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SNP discovery and association of S100A8 allelic variants with incidence of clinical mastitis in Sahiwal and Karan Fries cattle

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Abstract
The present study was carried out to identify single nucleotide polymorphisms (SNPs) in bovine S100A8 gene and associate novel allelic variants with mastitis incidence in Sahiwal and Karan Fries cattle. A panel of unrelated cattle was subjected to targeted sequencing to detect a total of 23 novel SNPs within S100A8 gene. A novel PCR-RFLP assay (HpyCH4V) was developed to genotype -2 (A/G) SNP locus in 148 Karan Fries cattle and 147 Sahiwal cattle. The genotype and allele frequencies were calculated for each of the four groups of animals viz. affected Sahiwal cattle, unaffected Sahiwal cattle, affected Karan Fries cattle, unaffected Karan Fries cattle. The overall frequency of GG, GA and AA genotypes were 0.884, 0.102 and 0.013 respectively in Sahiwal cattle while it was 0.973, 0.02 and 0.007 respectively in Karan Fries cattle. No significant association of S100A8 genotypes with incidence of clinical mastitis (P>0.05) was observed.in investigated cattle breeds.

Keywords: S100A8, SNPs, PCR-RFLP assay, association study, mastitis incidence

Introduction
Mastitis is inflammation of mammary gland and is one of the most common diseases affecting dairy cattle causing huge economic losses to farmers [1, 2]. It is caused by introduction and multiplication of pathogenic microorganisms such as *Staphylococcus aureus* and *Streptococcus agalactiae* in the udder. Costs due to clinical mastitis include veterinary and treatment costs, reduced milk production in the remaining lactation period, decreased milk quality, early culling and potential negative impact on productive life of cows. Further, public health issues arising out of increased risk of antimicrobial residues in milk and emergence of resistant bacteria is a cause for concern [3]. The prevention and treatment of mastitis represent a serious burden to producers and are primary concerns for the dairy industry. Despite the preventive and control measures, the incidence of mastitis continues to be one of the highest of all cattle diseases. The annual loss due to clinical and subclinical mastitis in Indian dairy cattle was estimated to be USD226 and USD586, respectively [4].
Selective breeding of dairy cattle for reduced susceptibility/increased resistance rates against mastitis is difficult as it is a polygenic trait with very low heritability [5]. Mastitis resistance in cattle is influenced by many genes that are involved in various processes of host immune system including response to infection, inflammation and recovery of udder health [6]. Nevertheless, breeding cattle through indirect selection approaches such as somatic cell count and candidate gene markers can help reducing the incidence of mastitis in the long run. Identifying candidate genes affecting mastitis resistance and association of allelic variants with somatic cell score or mastitis incidence will provide novel strategies to improve udder health and quality of dairy cow populations. A fair amount of genetic research related to udder health has been performed and many fine mapping experiments have resulted in identification of several QTLs in cattle affecting milk production and udder health [7] [8] [9]. These QTLs cover large chromosomal regions involving hundreds or thousands of genes. Several chromosomal regions have been linked to marked effects on mastitis resistance. However, beyond fine mapping, the ultimate target of QTL analysis is the identification of causal gene itself which facilitate identification of resistance genes and alleles [10].
Ogorevc *et al*., (2009) [9] developed a database on different candidate gene loci in mammary gland development, milk production and resistance or susceptibility to mastitis.

S100A8 (calgranulin A), a member of the S100 family of Ca2+ binding proteins is one of the important candidate genes related to mastitis resistance in cattle. S100A8 and S100A9 together form a heterodimer called calprotectin which has antimicrobial properties and plays an important role in innate immunity. S100A8 has also been reported to be differentially expressed with respect to mastitis in Holstein cattle [11]. The present study was undertaken with the following objectives: (i) targeted sequencing of S100A8 gene in a panel of unrelated Indian cattle (*Bos indicus* and *Bos indicus X Bos taurus crossbreds*) for the detection of novel single nucleotide polymorphisms and (ii) association of novel S100A8 allelic variants with mastitis incidence in Indian cattle.

