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Use of Microsatellite Polymorphisms in *Ovar-DRB1* Gene for Identifying Genetic Resistance in Fat-Tailed Ghezel Sheep to Gastrointestinal Nematodes

Research Article

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ABSTRACT

This study was designed to identify genetically resistant animals to gastrointestinal nematode (GIN) infections using microsatellite polymorphisms of *Ovar-DRB1* gene in Iranian Ghezel sheep breed lambs. In the present study 120 male Ghezel lambs were at 4 to 6 months of age randomly selected from six different sheep flocks in East Azerbaijan province (n=20 per flock). These lambs were naturally infected with GINs, and individual fecal samples were collected twice with a week interval to evaluate fecal egg counts (FEC). Blood samples were also collected for DNA isolation and PCR was performed to amplify the second exon and microsatellites within the second intron of the *Ovar-DRB1* gene. The data were analyzed using a mixed model of SAS software. The present study identified 24 genotypes and 20 alleles on *Ovar-DRB1* gene. Results indicated that the presence of 510 bp (base pair) allele (called allele F) in both homozygote and heterozygote animals had a strong association ($P<0.01$) with lower FEC; while, presence of 506 bp allele (called allele E) in homozygote animals was significantly associated ($P<0.01$) with higher FEC. Thus, this study showed a strong association between microsatellite polymorphism of *Ovar-DRB1* gene and resistance to GIN infections in Ghezel sheep lambs.

KEY WORDS gastrointestinal nematodes, Ghezel sheep, microsatellite polymorphisms, *Ovar-DRB1* gene.

INTRODUCTION

Ghezel, an Iranian native fat-tailed sheep breed, is mainly inhabited in North West regions of Iran (east and west side of Azerbaijan provinces), (Baneh *et al*. 2010). Sheep rearing is based on pasture grazing; therefore, animals are very susceptible to the widespread gastrointestinal nematode (GIN) infections (Baneh *et al*. 2010). Thus, GIN infections pose a high economic loss in terms of medical expenses for the treatment, high mortality rate and low productivity in young lambs (Amarante *et al*. 2009). Although anthelmintic drugs are common in use for the treatment of internal parasite infections, development of anthelmintic resistance by GINs worldwide and also in Iran, has driven the investigation toward finding a new strategy to overcome GIN infections in order to improve the economy of farmers in the area (Le Jambre, 1976; Hazelby *et al*. 1994; Gholamian *et al*. 2006). GIN infections vary among animals based on their genetic resistance. The genetically resistant animals to the infection have higher viability and productivity than the animals with lower degree of genetic resistance (Amarante *et al*. 2009). Accordingly, Matika *et al*. (2011) identified QTL regions for resistance to gastrointestinal nematode on ovine chromosomes (OAR) 3 and 14 in Suffolk and Texel sheep breeds using the multiplex microsatellite panels. Also, Salle *et al*. (2012) found four QTL regions on ovine

