Microsatellite Polymorphism in DRB2 Gene and its Relation to *Haemonchus Contortus* Parasites Fecal Egg Count in Iranian Ghezel Sheep

Hosseinzadeh, S.[1]; Rafat, S.A.[1*]; Moghaddam, Gh.[1]; Shodja, J.[1]; Nematollahi, A.[2]; Shamsuddin, M.[3]; Periasamy, K.[3]; Ebrahimi, M.[1]

1- *Department of Animal Science, College of Agriculture, University of Tabriz, Tabriz, Iran*
2- *Faculty of Veterinary, University of Tabriz, Tabriz, Iran*
3- *Animal Production and Health Section, International Atomic Energy Agency, Vienna, Austria*

[*]**Corresponding Author**: Rafat, S.A
E-mail: abbasrafat@hotmail.com

Abstract

Gastrointestinal parasites are one of the main sources of economic decline in sheep production around the world. Resistant sheep do not completely reject the disease; they only harbor fewer parasites than susceptible sheep and therefore have a lower fecal egg count. The present research was designed to evaluate microsatellite polymorphism in intron 5 of DRB2 gene and its correlation with *H. contortus* fecal egg counts in Ghezel sheep breed. For this reason, blood samples were taken from 80 male lambs between 4-6 months of age and some phenotypic traits including fecal egg counts (FEC), FAMACHA test, and packed cell volume (PCV) were measured. Blood DNA was extracted using chloroform-isoamyl alcohol protocol; then, microsatellite regions in intron 5 of DRB2 gene were replicated. Afterward, 3% agarose gel electrophoresis using ethidium bromide staining was performed to evaluate 25 base pairs allele size products using UVidoc software. Finally, the correlation between genotype and phenotypic traits was analyzed by linear and nonlinear mixed models using SAS software. The results showed that lambs having present genotype homozygous 300 were low FEC with lower prevalence of this kind of parasite than lambs having other types of homozygous genotypes. Based on the results, correlation between FAMACHA and PCV was -0.625 ($p< 0.01$); while, the correlation between FAMACHA and FEC was 0.731 ($p< 0.01$). Accordingly, the presence of allele 300 could be considered as a selecting indicator for animals with relatively higher resistance to *H. contortus* parasite. Also, the results suggested that PCV and FEC as well as FAMACHA can be used as reliable indicators for detecting *H. contortus* infection rate.

Keywords: DRB2 gene, FAMACHA test, Ghezel Breed, *H. contortus*, Microsatellite.

Introduction

Ghezel is one of the Iranian fat-tailed sheep breeds which is native of East Azerbaijan province. Their wool color changes from light brown to dark brown. Average body weight of Ghezel Sheep varies from 85 to 90 kg in males and 55 to 65 kg in females (Nasiryan *et al*. 2009). Since gastrointestinal nematode infections are one of the main global health and economic issues in sheep industries, Ghezel sheep also suffer from these infections. On the other hand, the high prevalence of resistant nematodes to anti-parasitic drugs (Geurden *et al*. 2014) required new strategies to control

worms in sheep. One of the possible alternative methods to reduce the problem of drug resistance is breeding those animals which are genetically resistant to nematodes. Genetic variation in resistance to gastrointestinal parasitic infections within and between breeds has been validated recently (Saddiqi *et al*. 2012). In sheep industry, farmers are then looking for evaluating the effect of selecting genetically resistant animals on the control of nematode infections in their flocks (Karrow *et al*. 2014). As DNA markers, related to quantitative trait loci (QTL), they influence host resistance to gastrointestinal parasitic infections; therefore, they are ideal targets for marker-assisted selections (Kim *et al*. 2014). Microsatellites are repeated short sequence motifs ranging from 1-6 base pairs of DNA which can be repeated in each place from few base pairs to up to 30 base pairs (Zane *et al*. 2002). Microsatellites are abundant throughout the genome. High rate of polymorphism and relatively simple scoring and data analysis are important features that make microsatellites markers of large interest for many genetic studies (Zane *et al*. 2002). The major histocompatibility complex (MHC) is a set of cell surface molecules encoded by a large gene family which is placed on chromosome 20 in sheep (Hajializadeh Valilou *et al.* 2015). MHC genes usually coexpress as the characteristic of each individual; in other words, for each MHC gene, each individual expresses alleles which are inherited from each parent (Gruszczynska *et al*. 2002). The MHC gene family is divided into two main subgroups including class I and class II, which encoded proteins that present different antigens to the appropriate T-cells (Hohenhaus and Outteridge, 1995). MHC regions have many pseudo genes. Ovar DRB 2 gene is one of the pseudo genes which lacks exon 1 and 2, while it has two aberrant coding ends (Gruszczynska *et al*. 2002). Then, in the present study we evaluated microsatellite polymorphism in intron 5 of DRB2 gene in order to detect genetically resistant Ghezel Sheep Breed against *H.* contortus. *H. contortus* is one of the most pathogenic nematodes of ruminants found out to be responsible for anemia, bottle jaw, and malfunction of abomasum which in term causes an increase in pH of abomasum and decrease in protein digestion, and finally it causes the death of infected sheep (Roberts *et al*. 2000; Notter *et al.* 2003). In this experiment, some phenotypic traits were used as an indicator for detecting resistant animals against H. contortus including: fecal egg count (FEC) (Papadopoulos *et al*. 2001; Riggio *et al*. 2014).

