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Association Analysis of MBL1 Gene SNPs, Genotype and Haplotypes with Clinical Mastitis in Murrah Buffaloes

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Mannose-binding lectins (MBL1) gene a most important constituent of immune response system of an organism, the primary role of this gene in the classical and lectin-activation pathways is to provide protection against various dieases or bacterial pathogens. In this study, eight novel SNPs identified in the promoter region of MBL1 gene were 154C>T, 235G>A, 252A>T, 265C>A, 268T>C, 282G>A, 431G>A and 551C>G, when compared to reference sequence of *Bubalus bubalis* **(**NCBI accession number KC415281). Genomic DNA isolated from 200 lactating Murrah buffaloes was amplified for the targeted sequence of MBL1. PCR products were custom sequenced, edited and used for further analysis. The results indicated that only 551C>G polymorphism locus met Hardy–Weinberg equilibrium (χ^2 (2df) = 4.4: P=0.11). Pair linkage disequilibrium analysis and haplotype construction of MBL1 gene were performed using SHEsis software. Pair linkage disequilibrium analysis revealed moderate to strong linkage disequilibrium between the eight SNPs loci A total of 17 haplotypes were generated from eight SNPs in promoter region of MBL1 gene. Association between eight SNPs of MBL1 gene in Murrah buffalo has been analyzed and relative risks of the alleles for clinical mastitis were estimated with an odds ratio. Allelic association analysis showed

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that T allele of 154C>T, A allele of 235G>A, A allele of 252A>T, A allele of 265C>A , T allele of 268T>C, A allele of 282G>A , A allele of 431G>A and G allele of 551C>G had significant association with increased risk of clinical mastitis (P< 0.01). Haplotype analysis showed that Hap15 (CAAATAAG) and Hap16 (TAAATAAG) i.e. the haplotypes containing all 8 “at-risk” alleles, were significantly associated with clinical mastitis (P< 0.01). Hap6 (CGAATAAC) and Hap8 (CGTCCGGC) were significantly associated with a lower risk of clinical mastitis in Murrah buffaloes (P< 0.01).On the other hand, the Hap6 (CGAATAAC) and Hap8 (CGTCCGGC) which are unrepresented in affected group and without “at-risk” alleles were significantly associated with a lower risk for clinical mastitis (P<0.01).These findings indicated a MBL1 gene polymorphism could be used as genetic marker for mastitis resistance in Murrah buffaloes.

Keywords: Genetic marker; mastitis resistance; murrah buffaloes.

1. INTRODUCTION

Mastitis is one of the most common diseases in milch animals and leads to huge economic losses to the dairy industry worldwide. The incidence rate of clinical mastitis ranges from 25% to 60% [1]. As mastitis being the major reason for low productivity, it is necessary to understand the target genes involved in disease resistance and to immune response mechanism to develop proper preventive measures. Thus, an approach based on improving the host genetics in resistance to infectious diseases through selective breeding by is becoming widely accepted. Resistance to mastitis is a complex trait and genes involved in the immune response have been indicated as strong candidates for marker-selective breeding. Mannose-binding lectin (MBL) is one of the most important constituent and is a pattern recognition molecule of the innate immune system [2, 3]. It binds to a range of sugars including N-acetyl-D-glucosamine, mannose, N-acetyl-mannosamine, fucose and glucose. This permits the protein to interact with a wide selection of microorganisms coated with such sugars. MBL bound to microbial surfaces is able to activate the complement system in an antibody and C1-independent manner. Most mammals have two forms of MBL, MBL-A and MBL-C, which are encoded by two distinct genes named MBL1 and MBL2, respectively [4]. The bovine MBL1 gene located on chromosome 28 and spans over 5223 base pairs (bp) long consisting of four introns and five exons encoding 248 aa. MBL2 gene spans over 5261 base pairs (bp) long which has three introns and four exons encoding 249 aa and is located on chromosome 26 [5]. SNP rs110326717 (g.2651G>A) was identified as a non-synonymous mutation GTT (Val)>ATT (Ile) at position 24th aa of *MBL*-A [6]. *MBLs* have been associated with susceptibility to various bacterial and viral diseases [7]. Impaired disease resistance was found to correlate with three SNPs within the coding region of *MBL1* in various breeds of pigs [8]. In Chinese Holstein cattle, a total of 5 SNPs have been reported for MBL1 gene, and the combined genotype ATGGC/ACAAC exhibited the lowest, thus *MBL1* gene possibly contributes to bacterial infection resistance and was proposed as a molecular marker of milk production traits to control mastitis [9]. In Vrindavani crossbred cattle, SNPs have been reported to be polymorphic in both the affected as well as unaffected groups of cows but no significant association has been found with the clinical mastitis [10,11]. Three SNPs at (c.1252G>A in intron1, c.2534G>A and c.2569T>C in exon2) have been reported in MBL1 gene. The SNP c.2534G>A showed significant association with milk SCS and cows with genotype AA having the highest milk Somatic Cell Score were prone for mastitis, whereas GG genotype with the lowest milk SCS were favorable for the mastitis resistance, thus MBL1 polymorphism was appeared to be a promising indirect marker to improve dairy mastitis resistance traits in cattle (Yuan *et al*., 2013). However, there is no report available in Murrah buffaloes. Hence this study was carried out with the aim to elucidate genetic control of immune response and clinical mastitis through selective breeding by using molecular marker in Murrah buffalo.

