Original Article

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SINGLE NUCLEOTIDE POLYMORPHISM, HAPLOTYPES AND GENOTYPES OF MANNOSE-BINDING LECTIN (MBL1) GENE AND THEIR ASSOCIATION WITH CLINICAL MASTITIS IN MURRAH BUFFALO

Shahid Ahmad Shergojry[1,*], Archana Verma[2], Ishwar Dayal Gupta[2] and Nazir Ahmad Mir[1]

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ABSTRACT

The Mannose-binding lectin (MBL1) gene is an important component of immune response system. It plays vital role in activation of the complement system and act as chief defense molecule of host cell to provide protection against various diseases. In the recent study, six novel single nucleotide polymorphisms (SNPs), at 2689G>C, 2751A>G, 4822T>C, 4853A>G, 4855T>C, and 4978T>C resulted in 2 non synonymous type of change at 4853A>G, 4855T>C resulting in one amino acid substitution Ser150Gly of MBL1 protein and their association with clinical mastitis were investigated in two hundred (200) Murrah buffaloes. These SNPs in MBL1 were genotyped by using the polymerase chain reaction (PCR) and Deoxyribose nucleic acid (DNA) sequencing methods in order to reveal the association with clinical mastitis. SHEsis software tool was used for construction of haplotypes and Linkage disequilibrium analysis. Twenty one (21) haplotypes were constructed. Allelic association analysis showed that C allele of 2689 G>C, A allele of 2751 A>G, C allele of 4822 T>C, A allele of 4853 A>G, C allele of 4855 T>C, and C allele of 4978 T>C had significant association with increased risk of clinical mastitis in Murrah buffaloes ($P<0.05$). Murrah buffaloes with CG genotype of 2689 G>C, AG or GG genotype of 2751 A>G, CC genotype of 4822T>C, AG or GG genotype of 4853 A>G, TC or CC genotype of 4855 T>C and CC genotype of 4978 T>C loci had significantly lower incidence of clinical mastitis compared to their counter genotypes. Haplotype association analysis showed that Hap21 (TATTGA) was found significantly related to higher risk of clinical mastitis ($P<0.05$). Hap14 (TGTTGG) and Hap5 (CGCCCA) were found significantly associated with lower risk of clinical mastitis in Murrah buffalo ($P<0.05$).

Keywords: *Bubalus bubalis*, buffaloes, immumne, nucleotide, polymorphism, haplotypes

INTRODUCTION

Bovine mastitis is the most common production disease of cattle and buffalo that causes huge economical losses to dairy entrepreneurs and farmers. The present economic conditions demand that the individual dairy animal should not only

[1]Sher-e-Kashmir University of Agricultural Sciences and Technology of Kashmir, Kashmir, India, *E-mail: drshahid21uk@gmail.com

[2]National Dairy Research Institute, Haryana, India

be high producer but also profitable. So to meet this ever increasing demand animal researchers/ breeders have to put continuous efforts to find molecular markers responsible for udder health traits and develop disease resistant breeding programme on fast track basis. Deoxyribose nucleic acid (DNA) based polymorphism, commonly known as DNA markers can be used for genetic improvement through selection for favorable health traits such as disease resistance. Mastitis is one of most complicated production disease in dairy animals and has caused tremendous loss to the dairy production worldwide. Different strategies have been formulated to minimize the impact of this complex disease by utilizing the number of therapeutic and prophylactic agents. Although these treatments have very good clinical outcome, emergence of drug-resistant strains and antibiotic residues in milk have brought great concerns. Detection of immune related genes influencing clinical mastitis is another way to improve the mastitis resistance in dairy animals. Thus, today, researchers are more interested in identifying the molecular markers in immunity related genes that will provide a guideline for early selection of dairy animals for mastitis resistance breeding programs. Mannose-binding lectin genes play a key role in defense system of host towards the susceptibility/ resistance to many infectious diseases. Genes involved in the immune response in mammals are Mannose-binding lectins (MBL1and MBL2) genes. They are important constituent of immune response system of an organism, the primary role of these genes are to provide protection against various diseases and mastitis. (Eisen and Minchinton, 2003; Holmskov *et al*., 2003; Van de Wetering *et al*., 2004; Gjerstorff *et al*., 2004). Association of Single nucleotide Polymorphism of Mannose-binding lectin (MBL) genes has been found with mastitis in dairy animals (Wang *et al*., 2011; Shergojry *et al*., 2021; Shergojry *et al*., 2023. Studies on association of immunity related genes with occurrence of mastitis will provide a guideline for early selection in a dairy herd. To date, the corresponding studies in Indian dairy buffalo breeds haven't been reported widely. So, it is worthwhile to study the association between immunity related genes polymorphism and clinical mastitis in Murrah buffalo. Therefore, the objective of study was carried with the aim to identify the genetic polymorphism in the MBL1 gene and its association with clinical mastitis in Murrah buffaloes.

