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Single nucleotide polymorphisms in *MBL1* gene of cattle and their association with milk production traits and somatic cell score

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ABSTRACT

Mannose-binding lectin (MBL), a pattern recognition molecule, is an important component of innate immune system. With multiple carbohydrate-recognition domains, it is able to bind to sugar groups present on the surfaces of a wide range of microorganisms and thereby provide first-line defence. *MBL* gene possibly contributes to bacterial infection resistance and was proposed as a molecular marker for milk production traits to control mastitis. A total of 100 cows (Hariana and Sahiwal) in milk were randomly selected and screened for the presence of one reported SNP (g.855G>A) in intron I and two SNPs (g.2686T>C and g.2651G>A) in exon 2 region of *MBL1* gene. A 588 bp PCR fragment encompassing the intron I as well as 401 and 162 bp of containing the exon 2 of *MBL1* gene were amplified and digested with *Apa*I, *Hae*III and *Sty*I, respectively. Genotype analysis using PCR-RFLP revealed a polymorphic banding pattern for g.855G>A and g.2686T>C while a monomorphic pattern for g.2651G>A. Association study of observed genotypes with milk production traits and somatic cell score (SCS) revealed a significant difference among g.2686T>C genotypes with age at first calving and SCS in Hariana cattle only. Although, the association study in present work was based on a relatively small sample size, further studies with large sample size are required to validate the impact of these SNPs.

Keywords: Hariana, Mastitis, *MBL1,* PCR-RFLP, Sahiwal, Somatic cell score, SNP

The total cattle population in India is 192.49 million, out of which indigenous cattle population is 142.11 million and exotic/crossbred cattle population is 50.42 million (20th Livestock Census 2019). India has a rich repository of well descript breeds of cattle with 50 registered native cattle breeds (NBAGR 2020). One of the best milch breed of cattle in India is Sahiwal, which has the capacity to give high quality milk on an average of 2,350 kg/lactation and Hariana is best dual purpose cattle breed of India producing on an average 1,700 kg of milk per lactation (Sharma *et al.* 2018). It is observed that the milk yield per head is low in India and to deal with the growing demand of milk and milk products due to constantly increasing human population of the country, it is essential to increase the production potential of indigenous livestock especially the cattle. Among the numerous logjams in accomplishing the milk production targets, disease occurrence continues to stay the most challenging obstacle. Bovine mastitis is an inflammatory and complex disease of the mammary gland most often caused by intramammary infections with environmental and/or contagious pathogenic bacteria (Chaneton *et al.* 2008, Verschoor *et al.* 2009). Previous reports has confirmed that mastitis is not only the most prevalent disease affecting the dairy industry, but also the most costly disease (Liu *et al.* 2011, Yuan *et al.* 2012), with economic losses attributed to decreased milk production and quality, increased labour due to treatment and herd management strategies, and premature culling of highly susceptible animals (Halasa *et al.* 2007, Verschoor *et al.* 2009). Due to high genetic correlation between mastitis and milk somatic cell count (SCC) or somatic cell score (SCS), SCC and SCS can serve as a surrogate trait for dairy mastitis resistance (Koivula *et al.* 2005, Tal-Stein *et al.* 2010, Yuan *et al.* 2011). The selection of lower SCC and SCS for dairy breeding strategy could reduce the occurrence of dairy mastitis (Yuan *et al.* 2011, 2012; Chu *et al.* 2012). In a way to mend this obstacle, genetic improvement through selection for mastitis resistance was explored for dairy cows primarily, then for dairy ewes and goats (Rupp and Boichard 2003). For integration of disease tolerance traits, it is crucial to get insights into the genetic traits underlying such diseases.