2. MATERIALS AND METHODS

2.1 Experimental Animals and DNA Extraction

About 10 ml of blood was collected in EDTA coated vacutainer tube from each of the 200 Murrah buffaloes maintained at Animal Health Complex, National Dairy Research Institute, Karnal. The samples were stored at -20°C until DNA isolation. Phenol-chloroform method, as

described by Sambrook and Russsel [12] with minor modifications was used for DNA isolation from blood samples of Murrah buffaloes. The quality and quantity of DNA were checked by UV- spectrophotometer. The DNA samples were diluted to 100ng/µl for utilizing as DNA template in PCR. The information on the mastitis incidence was collected from the records maintained at Animal Health Complex, National Dairy Research Institute, Karnal. Animals which had suffered at least once with mammary gland inflammation in their productive life were considered as mastitis animals, while those which did not suffer from mastitis till fourth lactation were taken as non-mastitis animals.

2.2 PCR Primers and Amplifications

Primer was taken as described by Wang *et al.* [6] and was procured from M/s. Eurofins Genomics India Pvt. Ltd, Bangalore, India. A 588 bp fragment of Promoter region of MBL1 gene was amplified using PCR Forward:5'-CCCTTCCAACTCATTGCTTC-3'andReverse: R-AGTCCCAACCACCCTCA-3'primers.The amplifications were carried out in 0.2 ml PCR reaction tubes using a programmable thermal cycler (MJ Research-PTC200).The thermal cycling conditions involved an initial denaturation at 94°C for 5 min, followed by 30 cycles with initial denaturation at 94°C for 30 sec, annealing temperature 60.5°C for 30 sec, extension at 72°C for 30 sec followed by a final extension at 72°C for 5 min.PCR products were detected by electrophoresis at 90 V in 1.5% agarose gels.

2.3 Sequencing and Data Analysis

PCR products showing intact sharp DNA bands were sent to 1st BASE Sequencing INT, Singapore for custom sequencing by using forward and reverse primers. Sequence data were analyzed using, Bioedit software [13]. The Quality of each chromatogram was checked by using DNA Baserv4 software. ClustalW software was used to analyze the sequencing results for detecting single nucleotide polymorphisms of MBL1 gene in Murrah buffalo.

2.4 Statistical Analysis

The genotypic, allelic frequencies, polymorphism information content (PIC), heterozygosity (He),effective number of alleles (Ne) and Hardy–Weinberg equilibrium (HWE) were estimated by using Popgene software [14]. Pairwise linkage disequilibrium (LD) and haplotype construction analysis was performed by using the online web based SHEsis software (http://analysis2.bio-x.cn/myAnalysis.php) [15] The associations between allele, haplotype of MBL1 gene and Clinical mastitis were analyzed through Chi-square test and relative risk for clinical mastitis were estimated using the odds ratios (ORs) with their 95% confidence intervals (CIs). All statistical analyses were performed using SAS v8.0 statistical package (SAS Institute, NC, USA). A P value <0.05 was defined as statistically significant.

