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Association of STAT1 gene with milk fat and protein yield in Holstein Friesian crossbred cattle maintained in the sub-tropical climate of India

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Research Article

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Abstract

Signal transducers and activators of transcription (STAT) genes are involved in signal mediation of various hormones and cytokines. STAT1 located on chromosome number 2 is involved in mammary gland development and is associated with milk composition traits in bovines. This study aimed to find any relationship and impact of STAT1/*BspHI* gene with milk fat and protein yields in a herd of Holstein Friesian (HF) crossbred cattle of sub-tropical climate of Northern India. Milk composition data of 535 adult HF crossbred cows for a period of 12 years was collected from the records maintained at Livestock Farm, Guru Angad Dev Veterinary and Animal Sciences University. First lactation data of 222 animals was chosen for further analysis. After data correction for non-genetic factors (season of calving, period of calving, interaction effect of season and period of calving and age at first calving) these animals were categorised into two groups based on corrected high and low milk fat and protein yields. Forty animals were then selected for blood collection and further laboratory analysis. Amplified using PCR-RFLP technique, the 314 bp STAT1 gene was digested using *BspHI* restriction enzyme. C-T polymorphism at nucleotide position 201 and 260 of the STAT1 amplicon was observed. At 201, for genotype AA and Aa, the genotypic frequencies were 0.80 and 0.20%. At 260, for genotype BB and Bb, the genotypic frequencies were 0.25 and 0.75%. Least square analysis showed a significant association of all genotypes with milk fat and protein yields. Hence, STAT1 can be used as a potential candidate gene to aid in better animal selection in breeding programmes.

Livestock are a vital part of the Indian economy. With 537 million total livestock population, India stands at first position globally. Of the 303 million total bovine population of the country, 193 million i.e. 36% is constituted by cattle. The exotic/crossbred comprises 51 million (27%) of the total cattle population, out of which 39% alone is contributed by Holstein Friesian crossbred cattle. In the state of Punjab, out of all the cattle population 83% is contributed by exotic/crossbred animals, a large share of which comes from Holstein Friesian crossbred cattle (Breed-wise Report of Livestock and Poultry- Based on 20th Livestock Census). The point of these numbers is to emphasise the dominance of the crossbred population, whose average milk yield (in Punjab) is 13.49 kg/d in 2022–23. (Basic Animal Husbandry Statistics, 2023, DAHD). There is a need for improvement.

Noticeable advances have been made in dairy cattle breeding in the past few decades. Conventional breeding practices are constrained by sex-limited, low heritability and late expressed traits. Now, utilisation of genetic markers allied with traditional breeding strategies for improved selection of genetically superior breeding animals is on the rise. Genetic markers are beneficial for evaluating the genetic basis of observed phenotypic differences (Teneva and Petrovic, 2010). These are any detectable sequences of DNA present on chromosomes linked with individual or species-specific variations which can be used to determine traits of interest. The genetic architecture of traits has proven to be an important tool to heighten genetic improvement. As molecular markers are reported to be more reliable in determining the genetic structure, these are widely used to achieve the purpose of continuation of the existence of species along with preserving the genetic diversity among livestock (Grechko, 2002). Many different genomic regions active on milk yield traits in dairy cattle breeds have been mapped in the last decade (Khatkar *et al.*, 2004). Milk composition traits which are of economic relevance in dairy cattle are controlled by several genes. Therefore, their improvement is of significance for animal breeders. In many studies, the relationship between different alleles of the candidate gene and phenotypic traits in the population have been investigated (Kwon and Goate, 2000).

