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Caprine MHC gene polymorphism and its association with endoparasitic infestation (*Haemonchus contortus*) in Indian goat breeds

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Abstract: The present study examined the variability of MHC class II DRB exon 2 gene using restriction fragment length polymorphism analysis of PCR-amplified fragments PCR-(RFLP), and its association with *Haemonchus contortus* infestation in Salem Black and Tellicherry goat population. Animals were naturally exposed to mixed infestation of endoparasites, predominantly *Haemonchus contortus*. Pooled fecal coproculture and larval identification showed predominant presence of haemonchus (L3) larva. Fecal egg count (FEC) and packed cell volume (PCV) were used as indicator traits. All the three studied loci, *TaqI*, *BsaI*, and *BsaHI*, were polymorphic having two alleles and three genotypes. The loci showed low to moderate values of polymorphic information content in both breeds. The mean fecal egg count estimates were 477.12 ± 34.14 and 730.42 ± 41.19 eggs per gram of feces for Salem Black and Tellicherry goats, respectively. The mean PCV values were within the normal range in both breeds; however, they showed negative correlation with FEC values. There was variation among genotypes in mean values of FEC and PCV for all loci; however, the effect of genotypes on indicator traits was found to be statistically nonsignificant ($P \leq 0.05$).

Key words: Endoparasite, goat, MHC gene, PCR-RFLP, polymorphism

1. Introduction

Haemonchus contortus is a blood-sucking parasite, mainly found in abomasum of sheep and goats; it causes massive hemorrhage, anemia, and mortality especially in young animals [1]. Classically, anthelmintics are the first line of defense for endoparasitic infestations. However, their widespread use led to the development of drug resistance in the parasites [2]. Selection and breeding for increased resistance is effective because it is manageable and permanent [3] and does not have any residual ill effects. In sheep, there are a number of breeds that have innate resistance towards gastrointestinal nematodes like Barbados Blackbelly, U.S. St. Croix, Indonesian Thin tail, Indian Garole and African Red Maasai breeds [4,5]. Among goats, there is one certain example of "haemonchotolerant" breed, which is the Nigerian West African Dwarf goat that has developed an innate ability to resist and launch a relatively quick immune response against *Haemonchus* infestation [6,7]. Some Indian breeds like Avikalin [8], Barbari and Jamunapari [9], Munjal, Malpura, and Coimbatore [10,11] have also shown resistance towards gastrointestinal nematodiasis in various studies.

A number of genes and variation within them have been attributed to be associated with resistance or susceptibility to gastrointestinal nematodiasis, among which the major histocompatibility complex (MHC) is the most widely studied one in disease resistance research in small ruminants [12]. MHC class II genes are central in conferring resistance/susceptibility to parasitic infestation [13]. Caprine MHC is located on chromosome number 23 also known as caprine lymphocyte antigen (CLA) or goat lymphocyte antigen (GoLA) which has three subgroups: MHC class I, MHC class II, MHC class III. Among these, the class II molecule plays a pivotal role in the initiation of the immune response by presenting exogenous antigens to helper T-lymphocytes [14]. It is classified into two subtypes: DQ and DR [15]. Among these two subtypes, the DRB locus is the most polymorphic [16] and is functionally

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responsible for the differences among individuals in the immune response against infectious agents [17,18]. The DRB fragment has been used as a putative genetic marker in Suffolk and Texel sheep breeds for resistance/susceptibility against gastrointestinal nematodes [19]. In goats, polymorphism in exon 2 of caprine MHC class II DRB gene has been reported in Chinese goats [14], Raeini Cashmere goats [20], Jamunapari goats [21], Rohilkhandi goats [22, 23] and Marwari goats [24] at different SNP loci. This may help in the development of markers for identification of resistant animals and formulation of breeding strategy for production of disease-resistant livestock [25].

In the current study, two indigenous goat breeds namely Salem Black and Tellicherry were used as the model organisms. Both these breeds are found in hot and humid climate of India (Tamilnadu and Kerala states). They are well-adopted in the local climate and thus are continuously and naturally exposed to a variety of gastrointestinal parasitic infestations. Therefore, the current study was aimed to investigate the genetic polymorphism in MHC class II DRB exon 2 gene in two tropical Indian goat breeds and to study the effects of different genotypes on indicator traits associated with gastrointestinal parasitism, especially *Haemonchus* spp.