3. RESULTS AND DISCUSSION

3.1 Sequencing and SNP Identification

Amplified PCR products were subjected to direct sequencing from both ends (5' and 3' ends). A total of 200 amplicon of MBL1 gene were subjected to sequencing by an automatic DNA sequence (1st BASE Sequencing INT, Singapore) .The DNA sequences obtained after editing with BioEdit software and alignment were further analyzed for detection of SNPs and comparative sequence analysis. For determining the SNPs in promoter region of MBL1 gene in Murrah buffalo reference sequence of *Bubalus bubalis* **(**NCBI accession number KC415281) were compared and aligned with the edited sequences of Murrah buffalo by ClustalW software, which resulted in a total of 8 nucleotide variations in Murrah buffalo The eight novel SNPs identified in the promoter region of MBL1 gene were 154C>T, 235G>A, 252A>T, 265C>A, 268T>C, 282G>A, 431G>A and 551C>G, when compared to reference sequence of *Bubalus bubalis* **(**NCBI accession number KC415281) (Table 1). The sequencing results indicated that these eight SNPs (Figs. 1–6) included five transitions (154C>T, 235G>A, 268T>C, 282G>A, and 431G>A) and three transversions (252A>T, 265C>A, and 551C>G). The raw nucleotide sequence was assembled to generate the promoter region sequence of MBL1 gene in Murrah buffalo. The promoter region sequence generated for the Bubaline MBL1 gene has been submitted to the NCBI for which GenBank accession No. KM087782.1 was assigned.

3.2 Genotypic and Allelic Frequencies

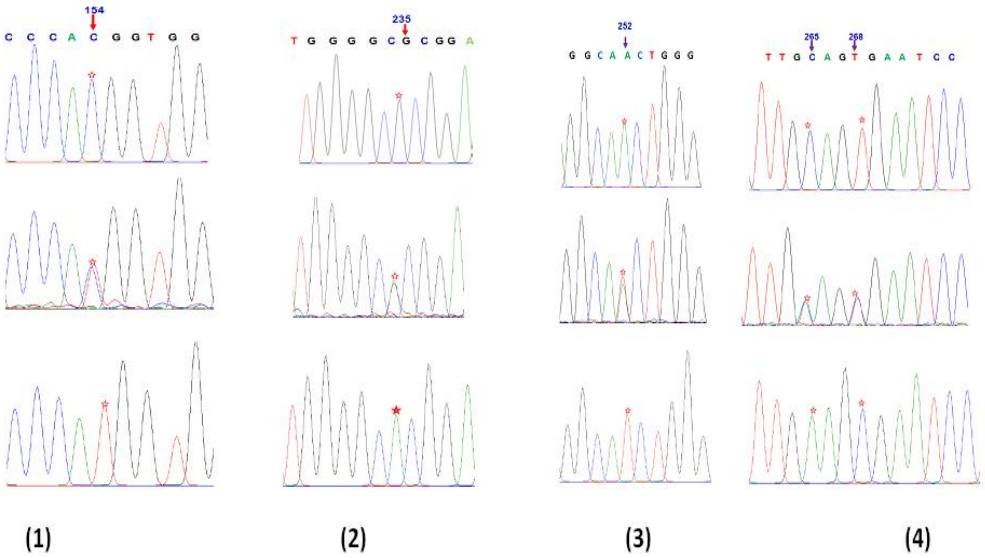
The allelic and genotypic distributions of eight SNPs identified in the promoter region of MBL1 gene in affected and unaffected animals have been summarized in Table 2 and 3.

Table 1. Summary of nucleotide changes in promoter region of MBL1 gene of Murrah buffalo as compared to *Bubalus bubalis* (NCBI Accession No. KC415281)

S.NO	Position	Bubalus bubalis	Murrah
1	154C>T	C	T
2	235G>A	G	A
3	252A>T	A	T
4	265C>A	C	A
5	268T>C	T	C
6	282 G>A	G	A
7	431 G>A	G	A
8	551 C>G	C	G

Table 2. Allelic frequencies distribution of 8 SNPs loci of MBL1 gene and their association with clinical mastitis in Murrah buffaloes

Locus	Allele frequency			Odds Ratio	χ^2 (1df)	P value
	Allele	Affected (n=100)	Unaffected (n=100)			
154C>T	C	34.5%	67.2%	0.256 %95	41.92	P<0.01
	T	65.5%	32.8%	CI =[0.168~0.39]		
235G>A	A	67.2%	32.2%	4.3126 %95	48.98	P<0.01
	G	32.8%	67.8%	CI=[2.83~6.55]		
252A>T	A	63.5%	52.1%	1.594 %95	4.83	P<0.05
	T	36.5%	47.9%	CI=[1.050~2.41]		
265C>A	A	63.2%	27.7%	4.473 %95	48.08	P<0.01
	C	36.8%	72.3%	CI=[2.89~6.914]		
268T>C	C	18.5%	66.7%	0.113 %95	93.42	P<0.01
	T	81.5%	33.3%	CI=[0.071~0.180]		
282G>A	A	65.6%	38.0%	3.117 %95	30.53	P<0.01
	G	34.%4	62.0%	CI=[2.07~4.69]		
431G>A	A	79.2%	41.4%	5.383 %95	56.84	P<0.01
	G	20.8%	58.6%	CI=[3.41~8.48]		
551C>G	C	45.3%	58.0%	0.601 %95	4.34	P<0.05
	G	54.7%	42.0%	CI=[0.37~0.972]		