MATERIALS AND METHODS

Animals

The samples were collected from 200 Murrah buffaloes maintained at Animal Health Complex, National Dairy Research Institute, Karnal. The blood samples were collected with anticoagulant Acid Citrate Dextrose (ACD) and stored at -20ºC.

Genomic DNA extraction and PCR amplification

Genomic DNA was extracted with phenol chloroform protocol as described by Sambrook and Russsel (1989) with minor modifications was used for DNA isolation from samples. The quantity and quality of DNA were measured by a spectrophotometer at 260/280 nm, and the genome DNA was diluted to 100 ng/ml.

Primers were designed by using Primer3 software based on the 5223 bp sequence for MBL1 (NCBI GenBank AC_000185.1). Primers were designed to include intron-exon boundaries, in order to evaluate possible mutations in target

region. Primers were procured from M/s. Eurofins Genomics India Pvt. Ltd, Bangalore. The sequence of primers, their respective nucleotide numbers, target region and amplicon sizes are given in Table 1.

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 minutes, followed by 33 cycles with initial denaturation at 94°C for 30 seconds, primer specific annealing temperatures of 57.5°C for 30 seconds to specifically amplify Exon 2, 53°C for 30 seconds to specifically amplify Exon 5 respectively, extension at 72°C for 30 seconds followed by a final extension at 72°C for 5 minutes.

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 (Hall, 1999). 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.

Statistical analysis

The genotypic and allelic frequencies, polymorphism information content (PIC), heterozygosity (He), effective number of alleles (Ne) and Hardy–Weinberg equilibrium (HWE) were estimated by using Popgene software (Yeh *et al*., 1999). 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) (Shi and He, 2005). The associations between allele, haplotype of MBL1 gene and Clinical mastitis were analyzed through Chi-square test and relative risk for clinical mastitis susceptibility 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 $P<0.05$ was defined as statistically significant.

RESULTS AND DISCUSSIONS

SNP Identification

The genetic polymorphisms of MBL1 gene was detected by PCR-direct sequencing method. The six novel SNPs, 2689 G>C and 2751 A>G were detected in the in intron 3 region of MBL1, and 4822 T>C, 4853 A>G, 4855 T>C and 4978 T>C were detected in exon 5 coding region of MBL1 gene. SNPs, 2689 G>C and 2751 A>G, were found in the fragment of primer 2 amplification, SNPs 4822 T>C, 4853 A>G, 4855 T>C and 4978 T>C were in the fragments of primer 5, respectively. The sequencing results indicated that these six SNPs included five transitions, 2751 A>G2689 G>C and one transversions4822 T>C, 4853 A>G, 4855 T>C and 4978 T>C (Figure 1) and Table 2.

The sequences of *Bubalus bubalis* partial coding region of MBL1 gene, was submitted to the NCBI for which GenBank accession No. KM087783 was assigned. The nucleotide sequence was analyzed using BLAST programme and the raw sequence was annotated with *Bos taurus* MBL1 gene reference sequence. The multiple sequence alignment was performed using the ClustalW software programme. ExPAsy translate tools was used to translate coding sequences into amino acid sequence and the resulting amino

acid sequence was aligned with corresponding reference sequence.

Two of the 4 SNPs observed in exon 5 were non-synonymous type of changes at nucleotide positions 4853 A>G and 4855 T>C and resulted in one amino acid substitution from serine to glycine at 150th position of MBL1 protein Table 3. SIFT (Sorting Intolerant From Tolerant) software tool predicted the amino acid substitution to affect the protein function, however, in what way the function will be affected, needs to be explored further through proteomics studies.

However, Liu *et al*. (2011) reported three novel SNPs at g.-2194 A>C, g.-1446 T>C and g.-1330 G>A in 537 animals of Chinese Holstein, Luxi Yellow and Bohai Black cattle. Their results revealed that one SNP g.2686T>C in exon 2 was synonymous mutation [GCT (Ala)>GCC (Ala) at amino acid position 35], whereas g.2651 G>A was non-synonymous mutation resulting in change in amino acid GTT (Val)>ATT (Ile) at position 24 of MBL 1 gene. In a similar study, including 596 animals of these three cattle breeds, Wang *et al.* (2011) also reported three SNPs: g.855 G>A in intron1, g.2651 G>A and g.2686T>C in exon 2 of the MBL1 gene with same amino acid substitution i.e. p. Val24Ile. Yuan *et al.* (2013) identified three SNPs c.1252G>A in intron1, c.2534 G>A and c.2569 T>C in exon 2 in 404 animals of three Chinese native cattle breeds (Sanhe, Holstein and Simmental), c.2534 G>A resulting in non-synonymous mutation Valine (Val) to Isoleucine (Ile) amino acid replacement, p.Val24 (Ile) of the bovine MBL1 gene.

