinical disease was diagnosed at 42% in Merino and 36% in Merino × Suffolk crossbred, whereas 12 and 11% in Border Leicester and Poll Dorset, respectively.[16] Despite the lack of information about economic loss, OJD is also prevalent in Turkish sheep breeds and there are some serosurvey studies reporting OJD seroprevalence from 8.3[17] to 55.8%.[18]

Eradication of OJD from sheep with classical measures is time-consuming, expensive and not completely effective. Although some success has been achieved by vaccination for reducing the disease prevalence, shedding of MAP from vaccinated animals would probably continue. Consequently, vaccinated and unvaccinated sheep affected with OJD will remain as source of infection. If available, selective breeding for resistance to OJD could be an effective method when used as a complementary part of the classical eradication strategy. A number of candidate genes associated with individual resistance or susceptibility to OJD have been reported in sheep. *NRAMP (SLC11A1)* and MHC locus,[19] *TLR1*, *TLR2*, and *TLR4*[20] genes were found to be associated with OJD prevalence. More recently, results of a Genome-Wide Association (GWA) study showed single nucleotide polymorphisms (SNPs) within *SEMA3D*, *CD109*, *PCP4*, *PRDM2,* and *ITFG2* genes were associated with OJD.[21] Two missense variants in the coding region of the *PCP4,* and one in the *CD109* genes were identified with the latter showing a strong linkage disequilibrium (LD) with an OJD-associated SNP on the Illumina OvineSNP50 BeadChip.[22]

In the present study, we used a retrospective cohort to determine OJD serostatus in four crossbreds and four native Turkish sheep breeds. Our aim was to use a matched case-control design to evaluate the potential association of previously reported SNP's[21,22] within the *CD109*, *PCP4* and *SEMA3D* genes with the serostatus of OJD in Turkish sheep.

Materials and methods

Animals

A total 1801 sheep from three different flocks from Sheep Research and Breeding Institute (SRI) (Bandirma/Balıkesir/Turkey) were included the study. Two tubes of whole blood samples with and without EDTA were collected for genetic and serological analysis from April to September in 2017. To allow sufficient time from seroconversion, only 2-year-old and older sheep were included the study. Four native Turkish breeds; Karacabey Merino, Kivircik, Imroz (Gokceada), Chios (Sakiz) and four composite breeds; Ramlic (Rambouillet × Native Daglic), Hampshire crosses (Hampshire Down × Karacabey Merino), SBA (Black Head Merino × Karacabey Merino) and Bandirma (Black Head Merino × Kivircik) were sampled. The composite breeds except Ramlic have been developed in SRI to improve the meat yield. The native breeds; Kivircik, Imroz and Chios are conserved as genetic resources under the national 'Conservation and Sustainable Use of Animal Genetic Resources' project conducted by the Turkish government. These breeds are well adapted to their arid/semi-arid extreme environments and are sustaining their productivity throughout hundreds of years at Aegean and Marmara regions of Turkey. Among them, the Imroz breed is native to Imroz island at the Marmara Sea and reared almost only on this island.

Serological tests

After centrifugation of fresh whole blood, serum samples were separated and kept at −20 °C until laboratory tests. All serum samples were subjected to indirect Enzyme-Linked Immunosorbent Assay (ELISA) using commercial test kits (Idexx Laboratories, Inc. Westbrook, USA). ELISA test was

Table 1. Seropositive and seronegative matched pairs according to breed and age.

Age (yr)	Karacabey Merino	Imroz	Kivircik	Bandirma	Ramlic	Hamph. Cross	SBA	Total
8	–	–	–	1	–	–	–	1
7	6	1	–	3	1	–	–	11
6	5	–	2	1	1	–	–	9
5	18	–	5	2	–	–	–	25
4	15	–	2	8	–	2	–	27
3	18	–	1	–	–	–	2	21
2	10	1	2	3	–	1	–	17
Total	72	2	12	18	2	3	2	111
	65%	1.8%	11%	16%	1.8%	2.7%	1.8%	100%

performed following the manufacturer's instructions. ELISA plates were read at 450 nm wavelength using an ELISA plate reader (BioTek Instruments, Winooski, USA). Samples with ambiguous ELISA results were tested once again and the samples that still had ambiguous results were excluded from the study.

