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Genetic variation of TLR-4 gene and its association with mastitis in crossbred cattle

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Abstract
TLR4 is a candidate gene for providing innate resistance towards mastitis infection. The present study was conducted to find polymorphism in exon 3 of TLR4 gene of Jersey crossbred cattle and to find association if any with somatic cell count, an indicator trait of mastitis resistance or susceptibility. Alu I RE digestion revealed three genotypic patterns AA, AB and BB with genotypic frequency of 0.28, 0.52 and 0.20 respectively. PIC value and value of heterozygosity indicate marker of moderate importance. Nei and Shannon information index suggests medium gene diversity. All the animals included in the association study showed only one genotype AB (except two) with respect to Alu I/ PCR-RFLP. Therefore, no association could be established between polymorphism of TLR-4 gene and incidences of mastitis. Using more sensitive techniques like SSCP and sequencing, exploring total sequence of TLR4 for variation and polymorphism study of other candidate genes like MHC can give more insight in host resistance towards mastitis.

Keywords: TLR-4, PCR-RFLP, polymorphism, somatic cell count

1. Introduction

Among infectious diseases mastitis is one of the most prevalent diseases of dairy cattle, which causes huge economic losses in terms of decreased production and inferior milk quality to the dairy industry worldwide (Rueg, 2003) [8]. It is an inflammatory disease of the mammary gland generally caused by a broad spectrum of bacterial and fungal intramammary infections and is the most frequent occurring and costly disease in dairy industry (Sordillo & Streicher, 2002) [10]. Besides loss in milk production treatment cost also account for economic burden. One of the altenative to reduce loss due to mastitis is host resistance and selection for candidate genes for mastitis resistance. One of the components of immune response is innate immunity of which TLR-4 (Toll like receptor 4) is as excellent example. The toll like receptors (TLRs) are a multigene family of cell-surface signaling molecules that play a fundamental role in the immune response to recognize pathogen that bind to specific pathogen associated molecular patterns (PAMPS). TLR-4 is the only important pattern recognition receptor of TLRs family that recognizes endotoxins associated with gram negative bacterial infections (Takeda *et al*., 2003) [11]. Currently, at least 13 members of the TLR family have been identified in mammals; genes encoding 10 of these receptors have recently been mapped to the bovine genome (McGuire *et al*., 2005) [6].

Somatic cell score (SCS) is commonly used as an indicator trait in the selection for mastitis resistance (Koivula *et al*., 2005; Herigstad *et al*., 2006) [4, 2]. The invading pathogens activate the immune defense system in the udder which is manifested by an increase in the number of somatic cells in milk.

2. Materials and Methods

2.1 Experimental Animals

The present study was carried out on crossbreed cattle maintained at Central cattle breeding farm, Belicharana, Jammu. A total of 50 animals were taken for study. Both blood and milk samples of these crossbred cattle were collected.

2.2 Collection of blood samples

Under sterile conditions, 10 ml of venous blood was collected from the jugular vein of the existing milch animals in a 15ml polypropylene centrifuge tube containing 0.5 ml of 0.5M EDTA solution. The tube was tightly capped and shaken gently to facilitate thorough mixing

of blood with the anti-coagulant. The tubes containing blood samples were transported to the laboratory in an icebox containing ice packs and were kept at –20 °C till the isolation of DNA was done.

2.3 Isolation of Genomic DNA
Genomic DNA was isolated from the venous blood samples by HiPurA^{TM} SPP Blood DNA Kit (Himedia). DNA pellet was dissolved by adding 100µl of elution buffer by vortexing for 1 minute and the tube was incubated at 65 °C for 10 minutes and then stored at -20 °C.