Asaf*et al.* (2014a) identified three genotypes AB, AA and BB in 112 bp fragment of targeted SNP, rs110326717 (g.2651 G>A) of MBL1gene in Vrindavanicrossbred (Holstein Friesian/ Brown Swiss/Jersey × Hariana) cattle. They reported polymorphism in affected as well as unaffected groups of cows but non-significant association with the clinical mastitis. In another study same authors identified two distinct genotypes GG and GA by *Apa*I-RFLP of 588 bp fragment and reported non-significant association between targeted SNP "rs109231409" with clinical mastitis in Vrindavani crossbred cattle (Asaf *et al*., 2014b).

Distributions of alleles and genotypes

The allelic and genotypic distributions of six SNPsidentified at 2689 G>C and 2751 A>G in intron 3 and 4822 T>C, 4853 A>G, 4855 T>C and 4978 T>C in exon 5 coding region of MBL1 gene in affected and unaffected animals are summarized in Table 4 and 5.

As for the SNP at 2689G>C locus the allele and genotype frequencies of the affected group (for allele C: 50.9% and G: 49.1%; for genotype, CC: 37.7 %, CG: 26.5% and GG: 35.8%) showed differences from those of the unaffected animals (for allele C: 47.3% and G: 52.7%; for genotype, CC: 23.8 %, CG: 46.9% and GG: 29.3%) the differences, however, not being statistically significant (for allele, P=0.51746; χ^2=0.41; but were significant for genotype: P<0.05, χ^2=7.24). The Chi-square analysis revealed that genotypic distribution of SNP at 2689 G>C locus in affected group was not in HWE (P<0.01; χ^2=11.78) but in an unaffected group the genotypic distribution was in HWE (P=0.4786; χ^2=0.50). For the SNP at 2751 A>G locus, the allele frequencies of the affected group (A: 72.2% and G: 27.8%) were significantly different from those of the unaffected group (A: 52.4% and G: 47.6%: P<0.01; χ^2=16.52). Whereas, the genotype frequencies in Affected group (AA: 57.7%, AG: 28.9% and GG: 13.4%) were significantly different from those of the unaffected group (AA: 33%, AG: 38.8% and GG: 28.2%,

P<0.01; χ^2=13.42). The genotypic distribution of SNP at 2751 A>G locus in affected group as well in unaffected were not in HWE (P<0.01; χ^2=7.68 in affected: P<0.05; χ^2=5.5 in unaffected). For the SNP at 4822 T>C locus the allele frequencies of the Affected group (C: 27.5% and T: 72.5%) were significantly different from those of the unaffected group (C: 54% and T: 46%, P<0.01; χ^2=29.08). Whereas, the genotype frequencies in affected group (CC: 10%, CT: 35% and TT: 55%) were significantly different from those of the unaffected group (CC: 40%, CT: 28 and TT: 32%; P<0.01; χ^2=24.85). The genotypic distribution of SNP at 4822 T>C locus in affected group was in HWE (P=0.2215; χ^2=1.49) but in Unaffected group the genotypic distribution was not in HWE (P< 0.01; χ^2=19.04). For the SNP at 4853 A>G locus, the allele frequencies of the affected group (A: 71% and G: 29%) were significantly different from those of the Unaffected group (A: 32.8% and G: 67.2%, P<0.01; χ2=58.42). Whereas, the genotype frequencies in Affected group (AA: 56.1%, AG: 29.9% and GG: 14%) were significantly different from those of the Unaffected group (AA: 11.8%, AG: 41.9% and GG: 46.3%, P<0.01; χ2=47.27). The genotypic distribution of SNP at 4853 A>G locus in Affected group was not in HWE (P<0.01; χ^2=7.99) but in an Unaffected group the genotypic distribution was in HWE (P=0.63893; χ^2=0.22).

For the SNP at 4855 T>C locus, the allele frequencies of the Affected group (C: 26.7% and T: 73.3%) were significantly different from those of the Unaffected group (C: 58.2% and T: 41.8%, P<0.01; χ^2=40.82). Whereas as the genotype frequencies in Affected group (CC: 16.5%, CT: 20.4% and TT: 63.1%) were significantly different from those of the Unaffected group (CC: 37.1%, CT: 42.3 and TT: 20.6%; P<0.01; χ^2=36.93), the genotypic distribution of SNP at 4855 T>C locus in affected group was not in HWE (P<0.01; χ^2=23.64) but in Unaffected group the genotypic distribution was in HWE (P=0.1970; χ^2=1.66).