Mannose binding lectin (MBL), is a member of the collectin family of proteins and a constituent of innate

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immune system (Bouwman 2006). It is characterized by the presence of collagen-like domains and carbohydrate recognition lectin domains. MBL binds to high mannose and N-acetyl-glucosamine oligosaccharides, permits the protein to interact, with a wide selection of microorganisms coated with such sugars such as yeasts, bacteria and viruses and serves as the initiator of the lectin pathway of complement system (Neth *et al.* 2000). *MBL1* has been isolated from cattle (Kawai *et al.* 1997) and its mutations has been reported to contribute to the variation of animal susceptibility to different types of infections counting dairy mastitis also (Shi *et al.* 2004, Lillie *et al.* 2005, 2007; Capparelli *et al.* 2008, Wang *et al.* 2011, Liu *et al.* 2011). The bovine *MBL1* has been mapped to BTA28 (Gjerstorff *et al.* 2004) and contains five exons, encoding a 248 aa protein. Previous studies (Liu *et al.* 2010, 2011; Wang *et al.* 2011, Yuan *et al.* 2013) have reported that *MBL1* gene polymorphisms were associated with milk SCS in cattle. The bovine *MBL1* may be considered as a functional and positional candidate gene for resistance to mastitis. Though, relatively a smaller amount of information is obtainable about the bovine *MBL1* SNPs and its relationship with dairy mastitis resistance. The objective of the present study was to screen SNPs in *MBL1* gene of Hariana and Sahiwal cattle, and to analyse the association of these polymorphisms with milk production traits and SCS which reflects mastitis resistance in dairy cattle.

MATERIALS AND METHODS

Animals and sample collection: The experimental animals enrolled in the present study were maintained at the Instructional Livestock Farm Complex (ILFC) of the College of Veterinary Science and Animal Husbandry, Mathura. Blood samples were collected randomly from 50 Hariana and 50 Sahiwal cattle. Approximately 5 mL of blood was collected from the jugular vein in vacutainer tubes containing EDTA as an anticoagulant, and genomic DNA was isolated as per the standard phenol chloroform isolation protocol (Sambrook and Russell 2001). The quality and quantity of DNA was determined using a spectrophotometer, by measuring the optical density ratio at a wavelength of 260 and 280 nm.

PCR amplification: Reported set of specific primers (Wang *et al.* 2011) were used to amplify targeted genomic regions of *MBL1* gene for screening of g.855G>A point mutation in intron I and g.2686T>C as well as g.2651G>A mutations in exon 2. The sequence of primers, target region, amplicon size, name of restriction enzymes (RE) is given in Table 1. A total of 50–100 ng of template DNA was amplified in a total volume of 25 µL PCR mix in the thermocycler (Bio-Rad, USA). The PCR mix contained: 2.5 µL PCR dream buffer 10×, 2.5 µL of 2 mM dNTPs, 0.5 pM from each primer and 1 U *Taq* DNA polymerase. The thermal cycling was standardized at 94°C for 5 min for initial denaturation with 35 cycles of the following parameters: denaturation at 94°C for 30 sec for g.855G>A/ intron1 and g.2686T>C/exon2 while 94°C for 20 sec for g.2651G>A/exon2; annealing at 59, 62 and 53°C for 30 sec (Table 1), elongation at 72°C for 30 sec. The reaction was continued for a final extension at 72°C for 10 min.

PCR-RFLP assay of MBL1 gene: In the present study, genotype analysis of targeted SNPs, viz. g.855G>A, g.2686T>C and 2651G>A *of MBL1 gene* was carried out using the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method in Hariana and Sahiwal cattle. The amplicon of 588 bp obtained from intron I region specific primers was digested using restriction enzyme (RE) *Apa*I (New England Biolab). The restriction digestion was carried out at 25°C for 12–14 h. The amplified product of 401 bp and 162 bp obtained from two different set of exon 2 region specific primers was digested using RE *Hae*III (New England Biolab) and RE *Sty*I (New England Biolab), respectively and the digestion was carried out at 37°C for 12–14 h. A reaction mix of 15 mL volume containing 5 mL PCR products, 0.5 mL *Apa*I/ *Hae*III/*Sty*I (10U=mL) and 1.5 mL 10× RE buffer (specific for each RE) was prepared for each restriction digestion reaction. For genotyping, digested products were checked on 2.0% agarose gel in 1× TBE buffer. The fragments were visualized under UV light after staining with ethidium bromide.

