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Molecular analysis of TLR4 gene and its association with intra-mammary infections in Sahiwal cattle and Murrah buffaloes

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Toll-like receptors (TLRs) are a multigene family of pathogen recognition and play a central role in the initiation of inflammation response and subsequent adaptive immune system. In the present study, genotypes of TLR4 variants were analysed in mastitic cattle and buffaloes. Genome of selected animals (*n*=218) comprising of Sahiwal (*n*=110) cattle and Murrah (*n*=108) buffaloes on the basis of their health history as well as on the causative agents screening, were analysed for polymorphisms in TLR4 using PCR-RFLP. Molecular analysis of TLR4 gene revealed two alleles (*A* and *B*) and three genotypes (*AA*, *BB* & *AB*) in both the breeds. Incidence of mastitis showed significant association of TLR4 variants with non-staphylococci microbes. Genotype *BB* (Sahiwal, 0.613; Murrah, 0.703) was found significantly associated with the mastitis irrespective of microbial or other factors, whereas *AB* predominated (Sahiwal, 0.714; Murrah, 0.772) in unaffected cases. Statistically, association between polymorphism of TLR4 variant and mastitis in both the populations emphasized that genotype *AA* were significantly lower than *BB* ($P < 0.05$). Additionally, *AA* genotype remained prevalent only in case of mixed microbial infection in Murrah, and streptococcal and mixed microbial infection in case of Sahiwal. Allele *A* might be favourable for mastitis resistance. Chi square test revealed that the two loci of both the populations were in Hardy-Weinberg equilibrium. The observations of present may be helpful in identifying markers of natural resistance against mastitis that could contribute in its containment programme in cattle and buffaloes.

Keywords: Buffalo, cattle, mastitis, PCR-RFLP, TLR4

Introduction

Over the years, efforts are in vogue to improve breeding and management practices in dairy animals to enhance the milk production. However, dairy animals with improved milk production are usually susceptible to intra-mammary infections (IMI) and/or mastitis[1,2]. Huge economic losses due to mastitis have been reported (approx. US $35 billion) in dairy industry worldwide[3]. Apart from the management practices, microbial pathogens are main etiological agents causing mastitis, which induce the dissimilar inflammatory responses due to unlike virulence potential[4-7]. The virulence factors of these microorganisms evoke the host immune system through various routes[5-8]. The analysis of changes in host immune system due to pathogenic attack could be helpful in understanding pathogenesis mechanism as well as to establish the early diagnostic method[8]. In recent years, many diagnostic approaches and control strategies have been adopted for reduction of mastitis[9,10]. Various genetic determinants (DRB3 of major histocompatibility complex, β-defensin, lactoferrin, neutrophils chemokine receptors, interleukins, TLR, etc) have been reported in animals that could be associated with susceptibility/resistance to mastitis[8,10].

Among these genetic determinants, TLR gene family has been reported as a promising molecular marker for correlation between host immune responses and bacterial pathogens in cattle mastitis. TLR family members have been shown to recognize a unique set of pathogen-associated molecular patterns (PAMP) and express the adaptive immune response to microorganisms[11,12]. Such attributes of TLR make it suitable candidate gene for mastitis detection in dairy cattle[13]. So far 13 members of the TLR family have been identified in mammals and TLR4 has been explored more in mastitic cattle. TLR4 has been reported to recognize lipopolysaccharide (LPS) endotoxin of microorganism[14,15]. Evidences show that concentrations of important facilitators of TLR2 and TLR4 signalling, specifically soluble CD14 and LPS-binding protein, are increased in milk during

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IMI caused by *Escherichia coli* and *Staphylococcus aureus*[16]. The strong expression of TLR4 gene during mastitis caused by *S. aureus*[17] suggests that TLR4 might be playing a role in the host response to IMI. Several studies have been conducted on polymorphism of TLR4 and the association of its variants with the occurrence of mastitis in *Bos taurus* cattles[18-20]. However, there is paucity of information on the role of this gene in the incidence of mastitis in Indian cattle (*B. indicus*) and buffaloes (*Bubalus bubalis*). The present study was carried out with an objective to find out the TLR4 in mastitic cattle and buffaloes caused by different microbes.

