Toll like receptor 4 gene polymorphism in exon 3 of Malnad Gidda Cattle

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Abstract

Toll-like receptor 4 (TLR4) as the ability of identifying diversified pathogenic ligands. It is a cell surface receptor that recognizes lipopolysaccharides (LPS) of gram-negative bacteria and it can act as candidate marker for marker assisted selection for disease resistance or susceptibly in the dairy cattle. Mutations in TLR4 can compromise the host immune response to certain pathogens, so it may be a potential candidate for marker assisted selection to enhance disease resistance in dairy cattle. In the present study, polymorphic pattern of exon-3 of TLR4 gene was studied in Malnad Gidda cattle which is a unique recognized dwarf cattle breed of western ghat region of Karnataka which is famous for its dwarfness and disease resistance characters.

Polymorphic analysis of exon-3 which was fragmented into three fragments to explore the genetic structure and sequence analysis and comparing the same with Bos taurus and other cattle breed to study the variations if any.

Keywords: Malnad Gidda, disease resistance, polymorphism, RFLP.

Introduction

Toll-like receptors (TLRs) are the major group of pattern recognition receptors (PRRs) which play a very important role in immunity of the individual. They are a family of germ line encoded receptors of the innate immune system and play an important role in disease resistance through their recognition of pathogen-associated molecular patterns (PAMPs) and provide critical host defence during microbial infection. In addition to their role in defense against pathogens, the deregulation of TLRs results in increase of uncontrolled inflammation and metabolic syndromes, which contributes to the development of chronic bacterial, viral and parasitic diseases[12]. *TLR4* is the important receptor of the TLRs family that recognizes endotoxins associated with gram-negative bacterial infections.[6,9,10,12]

TLR4 plays an important role in the recognition of bacterial pathogens and plays a key role in linking innate and adaptive immunity[1]. *TLR4* is considered as a 'candidate gene' for resistance to many of the devastating bovine disorders like mastitis[5,9,11], bovine tuberculosis[2,3] Johne's disease[9,13],

bovine respiratory disease complex. *TLR4* gene also acts as an ideal model to study the consequences of genetic variation and their relation to the function of receptor and their susceptibility to diseases.

Malnad Gidda is one of the unique recognized cattle breed which belongs to Malnad and coastal region of Karnataka. They are famous for their small body size and strength to adopt and withstand wide range of local climatic conditions of the hilly terrain of Western Ghat. They play a significant role in contribution to local economy by providing livelihood security by providing milk, draught power and manure with minimum or zero inputs. Malnad Gidda cows are small in size with compact body frame, weighing between 80 and 120 Kg. They are well adapted to hot humid and hilly region and are generally let for grazing on their own in forests and open areas. The milk yield of a well-built Malnad Gidda cow varies from 1.5 liters a day to 4 liters a day and the milk and milk products are known for a unique taste and creaminess[8]. These cattle are highly resistant to diseases when compared to crossbred cattle and have ability to withstand stressful environmental conditions[4,8].

Very unique characteristics of this breed of cattle are the incidences of many tropical diseases are rare. They have been identified to be better resistant against foot and mouth disease in comparison to any other breed of the region[4] and also resistant to some bacterial, viral and parasitic diseases. So with this background, the present study is taken up for identification of genetic markers of disease resistance or susceptibility and these genetic markers can be utilized as a tool for rapid genetic improvement in selection programme and to improve both health status of the cow which invariably support the economic condition of the farmer. Hence, the present investigation was underpinned with the objective to explore polymorphic patterns in coding region of exon-3 of *TLR4* genes in Malnad Gidda cattle.

Material and Methods

Blood samples were collected randomly from 110 unrelated Malnad Gidda cattle from different parts of Malnad area where these animal breeding tracts are distributed. DNA isolation was carried out by adopting the high salt method with minor modifications. The gDNA samples that were utilized for downstream analysis yielded satisfactory results. Exon-3 of *TLR4* was amplified as three separate fragments and designated as *TLR4*-E3F1, *TLR4*-E3F2 and *TLR4*-E3F1. While designing the primers, part of introns was included so as to cover the complete exonic regions and also to avoid the

presence of variations at the primer binding sites while sequencing.