STATs (signal transducers and activators of transcription) encompass a 7-member family of latent cytoplasmic transcription factors (STATs 1, 2, 3, 4, 5a, 5b and 6) which facilitate the actions of many cytokines and peptide hormones within target cells (Darnell, 1997). They were first found during the interferon signalling pathway study. These get their name as transcription factors as they allow the proteins to control turning on and off these genes by attaching to the regulatory regions, (Meyer *et al*., 2003). STAT genes have a general structure showing a functional modular domain type arrangement, where STAT1 and STAT3 genes are attached to DNA in a clamp-like fashion including a coiled domain of 4 β sheets. These precede the DNA-binding domain facilitating numerous protein–protein interactions. STAT1, STAT4 and both STAT5s show N-domain interactions among two activated STAT dimers which aid in the creation of stable tetramers. All STAT genes, other than STAT2, form greater-order tetramer complexes. STAT1 is an interesting protein with varying transcriptional functions. On being activated, it allows the expression of numerous genes but also supresses the transcription of some of the genes. Therefore, it is involved in both synergistic activation as well as the inhibition of gene expression. STAT1 is highly regulated during the development and differentiation of mammary gland and hence is significant in the process. Reports suggest that STAT1 plays an essential role in development and differentiation process of the mammary gland (Boutinaud and Jammes, 2004). It is located on chromosome number 2 in bovines and is found to be associated with milk composition traits as well as contributing to improved milk yield. Band *et al.* (2000) reported that it is linked with milk fat and protein synthesis as well as fat metabolism along with regulation of gene transcription. Ashwell *et al.* (2004) reported the 3′UTR region of STAT1 to be associated with milk fat yield related polymorphism. Significant associations between microsatellite markers and production traits in the vicinity of STAT1 have been observed in whole-genome scans. Ron *et al.* 2004 reported that STAT1 is regulated in the process of mammary gland development and apoptosis and is involved as an oncogene in the mammary gland. Cobanoglu *et al.* (2006) reported a highly significant association between STAT1 gene polymorphism and milk production traits in Holstein dairy cattle where they found CC and CT genotypes to be associated with higher milk, fat and protein yield of dairy cattle. Khandare *et al.* (2020) reported monomorphic as well as polymorphic SNP sites within STAT1 gene in association with milk traits in Gaolao cattle of India. Deng *et al.* (2015) also reported that SNPs in STAT1 possess a significant association with milk production traits.

Crossbreeding, being the most rapid and effective approach for genetic improvement of non-descript zebu cattle, is one of the major steps followed in the country resulting in an increase of milk production by 5–8 fold (Wakchaure *et al*., 2015). The upgrading and genetic improvement of the indigenous cattle in the country for better milk quantity and quality is mainly done by crossbreeding with Jersey and Holstein Friesian cattle breeds. Although being one of most important gene of STAT family, very little work has been done on STAT1 in respect of crossbred cattle. The Holstein Friesian crossbred cattle pertaining to Northern India are our study population, with the objective of identifying polymorphism(s), if any, and to study the relationship between STAT1 gene and milk fat and protein yields in these crossbred animals. Our data should aid in better animal selection which will certainly help in understanding the advanced role of this gene in milk related traits of the animals.

Materials and methods

The study had approval from the Institutional Animal Ethics Committee of Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana (Punjab), INDIA (GADVASU/2021/IAEC/60/14).

Animal records

The milk composition data pertaining to 535 HF crossbred cows were collected for a span of 12 years (2009–2020) from the records and history sheets maintained at Livestock Farm, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana (India). Ludhiana is the most densely populated city of the state of Punjab. It is situated between 30°-34' and 31°-01' north latitude and between 75°-18' and 76°-20' east latitude. As per Koppen climate classification, Ludhiana has a humid subtropical climate. The relative humidity ranges from 46 to 82%. The average temperature ranges from a highest of 42.5°C to a lowest of 16.7°C. The average annual precipitation in the city is around 890 mm each year. Out of these animals, first lactation records of 222 adult HF crossbred cows were used for this study. These records were subjected to correction for non-genetic factors. The non-genetic factors taken into consideration were season of calving, period of calving, interaction of season and period of calving and age at first calving. Season of calving was classified into 5 seasons: Spring (March to Mid-April), Summer (Mid-April to June), Rainy (July to September), Pre-Winter (October to November) and Winter (December to February). Period of calving was classified into 4 January to December two-year periods: 2009 to 2011, 2012 to 2014, 2015 to 2017 and 2018 to 2020. Age at first calving was divided into 3 groups: AG1 (<26.04 months), AG2 (from 26.04 to 31.08 months) and AG3 (>31.08 months).

