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Candidate Gene Analysis of Genetic Resistance to Gastrointestinal Nematodes in Sheep Through Association of Single Nucleotide Polymorphism with Phenotypic Traits

R. Selvam[1], N. Murali[2], A.K. Thiruvenkadan[3], G. Ponnudurai[4], K. Thilak Pon Jawahar[5], P. Kathiravan[6]

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ABSTRACT

Background: A study was executed to analyze the genetic resistance to gastrointestinal nematodes through the association of single nucleotide polymorphism markers in Toll-like receptor genes with phenotypic indicator traits in Kilakarsal and Vembur sheep breeds.

Methods: The phenotypic traits for analyzing the gastrointestinal nematode infection namely FEC, change in PCV and change in body weight were recorded. The SNP markers in TLR3, TLR5, TLR6, TLR9 and TLR10 genes were employed for genotyping. Competitive allele-specific PCR-based endpoint genotyping was used to type the SNPs. The gene and genotype frequencies were estimated by using the PEAS software program. A complete fixed effects model was utilized for analysis of the association of various genotypes at each SNP with phenotypic indicator traits.

Result: The global minor allele frequency of different polymorphic SNP loci ranged from 0.06 to 0.48 with a mean of 0.23, signifying their fitness for the association study. The effect of the farm had no significant influence on FEC and change in body weight, however, had a significant effect on change in PCV ($P<0.05$). No significant difference was detected between the sexes with FEC, change in PCV and change in body weight. The TT genotype in the TLR9_1769_CT locus showed the lowest least-squares mean FEC. The remaining 22 SNP loci showed no significant difference ($P>0.05$) with mean FEC. Association of 23 TLR SNP genotypes with change in PCV and change in body weight revealed no significant effect ($P>0.05$).

Key words: Competitive allele-specific PCR, Gastrointestinal nematode, Genetic resistance, Single nucleotide polymorphism, TLR genes.

INTRODUCTION

Gastrointestinal nematodes (GIN) such as *Haemonchus contortus* impose severe constraints in small ruminant production. Conventional approaches including deworming and other medication are not effective because of the anthelmintic resistance developed by the parasites. A potential alternative measure to alleviate the problem is breeding for disease resistance. The genetic variations within and among livestock breeds against the diseases were reported, in which some breeds or individuals within a breed are more resistant to the disease than others in the same population (Jovanovic *et al*., 2009). Indigenous sheep breeds of India exhibit enhanced resistance to various diseases. There are well-documented shreds of evidence for within and between-breed genetic dissimilarities in resistance to GIN infection and offers the opportunity to select animals for enhanced genetic resistance to the diseases (Zvinorova *et al*., 2016).

In India, the native sheep breeds have been found to have better resistance against parasites compared to crossbreds and exotic sheep. There are reports for genetic variation between indigenous sheep and exotic crosses in resistance to gastrointestinal nematodes such as *Haemonchus contortus*, *Trichostrongylus colubriformis* and *Ostertagia circumcinta* (Arora and Prince, 2004). Several studies have been carried out to detect susceptible and

[1]Department of Animal Genetics and Breeding, Veterinary College and Research Institute, Tamil Nadu Veterinary and Animal Sciences University, Theni-625 534, Tamil Nadu, India.
[2]Alambadi Cattle Breed Research Centre, Karimangalam, Dharmapuri-635 111, Tamil Nadu, India.
[3]Department of Animal Genetics and Breeding, Veterinary College and Research Institute, Namakkal-637 002, Tamil Nadu, India.
[4]Department of Veterinary Parasitology, Veterinary College and Research Institute, Namakkal-637 002, Tamil Nadu, India.
[5]Livestock Farm Complex, Madhavaram Milk Colony, Chennai-600 051, Tamil Nadu, India.
[6]Animal Production and Health Laboratory, Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture, International Atomic Energy Agency, Vienna, Austria.

