e - ISSN - 2348-2206
Print ISSN - 2348-2192

European Journal of Molecular Biology and Biochemistry

Journal homepage: www.mcmed.us/journal/ejmbb

ASSOCIATION OF TOLL-LIKE RECEPTOR GENE POLYMORPHISM WITH SOMATIC CELL SCORE IN INDIGENOUS, CROSSBRED JERSEY AND CROSSBRED HOLSTEIN FRIESIAN CATTLE

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Article Info

Received 27/08/2015
Revised 06/09/2015
Accepted 19/09/2015

Key words:- TLR gene polymorphism, Somatic cell score, Resistance to mastitis, Cattle.

ABSTRACT

Mastitis is a disease of major economic importance, causing reduced milk quality and leading to loss of milk production in cattle. The Toll-like receptors (TLRs) 2 and 4 play a role in the host response to inflammatory mastitis. The present investigation was carried out to study the polymorphism of TLR2 and TLR4 genes in cattle, to estimate the somatic cell count in milk and to study the relationship between TLR genes polymorphism and somatic cell score in mastitis. A total of 221 blood and milk samples were collected from 114 Jersey crossbred cows and Holstein Friesian crossbred cows suffering from mastitis and from 107 healthy indigenous, Jersey crossbred and Holstein Friesian crossbred cows with no previous history of mastitis. The PCR products were sequenced and screened for SNPs. There were 24 mutations (seven non-synonymous) in TLR2 gene and 11 mutations (one non-synonymous) in TLR4 gene. The SNPs detected were genotyped using both PCR-RFLP and tetra-primer ARMS-PCR. The mean SCC ($x10^5$/ml) was 2.31 ± 0.10 in normal milk samples and it was 68.85 ± 3.90 in mastitis samples. The SCS was 4.01 ± 0.031 and 9.43 ± 0.69 respectively. The most common pathogens found in the milk samples were *Staphylococcus*, *E.coli*, *Streptococcus*, *Klebsiella* and *Pseudomonas*. Samples harboring *E.coli* had the highest SCC (114.57± 4.2 $x10^5$/ml) when compared to others. The SNP 10095G>T of TLR2 gene influenced the SCS highly significantly. Among the three genotypes in 10095G>T, 'GG' and 'GT' genotypes were found to have lower mean SCS than the 'TT' genotypes. Therefore, the GG genotypes would be resistant to mastitis.

INTRODUCTION

The dairy sector in India has shown remarkable development in the past decade and is the largest producer of milk in the world with 137.7 million tonnes of milk during the year 2013-14. This improvement in milk production is mainly because of the crossbreeding policy adopted throughout the country. At the same time, the Indian dairy sector does face a few challenges which hamper the production and thereby the economy of the dairy farmers. Of these challenges, mastitis has been shown to be one of the diseases with the most significant adverse effect on economic dairying. Mastitis is a multifactor disease and resistance is influenced by many genes. The economic losses from clinical mastitis are due to reduced milk production as well as premature culling of animals. Higher losses were observed in crossbred cows due to their high production potential that was affected during mastitis period. Mastitis is characterized by physical, chemical and bacteriological changes in the milk and pathological changes in the glandular tissue of the udder. A variety of factors, such as pathogens, poor management practices, genetic factors and health of dairy cattle cause mastitis; out of these, the major part is played by pathogens. The most common pathogens are

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Escherichia coli, *Streptococcus dysgalactiae*, *Streptococcus uberis*, *Staphylococcus aureus* and *Streptococcus agalactiae* [1].

Somatic cell count (SCC) is an indicator for clinical mastitis and milk quality. Clinical mastitis is easy to detect, while cows suffering from sub-clinical mastitis do not show signs of local inflammation. Both types of mastitis affect milk quality. Presence of intra-mammary infections may be diagnosed indirectly by measuring markers on inflammation in milk. The most important marker is SCC, which can be detected by both direct and indirect methods. The genetic correlations between SCC and clinical mastitis were found to be in the order of 0.6 to 0.8 in dairy cattle in USA [2]. Earlier study had revealed that SCC has a higher heritability (0.05 to 0.25) than clinical mastitis (<0.10), allowing genetic progress to be more easily made in reducing the former than the later [3]. In the mammary gland, cells from the immune system together with epithelial cells are responsible for recognizing the invading pathogens *via*. toll-like receptors (TLRs). Among the TLRs, TLR2 and TLR4 have been found associated with mastitis in cattle and play a central role in innate immunity. Correlation between somatic cell score (SCS) and TLR2 gene polymorphism in Xinjiang Brown cattle had been reported [4]. TLR4 has been found associated with lipo-polysaccharide responsiveness. Association between TLR4 gene polymorphism with SCS and lactation persistency in Holstein cows had also been reported [5]. Such attributes make TLR4, a suitable candidate gene for mastitis detection in dairy cattle. Since there is dearth of reports on the role of TLR2 and TLR4 genes in resistance to mastitis in crossbred cattle in India, the present study was carried out in indigenous, crossbred Jersey and crossbred Holstein Friesian cattle.

