Association of caprine lymphocyte antigen-DRB3 gene with gastrointestinal nematode resistance in Sirohi and Barbari breeds of goat

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ABSTRACT
The present study was conducted on Sirohi and Barbari goat breeds to investigate the association of polymorphic variants with gastrointestinal nematode resistance in these goats. A total of 60 animals comprising 30 each of the two breeds selected randomly were included in the study. Genomic DNA isolated from venous blood was amplified for caprine lymphocyte antigen-DRB3 gene with specific primer by standardizing and optimising the PCR protocols. The PCR product of genomic DNA isolated from kids of Sirohi and Barbari breeds of goat on digestion with restriction enzyme Pst1 revealed three genotypes viz., AA, AB and BB with genotypic frequencies of 0.53, 0.37 and 0.10, respectively in Sirohi and 0.73, 0.20 and 0.07 in Barbari breed. The frequencies of A and B alleles were 0.72 and 0.28 in Sirohi and 0.83 and 0.17 in Barbari breed, respectively. Both the breeds were in Hardy-Weinberg equilibrium for these variants and were homogeneous with respect to their distribution. The effects of breed, genotype as well as their interaction were found to be non-significant. There were no significant association between genotypes at CLA-DRB3 locus and eggs per gram (EPG) count in both the breeds. It was concluded that this CLA-DRB3 gene locus cannot be used for selection of goats for nematode resistance in the present herd.

Key word: Barbari, CLA-DRB3 gene, PCR-RFLP, Sirohi.

INTRODUCTION
Gastrointestinal nematodes are perhaps the most important parasites of domestic goat and sheep world-wide causing significant morbidity and loss of production. These infections can be treated by anthelmintic chemotherapy; however, treatment is costly and drug resistance has evolved in all major parasite species. For these reasons, selection for parasite resistance in domestic goat and sheep has been undertaken in many countries (Beh and Maddox, 1996). The genetic basis of host variation in resistance to parasitic infection is of specific interest to animal breeders (Beh and Maddox, 1996 and Woolaston and Baker, 1996) and breeding for resistance to nematode infection provides an additional strategy to complement the use of anthelmintics in sheep and goat husbandry practices. Resistant animals can be selected on the basis of low faecal egg count (Eady et al., 2003 and Kahn et al., 2003). A number of genes have been associated with nematode resistance in sheep and goat. The major histocompatibility complex (MHC) has been known as the key locus for disease resistance. High degree of polymorphism for MHC genes in a population is itself an explanation of the capability of host immune systems to attack such a wide variety of antigens by recognizing them as not of their own.

The most polymorphic among the MHC gene is DRB locus (Amills et al., 1996). The MHC of the goat, also named the caprine lymphocyte antigen (CLA) or goat lymphocyte antigen (GoLA) system has been shown to be similar to that of sheep and cattle which have two expressed class II antigens, DQ and DR. Class II MHC genes have been extensively characterized in sheep and cattle, whereas in goats only four goat class II genes (Cahi-DRA, Cahi-DRB, Cahi-DYA Cahi-DIB) have been identified to date (Amills et al., 2004). In view of paucity of information on MHC gene polymorphism and its association with nematode resistance in goats, the present study was taken up to determine association of various polymorphic variants of exon 2 of caprine lymphocyte antigen-DRB3 gene with gastrointestinal nematode resistance using PCR-RFLP method in Sirohi and Barbari breeds of goat.

MATERIALS AND METHODS
Experimental animals and genomic DNA isolation: The present investigation was conducted on Sirohi and Barbari goat breeds maintained at Goat Rearing Farm, Amanala under RKVY Project on goat, Department of Animal Genetics and Breeding, College of Veterinary Science and Animal Husbandry, NDVSU, Jabalpur. A random sample of 100 goats

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above three months of age which had not been given any anthelmintic treatment during last sixty days comprising 50 each of Sirohi and Barbari breeds was screened for faecal egg count by the McMaster method (Soulsby, 1982) at monthly interval for three consecutive months. The animal showing more than 1000 EPG continuously for three months was considered as high worm load or 'susceptible group' and those showing less than 500 EPG continuously for three months were considered as low worm load or ‘resistant group’. From these animals 60 animals comprising 30 each of the two breeds were selected randomly for study of MHC class II CLA-DRB3 gene for association studies.