Corresponding Author: R. Selvam, Department of Animal Genetics and Breeding, Veterinary College and Research Institute, Tamil Nadu Veterinary and Animal Sciences University, Theni-625 534, Tamil Nadu, India. Email: selvam.r@tanuvas.ac.in

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resistant individuals based on phenotypic indicator traits like fecal egg counts (FEC), packed cell volume (PCV) (Nimbkar *et al*., 2003). ICAR-Central Sheep and Wool Research Institute, Avikanagar, Rajasthan developed the resistant line for *Haemonchus contortus* in Malpura and Avikalin sheep through regular screening for fecal egg counts at naive and exposed stage of infection in flocks and selection of progenies. The resistant line had lower fecal egg counts compared to their counterparts in susceptible line (Gowane *et al*., 2020). Over the last few years, various studies conducted to analyze the role of Toll-like receptor (TLR) in disease resistance against infectious diseases across the world, but studies in Indian breeds for analyzing the host genetic resistance against the parasites at molecular level are limited. This study was executed to analyze the genetic resistance to GIN infection through SNP markers within TLR genes in Kilakarsal and Vembur breeds of sheep.

MATERIALS AND METHODS

A sum of 50 Kilakarsal sheep breed from Livestock Farm Complex, Veterinary College and Research Institute, Tirunelveli and District Livestock Farm, Abishegappatti, Tirunelveli and 50 Vembur sheep breed from Livestock Farm Complex, Veterinary College and Research Institute, Tirunelveli and Government Sheep Farm, Sattur, Virudhunagar, of both sexes, 6 to 18 months of age were randomly selected. They are maintained under natural free grazing. The animals were dewormed as per the standard procedure. The phenotypic indicator traits for GIN infection *viz*., FEC, change in PCV and change in body weight were recorded at 0, 30 and 60 days after deworming for a period of two years from 2015 to 2017. The eggs and third-stage infective larvae of GIN (*Haemonchus contortus, Oesophagostomum sp*., *Strongyloides sp*.) were examined from fecal samples of naturally infected sheep. DNA samples were extracted from the whole blood using a modified high-salt method (Miller *et al*., 1988). A sum of 25 SNP markers from five TLR genes *viz*., TLR3, TLR5, TLR6, TLR9 and TLR10 were applied for genotyping (Table 1). Competitive allele-specific PCR-based endpoint genotyping was accomplished to type the SNPs. Endpoint allelic discrimination module implemented in ILLUMINA Eco Real-Time PCR was employed to call the genotypes formed on fluorescent intensity recorded for individuals of the two alleles. The emission data of all the samples were plotted in cluster plots for respective alleles and the genotypes were called based on distinct clustering (Fig 1). Basic diversity indices namely, allele frequency and genotype frequency were assessed by PEAS -Package for Elementary Analysis of SNP data software program (Xu *et al*., 2010). A complete fixed effects model was used for the analysis of the association of various genotypes at each SNP with FEC, change in PCV and change in body weight. The data on FEC were converted to log transformation for applying the LSMLMW program of least squares.

RESULTS AND DISCUSSION

Indicator traits for GIN infection

In the present study, the mean of post-deworming FEC values ranged from 0 to 4,500 in Kilakarsal sheep and from 0 to 3,150 in Vembur sheep. The post-deworming change in PCV values varied from -11 to 7% in Kilakarsal sheep and -7 to 5% in Vembur sheep. The post-deworming change in body weight varied from -4.25 to 3.70 kg in Kilakarsal sheep and -2.2 to 6.25 kg in Vembur sheep.

Competitive allele specific PCR based end point genotyping

In the sum of 25 SNPs, 22 SNPs were polymorphic, while SNPs TLR3_1081_AC and TLR9_2036_CT were monomorphic in Kilakarsal and Vembur sheep, whereas TLR10_1180_AG SNP was monomorphic in the Kilakarsal population only. The global minor allele frequency (MAF) for 22 polymorphic SNP loci ranges from 0.06 to 0.48 (mean: 0.23). However, 49.8% of SNP loci showed MAF of 0.20, 29.7% of SNP loci showed MAF of 0.30 and 18.1% of SNP loci showed MAF of 0.40.

