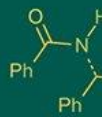


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Genotyping of crossbred cattle for SNP G22231T and SNP T25025A of BRCA1 gene and its association with somatic cell count

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Abstract

Mastitis is one of the important diseases of cattle, causing great economic losses to the dairy business. Considering the importance of mastitis in dairy industry and to know the involvement of tumor suppressor genes, the crossbred cattle population was targeted to find out the association of two SNPs (G22231T and T 25025A) of *Breast Cancer* gene-1 (*BRCA1*) and its association with somatic cell count (SCC). A total of 60 crossbred cows between 3rd and 5th lactation were selected after confirmation of subclinical mastitis with the California Mastitis Test (CMT). The estimated SCC were ranging from 1.21 to 8.20 $\times 10^5$ per ml of milk. The PCR products of 321 bp and 209 bp were successfully amplified for SNP's G22231T and T25025A. The PCR-PFLP with *HhaI* for G22231T produced two genotypes, AB and AA, while PCR-PFLP with *MaeI* for T25025A produced DD, CD and CC genotypes. It indicated that the crossbred cows were found to be polymorphic for both the SNPs. Association studies between genotypes of both the SNPs and their mean SCS were found to be non-significant. However, cows with AB genotype had the lowest SCS value for SNPG22231T as compared to AA. Whereas, lowest SCS value was observed in cows with CD genotype for SNP T25025A compared to genotypes DD and CC. The heterozygous genotypes AB and CD from SNPs had the lowest SCS. Highest SCS was observed in cows with genotype AB, CC and lowest SCS in animals with genotype ABCD. There was no significant association between the combined SNP genotypes and SCS.

Keywords: BRCA1 gene, mastitis, single nucleotide polymorphism, somatic cell count, somatic cell score

Introduction

Livestock sector in India is very important as it contributes to about 20.5 million of people for their livelihood. It provides an income source to about two-thirds of the rural community. According to the 20th Livestock Census, this sector employs 8.8% of the country's total population and accounts for 25.6% of India's agricultural GDP and 4.11% of the country's overall GDP. Indigenous cattle breeds are known for their resistance to tropical diseases compared to exotic and crossbred cattle, and comparatively lower milk production. Crossbred cattle have a significant role in increased milk production due to their high milk production efficiency. However, they are more susceptible to mastitis than the low producers. Mastitis is a global problem which causes huge economic losses by decreasing milk production. In India, the projected annual losses attributed to mastitis were approximately Rs.7165.51 crore per annum (Bansal *et al.*, 2009) [1] and Rs. 16,702 million per annum (Deb *et al.*, 2014) [4, 5]. Mastitis is a multi-etiological disease, but genetic causes are very common. There are many genes responsible for mastitis, but BRCA1 is one of the important genes. The length of the Bovine BRCA1 gene is 68.69 kb and is located on the 19th chromosome. It encodes 1849 amino acids, consisting of 22 exons and 22 introns. A mutation in BRCA1 itself and errors that occur in the repair of BRCA1 may cause damage to DNA, which eventually increases the risk of cancer. Somatic Cell Count below 1×10^5 /ml is considered normal milk, and above 1×10^6 /ml is considered infected milk. With advances in molecular genetics techniques, various molecular markers have been identified that can help target candidate genes responsible for production-related diseases such as mastitis.

Selection through the markers in breeding will eventually increase production. The genotypes linked with mastitis resistance can be incorporated in selection. Identifying SNPs linked to mastitis and using them for selection offers hope of producing mastitis-resistant animals with improved production in the near future. Considering the above-discussed facts, the present study is focused on analysing the BRCA1 gene and its association with SCC in the milk of crossbred cattle.

Materials and Methods

The present study comprised 60 unrelated crossbred cows (HF × Gir and Jersey × Gir) reared at Bombay Gorakshak Mandali, Kandivali, Mumbai. All the cows were apparently healthy and between 3rd to 5th lactation, tested for CMT positive (Ganguly *et al.*, 2008) [7]. Milk samples were collected for estimation of somatic cell count by DeLaval Cell counter and further converted into somatic cell score by using formula $SCS = \log_2 [SCC/100] + 3$ given by Rupp and Boichard (1999) [15]. The blood samples were also collected for isolation of genomic DNA using traditional Phenol: Chloroform: Isoamyl alcohol (P: C: I) method as described by Sambrook and Russell (2006) [16]. Purity and integrity of the isolated genomic DNA samples were checked on 0.8 percent agarose gel at 90 V for 60 minutes. The DNA fragments were amplified with 25 µl of PCR reaction mixture; nuclease free water-10.5 µl, PCR Master Mix-12.5 µl, forward primer-0.5 µl (10 pmole), reverse primer-0.5 µl (10 pmole) and template DNA-1.0 µl (50~100