2. Materials and methods

2.1. Selection of animals and sample collection

Two Indian goat breeds were considered for this study viz., Salem Black and Tellicherry. A total of 207 animals consisting of 100 Salem Black and 107 Tellicherry goats were selected from Government Sheep and Goat farm, Chinnasalem, Villupuram District of Tamilnadu, India. The animals were first screened for the presence of gastrointestinal infestation. Once it was established that the herd carried gastrointestinal nematode infestation, further sample collection was performed. Final sample collection and analysis was carried out between November 2016 and January 2017. The scheduled deworming was delayed and sample collection was planned 120 days postdeworming so that the animals can naturally acquire the infestation from grazing grounds. Bucks and does were selected from a randomly mating population; however, animals in periparturient period or advance pregnancy stage, or young ones up to the age of 4 months were excluded from the study. Care was taken to include the animals from the same age group and physiological status during sample collection. Blood and fecal samples were collected from all animals. Blood samples were collected as per approval of institutional animal ethical committee for isolation of genomic DNA and packed cell volume estimation. The fecal samples were used for estimation of fecal egg count.

2.2. Estimation of indicator trait

About 5 mL of blood was collected under sterile conditions from the jugular vein of goats using 2.7% EDTA as an anticoagulant in a 15-mL polypropylene centrifuge tube. One milliliter of blood was used for PCV estimation and the remaining amount was kept at –20 °C till further processing for DNA extraction. All the samples for estimation of indicator traits were collected on the same day. As the eggs of order Strongylida are mostly identical, pooled fecal samples were subjected to coproculture, and larval identification technique was used for identification and quantification of L3 larva of *Haemonchus* spp. as per the protocol described in RVC/FAO Guide to Veterinary Diagnostic Parasitology [26]. Once it was established that the majority of infestation was of *Haemonchus* spp., individual sampling was carried out. Fecal samples (3–4 mg) were collected from each animal and stored in clean plastic air lock bags at 4 °C. The samples were brought to the laboratory and subjected to McMaster egg floatation technique to ascertain FEC. The results were expressed as eggs per gram (epg) of feces [27]. PCV was estimated using standard Wintrobe's tube method [28]. It gives percentage of packed cell (RBC and WBC) and plasma in the blood and hence acts as an indicator trait for haemonchosis-induced anemia.

2.3. Isolation of genomic DNA

Genomic DNA was isolated from whole blood [29]. The quality of DNA was checked using 0.8% agarose gel electrophoresis and the quantity of DNA was estimated using a spectrophotometer. A total of 207 DNA samples comprising Salem Black (N = 100) and Tellicherry (N = 107) goats were used for the polymorphism study.

2.4. Locus under investigation

The locus under investigation was selected from NCBI GenBank database. Two sequences with accession numbers KP888556 and KP888557 [23] were utilized for the study. The sequences were aligned using MEGA 6.0 software to get variations among the full length of sequences. The SNP (C/G) at *TaqI* position, (T/C) at *BsaI* position, and (C/G) at *BsaHI* position were selected from 285 bp fragment using NEBcutter V2.0 tool.

2.5. PCR- RFLP

The PCR was performed using the forward (5′-TATCCCGTCTCTGCAGCA CATTTC-3′) and reverse (5′-TCGCCGCTGCACACTGAAACTCTC-3′) primers [30]. The 25-µL PCR reaction mixture consisted of 10 pmol of each primer, 12.5 µL of 2X PCR master mixes (Thermo Scientific), and 1 µL of 50 to 100 ng/µL of genomic DNA as a template. Reaction conditions included initial denaturation at 95 °C for 5 min followed by 40 cycles each of denaturation at 95 °C for 1 min, annealing at 59.5 °C for 45 s, extension at 72 °C for 1 min, and then

a final extension at 72 °C for 5 min. Amplified products were checked by 1.5% agarose gel electrophoresis for their quality. Restriction digestion of PCR products was carried out using three different restriction enzymes, i.e. *TaqI*, *BsaI*, and *BsaHI*. Reaction conditions for restriction digestion were followed as per manufacturer's instructions. In brief, about 10 µL of PCR products were digested by restriction endonuclease (2U) with the appropriate buffer supplied with the enzyme and kept for overnight incubation at 65 °C for *TaqI* (Thermo Scientific), 37 °C for *BsaI* (New England Biolabs), and 37 °C for *BsaHI* (Thermo Scientific). The digested products were separated on 2% agarose gel with suitable DNA marker. Finally, the agarose gel pictures were photographed with a gel documentation system (Syngene, USA).