Effect of non-genetic factors on phenotypic indicator traits for resistance to GIN

The effect of the farm had no significant influence on FEC and change in body weight, however, had a significant effect on change in PCV ($P<0.05$; Table 2). The sex of the sheep population also results in no significant differences with respect to the phenotypic indicator traits such as FEC, change in PCV and change in body weight ($P>0.05$). As reported earlier, in the present study also the indicator traits were neither influenced by the breed (Kathiravan *et al*., 2014) nor by other non-genetic factors (Varadhrajan and Vijayalakshmi, 2015). Since markers with MAF >0.05 were reported to be suitable for association studies (Tabangin *et al*., 2009), 22 polymorphic SNP loci with MAF >0.06 in this study are suitable for such studies.

Association of SNPs in TLR genes with resistance to GIN infection

All three possible genotypes were observed in the 22 polymorphic SNP loci except TLR10_1180_AG in which only two genotypes GG and AG were present (Table 3). In the TLR9_1769_CT locus, a significant difference ($P<0.05$) was observed with mean FEC of 460.36±1.16, 23.42±2.72 and 400.39±1.55 for CC, TT and CT genotypes, respectively (Table 4). The TT genotype in TLR9_1769_CT locus showed the lowest least-squares mean FEC and showed a significant difference, hence the individual with this genotype is considered resistant to GIN infection in these breeds. The remaining 22 SNP loci showed no significant difference ($P>0.05$) with mean FEC ranging from 184.53±1.72 to 3701±4.21 in Kilakarsal and Vembur sheep breeds taken together. The highest least-squares mean FEC was observed in AA genotype of TLR10_595_AG SNP and TT genotype of TLR10_771_CT with a mean of 3701±4.21.

The association of genotypes with PCV change revealed no significant effect (P>0.05) at all the SNP loci studied. With respect to change in body weight, all the SNP loci studied showed no significant association of genotypes (P>0.05).

Among the 23 SNP, analyzed for the association with resistance to GIN infection the TLR9_1769_CT locus had a significant difference and the TT genotype showed a significant difference, hence, the individual with this

Table 1: Details of TLR gene SNP loci used in the study.

	Gene ID	Chromosome	Genomic position	Strand	Alleles	AA change
TLR3_1081_AC	554258	26	14917743	+	A/C	A-Ile; C-Leu
TLR3_265_CT	554258	26	14916927	+	C/T	C-Arg; T-Trp
TLR3_340_CT	554258	26	14917002	+	C/T	C-Arg; T-Cys
TLR3_370_AG	554258	26	14917032	+	A/G	A-Asn; G-Asp
TLR3_631_AG	554258	26	14917293	+	A/G	A-Arg; G-Gly
TLR5_1354_AG	554256	12	24703539	-	A/G	A-Lys; G-Glu
TLR5_1578_CT	554256	12	24703315	-	C/T	CT-Asp
TLR5_2037_CT	554256	12	24702856	-	C/T	CT-Tyr
TLR5_276_CT	554256	12	24704617	-	C/T	CT-Ser
TLR5_786_CT	554256	12	24704107	-	C/T	CT-Ser
TLR6_1301_AG	554257	6	58035513	-	A/G	A-Met; G-Val
TLR6_229_GT	554257	6	58036585	-	G/T	G-Met; T-Ile
TLR6_49_CT	554257	6	58036765	-	C/T	CT-Phe
TLR6_589_AG	554257	6	58036225	-	A/G	AG-Thr
TLR6_814_AC	554257	6	58036000	-	A/C	A-Glu; C-Asp
TLR9_1308_GC	494547	19	48645210	+	G/C	G-Gly; C-Arg
TLR9_1769_CT	494547	19	48645664	+	C/T	CT-Val
TLR9_2036_CT	494547	19	48645931	+	C/T	CT-Cys
TLR9_2099_CT	494547	19	48645994	+	C/T	CT-Ser
TLR9_2504_CT	494547	19	48646399	+	C/T	CT-Asn
TLR10_1180_AG	554255	6	57993112	-	A/G	A-Ile; G-Val
TLR10_292_CG	554255	6	57992224	-	C/G	C-Leu; G-Val
TLR10_595_AG	554255	6	57992527	-	A/G	A-Ile; G-Val
TLR10_771_CT	554255	6	57992703	-	C/T	CT-Leu
TLR10_82_CT	554255	6	57992014	-	C/T	CT-Leu

Table 2: Mean (±SE) of FEC, change in PCV, change in body weight and least-squares ANOVA to estimate the effect of non-genetic factors on parasite resistance characteristics in Kilakarsal and Vembur sheep breeds.