The amplified PCR amplicons of exon-3 of *TLR4* gene were digested using different restriction enzymes by restriction fragment length polymorphism technique (RFLP). The details of the primer and restriction enzymes used are given in table 1. For identification of single-nucleotide polymorphism (SNP) in fragments, suitable restriction enzymes were identified using NEB-cutter V2.0 online tool (http://tools.neb.com/NEBcutter2/). The length of digested fragments was predicted *in silico* for each SNP locus along with its genotypes. The representative PCR products from each fragment of *TLR4* gene of Malnad Gidda cattle for different RFLP patterns were custom sequenced by using forward and reverse primers. The sequencing data was analyzed using DNA Star software (www.dnastar.com). Multiple sequence alignments were performed with BioEdit and Megalign software.

All the three PCR fragments of exon-3 of *TLR4* gene were subjected to basic local alignment search (BLAST) to identify the sequence homology with the corresponding regions of other breeds or species. Sequence data from variants of different regions were subjected to multiple sequence alignment software (CLUSTAL-W) to find out the SNPs. The annotated sequences of *TLR4* gene were translated into amino acid sequence and the same were compared with that of *Bos taurus TLR4* amino acid sequence available from NCBI database in order to identify the possible variation in functional properties of *TLR4* gene.

Results and Discussion
PCR-RFLP analysis of different fragments of exon-3 of *TLR4* gene: The PCR-RFLP analysis of fragment-1, corresponding to exon-3 of *TLR4* gene using *Bsr*I restriction enzyme was analyzed on agarose gel which revealed only AA genotype having band size of 663 bp exhibiting monomorphic pattern (Plate 1 and 2). The allelic frequency of A allele was 1.0 and the genotypic frequency of AA genotype was 1. Genotype AB and BB were not observed in the studied population.

Table 1
Details of Primer, restriction enzyme used for amplification and digestion of Exon-3 of *TLR4* gene in Malnad Gidda cattle

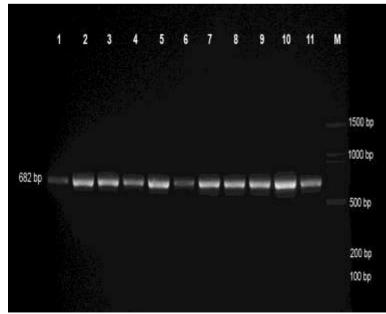| Gene	Primer's Name	F/R	5'-Primer sequence-3'	Length (bp)	Product Size (bp)	Region amplified	Restriction enzyme	Cutting sites
TLR4	*TLR4*-E3F1	F	GGAGACCTAGATGACTGGGTTG	20	682	Partial CDS, Partial intron-2	*Bsr*I	19bp, 663bp and 682bp
		R	CAATGGTCAGGTTGCACAGT	22				
	TLR4-E3F2	F	AGCTCAATGGATTGACTCTGC	21	996	Partial CDS	*Ava*II	172bp, 823bp and 996bp
		R	AGGCCATGATACGGTTGAAG	20				
	TLR4-E3F3	F	ACTCGCTCCGGATCCTAGAC	20	987	Partial CDS and Partial intron-3	*Apo*I	65bp, 922bp and 987bp
		R	GCACAATGCTTGGTACATGG	20				

Plate 1: PCR amplification fragment-1 corresponding to exon-3 of *TLR4* gene in Malnad Gidda cattle.
Lane 1 to 10: 682 bp amplicons, Lane M: 100 bp Molecular marker

The PCR-RFLP analysis fragment-2 corresponds to exon-3 using *Ava*II restriction enzyme revealed three genotypes namely AB genotype (996 bp, 823 bp and 172 bp), AA genotype (996 bp and 823 bp) and BB genotype (996 bp) exhibiting polymorphic pattern (Plate 3 and 4). The allelic frequencies of A and B allele were 0.52 and 0.47 respectively and the genotypic frequency of the AA genotype was 0.19, AB genotype was 0.66 and BB genotype was 0.14 with observed heterozygosity, PIC and allelic diversity of 0.66, 0.3744 and 0.4987, respectively.

