Indian Journal of Experimental Biology
Vol. 58, May 2020, pp.365-369

MHC-*DRB*1 exon 2 polymorphism and its association with faecal egg count of *Haemonchus contortus* in Munjal sheep

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Received 22 May 2019; *revised 21 December 2019*

Haemonchosis is an important disease of small ruminants. Anthelmenthic resistance has instigated the demand of other viable method for control of gastrointestinal parasite. Here, we investigated ovine major histocompatibility complex class II (Ovar MHC II) *DRB*1 exon 2 polymorphism and its association with faecal egg count (FEC) of *Haemonchus contortus* in Munjal population of sheep. Genomic DNA was isolated from blood samples of 46 lambs between 6-7 months of age. The polymorphism in *DRB*1 gene was analyzed using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) technique. PCR products of exon 2 of *DRB*1 gene were digested with *Hae*II, *Bsa*HI and N*ci*I restriction enzymes. Fragment of the *DRB*1 gene comprising 9 bp of the 5' intron and 270 bp of entire exon 2 was successfully amplified. On digestion of 279 bp PCR product with N*ci*I, three genotypes *viz*. A1A1, A2A2 and A1A2 were found with allele frequencies 0.65 and 0.35. *Hae*II enzyme revealed three genotypes A1A1, A1A2 and A2A2 with allele frequencies of A1 and A2 were 0.42 and 0.58. *Bsa*HI enzyme also generated three genotypes A1A1, A1A2 and A2A2 with allele frequencies of A1 and A2 was 0.42 and 0.58. We were able to found polymorphism in *DRB*1 gene but no association could be established between genotypes generated by different restriction enzymes and FEC of *H. contortus* in Munjal sheep.

Keywords: Anthelmenthic resistance, GIT infection, Haemonchosis, Lambs, Livestock, Meat, PCR-RFLP

Munjal is a mutton-type sheep popular among the farmers of Haryana, Rajasthan and Punjab. They are quite big in size, tall, rectangular and massive with a dark brown face. It is economical due to its earlier maturity, faster growth and shorter lambing interval[1]. They are continuously dwindling and the current population exhibit its threatened status[2]. The low productivity of Indian sheep is due to inadequate grazing resources, diseases causing high mortality, morbidity and consequent reduced production, and lack of organized effort for bringing genetic improvement. In India, losses due to gastroenteric roundworms in ruminants have been estimated more than 103 million dollars (USD) per year[3]. Due to its ubiquity and virulence, *Haemonchus contortus* is the most important gastroenteric nematode of sheep in many regions of the world[4]. Anthelmintic resistance is becoming a crucial obstacle for livestock producers worldwide, as there have been cases of resistance reported against every class of anthelmintic drug and at present no new class of anthelmintic drug is commercially available[5]. In light of the emerging anthelmintic resistance and consumer demand for animal products that are produced without the use of chemical products (organically produced), researchers have been engrossed on other viable methods for controlling helminthiasis in sheep.

Major histocompatibility complex (MHC) plays a keyrole in the immune response of vertebrates. It is located on chromosome 20 between bands q15 and q23. MHC class II molecules are found on the surface of antigen-presenting cells (APC), mainly on B lymphocytes, macrophages and dendritic cells, and are responsible for presenting antigens to $CD4^+$ T lymphocytes. Alleles of different MHC genes are known to be associated with disease resistance in sheep[6-9]. Resistance to haemonchosis is an inheritable genetic characteristic associated with sheep breeds[10]. Breeding for enhanced disease resistance offers numerous advantages compared to other methods of control. An increase in the number of resistant individuals should benefit the entire flock as it would reduce pasture contamination. As pasture contamination decreases, the number of larvae available for ingestion by the host animals would also decrease thereby reducing the parasite burden, resulting in lowered production losses[10,11]. As infectious organisms become resistant to the drugs used to control them, the cost of treatment and veterinary care increase faster than the value of animals, making breeding for disease resistance desirable. The most convenient, practical and reliable method for checking for resistance against gastrointestinal tract (GIT) infection is faecal egg count

(FEC)[12]. A generally accepted guideline is that 0-500 of trichostrongylid eggs per gram faeces (EPG) represents a low parasite burden; 500-2000 EPG a moderate burden; and >2000 trichostrongylid EPG is considered as a heavy burden[13,14]. For *H. contortus* infestation, a FEC >2000+ is considered clinically significant for drenching[15,16].