2.4 Gene amplification
Pair of primers reported by Wang *et al*., 2007 [12] was used to amplify T4CRBR2 region (exon 3) of TLR-4 gene, **f**orward 5'-AGACAGCATTTCACTCCCTC-3′ and reverse 5′-ACCACCGACACACTGATGAT-3′

Amplification was carried out in Thermal cycler (Eppendorf, USA). PCR was carried out in final volume of 25µl containing template DNA 1µl, 2X PCR master mix. 7.5µl, forward primer 1µl, reverse primer 1µl and 14.5µl distilled water. The PCR conditions were initial denaturation at 94 ° C for 4 min. followed by 35 cycles of denaturation (94 °C for 1 min.), annealing (51 °C for 30 sec.), extension (72 °C for 30 sec.) and final extension (72 °C for 10 min.). PCR product was checked on 2% agarose gel containing ethidium bromide @ 2 µl per 100ml of agarose gel to verify the amplification of the target region. 10µl of the PCR product and 2 µl of the 6X gel loading dye was mixed and loaded in the wells. 2µl of the 100 bp ladder (Himedia) was loaded as a marker in a separate lane to check size of the amplicon. The gel was run at 100 volts for 30 min. The amplified product in the gel was observed under UV- transilluminator and documented by photography.

2.5 Digestion of PCR product with *Alu* I
In order to detect the PCR-RFLP, the amplicon was digested with *Alu* I restriction enzyme. The restriction digestion was carried out in 25µl volume 15µl PCR product, R.E Assay Buffer C (10X) 2.5µl, *Alu*I (5U/µl) 3.0µl, autoclaved distilled water upto 25µl. The reaction mixture was incubated in the water bath at 37 °C for 5 hrs. After digestion, 5 µl of 6X loading dye was added to the reaction mixture and kept at 4 ºC till further use. Restriction fragments were resolved on 2.5% agrose gel horizontal electrophoresis and visualized by ethidium bromide staining. The ethidium bromide was added to the agrose gel @ of 1 µl/100ml of gel. The agrose gel electrophoresis was performed in 1X TBE buffer at 100volts for 30, 60, and 90 minutes till complete separation and visualization of all fragments of RE digested DNA fragments and DNA ladder. The restriction digested gene fragments were visualized on UV transilluminator and photographed with gel documentation system.

2.6 Somatic cell count (SCC)
Total somatic cell count was done as per the modified technique of leukocyte count described by Prescott and Breed (1910) [7]. 10µl of milk was drawn by micropipette and spread evenly on a clean grease free slide in 1 square cm area. The smear was allowed to dry in air at room temperature. Staining of dried milk smear was done by placing the slides for 2 minute with Newman's stain in a covered coupling jar. The excess stain was drained off by keeping the slides vertically on absorbent paper and air dried. The slides were then rinsed with tap water, drained and dried rapidly in air. 1 sq. cm area of milk smear was divided in four parts, by dividing at right angles and was examined under oil immersion for counting cells in five fields from each divided area. Counting of cells per ml of milk for the average number of cells per field was multiplied by microscopic factor that was determined as per Prescott and Breed (1910) [7].

2.7 Association study
For association study animals were divided into two groups viz., affected and non- affected. The animal with SCC above five lakhs/ ml was grouped as affected. Whereas animals with SCC lower than one lakhs/ml were grouped as non-affected. 6 milk samples studied had somatic cell count of less 1,00000cell/ml which were considered resistant, while the rest 15 samples having somatic cell count of more than 5,00000 cells/ml were considered susceptible and were selected for study with an objective of finding association between polymorphism of TLR-4 gene and mastitis. Rest of the samples having somatic count in between 1, 00000- 5, 00000 were discarded.