2.6. Statistical analysis

After enzymatic digestion, the allelic and genotypic frequencies at the locus, i.e. MHC class II DRB gene fragment were estimated using PROC ALLELE procedure of SAS 9.3. Test for Hardy–Weinberg equilibrium (HWE) and neutrality ratios were done using POP GENE v 1.32. The association between genotypes and indicator traits was ascertained using SAS 9.3 software.

3. Results

FEC and PCV were estimated for each animal as phenotypic indicator traits for ascertaining the extent of infestation. Results of pooled fecal sample coproculture showed predominant presence of *Haemonchus* spp. (L3) larva in both Salem Black (98%) and Tellicherry goats (99%). Therefore, for estimation of FEC value(s) only the strongyle eggs were counted.

A 285-bp region of MHC class II DRB gene in Salem black and Tellicherry goats was amplified (Figure 1). In Salem Black goats, the digestion of PCR product at three loci by restriction enzymes *TaqI*, *BsaI*, and *BsaHI* showed two alleles (A and B) and three genotypes (AA, AB, and BB) (Figures 2a–2c). The allelic and genotypic frequency at these three marker loci (*TaqI*, *BsaI*, and *BsaHI*) was calculated (Table 1).

In Salem Black goats, at *TaqI* locus, allele B (0.535) had a higher frequency than A (0.465), with heterozygote genotype AB (0.490) having highest frequency. For locus *BsaI*, allele A (0.960) had a higher frequency than allele B (0.040). A similar trend was also observed at locus *BsaHI* (Table 1). At locus *BsaI*, the genotype AA (0.930) had maximum frequency and at locus *BsaHI*, the genotype AB (0.440) was predominant (Table 1). The polymorphic information content, which is the measure of informativeness of a marker, was low for locus *BsaI* (0.074) and moderate for *TaqI* and *BsaHI* (Table 1). Similarly, heterozygosity and allelic diversity values were also low (*BsaI*) to moderate (*TaqI* and *BsaHI*) (Table 1). The loci *TaqI* and *BsaHI* were in HWE, whereas *BsaI* significantly ($P \leq 0.05$) deviated from HWE (Table 1).

In Tellicherry goats, the digestion of PCR product by three restriction enzymes (*TaqI*, *BsaI*, and *BsaHI*) produced two alleles (A and B) and three genotypes (AA, AB, and BB) (Figures 2d–2f). The locus *BsaI*, however, showed only two genotypes: AA and AB (Figure 2e). The allelic and genotypic frequency at these three marker loci (*TaqI*, *BsaI*, and *BsaHI*) was calculated (Table 1). At loci, *TaqI* and *BsaHI*, the alleles A and B had close to equal proportions; however, at loci *BsaI*, the allele A was predominant with a frequency of 0.939 (Table 1). Similarly, the loci *TaqI* and *BsaHI* had moderate PIC values of 0.374 and 0.369, respectively. A similar trend was observed for genotypes, AA and AB, with low PIC (0.108), low heterozygosity (0.122), and low allelic diversity (0.114) (Table 1). All the loci at Tellicherry goat population were found to be in Hardy–Weinberg ($P \leq 0.05$) equilibrium (Table 1).

FEC and PCV were the indicator traits estimated to ascertain the extent of *Haemonchus* infestation in both breeds. The mean value of FEC in Salem Black goat population was 477.12 ± 34.14 eggs per gram (varying from 0 to 1550) and 730.42 ± 41.19 eggs per gram (varying from 0 to 1600) in Tellicherry goats. Because the data showed more standard error due to the wide range in values, it was transformed using logarithmic transformation of $\log_{10}(x+1)$ in order to normalize the data. The mean of log transformed FEC (LFEC) was 2.32 ± 0.09 and 2.66 ± 0.07 in Salem Black and Tellicherry goats, respectively (Table 2). In Salem Black goats, the mean value of PCV was 25.86 ± 0.62, varying from 9.00 to 39.00, and in Tellicherry goats the mean value of PCV was 21.92 ± 0.73, varying from 10.00 to 38.00 (Table 2). There was a negative correlation between PCV and FEC values in both breeds.

