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Research Article

DETECTION OF SINGLE NUCLEOTIDE POLYMORPHISM USING TARMS-PCR IN *TLR*2 GENE IN LOCAL CATTLE OF JAMMU

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ABSTRACT: The local cattle of Jammu is one of the unique cattle which belongs to hilly areas of Jammu. A major group of Pattern recognition receptors (PRRs) are the Toll like receptors (*TLR*s) which play a very important role in immunity of the animal. Therefore, the study was aimed to detect the polymorphism in *TLR*2 gene. The genomic DNA was isolated from blood samples of 50 unrelated local cattle of Jammu from different locations.Tetra primer amplification refractory mutation system- polymerase chain reaction (TARMS-PCR) using self-designed primers was performed to detect the SNPs. TARMS-PCR for *TLR2* revealed three bands on gel (158 bp control fragment, 120 bp B allele, 100 bp A allele). The genotypic frequencies for AA, AB and BB genotypes and allele frequency of A and B alleles were 0.12, 0.64 and 0.24, 0.44 and 0.56, respectively. The calculated observed number of alleles (n_a), effective number of alleles (n_e), observed homozygosity (H_o), expected heterozygosity (H_e), Shannon's information index (I),Wright'sfixation index (F_{IS}), PIC values were 2.00, 1.9716, 0.5022, 0.497, 0.6859,-0.2987, 0.3713, respectively. The $\chi 2$ and G^2 values for genotypes were significant. From the present study it can be concluded that there are substantial genetic variations and heterozygosity present within the local cattle population of Jammu province, which can be explored to formulate the effective breeding policies for genetic improvement, propagation and conservation of this important local cattle population.

Key words: Local Cattle, *TLR*2, TARMS-PCR, Heterozygosity, PIC, F_{IS}

INTRODUCTION

Local cattle of Jammu is one of the unique cattle which belongs to hilly areas of Jammu like Poonch, Rajouri, Ramban, Reasi, Banihal etc. They are very popular for their strength to adopt and withstand a wide range of local climatic conditions of the hilly terrain and play significant role in contributing to the local economy and livelihood security to its owners by way of providing milk, draught power and manure with minimum or zero input. Local cattle are found resistant to many diseases as compared to crossbred cattle and have the ability to withstand stressful environmental conditions [1]. The ability of a dairy cow to resist contagious disease is in part related to the activity of its immune system which consists of a variety of mechanisms and processes which are capable of both non-specifically and specifically targeting invading microorganisms [2].

Pattern recognition receptors (PRRs) are known to play an important role in the innate or nonadaptive immune response as they detect the presence of pathogens that breach an animal's surface defenses [3]. A major group of PRRs are the Toll like receptors (TLRs) which play very important role in immunity of the individual. TLR2 can sense specific structures expressed by pathogens, such as bacteria and fungi. These structures include pathogen-associated molecular patterns (PAMPs) and some endogenous ligands. The TLRs consist of a large extracellular domain responsible for PAMP binding, a transmembrane domain and an intracellular Toll/interleukin-1 receptor (TIR) domain which binds molecules and initiates cellular immune responses [4]. The extracellular domains are composed of about 20 leucine-rich repeats (LRRs) motifs of 20–30 amino acids (AA) and form a solenoid shape with the potential to bind the TLR specific PAMP [5]. After pathogen-associated molecular patterns (PAMP) recognition, TLRs activate cellular signaling pathways to induce immune response genes, including

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inflammatory cytokines [6]. Ten different Toll-like genes have been identified within the cattle genome. Among them TLR2 has been first identified as antibacterial molecule that recognizes peptidoglycan of grampositive bacteria which is activated inside the phagosome by peptidoglycans [7]. It was reported that the expression of *TLR2* gene strongly increased in different body tissue following infection in cattle [8].Toll-like receptors (TLRs) are important pattern recognition receptors and are widely expressed on the surfaces of innate immune system cells, such as monocytes and macrophages. More than 10 family members of TLRs have been identified [9] since the discovery of the first TLRs in humans [10]. *TLR*2 proteins can transmit pathogenic microorganism invasion signals, activate innate immune response generation and further induce adaptive immune response [11,12]. *TLR*2 can combine with *TLR*1 or *TLR*6 to form heterodimers, which can recognize the peptidoglycan, phosphoprotein acid and lipoprotein of Gram-positive bacteria [13]. Moreover, *TLR*2 can induce interleukin-1β production through the NF-κb pathway [14]. *TLR2* gene is located on chromosome 17 and has 2355bp long DNA. It has 6 exons.It codes a protein having 784 amino acids. Therefore, the present study was undertaken to detect the SNPs in *TLR2* gene which may be used as the main candidate gene for genetic variation studies and marker-assisted disease resistance breeding.