Materials and Method

Sampling and DNA extraction

Blood samples were collected from 147 Sahiwal and 148 Karan Fries cattle maintained at Cattle Farm, National Dairy Research Institute (NDRI), Karnal. About 10 ml of blood was collected in a sterile 0.5% EDTA (10 µl/ml of blood) coated vacutainers by jugular venipuncture method. The collected blood was stored in the refrigerator at 4 ˚C till further processing for extraction of DNA. DNA was isolated from blood using standard phenol-chloroform method as described in Sambrook and Russell (2001) [12] with minor modifications. The quality of DNA was checked by comparing the bands with reference DNA in 0.8% agarose gel electrophoresis and the quantity was determined using Nanodrop.

Screening unrelated panel of cattle to detect novel SNPs

S100A8 has been reported to be differentially expressed with respect to mastitis in Holstein cattle [11]. Hence, the 5' flanking region of S100A8 gene was selected in order to identify single nucleotide polymorphisms (SNPs) that have potential role in altering the expression of S100A8 gene through possible differences in the potential regulatory region. A panel of unrelated animals from three breeds of cattle viz. Sahiwal, Tharparakar and Karan Fries cattle were screened to detect novel single nucleotide polymorphisms. Four animals from each of the above mentioned three breeds of cattle were selected in such a way that they are unrelated without any common ancestor. Three fragments of S100A8 gene viz. S100A8-I, S100A8-II and S100A8-III were PCR amplified [13] and subjected to sequencing from one end. The sequences were edited and subjected to secondary peak calling using Codon Code Aligner and subsequently for multiple alignments for SNP detection.

Development of PCR-RFLP genotyping assay to type -2 (A/G) SNP locus

The newly identified SNP (-2 A/G) within S100A8 fragment-III was genotyped in a large panel of animals for association of S100A8 allelic variants with incidence of mastitis in Sahiwal and Karan Fries cattle. Considering the significance of its location near transcription initiation site, the SNP locus at position -2 bp upstream to start codon was selected for association study. The complete sequence of S100A8 gene fragment-III was subjected to NEB cutter 1.0 analysis to identify the overlapping restriction site for designing a PCR-RFLP (Polymerase Chain Reaction-Restriction Fragment Length Polymorphism) based genotyping assay. The results of NEB cutter analysis revealed the location of -2 (G/A) SNP locus within the restriction site of type III enzyme HpyCH4V (Figure 1). The presence of two additional sites for the enzyme HpyCH4V within S100A8 fragment-III, resulted in the availability of three restriction sites for allele G and two for allele A.