The PCR-RFLP analysis of fragment-3 corresponding to exon-3 of *TLR4* gene   analysis using *Apo*I restriction enzymes revealed three genotypes namely AB genotype (987 bp, 922 bp and 65 bp), AA genotype (987 bp and 65 bp), BB genotype (65 bp), exhibiting polymorphic pattern (Plate 5 and 6). The allelic frequency of A and B allele was 0.57 and 0.42 respectively and the genotypic frequency of AA was 0.31, AB was 0.51 and BB was 0.16 with observed heterozygosity, PIC and allelic diversity of 0.51, 0.3695 and 0.4891 respectively.
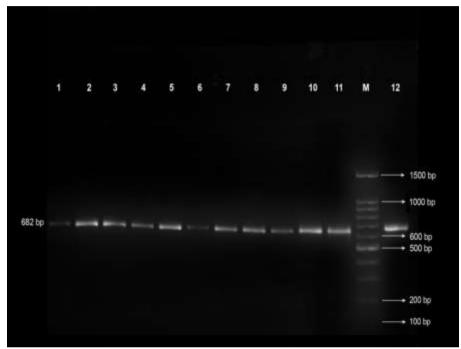

Plate 2: PCR-RFLP profile of *TLR4*-E3F1/*Bsr*I in Malnad Gidda cattle. Lane 1–11: AA genotype (663 bp), Lane M: 100 bp Molecular marker, Lane 12: PCR product of 682 bp
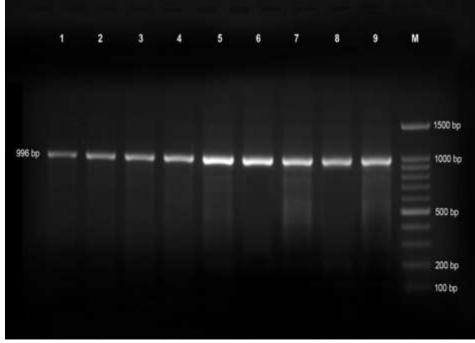

Plate 3: PCR amplification fragment-2 corresponding to exon-3 of *TLR4* gene in Malnad Gidda cattle. Lane 1 to 10: 996 bp amplicons, Lane M: 100 bp Molecular marker
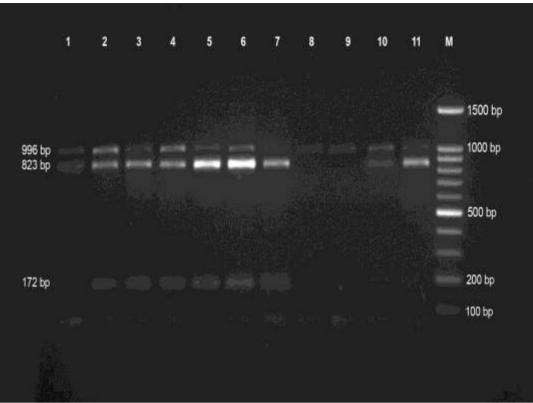
Plate 4: PCR-RFLP profile of *TLR4*-E3F2/*Bsr*I restriction enzyme in Malnad Gidda cattle. Lane 2 to 7: AB genotype (996 bp, 823 bp and 172 bp), Lane 1, 10 and 11: AA genotype (996 bp and 823 bp), Lane 8 and 9: BB genotype (996 bp), Lane M: 100 bp Molecular marker
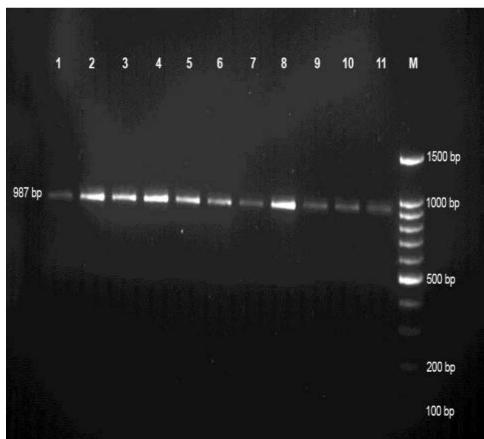