### PCR amplification and restriction digestion: Genomic DNA was extracted from venous blood as per the method described by John et al. (1991) with minor modifications. The concentration and purity of DNA were checked by UV-spectrophotometry. DNA samples with an OD 260/280 ratio of 1.7 to 1.9 were further subjected to agarose gel electrophoresis for quality check. The DNA having good quality intact bands with no smearing was used for further analysis. The following primer sequence was used for amplification of exon 2 of CLA-DRB3 gene as described by Amills et al. (1995).

**Forward primer 5’-TATCCGTCCTCTGCAGCACCATTTC-3’**

**Reverse primer 5’-TCGCCGCTGACACTGAAACTCTC-3’**

PCR amplification of MHC class II CLA-DRB3 gene was carried out in a final reaction volume of 25 μl. A master mix for desired number of samples was prepared and aliquoted 22 μl in each PCR tube. 3 μl genomic DNA (30 ng/μl) was added in each tube to make the final volume 25 μl. The best results were obtained when amplification was performed in PCR thermal cycler (Eppendorf Germany) programmed for 32 cycles with an initial denaturation at 94°C for 10 minutes, final denaturation at 94°C for 50 second, annealing at 57°C for 50 second and extension at 72°C for 1 minute with a final extension at 72°C for 10 minutes. The amplified product was visualized as a single compact band by UV transilluminator and photographed. The restriction enzyme PstI was used in the present study to digest the PCR product. Restriction digestion of the PCR product was performed in a total 30μl reaction mixture having 10X Buffer Tango 2 μl, PCR reaction mixture 10 μl, restriction enzyme (10units/μl) 1 μl and 17 μl nuclease free water. The reaction mixture was spun for few seconds for uniform mixing and then incubated at 37°C for 30 minutes in the water bath. After restriction enzyme digestion, the digest product mixture was electrophoresed on 2 % agarose gel and PCR-RFLP bands were visualized under UV light and documented by Geldoc gel documentation system (Bio-Rad, USA) and recorded after comparing with band size reported by previous workers. Genotyping of MHC Class II CLA-DRB3 gene locus was carried out according to the band pattern of respective genotypes.

### Statistical analysis of PCR-RFLP data: Gene and genotype frequencies were estimated using Popgene 32(version 1.32), microsoft Windows-based freeware for population genetic analysis (Yeh et al., 1999) and the population was tested for genetic equilibrium at this locus. Homogeneity of distribution of various polymorphic variants at exon 2 of CLA-DRB3 gene across the two breeds was studied using Chi-square test (Steel and Torrie, 1980). The association between polymorphic variants at exon 2 of CLA-DRB3 gene and EPG classes (high worm load and low worm load) was studied using Chi-square test of independence (Steel and Torrie, 1980). If any expected cell frequency fell below five, it was pooled with adjacent cell frequency. The significance of effect of polymorphic variants at this locus on EPG counts was studied by least squares analysis of variance (Harvey, 1990) employing the following statistical model.

$$Y_{ij} = \mu + a_i + B_j + (aB)_{ij} + e_{ijk}$$

Where, $Y_{ij}$ is the EPG of $k^{th}$ Goat of the $i^{th}$ breed and $i^{th}$genotype. $\mu$ is overall mean. $a_i$ is set of random cross classified effects due to genotypes. $B_i$ is set of fixed effects due to breeds. $(aB)_{ij}$ is interaction between $i^{th}$ genotype with $j^{th}$ breed. $e_{ijk}$ is random error assumed to be normally and independently distributed with mean zero and a common variance.