Somatic cell count: Milk samples of the cattle and buffalo population under polymorphic study were collected and analysed using LACTOSCAN somatic cell counter based on florescent microscope technique of counting cells. Since the distribution frequency of somatic cell count is usually

Table 1. Primer sequences and RE used in PCR-RFLP assay for genotyping *MBL1* SNPs

SNP/location	Primer Sequence (5′→3′)	SAF (bp)	AT (°C)	RE	Possible genotypes
g.855G>A	F: CCCTTCCAACTCATTGCTTC	588	62	*Apa*I	GG: 311 and 277 bp
Intron I	R: AGTCCCAACCACCCTCA				GA: 588, 311 and 277 bp AA: 588 bp
g.2686T>C	F: GCAGAGGTGGTGGCAAATGT	401	59	*Hae*III	TT: 274 and 127 bp
					TC: 274, 184, 127 and 90 bp
					CC: 184, 127 and 90 bp
Exon 2	R: CATCTTTAGAGAGAATGCCCC				
g.2561G>A	F: GGTGGCAAATGTTGGCTA	162	53	*Sty*I	GG: 162 bp
Exon 2	R: GTCTTCTGAGCATCCTCCA				GA: 162, 141 and 21 bp
					A: 141 and 21 bp

SAF, size of amplification fragment; AT, annealing temperature; RE, restriction enzyme; bp, base pair.

skewed (Ali and Shook 1980), we calculated the SCC (cells/µl) based parameter SCS using the following equation: SCS = log2 (SCC/100) + 3 (Rupp and Boichard, 1999).

Statistical analysis: The genotypic and allelic frequencies were estimated by standard procedure (Falconer and Mackay 1996). Association study of genotyped animals with somatic cell count of milk and other milk production traits including age at first calving (AFC), lactation period (LP), total milk yield (TMY) and milk yield at 300 days (MY300) was carried out to analyse the effect of polymorphisms. Statistical analysis was carried out using the General Linear Model (GLM) using SPSS software (version 16.0). The following linear model was applied:

$$Y_{ij} = \mu + G_i + e_{ij}$$

where Y_{ij}, observed trait value in animal; µ, mean trait value; G_i, effect of genotype; e_{ij}, random error. Significant differences among least square means of different genotypes were calculated using Duncan's multiple-range test, and P values of 0.05 were considered statistically significant.

RESULTS AND DISCUSSION

To improve disease resistance, the selection criterion has been shifted from phenotypically expressed disease status to allelic status at the DNA level (Pal and Chakravarty 2020). The information utility from candidate genes in breeding programs has potential effect in accuracy of selection and increasing selection differences (Mandal *et al.* 2018). The use of DNA markers to define the genetic makeup (genotype) and foretell the performance of an animal is an influential support to animal breeding. There has been growing amount of interest in selection of disease resistance genotype/haplotype in the dairy animals (Hou *et al.* 2010). Moreover, association of identified genotypes with various economic traits and analysis of gene function can be used to determine which polymorphisms will be useful as markers for desirable traits. The present study screened reported genetic variants, viz. g.855G>A mutation in intron I and g.2686T>C as well as g.2651G>A mutations in exon 2 region of *MBL1* gene by PCR-RFLP and analysed their association with milk production traits and SCS in Hariana and Sahiwal breeds of cattle. In this study, PCR products of 855 bp containing g.855G>A mutation in intron I was digested with *Apa*I restriction enzyme and produced three types of genotypes, viz. GG (311 and 277 bp), GA (GG, 588, 311 and 277 bp) and AA (undigested; 588 bp) in all the screened animals (Fig. 1).