For comparative analysis, two groups comprising of animals with high milk fat and protein yield (225 and 163 kg, respectively) and low milk fat and protein yield (147 and 141 kg, respectively) were formed. Out of 222 cows, two groups of animals (20 in each group) with highest and lowest milk fat and protein yield in the respective groups, making a total of 40 animals were selected for blood sample collection and further laboratory analysis.

Genotyping

Approximately 5 ml blood was taken by jugular venepuncture into EDTA tubes using aseptic measures and stored at 4°C. Genomic DNA was extracted using Qiagen DNA extraction kit (QIAamp DNA Blood Mini Kit). DNA quality was checked using gel electrophoresis assembly method. The concentration range (ng/µl) and purity (absorbance at 260–280 nm) of the DNA samples were measured using a spectrophotometer (Nanodrop). The 314 bp fragment of STAT1 gene was amplified using the reported set of forward and reverse primers; STATF: 5′-GCCTCAAG TTTGCCAGTGGC-3′ and STATR: 5′-GGCTCCCTTGATAG AACTGT-3′ (Cobanoglu *et al*., 2016). The amplification was performed in a 25 µl reaction volume. This comprised of 12.5 µl of Go Taq Green PCR Master Mix (Promega), 0.5 µl each of forward and reverse primers, 1 µl of genomic DNA, 10.5 µl of nuclease free water. The PCR amplification programme used consisted of the following time & temperature combinations: Initial denaturation at 95°C for 5 min (1 cycle) followed by 30 cycles each of denaturation, annealing and extension at 95, 55 and 72°C for 45, 30 and

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30 s respectively. This was followed by a final extension at 72°C for 5 min (1 cycle). After amplification, the PCR products were stored at 4°C. Agarose gel electrophoresis was carried out to check the amplified PCR products. The gel was then viewed under gel documentation system. The PCR products were digested using *BspHI* restriction enzyme at 37°C for 1 h. The RE digests were inactivated by incubation at 65°C for 15 min and stored at 4°C thereafter. The PCR products were subjected to Sangers Sequencing. The available sequence with accession number XM_025000697.1 at NCBI for *Bos taurus* was used as the reference sequence for STAT1 gene. The chromatograms received were first checked for the gene specificity using BLAST software. These were then viewed and interpreted using Finch TV and Clustal Omega (Multiple Sequence Alignment Tool: https://www.ebi.ac.uk/Tools/msa/clustalo/) for detection of SNP.

Statistical analysis

General linear model procedure of statistical analysis system software programme SAS, 2011 (version 9.3) was used for statistical analysis of corrected data (for genetic and non-genetic factors). Association between the genotypes obtained with milk composition traits was studied using least square analysis for fixed effect model with the assumption that the various components that were being fitted into the model were linear, independent and additive.

Statistical model used for least square analysis for the estimation of genetic factors:

$$Y_{no} = \mu + M_n + e_{no}$$

where, $Y_{no}$is the character variable ofO^{th}cow, μ is the overall mean of population, M_n is the random effect of n^{th}sire and e_{no} is the residual term = ∼NID $(0,\sigma_e^2)$.

Results

Clear bands at 314 bp of STAT1 gene PCR products were observed on a 2% agarose gel after subjecting them to gel electrophoresis (online Supplementary Fig. S1). This confirmed that the extracted DNA is of good quality. The PCR-RFLP results and the sequence alignment tool, Clustal Omega revealed two SNPs at nucleotide position of 201 and 260 of the STAT1 amplicon. All the genotypes were scored based on the banding pattern observed in gel electrophoresis. At position 201, AA and Aa genotypes were observed where, AA genotype was found to be homozygous for two positions i.e. at 2 015 954 and 2 602 015 954 bp DNA fragments. Aa genotype was observed at 3 142 605 954 bp DNA fragments. At position 260, BB and Bb genotypes were observed where, BB genotype was found to be homozygous at one position i.e. at 2 015 954 bp DNA fragments. The Bb genotype was found to be heterozygous for two positions i.e. at 3 142 605 954 and 2 602 015 954 bp DNA fragments. (online Supplementary Fig. S2). Clustal Omega interpretations and results for the two observed SNPs are shown in (online Supplementary Fig. S3). This revealed that SNP at position 201 and 260 aligned that with positions 3173 and 3232 respectively of the reference sequence. The coding sequence (CDS) region of STAT1 gene ranges from 308 to 2557 bp. Thus, these SNPs were found to be located in the 3″ untranslated region (3″UTR) of the gene. At both positions; 3173 and 3232 of the 3″ UTR, 'C' nucleotide has been substituted by 'T' nucleotide (online Supplementary Fig. S3). The very close proximity of the two SNPs observed here is suggestive of the presence of linkage of alleles A and B in the studied population.