In Salem Black and Tellicherry goat populations, association between genotypes and indicator traits was ascertained. In Salem Black goats, at *TaqI* locus, the mean FEC was higher in AA (561.41 ± 55.22 epg) genotype; however, at other two loci, *BsaI* and *BsaHI*, AB (766.67 ± 184.24 epg) genotype and BB (578.92 ± 70.64 epg) genotype had the highest FEC, respectively. Expectedly, the mean PCV value at *TaqI* locus was the lowest in AA genotype. Similarly, for other two loci *BsaI* and *BsaHI*, the genotypes AB and BB were having the lowest PCV values (Table 2).

In Tellicherry goats, at *TaqI* locus, the mean FEC was maximum in AB (755.49 ± 55.23 epg) genotype; however, at *BsaI* and *BsaHI* loci, the genotype AA had the maximum FEC value(s) of 745.78 ± 43.67 epg and 790.90 ± 84.87 epg, respectively (Table 2). The mean PCV value at *TaqI* locus was the lowest in AB genotype, and for other two loci, *BsaI* and *BsaHI*, the AA genotype had the lowest PCV (Table 2). The trend in FEC and PCV values clearly indicated

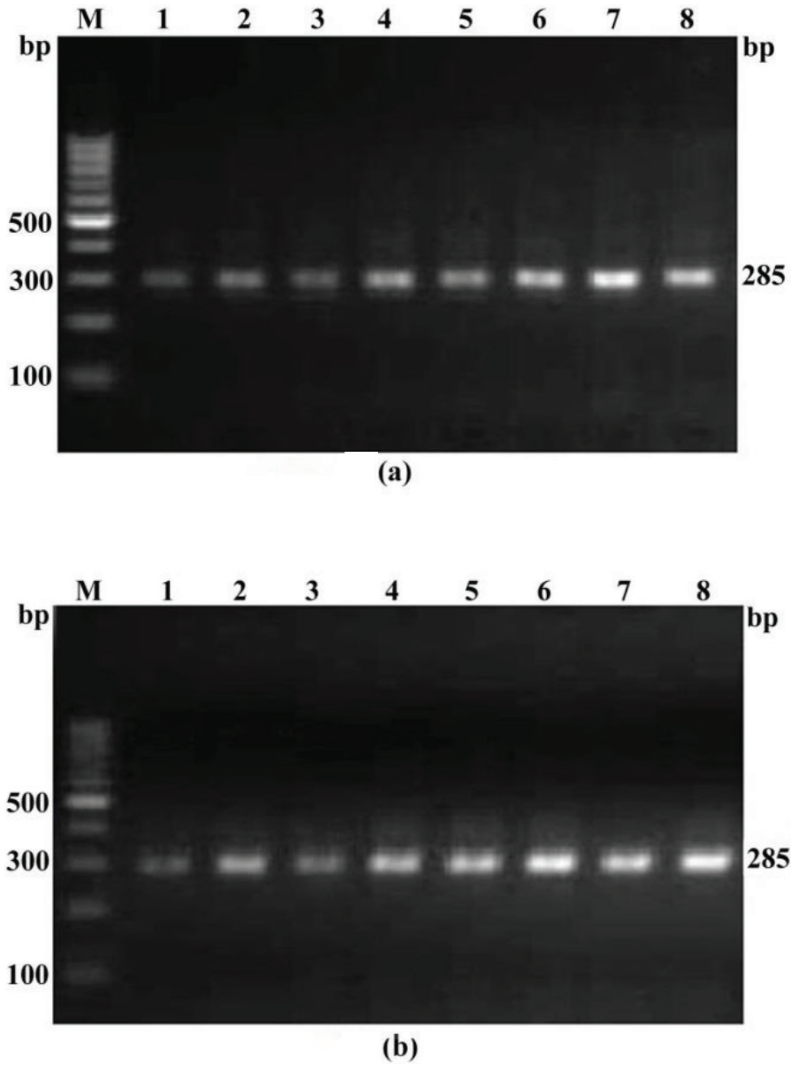

Figure 1. Amplification of 285 bp fragment of MHC class II DRB gene exon 2; Salem Black goats (a) and Tellicherry goats (b). Lane M - 100 bp DNA marker; lanes 1–8 - Amplified products.

that the increase in FEC was accompanied by a decrease in PCV values. In both breeds, there was difference in FEC and PCV values but multiple comparison procedure did not statistically associate any genotype with higher or lower value of indicator traits (Log FEC and PCV). Hence, the effect of genotypes on indicator traits was found to be statistically nonsignificant ($P \leq 0.05$) (Table 2).