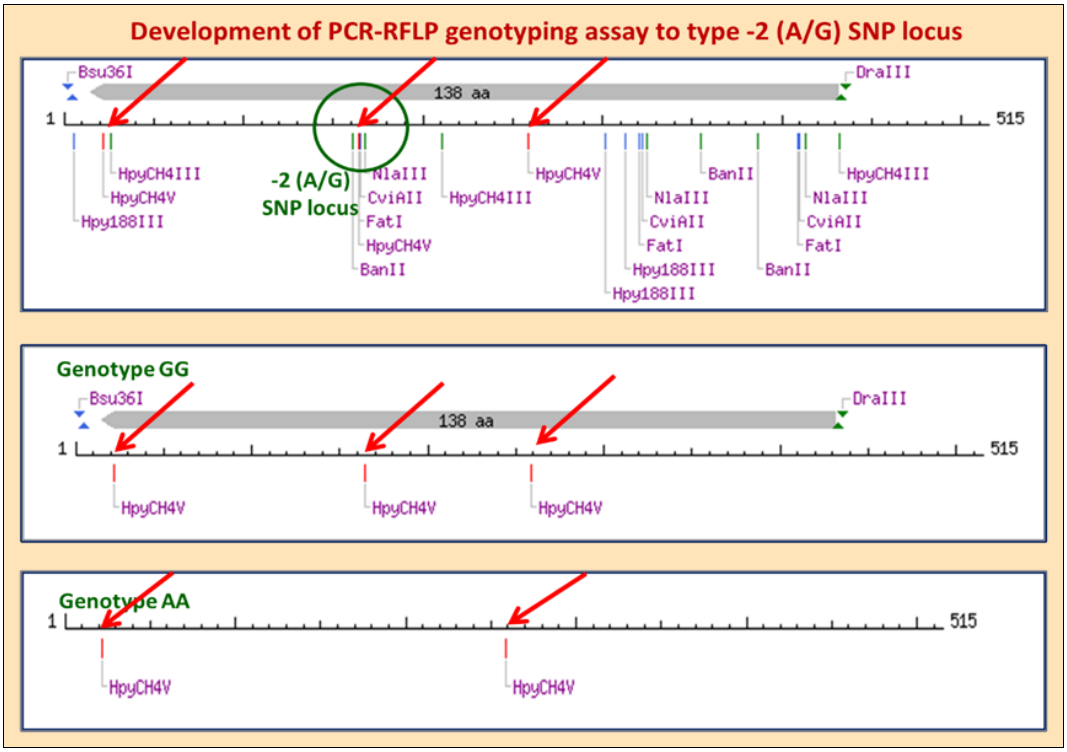

Fig 1: Results of NEB cutter analysis to develop PCR-RFLP genotyping assay for typing S100A8 -2 (A/G) SNP locus

Thus, GG genotype was expected to produce four fragments (255bp, 142bp, 95bp, 23bp) after restriction digestion while AA genotypes were expected to produce three fragments (255bp, 237bp, 23bp). The heterozygous genotypes were expected to produce five fragments (255bp, 237bp, 142bp, 95bp, 23bp) due to the presence of three restriction sites for

HpyCH4V in one chromosome and two in the homologus chromosome. For the PCR-RFLP genotyping assay, the PCR primer pair (S100A8-III) used for sequence characterization was utilized. The restriction digestion reaction was carried out in a final reaction volume of 20 µl containing 6 µl of PCR amplicon, 0.2 µl of restriction enzyme, 2 µl of buffer and 11.8 µl of water. The reaction mixture was kept for incubation at 37 ^{0}C temperature for 14-16 hours. The restriction digested fragments were resolved on 2.5% agarose gels stained with ethidium bromide. Photographs were taken using gel documentation system to score the gel manually for different genotypes.

Association of S100A8 allelic variants with mastitis

Data on incidence of clinical mastitis were collected from treatment registers for Sahiwal and Karan Fries cattle from National Dairy Research Institute, Karnal cattle farm. The animals treated once or more than once were classified under affected category irrespective of the repeated number of treatments. The genotype and allele frequencies were calculated for each of the four groups of animals viz. affected Sahiwal cattle, Unaffected Sahiwal cattle, Affected Karan Fries cattle, Unaffected Karan Fries cattle. The observed and expected genotypic frequencies were calculated for each of the Sahiwal and Karan Fries cattle and utilized for chi-square test of association between genotypes and mastitis incidence. The chi-square statistic was calculated following Snedecor and Cochran (1989) [14]. The calculated chi-square value was compared to the table P-value at two (3-1) degrees of freedom to ascertain the significance of association between genotype frequency and mastitis incidence in cattle.

Results and Discussion

Identification of allelic variants within S100A8 gene

SNP variations within S100A8 fragment-I: A total of 11 S100A8 fragment-I sequences covering ~350 bp of 5' flanking region were generated from four Karan Fries cattle, four Sahiwal cattle and three Tharparkar cattle and are available at NCBI-GenBank accession numbers JX312843-JX312853. A total of 12 single nucleotide polymorphic sites was observed within S100A8 fragment-I. Apart from this 12 SNPs, deletion of a stretch of 14 nucleotides was observed in Sahiwal and Tharparkar cattle. This deletion mutation was found to be absent both in *Bos taurus* cattle and *Bos taurus X Bos indicus* (Karan Fries) cattle. However, only four out of seven *Bos indicus* cattle (Sahiwal and Tharparkar) were found to possess this stretch of deletions in the 5' flanking region of S100A8 gene. Five Guanidine-Adenine (G-A) nucleotide variations were observed at positions -908, -889, -711, -644 and -573 bases upstream to start codon. Cytidine-Thymidine (C-T) mutation was found at seven polymorphic sites at positions -864, -814, -709, -690, -645, -597 and -583 bases upstream to start codon. Among the 12 SNPs, only one at position -889 showed all the three genotypes viz. AA, AG and GG.