Genetic analysis

One-hundred eleven matched pairs (case-control) were identified among the retrospective cohorts and genetic analyses were performed on these sheep. Genomic DNA was extracted from whole blood with EDTA using commercial kits (General Biotechnology Co., Ltd., Seoul, Korea). DNA quality was confirmed via spectrophotometer (Optizen-NanoQ, Mecasys Co., Ltd, Daejeon, Korea). A multiplex Single Nucleotide Primer Extension (SNuPE) assay was designed for genotyping of the samples. Briefly, a multiplex Polymerase Chain Reaction (PCR) was used to amplify the targeted regions of DNA. The extension primers without fluorescent dye were designed in a specific length for each SNP and was expected to bind to preceding nucleotide of the targeted SNP. The 'T' tails were added to ensure that the extension primers were of different lengths for each SNP. Amplification and extension primers and rs numbers of the analyzed SNPs are provided in Supporting Information Table S1. The SNuPE reaction was performed using SNaPshot™ Multiplex Kit (Thermo Fisher Scientific Inc., USA) and capillary electrophoresis was used for fragment analysis protocol on Applied Biosystems 3500 genetic analyzer. To validate SNuPE genotyping assay, approximately 5–10% of the samples for each SNP were directly sequenced and the results were in 100% concordance.

Statistical analysis

Allele frequencies and Hardy–Weinberg equation was calculated using PLINK 1.07 software.[23] Commingled ewe flocks in SRI have been reared under the same environmental and management conditions for more than 20 years, thus, exposure intensity and exposure durations to the MAP are assumed to be similar for all breeds. In an attempt to standardize the environment and exposure conditions, association analysis was performed only in flock 1 and flock 2 of SRI. The exception is Chios sheep (flock 3; $n = 51$) that are reared as a separate flock under different management conditions in SRI, therefore, this breed was not included in the association analysis.

Only ewes were included in the association study since exposure conditions of the rams can be different. Matched pairs of seropositive and seronegative ewes were constructed according to the age to minimize the differences between each animal's duration of exposure to MAP. Breed type was also added to pairing criteria to control the breed effect in the association study. As a result, a seropositive ewe was matched with a seronegative ewe with the same age, and breed type. In this way, 111 case-control matched pairs were constructed from a total of 1750 commingled SRI ewes. Age of sampled sheep was ranging from two to eight years (Table 1). A McNemar's test for correlated proportions was conducted[24] on the matched pairs for both recessive and dominant models.

Results

Distribution of OJD seroprevalence in breeds/flocks

The overall seroprevalence of 1801 Turkish native and crossbred sheep tested was 7.2% (Table 2). All native and composite breeds tested were infected with OJD. Among the native and composite breeds with the highest seroprevalence was the SRI Chios flock '3' (29.4%), which was reared in an isolated pen for 20 years and without direct contact with the other flocks, whereas the lowest prevalence detected in Imroz breed (2.3%). Chios breed is considered to be high milk-yielding breeds. Taken together, the results suggest that OJD infection is widespread in Turkish

sheep breeds, with the prevalence in some flocks exceeding 25% prevalence.

Association of the CD109, PCP4 and SEMA3D variants with OJD serostatus in Turkish sheep

Genetic analyses were performed with 111 matched case-control pairs ewes from SRI flocks which are K. Merino, Bandirma, Kivircik, Imroz, Hamph. Cross, Ramlic and SBA. Genotypes were obtained for all 222 samples (111 pairs) for *CD109* and *PCP4* SNP's, and all but 2 pairs for *SEMA3D*. The genotype frequencies revealed a number of SNPs are monomorphic in multiple breeds (Table 3). All but one SNP (*CD109-intron 8)* met Hardy–Weinberg expectations within breeds. The *CD109-intron 8* SNP deviated in Karacabey Merino sheep from HW with a p value of .003. Informative matched case-controls pairs were assigned for each SNP where only one member of the pair had the genetic risk factor (i.e., the case had 2 copies of the risk allele but the control did not). Typically, a minimum of 25 informative pairs are needed for a statistically significant analysis. When the genetic risk factor was defined as exactly one copy of the assigned allele (i.e., dominant), there were more than 25 informative pairs for each candidate SNP except *PCP4* 3'UTR marker. When the genetic risk factor was defined as one or two copies (i.e., additive or dominant) of the assigned SNP allele, there were more than 25 informative pairs for each candidate SNP except *PCP4-3'UTR*. When the genetic risk factor was defined as having two copies (i.e., recessive) of the SNP allele, there were more than 25 informative pairs for all but *PCP4-3'UTR and SEMA3D* (Table 4).

