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Genetic polymorphism of TLR-4 gene and its association with mastitis in Deoni and Holstein Friesian crossbred cows

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Abstract
The present study was conducted to determine the polymorphism of TLR-4 gene and its association with mastitis in Deoni and HF crossbred cattle. Milk and blood samples were collected from 244 animals comprising of 152 HF crossbred and 92 Deoni cows. The milk samples were screened for subclinical mastitis using three indirect diagnostic tests viz., California Mastitis Test (CMT), Electrical Conductivity (EC) and Somatic Cell Count (SCC). However, the animals were classified into mastitis free and mastitis affected groups based on SCC alone because of its higher sensitivity and specificity. Miller's high salt method was followed to isolate genomic DNA from venous blood. The quantity and quality of DNA was ascertained by spectrophotometer and 0.8 per cent agarose gel electrophoresis. A promoter region and exon 3 region of TLR-4 gene was amplified by PCR using published primers. On PCR-RFLP analysis, genetic variability was observed in TLR4 (promoter) and TLR4 (exon 3) genes in HF crossbred and Deoni cows. The TLR4 (promoter and exon 3) gene variants were not significantly associated with mastitis in both HF crossbred and Deoni cattle populations.

Keywords: Deoni, mastitis, TLR-4 gene, polymorphism

Introduction
Mastitis is considered the most frequent, important, complex, major health and costly disease of dairy cattle due to its huge economic implications and great impact on the dairy industry worldwide (Shook, 2006; Halasa *et al.*, 2007) [17, 3]. Mastitis resistance is a complex trait depending on genetic components as well as environmental and physiological factors. The direct selection of mastitis resistant animal is a very difficult approach in dairy breeding because of the low value of heritability as well as the limited progress in improving udder health by conventional selection procedures (Chen *et al.*, 2011) [2]. Utilization of Marker-assisted selection (MAS) in dairy cattle produces additional genetic and economic gains. In view of this, researchers have focused on identifying more informative genetic markers to allow faster and more accurate selection of cattle resistant to mastitis (Wiggans *et al*., 2011) [20]. The identification of genes having significant association with the mastitis would facilitate the inclusion of mastitis resistance in cattle breeding programmes. Ogorevc *et al.* (2009) [12] mentioned that about 934 candidate genes are involved in mammary gland development, milk production, sensitivity and resistant to mastitis. They also pointed out 15 candidate genes (TLR4, CD14, LTF, bola-13, IFNG, IL4, IL6, IL8, LBP, SAA3, CCR2, IL1B, C5AR1, TLR2 and β-4 defensin) useful in monitoring the mechanism of the development of an infectious disease as well as natural resistance of cows to mastitis. Polymorphisms in genes associated with the innate immune system are strong candidates to be evaluated as genetic markers. TLR4 (Toll Like Receptor 4) is responsible for initial recognition of invading organisms and is considered as a candidate gene for mastitis susceptibility. The bovine TLR family consists of 1–10 members and enables recognition of bacterial, viral and signals by individual cells (Ingham and Menzies, 2006) [5]. It is mapped on chromosome 8 (McGuire *et al*., 2005) [8], and consists of three exons and two introns. Toll-like Receptors (TLRs) play an important role in the recognition of components of pathogens and subsequent activation of the innate immune response, which then leads to the development of adaptive immune responses (Medzhitov and Janeway, 2000) [9]. TLR4 plays an important role in the innate immune status of cows during periods of risk from intramammary infection by gram- negative organisms (Miller *et al*., 2005) [11].

Bovine TLR4 gene is polymorphic, with 36 SNPs observed across 14 breeds of cattle. Of these, several SNPs were identified to have association with phenotypic or Estimated Breeding Value (EBV) for somatic cell count (Opsal *et al*., 2006; Sharma *et al*., 2006) [13, 15]. The present study was conducted to determine the variability of TLR-4 gene and to explore its possible association with mastitis in HF and Deoni cattle.

Materials and Methods

Animals and screening tests

A total of 244 unrelated animals were selected for the study comprising of 152 HF crossbred and 92 Deoni cows from different talukas surrounding Bengaluru and Bidar districts, respectively. Milk samples collected from these animals were screened for subclinical mastitis using three indirect tests viz., California mastitis test (CMT), electrical conductivity and somatic cell count (SCC). The animals were classified into mastitis free and mastitis affected based on results of SCC, because of its high sensitivity and specificity compared to the other two test.