4. Discussion

Major histocompatibility complex genes are important candidate genes for disease resistance. The MHC DRB genes have received a greater attention due to their functional significance in antigen presentation and high level of polymorphism. In sheep, the Ovar-DRB gene plays an important role in reduced resistance to parasitic infestation in Suffolk breed [19]. A restriction fragment length polymorphism analysis of PCR-amplified fragments (PCR-RFLP) study of DRB gene in Chinese native sheep and goats reported 7 alleles and 18 genotypes [31]. In goats, a nested PCR strategy was employed to amplify the exon 2 region of MHC DRB gene and a number of restriction digestion sites were identified that can be used for polymorphism and association studies [30]. This locus was also studied in variety of goat breeds viz. Rohilkhandi [22,23] Marwari [24], Gaddi [32], Chegu [32], Jamunapari [33], Changthangi [34], Raeini Cashmere [35], Egyptian [36], and Chinese native breeds [37].

In the present study, Tellicherry and Salem Black goat breeds were studied as the model organisms and exon 2 region (with some part of adjoining introns) of MHC DRB gene was amplified [30]. The products were digested by three different restriction enzymes viz., *TaqI*,

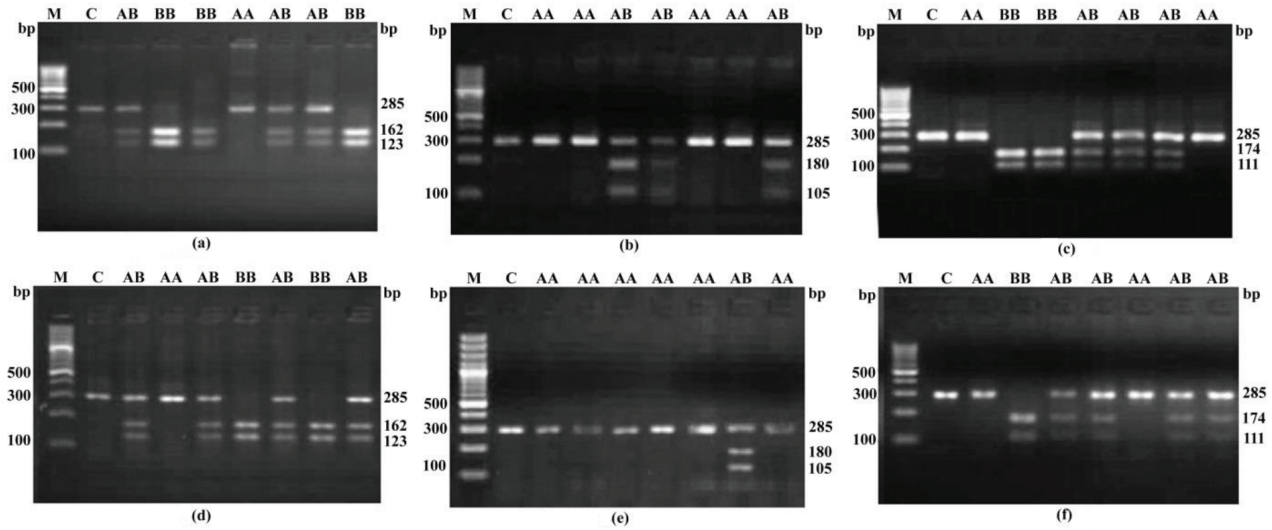

Figure 2. Restriction enzyme digestion of amplified products. (a) *TaqI* digestion in Salem Black goats (Lane M – 100 bp DNA marker, Lane C – undigested PCR product, Genotype AA – 285/285 bp, Genotype AB- 285/ 162,123 and Genotype BB -162,123/ 162,123). (b) *BsaI*digestion in Salem Black goats (Lane M – 100 bp DNA marker, Lane C – undigested PCR product, Genotype AA – 285/285 bp and Genotype AB- 285/ 180,105). (c) BsaHI digestion in Salem Black goats (In the fig: Lane M – 100 bp DNA marker, Lane C – undigested PCR product, Genotype AA – 285/285 bp, Genotype AB- 285/ 174,111 and Genotype BB – 174,111/ 174,111). (d) *TaqI*digestion in Tellicherry goat (Lane M – 100 bp DNA marker, Lane C – undigested PCR product, Genotype AA – 285/285 bp, Genotype AB- 285/ 162,123 and Genotype BB – 162,123/ 162,123). (e) BsaIdigestion in Tellicherry goat (Lane M – 100 bp DNA marker, Lane C – undigested PCR product, Genotype AA – 285/285 bp and Genotype AB- 285/ 180,105) (f) BsaHI digestion in Tellicherry goat(In the fig: Lane M – 100 bp DNA marker, Lane C – undigested PCR product, Genotype AA – 285/285 bp, Genotype AB- 285/ 174,111 and Genotype BB – 162,123/ 174,111).