chromosomes (OAR) including 5, 12, 13 and 21 which were responsible for resistance to *Haemonchus contortus* nematode in Romane × Martinik Black Belly backcross lambs. Other studies using the ovine 50 k SNP Chip identified association between QTLs on chromosomes 1, 6, 7 and 14 and resistance to gastrointestinal nematodes in Scottish Blackface, Romney, Perendale, Red Maasai × Dorper and Churra sheep breeds (Riggio *et al*. 2013; McRae *et al*. 2014; Benavides *et al*. 2015). The major histocompatibility complex (MHC) is a cell surface molecules which are encoded by a large gene family placed on chromosome 20 in sheep. There are two main groups of MHC gene family. MHC class I and class II. Ovar-DR gene is one of MHC class II genes and codes heterodimeric proteins (DRα and DRβ, each one of these proteins including α1, α2 and β1, β2 domains) in the membrane of B cells and antigen presenting cells such as macrophages, dendritic cells and Langerhans cells (Tizard, 2013). Charon *et al*. (2002) showed a relationship between microsatellite polymorphisms in the second intron of *MHC-DRB1* gene and resistance to *Teladorsagia circumcincta* in Polish Heath sheep. Dominik (2005) in a study indicated a list of QTLs and candidate genes for resistance to gastrointestinal nematodes in sheep. Furthermore, he found a strong relationship between MHC gene loci and genetic resistance to GINs. Among all the candidate genes (interferon γ, interleukin-4, MHC-I and MHC-II) responsible for GIN resistance, *Ovar-DRB1* gene (MHC class II) plays an important role in immunity to GINs and other internal parasites (Dominik, 2005). Several studies have proved the role of polymorphisms in *Ovar-DRB1* gene on resistance to numerous internal parasites and bacterial diseases in several sheep breeds (Hajializadeh *et al*. 2015; Schwaiger *et al*. 1995; Castillo *et al*. 2011; Larruskain *et al*. 2012; Shen *et al*. 2014). In our previous study, a significant association was found between gastrointestinal fecal egg counts (FEC) and the second exon of *Ovar-DRB1* gene using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP), (Hajializadeh *et al*. 2015). Since PCR-RFLP technique has some limitations for identifying all variations within an amplified gene fragment (Ferreira *et al*. 2014), new simpler and more accurate technique is required. Simple tandem repeats (STRs) or microsatellites are repeated short sequence motifs (Zane *et al*. 2002) which are a very powerful technique for studying the genetic diversities within and between populations and pedigree analysis in farm animals and livestock breeding (Abdul Muneer, 2014).

Moreover, high rate of polymorphism and relatively simple scoring and data analysis, are important features that makes microsatellite markers of large interest for many genetic studies (Abdul Muneer, 2014). As selecting resistant animals is usually based on low FEC (as a phenotypic trait), thus can be used as a standard method for assessing the level of resistance to GIN (Eady *et al*. 2003). Based on the above information and as mechanisms responsible for genetic resistance to GIN infections are not totally understood and also because of environmental resemblance between natural challenges with gastrointestinal nematodes and actual rearing conditions of sheep in pastures, the present study was designed to detect resistant animals to GIN infections (based on natural challenges) using microsatellite polymorphisms in the *Ovar-DRB1* gene in Iranian Ghezel sheep lambs.

MATERIALS AND METHODS

Animals and sampling

This study was conducted during the period from May to June, 2014 when animals were potentially at the highest risk of becoming infested with GINs. For this reason, 120 male weaned Ghezel lambs were at 4 to 6 months of age randomly selected from lambs in six flocks (n=20 per flock) in east Azerbaijan province, Iran. All procedures used in this experiment were warranted based on University of Tabriz Animal Care and Ethics Committee. Before the start of experiment on selected lambs deworming procedure was performed to ensure elimination of all the parasites. After 28 days, lambs feces were examined to confirm their parasite-free conditions. Afterward, all selected lambs from six flocks were allowed 28 days grazing on contaminated pastures during which they did not receive any deworming drugs (in order to naturally parasitize animals). On day 31 post infection, individual blood and fecal samples were collected twice with one week interval. Blood samples were obtained from jugular vein using sterile vacuum tubes coated with anticoagulant (EDTA) and were frozen at -20 ˚C for future DNA isolation. Fecal samples were collected individually from the rectum of each lamb for determining fecal egg counts (FEC) using modified McMaster (Clayton Lane) method (Anonymous, 1977; Zajac and Conboy, 2012). During FEC evaluation, nematode eggs were classified into four major species as following: 1- Strongyles (including: *Haemonchus contortus*, *Teladorsagia circumcincta*, *Ostertagia occidentalis* and *Trichostrongylus axei*, *colubriformis*, *vitrinus* and *rugatus*), 2- *Nematodirus* spp., 3- *Trichuris ovis*. and 4- *Marshallagia marshalli*. The total summations of four parasite classes counted in each lamb's feces were reported as FEC.