MATERIALS AND METHODS

Ten ml of blood and 15 ml of milk samples were collected from 114 Jersey crossbred and Holstein Friesian crossbred cows affected with mastitis from various veterinary hospitals in and around Chennai city of Tamil Nadu State, India. There was no case of mastitis among the indigenous cows. Additionally, 107 blood and milk samples were collected from healthy indigenous cows, Jersey crossbred cows and Holstein Friesian crossbred cows with no previous history of mastitis. Details like breed, age, parity, stage of lactation, previous history of mastitis and test day milk yield were collected.

Genomic DNA was isolated by modified phenol-chloroform method using DNA zol. The primers were designed according to published bovine genomic TLR2 sequence (Gen Bank Accession No.: NW_001493469) and TLR4 sequence (Gen Bank Accession No.: NM 174198). Primer 3 software (http://bioinfo.ut.ee/ primer3-0.4.0/) was used for designing the primers. PCR was carried out in 15µl volume and the amplicons were sequenced by automated ABI PRISM 3730XL Genetic Analyzer (Applied Biosystems, U.S.A.) from both ends. Sequence data were analysed using the Edit seq programme of LASERGENE software (DNASTAR Inc., Madison, WI, USA). The *Bos taurus* gene sequences for TLR2 and TLR4 genes obtained from NCBI were used as the reference sequences for analysis. The sequences were assembled and screened for SNPs. The SNPs thus detected were genotyped using both PCR-RFLP and tetra-primer ARMS-PCR.

The milk samples were subjected to California Mastitis Test, microbiological identification and estimation of SCC. The somatic cells in fresh milk were counted by the method of the International Dairy Federation with few modifications [6]. The somatic cell counts were then log transformed into somatic cell score, using the formula: $SCS = \log_2 (SCC/100000) + 3$. Most of the mastitis causing bacteria grew on 5 per cent calf or sheep blood agar. Briefly, 0.01 ml of milk was streaked vertically across the diameter of an agar plate. Milk samples plated on MacConkey agar detected coliforms and Gram-negative bacteria. *Klebsiella* spp. grew as pink colonies while *Pseudomonas* showed up as colourless colonies. Modified Edward's medium detected *Streptococci* and Mannitol salt agar was used for *Staphylococcus* identification. The EMB agar medium was the selective medium for *E.coli.*

Mean somatic cell count and breed (indigenous, Jersey crossbred and HF crossbred cows) were considered for association of different SNPs of TLR2 and TLR4 genes. The least-squares method [7] was used to estimate the association between SNPs of TLR2 and 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, j^{th} SNP

μ = Overall mean

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

G_j = Effect of j^{th} SNP (j =1 to 33)

e_{ij} = Random error normally and independently distributed with mean 0 and variance σ^2_e.

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

RESULTS AND DISCUSSION

A total of seven amplicons were generated using the primers covering the two exons of TLR2 gene. The sequence analysis revealed 24 mutations; one in exon 1 and the rest in exon 2. Out of these 24 mutations, 22 were identified as SNPs and seven were non-synonymous mutations. The non-synonymous type of variations found in the cows were 10095G>T, 10108G>A, 10537A>G, 10884A>T, 10916A>G, 11720C>T and 11901C>G. The variation 10095G>T resulted from transversion, replacing the amino acid glutamic acid with aspartic acid. For TLR4 gene with three exons, a total of nine amplicons were generated. The sequence analysis revealed 11 SNPs, one

SNP in 5'UTR; two SNPs in intron 1; one SNP in exon 2 and the rest in exon 3. Out of the 11 SNPs, only one SNP resulted in non-synonymous mutation.

