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TLR4 gene polymorphism and its association with mastitis resistance in indigenous and crossbred cattle of Tamil Nadu, India

Abstract

TLR4 gene is one of the candidate genes for mastitis resistance in cattle. This gene has three exons and PCR with nine sets of primers produced nine amplicons. The amplicons were sequenced from both ends, assembled and screened for SNPs. Totally, 221 dairy cows (Jersey crossbreds, Holstein Friesian crossbreds and indigenous breeds) were genotyped either by PCR-RFLP or tetra-primer ARMS-PCR. Genotyping results showed that one SNP was in 5'UTR region, two SNPs in intron 1, one in exon 2 and seven in exon 3. Out of 11 SNPs, allele frequencies were found to differ between healthy and mastitic cows in six SNPs viz. SNPs 245, 5053, 5134, 9422, 9787 and 9794. At SNP position 5134, the *G* allele frequency got reduced from 0.71 in healthy cows to 0.50 in mastitic cows but the A allele frequency increased from 0.29 in healthy cows to 0.50 in mastitic cows. The SNP 5053T>G resulted from transversion while the SNPs 5087G>A and 5134G>A resulted from transitions. In SNP 5134G>A, the frequency of both the alleles were in equal proportions in mastitic animals. The SNP 9787C>T in exon 3 had shown no appreciable change in the allele frequencies in both normal and mastitis animals. Homology modeling of TLR4 gene revealed no change in the length of the amino acids of TLR4 protein. The amino acid tyrosine present in the position 674 in the protein chain of *Bos taurus* was replaced lysine in crossbreds. No association was found between TRL4 genotypes and somatic cell scores in the breeds studied.

Keywords: tlr4 gene, sequence analysis, mutations, mastitis, SCS, association

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Introduction

Research in the field of natural resistance of animals and humans has increased the chances of controlling the pathogenic, metabolic and hereditary diseases. Toll-like receptors (TLRs) are important component of innate immune system. TLR gene family has been reported as a promising molecular marker for correlation between host immune responses and bacterial pathogens in mastitis. TLRs are the key sensors of microbial infections. They are ultimately responsible for recognition of most of the pathogens. The discovery of TLRs created not only a chance for better elucidation of defensive mechanism of the organism against pathogens but also facilitated identification of genes which decide on the resistance of animals to mastitis. Genes responsible for the expression of TLRs show considerable variability.[1] The polymorphism of TLR genes may affect the differentiation properties of individual alleles and thus change their function. Several studies have shown that mutations in the TLR genes may reduce the ability of the protein to recognize the pathogen associated molecular patterns (PAMPs) and hence interfere with innate immunity activation. McGuire et al.[2] recognized 13 TLRs in the mammalian system.[2] The bovine genome sequence has recently been completed and annotated DNA sequence information are available for genes encoding TLRs 1 to 10.[3] Bovine TLR4, like human TLR4, is highly polymorphic.[4] TLR4 has been found associated with lipo-polysaccharide responsiveness. Sharma et al.[5] had reported association between TLR4 gene polymorphism with somatic cell score (SCS) and lactation persistency in Holstein bulls.[5] Such attributes make TLR4, a suitable candidate gene for mastitis detection in dairy cattle. With this background, the present study was planned to investigate the variation in the nucleotide sequence of the TLR4 gene in indigenous and crossbred cattle and the association of such single nucleotide variation with the innate immunity to udder infection.

Materials and methods

Experimental animals and isolation of DNA

Ten ml of blood was collected aseptically by jugular vein puncture in a sterile vacutainer (Becton-Dickinson) containing 15percent of 0.12ml EDTA solution from 29 healthy indigenous cows, 50 healthy and 71 mastitic Jersey crossbred cows and, 28 healthy and 43 mastitic Holstein Friesian crossbred cows. There was no case of mastitic cow among the indigenous cows. The genomic DNA was isolated by modified Phenol-Chloroform method using DNAzol. The quality and quantity of DNA was assessed by nanodrop and diluted to obtain the template DNA (working DNA) concentration of approximately 20 to 50ng perµl and stored at -20°C.

Primer sequences and PCR amplification

The primers were designed using Primer 3 software (http://bioinfo.ut.ee/primer3-0.4.0/) according to published bovine genomic TLR4 sequence (GenBank Accession No.: NM 174198). For the amplification of three exons of TLR4 gene, nine primers were used and the regions covered were -128bp upstream to 617bp for exon 1 and 4962 bp to 5485bp for exon 2 which includes part of intron 1 and from 7933bp to 11183bp for exon 3. The PCR reactions were

carried out in a reaction volume of 15µl and the annealing temperature varied from 54.3 to 66.8°C. Around 5µl of the PCR product was run along with a 50bp DNA ladder (Gene Ruler™, Fermentas) in 2percent agarose gel, viewed in a UV trans-illuminator and documented by a gel documentation system (Bio-Rad, USA).