### RESULTS AND DISCUSSION

An amplified PCR product of 285bp size was observed in both the breeds i.e. Sirohi and Barbari on amplification of exon 2 of CLA –DRB3 gene (Plate 1a, b). The PCR product of similar bp size has also been reported by Ahmed and Othman (2006) in Egyptian Goat, Baghizadeh et al. (2009) in Raeini Cashmere goat, Hernandez (2011) in goats of the central highlands of Veracruz, Zhao et al. (2011) in ten domestic goats in southwest china and Singh et al. (2012) in Jamunapari breed of goats.

The PCR product digested with restriction endonuclease PstI revealed the existence of three different restriction patterns for PstI (241bp/44bp, 252bp/33bp, and 252bp/241bp/44bp/33bp) and two alleles (A and B) indicating that CLA –DRB3 gene locus under study was polymorphic for restriction endonuclease PstI (Plate 2a, b). Small fragments of 44bp and 33bp sizes were invisible on gel. This PstI restriction pattern is in congruence with finding
of Zhao et al. (2011) in ten goat breeds of China. However, besides three band patterns and two alleles as obtained in present study, an additional band pattern (158bp/79bp/48bp) and allele (C) was reported in their study. Further, Ahmed and Othman (2006), Baghizadeh et al. (2009) and Singh et al. (2012) have also reported three band pattern (270bp/15bp, 270bp/226bp/44bp/15bp and 226bp/44bp/15bp) and two alleles in Egyptian Goat, Raeini Cashmere goat and Jamunapari breed of goat, respectively. These differences in number of alleles and sizes of restriction fragments in various breeds reflect the existence of extensive polymorphism at the CLA-DRB3 locus resulting from multiple nucleotide substitutions between alleles.

Frequencies of genotypes and alleles: Only three genotypes were encountered in the samples of two breeds included in the present study i.e. AA, AB and BB. In Sirohi breed the frequencies of genotype AA, AB and BB were found to be 0.53, 0.37 and 0.10, respectively. Whereas, the corresponding genotypic frequencies in Barbari breed were found to be 0.73, 0.20 and 0.07, respectively. The frequencies of alleles A and B at the locus under study were 0.72 and 0.28, respectively in Sirohi and 0.83 and 0.17, respectively in Barbari goats. Survey of relevant literature revealed marked differences in genotypic and allelic frequencies at this locus in different breeds /populations. In Changthangi goat, the respective genotypic frequencies of AA, AB and BB have been reported to be 0.07, 0.72 and 0.20 with allelic frequencies of A and B to be 0.43 and 0.57, respectively (Sheikh et al., 2006), in Egyptian goat, Raeini Cashmere goat and Jamunapari goats, the corresponding genotypic frequencies were reported to be 0.00, 0.705 and 0.295; 0.21, 0.59 and 0.20; and 0.054, 0.22 and 0.724, respectively. Further the allelic frequencies of A and B in the above three breeds have been reported to be 0.352 and 0.648; 0.505 and 0.495 and 0.165 and 0.83, respectively (Ahmed and Othman, 2006; Baghizadeh et al., 2009 and Singh et al., 2012).

In the study of Zhao et al. (2011) on ten goat breeds of China the genotypic frequencies of AA varied from 0.0 to 0.59, frequency of AB varied from 0.10 to 0.80 and of BB varied from 0.20 to 0.86. In one breed fourth genotype i.e. CC was also reported with a frequencies of 0.57. These differences in allelic frequencies might be due to the fact that the different breeds populations maintained under the different sets of environmental conditions are subjected to different evolutionary forces to varying degree. In addition, sampling fluctuations may also contribute to the differences in allelic frequencies in different breeds and populations. Futher, mixing of populations from different geographical locations and hybridization accompanied by genetic difference might
have also contributed to this high genetic diversity among breeds.

**Test for genetic equilibrium:** The test for genetic equilibrium was carried out by comparing observed genotypic frequencies with expected genotypic frequencies calculated from gene frequencies. The non-significant Chi-square value observed in the present study for both the breeds revealed that these goat breeds (Sirohi and Barbari) were in Hardy-Weinberg equilibrium. Similar findings have been reported by Baghizadeh et al. (2009) in Raeini Cashmere goat; Hernandez (2011) in goats of the central highlands of Veracruz and Singh et al. (2012) in Jamunapari breed of goats. Zhao et al. (2011) also reported six goat breeds to be in Hardy-Weinberg equilibrium for the locus under consideration. However, other five breeds were not founded to be in Hardy-Weinberg equilibrium in their study.