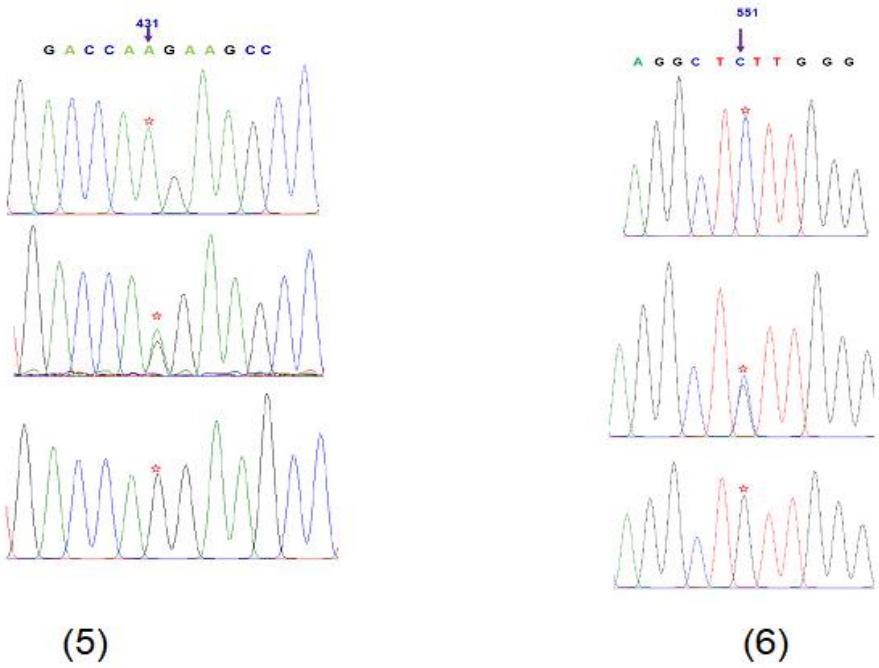


Fig. (1-6). Chromatogram showing sequencing results at 154C>T, 235G>A, 252A>T, 265C>A, 268T>C, 282G>A, 431G>A and 551C>G loci of MBL1 gene promoter; Arrow and star indicates single nucleotide substitutions in promoter region of MBL1 gene

The genotype CC, CT and TT for the SNP 154 C>T were 22.6%, 23.8% and 53.6% in affected animals and 50.9%, 32.8% and 16.4% in unaffected animals respectively, with significant differences between affected and unaffected animals (P<0.01). The genotype frequencies for AA, GG and AG of SNP 235G>A were 48.5%, 37.4% and 14.1% in affected animals and 19.8%, 24.8% and 55.4% in unaffected animals respectively. The genotype frequencies for AA, AT and TT of 252A>T were 53.8%, 19.2%, and 26.9% in affected animals and 17.1%, 70% and 12.9% in unaffected animals respectively. The genotype frequencies for AA, AC and CC of 265C>A were 56.9%, 12.5% and 30.6% in affected animals and 10.2%, 35.2% and 54.6 % in unaffected animals respectively. The genotype frequencies for CC, CT and TT of 268T>C were 13%, 10.9% and 76.1% in affected animals and 38.8%, 55.6% and 5.6% in unaffected animals respectively. The genotype frequencies for AA, AG and GG of 282G>A were 50%, 31.2% and 18.8% in affected animals, and 14.4%, 47.1% and 38.5% in unaffected animals respectively. The genotype frequencies for AA, AG and GG of 431G>A were 71.4%, 15.5% and 13.1% in affected animals and 16.4%, 50.00% and 33.6% in unaffected animals respectively. The genotype frequencies for CC, CG and GG of 551C>G were 32.6%, 25.6% and 41.9% in affected animals and 41.4%, 33.1% and 25.5% in unaffected animals respectively. Liu *et al*. [9] reported frequencies of A allele at locus g.−2194A>C as 0.900, 0.7273 and 0.9375, C allele at locus −1446T>C as 0.6098, 0.7841 and 0.6250, G allele at locus g. −1330G>A as 0.7111, 0.8523 and 0.8958 and G allele at locus at g.2651G>A as 0.6130, 0.7614 and 0.7500 in Chinese Holstein, Luxi Yellow and Bohai Black cattle respectively. Wang *et al.* [6] also reported G allele frequency of Chinese Holstein at locus g.855G>A as 0.87, 1.0 and 1.0, G allele Luxi Yellow frequency at locus g.2651G>A as 0.58, 0.75 and 0.76 and C allele of Bohai Black frequency at locus g.2686T>C as 0.57, 0.74 and 0.63.