The genotype frequencies for AA and Aa were observed to be 0.8 and 0.2% and for BB and Bb were observed to be 0.25 and 0.75%, respectively (Table 1). The presence of these different genotypes was also confirmed by DNA sequence analysis for STAT1/*BspHI*. The association study using least square analysis showed a significant association of AA and Aa genotypes for SNP (201, C to T) and BB and Bb genotypes (260, C to T) for STAT1 with milk fat yield as well as milk protein yields (Table 1).

Discussion

The STAT1 gene is involved in mammary gland function and development. This study reports the association between STAT1 gene and milk fat and protein yields in Holstein Friesian crossbred cattle. The cattle population chosen for blood sample collection and laboratory analysis was maintained in a uniform environment of the livestock farm complex of Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, Punjab where standardised feeding and managemental conditions were followed. Hence, all the animals can easily be considered to be of a homogeneous distribution. The animal grouping based on high and low milk fat and protein yields aimed at analysing any differences among the animals of these groups in relation to STAT1/*BspHI* as this gene plays a vital role in fat and protein metabolism. Various researchers have conducted experiments using the pooled DNA sequencing approach for detection of polymorphism as well as for the proper estimation of allele frequencies. In prior studies, the pooled DNA samples have been used to detect SNPs in coding and noncoding regions in different genes related to milk production traits (Khatib *et al.*, 2005, 2006). In our study, using the DNA sequence-based method, 2 SNPs (C to T) have been detected in the 314 bp fragment of STAT1 gene.

In our experiment, the PCR-RFLP and DNA sequencing results revealed the presence of two SNPs at position 201 and 260 (C to T) of the 314 bp STAT1 gene fragment. This also revealed the presence of a total of four genotypes (AA, Aa; BB, Bb). Two genotypes were observed at each SNP locus instead of the usual three. This can be attributed to the fact that both the observed SNPs lie in very close proximity to each other. This leads to observance of overlapping of the DNA bands in gel electrophoresis picture. Hence, banding pattern common to both the SNPs were observed. This further leads to observance of the AA genotype associated with SNP at 201 being homozygous for two positions while for SNP at 260, the genotype Bb was observed to be heterozygous for two positions. All these results are suggestive of presence of linkage between alleles A and B. Such proximity of the SNPs becomes predominantly the reason for easily heritable patterns in the population. Cobanoglu *et al.* (2016) in their study on Turkish cattle using the similar restriction enzyme *BspHI* reported a DNA fragment at a similar position to our study i.e. at 201 position. They identified 314, 201 and 113 bp fragments for CT genotype, 201 and 113 bp for CC genotype and an intact 314 bp band for TT genotype. Similarly, Askari *et al.* (2013) reported 201 260 and 54 bp DNA fragments for the 7 genotypes found in their experiment. Ashwell *et al.* (2004) and Ron *et al.* (2004) in their experiments reported quantitative trait loci affecting milk fat percentage in linkage with microsatellites (38.0 to 60.3 cM) and one affecting milk protein percentages respectively (61.7 to 70 cM). The observed genotypic frequencies

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Table 1. Observed frequencies of the obtained genotypes and least square means and standard errors for 305-day milk fat and protein yields of the different genotypes for STAT1 gene