Sequencing of PCR products

The amplicons of 10 representative samples were sequenced both in forward and reverse directions in an automated ABI PRISM 3730XL Genetic Analyzer (Applied Biosystems, USA) from both ends. Sequence data were analysed using the Editseq program of LASERGENE software (DNASTAR Inc., Madison, WI, USA). The *Bos taurus* gene sequence for TLR4 gene obtained from NCBI was used as the reference sequence for analysis. The sequence was assembled and screened for SNPs.

Genotyping of SNPs

The SNPs thus detected were genotyped using both PCR-RFLP and tetra-primer ARMS-PCR. The restriction sites and the corresponding enzymes were analysed using the online tool NEB cutter (http://tools.neb.com/NEBcutter2). Tetra-primer ARMS-PCR primers were designed using online tool Primer1 (http://primer1.soton.ac.uk/ primer1.html). The PCR products were digested with the corresponding restriction enzymes to identify SNPs. The digested products were resolved in three per cent agarose gel along with 50 bp DNA ladder and stored in a gel documentation system (Bio-Rad, USA).

Protein prediction

The nucleotide sequences of coding domains from 471 to 563 bp for exon1, 5111 to 5277 bp for exon 2 and 8027 to 10292 bp for exon 3 of TLR4 gene were submitted to EMBOSS (http://www.ebi.ac.uk/Tools/services/web_emboss_transeq/toolform.ebi), an online tool which translates the given nucleotide sequence into amino acid sequence. The amino acid sequence was submitted online to PSIPRED to predict its structure and the 3D structure is viewed with the help of Jmol, an open source molecular viewer (http://www.jmol.org/).

Estimation of gene and genotype frequencies

The genotypes were assigned on the basis of restriction digestion pattern of the PCR products. The allele and genotype frequencies were calculated by standard formula.[6]

Association studies

Mean somatic cell count and breed (HF crossbred, Jersey crossbred and native cows) were considered for association of different SNPs of TLR4 gene. The least-squares method as described by Harvey[7] was used to estimate association between SNPs of TLR4 with SCC using the following model:

$$Y_{ij} = \mu + B_i + G_j + e_{ij}$$

where,

Y_{ij}: Somatic cell count of an animal in i^{th} breed and j^{th} SNPs

μ: Overall mean

B_i: Effect of i^{th} breed (i =1 to 3)

G_j: Effect of j^{th} SNPs (j =1 to 3)

e_{ij}: Random error normally and independently distributed with mean 0 and variance σ^2_e (NID(0, σ^2_e)).

The difference of means between SCS within breed and various SNPs were tested for significance by applying Duncan's Multiple Range Test as modified by Kramer.[8]

Results and discussion

Nucleotide sequence of bovine TLR4 gene

A total of nine amplicons were generated for the three exons. The product length of primer covering the exon 1 was the longest of all (745bp), the major part of it being 5'UTR region. The sizes of other amplicons ranged from 450bp to 650bp except for the first primer of exon 3 which was 726bp long. In the present study, 11 SNPs were found after sequencing 5409bp out of the total gene length of 11013bp. Of the 11 mutations identified, one was in 5'UTR, two were in intron 1, one in exon 2 and seven in exon 3. There was a non-synonymous mutation in exon 3 (SNP 9787C>T) which had changed amino acid tyrosine to lysine. All these SNPs were reported earlier (seven SNPs by Wang et al.[9] one SNP by Mesquita et al.)[10] There was no novel SNP found in TLR4 gene in the present study. The SNPs and other variations found in the nucleotide sequences of expressed regions of bovine TLR4 gene among the crossbred populations are presented in Table 1.

SNP genotyping of TLR4 gene

Out of the 11 SNPs, six were genotyped by PCR-RFLP as they had restriction sites for *BstUI, MsPA1, AluI, TseI, BsiHKAI* and *BsaHI* enzymes; and five SNPs genotyped by tetra-primer ARMS-PCR. Out of 11 SNPs, allele frequencies were found to differ between healthy and mastitic cows in six SNPs viz. SNPs 245, 5053, 5134, 9422, 9787 and 9794 (Table 2). The change in allele frequencies were more pronounced in SNP position 245, 5053 and 9422. At SNP position 5134, the *G* allele frequency got reduced from 0.71 in healthy cows to 0.50 in mastitic cows but the *A* allele frequency increased from 0.29 in healthy cows to 0.50 in mastitic cows, thus making the allele frequencies of *G* and *A* exactly equal.

The variation was found at position 245G>C with the frequency of *G* allele being higher in the overall population (0.58). The size of amplified region was 524 bp with the primer spanning from 4962 to 5485 bp of the TLR4 gene covering entire exon 2 and parts of intron1. Two SNPs (5053T>G and 5087G>A) were found in the intron 1 and one in exon 2 (5134G>A). The SNP 5053T>G resulted from transversion while the SNPs 5087G>A and 5134G>A resulted from transitions. In SNP 5134G>A, the frequency of both the alleles were in equal proportions in mastitic animals. The SNP 9787C>T in exon 3 had shown no appreciable change in the allele frequencies in both normal and mastitis animals. The minor allele frequencies ranged from nine percent (9794T>C) to 43 per cent (9758C>A) in the mastitis affected animals.