3.3 Population Genetic Indices

Test of genotype frequencies for deviation from Hardy-Weinberg equilibrium (HWE) were analyzed by Chi-square analysis and were found significant (p<0.01) for all the SNPs at 154C>T, 235G>A, 252A>T, 265C>A, 268T>C, 282G>A and 431G>A loci in the studied population indicated that these genetic variants were not in the HWE (P <0.01). Whereas as SNP at

551C>G locus was in the HWE (χ^2 (2df) = 4.4: P=0.11). The value of PIC for 154C>T, 235G>A, 252A>T, 265C>A, 268T>C, 282G>A, 431G>A and 551C>G loci were 0.37463, 0.37596, 0.36587, 0.36384, 0.37136, 0.37596, 0.37311and 0.37287 respectively. According to the classification of polymorphism information content (PIC), the PIC value <0.25, 0.25-0.5 and >0.5, indicates low polymorphism; intermediate polymorphism; high polymorphism respectively. It clearly indicate that there was an intermediate genetic diversity for these SNPs of MBL1 gene in Murrah population. The Chi-square analysis showed that SNPs were not in HWE (P<0.05), except for 551C>G locus. The value of population genetic indices, including He, Ne, I, PIC and Chi-square value are shown in Table.4.

3.3.1 Haplotype distribution of MBL1 promoter region gene in Murrah buffalo

Haplotype frequencies in all mastitis affected and unaffected Murrah buffaloes for the eight SNPs at 154C>T, 235G>A, 252A>T, 265C>A, 268T>C, 282G>A, 431G>A and 551C>G loci were analyzed for LD analysis and highly correlated SNPs were used for haplotype construction .The haplotypes were constructed by using SHEsis software program. A total of 17 haplotypes were generated from eight SNPs; 10 were rarer i.e. the frequencies of these ten haplotypes were less than 3% and were excluded from the association analysis due to the small (<3) sample sizes. The frequencies of the remaining 7 haplotypes were compared in all mastitis affected and unaffected animals. The most common haplotype was Hap1 (CAAACAAC) observed in both affected and unaffected animals with frequency of 39% and 40% respectively. The haplotype Hap14 (CAAATAAC) , Hap15 (CAAATAAG) and Hap16 (TAAATAAG) with frequency of 6% , 22% and 30% in affected animals and haplotype Hap8 (CGTCCGGC), Hap6 (CGAATAAC) and Hap13 (TGTCTGAC) with frequency of 34% , 10% and 4% in unaffected animals respectively. Five haplotypes Hap6, Hap8, Hap14, Hap15 and Hap16 showed significant difference in frequencies between affected and unaffected animals were used for association analysis (Table.5).

However, Liu *et al*. [9] reported 28 different haplotypes constructed from the five SNPs loci of MBL1 gene in Chinese cattle breeds. They also observed that the H16 showed highest haplotype frequencies (25.7%), whereas the haplotypes H18 and H26 were not observed in the population. Wang *et al*. [6] reported that eight different haplotypes in Chinese Holstein, Luxi Yellow and Bohai Black breeds as H1: GGT, H2: GAC, H3: GGC, H4: AGC, H5: GAT, H6: AGT, H7: AAC and H8: AAT, with estimated frequencies of 0.366, 0.363, 0.110, 0.071, 0.052, 0.026, 0.010 and 0.002 for H1–H8, respectively. Their results revealed that among these eight haplotypes, H1 showed the highest and H8 had the lowest frequency.