Materials and Methods

Selection of Animals

Animals (*n*=168) were screened for mastitis and milk samples (*n*=112) were collected from infected cases comprising of Sahiwal (*n*=58) cattle and Murrah (*n*=54) buffaloes. Initially screening of animals for detection of mastitic infection was carried out using California mastitis test. Then milk samples were collected and subjected to microbiological identification. Milk sample (100 µL) was spread over blood agar medium and incubated at 37°C for 24 h. Grown out colonies were selected and further characterized using biochemical test to distinguish the types of streptococci and staphylococci[5,21]. Primary identification of isolates was confirmed using the molecular assay[22]. The animals for the study were selected on two criteria: first group of animals was based on the type of infection (streptococci and staphylococci), and the second group on information of disease and treatment record maintained in Animal Health Complex, National Dairy Research Institute, Karnal, India. The animals that had completed two or more lactations were kept in the second category. Thus, identified animals were made of affected and unaffected (control without history of mastitis and absence of microbes in test) groups.

Molecular Analysis of TLR4 Gene

Blood samples (5 mL each) were collected from jugular vein aseptically from the selected animals; 110 samples from Sahiwal and 108 from Murrah. DNA was extracted from collected blood samples using routine standard phenol-chloroform extraction method[8,23] and quality of DNA was assessed by 0.8% agarose gel electrophohoresis. Oligonucleotide primers (TLR32 & TLR31) were designed using the reference sequence of TLR4 gene (Acc. No.: AY297041 to AY297043) of *B. taurus* from NCBI GenBank. The amplicons for TLR32 were selected from 3257-3749 bp covering exon 3 region, and for TLR31 from 1578-1920 bp covering a region of exon 3 (1636-1920 bp) and a part of intron 2 (1578-1635 bp). The sequence of oligonucleotide primers used is given in Table 1. Amplification of these fragments was carried out using the PCR assays and the reaction mixture (25 µL) was consisted of 2.5 µL of 10× Taq buffer containing 25 m*M* $MgCl_2$ (Bangalore Genei, India), 2 µL dNTPs (200 µm/µL), 1 µL of each oligonucleotide primer forward and reverse (10 pm/µL; Sigma Aldrich, India), 1 unit of *Taq* DNA polymerase (3 U/µL; Bangalore Genei, India), 3 µL DNA (approx., 25 ng/µL) and rest part was sterile water. The amplification was carried out in 0.2 mL PCR tube in a thermal cycler (Biometra, Germany). The amplification conditions are also shown in Table 1. The amplified PCR products were visualized in 1.5% agarose gel containing 0.5 g/mL ethidium bromide under UV light. The amplified fragments were digested with restriction endonucleases, *Hae*III and *Taq*I. The restriction digestion was carried out using a mixture (10 µL) containing 1 µL of 10× restriction enzyme buffer, 2 U of restriction enzyme (3 U/µL), 6 µL PCR product and the rest part sterile water. Digestion was carried out at 37°C and 65°C for *Hae*III and *Taq*I, respectively. The restriction fragments were subjected to separation in 3% agarose gel electrophoresis.

Table 1—Oligonucleotide primers and their amplification conditions

Primer	Sequences	Acc. no.	Annealing temperature* (°C)	Product size (bp)
TLR32	F- TGTCAGCTGAGCAAGACGAT R- TGCTCAGAAGGCGATAGAGC	AY297043	58	493
TLR31	F- CATTTTGGTTTCCTATTCAGCA R- GATCCAAGTGCTCCAGGTTG	AY297043	56.5	343

*Initial denaturation step (5 min at 94ºC) followed by 30 cycles of amplification [denaturation for 60 sec at 94ºC, annealing temperature (given in table) for 60 sec and elongation for 60 sec at 72ºC] terminated with a 5 min incubation step at 72 ºC.