ng/µl) for SNP G22231T and SNP T25025A of BRCA1 gene, respectively using primer (Table.1) sequences by Deb *et al.* (2014) [4, 5]. Cycling conditions for PCR amplification of SNP G22231T and SNP T25025A of BRCA1 were standardized as: initial denaturation at 94 °C for 5 min, denaturation at 94 °C for 30 sec, annealing temperature at 55 °C (SNP 1)/60 °C (SNP 2) for 30 sec, Synthesis at 72 °C for 30 sec, and final Extension was done at 72 °C for 10 min. The restriction enzymes used in the present study were *HhaI* for intron-6 (SNP G22231T) and *MaeI*/(*Bfal*) for exon-9 (SNP T25025A). The PCR products were digested with restriction enzymes in the final reaction volume of 15 µl (Table 2). The reaction mixture was centrifuged for a few seconds for uniform mixing and incubated at 37 °C for 3 hours in the water bath, and then electrophoresed on 2.5 percent agarose gel for both intron-6 and exon-9. According to the expected fragment sizes, electrophoresis was performed at a constant voltage of 90V for 60 minutes, and a 100 bp ladder was used; bands were visualised under UV light and documented by a gel documentation system. RFLP analysis of SNP G22231T and SNP T25025A of BRCA1 gene revealed three genotypes of each. The somatic cell score of each genotype was estimated, and association studies were established between genotypes and corresponding somatic cell scores. Further, breed-wise and combined genotypes of SNP G22231T and SNP T25025A association were established. Single-factor one-way ANOVA was applied for further statistical analysis of the data for the association study.

Table 1: Primer sequences for amplification of SNP G22231T and T25025A.

Sr. No	Gene/locus	Primer sequence (5' 3')	Size bp	Authors
1	Intron 6	F:5'-CTTCAGAACCTGTACTTGTAAACC-3' R:5'-CAAGGAATATTTACTGAGCACC-3'	321 bp	Deb <i>et al.</i> (2014) [4, 5]
2	Exon 9	F:5'-GCAGTGGAAATTCCAAATAAACT-3' R:5'-GAATTAGATCTTCAGCTATGTGGC-3'	209 bp	Deb <i>et al.</i> (2014) [4, 5]

Table 2: RFLP reaction mix (BRCA1 G22231T)

Sr. No.	Reagent	Quantity
1	RE-enzyme-HaeI (1U/µl)	0.5µl
2	RE-buffer (10x buffer)	1.0 µl
3	PCR-product	5.0 µl
4	Nuclease-free water	8.5 µl
Total reaction volume		15.0 µl

Results and Discussion

This study has been planned in crossbred cows (HF × Gir and J × Gir) to study polymorphism of SNP G22231T and SNP T25025A of BRCA1 gene and its association with somatic cell count (SCC). PCR amplification of SNP G22231T and SNP T25025A of BRCA1 gene fragments of 321 bp (Fig.1) and 209 bp (Fig.2) were successfully obtained at an annealing temperature of 55 °C and 60 °C for 30 seconds, respectively which coincided with Deb *et al.* (2014) [4, 5] and Yuan *et al.* (2012) [21, 22] used 54.5 °C for intron-6 while for exon-9 it was 60 °C. *HhaI*/PCR-RFLP of SNP G22231T analysis revealed two-band patterns as three bands of size 321 bp, 272 bp, 49 bp and two bands 272, 49 bp (Fig. 3). Based on RFLP band pattern observed, crossbred cows were genotyped as AB (321 bp, 272 bp, 49 bp) and AA (272 bp and 49 bp) as reported by Yuan *et al.* (2012 b) [21, 22]. The allelic frequencies of SNP G22231T for A and B were 0.769 and 0.230, and only two genotypes were observed, i.e., AB and AA; genotype BB was absent.

Genotypic frequencies were 0.461(AB) and 0.538(AA), respectively, for HF crossbred cows under investigation. In Jersey crossbred cows, it was 0.323 for B and 0.676 for A, and the genotypic frequencies of BB, AB and AA were 0.088, 0.470 and 0.441, respectively.