3.3.2 Linkage disequilibrium analysis of MBL1 gene in Murrah buffalo

Pairwise Linkage Disequilibrium (LD) analyses between eight SNPs at 154C>T, 235G>A, 252A>T, 265C>A, 268T>C, 282G>A, 431G>A and 551C>G loci were estimated using SHEsis software programme and found that some SNPs loci exhibited weak to moderate pairwise LD as the pairwise Lewontin's D' value were less than 0.75 and pairwise r^2 value was less than 0.40 indicating weak to moderate LD. While as some SNPs loci exhibited moderate to strong LD between SNPs loci as Lewontin's D' value ranged between 0.75 to 1.00 and r^2 0.49 to 1.00 (as D' value >0.75 and r^2 > 0.49 indicated moderate to strong LD). The results from this study indicated that the Murrah buffalo possessed weak, moderate to strong LD between SNPs loci. Red squares represent high pairwise LD, coloring down to white squares of low pairwise LD. Dark red squares represent high association (D' ≥0.85), moderate red represent D' value 0.80 - 0.75 and light red represent D' of < 0.70. The D' and r^2 values are presented in Table 6 and Fig. 7.

3.4 Association Analysis

3.4.1 Association between SNP, haplotype of MBL1 gene and clinical mastitis in Murrah buffalo

Association between eight SNPs (154C>T, 235G>A, 252A>T, 265C>A, 268T>C, 282G>A, 431G>A and 551C>G) in the promoter region of MBL1 gene in Murrah buffalo has been analyzed and relative risks of the alleles for clinical mastitis were estimated with an odds ratio.

3.4.2 Allelic association analysis

Allelic association analysis showed that T allele of 154C>T, A allele of 235G>A, A allele of 252A>T, A allele of 265C>A, T allele of 268T>C, A allele of 282G>A, A allele of 431G>A and G allele of 551C>G loci, were more frequent in

affected animals as compared to unaffected animals, with an odds ratios of 0.256 for alleles of 154C>T (95% CI: 0.168~0.39), 4.31 for alleles of 235G>A (95% CI: 2.83~6.55), 1.594 for alleles of 252A>T (95% CI: 1.050~2.41), 4.47 for alleles of 265C>A (95% CI: 2.89~6.914),0.113 for alleles of 268T>C (95% CI: 0.071~0.180), 3.117 for 282G>A (95% CI: 2.07~4.69), 5.38 for alleles of 431G>A (95% CI: 3.41~8.48) and 0.601 for alleles of 551C>G (95% CI 0.37~0.972) respectively. These alleles showed significant association with increased risk of clinical mastitis in Murrah buffaloes (P< 0.01) and may be designated as "at-risk" alleles (Table.2).

3.4.3 Genotypic association analysis

The Chi-square analysis revealed that Murrah buffaloes with CC or TC genotype of 154C>T, GG of 235G>A, AT genotype of 252A>T,CC or AC genotype of 265C>A, TC or CC genotype of 268T>C, AG or GG genotype of 282G>A and AG or GG genotype of 431G>A loci had significantly lower incidence of clinical mastitis compare to their counter genotypes. Buffaloes with CC or CG genotype had non-significantly lower clinical mastitis than that of GG genotype (P=0.1108) at position 551C>G (Table.4).

Wang *et al.* [6] reported that the animals with genotype AA at position 2651 had significantly (P < 0.05) higher SCS than those with GG and GA genotypes of (P<0.05). The correlation between g.855G>A/G.2686T>C polymorphism and somatic cell score/milk traits was non-significant (P > 0.05). They also suggested that the cows with combined genotypes of GGC/AAC had the lowest SCS. Yuan *et al*. (2013) found a significant association between genotypes of locus c.2534G>A and SCS they reported that animals with genotype GG had significantly (P<0.05) lower SCS than those with GA and AA genotype. However, genotypes at c.1252G>A and c.2569T>C loci were not found significant.

Table 3. Genotypic frequencies distribution of 8 SNPs of MBL1 gene and their association with clinical mastitis in Murrah buffaloes

Locus	Genotype frequency			HWE χ^2 (1df) (P=6.63)			P value
	Geno-type	Affected (n=100)	Unaffected (n=100)	Affected	Unaffected	χ^2 (2df) (P=9.21)	
154	CC	22.6%	50.9%	18.82	7.62	32.37	P<0.01
C>T	CT	23.8%	32.8%	P<0.01	P<0.01		
	TT	53.6%	16.4%				
235	AA	48.5%	19.8%	18.92	2.30	39.035	P<0.01
G>A	AG	37.4%	24.8%	P<0.01	P = 0.129		
	GG	14.1%	55.4%				
252	AA	53.0%	17.1%	44.53	11.34	50.73	P<0.01
A>T	AT	19.2%	70.0%	P<0.01	P<0.01		
	TT	26.9%	12.9%				
265	AA	56.9%	10.2%	38.50	1.93	93.42	P<0.01
C>A	AC	12.5%	35.2%	P<0.01	P=0.16426		
	CC	30.6%	54.6%				
268	CC	13.0%	38.8%	37.59	6.75	105.67	P<0.01
T>C	TC	10.9%	55.6%	P<0.01	P<0.01		
	TT	76.1%	5.6%				
282	AA	50.0%	14.4%	9.07	0.001	30.53	P<0.01
G>A	AG	31.2%	47.1%	P<0.01	P= 0.99		
	G G	18.8%	38.5%				
431	AA	71.4%	16.4%	23.66	0.108	61.94	P<0.01
G>A	AG	15.5%	50.0%	P<0.01	P= 0.741		
	GG	13.1%	33.6%				
551	CC	32.6%	41.4%	10.06	16.11	4.40	P=0.1108
C>G	CG	25.6%	33.1%	P<0.01	P<0.01		
	GG	41.9%	25.5%				