Table 1. Allelic frequencies, genotypic frequencies, and heterozygosity statistics of Salem Black and Tellicherry goats at *TaqI*, *BsaI*, and *BsaHI* loci.

Breed	Locus	Alleles	Allelic frequency	Genotypes	Genotypic frequency	PIC	Heterozygosity	Allelic diversity	HWE Chi square probability*	Pr>ChiSq	Prob exact
Salem Black goats	*TaqI*	A	0.465	AA	0.220	0.374	0.490	0.498	0.023	0.879	0.836
		B	0.535	AB	0.490						
				BB	0.290						
	BsaI	A	0.960	AA	0.930	0.074	0.060	0.077	4.785	0.029	0.139
		B	0.040	AB	0.060						
				BB	0.010						
	BsaHI	A	0.520	AA	0.300	0.375	0.375	0.499	1.406	0.236	0.237
		B	0.480	AB	0.440						
				BB	0.260						
Tellicherry goat	*TaqI*	A	0.463	AA	0.206	0.374	0.514	0.497	0.122	0.727	0.849
		B	0.537	AB	0.514						
				BB	0.280						
	BsaI	A	0.939	AA	0.879	0.108	0.122	0.114	0.448	0.504	1.000
		B	0.061	AB	0.121						
				BB	-						
	BsaHI	A	0.421	AA	0.206	0.369	0.429	0.487	1.488	0.223	0.237
		B	0.579	AB	0.429						
				BB	0.365						

Table 2. Summary of descriptive statistics of indicator traits at *TaqI*, *BsaI* and *BsaHI* loci of Salem Black and Tellicherry goats

Breed	Locus	Genotype	N	FEC (epg)	Log FEC	PCV
Salem Black	*TaqI*	AA	22	561.41 ± 55.22[a]	2.58 ± 0.14[a]	24.59 ± 1.14[a]
		AB	49	477.73 ± 57.58[a]	2.17 ± 0.16[a]	25.69 ± 1.06[a]
		BB	29	412.14 ± 50.27[a]	2.36 ± 0.14[a]	27.10 ± 0.81[a]
	BsaI	AA	93	459.81 ± 34.14[a]	2.29 ± 0.09 [a]	26.16 ± 0.63[a]
		AB	6	766.67 ± 184.24[a]	2.73 ± 0.21 [a]	20.83 ± 2.29[a]
		BB	1	350.00 ± 0.00[a]	2.54 ± 0.00[a]	28.00 ± 0.00[a]
	BsaHI	AA	30	491.77 ± 57.06[a]	2.39 ± 0.16 [a]	25.27 ± 1.14[a]
		AB	44	406.97 ± 51.24[a]	2.16 ± 0.15 [a]	27.18 ± 0.87[a]
		BB	26	578.92 ± 70.64[a]	2.50 ± 0.16[a]	24.30 ± 1.32[a]
	Total = 100			477.12 ± 34.14	2.32 ± 0.09	25.86 ± 0.62
Breed	Locus	Genotype	N	FEC	Log FEC	PCV
Tellicherry	*TaqI*	AA	22	668.27 ± 92.34[a]	2.54 ± 0.18[a]	23.32 ± 1.73[a]
		AB	55	755.49 ± 55.23[a]	2.70 ± 0.08[a]	21.51 ± 0.98[a]
		BB	30	730.03 ± 83.81[a]	2.65 ± 0.12[a]	21.63 ± 1.40[a]
	BsaI	AA	94	745.78 ± 43.67[a]	2.68 ± 0.07 [a]	21.59 ± 0.77[a]
		AB	13	619.31 ± 123.81[a]	2.49 ± 0.24 [a]	24.23 ± 2.06[a]
		BB	-	-	-	-
	BsaHI	AA	52	790.90 ± 84.87[a]	2.82 ± 0.07 [a]	19.91 ± 1.63[a]
		AB	90	694.65 ± 61.64[a]	2.56 ± 0.13 [a]	23.13 ± 1.07[a]
		BB	65	738.49 ± 73.06[a]	2.68 ± 0.09[a]	21.62 ± 1.23[a]
	Total = 107			730.42 ± 41.19	2.66 ± 0.07	21.91 ± 0.72