Material and methods

In the present study, we used 80 Ghezel ram lambs with the average age between 4- 6 months which were collected from four flocks in the East Azerbaijan province. Data were collected twice in one-week interval. During these samplings, FAMACHA test was performed for identifying animal's anemia (Torres-Acosta *et al*. 2012) in a way that the color of the ocular mucous membranes from each animal was examined and classified according to the FAMACHA© eye color chart: 1 = red, non-anemic; 2 = red-pink, non-anemic; 3 = pink, mildly-anemic; 4 = pink-white, anemic; 5 = white, severely anemic. Fecal samples were collected separately from the rectum of each lamb in order to determine FEC using Clayton Lane technique and then, results were reported as eggs number per gram of feces. Flotation

methods are based on the difference in specific weight of eggs, cysts and baby or particles in stool. Specific weight of some parasite eggs is equal to 1.1 to 1.2 grams per milliliter, while the specific weight of water is a little more than 1. In order to make floating cream eggs, a solution with a specific weight greater than that of the cream eggs, has to be used. These solutions are called floating-maker solution that is supplemented by some sugar or salt to raise their specific weight. Usually float-maker solutions have specific weight between 1.2 - 1.25. At this range, the particles of feces would not float since they have specific weight equal to 1.3 or higher. By using these solutions, the parasites eggs remain floating on the liquid surface and other materials of feces are deposited at the bottom of the solution. Usually, in veterinary medicine, a combination of sugar, sodium nitrate and salt has been used as float-maker solution (Hendrix, 1998). Blood samples of all 80 lambs were taken during both samplings from the jugular vein in sterile vacuum tubes coated with anticoagulant (EDTA). Then, blood samples were transferred to the lab and PCV (%) was determined on the same day of collection using the micro-hematocrit method, packet cell volume (PCV), as an indicator for high presence of blood suckling parasites in abomasal wall (Saddiqi *et al*. 2010; Bishop, 2012). Blood samples' DNA was extracted using chloroform-isoamyl alcohol protocol (Samadi Shams *et al*. 2011). After determining the quality and quantity of extracted DNA, intron5 of Ovar-DRB2 gene was amplified by the polymerase chain reaction using its forward and reverse primers (Castillo *et al*. 2011).

OLADRB2 F 5´-CTGCCAATGCAGAGACACAAGA-3´
OLADRB2 R 5´-GTCTGTCTCCTGTCTTGTCTCAT-3´

Then, polymerase chain reaction (PCR) was performed using a 25 µl master mix kit (Ampliqon Company) in a T-Personal thermo-cycler (Biometera Personal Cycler Version 3.26 co. Germany). The PCR mixture was composed of 50–100 ng of DNA, 2.5 µl of 10X PCR buffer (200 mM (NH4)2SO4), 0.1 mM Tween 20%, 750 mM TrisHCl (pH 8.8), 2.5 mM MgCl2, 200 µM dNTPs, and 3 µl mix of oligonucleotids (10 pmol from each primer), 1U Taq DNA polymerase (Dream Taq polymerase, Ampliqon company) and 11 µl ddH2O. A total of 35 cycles was adapted for denaturation at 94^0C/30 second, annealing at 59^0C/30 second, and extension at 72^0C/50 second, and one cycle for the final extension (termination) at 72^0C/5 min. Then, PCR products for each sample were electrophoresed at 85 V for 45 min in 3% agarose gels (PAC1000, BioRad company, USA), and visualized under UV light (Castillo *et al*. 2011). The size of the alleles was determined based on a 25 bp DNA size standard (Ampliqon Company) using the UVIDOC software. The digested products were separated in a 2 and 3% agaros gel for 1 h at 85 V. Then, these gels were stained with ethidium bromide. Afterward, the correlation between allele size and trait of FEC, as a threshold trait, was evaluated. Finally, because of the repeated nature of data, the effect of genotype on phenotypic traits was analyzed by linear and nonlinear mixed model of SAS software (Little *et al*. 1998) using the following statistical model:

$Y_{ijk} = \mu + H_i + G_j + a_{ij} + e_{ijk}$

in which Y_{ijk} is the dependent variable, μ is the overall mean, H_i is the effect of flock, G_j is the effect of genotype, a_{ij} is the random effect of animal and e_{ijk} is the experimental error for i^{th} (1, 2, 3, 4) flock, j^{th} (1, 2, 3… 13) genotype, and k^{th} (1, 2, 3… 80) animal. The

Correlation between phenotypic traits was calculated by Spearman's correlation coefficient test.