Trait	Factor	Details	N	Mean±SE	d.f.	Sum of squares	Mean sum of squares	F	P value
FEC	Farm	ILFC	49	523.57±1.27	2	15309817.3	7654908.6	0.19	0.825
		DLF	26	635.50±1.38					
		GSF	25	620.35±1.43					
	Sex	Male	14	891.92±1.50	1	144983300.0	44983300.0	3.66	0.059
		Female	86	391.58±1.17					
Change in PCV	Farm	ILFC	49	-0.04±1.03	2	5103760.6	2551880.3	5.55	0.005*
		DLF	26	-1.65±1.04					
		GSF	25	1.24±1.04					
	Sex	Male	14	0.52±1.04	1	1053741.5	1053741.5	2.29	0.133
		Female	86	-0.87±1.02					
Change in body weight	Farm	ILFC	49	1.06±1.04	2	2508813.7	1254406.9	1.50	0.229
		DLF	26	0.87±1.05					
		GSF	25	0.35±1.05					
	Sex	Male	14	0.69±1.06	1	65521.3	65521.3	0.08	0.781
		Female	86	0.83±1.02					

N: Number of animals, d.f.: Degrees of freedom.

genotype is considered resistant to GIN infection. Similarly, Kathiravan *et al*. (2014) reported significant variances (P<0.05) in FEC, change in body weight and PCV at SNP loci in Corriedale, Pampinta, Indonesian Thin Tail and Indonesian Fat Tailed sheep and concluded that the SNP loci detected had the high potential for imminent association studies. Among different breeds, Indonesian fat-tailed sheep showed the lowest mean FEC (mean log FEC 3.2360.16) and Corriedale sheep exhibited the highest mean logFEC 3.5860.079, but this breed variation is not significantly different (P>0.05). It is in accordance with our results, in which no breed differences were found between Kilakarsal and Vembur sheep breeds. They also stated that genotypes at NAV3_591 and GLI1_576 were found to have significant differences in their FEC, whereas genotypes at ZBTB39_51, IL20RA_422, PIK3CD_433 and TLR7_2491 showed no significant differences in their mean log-transformed FEC. Also, they reported that significant association of genotypes with change in PCV at SNP loci namely, ANKRD52_113, CSRNP2_65, ESYT1_157, NAV3_591, TIMP3_716 and IL2RA_388, however, the similar SNPs to our present study did not show any significant association with change in PCV. Also, they stated that four SNP loci *viz*., ACVRL1_445, GPR84_520, SMCR7L_517 and TARBP2_97 had a significant association (P<0.05) and other SNPs including TLR5_2276, TLR7_2491 and TLR8_1045 did not show any significant association with change in body weight. This is comparable with our study in which the SNPs located in TLR5, TLR7 and TLR8 genes were not significantly associated with phenotypic indicator traits.

Similarly, McRae *et al*. (2014) analyzed the genome-wide SNP data for loci associated with genetic resistance to GIN infection in Romney and Perendale sheep and revealed candidate genes for cytokine response and chitinase activity were interned within QTLs associated with resistance. Mohammad *et al*. (2019) identified genomic regions on OAR2, 6, 18 and 24 which were associated with GIN resistance in Australian sheep. Ahbara *et al*. (2021) analyzed SNP genotypes in Tunisian sheep and reported that the candidate genes IL-4, IL-13, SLC22A4 and SLC22A5 were