Plate 5: PCR amplification fragment-3 corresponding to exon-3 of *TLR4* gene in Malnad Gidda cattle. Lane 1 to 10:987 bp amplicons, LaneM: 100bp Molecular marker
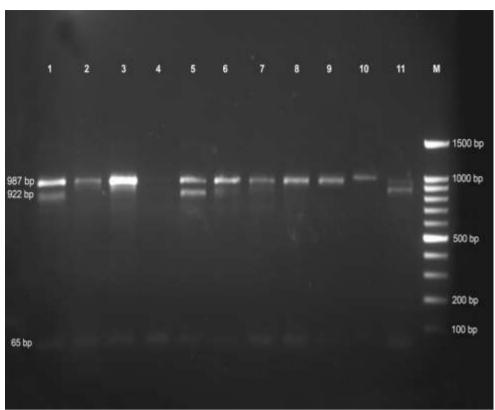
Plate 6: PCR-RFLP profile of *TLR4*-E3F3/*Apo*I in Malnad Gidda cattle. Lane 1, 5 to 7: AB genotype (987 bp, 922 bp and 65 bp), Lane 2, 3, 8and10: AA genotype (987 bp and 65 bp), Lane 4: BB genotype (65 bp), Lane M: 100bp Molecular marker
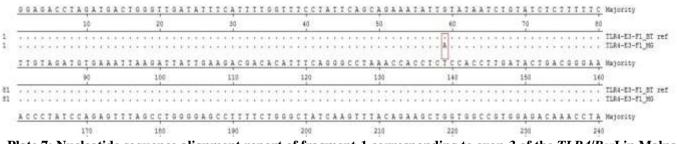

Plate 7: Nucleotide sequence alignment report of fragment-1 corresponding to exon-3 of the *TLR4/Bsr*I in Malnad Gidda cattle

In Malnad Gidda cattle population, the present investigation revealed a significant departure of *TLR4*-E3F1/*Bsr*I and *TLR4*-E3F2/*Ava*II markers from Hardy-Weinberg Equilibrium (HWE). However, *TLR4*-E3F3/*Apo*I marker was in HWE.

Sequence analysis of exon-3 of *TLR4* gene: Two of the representative PCR products of different fragments of *TLR4*-E3 were sequenced bi-directionally to confirm the variations in restriction site and also to identify any novel SNPs in exon-3 of the *TLR4* gene. Sequence analysis *TLR4*-E3F1 confirmed the presence of 682 bp partial coding sequence, partial intron-2 and sequence analysis has revealed no novel SNPs in the restriction sites of *Bsr*I enzyme. When compared to *Bos taurus* sequence, one new SNP was identified at 59th (Plate 7) position where in transition G is replaced by A in Malnad Gidda cattle (Plate 8) and it is synonymous SNP, there was no change in the pattern of amino acid sequence.

Basic Local Alignment Search Tool (BLAST) analysis of the annotated sequence of *TLR4*-E3F1 was used as query and subjected to nucleotide blast at NCBI which revealed 100 per cent identity with *Bos taurus* (AH013178.2) and crossbred cattle (KM114870.1), 99.85 with Sahiwal (HM219872.1), 99.83 per cent with Tharparkar (KM102982.1), 99.16 per cent with Vechur cattle (KX138607.1), 97 per cent with other *Bos indicus* cattle (EU386357.1), 97.80 per cent with *Bubalus bubalis* (JN786600.1) and *Capra hircus* (HM627213.2), 97.51 per cent identity with *Ovies aries* (GU461886.1) respectively.