The results showed a polymorphism including 9 alleles in intron 5 of DRB2 gene which ranged from 274 to 318 bp with different frequencies from 0.04 to 0.21 (Table 1 and figure 1).

Table 1. Allele frequencies

Alleles	274	278	283	288	300	303	307	312	318
Frequencies	0.04	0.05	0.09	0.13	0.19	0.04	0.13	0.12	0.21

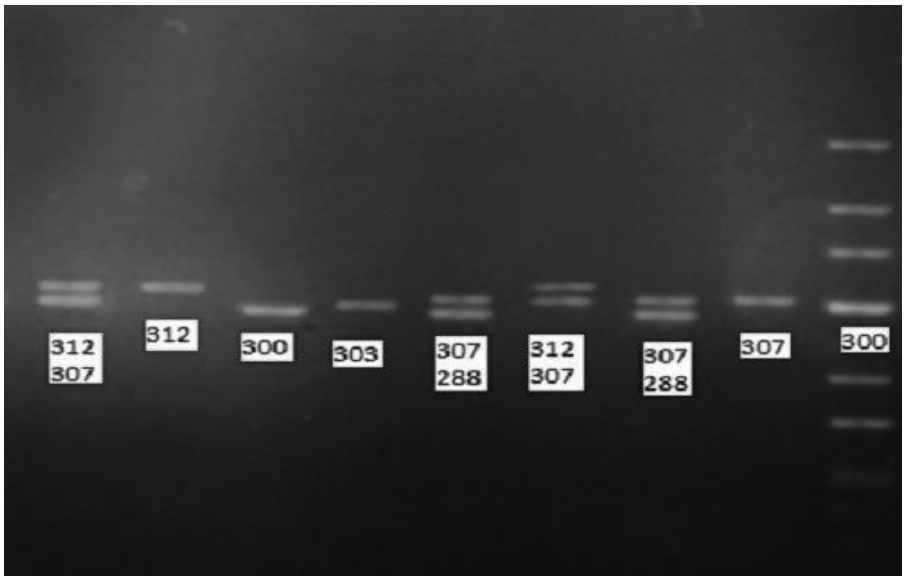

Figure 1. The observed polymorphism of DRB2 gene including 9 alleles in intron 5.

Table 2. Correlation between phenotypic traits based on spearman's correlation constant test.

	FEC	PCV
Famacha	0.731**	-0.625**

* P<0.05, ** P<0.01

Spearman correlation analysis among the phenotypic traits showed a high negative correlation (r=-0.625) between FAMACHA and PCV (Table 2).

The results also showed a high positive correlation (r=7.31) between FEC and FAMACHA (Table 2). There was significant difference between the levels of contamination among different flocks (Table 3). In the present study, microsatellite polymorphism in intron 5

genes DRB2 and its relation to Haemonchus contortus parasites faecal egg count in Ghezel sheep was examined and the results indicated that lambs with 300 homozygous genotype have lower FEC in comparison with other genotypes (Table 4).

Table 3. The differences between the levels of contamination among flocks.

	Flock2	Flock3	Flock4
Flock1	**	**	**
Flock2		**	**
Flock3	**		**

*(P<0.05); ** (P<0.01)

Table 4. Association between genotypes of the MHC microsatellites and the least square means of assessed phenotypic value in *H. contortus* infected Ghezel lambs

Locus	Allele	Allele copy number	Fecal egg count ±S.E	
OLADRB2	300	300	27.34 ± 1.1	P < 0.01

Discussion

In the other studies the number of alleles observed in intron 5 of DRB2 was: 13 alleles in Merino Corriedale Bosworth, Southdown Suffolk, and Border Leicester (Blattman *et al*. 1992); 8 alleles in German Rhonschaf (Janssen *et al*. 2004); 6 alleles in Soay (Patterson *et al*. 1998); and 15 alleles in Peluopy (Castillo *et al*. 2011). Different number of alleles might be due to the presence of polymorphism in intron 5 of DRB2 gene, as reported by Gruszczynska *et al*. (2002) with comparison of microsatellite polymorphism in intron 5 of DRB2 between Heatherhead and Zelaznienska sheep breeds in Poland that they had 11 and 8 alleles in the loci, respectively.

