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Article

Allelic polymorphism of Ovar-DRB1 exon2 gene and parasite resistance in two dairy sheep breeds.

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ABSTRACT.

The Ovar-DRB1 gene locus is one of the most polymorphic genes of the Major Histocompatibility Complex (Ovar-MHC) and holds a functional role to antigen presentation. The aim of this study was: a) to describe the Ovar-DRB1 locus variability in two dairy Greek sheep breeds and b) to investigate associations between this variability with resistance to gastrointestinal parasitosis. Blood and faecal samples were collected from 231 and 201 animals of Arta and Kalarrytiko breeds, respectively. The identification of alleles was performed using the sequence–base method. Faecal egg counting (FEC) of the gastrointestinal parasites and measures of blood plasma pepsinogen levels were performed in order to evaluate parasitological parameters. From this study in the overall examined animals, thirty-nine Ovar-DRB1 alleles were identified, among them, ten new alleles, reported for the first time in the literature. In Arta breed a total of twenty-four alleles were found. Among the detected alleles, ten were breed specific and five were new. Regarding the Kalarrytiko breed, twenty-nine alleles were found, fifteen of them were unique and nine were new. The studied breeds differed in their allelic profile, with only 12 common from the total of 134 different recorded genotypes. A higher number of animals with high parasitic load and high plasma pepsinogen values were found in Kalarrytiko. Associations between Ovar-DRB1 alleles with FEC values were found with certain heterozygous genotypes to present significantly reduced FEC values. The large number of detected alleles with low frequencies and the fact that the majority of animals were heterozygous, make hard to find strong associations.

KEYWORDS

Dairy sheep; Greek breeds; Ovar-DRB1 exon2 alleles; genetic polymorphism; parasites

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1 Introduction

The Major Histocompatibility Complex of sheep (Ovar-MHC) is a gene complex associated mainly with immunological properties. Ovar-DRB1 encodes the b1 structural core of an Ovar-MHC class II molecule, forming a large part of the binding region of the antigenic peptide (PBR) of the molecule (Marsh and Bodmer, 1993). Compared to all other genes of the Ovar-MHC, the Ovar-DRB1 gene locus exhibits the largest variability (Andersson and Rask, 1988). This variability is localized at specific positions of exon2 (270 bp).

Ovar-MHC class II molecules present antigenic peptides derived from extracellular proteins and parasites on the T-cell receptor (TCR) for CD4+T-lymphocytes (Germain and Margulies, 1993; Fremont *et al.*, 1996) which are expressed at higher concentrations on the surface of macrophages and B-lymphocytes (Outteridge *et al.*, 1996). Since the polymorphic pattern of PBR amino acids determines its conformation and electric charge, strong hydrophobic bonds are created only with specific antigenic peptides (Thorsby, 1999). Thus, different Ovar-DRB1 alleles favour the binding of different antigenic peptides in the PBR Ovar-MHC class II molecules (Rudensky *et al.*, 1991). Consequently, the polymorphism of Ovar-DRB1 exon2 locus is associated with the variability in resistance or susceptibility to diseases of a sheep population.

Many studies have shown that there is a correlation between Ovar-DRB1 alleles and parasite resistance, particularly to nematodes (Outteridge *et al.*, 1996; Paterson *et al.*, 1998; Charon *et al.*, 2002; Sayers *et al.*, 2005; Hassan *et al.*, 2011). This is also the case against other infectious agents, such as pathogenic bacteria and viruses (Larruskain *et al.*, 2010). Gastrointestinal nematodes are the most important source of infestation in sheep worldwide. Anthelminthic treatment with pharmaceutical compounds is a relatively expensive method of combating the infestation, which leads in many cases to the development of anthelminthic resistance (Roos, 1997). Additionally, a growing concern related to residues of pharmaceutical compounds which pass down into the food chain, have driven a large number of farmers to adopt breeding programs which rely less on drug delivery. For these reasons, the selection of sheep breeds and individuals resistant to parasitic infestation is promoted in many countries.

Recently, Ovar-MHC loci have attracted the research interest as candidate DNA markers since several genes from all classes of Ovar-MHC are involved in resistance or susceptibility to various sheep nematodes (Dukkipati *et al.*, 2006). Generally, all research studies have mainly been carried out in breeds of meat and wool type. Since genetic differences exist between the indigenous Greek and foreign sheep breeds (Rogdakis, 2002), there was great interest in exploring the Ovar-DRB1 genetic variability and the possible identification of new alleles in the Greek breeds. For the purposes of this study, two dairy Greek breeds with different management and productive characteristics were selected in order to describe the variability of Ovar-DRB1 gene locus: i) Arta, a breed with high milk yield and litter size and ii) Kalarrytiko, a rare breed, with low milk yield but well adapted to harsh environmental conditions. Considering that associations have been documented between certain Ovar-DRB1 alleles and parasitological parameters, there was further interest to investigate, whether similar associations among Ovar-DRB1 alleles and parasites could be found in order to use this knowledge as a criterion for selection programs to create animals resistant to gastrointestinal parasites, avoiding the administration of antiparasitic drugs.

  ISSN: 2283-3927

2 Materials and Methods

2.1 Animals and sample collection

A total of 432 blood samples were taken randomly from two dairy Greek sheep breeds. The breeds reared at the region of Western Greece, in flocks with a mean size of approximately five hundred animals. The flock of Arta breed (sample size=231) was kept at 414 m altitude (Vassiliki, Etoloakarnania Prefecture), grazing in private pastures throughout the year under a semi-extensive farming system. The Arta ewes were fed supplementary concentrates according to the requirements of their productive stage, milked mechanically twice per day and produced on average 276.08 ± 9.73 L of milk within a whole lactation. The flock of Kalarrytiko (sample size= 201) was kept at 838 m altitude (Small Gotista, Ioannina Prefecture) grazing in communal pastures under an extensive traditional farming system. During the winter sufficient herbage is not available and supplementary feed was given from October to March. Kalarrytiko ewes were milked by hand and produced on average 80.45 ± 3.02 L of milk.

