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Genotype based transcript abundance of TLR2 among crossbred cattle and their relation with mastitis

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Abstract

Mastitis is the most severe disease for dairy cattle based on traditional cost analysis. Genetic factor is one of the several factors affecting the occurrence of mastitis. Toll like receptor2 (TLR2), a key component of innate immune system, plays an important role in the initiation of the inflammatory responses to foreign pathogens. This present study was aimed to identify single nucleotide polymorphisms (SNPs) in the TLR2 gene and analyze the association of expression in different genotypes with mastitis. A total of two hundred nine crossbred cows (140 mastitis tolerant and 69 mastitis group) were selected to explore the genotypic and expression profiling of TLR2 gene and their association with mastitis susceptibility. Two fragments of TLR2 gene (179 bp and 313 bp) were amplified by Polymerase Chain Reaction (PCR). The different genotypic analysis by SSCP revealed that 179 bp fragment was monomorphic where in 313 bp was polymorphic. Association studies revealed that, AA genotype of 313 bp were less susceptible to mastitis and the mRNA expression of TLR2 was significantly ($P<0.05$) higher in AB and BB genotypic animals compare to AA. This suggested that genotype AA can be useful for selecting disease resistant traits.

Keywords: TLR2, polymorphism, mastitis, expression, SSCP

Introduction

The innate response to pathogen invasion of the mammary gland is likely to be dictated by a series of effecter cells activated by their pattern recognition receptors (Takeda *et al.* 2003). Toll like receptors represent the best described family of such "pattern recognition receptors" (Akira and Takeda 2004). They are a family of proteins that perform two functions: recognition of pathogen ligands and signaling to initiate innate and adaptive immune responses. Mammals are equipped with 13 different toll like receptors and each toll like receptor distinguishes unique patterns from bacterial cell wall components (Werling *et al.* 2006), so called pathogen-associated molecular patterns (PAMP). Their central role in regulation of the immune response to pathogens makes them promising candidate genes for genetic susceptibility studies. Each member of the TLR family is capable of recognizing a unique set of PAMP and other gram negative/positive bacteria.

TLR2 recognizes many bacterial, fungal, viral, and certain endogenous substances. Among these, peptidoglycan and lipoprotein, which are components in the cell walls of Gram-positive and Gram-negative bacteria, are the ligands for TLR2. TLR2 is a membrane protein, which is expressed on the surface of certain cells and recognizes foreign substances and passes on appropriate signals to the cells of the immune system. TLR2 is capable of detecting a variety of pathogen-associated molecular patterns (PAMPs) associated with gram positive or gram negative bacteria, mycobacteria, fungi, viruses, and parasites (Texereau *et al.* 2005). In the case of mastitis, TLR2 mRNA expression is strongly increased in mammary tissue of cattle (Goldammer *et al.* 2004). TLR2 is highly polymorphic in the bovine species and its expression is associated with intra-mammary infections (IMI), hence this gene may be a potential candidate for use in marker assisted selection to enhance mastitis resistance in dairy cattle. Bovine TLR2 was assigned to *Bos taurus* (BTA) chromosome 17. Bovine TLR2 gene includes two exons and spans about 13.2 kb of genomic DNA. Mariotti *et al.* (2009) identify eight SNPs in genes of great interest in cattle management by screening the nucleotide sequences of bovine TLR2, TLR4 and TLR6 genes. The present work was designed to identify genetic polymorphism in exon 2 region of TLR2 and further to establish the relationship of genotype wise differential expression profile of TLR 2 among crossbred cattle with occurrence of mastitis.

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2. Materials and methods

2.1. Experimental animals

A total of 209 crossbred cows (crosses of Holstein Friesian/Brown Swiss/Jersey with Hariana), maintained at Cattle and Buffalo Farm, IVRI, Izatnagar, were randomly selected for this study. These cows were maintained under similar feeding and managemental practices. The cows which had never been affected by clinical mastitis during their productive life, tested negative for California Mastitis Test (CMT) and Somatic Cell Count (SCC) were kept in the mastitis tolerant group. Whereas, the cows affected with clinical mastitis at least once on the basis of history of animals, positive for CMT and SCC were kept in the clinical mastitis group.