Statistical power analysis was conducted by G*Power 3.1[26] using the real proportion of the discordant pairs with 0.95 of detection of power, OD $\geq$3, and $p<.05$ criteria for each analyzed SNP. None of the candidate SNPs reached significance in McNemar's test (p>.05) except *CD109* Int.8 in the 'one allele is protective' model (p value, .02). However, the OR did not meet our significance threshold of 3.0 for a risk factor, or 0.333 for a protective SNP (OR, 0.6). Consequently, these SNPs in the candidate genes (*CD109, PCP4 and SEMA3D*) were not detected to be associated with OJD serostatus in Turkish sheep.

Discussion

In this study, we performed a serological survey for OJD in four crossbred populations and four native sheep breeds from the SRI research flock in south Marmara region of Turkey. Due to chronic, subclinic nature of infection, most of seropositive sheep were asymptomatic. The native breeds; Kivircik, Imroz and Chios, have currently been conserved under a project of Republic of Turkey Ministry of Agriculture and Forestry entitled, 'Conservation and Sustainable Use of Animal Genetic Resources' and it is forbidden by regulation to cross them with other breeds. Unfortunately, OJD was prevalent among Turkish native, composite and dairy breeds ranging from 2 to 29%.

Animal husbandry management types (extensive or intensive), stock density, pasture usage, climate, and so forth are important factors affecting exposure

Table 2. OJD seroprevalence of sampled native and composite Turkish sheep.

Breeds	Flock ID	Locations	OJD serostatus n	Neg	Pos	Prevalance (%)
K. Merino	1	Bandırma/ SRI[a]	901	827	74	8.2
Kivircik	2	Bandırma/ SRI	208	196	12	5.8
Imroz	2	Bandırma/ SRI	86	84	2	2.3
Bandirma	2	Bandırma/ SRI	366	348	18	4.9
Hampshire cross	2	Bandırma/ SRI	102	98	4	3.9
Ramlic	2	Bandırma/ SRI	51	49	2	3.9
SBA	2	Bandırma/ SRI	36	34	2	5.6
Chios	3	Bandırma/ SRI	51	36	15	29.4
		Total	1801	1672	129	7.2

[a]SRI: Sheep Research and Breeding Institute-Balikesir/Bandirma.

Table 3. Minor allele frequencies (MAF) for candidate SNPs in Turkish sheep.

Overal minor allele frequencies				MAF according to breeds						
SNP	Allele1[a]	Allele2	MAF	K. Merino	Bandirma	Kivircik	Imroz	Hamph. Cross.	Ramlic	SBA
PCP4-Int. 1[b]	T	G	0.09	0.13	0.01	–[c]	0.38	–	–	–
PCP4-3'UTR[b]	A	G	0.04	0.05	–	–	–	–	0.13	–
SEMA3D	A	G	0.09	0.08	0.16	–	–	0.25	–	–
CD109-Int.2	G	A	0.47	0.45	0.40	0.21	0.38	0.33	0.50	0.25
CD109-Int.8	A	C	0.46	0.41	0.14	0.15	0.25	0.33	0.50	0.25

[a]Minor allele.
[b]Antisense strand.
[c]The minor allele was not detected.

Table 4. McNemar's test for OJD association with SNP risk and/or protective alleles.