Blood samples were taken from each animal by jugular vein puncture, in order to use whole blood for genomic DNA isolation and plasma for evaluation of pepsinogen levels. Faecal samples were obtained from the rectum of each animal to estimate parasitic burdens.

2.2 Genotyping of Ovar-DRB1 exon2 region

2.2.1 Identification of alleles by direct sequencing

A touchdown PCR, using as substrate the genomic DNA from each animal, was used for the amplification of a 340 bp long DNA fragment containing the exon 2 of the Ovar-DRB1 locus. The total reaction volume was 25 μl and contained 110 -120 ng genomic DNA, 10 pmol/μl of the DRB1-330 primers (forward primer): ATTAGCCTCYCCCCAGGAGKC and DRB1-329 (reverse primer): CACCCCCGCGCTCACCTCGCCGC (Ballingall and Tassi, 2010) and 0.5 U Phusion® High-Fidelity DNA polymerase (NEB). Initially, the reaction occurred in two hybridization cycles at the following temperatures: 67°C, 66°C, 65°C, 64°C and continued with 30 cycles at 63°C. Touchdown PCR products were directly sequenced at Laboratories of CEMIA SA (Larissa), with the Genetic Analyzer ABI3730xl (Applied Biosystems). The sequencing cycle kit BigDye Terminator v3.1 (Applied Biosystems) was used for the reaction, according to manufacturer's guidelines. Two suitable software packages: "GeneStudioTMProfessional sequence Analysis Software" and "Nucleotide BLAST Basic Local Alignment Search Tool" were used for the identification of alleles after sequencing.

2.2.2 Identification of novel alleles by cloning and sequencing

Regarding the identification of candidate alleles (new alleles), cloning and sequencing was performed in three successive steps: At the first step, for those animals presenting new alleles, a simple PCR was performed with a different reverse primer compared to the respective primer used in the Touchdown PCR. A 364 bp long DNA fragment containing the exon2 locus of the Ovar-DRB1 gene was amplified. The total reaction volume was 50 μl containing : a) 85-95

  ISSN: 2283-3927

ng genomic DNA, b) 10 pmol/μl of the forward primer DRB1-330, c) 10 pmol/μl of the reverse primer DRB1-313: ACACACTGCTCCACACTGG (Ballingall and Tassi, 2010) and d) 3,5 U Taq DNA polymerase (Fermentas). The reaction was performed at +62°C, for 37 cycles. At the second step, due to the fact that the PCR product consisted of the allelic pair of genotypes, cloning was performed, using a culture of susceptible bacterial cells, in order to initially segregate them, and subsequently to find the candidate allele considered as new. Using the ligation reaction, the isolated 364 bp long DNA fragment containing the exon2, was inserted to the plasmid pBluescript SK (2961 bp) and used for the transformation of susceptible *Escherichia coli* cells of the JM109 strain. The localization of cloned alleles of exon2 within a bacterial culture was performed with a Colony PCR. The products of Colony PCR were incubated with 10U of the restriction enzyme RsaI (NEB) in order to ascertain, from the digestion patterns in which of the received transformed bacterial colonies, clones of the two alleles were contained. The third and final stage of the process was the selection of the colonies containing the new alleles, from which the recombinant plasmids were isolated, in order to sequence the inserts and to finally confirm the sequence of the alleged new allele as new indeed. The isolation of the plasmids was performed using standard reagent kitsNucleoSpin® Plasmid (Macherey-Nagel).

2.3 Parasitological analyses

2.3.1 Count of faecal parasite reproductive elements

Faecal egg counts (EPG) were conducted for strongyle-type eggs cumulatively and for *Nematodirus* sp., *Strongyloides* sp., *Trichuris* sp., and *Moniezia* sp. eggs separately (Thienpont *et al.*, 1986). Faecal counts of coccidian oocysts were also enumerated.

2.3.2 Estimation of pepsinogen levels in blood plasma

Plasma pepsinogen in each animal was determined by the method of Hirschowitz (1955) as modified by Korot'ko & Islyamova (1963). Pepsinogen is the inactive precursor of pepsin, one of the main proteolytic enzymes of digestive system. Pepsin acts as peptidase, catalysing the hydrolysis of peptide bonds from the aromatic amino acids (tyrosine, phenylalanine, tryptophan). In the presence of parasites larvae the intense inflammation of the gastric mucosa of the abomasum, affects the function of the gastric glands and HCl production is decreased. As a consequence pepsinogen could not be converted to pepsin and inserts in blood circulation, increasing the plasma pepsinogen levels. In brief, blood plasma samples were collected from the supernatant after centrifugation (3000 rpm, 30 min) and six standard solutions of tyrosine were prepared. A staining solution of Folin-ciocalteau was added in each sample. The optical density of blank, standard tyrosine solutions and samples were measured at 560 nm in a spectrophotometer. The concentration of samples in pepsinogen was given as values of mI.U. tyrosine.

ISSN: 2283-3927

2.4 Statistical analysis

The genotypic frequencies (observed and expected estimations) and the allelic frequencies were estimated with GENEPOP (Rousset, 2008). The above software was also used to measure the observed and expected heterozygosity and homozygosity. The appropriate sampling function z was used to test for significant differences between ratios of heterozygosity. The null hypothesis was H_o: $p_A - p_K = 0$ while the alternative one was H_1: $p_A \neq p_K$. Sample sizes were large enough (>100) and the null hypothesis was rejected when $z > z_{0,05}$ or $z < -z_{0,05}$.

Associations between genotypes and parasite categories were performed at first, with a χ^2 contingency Pearson test but then the simulated Monte Carlo method was preferred because in many cases the number of observations was fewer than 5. The EPG values or FEC (Faecal Egg Counts) and pepsinogen concentration values (mI.U. tyrosine) declined from normality. Their analysis was done using the Bartlett's test for checking homogeneity of the sample and the non parametric test of Kruskal-Wallis.