2.2. Sample collection

About 6 ml of blood was collected from the jugular vein of each animal in a sterile polypropylene vials containing 2.7% EDTA (0.5ml/10 ml of blood) as an anti-coagulant under sterile conditions. After collection of blood, the vials were tightly capped and shaken gently to facilitate thorough mixing of blood with the anti-coagulant. The vials were then placed immediately in icebox containing ice and gel cool packs and were transported to the laboratory immediately. The genomic dna was isolated from the blood samples by 'Phenol:Chloroform isolation' method as described by Sambrook and Russell (2001). The purity of genomic dna was checked using Spectrophotometer.

2.3. Primer designing and amplification

Primers of TLR2 gene were designed for amplification of two fragments, TLR2 A (179 bp) and TLR2 B (313 bp) gene encompassing partial exon 2. All the pairs of primers for amplification of fragments were designed on the basis of sequences of bovine and other species available publicly at NCBI (Gene Bank) with the help of Integrated DNA Technology (IDT) online computer software. Sequences of the primers used in this study are given in table 1. The polymerase chain reactions (PCR) were carried out in a total volume of 25μl solution containing 50 ng/μl of template DNA, 1X buffer (Tris-HCl 100 mmol/l, pH 8.3; KCl 500 mmol/l), 0.25 μmol/l primers, 2.0 mmol/l $MgCl_2$, 0.25 mmol/l dNTPs, and 0.5U *Taq*DNA polymerase (Sigma-aldrich, USA). The mixture was heated at 94°C for 5 min, followed by 35 cycles of 94°C for 30 seconds, annealing at 55^0C for 30 seconds and 72°C for 30 seconds and a final extension at 72°C for 8 min. The PCR products was separated on 1.0% agarose gel (Sigma-aldrich, USA) including 0.5 μg/ml of ethidium bromide, photographed under Gel Documentation system (Alpha imager® EP).

2.4. SSCP based genotyping, cloning and sequencing

15% polyacrylamide gel was prepared to carry out the Polymerase Chain Reaction-Single Strand Conformation Polymorphism (PCR-SSCP) analysis as per standard protocol described elsewhere. SSCP gels were scored for different banding patterns observed for the different regions of the TLR2 genes. The PCR products showing different patterns were purified and cloned in pGEM®-T Easy vector system (Promega, USA) as per the manufacturer's protocol. The different genotypes detected by PCR-SSCP of two fragments were cloned in pGEM®-T and sent for DNA Sequencing Facility, South Campus, New Delhi. Both forward and reverse sequences were carried out by M13 universal primers present in pGEM®-T Easy Vector for each allelic pattern. Resultant sequences were analyzed by DNASTAR programme with available sequences of different species at NCBI Gene Bank (www.ncbi.nlm.nih.gov/BLAST) database.

2.5. Estimation of gene and genotype frequencies

The gene and genotype frequencies of different fragments of gene were estimated by standard procedure (Falconer and Mackey, 1996). Genotype of each animal was recorded manually. Genotyping involved the recording of the homozygous or heterozygous state of the animal, as well as the size of the respective alleles in base pairs.