Among *Ovar* MHC class II genes, the expressed *DRB*1 locus has been found to be highly polymorphic[17]. The allelic diversity of Ovar-*DRB1* exon 2 had been well studied and a DNA-based genotyping method was developed for rapid analysis of allelic diversity at the *DRB1* locus in sheep[18]. Its polymorphism and associations with body weight, milk contents and weight traits have also been studied in different breeds[19,20]. The polymorphism of *DRB* 1 gene plays important role in resistance to nematode infection in Suffolk sheep[21]. In this study, we analyzed the relationship between ovine MHC-*DRB*1 gene polymorphism and FEC of *Haemonchus contortus* infection by PCR-RFLP method in Munjal sheep.

Material and Methods

Collection of faecal and blood samples

The study was conducted on 6-7 month old Munjal lambs (46 animals) maintained at sheep breeding farm of Department of Animal Genetics and Breeding, College of Veterinary Sciences, Lala Lajpat Rai University of Veterinary and Animal Sciences, Hisar, Haryana. Lambs were derived from 25 rams and 46 different ewes. Rams were not used to produce ewes. Faecal samples were collected before and after deworming (albendazole 5 mg/kg body wt.). Faecal samples were collected from rectum manually to estimate the FEC and maintained at 4°C. FEC was done immediately after collection of samples by modified McMaster's technique. Average of all readings was taken to estimate egg per gram (EPG). Coproculture was done on the day of collection of faeces. The infective larvae (L_3) were collected according to Baermann technique[22] and identified[23]. About 5 mL of peripheral blood was also collected from same animal from which faecal samples were collected. Approval of samples collection was taken from Institutional Animal Ethical Committee (Agenda item no.16 dated 16/1/13).

PCR-RFLP

Genomic DNA was isolated from 5 mL of blood samples as described by Sambrook & Russel[24] with minor modifications. The purity of DNA was evaluated by UV spectrophotometer (Nanodrop, Thermo scientific, USA). The published primer pair (forward primer: 5'-TCT CTG CAG CAC ATT TCC TGG-3', Reverse Primer: 5'-CTC GCC GCT GCA CAG TGA AAC-3') were used to amplify exon 2 of *DRB*1[25]. PCR was carried out in a final volume of 25 μL reaction mixture containing 200 ng of template DNA, 10X PCR buffer, 4 mM of dNTPs, 2 μM of each primer and 1.25 U of *Taq* DNA polymerase. Amplification was carried out in thermal cycler. The conditions of PCR were one cycle for 3 min at 95°C, followed by 35 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s with final extension at 72°C for 10 min.7.5 μL of the mixture PCR were digested at 37°C with 5 U of *Hae*II (New England Biolabs,Canada), 2.5 U *Bsa*HI (New England Biolabs) for 5 h and 2.5 U *Nci*1(New England Biolabs) for 30 min. Digested samples were resolved by a 3% agarose gel electrophoresis for 2-3 h at 5 V/cm.

Cloning and sequencing of PCR products

PCR amplified *DRB*1 gene was cloned using the pJET1.2/blunt cloning vector (Fermentas,USA). The JM107 strain of *Escherichia coli* (Fermentas) was used as a host system for transformation. Clones were screened by colony touch PCR. Plasmid was isolated by alkaline lysis method. Recombinant plasmid was sequenced using BigDye® Terminator v3.1Cycle Sequencing Kit at Applied Biosystem 3130 XL Genetic Analyzer plateform available in the Dept of Animal Biotechnology, LUVAS, Haryana.

Statistical analysis

The genotype frequencies association with low and high FEC was analyzed by chi- square test (with p value less than 0.05 for significant association).

Results and Discussion

Measurement of FEC is the standard method for assessment of the level of resistance to gastrointestinal nematodes (GIN)[26]. In this study, mean FEC was found to be 2611.30±340.33 (Fig. 1). Larval composition after coproculture was found to be 91% *H. contortus*, 7% *Trichostrongyles* spp. and 2% *Strongyloides* spp. Thus, indicating *H. contortus* to be the major nematode affecting the lambs. Animals were grouped on clinical significant limit of FEC, for high FEC >2000 EPG and low FEC <2000 EPG.