Locus	Genotype	*N*	Observed frequency (%)	305-day fat yield (kg)	305-day protein yield (kg)
201	AA	32	0.8	$148.38^{b} \pm 2.23$	$129.00^{b} \pm 2.17$
	Aa	8	0.2	$193.60^{a} \pm 4.70$	$160.89^{a} \pm 4.59$
260	BB	10	0.25	$154.09^{b} \pm 4.42$	$129.90^{b} \pm 4.31$
	Bb	30	0.75	$187.89^{a} \pm 2.41$	$159.99^{a} \pm 2.35$

Least square means with different superscripts for the same site differ significantly ($P < 0.05$) while those with same superscripts show no significant difference.

for AA, Aa; BB, Bb in our study, were 0.8, 0.2%; 0.25, 0.75% respectively. The frequency of the genotype TT in Holstein cattle has been reported in the range of 7.92–10.63% (Cobanoglu *et al.*, 2006; Khatib *et al.*, 2009). Similarly, the minor allele T was observed at a frequency of 15.27%. The frequency of the rare genotype TT was found to be 2.15% by Rychtářova *et al.* (2014).

In this study, we observed a significant association of AA and Aa genotypes for SNP (201, C to T) and BB and Bb genotypes (260, C to T) for STAT1 gene with milk fat yield as well as milk protein yield for all the animals of the studied population. Animals of both the groups, i.e. high and low milk fat and protein yields showed a significant association with all the genotypes observed in the study. This is indicative of the essential role of this gene in relation to milk fat and protein metabolism. The results of this study are consistent with another study on STAT1 gene using *PagI* restriction enzyme-based PCR-RFLP method in two studied cattle populations by Cobanoglu *et al.* (2006) where, in one they found that the CC & CT genotypes were observed to be significantly associated with increases in milk fat, and protein yields while in the other population C allele was associated with significant increases in milk fat and protein percentages. Similarly, Askari *et al.* (2013) in their experiment on Esfahan Holstein cattle reported 7 homozygous genotypes where the frequencies of A, B, C & D alleles were observed to be 0.021, 0.101, 0.332 and 0.546, respectively. They also reported a significant association of all the STAT1/*Pag1* genotypes with milk fat percentage. Similarly, Rychtarova *et al.* (2014) observed significant differences between CC and CT genotypes of STAT1 gene for protein percentage as well as in estimated breeding values for fat and protein percentages in Czech Fleckvieh cattle population. However, Cobanoglu *et al.* (2016) in their study on STAT1/*BspHI* polymorphism reported three genotypes, with non-significant effects in Holstein cows. Contrary to this, significant effects were observed for Jersey cows in respect of test day fat and protein yields. Also, Ardicli *et al.* (2018) reported non-significant differences among STAT1 genotypes with milk fat and protein contents in Holstein cows of Turkey. Vafaee and Mashhadi (2016) also stated a non-significant association of milk fat and protein yields with three genotypes they found in Brown Swiss cattle. Khandare *et al.* (2020) reported one monomorphic and three polymorphic sites in respect of milk traits in Gaolao cattle of India using STAT1 gene variants and *PagI* restriction enzyme. STAT1 gene has also been explored a little in the buffalo population. Kumar *et al.* (2015) studied STAT1/*HaeIII* relationship with milk production traits and reported monomorphic sites in the 3′UTR region of Murrah, Gojri and Chhattisgarhi buffalo breeds of India. Hasan *et al.* (2021) in their experimental study on Indonesian water buffaloes found polymorphisms at two loci of the STAT1 gene in association with milk production traits. Deng *et al.* (2015) reported that SNPs in STAT1 possess a significant association with milk production traits.

In conclusion, we report two STAT1 SNPs and two genotypes associated with each SNP in HF cross-bred cows. Also, linkage between the two observed alleles was found. Least square analysis showed a significant association of all genotypes with milk fat and protein yields. The slight differences in the patterns observed in this study and those reported by other authors can be attributed to the fact that crossbreeding through artificial insemination method of *Bos taurus* with indigenous cattle is on the rise. We conclude that this gene can be used as a potential candidate for various marker-assisted selection programmes in HF crossbred cattle in sub-tropical climates.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S0022029924000530

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