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Exploring the relationship of STAT5A gene with milk fat and protein yield in Holstein Friesian crossbred cattle

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ABSTRACT

Signal transducers and activators of transcription (STATs) arbitrate signals of various cytokines and hormones. STAT5A is a crucial intermediary of prolactin signalling mechanism which set in motion the transcription of milk protein genes. This research study was amied to explore the relationship and/or effect of STAT5A/*AvaI* gene with milk fat and protein content in a North Indian crossbred cattle herd of Holstein Friesian. Milk composition data of 535 adult HF crossbred cows was collected for over a period spanning 12 years from the records available at Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, Punjab. Thereafter data of first lactation length of 222 animals was selected for further analysis. Data correction was applied for non-genetic factors i.e SoC :season of calving, PoC : period of calving, SoC*PoC :the interaction effect of season & period of calving & AFC: age at first calving. Corrected high and low milk fat and protein yields was used to form two groups. Forty animals were selected for further blood collection and laboratory assessment. *AvaI* restriction enzyme was used for digestion of the 215bp STAT5A gene. Animals of both groups exhibited a uniform monomorphic pattern with DNA fragments of 181bp and 34 bp. The results indicated presence of a fixed locus for STAT5A gene in the studied population. Considering the results, more exploratory studies need to be conducted to further explicate the profound role of STAT5A in various milk related traits among the bovine species.

Keywords*: AvaI,* Holstein Friesian crossbred cattle, PCR-RFLP, STAT5A

Livestock plays a vital role in the national economy. With a total of 536.76 million livestock animal population, India ranks first globally. This includes 193.46 million cattle, out of which. 51.36 million (26.5 %) of the total cattle population is constituted by exotic/crossbred cattle, which majority comprised by Holstein Friesian 39.3 % (HF) admixture (crossbred) cattle. In Punjab, 21,05,587 (83.1%) headcounts of exotic/crossbred cows, belong to HF crossbred cattle type (Breed-wise Report of Livestock and Poultry- Based on 20th Livestock Census).

India contributes 22% (230.58 million tonnes in 2022-23) of the global milk production, making it the largest milk producer in the world. Crossbred cattle population contributes nearly 68.7 million tonnes (29.81%) with an average milk yield of 8.55 kgs/day. With 14.3 million tonnes of milk being produced in the state, Punjab holds 7th position in milk production in the country with its exotic/crossbred animals contributing around 5.24 million tonnes. The average milk yield of a crossbred cow in Punjab was 13.49 kg/day in 2022-23. With a figure of 1283 g/day, the state ranked 1st in per capita milk availability in the country, which was much higher as compared to the national average of 459 g/day (BAHS-Basic Animal Husbandry Statistics-2023, DAHD).

Milk composition and related traits hold an economic importance in dairy cattle especially in commercial dairy sector. These traits are governed by multiple genes. For an animal breeder, improving these traits or selecting such genes becomes a key objective. However, traditional breeding methods are bound to several limitations such as the traits being sex-limited, having low heritability, and late expression in life. To overcome these limitation, molecular markers have become an essential tool in genetic improvement when used alongside conventional breeding approaches. These genetic markers are basically identifiable set of DNA arrangements present on the chromosomes associated with an individual as well as species-specific variations. According to Grechko (2002), these molecular markers are highly reliable tools for assessing genetic structure and predicting an individual's phenotype. Recent advances like marker based selection have created new opportunities for genetic improvement in livestock by helping in accurate identification of these genomic regions which are linked to economically important traits (Dekkers and Hospital, 2002). Genetic markers also tend to be beneficial tools for understanding the basic genetic foundations of the observable phenotypic

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variations (Teneva and Petrovic, 2010). Additionally, genomic selection (GS) has emerged as one of the most promising approaches for augmenting economic traits governed by multiple major genes complexes (Ibtisham *et al*. 2017).

STAT translating to Signal Transducers and Activators of Transcription group encompasses seven latent cytoplasmic factors i.e STAT 1-4, STAT5a, STAT5b, and STAT6 that arbitrate the effects of various peptidyl hormones within target cells along with cytokines. (Darnell *et al*. 1994). Among these, STAT5 is of importance in prolactin signaling and milk protein gene transcription. It is also referred to as MGF i.e Mammary Gland Factor or MPBF i.e Milk Protein Binding Factor (Watson and Neoh, 2008). The STAT5 gene consists of 19 exons and is involved in the coding protocol for a 794 length amino acid based protein (Seyfert *et al*. 2000). It was identified in sheep as a single gene for the first time. Its two isoforms i.e STAT5A and STAT5B were later on discovered in humans, cattle, rats and mice. In cattle, the STAT5A gene is sited on chromosome number 19 and is found to be crucial in various mechanisms in uterine epithelial as well as mammary gland (Khatib *et al*. 2008). STAT5A has been allied with variations in milk production as well as milk composition in dairy cattle. It thus plays a significant role in response to lactogenic hormones (Ogorevc *et al*. 2009).