For the SNP at 4978 T>C locus, the allele frequencies of the Affected group (C: 35.5% and T: 64.5%) were significantly different from those of the Unaffected group (C: 57.5% and T: 42.5%, P<0.01; χ^2=19.31). Whereas, the genotype frequencies in Affected group (CC: 9.7%, CT: 51.6% and TT: 38.7%) were significantly different from those of the unaffected group (CC: 36.4%, CT: 42.1% and TT: 21.5 %; P<0.01; χ^2=20.83). The genotypic distribution of SNP at 4978 T>C locus in affected as well as in an Unaffected group was in HWE (P=0.21974; χ^2=1.50 in Affected group; P=0.1486; χ^2=2.08 in Unaffected group).

The results of the χ2 analysis for 2689 G>C, 2751 A>G, 4822 T>C, 4853 A>G, 4855 T>C and 4978 T>C SNP loci in the overall Murrah population indicated that these genetic variants were not in the HWE (P<0.05). The PIC ranged from 0.3537 to 0.37636 indicated that there was moderate degree of polymorphism for these SNPs loci of MBL1 gene in Murrah population. The value of population genetic indices, including He, Ne, I, PIC and Chi-square value are showed in Table 6.

Liu *et al.* (2011) reported frequencies of A allele at locus g.-2194 A>C as 0.900, 0.7273 and 0.9375, C allele at locus -1446 T>C as 0.6098, 0.7841 and 0.6250, G allele at locus g.-1330 G>A as 0.7111, 0.8523 and 0.8958 and G allele at locus at g. 2651 G>A as 0.6130, 0.7614 and 0.7500 in Chinese Holstein, Luxi Yellow and Bohai Black cattle respectively. Wang *et al.* (2011) also reported G allele frequency at locus g.855 G>A as 0.87, 1.0 and 1.0, G allele frequency at locus g.2651 G>A as 0.58, 0.75 and 0.76 and C allele frequency at locus g.2686 T>C as 0.57, 0.74 and 0.63 in these three Chinese Holstein, Luxi Yellow and Bohai Black

breeds respectively.

Linkage disequilibrium analysis other than promoter region of MBL1

The pairwise LD between the six SNPs at 2689 G>C, 2751 A>G, 4822 T>C, 4853 A>G, 4855 T>C and 4978 T>C in the Murrah buffaloes were estimated using SHEsis software programme. The pairwise LD values measured by D‘ and r^2 values suggest that SNP loci visualized in figure as 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' between 0.80 and 0.75, and light red represent D' of<0.70. Our results indicated that the Murrah buffalo possessed moderate to strong LD between the SNPs loci of MBL1 gene in Murrah. The D' and r^2 values for the six SNPs are presented in Table 7 and Figure 2.

Haplotype distribution other than promoter region of MBL1 gene in Murrah buffalo

Six SNPs at 2689 G>C, 2751 A>G, 4822 T>C, 4853 A>G, 4855 T>C and 4978 T>C were used for haplotype construction, by using SHEsis software programme.

The haplotype analysis showed that 21 haplotypes were generated from six SNPs among the Affected and Unaffected group composed of 200 Murrah buffaloes. For convenience the 21 haplotypes were given haplotypes ID as Hap1 to Hap21 to simplify the term usage. Eleven haplotypes were rarer *i.e*., the frequencies of these 11 Haplotypes were less than 3% and were excluded from the association analysis. The frequencies of the remaining 10 haplotypes were compared in all mastitis affected and unaffected animals. The most common haplotype was Hap1 with frequency of 39% in affected and 33% in unaffected animals. The estimated haplotype frequencies for Hap14 (TGTTGG), Hap5 (CGCCCA), Hap13 (TGTTCG) and Hap12 (TGTCCG) were 28%, 22%, 5% and 4% respectively, which were observed only in unaffected animals. Whereas the Hap21 possessed the highest frequency (30%), followed by Hap19 (TATCGA), Hap18 (TACTCA), Hap17 (CATCGA) and Hap15 (CACCGA) with haplotype frequencies of 8%, 7%, 7% and 4% respectively, in mastitis affected animals (Table 8).