Genotype and molecular analysis

Blood DNA was isolated using Samadi Shams *et al*. (2011) protocol. Polymerase chain reaction (PCR) was performed using PCR master mix kit (Ampliqon Company) in a T-personal thermo-cycler (Biometera Personal Cycler Version

3.26 co., Germany). The 25 µL PCR mixture contained: 50-100 ng of DNA, 2.5 µL of 10X PCR buffer (200 m*M* $(NH_4)2SO_4$), 0.1 m*M* Tween 20%, 750 m*M* Tris-HCl (pH=8.8), 2.5 m*M* $MgCl_2$, 200 µ*M* dNTPs and 3 µL mix of oligo nucleotides (10 p mol from each primer), 1U Taq DNA polymerase (Dream Taq polymerase, Ampliqon company) and 11 µL ddH_2O. Forward and reverse sequences of primers (Bioneer, Korea) used for amplification of the second exon and microsatellite sequences in the second intron of *Ovar-DRB1* gene included F: 5'-TCTCTGCAGCACATTTCCTGG-3' (Ammer *et al.* 1992) and R: 5'-CGTACCCAGAGTGAGTGAAGTATC-3' (Schwaiger *et al.* 1993). PCR program included 36 cycles of amplification which were as following: 1 cycle of initial denaturation at 94 ˚C for 5m; 35 cycles included denaturation at 94 ˚C for 30 s, annealing at 55 ˚C for 60 s, extension at 72 ˚C for 60 s; followed by termination at 72 ˚C for 5 m. Then, PCR products were electrophoresed at 1200 V with 25 mA for 35 min in 4% acryl amide gel using Gel-Scan™ 3000 automated DNA Sequencer on a Real-Time Gel system (Corbett Robotic co., Australia). Size of the PCR products was determined based on a 25 bp DNA standard ladder (Thermo Scientific). The UVIdoc software (version 99.02 for windows) was used to identify microsatellite alleles in the second intron of *DRB1* gene.

Statistical analysis

For data analysis, total nematode egg count was considered as FEC (fecal egg count). Then, FEC values were considered as a residual deviation of flock × time interactions. Also, the distribution of residuals was tested for skewness (ω) and kurtosis (κ). Normality test was performed for FEC; then, the data which were not normally distributed, were transformed using Box–Cox transformation [(FECλ-1)/λ], (Box and Cox, 1994). Optimum values of λ between the range of -2 and 2 were determined using a maximum-likelihood criterion (Draper and Smith, 1981) in trans regression procedure of SAS (2002). Blood samples of 120 lambs in six flocks were used for evaluating microsatellite polymorphisms of Ovar-DRB1. During analysis of association between each allele and FEC, genotypes with frequencies lower than 2% were deleted. The effects of each genotype and also each allele on transformed FEC were analyzed by the mixed model (repeated measures analysis of variance) of SAS software (Hajializadeh *et al.* 2015; Castillo *et al.* 2011). The statistical model is described as:

$$Y_{ijklm} = \mu + Flock_i + Genotype_j + Time_k + Lamb_l (Flock_i \times Genotype_j) + e_{ijklm}$$

Where:

Y_{ijklm}: dependent variable.

µ: overall mean.

$Flock_i$: fixed effect of flocks (1, 2,...,6).

$Genotype_j$: fixed effect of genotypes (genotypes coded as 0, 1 and 2, based on number of copies in the determined alleles).

$Time_k$: fixed effect of times (sampling time 1 and 2).

$Lamb_l$: random effect of lamb (1, 2, 3,…,120).

$Flock_i \times Genotype_j$: interaction effect between Flock and Genotype.

e_{ijklm}: experimental error.

In the primary model, we considered the interaction effect between Flock and Time, but since this interaction was not significant, Flock × Time was deleted from the final statistical model presented in the above.

RESULTS AND DISCUSSION

The descriptive statistics regarding fecal egg count (FEC) of various classes of gastrointestinal nematodes from five flocks of Ghezel lambs are presented in Table 1. Based on these statistics, the prevalence of *Trichuris ovis* nematode in the investigated lambs was the lowest (6%); therefore, it was excluded from the data at the time of statistical analysis. On the other hand, *Nematodirus* nematode showed the highest prevalence in Ghezel lambs of six flocks (44.64%). Therefore, these results showed that pastures in east Azerbaijan province (Iran) had the highest level of contamination with *Nematodirus* nematode.