Associations between Ovar-DRB1 genotypes and FEC values of parasites were investigated using non parametric tests (Mann-Whitney or Kruskal-Wallis depending from the number of levels for the specific alleles) considering the fixed effect of genotype for the specific i allele with three levels (first level: animals without the specific allele, second level: animals with one copy of the i allele and third level: animals with two copies of the i allele). The same analysis was performed in order to reveal associations between Ovar-DRB1 genotypes and pepsinogen levels. The above analyses were carried out a) on the total examined animals for alleles which are common between the studied breeds and b) on a within breed basis for alleles which are unique for each breed.

3 Results

3.1 Genotyping analysis and polymorphism of Ovar-DRB1 exon2 gene

3.1.1 Genotypic frequencies

One hundred and thirty four genotypes were recorded in the total of 432 examined animals. Eighty-one (81) and sixty-five (65) different genotypes were found among the 231 and 201 individuals from Arta and Kalarrytiko breeds, respectively. Twelve common genotypes were found between breeds which are presented in supplementary Table A. Among the common genotypes only three were homozygous for alleles: DRB1*0402, DRB1*0901 and DRB1*2101. Homozygous genotypes among the examined animals were quite few, only eleven (11) and ten (10) in Arta and Kalarrytiko, respectively.

A different set of genotypes with relative high frequency was observed in each breed. The most common genotypes and in descending order for Arta breed were: DRB1*0101-DRB1*0102, DRB1*0102-DRB1*1001, DRB1*0102-DRB1*2101, DRB1*0101-DRB1*0901, DRB1*0102-DRB1*0901, DRB1*0102-DRB1*0402. On the other hand in Kalarrytiko breed the genotypes with higher frequency and in descending order were: DRB1*0402-DRB1*0702, DRB1*0702-DRB1*0702, DRB1*0102-DRB1*0402, DRB1*0702-DRB1*1602. Among the recorded genotypes the

  ISSN: 2283-3927

percentage of new alleles was 23 % and 54 % for Arta and Kalarrytiko breed, respectively. A percentage of 52 % and 49 % of Arta and Kalarrytiko genotypes, respectively, were represented by only one individual. Half of the genotypes in both breeds had extremely low genotypic frequencies below 0.01. Taking into account the above results it is evident that each breed is characterised by its specific genotypic profile.

3.1.2 Allelic frequencies

A set of thirty-nine (39) alleles for both breeds were identified. The allelic frequencies for each breed and for the total number of animals are presented in Table 1. Among the alleles found, a total of 29 are already known and registered in the Database for Ovar-MHC (IPD-MHC Database EMBL-EBI), while the remaining ten (10) are reported for the first time.

Table 1 illustrates the number of identified alleles in Arta (24 alleles) and in Kalarrytiko (29 alleles) flocks, the number of shared and the number of unique alleles for each breed. The unique alleles for Arta were ten out of 24 (DRB1*0104, DRB1*0302, DRB1*0308, DRB1*1001, DRB1*1003, DRB1*1501, DRB1*1901, DRB1*2001, DRB1*2201 and DRB1*12). Also, in Arta breed a subset of five alleles from the total of 24 (DRB1*1606, DRB1*1607, DRB1*1608, DRB1*2502 and DRB1*12) represented new alleles, reported first time in literature. The following alleles: DRB1*0102, DRB1* 0101 and DRB1*0402, were detected in descending order with frequencies of 0.232, 0.139 and 0.095, respectively. For the above alleles their cumulative incidence was equal to 0.465. In Kalarrytiko a set of fifteen alleles, from the total of 29 detected, were breed specific (DRB1*0403, DRB1*0702, DRB1*0803, DRB1*1102, DRB1*1301, DRB1*1302, DRB1*1502, DRB1*160202, DRB1*1604, DRB1*1605, DRB1*2003, DRB1*2401, DRB1*11, DRB1*26 and DRB1*30). A subset of 9 alleles, from the total of 29 detected, were new (DRB1*160202, DRB1*1606, DRB1*1607, DRB1*1608, DRB1*2003, DRB1*2502, DRB1*11, DRB1*26 and DRB1*30). In Kalarrytiko the most frequent alleles were: DRB1*0702, DRB1*0402 and DRB1*1606 with frequencies 0.211, 0.192 and 0.107 respectively, and cumulative incidence equal to 0.510.

3.1.3 Frequencies of homozygosity and heterozygosity - Deviations from Hardy-Weinberg equilibrium

The percentage of 84.49% in the overall examined animals (n=432) were classified as heterozygotes and the remaining 15.51% as homozygotes. Estimates of observed homozygosity and heterozygosity (mean ± S.E.M.) within the Arta breed was p_A=0.125±0.022 and q_A=0.875±0.022, respectively. For the Kalarrytiko breed the relative estimates were p_K=0.189±0.028 and q_K=0.811±0.028. The above estimates were not differentiated between breeds (z = -1.82>-1.96, P>0.05).

The unbiased estimation of expected heterozygosity according to Nei (1978) was H_{expA}=0.890 and H_{expK}=0.886 in Arta and Kalarrytiko breed, respectively and the above estimations were not different between them (P>0.05). Observed and expected estimations of heterozygosity differed significantly in Kalarrytiko breed reflecting deviation from Hardy-Weinberg equilibrium. More specifically, the observed heterozygote animals (q_K=0.811) were fewer than expected (H_{expK}= 0.886) (P<0.05).

ISSN: 2283-3927

Table 1. Frequencies of Ovar-DRB1 alleles in Arta breed (n=231), in Kalarrytiko breed (n=201) and in the total examined animals (n=432).