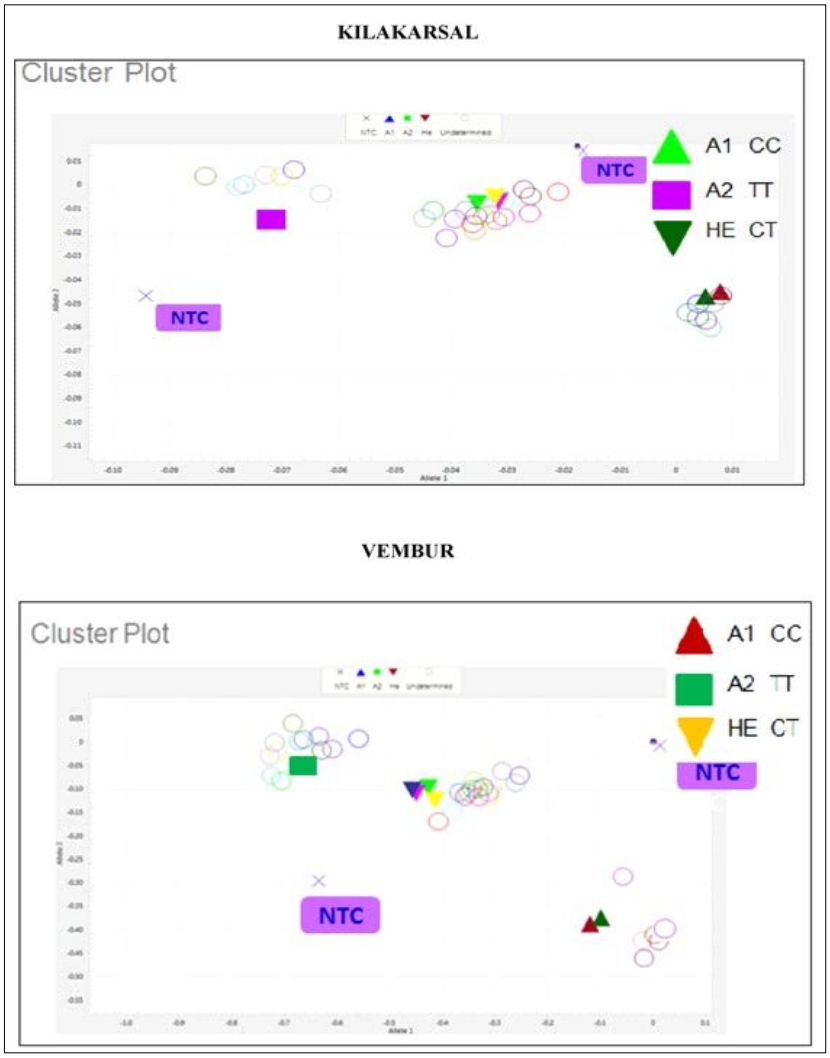


Fig 1: Cluster plot for TLR3_265_CT SNP locus for kilakarsal and vembur sheep after KASP SNP genotyping.

Table 3: Mean (±SE) of FEC for genotypes at different SNP loci three months post deworming in Kilakarsal and Vembur sheep.