SNP variations within S100A8 fragment-II: A total of ten S100A8 fragment-II sequences covering ~415bp of 5' flanking region were generated from four Sahiwal cattle and three each from Karan Fries and Tharparkar cattle (NCBI-GenBank accession numbers JX312833-JX312842). A total of four SNP variations were observed within the fragment viz. Cytidine-Thymidine (C-T) mutation at position -547, Adenine-Guanidine mutation at position -347, Thymidine-Cytidine mutation at position -301 and Guanidine-Adenine mutation at position -282. Among the four newly identified SNPs, SNP locus at position -347 showed all the three genotypes viz. AA, GG and A/G while the other three SNP loci showed the presence of only homozygotes in the studied animals. SNP at position -510 was found to be within the putative site for Elk-1 factor while none of the other SNPs were found to be within the putative regulatory regions.

SNP variations within S100A8 fragment-III: Eleven nucleotide sequence fragments of ~430bp covering 5' flanking region and partial exon1 of S100A8 gene were generated and are available at NCBI-GenBank accession numbers JX312854-JX312864. A total of seven novel SNP loci were detected at positions -2, +19, +81, +274, +289, +332 and +333 from start codon. Among these SNP loci, all the three genotypes were observed at positions -2 (GG, AA, AG), +81 (TT, CC, TC), +274 (GG, AA, AG) and +289 (CC, TT, CT). SNP locus at position +19 showed GG homozygotes and AG heterozygote while SNP at position +332 and +333 showed only homozygotes. SNP at position +19 was found to be within exon 1 region that also forms part of 5' untranslated region (5'UTR). Five SNP loci viz. at positions +81, +274, +289, +332 and +333 were found to be within intron 1 region.

Association of S100A8 allelic variants with incidence of mastitis in cattle

PCR-RFLP genotyping of 148 Karan Fries cattle and 147 Sahiwal cattle at -2 (A/G) SNP locus was performed. The representative gel picture of agarose gel electrophoresis of restriction digested PCR products is presented in Figure 2. The gels were scored manually and frequencies of genotypes and alleles within Karan Fries and Sahiwal cattle were estimated. The overall frequency of GG, GA and AA genotypes were 0.884, 0.102 and 0.013 respectively in Sahiwal cattle while it was 0.973, 0.02 and 0.007 respectively in Karan Fries cattle. In both breeds of cattle, frequency of GG was highest and the AA was the lowest. The frequency of heterozygous cattle was found to be higher (10.2%) in Sahiwal cattle as compared to that of Karan Fries cattle (2%). The estimated overall frequency of allele "G" and "A" in Sahiwal cattle was 0.94 and 0.06 while it was 0.98 and 0.02 in case of Karan Fries cattle. The present study revealed that the locus S100A8, -2 (A/G) was polymorphic with minor allele frequency ranging between 2% and 6% in Indian cattle.

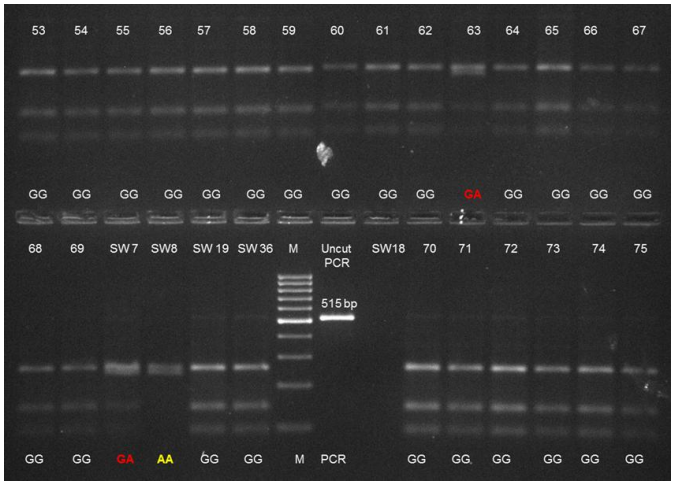

Fig 2: Restriction digested PCR products of S100A8 showing GG, AA and GA genotypes.