The similar results have been noted by Yuan *et al.* (2012 b) [21, 22] who reported three genotypes in three cattle breeds with genotypic frequency as AA: 0.2897, AB: 0.5327, BB: 0.1776 in Holstein cows, AA: 0.3035, AB: 0.5423 and BB: 0.1542, in Sanhe cattle and AA: .3020, AB: 0.5099 and BB: 0.1881 in Simmental cattle. The allele frequencies were 0.5561 and 0.4439 for A and B, respectively, in Holstein cattle; 0.5746 and 0.4254 in Sanhe cattle; and 0.5099 and 0.4792 in Simmental cattle. Magotra *et al.* (2020) [11] revealed three genotypes GG, GT and TT for SNP G43737229T with genotypic frequencies 0.255, 0.333 and 0.412 and three genotypes GG, AG and AA for SNP G43761121A with genotypic frequencies 0.392, 0.402 and 0.206. Sonwane *et al.* (2023) [19] reported two genotypes, BB and AB, for exon 3 of TLR4 gene in crossbred cattle, respectively, with genotypic frequencies of 0.775 and 0.225, while allelic frequencies for allele B and A were 0.887 and 0.112. Deb *et al.* in 2014 [4, 5] found only two genotypes in Frieswal cattle for SNP G22231T, and genotypic frequencies were AA: 0.22 and AB: 0.78, and the gene frequencies were 0.61 and 0.39. PCR-RFLP pattern of 209 bp fragment of SNP T25025A revealed three genotypes

(Fig. 4) as DD (209 bp), CD (209, 185, 24 bp) and CC (185, 24 bp). The genotypic frequencies for SNP T25025A recorded were CC, CD and DD, which were 0.04, 0.48 and 0.48. Gene frequency for HF crossbred cows was 0.288 and 0.711 for C and D. For Jersey crossbred, gene frequencies were 0.28 and 0.72 for C and D, and the genotype frequencies were DD, CD and CC, which were 0.485, 0.371 and 0.142. The association studies were established between the two genotypes of SNP G22231T (Intron-6) and somatic cell score of 60 crossbred cows. One-way ANOVA showed non-significant effect

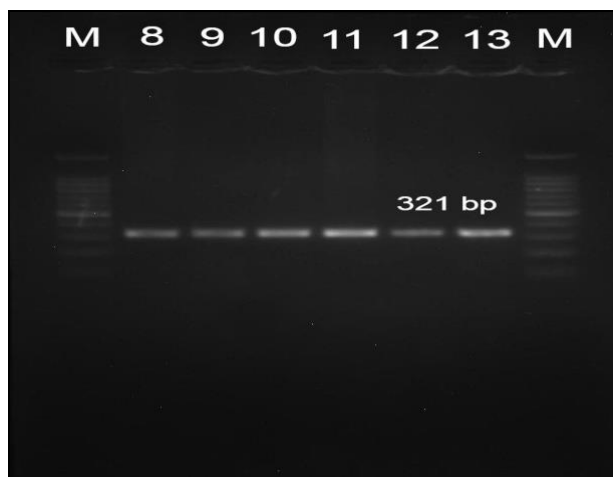


Fig 1: PCR amplification of SNP G22231T(321 bp)

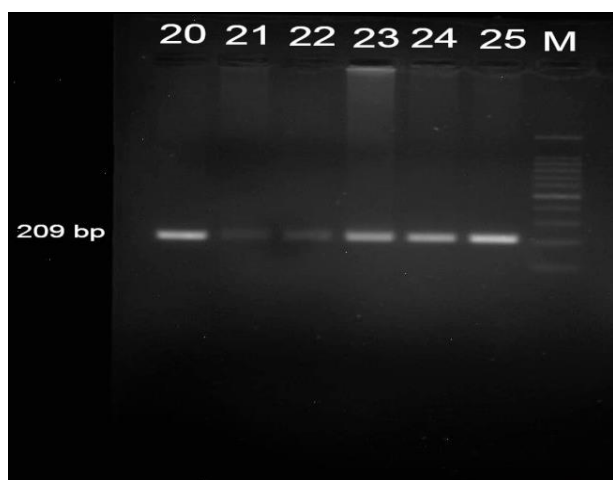


Fig 2: PCR amplification of SNP T25025A (209 bp)