Alleles	Allelic frequency		
	Arta breed	Kalarrytiko breed	Total
DRB1*0101	0.1385	0.0174	0.0822
DRB1*0102	0.2316	0.0647	0.1528
DRB1*0104	0.0043	-	0.0023
DRB1*0302	0.0022	-	0.0012
DRB1*0304	0.0498	0.0050	0.0289
DRB1*0308	0.0043	-	0.0023
DRB1*0311	0.0152	0.0522	0.0324
DRB1*0402	0.0952	0.1915	0.1400
DRB1*0403	-	0.0025	0.0012
DRB1*0601	0.0498	0.0025	0.0278
DRB1*0702	-	0.2114	0.0984
DRB1*0801	0.0238	0.0025	0.0127
DRB1*0803	-	0.0199	0.0104
DRB1*0901	0.0844	0.0398	0.0637
DRB1*1001	0.0887	-	0.0475
DRB1*1003	0.0130	-	0.0069
DRB1*1102	-	0.0025	0.0012
DRB1*1301	-	0.0025	0.0012
DRB1*1302	-	0.0025	0.0012
DRB1*1501	0.0065	-	0.0035
DRB1*1601	0.0130	0.0025	0.0081
DRB1*1602	-	0.0697	0.0336
DRB1*160202#	-	0.0373	0.0035
DRB1*1604	-	0.0075	0.0046
DRB1*1605	-	0.0100	0.0069
DRB1*1606#	0.0022	0.1070	0.0081
DRB1*1607#	0.0022	0.0697	0.0012
DRB1*1608#	0.0152	0.0075	0.0463
DRB1*1901	0.0130	-	0.0023
DRB1*2001	0.0152	-	0.0023
DRB1*2003#	-	0.0025	0.0035
DRB1*2101	0.0801	0.0075	0.0174
DRB1*2201	0.0043	-	0.0509
DRB1*2401	-	0.0050	0.0336
DRB1*2502#	0.0043	0.0025	0.0116
DRB1*11#	-	0.0473	0.0220
DRB1*12#	0.0433	-	0.0231
DRB1*26#	-	0.0025	0.0012
DRB1*30#	-	0.0050	0.0023

#: New alleles

-: No allele found

ISSN: 2283-3927

3.2 Parasitological analysis

3.2.1 Faecal examination

Faecal samples were collected from 157 Kalarrytiko and 224 Arta sheep to obtain the faecal egg count of gastrointestinal parasites. In the total examined animals (n=381), a percentage of 33% (126/381) was positively infected. Specifically, eggs from various parasites were detected in 55.41% (87/157) and 17.41% (39/224) animals from Kalarrytiko and Arta breed, respectively (Table 2). A greater variety of parasite reproductive elements was found in Kalarrytiko: eggs of strongyles, *Nematodirus* spp, *Strongyloides* spp., *Trichuris* spp., *Moniezia* spp. and oocytes of coccidia while in Arta breed were mainly detected, eggs of strongyles, *Strongyloides* spp., *Capillaria* spp. and coccidia. The above findings on FEC and pepsinogen values between the studied breeds could be considered as the possible effect of the farming system combined with the different pasture locations used by the flocks.

Table 2. Prevalence (%) and faecal egg counts (FEC) (epg) of gastrointestinal parasites and pepsinogen levels (m I.U. tyrosine) in sheep of the Arta and Kalarrytiko breeds.

Infection	Arta (N=224)				Kalarrytiko (N=157)			
	n	Prevalence %	FEC Median (Qr)	Pepsinogen Median (Qr)	n	Prevalence %	FEC Median (Qr)	Pepsinogen Median (Qr)
Helminths*	25	11.16	100 (200)	319.26 (356.46)	52	33.12	500 (1625)	228 (374)
Strongyles	13	5.80	100 (200)	405.20 (255.06)	46	29.30	500 (1175)	223 (345.5)
Strongyloides spp.	10	4.46	200 (200)	220.96 (517.99)	7	4.46	200 (1000)	138 (371)
Nematodirus spp.	0	-	-	-	1	0.64	N/A	N/A
Trichuris spp.	1	0.46	N/A	N/A	1	0.64	N/A	N/A
Capillaria spp.	1	0.45	N/A	N/A	0	-	-	-
Moniezia spp.	0	-	-	-	7	4.46	300 (400)	212 (374)
Coccidia	17	7.59	200 (100)	317.13 (442.95)	57	36.31	200 (350)	381.5 (571.5)

*: Infection with all the investigated parasites except coccidian
-: No parasite found
N/A: Not applicable

3.2.2 Pepsinogen levels

The percentage of animals with pathological values of tyrosine was higher in Kalarrytiko breed (Table 2). More specifically, 56.1% for Kalarrytiko (88 animals from the total of 157 examined) and 43.6% for Arta (89 animals from the total of 224 examined) exhibited >375 m

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I.U. tyrosine. However, in Kalarrytiko breed animals with values up to 500 m I.U. tyrosine showed a very sharp increase in high levels up to 6.315 m I.U, while for Arta breed the relative values showed a lower increase up to the value of 2.243 m I.U. tyrosine. The Spearman correlation coefficient between FEC and pepsinogen values was nearly zero.

3.3 Associations between genotypes, parasitic load, and pepsinogen levels

Investigation of possible associations between genotypes and FEC values was carried out for the genotypes of common alleles with relatively high frequency in the total examined animals or within each breed. We found few significant associations between certain genotypes and FEC values which are presented in Table 3. Animals heterozygous for alleles DRB1*0101, DRB1*0102 and DRB*0311 had significantly lower FEC values in comparison with the remaining genotypes. In the case of DRB1*0311 allele, within the group "heterozygotes", animals heterozygous for alleles DRB1*0302, DRB1*0304 and DRB1*0308, were also included, because the above three alleles have a tight nucleotide resemblance with DRB1*0311 (they belong to the same allelic family). On the opposite direction the heterozygous animals for DRB1*0402 allele had higher FEC values in comparison with the homozygotes and the remaining genotypes. Aditionally, animals carrying one or two copies of DRB1*0702 allele (with the highest frequency in Kalarrytiko breed), had the higher median FEC numbers in comparison with the remaining genotypes.