Sequence analysis of *TLR4*-E3F2 confirmed the presence of 996 bp partial coding sequence and revealed nil SNPs in the restriction sites of *Ava*II enzyme in *TLR4*-E2F2 (Plate 40). When compared to the *Bos taurus* sequence, no evidence of new SNP or no variation in the amino acid sequence was observed in Malnad Gidda cattle. BLAST analysis *TLR4*-

E3F2 revealed 100 per cent identity with *Bos taurus* (AH013178.2) and crossbred cattle (KM114870.1), 99.50 with Sahiwal (HM219873.1), 99.50 per cent with Tharparkar(KM102982.1), 99.80 per cent with Vechur cattle (KX138607.1), 99.90 per cent with other *Bos indicus* cattle (EU386357.1), 98.09 per cent with *Bubalus bubalis* (JN786600.1), 97.49 per cent with *Capra hircus* (DQ922635.1) and 95.68 per cent identity with *Ovies aries* (GU461886.1) respectively.

Sequence analysis of *TLR4*-E3F3 revealed nil SNPs in the restriction sites of *Apo*I enzyme and when compared to the *Bostaurus* sequence, one new SNP was identified at 137bp position (Plate 9 and 10) in AA genotype has transition C is replaced by T in Malnad Gidda cattle and it is a synonymous SNP. No change in the pattern of the amino acid sequence was observed.
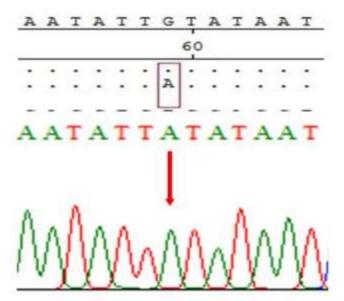

Plate 8: Chromatograph showing nucleotide G>A transition for *TLR4*-E3F1 gene at 59bp position
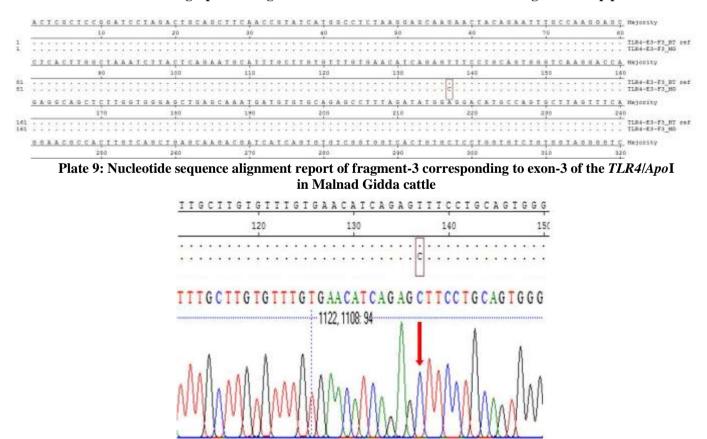

Plate 9: Nucleotide sequence alignment report of fragment-3 corresponding to exon-3 of the *TLR4*/*Apo*I in Malnad Gidda cattle

Plate 10: Chromatograph showing nucleotide T>C transition for the *TLR4*-E3F3 gene at 137bp position

Conclusion

The present study indicates that no observable and significant variations were seen in polymorphic pattern of Malnad Gidda *TLR4* genes when compared to Bos Taurus and sequence analysis also does not revealed any non synonymous SNP"s in Malnad Gidda cattle. The present study has contributed in enriching the knowledge on genomic resource of Malnad Gidda cattle, which may form base for further comprehensive studies with respect to *TLR2* and *TLR4* genes.

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