However, Liu *et al*. (2011) reported 28 different haplotypes constructed from the five SNPs loci of MBL1 gene in Chinese cattle breeds. They also observed that the H16 showed the highest haplotype frequencies (25.7%), whereas the haplotypes H18 and H26 were not observed in the population. Wang *et al*. (2011) reported 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 to H8, respectively. Their results revealed that among these eight haplotypes, H1 showed the highest and H8 had the lowest frequency

Association analysis

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

Six SNPs of MBL1 gene at 2689 G>C, 2751 A>G, 4822 T>C, 4853 A>G, 4855 T>C and 4978 T>C were analyzed for the association with clinical mastitis in Murrah buffalo. Statistically, significant association between all the six SNPs of the MBL1 gene and clinical mastitis in Murrah buffalo were detected by Chi-square tests. The relative risk of the alleles for clinical mastitis were estimated by SHEsis software for all the SNPs and represented in terms of odds ratio.

Allelic association analysis

An allelic association analysis showed that C allele of 2689 G>C, A allele of 2751 A>G, C allele of 4822 T>C, A allele of 4853 A>G, C allele of 4855 T>C, and C allele of 4978 T>C were more frequent in affected animals as compared to unaffected, with odds ratios of 1.157 for alleles of 2689 G>C (95% CI: 0.742~1.80), 2.35 for alleles of 2751 A>G (95% CI: 1.551~3.56), 0.323 for alleles of4822 T>C (95% CI: 0.212~0.490), 5.023 for alleles of 4853 A>G (95% CI: 3.28~7.687), 0.261 for alleles of 4855 T>C (95% CI: 0.171~0.397) and 0.406 for 4978 T>C (95% CI: 0.271~0.609) respectively. Association analysis indicated that these alleles are "at-risk" alleles and showed significant association with increased risk of clinical mastitis in Murrah buffaloes (P<0.01) (Table 2).

Genotypic association analysis

The Chi-square analysis revealed significant difference between affected and unaffected animals for genotypic distribution frequencies of the MBL1 gene. Genotypic association analysis showed that Murrah buffaloes with CG genotype of 2689G>C, AG or GG genotype of 2751A>G, CC genotype of 4822T>C, AG or GG genotype of 4853A>G, TC or CC genotype of 4855T>C and CC genotype of 4978T>C loci had significantly lower incidence of clinical mastitis compared to their counter genotypes (Table 3).

Wang *et al.* (2011) 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.855 G>A/G. 2686 T>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.2534 G>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.1252 G>A and c.2569 T>C loci were not found significant.

Haplotype association analysis

The haplotypes having frequency greater than 3% were used for association analysis. Association results for haplotypes analysis showed that Hap21, Hap19, Hap18, Hap17 and Hap15 represented only in affected animals and had a significant effect on increased risk for clinical mastitis in Murrah buffaloes (P<0.01). These haplotypes containing all the alleles which are "at-risk" alleles for clinical mastitis. On the other hand thehaplotypes, Hap14 (TGTTGG) and Hap5 (CGCCCA) observed only in unaffected animals were significantly associated with lower risk of clinical mastitis in Murrah buffalo (χ^2=37.23 and 27.34: P<0.01). In addition, haplotypes Hap12 and Hap13 was also found to be associated with lower risk of clinical mastitis (χ^2 =4.08 and 5.29: P<0.05). The haplotype association analysis revealed that haplotypes Hap14 (TGTTGG), Hap5 (CGCCCA), Hap12 (TGTCCG) and Hap13 (TGTTCG) played a significant protective role against clinical mastitis. Our results indicated that haplotype Hap14 (TGTTGG) and Hap 5 (CGCCCA) were significant and could act as favorable haplotypes against clinical mastitis in Murrah buffaloes (Table 6).

However, Liu *et al.* (2011) reported the association of haplotype combinations with SCS. They observed that the animals with the haplotype combinations H15H1 and H16H1 were lower SCS (P<0.05). Wang *et al.* (2011) reported that animals with haplotype combination H3H7 showed significantly lower SCS scores in comparison to

Table 1. Sequence of the primers for amplification of MBL1 gene in Murrah buffalo.

Primer code	5` Primer Sequence 3`		Target region	Amplicon size (bp)	TA (°C)
P2	F	GCAGAGGTGGTGGCAAAT	2380-2781 (Exon-2)	401	57.5
	R	GTCATCTTTAGAGAGAATGCCCC			
P3	F	GGCAGTTTCTTGGGGTCAAT	2941-3241 (Exon-3)	278	53
	R	CCACCTGGCCTGGAGTTC			
P4	F	TGGACGGAGGAGCGTAGTAG	3961-4261 (Exon-4)	281	53
	R	TGTAAGTTCTTTTCCACCCTTCC			
P5	F	CAACCCCAGATTCCCAGAG	4681-5221 (Exon-5)	509	53
	R	CAGAGCAGCAGGGAGACACT			

Table 2. Summary of nucleotide changes in MBL1 gene of Murrah buffalo.