2.8 Statistical analysis
2.8.1 Genotypic frequencies for each variant genotypes were calculated by using the formula:

$$\text{Genotype frequency} = \frac{\text{Number of animals with specific genotype}}{\text{Total number of animals}}$$

2.8.2 Allele (Gene) frequencies were calculated as follows:

A= (AA+1/2AB)
B= (BB+1/2AB)

Where, AA, BB= Genotypic frequency of homozygote
AB= Genotypic frequency of heterozygote
A, B= Allelic frequencies

2.8.3 POPGENE Software was used to calculate the following values:
SE, Chi-square (χ^2) test, Observed Homozygosity, Observed Heterozygosity, Expected Homozygosity, Expected Heterozygosity, na, ne, I, Nei, Fis, G^2.
Where,
SE = Standard error of the mean allelic frequency
Expected Homozygosity and Heterozygosity were computed using Levene (1949)
Na = Observed no. of alleles
Ne = Effective no. of alleles (Kimura and Crow, 1964)
I = Shannon's information index (Lewontin, 1972)
Nei = Nei's (1973) expected Heterozygosity
Fis = Wright's (1978) fixation index as a measure of heterozygosity deficiency or excess
G^2 = Likelihood ratio test for Hardy-Weinberg equilibrium

2.8.4 Polymorphic information content (PIC) values were calculated out to check the informativeness of the marker. PIC value was estimated by given formula, used for co-dominant marker like PCR- RFLP:

$$PIC = 1 - (\sum p_i^2) - \sum \sum 2\, p_i^2\, p_j^2$$

Where, p_i & p_j are the frequencies of the ith and j^{th} alleles at a locus in a population

3. Results and Discussions

A 382 bp fragment of TLR-4 gene of crossbred cattle comprising a part of exon 3 was amplified using specific primers as used by Wang *et al*., 2007 [12]. The PCR Product was digested with *Alu* I restriction enzyme and three genotypic pattern were revealed viz., AA, AB, and BB. As shown in the Fig. 1 below. The genotypic frequency of AA genotype was 0.28, AB genotype was 0.52 and BB 0.20. The gene frequency of A and B allele was 0.54 and 0.46 respectively. Wang *et al*., 2007 [12] also found three genotypes in T4CRBR2 region (exon 3) of TLR-4 gene with similar banding pattern. The genotypic frequency of genotype AA, AB and BB genotype was 0.1667, 0.5000, and 0.3333 respectively in Chinese Holstein. The frequency of allele A and B in Chinese Holstein was 0.4167 and 0.5833 respectively. Similar trend was observed in Sanhe cattle and Chinese Simmental.

Table 1: Chi square value

Genotype	O	E	O-E	$(O-E)^2$	$(O-E)^2/E$ (Chi-square)
AA	14	12.5	2.5	6.25	0.447
AB	26	25.0	1.0	1.00	0.038
BB	10	12.5	2.5	6.25	0.625
	$\sum(O-E)^2/E = 1.11$				

where, O= observed genotypes
E= expected genotypes

Chi-square value is non-significant which shows that the population under study is in Hardy-Weinberg equilibrium.

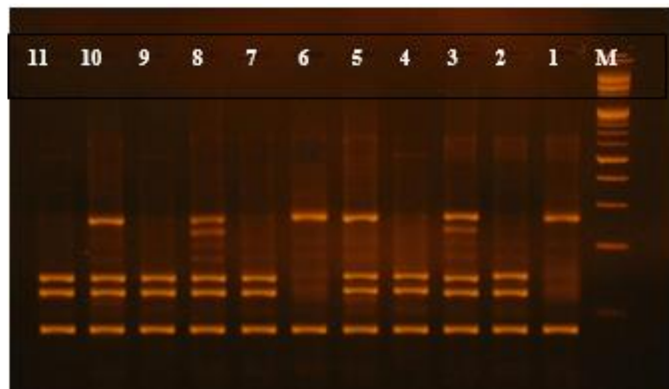

Fig 1: PCR–RFLP patterns by using *Alu* I restriction enzyme

Lane M	:	100bp DNA Ladder
Lane 1-11	:	Digested PCR products
Lane 1, 6	:	AA Genotype
Lane 3, 5, 8, 10	:	AB Genotype
Lane 2, 4, 7, 9, 11	:	BB Genotype

Using the equation of PIC for co-dominant markers, the estimated value was 0.373.