A number of studies around the world have attempted to identify relationships between genetic resistance to GIN and various genes and genetic markers Four QTL

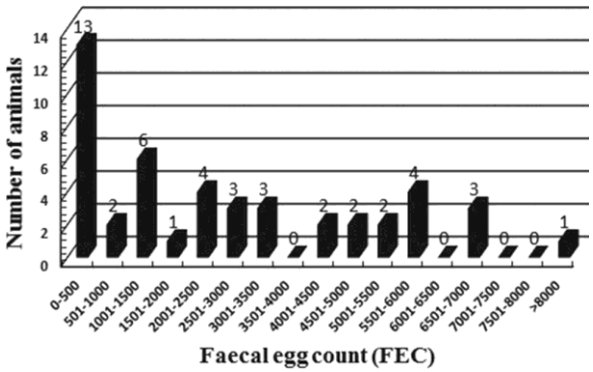

Fig. 1 — Faecal egg count of strongyles

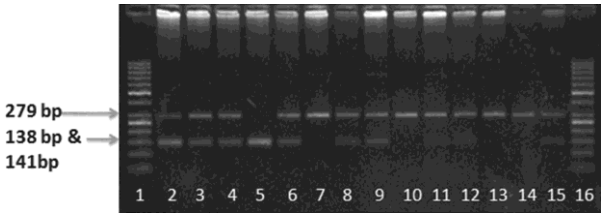

Fig. 2 — Resolution of *Nci*I digested PCR products on 3% agarose gel. [Lane 1 & 16: 50 bp ladder; Lanes 2,3,4,6,8,9,11,12,15: A1A2 (279 bp,138 bp,141 bp); Lane 5: A2A2 (138 bp,141 bp); and Lanes 7,10,13,14: A1A1 (279 bp)]

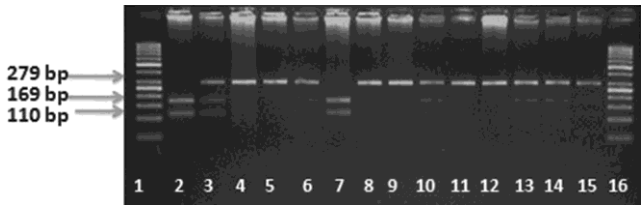

Fig. 3 — Resolution of *Hae*II digested PCR products on 3% agarose gel. [Lanes 1 &16: 50 bp ladder; Lanes 2,7: A2A2(169 bp, 110 bp); Lanes 4,5,8,9,11,12: A1A1 (279 bp); and Lanes 3,6,10,13,14,15: A1A2 (279 bp, 169 bp, 110 bp)]

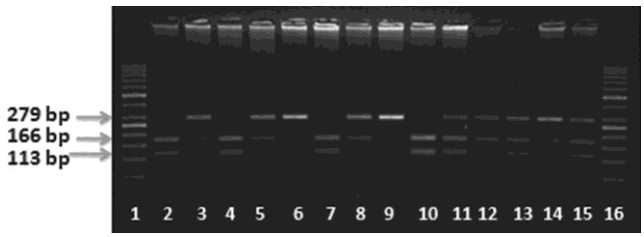

Fig. 4 — Resolution of *Bsa*H1 digested PCR products on 3% agarose gel. [Lane 1 & 16: 50 bp ladder; Lanes 2,4,7,10: A2A2 (166 bp, 113 bp); Lanes 6,9 A1A1 (279 bp); and Lanes 3,5,8,11,12,13,14,15: A1A2 (279 bp, 166 bp, 113 bp).

Table 1 — Genotypic frequencies of *DRB*1 with *Nci*I, *Hae*II, *Bsa*HI in Munjal sheep

Animals	*Nci*I			*Hae*II			*Bsa*HI		
	A1A1	A1A2	A2A2	A1A1	A1A2	A2A2	A1A1	A1A2	A2A2
High FEC	0.46 (11)	0.37 (9)	0.17 (4)	0.50 (12)	0.38 (9)	0.12 (3)	0.25 (6)	0.46 (11)	0.29 (7)
Low FEC	0.5 (11)	0.41 (9)	0.09 (2)	0.45 (10)	0.41 (9)	0.14 (3)	0.36 (8)	0.45 (10)	0.18 (4)
Chi Square test (P <0.05)	Non significant			Non significant			Non significant		

[Figures in parentheses are number of observations]

regions on sheep chromosome numbers 5, 12, 13, and 21 have been reported in Romane × Martinik Black Belly back cross lamb that had an important role in genetic resistance to *H. contortus*[8]. Similar study suggested other regions of 1, 3, 4, 5, 7, 19, 20 and 24 chromosomes that were involved in GIN resistance[9]. Likewise, evidences were found for QTL on chromosomes 2, 3, 14 and 20 that were associated with parasitic infections in Scottish blackface sheep[27].