McNemars pair status and test statistics[a]	McNemar's quadrants and equations[b]	SNP ID (risk or protective allele)				
		PCP4 Int.1 (T)	PCP4 3UTR (A)	SEMA 3D (A)	CD109 Int.2 (G)	CD109 Int.8 (A)
One copy of risk and/or protective allele[c]						
1,1	'a'	5	0	3	32	11
1,0	'b'	13	6	13	29	21
0,1	'c'	13	8	15	27	37
0,0	'd'	80	97	77	23	42
Total pairs	$a+b+c+d$	111	111	108	111	111
Discrdant pairs	$b+c$	26	14	28	56	58
OR	b/c	1.0	0.8	0.9	1.1	0.6
CI95 lower	–	0.5	0.3	0.4	0.6	0.3
CI95 upper	–	2.2	2.2	1.8	1.8	1.0
McNemar's $\chi 2$	$(\|b-c\|-1)^2/(b+c)$	0.0	0.1	0.0	0.0	3.9
p Value	–	.31	.37	.28	.20	.02
One or two copies of risk and/or protective allele						
1,1	'a'	5	0	4	63	51
1,0	'b'	15	7	14	16	15
0,1	'c'	14	8	15	23	24
0,0	'd'	77	96	76	9	21
Total pairs	$a+b+c+d$	111	111	109	111	111
Discordant pairs	$b+c$	29	15	29	39	39
OR	b/c	1.1	0.9	0.9	0.7	0.6
CI95 lower	–[d]	0.5	0.3	0.5	0.4	0.3
CI95 upper	–	2.2	2.4	1.9	1.3	1.2
McNemar's $\chi 2$[e]	$(\|b-c\|-1)^2/(b+c)$	0.0	0.0	0.0	0.9	1.6
p Value	–	.29	.39	.29	.14	.09
Two copies of risk and/or protective allele						
1,1	'a'	0	0	2	9	10
1,0	'b'	2	1	9	23	24
0,1	'c'	1	0	4	16	17
0,0	'd'	108	110	94	63	60
Total pairs	$a+b+c+d$	111	111	109	111	111
Discordant pairs	$b+c$	3	1	13	39	41
OR	b/c	2.0	na[f]	2.3	1.4	1.4
CI95 lower	–	0.2	na	0.7	0.8	0.8
CI95 upper	–	22.1	na	7.3	2.7	2.6
McNemar's $\chi 2$	$(\|b-c\|-1)^2/(b+c)$	0.0	0.0	1.2	0.9	0.9
p Value	–	.75	1.00	.17	.14	.14

[a]Each member of a case-control pair is assigned a value of '1' or '0' depending on whether the risk factor is present (1) or absent (0) and falls into one of four possible categories pair status categories: '1,1'; '1,0'; '0,1'; or '0,0'. All subsequent test statistics are calculated from these values.
[b]These are quadrants from the McNemar's contingency table for classifying pairs.
[c]The allele is defined as 'risk' if McNemar's '*b*' quadrant value is greater than or equal to the '*c*' quadrant value. The allele is defined as 'protective' if McNemar's '*b*' quadrant value is less than the '*c*' quadrant value.
[d]These were calculated from more complex equations. The CI95 intervals were calculated as $e^{(\ln(OR) - 1.96\sqrt{(1/b + 1/c)}}$ to $e^{(\ln(OR) + 1.96\sqrt{(1/b + 1/c)}}$. The two-sided exact *p* value was calculated as twice the binomial probability distribution function with '*b*' successes, '*b* + *c*' trials and the probability of success set to .5.
[e]Continuity corrected.[25]
[f]Not applicable, too few discordant pairs.

intensity and consequently affecting OJD prevalence. The SRI ewes from flock 1 and 2 were best suited to the matched, case-control association analysis due to rearing at the same environmental and exposure conditions for years.[22] have investigated the coding region variations of *PCP4* and *CD109* which might be in LD with associated anonymous markers. They found a missense mutation in exon 3 and four mutations in the 3′UTR of the *PCP4*, nevertheless, those mutations had low MAF and they were not in LD with the anonymous markers. On the other hand, a strong LD (0.79) between *CD109* intron 2 marker and a missense mutation in the exonic region of *CD109* was detected in Sarda and some other indigenous Italian sheep breeds. Allele distribution of the missense mutation in Sarda sheep was reported to be 0.25 for case and 0.54

for control (n=42). In the present study, one of the anonymous markers around the *CD109* gene reached significant p value with assumption that 'one allele is protective' (p, .02; OR, 0.6). The OR detection limits of our study design with a 95% confidence interval were from 3 to 30 (Table 4), thus, OR value of such association was lower than our detection limits.

In conclusion, our serosurvey indicates that OJD is prevalent in Turkish sheep and breed-based sero-prevalence has been detected up to 29.4%. Within our detection limits, results of case/control association analysis did not reveal any association between OJD serostatus and *PCP4-Intron 1, PCP4-3'UTR, SEMA3D, CD109-intron 2* and *CD109-intron 8* anonymous SNP markers. Further research including more candidate genes and/or chip-based GWAS are needed to understand underlying host genetic factors regarding immune response to OJD in Turkish sheep.

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).

Data availability

Analyzed genotype data of the study are available from the corresponding author on reasonable request.

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