Protein prediction

Three dimensional structure of TLR4 gene with identified SNPs were predicted with homology modeling. As mutation causing changes in amino acid may eventually lead to altered function of the protein and subsequently contribute to alteration in susceptibility or

resistance to pathogen invasion. Thus protein structures of reference sequence and the sequence with SNPs were predicted. There was no change in the length of the amino acids of TLR4 protein (841 amino acids). The amino acid tyrosine was present in the position 674 in the protein chain of *Bos taurus* while it was lysine in crossbreds; the molecular weight of *Bos taurus* protein was 96014.8 Da while that of crossbreds was 96026.9Da.

Association of TLR4 gene with SCS

In the present study, no association was found between TRL4 genotypes and SCS. Similarly, no association was found between TLR4 gene SNP 245G>C and SCS in earlier studies also.[5,11] On the contrary, associations were found between SNP 245 and SNP 5087 and SCS[10] and between SNP 9787 and SCS.[9]

Table 1 Identified SNPs and type of mutation in bovine TLR 4 gene

Sl. No.	SNP	Nucleotide		Type of mutation	Amino acid substitution	Change in property of amino acid
		Bos taurus	Crossbred cattle			
5'UTR						
1	245G>C	G	C	Transversion	No	---
Intron1						
2	5053T>G	T	G	Transversion	No	---
3	5087G>A	A	G	Transition	No	---
Exon2						
4	5134G>A	G	A	Transition	No	---
Exon3						
5	8885A>G	A	G	Transition	No	---
6	8933T>G	T	G	Transversion	No	---
7	9287A>G	A	G	Transition	No	---
8	9422C>T	C	T	Transition	No	---
9	9758C>A	C	A	Transversion		
10	9787C>T	C	T	Transversion	Tyrosine to Lysine	Neutral-polar to Neutral non-polar
11	9794T>C	T	C	Transition	No	---

Table 2 Genotype and allele frequencies of SNPs in bovine TLR 4 gene

Sl. No.	SNP	Genotype	Genotype frequency			Allele	Allele frequency		
			Healthy cows	Mastitic cows	overall		Healthy cows	Mastitic cows	Overall
		GG	0.21	0.62	0.42	G	0.42	0.72	0.58
1	245	GC	0.45	0.21	0.32	C	0.58	0.28	0.42
		CC	0.34	0.17	0.26				
		TT	0.29	0.51	0.41	T	0.5	0.68	0.6
2	5053	GT	0.43	0.35	0.38	G	0.5	0.32	0.4
		GG	0.28	0.14	0.21				
		GG	0.55	0.38	0.47	G	0.7	0.68	0.7
3	5087	GA	0.3	0.6	0.45	A	0.3	0.32	0.3
		AA	0.15	0.02	0.08				
		GG	0.48	0.2	0.34	G	0.71	0.5	0.6
4	5134	GA	0.46	0.6	0.53	A	0.29	0.5	0.4
		AA	0.06	0.2	0.13				
		AA	0.44	0.5	0.48	A	0.67	0.63	0.66
5	8885	AG	0.47	0.26	0.36	G	0.33	0.37	0.34
		GG	0.09	0.24	0.16				

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Table Continued..

Sl. No.	SNP	Genotype	Genotype frequency			Allele	Allele frequency		
			Healthy cows	Mastitic cows	overall		Healthy cows	Mastitic cows	Overall
		TT	0.46	0.38	0.41	T	0.69	0.61	0.65
6	8933	TG	0.47	0.46	0.48	G	0.31	0.39	0.35
		GG	0.07	0.16	0.11				
		AA	0.4	0.46	0.44	A	0.68	0.67	0.68
7	9287	AG	0.57	0.42	0.48	G	0.32	0.33	0.32
		GG	0.03	0.12	0.08				
		CC	0.17	0.4	0.29	C	0.4	0.62	0.52
8	9422	CT	0.47	0.45	0.46	T	0.6	0.38	0.48
		TT	0.36	0.15	0.25				
		CC	0.26	0.26	0.26	C	0.56	0.57	0.56
9	9758	CA	0.6	0.63	0.61	A	0.44	0.43	0.44
		AA	0.14	0.11	0.13				
		CC	0.56	0.31	0.43	C	0.76	0.61	0.68
10	9787	CT	0.41	0.6	0.5	T	0.24	0.39	0.32
		TT	0.03	0.09	0.07				
		TT	0.66	0.85	0.76	T	0.79	0.91	0.85
11	9794	TC	0.26	0.13	0.19	C	0.21	0.09	0.15
		CC	0.08	0.02	0.05				

Conclusion

In the present study, the TLR4 gene revealed 11 SNPs; one SNP at position 9787 resulted in non-synonymous mutation leading to replacement of amino acid tyrosine in *Bos taurus* at position 674 to lysine in the crossbred cows. The SNP at 5'upstream region in TLR4 gene, 245G>C found in this study is in potential DNA binding site and even though no association was found between this SNP and SCS, further study in large population is needed.

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None.

Conflict of interest

Author declares that there is no conflict of interest.

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