Statistical Analysis

The genotypes were directly obtained by counting the bands appearing in the agarose gels and their frequency was calculated as number of animals having a particular genotype/total number of animals screened. Gene frequencies were calculated as p=P+1/2H; q=Q+1/2H, where p and q are gene frequencies, P and Q are genotype frequencies of homozygote and H is genotype frequency of heterozygote. The difference in the observed and expected genotypic frequencies was evaluated using a chi-square (X^2) test. Chi-square=$\sum$ $(O-E)^2/E$, where O is observed frequency and E is expected frequency. Association between TLR4 variant with affected and non-affected mastitic cases was estimated using a chi-square (X^2) test[24].

Results

Selection of Animals

Animals (*n*=168) screened with microbiological tests for mastitis revealed that 112 animals (Sahiwal, 58 cattle and Murrah, 54 buffaloes) were positive for microbial infections (data published; Sentitula *et al*[22]). All these positive animals were kept in the first group or microbial mastitis cases. Based on health complex record, 106 animals (Sahiwal, 52 and Murrah, 54) were kept in the second group or random cases category. As per the health record maintained in health complex, of 52 animal of Sahiwal, 25 were mastitis affected and 27 were unaffected; while in 54 Murrah buffaloes, 22 cases were affected and 32 were unaffected. The unaffected animals of both Sahiwal and Murrah were used as control.

Molecular Evaluation of TLR4 Gene

The amplification of DNA samples revealed specific fragments of TLR32 (493 bp) and TLR31 (343 bp) locus of TLR4 gene and were confirmed by sequencing. Further, amplicons of TLR32 locus digested with *Hae*III generated two genotypes (*BB*: 222 & 271 bp; *AB*: 222, 271 & 493 bp) in Sahiwal, and three (*BB*: 222 & 271 bp; *AB*: 222, 271 & 493 bp; *AA*: 493 bp) in Murrah (Fig. 1). In Sahiwal cases, allele *B* was found in higher frequency (0.836) than allele *A* (0.164) and homozygous *BB* was observed to show the highest (0.672) genotype frequency. Similarly, in Murrah cases, allele *B* revealed higher frequency (0.634) than allele *A* (0.306) and *BB* predominated (0.481) as compared to *AA* genotype (0.093). The details of frequencies of allelic patterns and their combinations are shown in Table 2. Genotype-wise incidence of mastitis along with the causative agents and their association showed that cases showing homozygous *BB* were more susceptible to mastitis. In addition, *BB* genotype was found prevalent in mixed microbial infection that was 74.35 and 61.53% in Sahiwal and Murrah, respectively. Genotype *AA* was observed only in case of mixed infection mastitis in Murrah (Table 3). Analysis of random samples also revealed same fragments and combinations as microbial samples. Allele *B* was found to have higher frequency than allele *A* in both Sahiwal and Murrah cases, while homozygous *BB* was observed as the highest genotype frequency as indicated in Table 2. Incidences of mastitis showed that cattle with *BB* genotype were prone to suffer from mastitis that was observed to be 61.29 and 61.53% in Sahiwal and Murrah, respectively. Genotypic frequency *AB* remained high in unaffected animals of Sahiwal and Murrah, which was found to be 0.714 and 0.772, respectively as shown in Table 2.

Digestion of TLR32 locus amplicons with *Taq*I of microbial samples generated three genotypes (*BB*: 125, 163 & 205 bp; *AA*: 125, 163 & 288 bp; *AB*: 125, 163, 205 & 288 bp) in Sahiwal and two in case of Murrah (*BB*: 125, 163 & 205 bp; *AB*: 125, 163, 205 & 288 bp) as shown in Fig. 2. Frequencies of the allelic patterns and their combinations are given in Table 2. In Sahiwal cases, allele *B* was found in higher frequency (0.647) than allele *A* (0.353), while homozygous *BB* was observed to be dominant genotype (0.448). Likewise, allele *B* and genotype *BB* also remained prevalent in Murrah cases. The observed results revealed that *AA* genotype was found only in case of streptococcal and mixed infections that were found to be 11.11 and 8.88%, respectively. Molecular analysis of random samples also showed the similar pattern of results. Details of pattern of mastitis infection results for microbial cases are presented in Table 3.