This may be due to random mating for CLA-DRB3 genotypes over the generations in these breeds besides any one of the following causes which might have brought this equilibrium condition: the different CLA-DRB3 alleles have no selective advantage over each other, the different genotypes are having equal reproductive and survival rates, superiority of heterozygotes and state of balance between different forces which change the gene frequencies. The non-significant (p<0.05) Chi-square value in both the breeds of goat are the indication of no differences in their genotypic distribution with respect to gene frequency. However, the causes that might have been working to maintain equilibrium condition in addition to random mating could not be ascertained.

**Homogeneity of distribution of CLA-DRB3 genotypes in two breeds:** Distribution of various genotypes at any locus may vary in different breeds with varied gene frequencies. The test of homogeneity was used so as to reveal whether the two breeds differed significantly from each other in respect of distribution of genotypes at the locus under study which would tell likeness (homogeneous) or unlikeness (heterogeneous) of the two breeds viz., Sirohi and Barbari. Non-significant Chi-square value indicated that the two breeds were homogeneous with respect to genotype frequency at this locus. Contrary to our finding, Zhao et al. (2011) reported significant differences in genotype frequencies among ten goat breeds of China. These differences might be due to the fact that Sirohi and Barbari breeds have not phylogenetically diversified much whereas ten goat breeds of China under references have evolutionarily diversified much from one other.

**Association of MHC Class II CLA-DRB3 gene polymorphic variants with faecal egg count:** The animals under study were classified (observed frequencies) according to two attributes viz., genotype (AA, AB and BB) and the egg count groups (low EPG and high EPG) in the form of a contingency table. The association between these two attributes i.e. genotyped and egg count groups were tested employing Chi-square test of independence. Under the null hypothesis that the two attributes are independent the expected frequencies were calculated and Chi-square value was obtained. Non-significant Chi-square value indicated that there was no any association between the genotype CLA-DRB3 locus under study and the egg count groups.

**Effect of MHC Class II CLA-DRB3 gene polymorphic variants on faecal egg count:** To study the effect of CLA-DRB3 gene polymorphism on faecal egg count in the two breeds of goats, least squares analysis of variance was employed incorporating breed (fixed effect), genotype (random effect) and their interaction in the model. It was revealed that the effects of breed, genotype as well as their interaction were all non-significant. Mean egg counts in Sirohi and Barbari breeds were found to be 556.42±1.27 and 663.59±1.33, respectively, indicating slightly higher mean EPG in Barbari as compared to Sirohi breed. Highest mean egg count was recorded for animals of genotype AB followed by genotype BB and genotype AA. In Sirohi breed, genotype BB recorded lowest mean egg count whereas in Barbari breed lowest mean egg count was observed for genotype AA (Table 2).

In the present study, no association was found between genotypes of CLA-DRB3 locus and gastrointestinal nematode infection using egg per gram in faeces as a phenotypic indicator (Table1). The least squares analysis of variance also revealed non-significant effect of genotypes at this locus on egg per gram. The effects of breed and interaction between breed and genotype were also non-significant. The parallel studies in goats appear to be meagre. Khobra et al. (2013) also reported that there was no difference among A and B allelic pattern with respect to egg per gram in Jamunapari breed of goat. However, they also concluded that C allele pattern was found exclusively in high EPG class. Some interesting reports on association of alleles at DRB and other loci faecal egg count have appeared in sheep. In