Table 3. Associations between Ovar-DRB1 genotypes and faecal egg counts (FEC) (epg).

Allele	Ovar-DRB1 genotypes	n	FEC Median (Interquartile Range)	P
DRB1*0102	No copy of the allele	265	0 (200)	*
	Heterozygote	106	0 (25)	
	Homozygote	7	0 (200)	
DRB1*0101	No copy of the allele	316	0 (200)	**
	Heterozygote	57	0 (0)	
	Homozygote	5	0	
DRB1*0402	No copy of the allele	281	0 (100)	***
	Heterozygote	85	0 (350)	
	Homozygote	12	50 (175)	
DRB1*0311	No copy of the allele	331	0 (200)	**
	Heterozygote	47	0 (0)	
	Homozygote	0	N/A	
DRB1*0702	No copy of the allele	319	0 (100)	***
	Heterozygote	48	100 (300)	
	Homozygote	10	100 (425)	

*: Significant association p<0.05
**: Signifant association p<0.01
***: Significant association p<0.001

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Pepsinogen levels did not differ significantly among the various parasites in the studied breeds (Table 2). Furthermore, no significant association was found between genotypes and pepsinogen levels with one exception for the homozygote animals for DRB1*0402 allele in the total population which tend to have lower medians values of pepsinogen levels in comparison with the remaining genotypes ($P<0,07$).

4 Discussion

The studied breeds differ considerably in their morphological and productive traits. Arta is a breed of high milk production, kept in lowland of Western Greece in large flocks. Kalarrytiko is a rare breed of mountain type of medium productivity with a small population size in comparison to Arta breed (Rogdakis, 2002) and this fact could explain the significant deficit of heterozygote individuals found in Kalarrytiko. However, the fact that a greater number of alleles was found in Kalarrytiko as well as, more new alleles were identified in the above breed, confirms the necessity to preserve the small and rare breeds because in their genetic pool, a considerable amount of genetic variation, can be found. The large number of alleles and the high levels of heterozygosity found in both breeds are expected findings, due to the fact that Ovar-DRB1 gene locus is the most polymorphic among the Ovar-MHC loci (Andersson and Rask, 1988).

Generally, relying on the number of detected alleles, the results of the present study could be compared with relative studies using the sequencing method for the detection of Ovar-DRB1 genetic variability. Schwaiger *et al.* (1993, 1994) identified 47 alleles in flocks from Perendale, Coopworth, Landrace, Merino and Texel sheep. Paterson (1998) identified 5 alleles in a population of Soay feral sheep, while Gruszczynska (1999) found 36 and 28 alleles in two flocks of Merino sheep. From the same scientific team (Gruszczynska *et al.*, 2005) 36 and 30 alleles were found respectively, in two flocks of Polish Heath sheep. Additionally, Charon *et al.* (2002) in another flock of Polish Heath breed found 20 Ovar-DRB1 alleles, while Konnai *et al.* (2003) detected 28 alleles in Suffolk, 14 in Cheviot and 9 in Corriedale sheep breeds. Sayers *et al.* (2005) identified 8 alleles in Texel and 7 in Suffolk breeds. Finally, in the study of Balingall and Tassi (2010) 38 new alleles were identified in a sample of 214 animals from 15 different breeds. Considering all the above information, as well as the number of alleles found from the present study, it is justified to argue that the Greek sheep breeds contain considerable amount of genetic variation in Ovar-DRB1 locus.

There is substantial evidence that genetic factors affect the resistance of animals in gastrointestinal parasite numbers (Bishop *et al.*, 2004; Sayers *et al.*, 2005), although many other factors as the type, age and sex of the parasite, the intensity of infection, the age, diet and frequency of the host defecation, act on the counted number of reproductive elements of gastrointestinal parasites. Also, the FEC values are affected by time of year and weather conditions (Abbott *et al.*, 2009). Possibly, the large numbers of gastrointestinal parasites in Kalarrytiko animals occurred due to a relatively large number of fertile and adult parasites, which act within the organism to enhance the immune animal hypersensitivity reaction causing an increase of the permeability of abomasum mucosa, resulting in the estimation of very high values mI.U. tyrosine (Yakoob et al, 1983b). As already mentioned, the environmental factors

ISSN: 2283-3927

(climate and habitat) acting on the studied breeds were different and do not allow a further comparison between FEC values and the tyrosine values estimated for both breeds.

Pepsinogen levels in plasma were determined in order to assess the extent of damage in the gastric mucosa of animals by gastrointestinal parasites. The endoparasitic infection is associated with pathophysiological disorders such as the increase of plasma pepsinogen levels, the albumin catabolism and plasma protein losses in the gastrointestinal tract (Yakoob et al, 1983a). For this reason, the estimation of pepsinogen concentrations in the blood plasma has been proposed as an auxiliary diagnostic technique of endoparasitic infections (parasitic gastroenteritis) in ruminants (Anderson *et al.*, 1965). According to Stear *et al.* (1995a, b) the plasma pepsinogen concentration expresses the host response to infection by nematodes, while FEC values reflect the behaviour of pests in the host. In particular, the above researchers found that in adult ewes infected naturally by gastrointestinal nematodes, the increase of plasma pepsinogen concentration was merely an expression of the immunological host response to infection and had no relation to the parasitic load.