2.6. PBMC isolation, LPS induction, RNA isolation and cDNA synthesis

Freshly isolated peripheral blood mononuclear cells (PBMCs) from each genotypic animals were cultured in 6 ml RPMI medium-1640 (Sigma, USA) with heat inactivated 10% fetal bovine serum (Sigma, USA) and divided into two groups of 3 ml, one group without lipopolysaccharide (LPS) and other with 5 μl (0.1μg/ml) LPS (Sigma, USA) induction for expression study. Then the two groups were kept at 37°C at 5% CO_2 for 24 h. Supernatants from PBMC culture were removed after centrifugation, from 24 h priming with LPS and without LPS induced cell plate. Then the primed cells were washed twice in RPMI and immediately RNA isolation was done by using RNA purification kit (Thermo Scientific, USA), following the manufacturer's instruction. Total RNA was treated with DNase I, RNase-free (Fermentas, USA) for removal of genomic DNA from RNA, following the manufacturer's instruction. The purity of the RNA was verified by measuring absorbance of the RNA solution in UV Spectrophotometer at 260 nm and 280 nm. The RNA samples showing the $OD_{260}:OD_{280}$ value in between 1.9-2.2 was considered as good quality and used for further analysis. The purity of the extracted RNA was checked using 2.2 M formaldehyde denatured agarose gel (Sambrook *et al.* 1989) electrophoresis. Good quality RNA shows two intact bands (28s and 18s rRNA) with smearing in between (mRNA). Reverse transcription of total RNA was carried out using a ProtoScript first strand cDNA synthesis kit (New England Biolabs, Beverly, MA, USA) as per the manufacturer's recommendations employing the M-MuLV reverse transcriptase and random primers. The cDNA product was stored at –20 °C.

2.7. Real Time based analysis of transcript abundance

Gene specific primers for TLR2 and GAPDH (for internal control) were used for amplification of targets (Table 1). All PCR reactions were performed in triplicate. The amplification was carried out in 25 μl volume reaction mixture containing 12.5 μl, 2X master mix (Maxima SYBR Green qPCR Master Mix, ThermoScientific, USA), 1 μl (10 pmol) of gene specific forward and reverse primer, 3 μl of cDNA template and 8.5 μl nuclease free water. Negative control was included for the RT-qPCR assay. Real-Time PCR was carried out in CFX96 Touch™ (Bio-Rad, USA). The thermal profile used for this was as follows: 95 °C for 10 min then 40 cycles of 95 °C for 15 sec, 62 °C for 15 sec and 72 °C for 15 sec with fluorescence recording at the end of each cycle, followed by denaturation of products from 65 °C to 95 °C with fluorescence recording throughout the step. Fold changes in target transcript levels were determined by using the method described by Livak and Schmittgen (2001). GAPDH was used as a house keeping gene (endogenous control) for the analysis of data.

2.8. Statistical analysis

The PROC LOGISTIC procedure of SAS 9.3 was used to find out the overall association of the various genotypes of TLR2 gene fragments with mastitis occurrence. Investigation was done on 129 randomly selected animals on mastitis occurrence/tolerance on the basis of SSC and CMT (0 for non-mastitic group and 1 for mastitis affected group). Initially in univariate logistic regression analysis, the non-genetic factors like milk yield, lactation length and calving interval were fitted and found that none of these effects were significantly affecting the incidences of mastitis. Hence, further these non-genetic factors were dropped in logistic models fitted for studying effect of different genotypes on mastitis. qPCR data are presented as mean ± SEM and analyzed by using SPSS statistical program (SPSS 16.0 for Windows; SPSS, Inc., Chicago, IL, USA). Significant differences were determined by one-way ANOVA using the SPSS program.