The chi square values, genotype and allele frequencies are presented in Table 2. Allele G (0.89 and 0.86) was more frequently present than allele A (0.11 and 0.14) in Hariana and Sahiwal cattle, respectively. Similarly, allele G was dominant as compared to allele A in Chinese Holstein (CH) cattle reported by Liu *et al.* (2010) and Wang *et al.* (2011) as well as in Vrindavani crossbred cattle (Holstein Friesian/Brown Swiss/Jersey × Haryana) studied by Asaf *et al.*

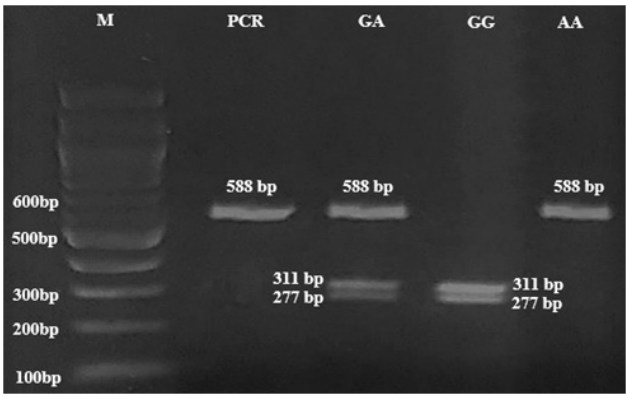

Fig. 1. RFLP products of *MBL1* intron I region digested with *Apa*I RE. Lane M: Marker (100 bp DNA ladder), Lane PCR: undigested PCR amplicon of 588 bp, Lane GA, GG and AA: RFLP products of size 588, 311 and 277 bp for genotype GA, 311 and 277 bp for genotype GG and 588 bp for genotype AA respectively.

(2014b), however, Asaf *et al.* (2014b) found only two genotypes (GG and GA) for this SNP and these variation might be due to the crossbreeding or selection history. Chi-square (χ^2) analysis revealed that the population of Hariana and Sahiwal cattle under study was in Hardy Weinberg equilibrium (HWE) (*P<0.05*) which were in agreement with the findings of Liu *et al*. (2010) and Wang *et al*. (2011) in CH cattle, while in contrast with Asaf *et al.* (2014b) in Vrindavani crossbred cattle. Though, intron sequences do not code for amino acids/proteins, they have been proved to play vital regulating parts in gene expression and its regulation (Nott *et al.* 2003), mRNA transcription and splicing (Zan *et al.* 2007).

For screening of the SNP g.2686T>C in exon 2 region of *MBL1* gene which is synonymous (g.2686T>C) with no amino acid change Ala35Ala in the N-terminus region (Wang *et al.* 2011), 401 bp amplified products were digested with *Hae*III restriction enzyme which produced all the three possible genotypes with banding pattern of 274 and 127 bp for genotype TT, 274, 184, 127 and 90 bp for TC genotype

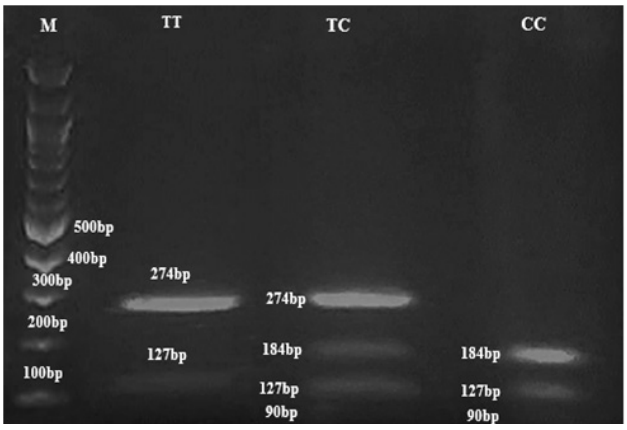

Fig. 2. RFLP products of exon 2 region of *MBL1* gene digested with *Hae*III RE. Lane M: Marker (100 bp DNA ladder), Lane TT, TC and CC: RFLP products of size 274, 127 bp for genotype TT, 274 and 184, 127 and 90 bp for genotype TC and 184, 127 and 90 bp for genotype CC, respectively.