N = Number of observations in particular class; *Means with the same letter are not significantly different ($P < 0.05$).

BsaI, and *BsaHI*. In both Tellicherry and Salem Black goat populations all the loci exhibited two alleles and three genotypes (except, *BsaI* locus in Tellicherry goats) with low to moderate levels of PIC values. A comparable previous study on Rohilkhandi goats of India using *TaqI* and *BsaI* restriction enzymes showed similar results with lack of one homozygote at *BsaI* locus [23] and low to moderate PIC values [22]. Furthermore, population study of the genotypic data showed the loci *TaqI* and *BsaHI* to be in HWE and *BsaI* to be significantly deviating from HWE in Salem Black goats. Previously, both loci were reported to be in HWE in Rohilkhandi breed [22]. Similarly, in Tellicherry goats, all the loci were found in HWE. There was low to medium heterozygosity at all the loci in both breeds with similar trends for allelic diversity and PIC value. Earlier PCR-RFLP studies using multiple restriction enzymes on this gene reported excess heterozygosity and significant deviations from HWE [25,38].

The genotypes obtained were then used for testing their association with indicator traits (FEC and PCV) for *Haemonchus* infestation [39,40]. PCV was negatively correlated with FEC [41], and is a useful parameter to ascertain the extent of anemia caused by blood-sucking parasites, especially *Haemonchus* [39,42]. In the present study, the FEC count varied from 0 to 1550 (Salem Black goat) and 1600 (Tellicherry goat) eggs per gram of feces, for naturally exposed population. However, the average FEC value for Salem Black goats (477.12 ± 34.14) was lower than Tellicherry goats (730.42 ± 41.19). The mean PCV values for both the breeds were within the normal range; however, it was negatively correlated with FEC. These indicator traits were used for the association study to ascertain the effect of each genotype and to identify the resistant/susceptible genotype(s). DRB fragment carries functional importance in host immune response, and it has been widely studied in sheep and goats. Previously, DRB gene was studied in Chinese Merino, Kazakh, and Duolang sheep for its possible association with hydatidosis, the results suggested its role for resistance to the infection [43]. A similar study reported its role for resistance to Cystic Echinococcosis (C.E) in Chinese Merino sheep [44]. Similarly, the effect of DRB genotype (Digested by *PstI* restriction enzyme) was found significant on egg counts of *Marshalla giamarshalli*, strongyle, and total nematode

FEC [45]. In the present study, genotypes at all the loci, in both breeds, were tested for association with indicator traits (FEC and PCV). As the FEC values showed more standard error they were log transformed (LFEC) to make the variance stable. The average values of FEC and PCV showed difference in different genotypes. Lower average PCV values were observed for higher average FEC at all the loci in both breeds; however, the clinical manifestation of anemia was not observed. The locus *BsaI* showed only two genotypes (AA and AB) in Tellicherry goats and single BB genotype in Salem Black goats. It was previously reported that locus *BsaI* was statistically associated ($P \leq 0.05$) with FEC in Rohilkhandi goats [22]. In the present study, although there was difference in mean values of FEC and PCV, the effect of genotypes on indicator traits (FEC and PCV) was found to be nonsignificant ($P \leq 0.05$) in both breeds.

5. Conclusion

Salem Black and Tellicherry goats were genotyped at MHC DRB exon 2 using PCR-RFLP. The gene was polymorphic for all the loci in both breeds. Additionally, FEC and PCV were studied to assess the extent of parasitic infestation and to study the effect of genotypes on these parameters. The FEC and PCV values showed negative correlation among both breeds. Although there was difference in mean values of indicator traits among all genotypes in both the breeds, statistically, the effect of genotypes on indicator traits was nonsignificant ($P \leq 0.05$).

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Conflict of interest

All the authors declare that there is no conflict of interest.

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