S. No.	Position	Region	Ref. Murrah	Variant Murrah	Type of variation
1	2689	Intron 3	G	C	Transversion
2	2751		A	G	Transition
3	4822	Exon5	C	T	
4	4853		G	A	
5	4855		T	C	
6	4978		C	T	

Table 3. Summary of amino acid change in MBL1 gene of Murrah buffalo.

Change	Synonymous	Non-synonymous	Synonymous
Nucleotide change	TAC 4822 TAT	GGT 4853 AGC GGT 4855 AGC	ACC 4978 ACTT
Amino acid	Tyr 139 Tyr	Gly 150 Ser	Thr 191 Thr
Charge		Nonpolar > Polar	

Table 4. Allelic frequencies distribution of six SNPs of MBL1 gene and their association with clinical mastitis in Murrah buffaloes.

Locus	Allele frequency			Odds ratio	x^2 (1df)	P-value
	Allele	Affected (n=100)	Unaffected (n=100)			
2689	C	50.9	47.3	1.157 %95	0.41	P=0.51
G>C	G	49.1	52.7	CI=[0.742~1.80]		
2751	A	72.2	52.4	2.352 %95	16.52	P<0.01
A>G	G	27.8	47.6	CI=[1.551~3.56]		
4822	C	27.5	54.0	0.323116 %95	29.08	P<0.01
T>C	T	72.5	46.0	CI=[0.212~0.490]		
4853	A	71.0	32.8	5.023797 %95	58.42	P<0.01
A>G	G	29.0	67.2	CI=[3.28~7.687]		
4855	C	26.7	58.2	0.261091 %95	40.82	P<0.01
T>C	T	73.3	41.8	CI=[0.171~0.397]		
4978	C	35.5	57.5	0.406911 %95	19.31	P<0.01
T>C	T	64.5	42.5	CI=[0.271~0.609]		

Table 5. Genotypic frequencies distribution of six SNPs of MBL1 gene and their association with clinical mastitis in Murrah buffaloes.

Locus	Genotype frequency			HWE x^2 (1df); (P=6.63)		x^2 (2df); (P=9.21)	P-value
	Geno-type	Affected (n=100)	Unaffected(n=100)	Affected	Unaffected		
2689G>C	CC	37.7%	23.8%	11.78	0.50 P=0.4786	7.24	P=0.0267
	CG	26.5%	46.9%				
	GG	35.8%	29.3%				
2751 A>G	AA	57.7%	33.0%	7.68	5.05 P=0.024	13.42	P<0.01
	AG	28.9%	38.8%				
	GG	13.4%	28.2%				
4822 T>C	CC	10.0%	40.0%	1.49 P=0.22	19.04	24.85	P<0.01
	CT	35.0%	28.0%				
	TT	55.0%	32.0%				
4853 A>G	AA	56.1%	11.8%	7.99	0.22 P=0.63893	47.27	P<0.01
	AG	29.9%	41.9%				
	GG	14.0%	46.3%				
4855 T>C	CC	16.5%	37.1%	23.64	1.66 P=0.1970	36.93	P<0.01
	CT	20.4%	42.3%				
	TT	63.1%	20.6%				
4978 T>C	CC	9.7%	36.4%	1.50 P=0.219	2.08 P=0.1486	20.83	P<0.01
	CT	51.6%	42.1%				
	TT	38.7%	21.5%				

Table 6. Population genetic indices of MBL1 gene in Murrah buffaloes.

Locus	Ne	I	He	PIC	HWE test
2689 G>C	1.9976	0.6925	0.50105	0.37636	Disequilibrium (P<0.01)
2751 A>G	1.8911	0.6641	0.46471	0.3537	
4822 T>C	1.9338	0.6759	0.48410	0.36751	
4853 A<G	1.9916	0.6910	0.48410	0.36015	
4855 T>C	1.9501	0.6803	0.48842	0.36974	
4978 T<C	1.9940	0.6916	0.49945	0.37521	

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

Table 7. Pairwise D' and r^2 value of the six SNPs in MBL1 gene.

Locus	2689 G>C	2751 A>G	4822 T>C	4853 A>G	4855 T>C	4978 T>C
2689 G>C	-	0.321	0.879	0.962	0.707	0.888
2751 A>G	0.061	--	0.269	0.158	0.070	0.824
4822 T>C	0.734	0.046	--	0.886	0.916	0.903
4853 A>G	0.705	0.019	0.629	-	0.641	0.721
4855 T>C	0.353	0.004	0.654	0.365	-	0.378
4978 T>C	0.327	0.461	0.352	0.281	0.105	-

Note: D' is above the diagonal for SNPs and r^2 is below the diagonal.