3. Results and Discussion

The study was designed to analyze the expression profile of bovine TLR2 gene among different genotypic animals based on identified SNP at exon 2 region among crossbred cattle and their relation with mastitis cases. Among the two target fragments on partial exon 2 region of TLR2, the 179 bp was found to be monomorphic by SSCP analysis. Whereas, the 313 bp fragment of TLR2 gene was found to be polymorphic, this revealed three genotypic patterns (Figure 1). Sequence analysis of this fragment also confirmed the results observed by SSCP banding patterns. Both the alleles of the fragment were compared with the sequences of *Bos indicus* (EU413951.1), *Bos taurus* (AY634629.1), *Bubalus bubalis* (HM756162.1), *Capra hircus* (HQ263214.1) and *Ovis aries* (EU580543.1), available at NCBI database. The sequence alignment report revealed single nucleotide polymorphism (SNP) in allele B. At 34th position, there is a change of nucleotide from adenine to thymine (A to T) in allele B as compared to allele A and other domestic species (Figure 2). But, there is no change in the amino acid sequence due to change is synonymous in nature. Allele frequencies were estimated by direct counting. The calculated genotype frequency for AA, AB and BB were 0. 157, 0.605 and 0.236 respectively and the allelic frequencies as 0.460 for A and 0.539 for B in mastitis affected cattle. Whereas, tolerant group having frequencies of 0.392, 0.244 and 0.186 for AA, AB and BB genotypes respectively and allelic frequencies as 0.514 for A and 0.282 for B. In the association study of various genotypes of TLR2 with mastitis occurrence, the odds ratio of happening/incidents for mastitis in genotype AB and genotype BB were 3.21 and 1.87, respectively as compared to genotype AA. Chi-square probability revealed significant difference between these genotypes and AA genotypes are comparatively less susceptible to mastitis than other genotypes. To shed light on the expression pattern of bovine TLR2 gene among different genotypic groups involved in the susceptibility phenotype to mastitis, we quantified the relative mRNA expression of the TLR2 gene in LPS induced PBMC cells from different genotypic individuals. Our results demonstrated that mRNA expression after LPS induction, increased 1.2, 2.9 and 1.9 times than un-induced cells among AA, AB and BB genotypes respectively (Figure 3). After LPS induction, comparative fold changes among three different genotypes were also studied. Taking AA genotype as a calibrator, the results indicated that relative fold changes of AB and BB genotypes were significantly ($P < 0.05$) up-regulated for 3.3 and 1.4 times respectively (Figure 4).

These finding are quite supportive with the finding that mastitis strongly increased (4 to 13 fold) the mRNA abundances of TLR2 (Goldammer *et al*. 2004). Ibeagha-Awemu *et al*. (2008) studied expression of TLR2 at both mRNA and protein levels by quantitative real-time PCR (qPCR) and flow cytometry, respectively. The mRNA of receptor was up-regulated by all concentrations of LPS used ($P < 0.01$). In the present investigation we observed that, genotypic variation among mastitis case and control groups may directly or indirectly linked with differential expression profile of TLR2 gene. But, in the present study, it was not known whether the expression levels of TLR2 gene reflect only mRNA or also at protein levels at different genotypic groups, as post-transcriptional regulation factors were not targeted here. Consequently, it would be stirring to carry out proteomic based studies to confirm the findings for selecting the SNP as biomarker for mastitis resistance / susceptibility.