Further, to carry out association of observed S100A8 allelic variants with incidence of clinical mastitis in cattle, the samples under investigation were classified into mastitis affected and unaffected animals based on the records maintained at National Dairy Research Institute, cattle yard, Karnal. Among 147 Sahiwal cattle included in the present study, 88 were affected by clinical mastitis at least once during their productive life period, accounting for 59.9%. In case of Karan Fries cattle, 82 out of 148 animals were affected by clinical mastitis at least once during their productive life accounting for 57.4%. This is contrary to the general belief that indigenous Sahiwal cattle are relatively more resistant to mastitis than Karan Fries cattle.

The frequency of "G" and "A" alleles in mastitis affected Sahiwal cattle was 0.982 and 0.018 respectively while it was 0.985 and 0.015 in mastitis unaffected Sahiwal cattle respectively (Figure 3).The frequency of "G" and "A" alleles in mastitis affected Karan Fries cattle was 0.920 and 0.080 while it was 0.958 and 0.042 in mastitis unaffected Karan Fries cattle, respectively.

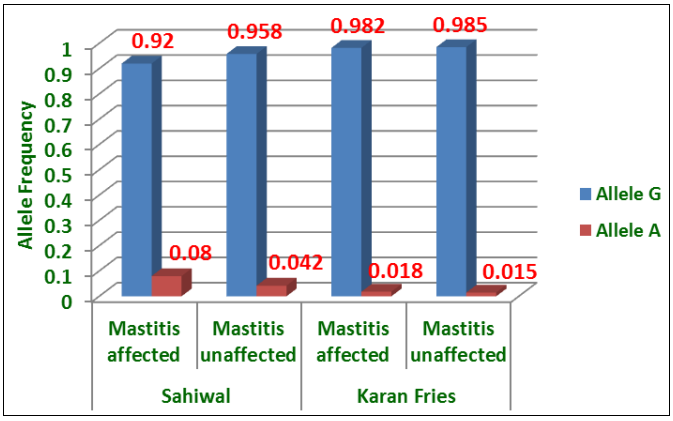

Fig 3: Frequency of "G" and "A" alleles in mastitis affected and unaffected Sahiwal and Karan Fries cattle

Table 1: Frequency of genotypes at S100A8 -2 (A/G) SNP locus and association with mastitis incidence in Sahiwal and Karan Fries cattle

Breed	S100A8 genotype	Mastitis Affected		Mastitis Unaffected	
		Number	Frequency	Number	Frequency
Sahiwal	GG	76	0.86	54	0.92
	GA	10	0.11	5	0.08
	AA	2	0.01	0	0.0
	Total	88	1.0	59	1.0
Karan Fries	GG	80	0.98	64	0.97
	GA	1	0.01	2	0.03
	AA	1	0.01	0	0.0
	Total	82	1.0	66	1.0

The frequency of S100A8 genotypes viz. GG, GA and AA within mastitis affected Sahiwal cattle were 0.86, 0.11 and 0.02 while it was 0.98, 0.08 and 0.00 in case of mastitis unaffected animals respectively (Table 1). Similarly, the frequency of GG, GA and AA genotypes in mastitis affected Karan Fries cattle was 0.98, 0.01 and 0.01 respectively while it was 0.97, 0.03 and 0.00 in mastitis unaffected animals respectively. The genotype frequencies within different groups in Karan Fries and Sahiwal cattle were subjected to chi-square test of association. The observed and expected frequencies were calculated to estimate chi-square value and the calculated chi-square value was found to be less than Table P-value in both Sahiwal and Karan Fries cattle indicating no significant association between S100A8 allelic variants and incidence of clinical mastitis.

Conclusion

In summary, the present study is the first report on SNP discovery and association of S100A8 allelic variants with incidence of clinical mastitis in Indian cattle. A total of 23 novel SNPs was identified and a PCR-RFLP assay was developed to genotype -2 (A/G) SNP locus in Sahiwal and Karan Fries cattle. No significant association of S100A8 genotypes with incidence of clinical mastitis (P>0.05) was observed.in investigated cattle breeds. However, expanding the study in a larger population with inclusion of additional phenotypes like somatic cell count may help to further investigate the association of S100A8 variants with resistance/susceptibility to mastitis in Indian cattle.

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