Indigenous cattle of India show high heat and disease resistance as they are very well adapted to the tropical conditions of the country. Crossbreeding using Holstein Friesian (HF) and Jersey breeds is done nationwide for the upgradation and genetic improvement of the indigenous cattle. This is mainly done to procure better milk quantity and quality. Crossbreeding studies related to STAT5A gene have been reported in literature, but meagre research based experiments have been done in respect to HF admixture (crossbred) type cattle. As crossbred cattle is being developed using HF for its better-quality milk, the main objective and focus of this research study was mainly to explore polymorphism (if any) and any possible relationship between STAT5A gene with milk fat and protein yields in this breed, thus aiding in better animal selection along with better insight into the unconventional role played by this gene in bovines.

MATERIALS AND METHODS

Data of milk composition related records of 535 HF (Holstein Friesian) crossbred animals were collected over a 12-year period (2009–2020) based on records maintained at the livestock farm of Guru Angad Dev Veterinary and Animal Science University. The institute is located in Ludhiana, a densely populated city in Punjab. Geographically, this city falls between 30°34'& 31°01' northern latitude and between 75°18'& 76°20' eastern longitude. The Köppen climatic classification states that the city is mostly of humid subtropical type climate, with a relative humidity range falling between 46% & 82%. The average temperature of the city lies mostly between 16.7°C & 42.5°C. It receives an average annual precipitation of nearly 890 mm. Out of these animals, the first lactation period data of 222 adult HF cows; was used for the experiment. Correction for non-genetic factors was then applied to these records. The non-genetic factors considered in this experiment were SoC i.e season of calving, PoC i.e period of calving, SoC*PoC i.e the interaction effect of season & period of calving and AFC i.e age at first calving. The Season of calving (SoC) was basically classified into 5 seasons as follows: March – Mid-April i.e Spring, Mid-April – June i.e Summer, July – September i.e Rainy, October – November i.e Pre-winter & December – February i.e Winter. Period of calving (PoC) was grouped as 4 periods i.e January 2009 - December 2011, January 2012 - December 2014, January 2015 - December 2017 & January 2018 - December 2020. Age at first calving (AFC) was divided into 3 groups i.e AG1 (<26.04 months), AG2 (From 26.04 to 31.08 months), AG3 (>31.08 months). These animals were classified with high milk fat and protein yield (225.02 kgs &162.61 kgs, respectively) and low milk fat and protein yield (147.06 kgs & 140.52 kgs, respectively) into two groups. Out of these 222 animals, 40 animals (20 animals from each group) were chosen for aseptic blood collection, DNA extraction and the further laboratory assessment.

About 5 ml of blood was aseptically collected from the jugular vein of adult HF crossbred cattle in proper EDTA-coated tubes and stored at 4°C. Qiagen -QIAamp DNA Blood Mini Kit (Qiagen, 50 samples) was used for genomic DNA extraction. The quality of this extracted DNA was assessed by using gel electrophoresis, while the purity (260/280nm absorbance) and concentration (ng/µL) were determined using a Nanodrop spectrophotometer. A reported set of sequence of forward & reverse primers i.e STATF(forward): 5'-CTGCAGGGCTGTTCTGAGAG-3 '& STATR(reverse): 5'-TGGTACCAGGACTGTAGCACAT-3' (Dario and Selvaggi 2011) was used to amplify the 215 bp STAT5A gene. A 25 µL reaction volume was used for carrying out this amplification process. This comprised of 12.5µL (1X concentration) of Go Taq coded Green PCR Master Mix from Promega, 0.5 µl (10 pmoles/µL) each of forward & reverse primers, 1 µL (100ng/µL) of the extracted genomic DNA & 10.5 µL of Nuclease Free Water (NFW). The programme for PCR amplification that was used is as follows: Initial denaturation process at 95°C for 5 min (1 cycle), cyclic denaturation, cyclic annealing and cyclic extension at 95°C, 55°C & 72°C for 45 sec, 30 sec and 30 sec, respectively for 30 cycles each. The final extension process was done at 72°C for around 5 min (1 cycle). A 4°C temperature was used for storage of the amplified PCR products. These were then screened and analysed using Agarose gel electrophoresis. The gel was then viewed using a proper gel documentation system. *AvaI* restriction enzyme was used to digest the PCR products at 37ºC for 1 hr. This restriction enzyme and gene combination was chosen on the basis of the reported set as used by Dario and Selvaggi (2011). The RE digest products were incubated