Table 8. Haplotype frequencies distribution of affected and unaffected of MBL1 gene in Murrah buffaloes.

Hap id	Haplotype definition	Frequency		x^2	Fisher'svalue	Odds ratio [95%CI]
		Affected	Unaffected			
Hap1	CC C CC A	39%	33%	1.12	P=0.28	1.313 [0.793~2.173]
Hap2	C A C CC G	0%	0%			
Hap3	C A C C G G	0%	0%			
Hap4	C A T C G G	1%	0%			
Hap5	C G C CC A*	0%	22%	27.34	P<0.01	
Hap6	C G C CC G	0%	2%			
Hap7	C G C C G G	0%	0.5%			
Hap8	C G T C G G	0%	0.8%			
Hap9	C G T T G G	1%	2%			
Hap10	T G C CC A	0%	1.7%	3.52	P=0.19	
Hap11	T G C CC G	0%	1%			
Hap12	T G T C C G*	0%	4%	4.08	P<0.01	
Hap13	T G T T C G*	0%	5%	5.29	P<0.01	
Hap14	T G T T G G*	0%	28%	37.23	P<0.01	
Hap15	C A C C G A*	4%	0%	7.36	P<0.01	
Hap16	C A C T C A	2%	0%			
Hap17	C A T C G A*	7%	0%	13.67	P<0.01	
Hap18	T A C T C A*	7%	0%	13.67	P<0.01	
Hap19	T A T C G A*	8%	0%	14.80	P<0.01	
Hap20	T A T T C A	1%	0%			
Hap21	T A T T G A*	30%	0%	63.83	P<0.01	