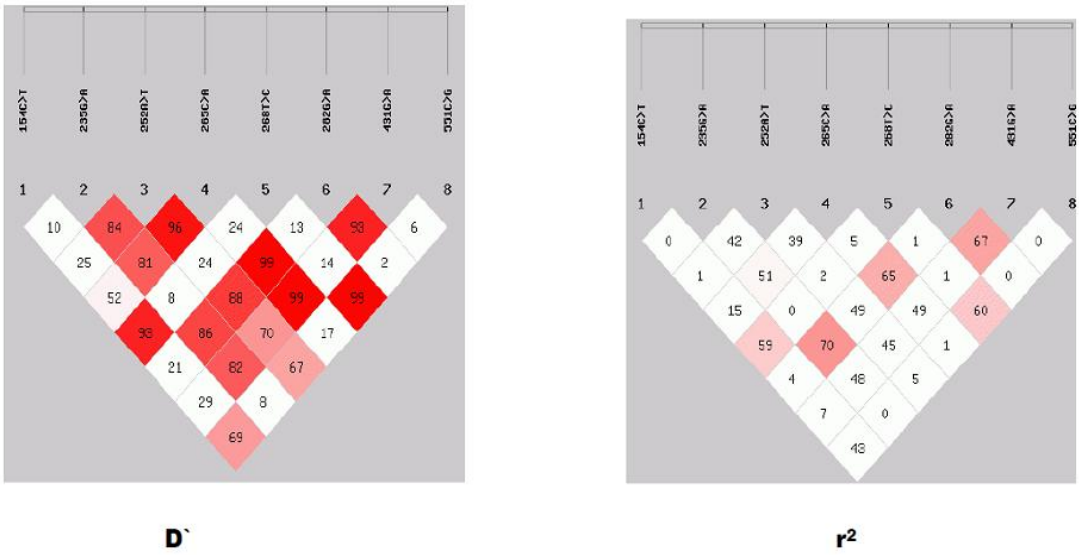


Fig. 7. Plot showing pair wise LD values of D` and r^2 of the eight SNPs in MBL1 gene

Table 4. Population genetic indices of Promoter Region of MBL1 gene in Murrah buffaloes

Locus	Ne	I	He	PIC	HWE test
154 C>T	1.9902	0.6907	0.49841	0.37463	Disequilibrium (P<0.01)
235G>A	1.9998	0.6931	0.50094	0.37596	
252A>T	1.9303	0.6750	0.48201	0.36587	
265C>A	1.9303	0.6750	0.47998	0.36384	
268T>C	1.9761	0.6871	0.49335	0.37136	
282G>A	1.9988	0.6928	0.50080	0.37596	
431G>A	1.9588	0.6826	0.49270	0.37311	
551 C>A	1.9782	0.6876	0.49513	0.37287	HW-equilibrium (P=0.11)

He= heterozygosities, Ne =effective of alleles, I = Shannon's Information index and PIC= polymorphism information contents

3.4.4 Haplotype association analysis

Association analysis of the five haplotypes showed that Hap14 (CAAATAAC), Hap15(CAAATAAG) and Hap16 (TAAATAAG) had significant effect on increasing risk of clinical mastitis. *i.e.*, the haplotypes containing all 8 "at-risk" alleles were represented in affected animals only and were associated with susceptibility to clinical mastitis in Murrah buffaloes. On the other hand, Hap6 (CGAATAAC) and Hap8 (CGTCCGGC), which is unrepresented in affected group and without all these 8 alleles were associated with a lower risk of clinical mastitis in Murrah buffaloes (P<0.01). it clearly indicates that Hap6 and Hap8 may be the favorable haplotypes for mastitis resistance. As haplotypes were deduced from the significant SNPs and it was inferred that Murrah buffaloes with Hap6 and Hap8 allelic combinations are least susceptible to mastitis (Table.6).