Analysis of microsatellite polymorphisms in Ovar-DRB1 showed 20 alleles and 24 genotypes. The frequency of each observed alleles are presented in Table 2. Based on these results, 10 genotypes had frequencies higher than 2% including: AA, BB, DD, EE, FF, HH, JJ, MM, OO and RR. Among these genotypes, F (20%), E (15%) and O (12.5%) alleles had the highest frequencies of microsatellite loci in *Ovar-DRB1* gene.

Results also showed an association between genotypes (with 0, 1 and 2 allele copy numbers) of the most frequent alleles and FEC (Table 3). Based on the results presented in Table 3, F and E allele frequencies had significant effects on FEC ($P<0.01$). Accordingly, the presence of E allele (506 bp) in homozygote (EE) animals, as a GIN susceptible allele, was accompanied with a significant increase in the means of FEC ($P<0.01$). Whereas the presence of F allele (510 bp), as a GIN resistant allele, in homozygote (FF) and heterozygote (FA) animals was accompanied with a significant decrease in the means of FEC in resistant animals ($P<0.01$). Meanwhile, other observed alleles with their various genotypes showed no significant association with FEC.

Moreover, the comparison of association related to FEC means in different flocks are presented in Table 4.

Table 1 The descriptive statistics of fecal egg count (FEC) for various classes of gastrointestinal nematodes

Nematode classes	Mean	SD	Minimum value	Maximum value
***Strongyles* FEC**	83.33	121.37	0	612.50
***Nematodirus* FEC**	108.53	147.15	0	805.00
***Trichuris ovis* FEC**	1.47	7.57	0	70.00
***Marshallagia marshali* FEC**	51.28	75.75	0	315.00
Total nematode FEC	243.14	251.33	0	1505.00

SD: standard deviation.

Table 2 Allelic and genotypic frequencies

Ovar-DRB1				
Allele	**Allelic frequencies**	**Genotypes**	**N**	**Genotypic frequencies (%)**
A	0.062	AA	7	5.83
B	0.041	BB	5	4.16
C	0.012	CC	1	0.83
D	0.054	DD	3	2.5
E	0.15	EE	18	15
F	0.204	FF	24	20
G	0.012	GG	1	0.83
H	0.083	HH	10	8.33
I	0.016	II	2	1.66
J	0.05	JJ	6	5
K	0.016	KK	2	1.66
L	0.016	LL	2	1.66
M	0.037	MM	4	3.33
N	0.016	NN	2	1.66
O	0.125	OO	15	12.5
P	0.020	PP	2	1.66
Q	0.016	QQ	2	1.66
R	0.054	RR	5	4.166
S	0.016	SS	2	1.66
T	0.008	FA	1	0.833
-	-	MC	1	0.833
-	-	PD	1	0.833
-	-	RG	1	0.833
-	-	TR	2	1.66

Analysis results showed a significant effect of flock (as a fixed effect) on means of FEC ($P<0.01$). While, no significant effect of time on means of FEC was observed.

In the present study microsatellite polymorphisms of the second intron in *Ovar-DRB1* gene of Iranian Ghezel sheep breed was evaluated to identify intra-breed variation in genetic resistance to GIN infections. In a previous study, using PCR-RFLP technique in Ghezel sheep showed polymorphisms in the second exon of DRB1 gene and its association with FEC for the first time in Iran (Hajializadeh *et al*. 2015). Since using PCR-RFLP technique has some limitations, in the present study microsatellites were used as a simpler, more applicable and more accurate technique for identifying genetic variations.

Simple sequence repeats (STRs) play important roles in natural evolution (Awadalla and Ritland, 1997) and genome evolution (Moxon and Wills, 1999). Also microsatellites were mainly used in genomic selections of farm animals and livestock breeding as well as determining the location of mutations in genetic disorders with the help of disease markers (Eady *et al*. 2003; Abdul Muneer *et al*. 2009; Abdul Muneer, 2014; Teneva *et al*. 2013). Researchers also showed significant functional role of STRs in regulation of gene expression, binding to nuclear proteins and its function as transcriptional activating elements (Li *et al*. 2002).