DNA extraction and PCR amplification

High salt procedure as described by Miller *et al*. (1988) [10] was employed for isolation of genomic DNA from venous blood. The DNA concentration and quality were assessed based on the absorbance of UV light at 260 and 280 nm (A260/280) using spectrophotometer and 0.8 per cent agarose gel electrophoresis. The genomic DNA was diluted to a final concentration of 50 ng/µl using TE buffer and stored at -20 °C. Amplification of Promoter Promoter and exon 3 region of TLR-4 gene spanning 546 and 695 bp was done by utilizing published primers as below.

TLR4 (Promoter)	F: 5' TTCTTCAACCCAACCCACCT 3' R: 5' GCCCTGGCTCACCACAACTA 3'	546	(Zhixiong *et al*., 2014) [21]
TLR4 (Exon 3)	F: 5' TGCTCCCTGACATCTTCACA 3' R: 5' GGCCACCCCAGGAATAAA 3'	695	(Wakchaure *et al*., 2012) [19]

Each PCR reaction was carried out in a total volume of 25 µl containing 12.5 µl of 2 X Red PCR Master Mix, 1 µl (5 pmol/µl) each of forward and reverse primers, 50 ng template DNA and 9.5 µl of PCR grade water. The PCR reaction for amplification of TLR4 (Promoter region) was carried out with initial denaturation at 95 °C for 5 min, 30 cycles of denaturation at 94 °C for 40 sec, annealing at 60.5 °C for 30 sec, and extension at 72 °C for 45 sec, followed by final extension at 72 ºC for 10 minutes. Similarly, for TLR4 (exon 3), the PCR protocol used is initial denaturation at 95 °C for 2.5 min, 35 cycles of denaturation at 94 °C for 30 sec, annealing at 60. °C for 30 sec, and extension at 72 °C for 1.5 min, followed by final extension at 72 ºC for 10 minutes. The PCR amplified products were confirmed by resolving on 1.5% agarose gel with 100 bp ladder.

RFLP analysis

The RFLP analysis of PCR amplified promoter region of TLR-4 gene was carried out using *Bgl*I and *Hinf*I restriction enzyme (Thermo Fisher Scientific, Bangalore), respectively. The restriction enzyme digestion was carried out in a reaction volume of 30 µl, which consisted of 18ìl of autoclaved triple distilled water, 2 µl of 10 X assay buffer, 1 µl (10U/ µl) of RE and 10 µl of PCR product. The reaction mixture was incubated at 37 °C for 12 hours. The digested products were resolved on 2.0 per cent agarose gel along with 100 bp ladder.

Statistical analysis

The genotypes were determined by scoring the bands under the gel documentation system. The allele number, allele frequency, genotype frequency and observed and expected heterozygosites were calculated as described by Rosner (2005) [14]. The association between genotypes and occurrence of mastitis was analyzed by using chi square test (Snedecor and Cochran, 2004) [18].

Results and Discussion

Classification of animals based on mastitis screening tests The milk samples were screened for mastitis by different diagnostic methods namely, CMT, EC and SCC (Shivashanker *et al*., 2018) [16]. Electrical conductivity of milk can only give useful information about udder health status, but are considered not reliable when used alone in the diagnosis of mastitis (Hillerton and Walton, 1991) [4]. Of the three tests, SCC is having high sensitivity and specificity as compared to CMT and EC (Badiuzzaman *et al*., 2015; Magotra *et al*., 2016; Iraguha *et al*., 2017) [1, 4, 6], hence, the experimental animals were classified into mastitis free and mastitis affected solely based on SCC results (Table 1).

Table 1: Classification of animals based on SCC

Sl. No.	Breed	Total no.	Mastitis free	Mastitis affected
1.	H.F crossbred	152	52	100
2.	Deoni	92	73	19

RFLP analysis of TLR4 (promoter) gene

Amplified product of TLR4 (Promoter) gene was digested with restriction enzyme *Bgl*I in HF crossbred and Deoni cows, which showed three fragments of 546, 423 and 123 bp, on 1.5 per cent agarose gel electrophoresis. These fragments could be categorized into three genotypes *viz*., GG (546 bp), AA (423 and 123 bp) and GA (546, 423 and 123 bp) (Figure 1) This is in agreement with the genotypic patterns reported by Zhixiong *et al*. (2014) [21] in Chinese Holstein dairy cows.