MATERIALS AND METHODS

Isolation of the genomic DNA and its preliminary analysis

A total of fifty (50) unrelated indigenous native cattle of Jammu region were selected randomly from natural breeding tracts i.e. different remote areas of districts of Jammu namely Poonch, Rajouri, Ramban, Doda, Udhampur. Blood samples were collected aseptically from jugular vein in 15 ml centrifuge tubes coated with 0.8MEDTA and stored at 4◦C for future use. Genomic DNA isolation was carried out by phenol-chloroform isoamyl (PCI)method.The concentration and purity of DNA was checked on Nano-drop. Samples with OD ratio from 1.7 to 1.9 were used for further study. 0.8% agarose gel was used for checking quality of DNA. The gel was photographed by UV transilluminator.

Retrieval and analysis of *TLR2* gene sequences of local cattle

The complete gene sequences of *TLR2* gene available on NCBI GenBank database [15] were retrieved for determining the consensus sequence and presence of single nucleotide polymorphisms (SNPs). Retrieved *TLR2* gene sequences of different cattle wereanalyzed for multiple sequence alignment through ClustalW Multiple alignment programme of BioEdit [16] Sequence Alignment Editor Version 7.0.

Primer Designing and TARMS PCR

The primers for TARMS PCR were designed using BioEdit software and designated as forward inner primer (FIP), reverse inner primer (RIP), forward outer primer (FOP) and reverse outer primer (ROP) as followsOuter forward 5´-ATTCCAGATCTTTAAACTC CATCCCCTCTGG-3´; 3´- Outer reverse CACTGTGTG AATTTCATTGGCCCCCAGC- 5´; 5´- Inner forward ACCTGTCCAACAACGAGATCACCTATGTCA- 3´; 3´- Inner reverse CACACCTCTGCAGGTCTCTGT TG CC-5´. TARMS-PCR reaction mixture was prepared for 15 µl comprising of master mix 6µl**,**template4µl, Outerforward primer 0.5µl, Outer reverse primer 0.5µl, Inner forward primer 1 µl, Inner reverse primer 0.5µl and distilled water 2.5µl.

PCR Amplification

Amplification reactions were carried out in Biorad thermal cycler, USA under optimized conditions which are as follows: Initial denaturation step at 95°C for 3 minutes followed by 30 cycles of cyclic denaturation at 95°C for 30 seconds, annealing at 64°C for 30 seconds, extension at 72°C for 40 seconds and final extension at 72°C for 5 minutes.

Checking of PCR product

The amplified PCR products were then checked on 3% agarose gel along with 50bp DNA ladder to know the exact size of the band.

Statistical analysis

Genotyping was done. Statistical analysis was performed using PopGene software [17] to calculate allele frequency, observed number of alleles (n_a), effective number of alleles (n_e) [18], Shannon's informative index (I) [19], observed heterozygosity (H_o), expected heterozygosity (H_e)[20],Polymorphic Information Content (PIC) and Wright's fixation indices (F_{IS}) [21]**.** Ewens-Watterson Test for neutrality was also done.

RESULTS AND DISCUSSION

DNA isolation and PCR amplification

DNA isolation was done from the venous blood samples using PCI method of isolation. Good quality bands of DNA were obtained without smearing and

Table 1: Genotype distribution and allelic frequencies at the *TLR2* gene in local Cattle population.

Locus	Genotypes			Gene/Allele		χ^2-test (HWE)	p-Value	G^2-test (HWE)	p-Value
	A/A	A/B	B/B	A	B				
TLR2	0.12 (06)	0.64 (32)	0.24 (12)	0.44	0.56	4.17	0.0412	4.27	0.0387

Figures in parentheses are number of observations.

Table 2: Summary of genetic variation statistics of *TLR2* gene in local cattle population.

Locus	n_a	n_e	I	F_{IS}
TLR2	2.00	1.97	0.6859	-0.2987

- n_a = Observed number of alleles
- n_e = Effective number of alleles [Kimura and Crow (1964)] [18]
- I = Shannon's Information index by Lewontin (1972), [19]
- F_{IS} = Fixation index of individual with sub-population by Wright's (1978) [21]

haziness on 0.8% agarose gel when viewed under UV trans-illuminator as shown in Fig 1. PCR amplification was carried out for *TLR2* genes at temperature of 64°C for 0.30sec.