Table 2. Genotype and allele frequencies and chi square values of SNPs (g.855G>A, g.2686T>C and 5.2651G>A) found in Hariana and Sahiwal cattle *MBL1* gene

Breed	g.855G>A						g.2686T>C						g.2651G>A					
	Genotype frequency/ sample size			Allele frequency		Chi square (χ^2)	Genotype frequency/ sample size			Allele frequency		Chi square (χ^2) 3.89 (p>0.01)	Genotype frequency/ sample size			Allele frequency		
	GG	GA	AA	G	A		TT	TC	CC	T	C		GG	GA	AA	G	A	
Hariana (N=50)	80.0 (n=40)	18.0 (n=9)	2.0 (n=1)	0.89	0.11	0.32 (*P<0.05*)	32.0 (n=16)	14.0 (n=7)	54.0 (n=27)	0.39	0.61	24.91 (*P>0.05*)	100.0 (n=50)	0.0	0.0	1.0	0.0	
Sahiwal (N=50)	74.0 (n=37)	24.0 (n=12)	2.0 (n=1)	0.86	0.14	0.004 (*P<0.05*)	36.0 (n=18)	12.0 (n=6)	52.0 (n=26)	0.42	0.58	25.12 (*P>0.05*)	100.0 (n=50)	0.0	0.0	1.0	0.0	
Total (N=100)	77.0 (n=77)	21.0 (n=21)	2.0 (n=2)	0.88	0.12	0.23 (*P<0.05*)	34.0 (n=34)	13.0 (n=13)	53.0 (n=53)	0.40	0.60	54.53 (*P>0.05*)	100.0 (n=100)	0.0	0.0	1.0	0.0	

N, Sample size; n, Number of animals of particular breed and genotype.

and 184, 127 and 90 bp for genotype CC in all the screened animals (Fig. 2). The chi square values, genotypic and allelic frequencies were calculated and presented in Table 2. The allele C (0.61 and 0.58) was more frequent as compared to allele T (0.39 and 0.42) in studied Hariana and Sahiwal cattle population, respectively. All the three genotype of SNP g.2686T>C were found in CH cattle by Liu *et al.* (2010), in CH, LY and BB cattle breeds by Wang *et al.* (2011) and Liu *et al.* (2011) as well as in Sahiwal cattle by Kamaldeep *et al.* (2017b).

Additionally, all these researchers found allele C as dominant when compared to allele T which was in agreement with the present findings. However, CC genotype was highly frequent in screened Hariana and Sahiwal cattle of present study which was not in agreement with the findings of Kamaldeep *et al.* (2017b) in Sahiwal cattle where the value of TC genotype was highest and these variations in the frequency of different genotypes might be due to the geographical distribution, population size and selection history. The population of Hariana and Sahiwal cattle under study was not in HW-equilibrium (*P>0.05*) for g.2686T>C mutation which is in agreement with the reports of Liu *et al*. (2010) in CH cattle, Wang *et al*. (2011) and Liu *et al*. (2011) in LY and CH cattle. On the contrary, BB cattle by Wang *et al*. (2011) and Liu *et al*. (2011), and Sahiwal cattle by Kamaldeep *et al*. (2017a, 2017b) were found in HW equilibrium and this difference might be due to the variation in sample size.

The other exonic SNP (2651G>A) which is a non-synonymous mutation causing substitution of Val24Ile in N-terminus region of bovine MBL1 was screened. The amplified products of 162 bp were digested with *Sty*I restriction enzyme and produced monomorphic pattern with only one genotype GG having undigested product of 162 bp (Fig. 3). However, several authors (Wang *et al.* 2011,

Fig. 3. RFLP products of exon 2 region of *MBL1* gene digested with *Sty*I RE showing monomorphic pattern. Lane PCR: Undigested PCR product (162 bp), M: Marker (100 bp DNA ladder), Lane 1–3: GG genotype (uncut band; 162 bp).