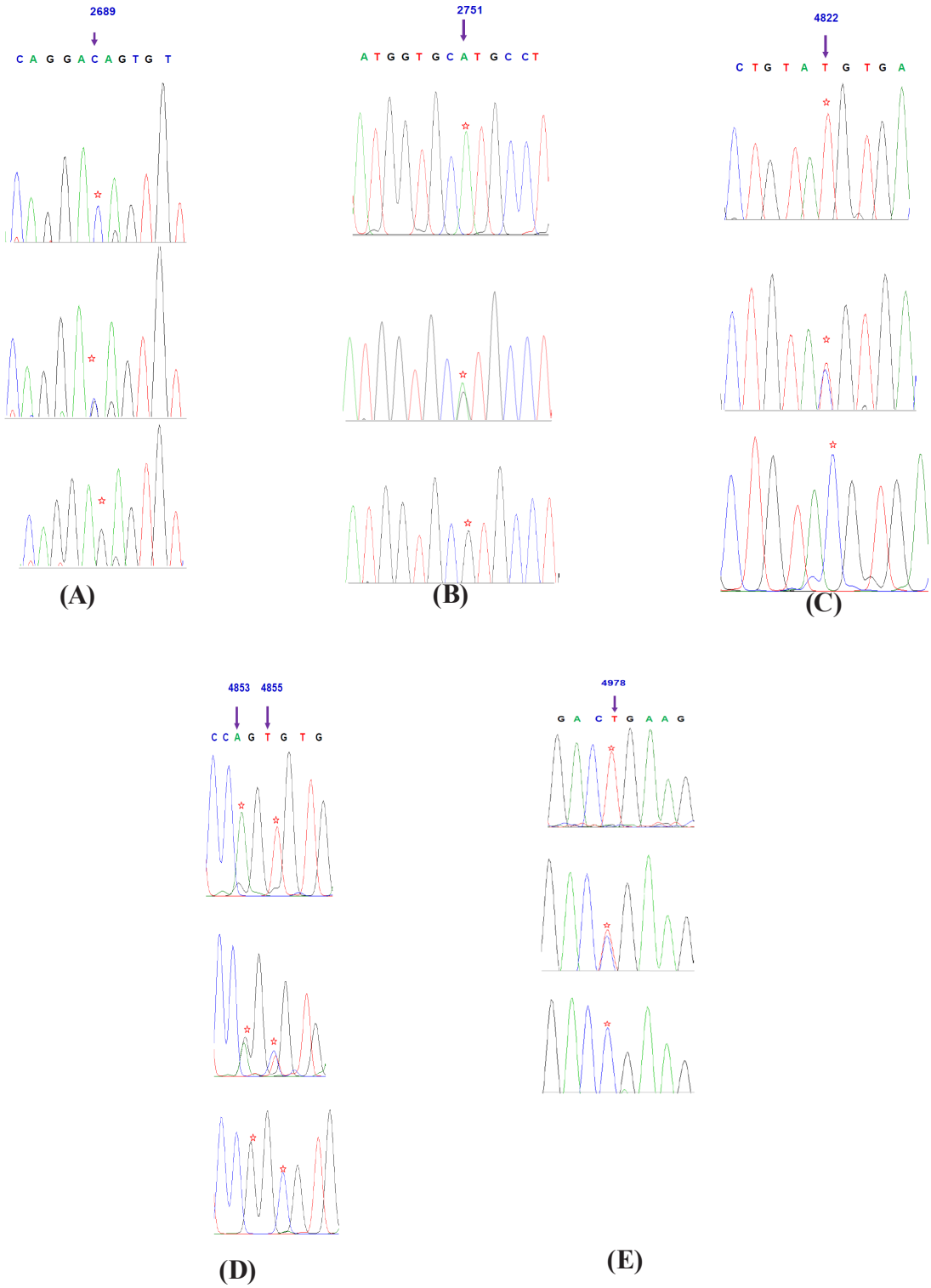

Figure 1. Chromatogram showing sequencing results at 2689 G>C and 2751 A>G in intron 3; 4822 T>C; 4853 A>G, 4855 T>C and 4978 T>Cin exon 5. Arrow and star indicates single nucleotide substitutions in intron 3 and exon 5 coding region of MBL1 gene.

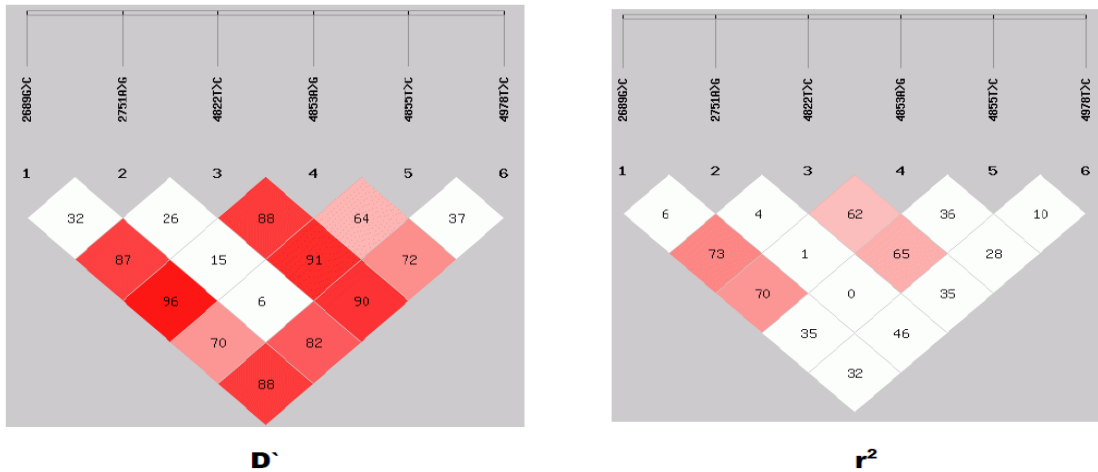

Figure 2. Pairwise D' and r^2 value of the six SNPs in MBL1 gene.

the haplotype combinations (P<0.05).

Our finding differs with the results reported by authors including (Wang *et al*., 2011; Liu *et al*., 2011; Yuan *et al*., 2013; Asaf *et al*., 2014a; Asaf *et al*., 2014b). Some of the reasons for these differences in our study may be due to differences in target region of the gene, due to genetic variation between the species and can also be due to the small population size in which study was conducted. However, the SNPs and haplotypes described in our study were novel and have not been reported till date.

CONCLUSION

In this study, the six novel SNPs (2689 G>C, 2751 A>G, 4822 T>C, 4853 A>G, 4855 T>C, and 4978 T>C) identified at MBL1 loci in Murrah buffaloes significantly associated with clinical mastitis Murrah buffaloes with CG genotype of 2689 G>C, AG or GG genotype of 2751 A>G, CC genotype of 4822 T>C, AG or GG genotype of 4853 A>G, TC or CC genotype of 4855 T>C and CC genotype of 4978 T>C loci had significantly lower incidence of clinical mastitis compared to their counter genotypes. Haplotype analysis showed that Hap14 (TGTTGG) and Hap5 (CGCCCA) were found significantly associated with lower risk of clinical mastitis in Murrah buffalo (P<0.01). Thus, these haplotypes / SNPs at the MBL1 loci could be used as a candidate marker for mastitis resistance selection in Murrah buffaloes. However, further research is required to confirm this conclusion in large population.

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