Nematode resistance includes the initiation and maintenance of a host response that prevents, reduces, or clears parasitic infection (Bricarello *et al*, 2004). Resistant animals do not completely reject the disease, but they have a lower parasitic load than susceptible animals, as measured by fewer eggs in their feces. This resistance is based on the immunological capabilities of each individual when challenged with the parasitoses (Gill *et al*, 1991). In another study, a number of phenotypic traits such as fecal egg count (Singleton *et al*. 2011; Saddigi *et al*. 2012), packet cell volume (PCV), (Saddiqi *et al*. 2010; Bishop, 2012), and FAMACHA test

(Torres-Acosta *et al*. 2012) were used to identify the presence of parasitic infections or measurement of animal's resistance against parasites *H. contortus* (Papadopoulos *et al*. 2001).

As high FEC is an indicator of high prevalence of parasites in the gastrointestinal system (Singleton *et al*. 2011), and since *H. contortus* is a blood suckling parasite inhabited in abomasum, it usually causes anemia in the animal (Roberts *et al*. 2000), and reduces hematocrit causing a low PCV (Saddiqi *et al*. 2010; Bishop, 2012). Higher FAMACHA score, more FEC and low PCV could be considered as the indicators of more intense anemia. Our correlation results were in agreement with what was reported by Kaplan *et al*. (2004).

One of the main gastrointestinal organs, where *H. contortus* lives in, is abomasum, which is seriously injured by parasite teeth at the time of blood suckling (Roberts *et al*. 2000). Injuries in the abomasum causes lower HCL secretion, higher pH in abomasum, lower conversion of pepsin into pepsinogen as a result of higher pH, lower protein digestion and finally, lower body weight gain (Roberts *et al*. 2000). Similar to our results, Zaros *et al*. (2014) reported higher FEC accompanied by lower body weight and PCV as a result of *H. contortus* infection in Brazilian Somalis crossbreed sheep. The animals with higher resistance to *H. contortus* have higher PCV, but lower FEC (Zaros *et al*. 2014). In all, from these correlations, it can be concluded that the presence of all these signs (higher FAMACHA scores, low PCV, and high FEC) should be considered for detecting animals under *H. contortus* infection and they can be used for detecting animals with higher resistance against *H. contortus*.

The significant difference among different flocks could be the result of genetic resistance and environmental contamination (Babar *et al*. 2013; Hajializadeh *et al*. 2015).

It has been shown that ovine major histocompatibility complex (MHC) is associated with nematode resistance (Schwaiger *et al*. 1995; Dukkipati *et al*. 2006). MHC consists of two classes (I and II) in which OLADRB2, a microsatellite locus, is located within the class II region of MHC gene (Crawford *et al*. 1995). In the present study, the microsatellite polymorphism in intron 5 of genes DRB2 and its relation to Haemonchus contortus FEC, was not in agreement with the results observed by Castillo *et al*. (2011) as they showed that allele 282 caused *H. contortus* FEC to reduce in Pelibuey sheep. In their experiment, they studied polymorphisms in three microsatellites located at the class I (OMHC1) and class II (OLADRB1, OLADRB2) regions of the MHC and they showed that MHC polymorphisms have an important role in resistance to parasitic infections and it could be used as genetic markers to assist selection and improve parasitic resistance to *H. contortus* (Castillo *et al*. 2011). The reason that we obtained results totally different from those presented by Castillo *et al*. (2011) might be because of difference in allele ranges of the two breeds. It means that allele ranges in Ghezel lambs were between 274 and 318, while allele ranges in Pelibuey lambs were between 262 and 296. In another study, in Ghezel sheep, Hajializadeh Valilou *et al*. (2015) showed a relation between polymorphism DRB1 gene located in class II of MHC complex to Haemonchus contortus parasites FEC.

Conclusion

The overall results of the present study showed that allele 300 could be used as an indicator for selecting animals being more resistant to *H. contortus* as one of the main gastrointestinal parasites in Ghezel sheep breed. Results showed that PCV, FAMACHA and FEC are suitable indicators for detecting animals suffering from *H. contortus* infections. Our results reinforce previous studies that some polymorph alleles of the ovine MHC are involved in determining levels of susceptibility or resistance to infection with gastrointestinal nematode parasites. The results also provide the opportunity to use these alleles as genetic markers of resistance to gastrointestinal nematode parasites including *H. contortus*, leading to the development of those that are better adapted to parasite infestations in the environment. The implication of this research is that polymorphic markers of Ovar-DRB2 can be used in applied animal breeding programs on sheep farms of the region, especially in animals infected with gastrointestinal nematode parasites and located in the similar regions of Asia. Assessment of the precision of genetic evaluations based on molecular information has potential to provide a new perspective on the design of sheep breeding schemes and selection programs.

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