Table 3. Association studies of *Apa*I/g.855G>A genotypes with milk production traits in Hariana and Sahiwal cattle

Breed	Lactation	Genotype	AFC (days)	TMY (litres)	LP (days)	MY300 (litres)
Hariana	I (N=50)	GG (40)	2140.0±61.4 (40)	1380.0±114.0 (40)	340.0±15.3 (40)	1218.0±81.1 (40)
		GA (9)	2060.0±259.0 (9)	1610.0±302.0 (9)	321.0±13.3 (9)	1470.0±192.0 (9)
		AA (1)*	2270 (1)	1355 (1)	285 (1)	1355 (1)
	II (N=44)	GG (36)	–	1630.0±164.0 (36)	326.0±12.4 (36)	1435.0±130.0 (36)
		GA (7)	–	1850.0±316.0 (7)	315.0±17.5 (7)	1770.0±261.0 (7)
		AA (1)*	–	1460 (1)	352 (1)	1240 (1)
Sahiwal	I (N=50)	GG (37)	1821.0±155.0 (37)	1832.0±149.0 (36)	376.0±21.2 (36)	1435.0±61.1 (23)
		GA (12)	1800.0±150.0 (24)	1910.0±304.0 (23)	338.0±45.5 (23)	1570.0±118.0 (23)
		AA (1)*	1750 (1)	2660 (1)	418 (1)	1910 (1)
	II (N=46)	GG (34)	–	1855.0±153.0 (34)	372.0±20.2 (34)	1469.0±63.1 (34)
		GA (11)	–	1940.0±309.0 (11)	351.0±51.0 (11)	1609.0±121.0 (11)
		AA (1)*	–	2831 (1)	433 (1)	2100 (1)

AFC, Age at first calving; TMY, total milk yield; LP, lactation period; MY300, Milk yield upon 300 days. *Only one AA genotype was present in screened population so it has not been included in association analysis.

Table 4. Association studies of *Hae*III/g.2686T>C genotypes with milk production traits in Hariana and Sahiwal cattle

Breed	Lactation	Genotype	AFC (days)	TMY (liters)	LP (days)	MY300 (litres)
Hariana	I (N=49)	TT (16)	1930.0±85.9[a] (16)	1570.0±124.0 (13)	352.0±12.0 (13)	1340.0±106.0 (13)
		TC (6)	2570.0±261.0[b] (6)	1250.0±154.0 (5)	329.0±16.4 (5)	1109.0±131.2 (5)
		CC (27)	2160.0±53.9[ab] (27)	1210.0±136.0 (23)	327.0±20.8 (23)	1040.0±89.8 (23)
	II (N=36)	TT (12)	–	1770.0±177.0 (12)	330.0±18.4 (12)	1580.0±152.0 (12)
		TC (4)	–	1810.0±223.0 (4)	314.0±12.4 (4)	1710.0±167.0 (4)
		CC (20)	–	1650.0±185.0 (18)	320.0±23.1 (18)	1430.0±145.0 (18)
Sahiwal	I (N=50)	TT (18)	1770.0±76.0 (18)	2000.0±246.0 (18)	396.0±23.2 (18)	1460.0±123.0 (18)
		TC (6)	2140.0±18.8 (6)	1640.0±89.3 (5)	299.0±36.9 (5)	1580.0±46.2 (5)
		CC (26)	1750.0±110.0 (26)	1860.0±289.0 (25)	317.0±18.6 (25)	1510.0±78.3 (25)
	II (N=42)	TT (15)	–	2142.0±103.0 (15)	385.0±25.2 (15)	1541.0±109.0 (15)
		TC (4)	–	1731.0±92.3 (4)	325.0±32.8 (4)	1482.0±48.3 (4)
		CC (23)	–	1905.0±262.0 (23)	331.0±19.2 (23)	1685.0±81.2 (23)

AFC, Age at first calving; TMY, total milk yield; LP, lactation period; MY300, Milk yield upon 300 days. *Only one AA genotype was present in screened population so it has not been included in association analysis.