The identification of resistant and susceptible sheep to nematodes is more effective when the estimation of FEC values is done in parallel with the estimation of plasma pepsinogen levels (Stear *et al.*, 1995a, b). In some regions where *Ostertagia* spp is detected, serum plasma analysis is a useful diagnostic tool. In general, pepsinogen levels > 375 mI.U. tyrosine correspond to clinical symptoms. However, interpretation problems may arise in immunized animals that are infected by gastrointestinal parasites. In these animals, there are no clinical signs but, as mentioned, plasma pepsinogen levels may be elevated because of immunological hypersensitivity reaction that occurs in the abomasum mucosa. Most studies on the concentration of pepsinogen in the blood plasma have been conducted in sheep experimentally infected with nematodes (Yakoob *et al.*, 1983b; Fox *et al.*, 1988; Mostofa *et al.*, 1990; Lawton *et al.*, 1996) while there are few studies in sheep infected with nematodes in a natural way (Yakoob *et al.*, 1983a). Besides the determination of plasma pepsinogen levels when the genus *Haemonchus* spp dominates, hematocrit measuring provides a rapid estimation of the degree of anaemia which is the characteristic clinical sign of nematodosis caused by the above parasite. Also, in some countries the serological diagnosis (ELISA) is used mainly for the species *Ostertagia* spp and *Cooperia* spp. However, so far there is insufficient information on the association between serological titres and parasitic load.

Generally, from this study neither significant differences of pepsinogen levels were found between heterozygote and homozygote Ovar-DRB1genotypes, nor any relationship between the various genotypes with high or low pepsinogen levels. However, there was a trend for homozygous animals carrying DRB1*0402 allele to have lower median values of mI.U. tyrosine in comparison with the other genotypes.

Genetic factors play an important role in resistance to nematodes (Bisset *et al.*, 1992). As the estimated coefficient of heritability for FEC values ranges from 0.2 to 0.4 in various sheep breeds which have previously been infected with parasites (Stear *et al.*, 1997a, b), this suggests that a sheep selection program of genetic resistance to nematodes can be effectively implemented (Stear *et al.*, 2001). According to research studies of Behnke *et al.* (2003, 2006) MHC polymorphism of class II genes and mainly of TNF gene regions determines the resistance or the host susceptibility to diseases caused by gastrointestinal parasites. In the study of Davies *et al.* (2006) in Scottish Blackface sheep, it was shown that specific QTLs in chromosomes 2, 3, 14, and 20 are related with the resistance to infection of *Teladorsagia*

ISSN: 2283-3927

circumcincta. The analysis of chromosome 20 revealed that the Ovar-MHC region is significantly correlated with resistance to gastrointestinal nematodes. Furthermore, QTLs related to the specific activity of the IgA immunoglobulins against nematodes are found on chromosomes 3 and 20.

Associations of MHC DRB1 alleles with resistance to nematodes have been found not only in sheep (Outteridge *et al.*, 1996; Sayers *et al.*, 2005) but also in cattle (Stear *et al.*, 1988 & 1990; Gasbarre *et al.*, 1993) and mice (Froeschke and Sommer, 2005). Early studies in Scottish Blackface lambs, based on the hybridization of oligonucleotides in exon 2 of Ovar-DRB1 gene, have shown that, lambs infected naturally with *Osterdagia circumcincta,* had FEC values associated with Ovar-DRB1 alleles (Buitcamp *et al.*, 1994; Schwaiger *et al.*, 1995; Stear *et al.*, 1996). In addition, the significant effect of Ovar-DRB1 alleles on the drastic reduction of FEC values, was detected by substitution of the common allele I, with G2 allele, identical with the allele Ovar-DRB1*0203, which confers resistance to nematode parasites. The association of G2 allele with low FEC values in Scottish Blackface sheep was confirmed by the study of Stear *et al.* (2005). There was also, an association of G2 allele with reduced number of adult parasites of *T. circumcincta* species, although no association was found with the length size of female parasites. A possible explanation is that the MHC effect on FEC values functions through the control of the number of adult parasites and not the control of their fertility. In the study of Hassan *et al.*, (2011) the G2 allele, renamed as Ovar-DRB1*1101, gave increased resistance against the nematode *T. circumcincta* in Suffolk lambs. Evidence was also, presented that the resistance of animals, due to Ovar-DRB1*1101 allele, was more acquired and less innate and furthermore it was depended from the excretion of adult parasites through the multiplication of mast cells of the mucosa.

In the study of Sayers *et al.* (2005) the allele Ovar-DRB1*0203 was associated with reduced FEC values in Suffolk breed. The above strong association seems to be due, not only to an antibody production that protects the animals carrying G2 allele, but also to the genetic linkage of G2 allele with an allele of resistance mounted on another locus position. This probably occurs because the loci close to one another on the same chromosome, tend to stay together during the reduction and therefore they are linked genetically (McCririe *et al.*, 1997). Additionally, the parasite *Trichostrongylus colubriformis* in sheep appears capable to negatively regulate many genes of immunologic importance, especially Ovar-DRB1 and DRA, of the migratory cells in lymph (Knight *et al.*, 2010).

Several genetic mechanisms have been proposed to explain the MHC allelic variability. New alleles appear through point mutations, genetic recombination or genetic transmutations (Janeway and Travers, 1996). The simultaneous antagonistic evolution (coevolution) between host and parasites is another proposed mechanism for maintenance of genetic variability both in host and parasitic species (Paterson *et al.*, 1998; Cutrera *et al.*, 2011). The hypothesis that the high levels of MHC genetic polymorphism of host species, driven by the parasites themselves, is the result of the action of balancing selection, interprets the reduction of homozygous and the increase of heterozygous frequencies in a particular population (Hedrick and Kim, 1998). The balancing selection maintains genetic polymorphism through three different genetic mechanisms: a) the heterozygote advantage (overdominance) b) the advantage of rare alleles and c) the variance in the levels of selective pressure (Charbonnel and Pemberton, 2005). In the case of overdominance, selection will not fix or eliminate one or the other allele, but will make a stable balance of heterozygotes, in which both alleles will be present in the population,