Table 1: Details of primers used for amplification of TLR2 gene fragments

| Sl. no.	Primer Name/ Identification	Primer sequence	Primer length	Amplicon size
1.	TLR2 A F	5' GCCTGTGAGTGGATATATAC 3'	20 bp	179 bp
	TLR2 A R	5' CTGGGATCTTAGAAGTAGAG 3'	20 bp	
2.	TLR2 B F	5' GACATAAAGGGACCTGAACC 3'	20 bp	313 bp
	TLR2 B R	5' CTCTGACCAGGCTTCTTCTC 3'	20 bp	
3.	RT-TLR2 F	5'GCTCCTGTGACTTCCTGTCC 3'	20 bp	149 bp
	RT-TLR2 R	5'GTGGCATTCAGAAAGGGAGA3'	20 bp	
4.	RT- GAPDH F	5'TGACCCCTTCATTGACCTTC3'	20 bp	143 bp
	RT- GAPDH R	5'GATCTCGCTCCTGGAAGATG3'	20 bp	
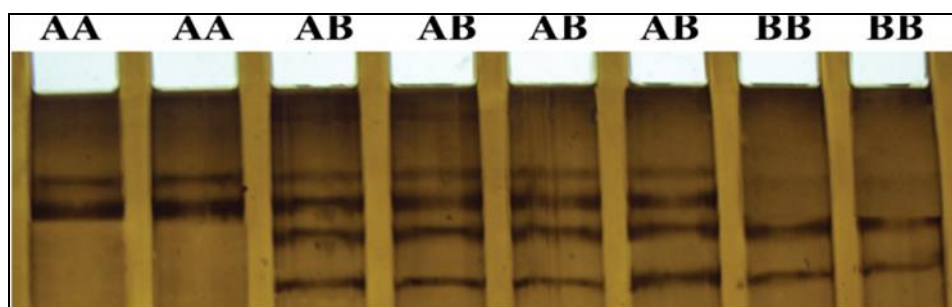
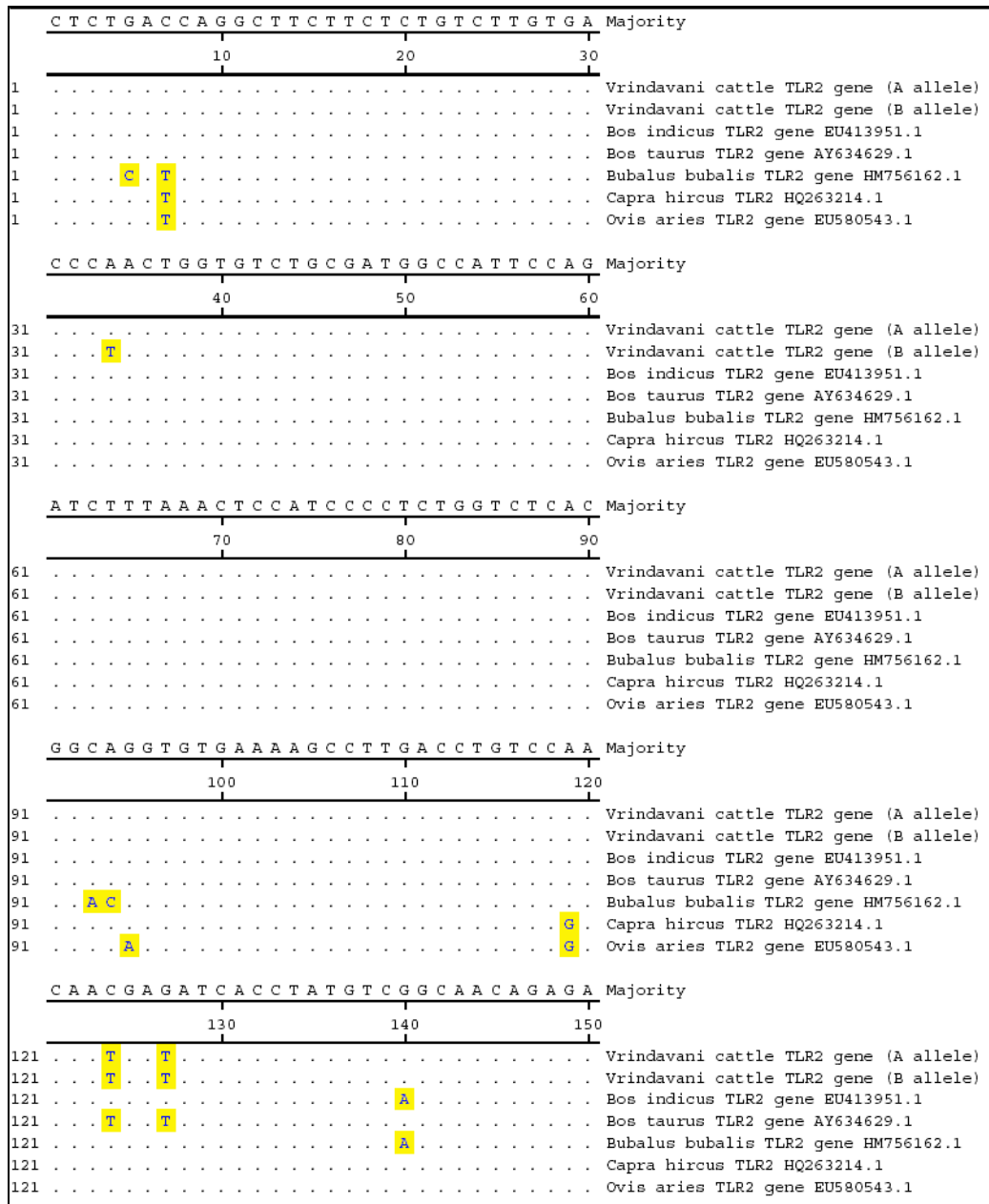**Fig 1**: SSCP pattern for 313 bp fragment of TLR2 gene