Table 5. Association studies of *MBL1* SNPs with SCS in Hariana and Sahiwal cattle

SNP	Genotype	Hariana cattle	Sahiwal cattle
g.855G>A	GG	675.0±223.0 (38)	747.0±362.0 (36)
	GA	630.0±236.0 (9)	1140.0±668.0 (10)
	AA*	90.3 (1)	554.0 (1)
g.2686T>C	TT	841.0±295.0[ab] (15)	401.0±79.6 (17)
	TC	1480.0±505.0[b] (7)	822.0±249.0 (6)
	CC	170.0±51.5[a] (26)	1190.0±648.0 (24)

*Only one AA genotype was present in screened population so it has not been included in association analysis.

Liu *et al.* 2011 and Asaf *et al.* 2014a) found polymorphic pattern for this SNP using different methods such as CRS-PCR and allele specific PCR, respectively.

Results of association study of two SNPs, g.855G>A and g.2686T>C of MBL1 gene with milk production traits including age at first calving (AFC), lactation period (LP), total milk yield (TMY) and milk yield at 300 days (MY300) as well as SCS are summarized in Tables 3, 4 and 5, respectively. The association study of g.855G>A with milk production traits and SCS revealed no significant difference ($P>0.05$) among genotypes which was in agreement with the findings of other researchers including Liu *et al.* (2010), Wang *et al.* (2011), Liu *et al.* (2011), Asaf *et al.* (2014b), Kamaldeep *et al.* (2017b). However, in case of g.2686T>C mutation, a significant difference ($P<0.05$) was observed for AFC and SCS in Hariana cattle where TC genotype was significantly higher (2570.0±261.0 days) than TT genotype (1930.0±85.9 days) for AFC and genotype TC was significantly higher (1480.0±505.0) than genotype CC (170.0±51.5) for SCS. Moreover, Wang *et al.* (2011), Liu *et al.* (2011) interestingly, found a significant difference between combined genotypes of the three SNPs (g.855G>A, g.2651G>A and g.2686T>C) and milk production traits, suggesting that the genotype of one SNP may be influenced by other SNPs. Therefore, the analysis of haplotype combination for marker assisted selection will be more valuable than the study of a single SNP which would be in consistent with the conclusion drawn in human SNP and inheritance studies by Fallin *et al.* (2001). Moreover, Yuan *et al.* (2013) observed no significant correlation between c.1252G>A and c.2569T>C and milk SCS in the analyzed cattle populations. However, a significant correlation with milk SCS was detected in c.2534G>A.

In present study, value of SCS for different genotype had very less difference hence it not be predicted that which genotype is favourable or susceptible to mastitis. *MBL1* is one of the most important constituent of the innate immune system. Several studies have reported that *MBL1* gene polymorphisms were associated with milk somatic cell score in cattle. The present study was carried out on best indigenous milch breeds (Hariana and Sahiwal) of cattle. PCR-RFLP was performed to genotype reported SNPs with the purpose to explore underlying genomic variation in indigenous germplasm. Two SNPs, viz. g.855G>A, g.2686T>C were found polymorphic with all the three possible genotypes and g.2651G>A SNP was found monomorphic in all the screened samples. Association study with milk production traits and SCS revealed a significant ($P<0.05$) difference among g.2686T>C genotypes for age at first calving and somatic cell score in Hariana cattle only. As the association study was conducted on relatively small sample size. Thus, the association of the targeted locus need to be explored further on large sample size to check their impact on milk production traits and mastitis resistance.

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