The pH of mastitis milk was higher than the normal milk. The mean pH for normal milk samples was 6.93±0.02 and for mastitis milk it was 7.42±0.04. Normal samples had CMT scores ranging from N (Negative) to T (Trace) (48.86 per cent). The CMT scores for mastitis milk ranged from 1 to 3 based on the severity of the infection. Most of the samples showed the maximum reaction of 3+ (31.50 per cent), followed by 2+ (15.53 per cent) and 1+ (4.11per cent). The CMT is a simple, inexpensive rapid screening test for mastitis. The test is based on amount of nuclear protein in the milk sample. Since inflammatory cells associated with mastitis are the predominant cell type present in the milk, the CMT reflects the SCC level quite accurately and is a reliable indicator of the severity of infection. At present, the only indirect mastitis test which can be used as the "cow side" tests is the CMT.

Infectious mastitis leads to a reduced synthetic activity, changes in the milk composition and elevated SCC. Normal counts of immune cells in healthy mammary quarters range between 20,000 and 100,000 cells / ml [9, 10]. Mammary quarters with lower cell counts tend to respond less efficient to an intra-mammary challenge and also show higher incidences of clinical mastitis. During bacterial infection of the bovine mammary gland, large numbers of leukocytes migrate into the udder, resulting in the establishment of a host response against the pathogen. The somatic cells present in the milk of a healthy cow are mainly macrophages (66-88 per cent); in addition, there are neutrophils and epithelial and mononuclear cells. The proportion of neutrophils is only 1-11 per cent in a healthy quarter but increases up to 90 per cent in a quarter with intra-mammary infection. More than 90 per cent of SCC in infected gland is composed of neutrophils.

The SCC values for normal samples ranged from 1.53 to 2.85 x 10^5/ ml with a mean of 2.31 ± 0.10 x 10^5 cells / ml and for mastitis samples it ranged from 26.35 to 179 x 10^5 cells / ml with a mean of 68.85 ± 3.90 x10^5 cells / ml. Normally in milk from a healthy mammary gland, the SCC is lower than 1×10^5 cells / ml, while bacterial infection can cause it to increase to above 1×10^6 cells / ml [11]. Elevated SCC primarily consists of leucocytes, which include macrophages, lymphocytes and neutrophils. During inflammation, major increase in SCC is because of the influx of neutrophils into milk and at that time over 90 per cent of the cells may be polymorphonuclear leukocytes. Higher the SCC, greater is the risk of raw milk contamination with pathogens and antibiotic residues. The reduction of bovine mastitis prevalence is a major goal of the dairy industry throughout the world. SCC has been included as a component of the definition of mastitis and the original limit for SCC of a healthy quarter is 500000 cells / ml [12].

Because of its extremely skewed distribution, SCC is log transformed. The mean SCS was 4.01 ± 0.031 for normal samples and 9.43 ± 0.69 for mastitis samples. The virulence factors of the microorganisms evoke the host immune system through various routes [13], one of which is manifested by an increase in the number of somatic cells in milk. This increment in SCC not only deteriorates udder health but also affects milk quality by releasing high amount of protease and lipase enzymes, which are highly heat resistant in nature and cause problem during processing of milk and milk products [14]. SCS associated loci had been reported to improve resistance to mastitis in dairy cattle [15]. Genetic evaluation and selection of sires for lower SCC or SCS may reduce the incidence of mastitis [3, 16]. Because of its high heritability and close association with clinical mastitis, SCC values have been widely used for selection for mastitis resistance in dairy cattle [17].

The culture results showed that the cows were infected with the most common pathogens like *E.coli*, *Staphylococcus* spp., *Streptococcus* spp., *Klebsiella* spp. and *Pseudomonas* spp. Some milk samples showed mixed infection, while majority of the samples had *Staphylococcus* spp. as the causative agent (43 per cent) followed by *E.coli* (22 per cent), *Streptococcus* spp. (11 per cent), *Klebsiella* spp. (7 per cent) and *Pseudomonas* spp. (5 per cent). About 12 per cent of milk samples were detected with mixed infections with the combinations of *Klebsiella* spp. with *E.coli*, *Klebsiella* spp. with *Staphylococcus* spp., *E.coli* with *Streptococcus* spp. and *E.coli* with *Staphylococcus* spp. Samples harboring *E.coli* had the highest mean SCC (114.57± 4.2 x 10^5 cells / ml) when compared to others (Table 1). This is consistent with the findings that the elimination of infection of *E.coli* is host dependent and during *E.coli* infection the function and number of neutrophils are compromised [18]. However, in a study on bovine mastitis, it was found that *S.aureus* was the major mastitis causing organism, followed by *Streptococci*, *E.coli*, *Corynebacterium* spp. and Klebsiella [19].This inflammation of the mammary gland is almost always caused by bacteria (both Gram-positive and Gram-negative) that invade the mammary gland by penetration through the teat canal. Gram-negative bacteria are responsible for approximately one-third of all clinical cases of bovine mastitis and almost 25 per cent of all these cases result in culling or death of the animal [20].