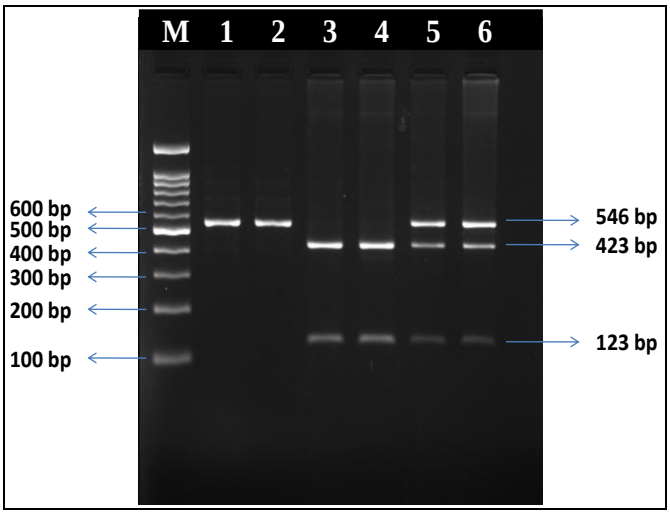

Fig 1: Agarose gel (1.5 %) electrophoresis showing PCR product after digestion with restriction enzyme *Bgl*I for detection of TLR4 (promoter) gene polymorphism in HF crossbred and Deoni cows. Lane M: Molecular Marker (100bp DNA ladder); Lane 1: Homozygous genotype AA in HF crossbred (546bp); Lane 2: Homozygous genotype AA in Deoni (546 bp); Lane 3: Homozygous genotype GG in HF crossbred (423 and 123 bp); Lane 4: Homozygous genotype GG in Deoni (423 and 123 bp); Lane 5: Heterozygous genotype GA in HF crossbred (546, 423 & 123 bp and Lane 6: Heterozygous genotype GA in HF crossbred (546, 423 & 123 bp).

RFLP analysis of TLR4 (exon 3) gene

Digestion of PCR amplified product of TLR4 (exon 3) gene by *Hin*FI exhibited four fragments of 124, 209, 362 and 571 bp, resolving into three genotypes in HF crossbred and Deoni cows. The three genotypes were CC represented by 571 and 124 bp fragments, CD represented by 571, 362, 209 and 124 bp fragments and DD represented by 362, 209, and 124 bp fragments (Figure 2). The results are in accordance with the reports of Wakchaure *et al.* (2012) [19] in Sahiwal cattle.

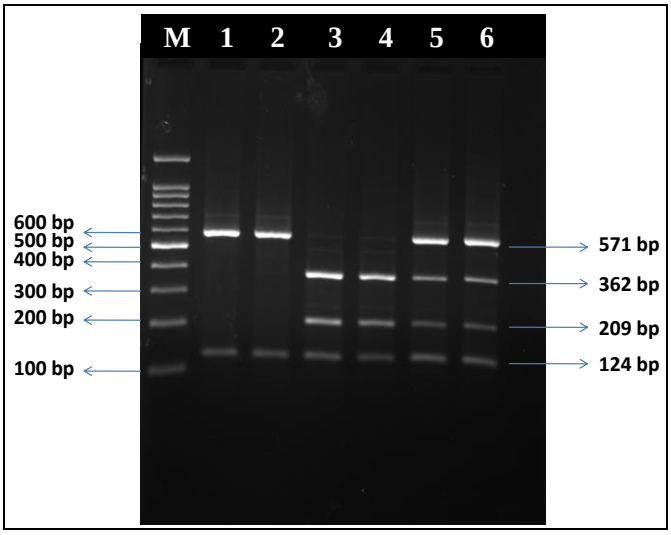

Fig 2: Agarose gel (1.5%) electrophoresis showing PCR product after digestion with restriction enzyme *Hinf*I for detection of TLR4 (exon 3) gene polymorphism in HF crossbred and Deoni cows. Lane M: Molecular Marker (100bp DNA ladder); Lane 1: Homozygous genotype CC in HF crossbred (571 and 124 bp); Lane 2: Homozygous genotype CC in Deoni (571 and 124 bp); Lanes 3, 4: Homozygous genotype DD in Deoni (362, 209 & 124 bp); Lane 5: Heterozygous genotype CD in HF crossbred (571, 362, 209& 123) and Lane 6: Heterozygous genotype CD in Deoni (571, 362, 209 & 123 bp).