Fig 2: Partial Alignment report of nucleotide sequence of 313 bp fragment of TLR2 gene with that of other species
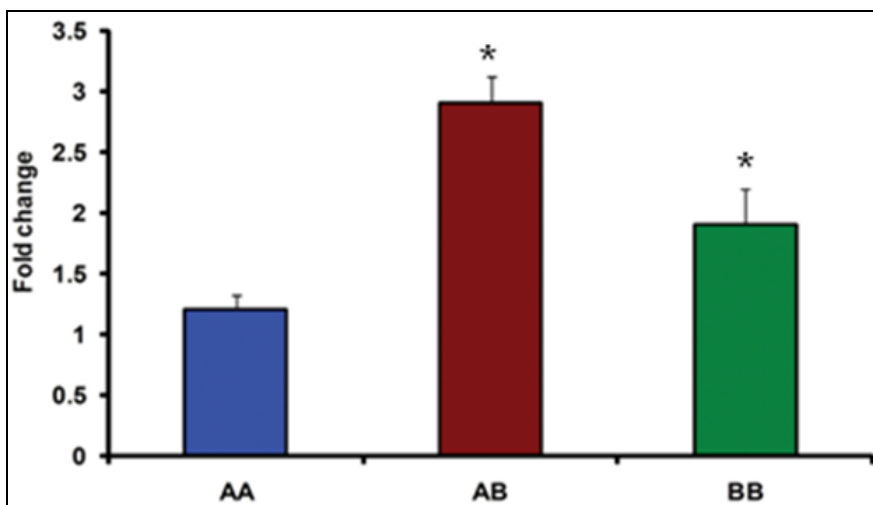

Fig 3: TLR2 mRNA expression profile of different genotypes (induced with LPS) keeping un-induced as calibrator. Bar with * indicates significant difference at $P<0.05$
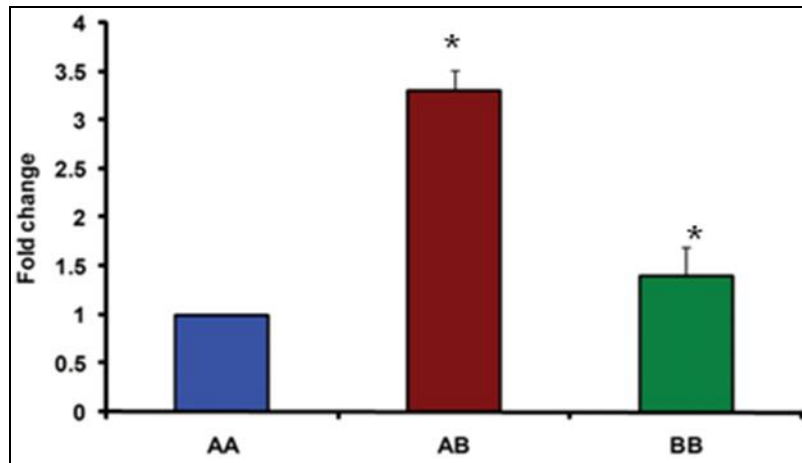
Fig 4: Comparative fold changes among different genotypes of TLR2 gene (after LPS induction) keeping AA genotype as calibrator. Bar with * indicates significant difference at P<0.05

Ethical standards
The experiments comply with the current laws of the country.

Conflict of Interest
The authors declare that they have no conflict of interest.

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