65°C for around 15 mins and then inactivated and stored at 4°C thereafter. Later, these products were sent for Sanger Sequencing. The chromatograms available after sequencing were firstly analysed for gene specificity by using the BLAST software. Finch TV and Clustal Omega softwares which are alignment tools for multiple sequences, were used to view and interpret these sequences thereafter for the possible detection of any SNP.

Ethics committee approval: The blood sample collection from 40 adult Holstein Friesian crossbred female cattle was permitted by IAEC i.e Institutional Animal Ethics Committee vide the proposal no. GADVASU/2021/IAEC/60/14 in the 60th IAEC meeting.

SAS, statistical analysis system programme, version 9.3, 2011 which used a GLM i.e General Linear Model to statistically analyse the corrected data for both the genetic as well as non-genetic factors). The following statistical model was designed for the LSA i.e least square analysis for estimating the genetic factors:

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

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

RESULTS AND DISCUSSION

This experimental research aimed to explore the relationship of STAT5A with milk fat and protein yields in a HF crossbred type cattle herd which are maintained in the Northern part of India. This gene was chosen to owing to its meaningful influence in milk production and composition traits as well as in signal transduction in mammary gland (Khatib *et al*. 2008). The results observed depicted clear restriction digestion bands in the 215 bp length of PCR products of STAT5A gene on a 2% agarose gel solution using gel electrophoresis procedure (Figure 1). The results of restriction fragment length polymorphism (RFLP) displayed an identical restriction pattern with noticeable bands at 181 bp and 34 bp for both the groups (Figure 2). All the animals revealed a uniform restriction pattern across both the groups. A monomorphic pattern and only one genotype (AA), for the cattle of both the groups was observed which is quite indicative of the homozygous nature of this gene and absence of any genetic variation for STAT5A in the crossbred population. Non-existence of variation indicates that the allele A is fixed in the animals of the HF crossbred population the said traits in this region. A similar study was performed by Krovvidi *et al*. (2021) on Murrah buffaloes, Ongole and crossbred cattle of Southern India where they found a higher content of milk fat for the CC genotype animals ($P > 0.05$) in the Jersey crossbred type cattle while in HF admixture and Ongole cattle, CT genotype showed a higher fat percentage as compared to CC genotypes. Their study revealed no significant associations of STAT5A with any of the studied traits in any of the studied breeds. Fixation of A allele in the studied population of crossbred animals was also observed. The difference in the research conducted by Krovvidi *et al.* (2021) and ours lies in the presence of a 34 bp fragment in our results which was common to animals of both the groups. A similar observation was stated by Daldaban *et al*. (2020) in Anatolian buffaloes. The fixation is attributable to environmental variations occurring overtime through several generations of the breed. However, STAT5A gene was reported to be polymorphic by Khatib *et al.* (2008) in cattle where they found two SNPs-SNP12195 and SNP14217 present in exon 8 and intron 9, respectively. The G allele of SNP12195 was found to be in a significant association with the decline in milk fat & protein percentages. Brym *et al*. (2004) reported noteworthy differences in milk fat & protein content in association with STAT5A in Jersey cattle. They found that the cows possessing AG and AA genotypes exhibited higher milk protein content while AA genotype animals had higher levels of content of milk fat compared to those with GG genotype. Selvaggi *et al*. (2013) in their experiment on STAT5A/*MslI,* PCR-RFLP on Jersey cattle of Italy observed insignificant differences among TC and TT genotypes considering milk protein and fat yields, despite of finding a T→C substitution in this gene. Similarly, Dario and Selvaggi (2011) studied STAT5A/*AvaI* polymorphism in Jersey cattle of Southern Italy and reported significant differences between yield of milk fat