However, Liu *et al*. [9] reported the association of haplotype combinations with SCS. They observed that the animals with the haplotype combinations H15H1 (n=6) and H16H1 (n=79) were significantly lower SCS than those with the haplotype combinations H8H5, H8H21, H16H8, H16H22, and H16H5 (P<0.05).

Wang *et al*. [6] reported that animals with haplotype combination H3H7 showed significantly lower SCS scores in comparison to the haplotype combinations of H1H1 (P < 0.05) and H2H2 (P=0.0098).

Table 5. Haplotype frequencies distribution of MBL1 gene and its association with clinical mastitis in Murrah buffaloes

Hap id	Haplotype definition	Frequency		$\chi 2$	Fisher's Value	Odds Ratio [95%CI]
		Affec-ted	Un-affected			
Hap1	C A A A C A A C	39 %	40%	0.72	P=0.39	0.783 [0.44~1.37]
Hap2	C A T A C A A C	0%	1%			
Hap3	C AT C C A A C	0 %	2%			
Hap4	CA T C C G A C	0 %	2%			
Hap5	C A T C C G G C	0 %	1%			
Hap6	C GA A T A A C*	0 %	10%	9.79	P<0.01	
Hap7	C G A C T A A C	0 %	1%			
Hap8	C G T C CG G C*	0 %	34%	41.92	P<0.01	
Hap9	C G T C T G G C	0 %	2%			
Hap10	T G A C TA A C	0 %	1%			
Hap11	T G T C T A A C	0 %	1%			
Hap12	TG T C TG A C	1 %	1%			
Hap13	T G T C T G A C*	0 %	4%	3.52	P<0.01	
Hap14	C AA A T A A C*	6%	0 %	7.43	P<0.01	
Hap15	C A A AT A A G*	22%	0 %	30.40	P<0.01	
Hap16	T A A A T A A G*	30%	0 %	43.21	P<0.01	
Hap17	T AA C T A AG	2%	0 %			

Table 6. Pairwise D` and r^2 value of the eight SNPs in MBL1 gene

	154 C>T	235 G>A	252 A>T	265 C>A	268 T>C	282 G>A	431 G>A	551 C>G
154C>T	-	0.109	0.255	0.527	0.933	0.213	0.296	0.692
235 G>A	0.010	-	0.846	0.820	0.080	0.869	0.828	0.088
252A>T	0.015	0.426	-	0.967	0.240	0.882	0.704	0.676
265C>A	0.159	0.515	0.399	-	0.249	1.000	1.000	0.179
268T>C	0.597	0.005	0.027	0.052	-	0.135	0.142	1.000
282G>A	0.041	0.704	0.496	0.656	0.014	-	0.934	0.026
431G>A	0.075	0.481	0.456	0.491	0.012	0.671	-	0.067
551C>G	0.439	0.003	0.057	0.015	0.609	0.000	0.003	-

Note : D' is above the diagonal for SNPs and r2 is below the diagonal

The results from this study differs with the earlier reports of Wang *et al.* [6]; Liu *et al*. [9]; Yuan *et al.* (2013) and Asaf *et al.* [10,11].These differences might be due to differences in target region of the gene, genetic variation between the species and can also be due to small population size in the present study . However, the SNPs and haplotypes described in our study were novel and have not been reported till date.

4. CONCLUSION

In this study, the eight novel SNPs (154C>T, 235G>A, 252A>T, 265C>A, 268T>C, 282G>A, 431G>A and 551C>G) detected at MBL1 locus significantly associated with clinical mastitis in Murrah buffaloes the CC or TC genotype of 154C>T, GG of 235G>A, AT genotype of 252A>T, CC or AC genotype of 265C>A, TC or CC genotype of 268T>C, AG or GG genotype of 282G>A and AG or GG genotype of 431G>A loci had significantly lower incidence of clinical mastitis compare to their counter genotypes. Haplotype analysis showed that Hap6 (CGAATAAC) and Hap8 (CGTCCGGC) were significantly associated with a lower risk of clinical mastitis in Murrah buffaloes (P< 0.01). Thus, these haplotypes / SNPs at the MBL1 loci could be used as a candidate marker for selection of mastitis resistance Murrah buffaloes. However, further research is required to confirm this conclusion in large population.

CONSENT

Not applicable.

ETHICAL APPROVAL

Animal Ethic committee approval has been taken to carry out this study.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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