The least-squares analysis of variance for the effect of SNP genotypes on SCS revealed that except SNP 10095G>T of TLR2, rest of the 33 SNPs (22 SNPs in TLR2 and 11 SNPs in TLR4) did not show any association with the SCS. The SNP 10095G>T influenced the SCS highly significantly ($P<0.01$). However, the genetic group (breed) did not show any variation in the SCS. Among the three genotypes in 10095G>T, GG and GT genotypes were found to have lower mean SCS (5.207 ± 1.18 and 6.633 ± 1.23) than the TT (10.380 ± 1.65) genotypes, the differences between the genotypes being significant ($P<0.01$). The SNP 10095G>T is located in the extracellular domain region which causes change in amino

acid from glutamic acid to asparagine and may possibly affect the function of TLR2 gene. Statistically GG genotype had the lowest SCS and GT and TT genotypes showed higher SCS and probably, GG genotype could be a mastitis tolerant genotype. In a similar study, the TT and TG genotypes of this SNP had larger SCS than those of GG genotype in three breeds of cattle [21]. In another study, the genotype TT had significantly lower SCS than the TG and GG genotypes [22]. The contradictory findings between the genotypes and SCS may be explained by differences between environmental conditions, traits characterized and bacterial flora and may also result from the different states of linkage disequilibrium in the populations under study. In the present study, no association was found between TRL4 genotypes of the 11 SNPs and SCS. Similarly, no association was found between TLR4 gene SNP 245G>C and SCS in an earlier study [23]. However, associations were found between SNP 245 and SNP 5087 of TLR4 gene and SCS [24] and between SNP 9787 and SCS [25].

Table 1. Causative agents and somatic cell counts

Sl. No.	Causative agent	Incidence (%)	SCC values ($x10^5$ cells / ml)
1	*E.coli*	22.00	114.57 ± 4.2
2	*Staphylococcus* spp.	43.00	33.08 ± 3.68
3	*Streptococcus* spp.	11.00	61.78 ± 2.50
4	*Klebsiella* spp.	7.00	47.79 ± 2.5
5	*Pseudomonas* spp.	5.00	29.17 ± 7.47
6	Mixed infections	12.00	70.59 ± 1.31

CONCLUSION

From the present study based on the somatic cell scores of cows suffering from mastitis and normal ones, it was observed that GG genotype of SNP 10095G>T of TLR2 gene could be mastitis tolerant genotype in the breeds studied. The heifer calves with GG genotype can be selected and these animals would be resistant to mastitis when they produce milk.

ACKNOWLEDGEMENTS

The authors wish to thank the Senior Research Fellows and post-graduate students of the department for lending their helping hands whenever needed. The authors also thank the staff of the Post-graduate Research Institute in Animal Sciences, Kattupakkam and the Veterinary Assistant Surgeons of the various Veterinary Hospitals for their assistance during collection of blood and milk samples.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