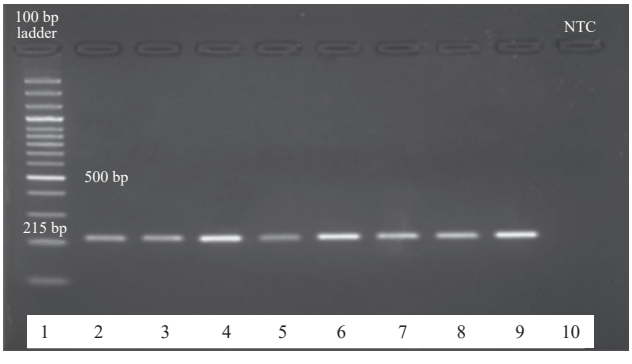

Fig. 1 Agarose gel (2%) picture showing bands at 215 bp of STAT5A PCR products (lane 2-9) along with a Non-Template Control (NTC, lane 10)

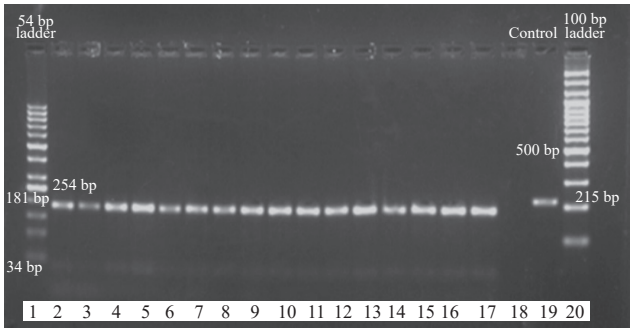

Fig. 2 PCR-RFLP pattern of STAT5A gene (lane 2-17 which reveal a monomorphic pattern with bands at 181bp and 34bp: AA genotype) and a Non-Template Control (NTC,lane 19) Lane 18 has been kept empty for spacing between previous lanes and NTC

at the level P<0.01 & milk protein at the level P<0.05 among CC and CT genotypes without much differences in their protein and fat contents. Also, Selvaggi *et al*. (2009) studied STAT5A/*AvaI* in Italian Brown cattle and observed significant differences among the two genotypes (CC and CT). They found that CC genotype cows produced milk with a higher protein content i.e 3.40% compared to that produced by cows with CT genotype i.e 3.21%. However, no noteworthy (significant) difference was reported in milk fat content of the two genotypes. In the experiment designed by Flisikowski *et al*. (2004) involving Polish Friesian cattle, they observed no relationship between STAT5A/*Msl*I and milk protein as well as fat content, and daily milk fat yield of the animals.

Al-Azzawi and Al-Dulaimi (2020) in their study on STAT5A gene in Holstein cattle in Iraq observed two genotypes CC and CT where they found that the milk fat & protein % of the CC genotype type animals was significant at the level P<0.05 for the month of July during their study. Along with this, no uncut DNA fragment was found in the studied population. Therefore, results observed among the animals of both groups were considered to be quite similar. The differences observed in our study as compared to both Azzawi and Dulaimi's studies can be attributed to the increasing practice of crossbreeding *Bos taurus* with indigenous cattle through artificial insemination, aimed at improving milk-related traits. This trend influences the genetic makeup of the crossbred population. Furthermore, the geographical and environmental variability across different regions of India contributes significantly to the observed diversity particularly between crossbred cattle in North and South regions of India. The animals used in our study were maintained under relatively controlled conditions at a livestock farm, which might have impacted the genetic composition across generations and may explain the presence of a fixed locus for the gene under investigation. Additionally, the history of selective breeding for desirable traits in various breeds is likely to alter allele frequencies in other parts of the genome due to the polygenic effects.

Although the STAT5A gene has a noteworthy role to play in milk fat & protein yields as well as physiological growth processes, limited research has explored the critical association between the STAT5Agene/AvaI RE polymorphism with milk related traits in particular with HF crossbred cattle. In our experimental research, all the individuals in the studied population revealed a fixed locus for STAT5A gene in relation to milk composition traits i.e milk fat & protein yields. STAT5A polymorphism/ monomorphism in HF crossbred cattle in relation to milk fat & protein yields could not be found in the literature. Although, STAT5A is potentially considered a strong candidate gene for understanding the complex mechanisms underlying milk composition traits and their exhibition in phenotypic expression in dairy animal species, the findings of the present research study, however did not support its use as a genetic marker for the milk fat & protein yield traits in connection to HF crossbred type (admixture) cattle. Further research is needed to explore the genomic architecture of this gene and to better elucidate its function as well as association with milk composition traits across different indigenous and exotic bovine breeds.

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