SNP_ID	Genotype	N	Mean±SE
TLR3_265_CT	CC	17	334.47±1.43
	TT	33	426.94±1.29
	CT	50	464.31±1.23
TLR3_340_CT	CC	63	464.15±1.20
	TT	7	184.53±1.72
	CT	30	436.34±1.30
TLR3_370_AG	AA	7	184.53 ±1.72
	GG	63	464.15±1.20
	AG	30	436.34±1.30
TLR3_631_AG	AA	3	745.93±2.35
	GG	67	412.06±1.20
	AG	30	437.73±1.31
TLR5_276_CT	CC	89	430.73±1.17
	TT	1	1074.69±4.32
	CT	10	361.46±1.59
TLR5_786_CT	CC	8	396.84±1.67
	TT	48	375.37±1.23
	CT	44	498.4±1.25
TLR5_1354_AG	AA	8	396.84±1.67
	GG	49	381.47±1.23
	AG	43	492.57±1.25
TLR5_1578_CT	CC	23	526.77±1.35
	TT	27	427.76±1.32
	CT	50	387.55±1.23
TLR5_2037_CT	CC	50	395.86±1.23
	TT	8	396.84±1.67
	CT	42	474.21±1.25
TLR6_49_CT	CC	1	3694.15±4.24
	TT	86	399.03±1.17
	CT	13	567.58±1.49
TLR6_229_GT	GG	86	399.03±1.17
	TT	1	3694.15±4.24
	GT	13	567.58±1.49
TLR6_589_AG	AA	86	399.03±1.17
	GG	1	3694.15±4.24
	AG	13	567.58±1.49
TLR6_814_AC	AA	87	403.07±1.17
	CC	1	3676.7±4.25
	AC	12	543.59±1.52
TLR6_1301_AG	AA	6	957.23±1.80
	GG	57	377.87±1.21
	AG	37	452.61±1.27
TLR9_1308_GC	GG	22	262.83±1.54
	CC	53	535.39±1.41
	GC	25	405.67±1.56
TLR9_1769_CT	CC	87	460.36±1.16
	TT	2	23.42±2.72
	CT	11	400.39±1.55
TLR9_2099_CT	CC	4	371.39±1.23
	TT	1	434.74±1.52
	CT	3	506.42±1.26
TLR9_2504_CT	CC	1	434.01±1.52
	TT	4	338.55±1.25
	CT	4	525.92±1.24
TLR10_82_CT	CC	1	399.1±1.49
	TT	3	416.32±1.30
	CT	5	441.37±1.23
TLR10_292_CG	CC	1	399.1±1.49
	GG	3	416.32±1.30
	CG	5	441.37±1.23
TLR10_595_AG	AA	1	3701.3±4.21
	GG	8	392.7±1.16
	AG	1	727.23±1.57
TLR10_771_CT	CC	8	392.7±1.16
	TT	1	3701.3±4.21
	CT	1	727.23±1.57
TLR10_1180_AG	AA	0	-
	GG	9	430.71±1.16
	AG	1	187.25±4.29

Table 3: Continue....

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involved in innate immune against GIN infection. Maria *et al*. (2021) reported the associations between candidate genes and FEC, PCV in Corriedale and Pampinta sheep infected with *Haemonchus contortus* and identified SNPs positioned on OARs 3, 6, 12 and 20, represented allelic variants from the MHC-Ovine Lymphocyte Antigen-DRA, IL2 receptor β, C-type lectin domain families, TLR 10 and NLR showed significant differences. Carracelas *et al*. (2022) identified genomic regions linked with GIN resistance in Corriedale sheep by and validated the association of SNPs in *TIMP3*, *TLR5*, *TLR9* and *LEPR* with FEC. Thorne *et al*. (2023) studied the association of SNPs with GIN indicator traits as FEC and PCV in Rambouillet and Dorper lambs and concluded that the candidate genes CAPZB, GALNT6, IGF1R, PTK2B and SCUBE1 involved in immunity and cellular signaling for coagulation and wound healing following epithelial damage in GIN infection.

In India, Arora and Prince (2004) reported that there is significant difference in fecal egg count, hematological and biochemical parameters between the indigenous sheep breeds and exotic crosses with respect to infection with gastrointestinal nematodes and concluded that the indigenous sheep excreted fewer worm eggs in faeces and had lower morbidity and mortality rates compared to exotic breed and their crosses and Garole sheep are naturally resistant to haemonchosis. Swarnkar *et al*. (2017) examined the host response in Malpura lambs selected for resistance or susceptibility to *Haemonchus contortus* that was measured by means of fecal egg count, body weight, biochemical and hematological parameter for post challenge in which, an increase of 0.3 kg was noticed in resistance line lambs than to a decrease of 4.1 kg in susceptible line

Table 4: Least-squares ANOVA of FEC at different SNP loci three months post deworming in Kilakarsal and Vembur sheep.