TARMS PCR (Tetra amplification refractory mutation system) successfully amplified different genotypes amplicons thereby detecting SNP at 201 bp (G to A).The amplified DNA revealed 3 bands of 158bp (control fragment),120 bp (B allele) and 90bp (A allele) as shown in Fig 2. T-ARMS PCR is a relatively fast and inexpensive method for SNP genotyping. The method requires only a single PCR amplification followed by gel electrophoresis, making it relatively simple to perform. T-ARMS PCR can accurately distinguish between different genotypes, even with small amounts of DNA and can be used to genotype various SNPs in different species.

Genotype distribution and allelic frequencies at the *TLR2* gene

Genotypic distribution and allelic frequencies presented in Table 1 revealed that there are heterozygosity present in the population for *TLR*2 gene. The genotype frequencies were 0.12, 0.64 and 0.24 for AA, AB and BB genotypes, respectively. Heterozygous frequency is maximum in the population. B allele (0.56) was predominant over the A allele (0.44) in the studied population. Polymorphisms in *TLR*2 gene was reported for G and T alleles in cattle population [22], in TLR2 promoter and coding region were reported in six different cattle breeds of China using PCR-RFLF and CRS-PCR (Created Restriction Site-PCR) methods [23], HF cross bred cattle using PCR-SSCP method [24], Vietnamese Holstein Cattle [25] and crossbred cattle in India [26]. On the other hand, monomorphic *TLR*2 gene was reported in Canadian Holstein cattle [27], Italian native cattle [28]. The highest frequency of the $TLR2^{T}$ allele (0.99) was found in the Ayrshire breed. The herd of Ayrshire cows is close to being monomorphic for the *TLR2* gene; 98% of the animals have the homozygous $TLR2^{TT}$ genotype [29]. Similar genotype frequencies (0. 157, 0.605 and 0.236 for AA, AB and BB, respectively) and allele frequencies (0.460 for A and 0.539 for B) were reported in mastitis affected cattle [26], whereas, higher frequencies for AA genotype (0.392) and A allele (0.514) were reported in mastitis tolerant group [26]. Lower heterozygous genotype frequencies were reported in HF cross bred cattle for TLR 2 gene [24]. The allelic frequency of A and B allele were 0.5 and the genotypic frequency of the AB genotype is 1.00 without any homozygous genotype [30].In the studied population heterozygosity was more than that of the homozygosity indicates that individuals carrying different alleles which can impact the function of the TLR2 protein, potentially altering its ability to recognize and respond to pathogens.

χ^2-test and G^2-test statics in Table 1 showed significant effect ($p<0.05$) which indicates population is not in Hardy Weinberg equilibrium and evolutionary forces have acted in population. Deviation from Hardy Weinberg equilibrium might be due to use of a smaller number of breeding bulls in the population or introduction of other inheritance from the different population.Similarly,$\chi2$ values showed a highly significantdeviation from HW equilibrium probabilitiesfor TLR2 genotypes in HF crossbred cattle [24]. On contrary, non-significant ($p> 0.05$) genotype frequencies of 15 SNPs fitted with the Hardy-Weinberg equilibrium in HF breed in China were reported [31].

Summary of genetic variation statistics of *TLR2* gene

The observed number of alleles (n_a) and effective number (n_e) of alleles for *TLR2*were 2.00 and. 1.97, respectively in local cattle of Jammu (Table 2). Lower

Table 3: Summary of Heterozygosity Statistic of *TLR2* gene in local cattle population.

Locus	Obs_Ho	Obs_He	Ho	He	Nei**	Ave_Het	PIC
TLR2	0.535	0.465	0.535	0.465	0.4928	0.4928	0.3713

- Obs_Ho = Observed homozygosity
- Obs_He= Observed heterozygosity
- Ho = Expected homozygosity,
- He = Expected heterozygosity (Levene, 1949), [20]
- ** Nei's (1973) expected heterozygosity
- Ave_Het = Average Heterozygosity
- PIC = Polymorphic information content

Table 4: Ewens-Watterson Test for neutrality of *TLR2* gene in local cattle population.

Locus	Obs. F	Min. F	Max. F	Mean	SE	L95	U95
TLR2	0.5072	0.5000	0.9802	0.8125	0.0272	0.5018	0.9802

- F- F-values.
- L95-Lower confidence limits at 95% confidence.
- U95-Upper confidence limits at 95% confidence.

estimate of n_e value was also reported in HF crossbred cattle [24].

Shannon index (I) in the studied population was 0.6859. Shannonindex value indicates the genetic diversity within a population. Specifically, it measures the allelic diversity i.e.how many different alleles are present at a locus and how evenly their frequencies are distributed. Lower Shannon index (I) values were estimated in HF crossbred cattle for different *TLR*2 SNPs [24].