In the present study, a total of 24 genotypes and 20 microsatellite alleles were identified in the second intron of Ovar *MHC-DRB1* gene and these results are in line with previous studies including Schwaiger *et al*. (1993), Charon *et al*. (2002) and Castillo *et al*. (2011).

It was also showed that the presence of allele called F (510 bp) in homozygote (FF) and heterozygote (FA) animals was strongly ($P<0.01$) associated with lower means of FEC in resistant animals.

On the other hand, the presence of E allele (506 bp) in homozygote (EE) animals was positively ($P<0.01$) associated with higher means of FEC in susceptible animals to GINs.

Table 3 Association between genotypes (allele copy numbers) and means of fecal egg count (FEC)

Allele	Genotype	Allele copy number	Least squares means of FEC ± SE	P-value
A	-	0	5.00±0.21	NS
	FA	1	5.12±0.21	NS
	AA	2	5.00±0.21	NS
F	-	0	5.20±0.05	
	FA	1	5.05±0.49	
	FF	2	4.48±0.09	(P<0.01)[1]
E	-	0	4.92±0.05	
	EE	2	5.56±0.13	(P<0.01)[2]
B	-	0	5.02±0.05	NS
	BB	2	5.17±0.25	NS
H	-	0	5.00±0.05	NS
	HH	2	5.25±0.18	NS
J	-	0	5.01±0.05	NS
	JJ	2	5.34±0.22	NS
M	-	0	5.02±0.05	NS
	MC	1	5.60±0.57	NS
	MM	2	4.98±0.28	NS
R	-	0	5.03±0.05	NS
	RT	1	4.63±0.58	NS
	RR	2	5.02±0.27	NS
D	-	0	5.01±0.05	NS
	PD	1	5.65±0.57	NS
	DD	2	5.14±0.33	NS
O	-	0	4.91±0.09	NS
	OO	2	5.04±0.09	NS

NS: non significant.
SE: standard error.
[1] Comparison with considering 0 and 1 allele copy numbers.
[2] Comparison with considering 0 allele copy number.

Table 4 Association between the fixed effect of flock and means of fecal egg counts (FEC)

Means of FEC ± SE (Flock)		Means of FEC (Flock)	P-value
4.35 0.17 (1)	*vs.*	4.04 (2)	NS
4.35±0.17 (1)	*vs.*	5.03 (3)	**
4.35±0.17 (1)	*vs.*	4.62 (4)	NS
4.35±0.17 (1)	*vs.*	5.70 (5)	**
4.35±0.17 (1)	*vs.*	6.12 (6)	**
4.04±0.14 (2)	*vs.*	5.03 (3)	**
4.04±0.14 (2)	*vs.*	4.62 (4)	**
4.04±0.14 (2)	*vs.*	5.70 (5)	**
4.04±0.14 (2)	*vs.*	6.12 (6)	**
5.03±0.14 (3)	*vs.*	4.62 (4)	*
5.03±0.14 (3)	*vs.*	5.70 (5)	**
5.03±0.14 (3)	*vs.*	6.12 (6)	**
4.62±0.14 (4)	*vs.*	5.70 (5)	**
4.62±0.14 (4)	*vs.*	6.12 (6)	**
5.70±0.13 (5)	*vs.*	6.12 (6)	*
6.12±0.14 (6)	-	-	-

* (P<0.05) and ** (P<0.01).
NS: non significant.
SE: standard error.