Amplicon size of 279 bp fragment of the *DRB*1 gene comprising 9 bp of the 5' intron and 270 bp of entire exon 2 was successfully amplified. On digestion of 279 bp PCR product with N*ci*I, three genotypes A1A1 (279 bp), A2A2 (138 bp, 141 bp) and A1A2 (279 bp, 138 bp, 141 bp) were found with allele frequencies 0.65 and 0.35 (Fig. 2 & Table 1). *Nci*I generated three genotype patterns which were also reported in the Suffolk sheep by PCR RFLP method[28]. *Hae*II enzyme revealed three genotypes A1A1 (279 bp), A1A2 (279 bp, 169 bp, 110 bp) and A2A2 (169 bp, 110 bp) with allele frequencies of A1 and A2 were 0.42 and 0.58 (Fig. 3 & Table 1). *Bsa*HI enzyme also revealed three genotypes A1A1 (279 bp), A1A2 (279 bp, 166 bp, 113 bp) and A2A2 (166 bp, 113 bp) with allele frequencies of A1 and A2 was 0.42 and 0.58 (Fig. 4 & Table 1). To the best of our knowledge, no information is available regarding use of last two enzymes for digestion of *DRB*1 gene in the literature. By applying chi-square test(P <0.05), no significant association was found between the genotypes generated by different restriction enzymes and animals of high and low FEC. The RFLP profiles of *Ovar-DRB*1 alleles were verified by sequencing the cloned products and the observed patterns of fragments matched exactly with those predicted from DNA sequences (Suppl. Fig. S1. *All supplementary data are available only online along with the respective paper at NOPR repository at http://nopr.res.in*). By using these enzymes, three transition sites were identified i.e., 140 (G → A), 166 (G → A) and 165 (A →G), marked in Fig. S1.

However, we found polymorphism in *DRB*1exon 2 but could not establish significant association between genotypes frequencies generated by these enzymes and FEC. There are various studies across the world where correlation had been found with specific breeds of sheep. In Suffolk and Texel sheep, variation at the ovine MHC-*DRB*1 locus was analyzed with variation in FEC and found that in Suffolk breed one *Ovar-DRB*1 allele was associated with a decrease in FEC and two alleles with an increase in FEC. But there was no evidence for an association between *Ovar-DRB*1 alleles and FEC in the Texel breed[21].

Reinforcing previous evidences also in Ghezel sheep, some alleles of the ovine MHC were involved in determining the levels of susceptibility or resistance to infection with GIN[29]. Similarly, in Merino sheep, the highly resistant sire was homozygous at the MHC, no significant association was found between any band or haplotype and FEC[30]. Analysis of the restriction patterns of exon 2 of *DRB*1 revealed significant association with hydatidosis resistance and susceptibility in Chinese Merino sheep, Duolang sheep, and Kazakh sheep[31]. In India, lack of association of resistance towards *H. contortus* infection with PCR-RFLP of MHC-DYA gene was found in Rambouillet crossbred sheep[32]. Polymorphism in exon 2 of DRB3 gene of native sheep and goat populations of Himachal Pradesh was studied using PCR-RFLP and DNA sequencing and observed significant correlation between SNP and disease resistance trait[33]. Ten SNP within six candidate genes (CIITA, ATP2A3, HSPA8, STAT5B, ESYT1 and SERPING1) were associated with FEC in goats with a nominal significance level of $P < 0.05$[35]. These findings suggested that polymorphism of the MHC is closely associated with disease resistance or susceptibility against nematode infection in some sheep breeds. We could not find any association may be due to difference in genetic background of the breed. Other reasons may be the difference in experiment designing, for example, the choice of the parasite, the method of genetic typing and the age of the animals.

Conclusion

There is no single strategy to breed sheep without GIT infection which are causing great loss to the farmers. Breeding resistant animals along with use of antihelminthics can help to overcome this problem until we find a permanent or more substantial solution. On the basis of PCR-RFLP using three restriction enzymes (*Hae*II, *Bsa*HI and*Nci* I), no association was found between resistance of *Haemonchus contortus* and *DRB*1 gene polymorphism in Munjal sheep. Low sample size could be the one of the reason for not finding any association. This is a preliminary work, and further thorough studies require ascertaining MHC-*DRB*1 suitability as genetic marker for resistance toward *H. contortus* infection in Munjal sheep.

Acknowledgement

We are grateful to DBT, Government of India (GOI) for providing grant to conduct research work under M.VSc (Animal Biotechnology) programme.

Conflicts of interest

Authors have declared no conflict of interests.

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