Polymorphic studies

Genotypic and allelic frequencies of TLR4 (promoter) gene

The genotypic frequencies were 0.40, 0.53 and 0.07 in HF crossbred and 0.48, 0.39 and 0.13 in Deoni cows for GG, GA and AA, respectively. Heterozygotes were found to be more in HF cross bred cows compared to Deoni cows. In contrast Zhixiong *et al.* (2014) [21] reported highest frequency of GG genotype (0.52) in Chinese Holstein cow. In present study, the frequencies of G and A allele were 0.67 and 0.33 for both HF crossbred and Deoni cows. A higher frequency of G allele (0.71) was also observed by Zhixiong *et al.* (2014) [21] in Chinese Holstein cow. The observed and expected heterozygosities were 0.532 and 0.444 in HF crossbred, respectively, whereas, the respective values were 0.391 and 0.440 in Deoni cows. In the studied populations, only Deoni population was in equilibrium Whereas, HF crossbred population was not in equilibrium. The deviation of HF crossbred population may be attributed to lower size of the population (especially the sires) or external forces acting on it.

Genotypic and allelic frequencies of TLR4 (exon 3) gene

In present study, only two genotypes *viz.*, CC and CD with frequency of 0.89 and 0.11, respectively were observed in HF crossbred cows whereas three genotypes *viz.*, CC, CD and DD with frequencies of 0.54, 0.40 and 0.06, respectively were observed in Deoni cows. Higher frequency of CC genotype was also reported by Wakchaure *et al*. (2012) [19] in Sahiwal cattle. The allelic frequencies were 0.94 and 0.06 in HF crossbred and 0.48 and 0.52 Deoni cows for C and D, respectively. The frequency of D allele was found to be higher in Deoni cows. In contrast Wakchaure *et al*. (2012)[19] reported higher frequency of C allele in Sahiwal cattle.The observed and expected heterozygosities were 0.105 and 0.100 in HF crossbred, respectively, whereas, the respective values were 0.250 and 0.119 in Deoni cows. Further, both the studied populations were found to be in equilibrium for the studied locus.

Association study:

Association of TLR4 (promoter) genotypes with mastitis

In the present study, no significant association was observed between TLR4 (promoter) genotypes and mastitis in HF crossbred and Deoni cows (Table 2 and 3). In contrast, Zhixiong *et al.* (2014) [21] reported the presence of association between AA genotype and lower somatic cell score.

Table 2: Observed genotypes of TLR4 (promoter) and their association with mastitis in HF crossbred cows

Groups	Total no. of Animals	Genotype frequency			χ^2 Value
		GG	GA	AA	
Mastitis affected	100	37	55	8	1.772^{NS}
Mastitis free	52	24	26	2	
Total	152	61	81	10	

Table 3: Observed genotypes of TLR4 (promoter) and their association with mastitis in Deoni cows

Groups	Total number of individuals	Genotype frequency			χ^2 Value
		GG	GA	AA	
Mastitis affected	19	9	6	4	1.552^{NS}
Mastitis free	73	35	30	8	
Total	92	44	36	12	

Association of TLR 4 (exon 3) genotypes with mastitis

In present study, there was no significant association between TLR4 (exon 3) genotypes and mastitis in HF crossbred and Deoni cows (Table 4 and 5). In contrast, Wakchaure *et al*. (2012) [19] reported higher incidence of mastitis in CC genotype in Sahiwal cattle.

Table 4: Observed genotypes of TLR4 (exon 3) gene and their association with mastitis in HF crossbred cows

Groups	Total number of animals	Genotype frequency			χ^2 Value
		CC	CD	DD	
Mastitis affected	100	88	12	-	0.6740^{NS}
Mastitis free	52	48	4	-	
Total	152	136	16	-	

Table 5: Observed genotypes of TLR4 (exon 3) gene and their association with mastitis in Deoni cows

Groups	Total number of animals	Genotype frequency			χ^2 Value
		CC	CD	DD	
Mastitis affected	19	11	5	3	0.589^{NS}
Mastitis free	73	39	32	2	
Total	92	50	37	5	

Conclusion

Genetic variability was observed in TLR4 (promoter) and TLR4 (exon 3) genes in HF crossbred and Deoni cows. The TLR4 (promoter and exon 3) gene variants were not significantly associated with mastitis in both HF crossbred and Deoni cattle populations.

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