Thus, it seems that F allele is an effective allele for reducing FEC in Ghezel breed lambs. In a similar study, Schwaiger *et al*. (1995) discovered an association between microsatellite polymorphisms of the second intron in Ovar-DRB1 and resistance to *Ostertagia circumcincta* in naturally infected Scottish Blackface sheep. They also identified nineteen alleles in which the presence of two alleles (G2 and I) was accompanied with lower FEC in sheep. In another similar study, Outteridge *et al*. (1996) using MHC-DRB1 microsatellite polymorphism in Merino sheep indicated eight alleles with high frequency in genotype of animals in which two allele were associated with low FEC. Furthermore, Paterson *et al*. (1998) reported an association between microsatellite polymorphisms in the second intron

of *MHC-DRB1* gene and resistance to Teladorsagia circumcincta. They also indicated that the presence of 257 bp allele was associated with low parasite resistance in lambs, while the presence of 263 bp allele was associated with high parasite resistance in yearling Soay sheep (Paterson *et al*. 1998). Charon *et al*. (2002) also showed that the presence of 468 bp, 482 bp and 530 bp microsatellite alleles in the second intron of *MHC-DRB1* gene was associated with lower FEC, while the presence of 568 bp was associated with higher FEC of *Teladorsagia circumcincta* in Polish Heath sheep. In another study in Mexico, Castillo *et al*. (2011) showed association between MHC (Ovar-MHC1, Ovar-DRB1 and Ovar-DRB2) microsatellites and FEC, blood packed cell volume, and blood eosinophilia in Pelibuey sheep which were artificially infected with *Haemonchus contortus* larvae. They also identified twenty alleles and found that alleles having 482 and 500 base pairs in length were associated with lower FEC, while a simultaneous higher blood eosinophilia and antibody levels in resistant lambs (Castillo *et al*. 2011). In the studies, Schwaiger *et al*. (1995), Charon *et al*. (2002) and Castillo *et al*. (2011) indicated significant association between microsatellites polymorphisms in the second intron of *DRB1* gene and lower FEC in Scottish Blackface sheep, Polish Heath sheep and Mexican Pelibuey sheep. In accordance with these results, in the present study the association between microsatellites polymorphisms in the second intron of *DRB1* gene and lower FEC was detected in Iranian Ghezel sheep.

Contradictory to the results of the present study, Cooper *et al*. (1989), Blattman *et al*. (1993) and Hulme *et al*. (1993) found no relationship between polymorphisms in MHC locus and susceptibility or resistance to GINs.

Davies *et al*. (2006) identified QTL regions on ovine chromosomes of 2, 3, 14 and 20 which were associated with FEC of GINs in Scottish Blackface sheep. Moreover, Riggio *et al*. (2014) used a joint (Meta) analysis for identifying genome-wide significant regions on OAR 4, 12, 14, 19 and 20. As OAR20 is near MHC regions, it is considered an important candidate gene for resistance to GINs in three European sheep populations. With considering these results (Davies *et al*. 2006; Riggio *et al*. 2014) and results of the present study, it seems that MHC regions still have more potential for finding the best candidate gene against GINs in sheep population. Furthermore, some complimentary studies using genome-wide association study (GWAS) found QTL regions on 1, 3, 6, 7, 14, 15 and 26 chromosomes which were also associated with resistance to gastrointestinal nematodes in different worldwide sheep breeds (Matika *et al*. 2011; Riggio *et al*. 2013; McRae *et al*. 2014; Benavides *et al*. 2015; Pickering *et al*. 2015); then, these new QTL regions can be considered for future studies in Ghezel sheep breed to find best candidate gene for resistance to gastrointestinal nematodes.

Since the presence of moderate heritability (0.2–0.6) of resistance to GINs in sheep (Baker, 1998; Stear *et al*. 2007), selective breeding can be used for developing a resistant sheep population in the area based on the presence of resistant alleles. Also, detected molecular markers responsible for resistance to GINs in sheep can be used in breeding programs in order to build a resistant population.

CONCLUSION

Results of the present study showed that microsatellite markers can be used as a powerful and accurate tool in recognizing resistant animals to GIN infections. Furthermore, our results showed that *Ovar-DRB1* gene can be used as a useful candidate gene in Ghezel sheep breeding programs for improving genetic resistance to GINs. According to the strong association detected between the presence of 510 bp allele (called allele F) and genetic resistance to GIN infections, this finding can be expanded to similar strains of this breed like Morkaraman in Turkey and Afshari in Iran and even in other related breeds.

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