SNP_ID	d.f.	Sum of squares	Mean sum of squares	F	P value
TLR3_265_CT	2	25462693.3	12731346.7	0.32	0.727
TLR3_340_CT	2	101401334.7	50700667.3	1.30	0.278
TLR3_370_AG	2	101401334.7	50700667.3	1.30	0.278
TLR3_631_AG	2	18693623.1	9346811.5	0.23	0.792
TLR5_276_CT	2	21423633.9	10711816.9	0.27	0.765
TLR5_786_CT	2	35442580.5	17721290.3	0.45	0.642
TLR5_1354_AG	2	29001380.4	14500690.2	0.36	0.696
TLR5_1578_CT	2	27916821.6	13958410.8	0.35	0.705
TLR5_2037_CT	2	14903226.6	7451613.3	0.19	0.830
TLR6_29_GT	2	113084155.3	56542077.7	1.45	0.239
TLR6_49_CT	2	113084155.3	56542077.7	1.45	0.239
TLR6_589_AG	2	113084155.3	56542077.7	1.45	0.239
TLR6_814_AC	2	104589049.5	52294524.7	1.34	0.267
TLR6_1301_AG	2	90340185.9	45170093.0	1.15	0.320
TLR9_1308_GC	2	65610889.5	32805444.8	0.83	0.439
TLR9_1769_CT	2	318237970.9	159118985.5	4.32	0.016*
TLR9_2099_CT	2	39398889.0	19699444.5	0.50	0.611
TLR9_2504_CT	2	80371018.3	40185509.2	1.02	0.364
TLR10_82_CT	2	2387647.9	1193823.9	0.03	0.971
TLR10_180_AG	1	12825231.3	12825231.3	0.32	0.571
TLR10_292_CG	2	2387647.9	1193823.9	0.03	0.971
TLR10_595_AG	2	150935679.6	75467839.8	1.96	0.147
TLR10_771_CT	2	150935679.6	75467839.8	1.96	0.147

(*P<0.05), d.f.: Degrees of freedom.

on single challenge and in most of the parameters for evaluation of infection by *Haemonchus contortus* was lower in resistance line than in susceptible line and It was concluded that the animals selected for resistance can tolerate parasite challenge effectively with reduced intensity of infection, higher body weight gain and reduced pathogenic effect. Gowane *et al*. (2020) studied the genetic structure of *Haemonchus contortus* resistance and susceptibility lines in Malpura and Avikalin sheep and analyzed the genetic parameters for fecal egg count. They reported that the log transformed FEC was significantly (P<.05) affected by sex, year and month of recording for all Malpura resistant, Malpura susceptible, Avikalin resistant and Avikalin susceptible and there was low permanent environment effect, low heritability for LFEC and Repeatability for LFEC were 0.05, 0.11, 0.07 and 0.06 for Malpura resistant, Malpura susceptible, Avikalin resistant and Avikalin susceptible, respectively.

CONCLUSION

In this study the genetic resistance to gastrointestinal nematodes through candidate gene analysis in Kilakarsal and Vembur breeds of sheep were assessed using the single nucleotide polymorphic markers within five toll like receptor genes TLR3, TLR5, TLR6, TLR9 and TLR10. Among 25 SNPs analyzed, 22 were found to be polymorphic in both Kilakarsal and Vembur sheep. The global minor allele frequency of the polymorphic SNP loci varied from 0.06 to 0.48 with a mean of 0.23. The effect of farm had no significant influence on FEC and change in body weight, however had significant effect on change in PCV (P<0.05). No significant difference was observed between the sexes with FEC, change in PCV and change in body weight. TLR9_1769_CT locus had significant difference (P<0.05) with mean FEC of 460.36±1.16, 23.42±2.72 and 400.39±1.55 for CC, TT and CT genotypes, respectively in Kilakarsal and Vembur sheep population. The TT genotype in TLR9_1769_CT locus showed the lowest least-squares mean FEC. The remaining 22 SNP loci showed no significant difference (P>0.05) with mean FEC ranging from 184.53±1.72 to 3701±4.21. Association of 23 TLR SNP genotypes with change in PCV and change in body weight revealed no significant effect (P>0.05). The findings of this study will aid in genetic selection against nematode infection in sheep breeds of Tamil Nadu as well as India, which will have positive effect on reduced use of anthelmintic drugs, pasture contamination, spread of anthelmintic resistance and provide a better knowledge for managing the problem in a sustainable manner.

Conflict of interest

The authors declared that there is no conflict of interest.

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