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>Degree of freedom</th>
<th>Mean Sum of squares</th>
<th>F-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Breed</td>
<td>01</td>
<td>0.044</td>
<td>0.23&lt;sup&gt;NS&lt;/sup&gt;</td>
</tr>
<tr>
<td>Genotype</td>
<td>02</td>
<td>0.106</td>
<td>0.55&lt;sup&gt;NS&lt;/sup&gt;</td>
</tr>
<tr>
<td>Breed X Genotype</td>
<td>02</td>
<td>0.101</td>
<td>0.52&lt;sup&gt;NS&lt;/sup&gt;</td>
</tr>
<tr>
<td>Error</td>
<td>54</td>
<td>0.193</td>
<td></td>
</tr>
</tbody>
</table>

<sup>NS</sup>=Non-significant
TABLE 2: Least squares means and standard errors for faecal egg count

<table>
<thead>
<tr>
<th>Effect</th>
<th>EPG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Overall mean</td>
<td>607.65±1.30(60)</td>
</tr>
<tr>
<td>Breed (B)</td>
<td></td>
</tr>
<tr>
<td>Sirohi (B₁)</td>
<td>556.42±1.27(30)</td>
</tr>
<tr>
<td>Barbari (B₂)</td>
<td>663.59±1.33(30)</td>
</tr>
<tr>
<td>Genotype (G)</td>
<td></td>
</tr>
<tr>
<td>AA (G₁)</td>
<td>520.83±1.18(38)</td>
</tr>
<tr>
<td>BB(G₂)</td>
<td>600.90±1.59(05)</td>
</tr>
<tr>
<td>AB (G₃)</td>
<td>716.97±1.29(17)</td>
</tr>
<tr>
<td>B x G Interaction</td>
<td></td>
</tr>
<tr>
<td>B₁G₁</td>
<td>589.93±1.29(16)</td>
</tr>
<tr>
<td>B₁G₂</td>
<td>437.93±1.79(03)</td>
</tr>
<tr>
<td>B₁G₃</td>
<td>666.96±1.36(11)</td>
</tr>
<tr>
<td>B₂G₁</td>
<td>459.83±1.24(22)</td>
</tr>
<tr>
<td>B₂G₂</td>
<td>824.70±2.05(02)</td>
</tr>
<tr>
<td>B₂G₃</td>
<td>770.73±1.51(06)</td>
</tr>
</tbody>
</table>

Figures in parenthesis are number of animals in that particular group

Suffolk breed of sheep one Ovar-DRB allele was associated with a decrease in faecal egg count and two alleles with an increase in faecal egg count (Sayer et al., 2005). But no such association was found in Texel breed of sheep. As revealed by least squares analysis substitution of most common allele (I) of MHC-DRB1 locus by allele G₂ resulted in a 58 fold reduction in faecal egg count in 6 month old lambs and a 22 fold reduction in 5 month old lambs in Scottish Blackface sheep (Schwaiger et al., 1995) suggesting that the MHC plays an important role in the development of resistance to nematode infection. In Texel breed of sheep B haplotype at interferon-γ locus was associated with resistance to nematode infection as measured by faecal egg count while no any such association could be found in Suffolk breed (Sayer et al., 2006). One of the probable reasons for the absence of an association between CLA-DRB3 exon 2 alleles and egg per gram in Sirohi and Barbari in present study may be that the linkage disequilibrium to locus under study has not been established in these breeds, besides various tangible and non-tangible environmental factors and interaction among them.

CONCLUSIONS

Both the breeds viz., Sirohi and Barbari were polymorphic for the exon 2 locus of CLA-DRB3 gene under study with respect to PstI restriction endonuclease. Two alleles (A and B) and three genotypes (AA, BB and AB) were revealed in both the breeds by PCR-RFLP analysis using PstI restriction enzyme. Both the breeds were in Hardy–Weinberg equilibrium for this region of CLA-DRB3 gene. Also the two breeds were homogeneous with respect to distribution of genotypes at locus under study. In the present study, no association was found between genotypes of CLA-DRB3 locus and gastrointestinal nematode infection using egg per gram in faeces as a phenotypic indicator. The least squares analysis of variance also revealed non-significant effect of genotypes at this locus on egg per gram. The effects of breed and interaction between breed and genotype were also non-significant.

REFERENCES