REFERENCES

1. Mason C. (2006). Basic mastitis bacteriology: untangling the pathogens. *Irish Vet J*, 59, 453-459.
2. Shook GE, Schultz MM. (1994). Selection on somatic cell score to improve resistance to mastitis in the United States. *J Dairy Sci*, 77,648-658.
3. Emanuelson U, Danell B, Philipson J. (1988). Genetic parameters for clinical mastitis, somatic cell counts, and milk production estimated by multiple-trait restricted maximum likelihood. *J Dairy Sci*, 71, 467-476.
4. Bai J, Lin J, Li W. (2012). Association of toll-like receptor 2 polymorphism with somatic cell score in Xinjiang Brown cattle. *Anim Sci J*, 83, 23-30.
5. Sharma BS, Leyva I, Schenkel F, Karrow NA. (2006). Association of toll-like receptor 4 polymorphism with somatic cell score and lactation persistency in Holstein bulls. *J Dairy Sci*, 89, 3626-3635.
6. International Dairy Federation, 1987. Bovine mastitis: Definition and guidelines for diagnosis. Bulletin of the IDF, Document 211.
7. Harvey WR. (1986). Mixed model least-squares and maximum likelihood computer program, PC version (PC-1) LSMLMW with Parmcard.
8. Kramer CY. (1957). Extension of multiple range tests to group correlated adjusted means. *Biometrics*, 13(1), 13-18.
9. Schepers AJ, Lam TJGM, Schukken YH, Wilmink JBM, Hanekamp WJA. (1997). Estimation of variance components for somatic cell counts to determine thresholds for uninfected quarters. *J Dairy Sci*, 80, 1833-1840.
10. Green LE, Schukken YH, Green MJ. (2006). On distinguishing cause and consequence: Do high somatic cell counts lead to lower milk yield or does high milk yield lead to lower somatic cell count? *Prev Vet Med*, 76, 74-89.
11. Bytyqi H, Zaugg U, Sherifi K, Hamidi A, Gjonbalaj M, Muji S, Mehmeti H. (2010). Influence of management and physiological factors on somatic cell count in raw cow milk in Kosova. *Veterinarski Arhiv*, 80(2), 173-183.
12. International Dairy Federation, 1971. A monograph on bovine mastitis. IDF Bulletin No.60.
13. Kumar H, Kawai T, Akira S. (2011). Pathogen recognition by the innate immune system. *Int Review Immunol*, 30, 16-34.
14. Barbano DM, Ma Y, Santos MV. (2006). Influence of raw milk quality on fluid milk shelf life. *J Dairy Sci*, 89, E15-E19.

15. Shook GE. (2006). Major advances in determining appropriate selection goals. *J Dairy Sci*, 89(4), 1349-1361.
16. Chu C, Yu C, Lee Y, Su Y. (2012). Genetically divergent methicillin-resistant *Staphylococcus aureus* and sec-dependent mastitis of dairy goats in Taiwan. *BMC Vet Res*, 8, 39-48.
17. Lund T, Miglior F, Dekkers JCM, Burnside EB. (1994). Genetic relationships between clinical mastitis, somatic cell count, and udder conformation in Danish Holsteins. *Liv Prod Sci*, 39(3), 243-251.
18. Bradley AJ, Green MJ. (2001). Adaptation of *Escherichia coli* to the bovine mammary gland. *J Clin Microbiol*, 39(5), 1845-1849.
19. Sharma N, Rho GJ, Hong YH, Kang TY, Lee HK, Hur TY, Jeong DK. (2012). Bovine mastitis: An Asian perspective. *Asian J Anim Vet Adv*, 7(6), 454-476.
20. Ebehart RJ. (1984). Coliform mastitis. *The Vet Clinics North America. Large Anim Prac*, 6(2), 287-300.
21. Zhang LP, Gan QF, Ma TH, Li HD, Wang XP, Li JY, Gao X, Chen JB, Ren HY, Xu SZ. (2009).Toll-like receptor 2 gene polymorphism and its relationship with SCS in dairy cattle. *Anim Biotech*, 20(3), 87-95.
22. Huang J, Liu L, Wang H, Zhang C, Ju Z, Wang C, Zhong J. (2011).Variants and gene expression of the TLR2 gene and susceptibility to mastitis in cattle. *Asian J Anim Vet Adv* 6(1), 51-61.
23. Wang XP, Xu SZ, Ma TH, Gao X, Ren HY, Chen JB. (2006). Genetic variation in the 5'flanking region of bovine TLR4 gene and correlation with mastitis. *Hereditas*, 28(12), 1520-1524.
24. Mesquita AQ, Rezende CSM, Mesquita AJ, Jardim EAGV, Kipnis APJ. (2012). Association of TLR4 polymorphisms with sub-clinical mastitis in Brazilian Holsteins. *Braz J Microbiol*, 43(2), 692-697.
25. Wang XP, Xu SZ, Gao X, Li JY, Ren HY, Luoren ZM. (2008). Cloning and SNP screening of the TLR4 gene and the association between its polymorphism and somatic cell score in dairy cattle. *South Afr J Anim Sci*, 38(2), 101-109.