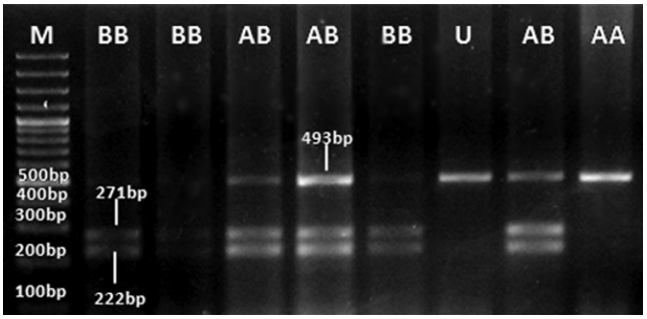

Fig. 1—Genotypes of TLR32 digested with *Hae*III (AA, AB & BB = Different genotypes; U = Unaffected cases)

Table 2—Genotypic and allelic frequencies of TLR4 gene in microbial and random mastitic cases

Breed	Locus of gene and restriction enzyme	Genotypic frequency						Gene frequency				$\chi 2_m$	$\chi 2_r$
		Microbial cases (n = 112)			Random cases (n = 106)			Microbial cases (n = 112)		Random cases (n = 106)			
		BB_M	AB_M	AA_M	BB_R	AB_R	AA_R	B_M	A_M	B_R	A_R		
Sahiwal	TLR32 (*Hae*III)	0.672	0.328	0.000	0.596^T	0.404^T	0.000	0.836	0.164	0.798^T	0.202^T	0.662	1.299
					0.613^A	0.286^A				0.567^A	0.285^A		
					0.387^U	0.714^U				0.433^U	0.714^U		
Murrah		0.481	0.426	0.093	0.481^T	0.408^T	0.111	0.634	0.306	0.685^T	0.315^T	1.049	0.165
					0.615^A	0.227^A	0.166			0.555^A	0.462^A		
					0.384^U	0.772^U	0.833			0.444^U	0.537^U		
Sahiwal	TLR32 (*Taq*I)	0.448	0.397	0.155	0.519^T	0.385^T	0.096	0.647	0.353	0.712^T	0.288^T	0.912	0.331
					0.703^A	0.250^A	0.200			0.558^A	0.606^A		
					0.296^U	0.750^U	0.800			0.346^U	0.394^U		
Murrah		0.537	0.463	0.000	0.741^T	0.259^T	0.000	0.769	0.231	0.870^T	0.130^T	2.034	0.280
					0.525^A	0.071^A				0.519^A	0.071^A		
					0.475^U	0.928^U				0.481^U	0.928^U		
Sahiwal	TLR31 (*Hae*III)	0.517	0.483	0.000	0.519^T	0.481^T	0.000	0.759	0.241	0.760^T	0.240^T	2.521	2.230
					0.555^A	0.400^A				0.529^A	0.400^A		
					0.444^U	0.600^U				0.471^U	0.60^U		
Murrah		0.519	0.481	0.000	0.537^T	0.463^T	0.000	0.760	0.240	0.769^T	0.231^T	2.348	2.031
					0.448^A	0.360^A				0.472^A	0.360^A		
					0.551^U	0.640^U				0.527^U	0.640^U		

T = Total cases in random; M = Microbial mastitic cases; R = Random cases; A = Affected cases of random group; U = Unaffected cases random group