Negative F_{IS} values (-0.2987) depicted in Table 2 indicates the heterozygosity excess in the population. It means there is more genetic diversity in population and less inbreeding has occurred. The negative F_{IS} value might be due to introduction of genetically dissimilar partners or admixture of populations. Higher values of F_{IS} were reported in different SNPS in *TLR*2 gene in HF crossbred cattle [24].

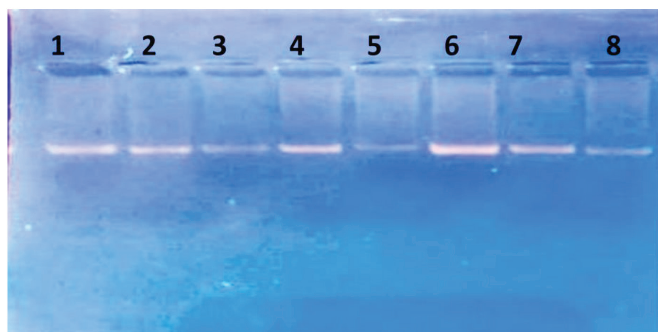
Summary of Heterozygosity Statistic of *TLR2* gene

For *TLR2* gene in the present study observed and expected homozygosity value was 0.535, observed and expected heterozygosity value was 0.465 and average heterozygosity was 0.4928, respectively (Table 3). The results show no overall loss of heterozygosity. The result indicates genetic diversity in the population. However, lower observed heterozygosity compared to expected heterozygosity were reported in different SNPS in *TLR*2 gene in HF crossbred cattle [24]. Observed heterozygosity (Ho) and Expected heterozygosity (He) of the loci determined from SNP frequencies ranged from 0.051 to 0.466 and from 0.060 to 0.493, respectively in cattle [32]. On the other hand, observed heterozygosity was reported to be 1.0 in Malnad Gidda cattle [30].

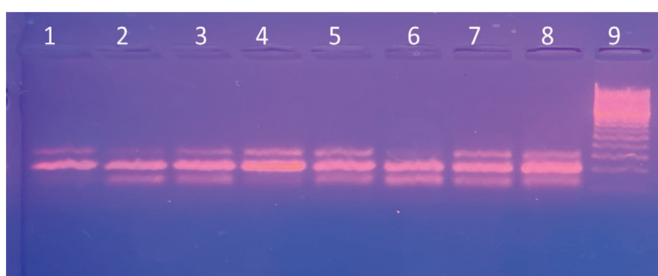
Nei's expected heterozygosity 0.4928 means that there is a 49.28% chance that two alleles randomly selected from the population are not the same. This reflects a moderate level of genetic variation in the population.The polymorphism information content (PIC) was 0.3713 (Table 3) showing diversity in the population.Nei's expected heterozygosity and PIC value indicates moderate level of genetic diversity within the population. Higher estimate of PIC value was reported in Malnad Gidda cattle [30]. However lower to similar PIC values were reported in cattle ranged from 0.058 to 0.372 [32].

Ewens-Watterson Test for neutrality of *TLR2* gene

Ewen Watterson test for neutrality showed F value of 0.5072 (Table 4) which is very close to lower bound (0.5018) of 95% confidence interval. Since observed F is within the confidence interval (within the range of L95-U95) it shows *TLR2* gene in consistent with neutrality. The non-significant Ewens-Watterson test suggests that the observed allele frequencies conform to expectations under neutral evolution. Therefore, no strong evidence of selection acting on this locus was detected. *TLR*2 is an immune gene often associated with disease resistance (and sometimes under selection). But the result shows that there might not be any current selective pressure which reflects the stable environment, low pathogen exposure, or historic balancing selection



Lane: 1-8 diluted DNA
Fig1: DNA bands 0n 0.8% Agarose gel



Lane 1, 4: B/B
Lane 2, 6: A/A
Lane 3, 5, 7 8: A/B
Lane 9: 50 bp DNA ladder
Fig 2: Genotyping after TARMS PCR

that has now stabilized. However, the fact that observed F is much lower than mean (0.8125) might suggest some deviation from neutrality probably indicating balancing selection, admixture, or gene flow.

However, the fact that observed F is much lower than mean (0.8125) might suggest some deviation from neutrality probably indicating balancing selection, admixture, or gene flow.

The functional details and prediction for structural model for *TLR2* in chicken model has been described earlier [33] and need to be undertaken in cattle breed.

CONCLUSION

The results from the present study showed polymorphic nature of *TLR2* gene in local cattle of Jammu that shows moderate level of genetic variation in the population and *TLR2*gene can be used for genetic improvement and marker assisted selection for disease resistance.

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