Table 2: POPGENE analysis

Parameter	Value	Parameter		Value
SE	0.026	Exp. Hom.		0.49
na	2	Exp. Hetero.		0.50
ne	1.99	Nei		0.49
I	0.69	Fis	A	-0.04
			B	-0.04
Obs. Hom	0.48	G^2		0.067
Obs. Hetero	0.52			

Since the allelic frequency both A and B are relatively similar ne (effective number of allele) is as good as observed number of allele (na). Chi Square value is not significant which means the population is in Hardy Weinberg equilibrium. PIC value was 0.373. PIC values can range from 0 to 1. At a PIC of 0, the marker has only one allele. At a PIC of 1, the marker would have an infinite number of alleles. A PIC value of greater than 0.7 is considered to be highly informative, whereas a value of 0.44 is considered to be moderately informative. A gene or marker with only two alleles has a maximum PIC of 0.375. Clearly markers with greater numbers of alleles tend to have higher PIC values and thus are more informative. The PIC value obtained in the study is moderately informative as a marker

Both Shannon Information index and nei have values which suggest a medium range of gene diversity. The cause of a medium range of diversity within population may be due to genetic drift, mutation and gene flow. As the population size at Belicharana farm, Jammu is quite low the effect of genetic drift can't be denied. Mutation can be ignored as the allelic frequency is quite high to be explained on mutation only. As the farm is utilising artificial insemination with semen from proven bull outside the farm role of gene flow is also there. Artificial selection is not practised in the farm, though natural selection may operate as because TLR4 gene is a candidate gene in providing innate resistance towards infectious diseases especially important for mastitis.

Total of 50 milk samples were taken and studied. A total of 6 milk samples studied had somatic cell count of less 1,00000cell/ml which was considered resistant, while the rest 15 samples having somatic cell count of more than 5,00000 cells/ml were considered susceptible and were selected for study with an objective to find association between polymorphism of TLR-4 gene and innate resistance to mastitis. Rest of the samples having somatic count in between 1, 00000- 5, 00000 were discarded. The reports were similar to Malinowski (2001) [5], as Somatic cell count in the milk of the healthy cows was generally less than 2,00000 cells/ml. In sub- clinical mastitis, the SCC was greater than 4,00000 cell/ ml while in clinical mastitis somatic cell count was between 10- 50 million cells/ml.

All the animals included in the association study showed only one genotype AB (except two) with respect to Alu I/ PCR-RFLP. One animal each in affected and non affected group respectively had genotype of AA and BB. Therefore, no association could be established between polymorphism of TLR-4 gene and incidences of mastitis. Though variation in response of somatic cell count was detected, no association was established.

Restriction enzymes detect polymorphism based on the presence or absence of restriction sites of particular RE and hence cannot detect all the variations present in the particular DNA sequence. Techniques like SSCP and sequencing are more sensitive and can detect almost all variation present. Also other regions of TLR-4 gene should be screened for variations. The TLR4-2021 (C/T) polymorphism, within the third exon, results in substitution of threonine with isoleucine at amino acid position 674, located within the transmembrane cytoplamic domain (White *et al*., 2003) [13]. The C allele associates with decreased SCS and higher lactation persistency in the daughters of Holstein Friesian bulls (Sharma *et al*., 2006) [9]

It is also possible that more than a single gene is responsible for determining resistance / susceptibility to mastitis. The role

of Major Histocompatibility Complex in the susceptibility or resistance to intramammary infection is well documented. MHC genes have been mostly studied with respect to mastitis susceptibility and resistance. A strong and significant association between BoLA classes I and II haplotypes along with alleles and mastitis has been reported by several workers (Aarestrup *et al.,* 1995; Kelm *et al.,* 1997) [1, 3].

4. Conclusions

It can be concluded from the present study that TLR-4 is polymorphic in Crossbred cattle population. Future studies need to be directed to explore polymorphism throughout the entire TLR-4 gene and to ascertain their suitability as potential class I genetic marker for innate resistance towards mastitis.

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