Table 3—Genotype-wise incidence of mastitis along with the causative agents

Breed	Locus of gene and restriction enzyme	Type of microbes	Genotypic incidence of mastitis (%) Microbial cases (n = 112)		
			BB_M	AB_M	AA_M
Sahiwal	TLR32 (*Hae*III)	Staphylococci	12.82	10.52	0.00
		Streptococci	12.82	15.78	0.00
		Mixed microbes	74.35	73.68	0.00
Murrah		Staphylococci	19.23	8.69	0.00
		Streptococci	19.23	4.37	0.00
		Mixed microbes	61.53	86.95	100.0
Sahiwal	TLR32 (*Taq*I)	Staphylococci	15.38	13.04	0.00
		Streptococci	19.23	13.04	11.11
		Mixed microbes	65.38	73.19	8.88
Murrah		Staphylococci	17.24	8.00	0.00
		Streptococci	6.90	16.00	0.00
		Mixed microbes	75.86	76.00	0.00
Sahiwal	TLR31 (*Hae*III)	Staphylococci	6.66	17.85	0.00
		Streptococci	16.66	10.71	0.00
		Mixed microbes	76.66	71.42	0.00
Murrah		Staphylococci	25.00	0.00	0.00
		Streptococci	14.28	7.69	0.00
		Mixed microbes	60.71	92.30	0.00

M = microbial mastitic cases

Analysis of the TLR 31 (343 bp) locus amplicons of microbial samples digestion with *Hae*III produced two genotypes (*BB*: 98 & 196 bp; *AB*: 98, 196, 245, 294 & 343 bp) in both the breeds studied (Fig. 3). In Sahiwal cases, allele *B* was found to have higher frequency (0.759) than allele *A* (0.241), while homozygous *BB* was found higher (0.517) in genotype frequency. In Murrah cases, distribution of allele *B* was most frequent (0.760), whereas allele *A* was least common (0.240). The predominant genotype was *BB* (0.519). Genotype *BB* also predominated in case of mixed infections (92.30%).

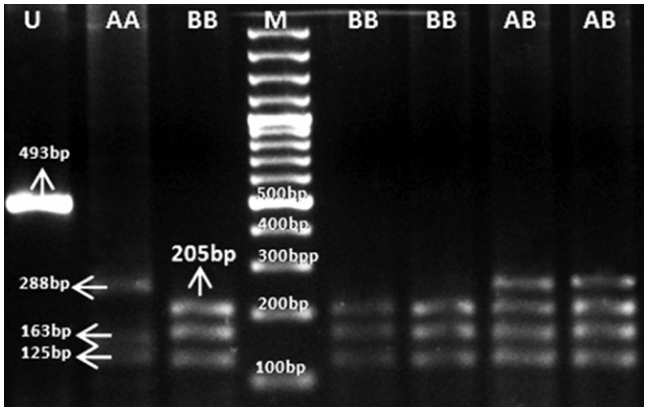

Fig. 2—Genotypes of TLR32 digested with *Taq*I (AA, AB & BB = Different genotypes; U = Unaffected cases)

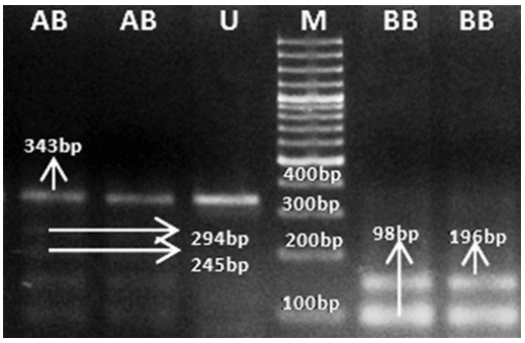

Fig. 3—Genotypes of TLR31 digested with *Hae*III (AA, AB & BB = Different genotypes; U = Unaffected cases)

Digestion of random samples produced similar fragments and combinations as microbial samples. In Sahiwal, allele *B* was found to have higher frequency (0.760) than allele *A* (0.240, while homozygous *BB* was found to have the highest (0.519) genotype frequency. Genotype-wise incidence of mastitis in Sahiwal revealed that homozygous *BB* (55.00%) was more inflicted with mastitis. In Murrah cases, allele *B* was found to have higher frequency (0.769) than allele *A* (0.231) in the herd, while homozygous *BB* was found to have the highest (0.537) genotype frequency. PCR-RFLP analysis of TLR31 with *Taq*I revealed no cutting site of the locus in samples of both the breeds. Chi-square test revealed that the two populations were in Hardy-Weinberg equilibrium for TLR32 and TLR31 locus (Table 2). Association of TR32 variant with mastitis in Sahiwal and Murrah revealed that there was significant difference between *BB* and *AB* genotype as well as *BB* and *AA* genotype. However, no significant difference was found between *AB* and *AA* genotype (Table 4). In TLR31 locus, no significant difference was found between *BB* and *AB* genotype in both the populations (Table 4).