ISSN: 2283-3927

at frequencies determined solely by the selection rates against the two homozygote genotypes. In this case, the degree of MHC heterozygosity increases the range of parasites which are recognized by the immune system and thus increases the relative Darwinian fitness of MHC heterozygote genotypes compared to that of the homozygote (Paterson *et al.*, 1998). In the case of the second mechanism (advantage of the rare allele) (Hamilton, 1980; Takahata and Nei, 1990), the Darwinian fitness of the allele is reduced when the frequency of allele in the population increases. Genotypes with rare MHC alleles present a strong selective advantage as fewer pathogens have been exposed and adapted to them (Clarke and Kirby, 1996). The MHC alleles are favoured at low frequencies, while an increase of their frequencies could only cause a shift in the genetic composition of parasitic population. Thus, according to the case of rare allele, the interaction of host-parasite is considered as a dynamic procedure. The action of parasites decreases the Darwinian fitness of the most common MHC alleles of the host with which interacts (Paterson *et al.*, 1998). The third proposed mechanism is related with variances on the rate of selective pressure. Variations in type or size of the parasite population in the area, lead to constant changes in the intensity of selection and thus to maintenance of the polymorphism. This phenomenon is dependent on the presence of the same pathogen (Hedrick, 2002; Charbonnel and Pemberton, 2005).

The possibility that an interaction between various genotypes, as this is reflected by the different breeds and the farming system could not be excluded although this was impossible to be investigated in the present study. Genetic selection schemes applied in Australian conditions by selecting Merino sheep with low faecal egg – counts gave good selection response and have not resulted in any negative correlation response of the economically important production traits. Sheep resource flocks helminth resistant (Rylington Merino) have been created providing significant contribution in understanding the breeding for disease resistance (Karlsson & Greeff, 2012). From the other hand the diversity of environmental conditions in this continent, highlights the need of various strategies and makes difficult to give universal recommendations in order to control gastro-intestinal parasites (Larsen, 2014).

5 Conclusions

Our results suggest the considerable amount of genetic variability in the Ovar-DRB1 locus for the two Greek dairy sheep breeds, which is underlined due to the high number of detected alleles as well as the finding of new alleles from this study. In the Kalarrytiko breed, a rare breed with small population size, a higher number of new alleles were identified than in the Arta breed. This finding suggests that rare Greek sheep breeds potentially are an important gene pool of new alleles. Our results also indicated that the majority of detected alleles have low frequencies and subsequently the majority of animals were in heterozygote state for both breeds. Although certain genotypes of Ovar-DRB1 alleles were associated with lower median FEC values, the above findings make more complicated the selection of desirable genotypes resistant to gastrointestinal parasites. Considering the central role that the major histocompatibility complex (MHC) plays in the immune system, our study contributes to the accumulated knowledge on MHC effects on parasite resistance, however further studies in the future will be required with other genes involved in MHC region in order to clarify the genetic background of parasite resistance.

ISSN: 2283-3927

Appendix: Supplementary table

Table A. Observed (obs) and expected (exp) genotypes for Ovar-DRB1 alleles detected in the studied breeds