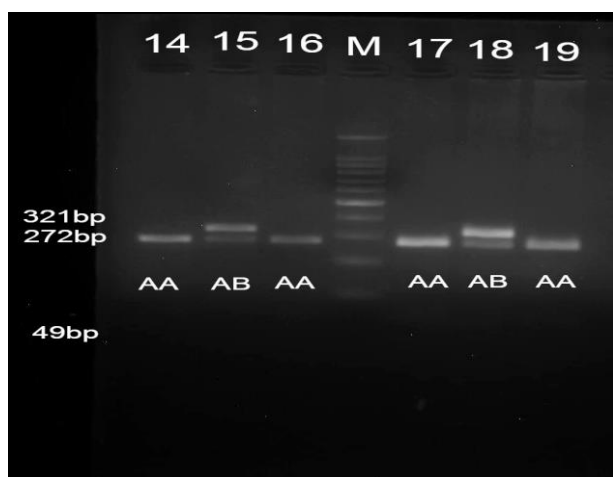


Fig 3: HhaI-PCR-RFLP of SNP G22231T



Fig 4: MaeI-PCR-RFLP of SNP T25025A

($p = 0.79$, $\alpha = 0.05$) of somatic cell score on two genotypes AB and AA. However, genotype AB had less SCS values, i.e. 4.38 ± 0.33 as compared to AA with SCS mean values as 4.70 ± 1.47 and 4.78 ± 0.27 , respectively. Similar findings have been noted by Yuan *et al.*, 2012^[21, 22], who observed a non-significant association between genotypes of SNP G22231T, i.e. AB, BB and AA and SCS. They reported highest SCS for genotype AB and lowest for AA and further justified that the (G22231T) introns are not the coding sequence, but previous research on introns has enlightened the role of introns in mRNA splicing, gene expression, etc. Sonwane *et al.* (2023)^[19] reported non-significant association between mean somatic cell score and genotypes BB and AB in exon 3 of TLR4 gene in crossbred cattle. For the confirmatory role of SNP G22231T locus of BRCA1 gene in Bovine mastitis, more work is suggested to be undertaken.

For the SNP T25025A (Exon-9) non-significant ($p = 0.30$, $\alpha = 0.05$) association was observed between the genotypes DD, CD and CC and somatic cell score. The mean value for SCS of CC genotype was found to be higher, i.e. 5.42 ± 0.86 followed by DD and CD with SCS values 4.78 ± 0.33 and 4.32 ± 0.30 , respectively. Similar results have been noted by Yuan *et al.* (2012)^[21, 22], who found a non-significant association of SNP T25025A locus of BRCA1 gene and the somatic cell count for the CC genotype compared to the CD and DD genotypes. However, they observed a lower somatic cell count compared to the CC genotype (3.68), and it was found close to a significant level ($p < 0.05$), and the highest SCS was for genotype DD (4.47). They also studied the locus C28300A of BRCA1 gene and found a significant association with SCS, in which animals belonging to EE (3.22) genotypes had significantly lower somatic cell count than genotypes EF (4.94) and FF (5.53) in Holstein, Sanhe and Simmental cattle breeds.

One-way ANOVA revealed no significant association between combined genotypes and corresponding somatic cell score. However, lowest SCS (4.00 ± 0.30) and highest SCS (5.32) were observed in ABCD and AACC genotypes, respectively. Whereas, Yuan *et al.* (2012 b)^[21, 22] observed a significant association between combined genotypes (24) of three SNPs genotypes and somatic cell score in Chinese commercial cattle. Yuan *et al.* (2012a)^[21, 22] studied SNPs of BRCA1 gene and their relation with mastitis in Chinese commercial cattle (Total-404 animals, Holstein-107, Sanhe-201, Simmental-96) and reported three SNPs (c.26198 C>T, c.5682 G>C, c.46126 G>T) in exon-9, in intron-2 and exon-

12 showed many genotypes by using PCR-RFLP. They reported that genotype KK had significantly lower SCS than genotypes KL and LL, and combined genotype analysis of three SNPs showed that HHLLNN genotype with the highest SCS has been associated with mastitis susceptibility, whereas GGKKMM genotype with the lowest SCS was associated with mastitis resistance. Deb *et al.* (2014)^[4, 5] studied the association between polymorphism of SNP regions G22231T and T25025A of BRCA1 gene and somatic cell score in 200 Frieswal cows for mastitis resistance; significant association was found between SCS and genotypic groups of two SNP regions, cows with AB genotype (4.31 ± 0.32) having significantly ($P \leq 0.01$) higher SCS than AA (3.89 ± 0.26) for G22231T. Wherein, for SNP T25025A, the genotype DD (3.21 ± 0.25) had significantly ($P \leq 0.01$) higher SCS than CD (3.86 ± 0.27). Combination of Genotypes for two SNPs in Frieswal cows leads to four kinds of haplotypes, and association analysis with SCS showed a significant difference among them. Combining different genotypes for each SNP results in four kinds of haplotypes viz. ABCD (63), ABDD (51), AACD (56) and AADD (30). After association studies with SCS, Magotra *et al.* (2015)^[10] observed that ABDD (4.28 ± 0.26) and AACD (3.35 ± 0.19) haplotypes had significantly ($P \leq 0.05$) highest and lowest SCS. No association was found in Sahiwal cows for the BRCA1 gene and SCS.

The crossbred cows under investigation were found to be polymorphic for both the SNPs with three genotypes. The association study between mean somatic cell scores and genotypes of SNP G22231T and T25025A of BRCA1 gene and SCS was found non-significant, indicating that there was no association between BRCA1 gene and somatic cell count in crossbred cows with subclinical mastitis. The non-significant association was found between the combination of genotypes of SNP G22231T and T25025A of BRCA1 gene and their corresponding somatic cell score. However, heterozygous genotypes, i.e. AB, CD and ABCD, showed lowest somatic cell score for both the SNPs and combined genotypes compared to the homozygous genotypes. It is suggested that if the number of cows increased, there might be chances for a desirable association between different genotypes and their somatic cell score.

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