Discussion

Present work was focused on TLR4 gene polymorphism and its association with bacterial infections. The incidence of bacterial infections in bovines has been described in many earlier studies[4-8,25] and these investigations reported 10-50% of mastitis cases due to bacterial infections. Staphylococci and streptococci have been reported as major causative agents of mammary gland infections[4-8,25]. Similar results were also obtained

Table 4—Correlation or association between TLR4 variant and mastitis incidence

Breed	Locus of genes	Genotypes	*Hae*III			*Taq*I		
			BB	*AB*	*AA*	*BB*	*AB*	*AA*
Sahiwal	TLR32	*BB*	-	5.37*	-	-	9.46**	4.57*
		AB	5.37*	-	-	9.46**	-	0.05
		AA	-	-	-	4.57*	0.05	-
Murrah	TLR32	*BB*	-	7.29**	3.94*	-	8.84**	-
		AB	7.29**	-	0.007	8.84**	-	-
		AA	3.94*	0.007	-	-	-	-
Sahiwal	TLR31	*BB*	-	1.26	-	-	-	-
Murrah	TLR31	*BB*	-	0.43	-	-	-	-

Significance level: **p<0.01; *p<0.05

in our previous study[22]. In addition TLR receptors in animal have been reported for recognition of pathogens and expression of immunological response[14,18-20].

TLR receptors have been observed to express polymorphism and/or mutations in PAMP, which could lead towards variation in properties of the receptors and functions[18,20]. The PCR-RFLP was employed to reveal polymorphism in such genetic determinants. The results of the present study showed polymorphism in TLR4 gene (Tables 2 & 3). Digestion of amplicons of TLR31 and TLR32 using *Hae*III (Figs 1 & 3) revealed that allele *B* was the predominant allele of this locus and *BB* genotype was present in greater frequency in mastitic cases in both the breeds. Genotype *AB* dominated in unaffected cases (Table 2). Further, analysis of TLR32 locus using *Taq*I enzyme showed the similar results (Table 2). The utility of TLR4 gene as a marker for identifying natural resistance against mastitis is well documented by many authors[13,18-20]. TLR4 variant was also able to distinguish the affected and unaffected cases in this investigation.

In the present study, a significant association was observed between mixed microbes and TLR4 genotypes, supporting the relationship between TLR alleles and the presence of causative agents (Table 3). However, breed or species specific association could not be found between TLR4 alleles and bacterial infections studied in Sahiwal cattle and Murrah buffaloes. The association between TLR4 variant and mastitis in both the populations showed that presence of *AA* genotype was significantly lower than that of *BB* ($P < 0.05$). The *A* allele might be favourable for mastitis resistance. The literature on TLR4 gene and its association with mastitis related traits is limited. It was observed that allele *AB* carrying animals showed less proportion of mastitis pathogens, which were prevalent in unaffected animals. The cases carrying *BB* were found more often associated with bacterial infections (Table 3) and the probability of occurrence of mastitis was higher. Similar findings have been reported in earlier study in ewes[18]. Other investigations also reported TLR4 gene in association with somatic cell score irrespective of microorganisms[19,20].

In conclusion, it is difficult to select candidate genes for mastitis resistance as a large number of pathogens and physiological mechanisms contribute to the observed variations. Breeding of animals for resistance to udder pathogens is important as a remedial tool to therapeutic and prophylactic measures. Production of resistant animals through genetic modification is possible. The present study has identified not only the genotypes associated with mastitis resistance/susceptibility but also the pathogens. Conversely, the association of pathogens was not found significant with few genotypes. However, observations provided the vital information. The information on pathogens is still useful in animal breeding programme. Unequivocally, TLR4 gene in the animal breeding programme may serves as genetic marker corresponding to major pathogens, such as, streptococci and staphylococci.

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