Genotypes	obs (exp)	Genotypes	obs (exp)	Genotypes	obs (exp)
		Arta			
DRB1*0101-DRB1*0101	6 (4.373)	DRB1*0901-DRB1*0304	1 (1.946)	DRB1*1901-DRB1*0101	1 (0.833)
DRB1* 0102-DRB1*0101	14 (14.855)	DRB1* 0901-DRB1*0311 #	2 (0.592)	DRB1*1901-DRB1*0102	1 (1.393)
DRB1*0102-DRB1*0102	8 (12.301)	DRB1*0901-DRB1*0402 #	4 (3.722)	DRB1*1901-DRB1*0402	2 (0.573)
DRB1*0304-DRB1*010	3 (3.193)	DRB1*0901-DRB1*0801	2 (0.931)	DRB1*1901-DRB1*0601	1 (0.299)
DRB1* 0304-DRB1*0102 #	7 (5.338)	DRB1*0901-DRB1*0901#	2 (1.607)	DRB1*2001-DRB1*0402	3 (0668)
DRB1*0304-DRB1*0304	2 (0.549)	DRB1*1001-DRB1*0101	7 (5.692)	DRB1*2001-DRB1*0901	3 (0.592)
DRB1*0308-DRB1*0101	1 (0.278)	DRB1*1001-DRB1*0102	12 (9.516)	DRB1*2101-DRB1*0101	1 (5.137)
DRB1*0311-DRB1*0102	1 (1.625)	DRB1*1001-DRB1*0302	1 (0.089)	DRB1*2101-DRB1*0102	12 (8.588)
DRB1*0402-DRB1*0101 #	3 (6.109)	DRB1*1001-DRB1*0304	1 (2.046)	DRB1*2101-DRB1*0311	1 (0.562)
DRB1* 0402-DRB1*0102 #	9 (10.213)	DRB1*1001-DRB1*0311	1 (0.623)	DRB1*2101-DRB1*0402	1 (0.532)
DRB1*0402-DRB1* 0104	1(0.191)	DRB1*1001-DRB1*0402	3 (3.913)	DRB1*2101-DRB1*0801	1 (0.883)
DRB1*0402-DRB1*0304	2 (2.195)	DRB1*1001-DRB1*0601	2 (2.046)	DRB1*2101-DRB1*0901	2 (3.130)
DRB1* 0402-DRB1* 0308	1 (0.191)	DRB1*1001-DRB1*0901	1 (3.469)	DRB1*2101-DRB1*1001	8 (3.291)
DRB1*0402-DRB1* 0311 #	1 (0.668)	DRB1*1001-DRB1*1001	1 (1.779)	DRB1*2101-DRB1*1901	1 (0.482)
DRB1* 0402-DRB1* 0402 #	4 (2.052)	DRB1*1003-DRB1*0101	1 (0.833)	DRB1*2101-DRB1*2101#	2 (1.445)
DRB1*0601-DRB1* 0101	3 (3.193)	DRB1*1003-DRB1*0102	5 (1.393)	DRB1*2201-DRB1*0102	2 (0.464)
DRB1* 0601-DRB1*0102	8 (5.338)	DRB1*1501-DRB1*0101	1 (0.417)	DRB1*2502-DRB1*2502	1 (0.002)
DRB1*0601-DRB1* 0304	1 (1.148)	DRB1*1501-DRB1*0402	1 (0.286)	DRB1*12-DRB1*0101	1 (2.777)
DRB1*0601-DRB1*0402	3 (2.195)	DRB1*1501-DRB1*0801	1 (0.072)	DRB1*12-DRB1*0102	5 (4.642)
DRB1*0601-DRB1* 0601	1 (0.549)	DRB1*1601-DRB1*0101	4 (0.833)	DRB1*12-DRB1*0104	1 (0.087)
DRB1*0801-DRB1*0102	2 (2.553)	DRB1*1601-DRB1*0402 #	1 (0.573)	DRB1*12-DRB1*0304	1 (0.998)
DRB1*0801-DRB1*0311	1 (0.167)	DRB1*1601-DRB1*0601	1 (0.299)	DRB1*12-DRB1*0601	1 (0.998)
DRB1*0801-DRB1* 0402 #	1 (1.050)	DRB1*1607-DRB1*0101	1 (0.139)	DRB1*12-DRB1*0901	1 (1.692)
DRB1*0801-DRB1*0601	1 (0.549)	DRB1*1608-DRB1*0101	1 (0.972)	DRB1*12-DRB1*1001	3 (1.779)
DRB1*0801-DRB1*0801	1 (0.119)	DRB1*1608-DRB1*0102	4 (1.625)	DRB1*12-DRB1*2101	4 (1.605)
DRB1*0901-DRB1*0101	10 (5.414)	DRB1*1608-DRB1*0304	1 (0.349)	DRB1*12-DRB1*1606	1 (0.043)
DRB1*0901-DRB1*0102 #	9 (9.052)	DRB1*1608-DRB1*2001	1 (0.106)	DRB1*12-DRB1*12	1 (0.412)
		Kalarrytiko			
DRB1*0304-DRB1*0102 #	1 (0.130)	DRB1*1601-DRB1*0402 #	1 (0.192)	DRB1*1607-DRB1*0102	1 (1.816)
DRB1*0402-DRB1*0101 #	1 (1.344)	DRB1*1602-DRB1*0102	2 (1.816)	DRB1*1607-DRB1*0311	4 (1.466)
DRB1*0402-DRB1*0102 #	11 (4.993)	DRB1*1602-DRB1*0702	11 (5.935)	DRB1*1607-DRB1*0402	3 (5.377)
DRB1*0402-DRB1*0311 #	7 (4.032)	DRB1*160202-DRB1*0402	2 (2.880)	DRB1*1607-DRB1*0702	5 (5.935)
DRB1*0402-DRB1* 0402 #	8 (7.297)	DRB1*160202-DRB1*0702	1 (3.180)	DRB1*1607-DRB1*1602	2 (1.955)
DRB1*0702-DRB1*0101	4 (1.484)	DRB1*160202-DRB1*1102	1 (0.037)	DRB1*1607-DRB1*1606	4 (3.003)
DRB1*0702-DRB1*0102	2 (5.511)	DRB1*160202-DRB1*160202	5 (0.262)	DRB1*1607-DRB1*1607	4 (0.943)
DRB1*0702-DRB1*0402	13 (16.322)	DRB1*1604-DRB1*0102	1 (0.195)	DRB1*1608-DRB1*0402	1 (0.576)
DRB1*0702-DRB1*0601	1 (0.212)	DRB1*1604-DRB1*0702	2 (0.636)	DRB1*1608-DRB1*0702	2 (0.636)
DRB1*0702-DRB1*0702	11 (8.903)	DRB1*1605-DRB1*1605	2 (0.015)	DRB1*2101-DRB1*2101 #	1 (0.008)
DRB1*0801-DRB1*0402 #	1 (0.192)	DRB1*1606-DRB1*0101	2 (0.751)	DRB1*2401-DRB1*0402	1 (0.384)
DRB1*0803-DRB1*0311	1 (0.419)	DRB1*1606-DRB1*0102	5 (2.788)	DRB1*2502-DRB1*0402	1 (0.192)
DRB1*0803-DRB1*0402	3 (1.536)	DRB1*1606-DRB1*0304	1 (0.215)	DRB1*11-DRB1*0402	3 (3.648)
DRB1*0803-DRB1*0702	1 (1.696)	DRB1*1606-DRB1*0311	4 (2.252)	DRB1* 11-DRB1*0901	1 (0.758)
DRB1*0803-DRB1*0803	1 (0.070)	DRB1*1606-DRB1*0402	3 (8.257)	DRB1* 11-DRB1*2003	1 (0.047)
DRB1*0901-DRB1*0102 #	1 (1.037)	DRB1*1606-DRB1*0403	1 (0.107)	DRB1*/11-DRB1*2101	1 (0.142)
DRB1*0901-DRB1*0311 #	1 (0.838)	DRB1*1606-DRB1*0702	9 (9.115)	DRB1* 11-DRB1*160202	1 (0.711)
DRB1*0901-DRB1*0402 #	5 (3.072)	DRB1*1606-DRB1*0803	1 (0.858)	DRB1* 11-DRB1*1606	1 (2.037)
DRB1*0901-DRB1*0702	1 (3.392)	DRB1*1606-DRB1*0901	2 (1.716)	DRB1*11-DRB1*/11	3 (0.426)
DRB1*0901-DRB1*0901#	1 (0.299)	DRB1*1606-DRB1*1602	5 (3.003)	DRB1*26-DRB1*1607	1 (0.070)
DRB1*1301-DRB1*0702	1 (0.212)	DRB1*1606-DRB1*2401	1 (0.215)	DRB1*30-DRB1*0702	2 (0.424)
DRB1*1302-DRB1*0702	1 (0.212)	DRB1*1606-DRB1*1606	2 (2.252)		

Symbol #: the common genotypes between the two breeds

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