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Association of milk production traits with genetic variants in exon 5 and intron 3 of bovine growth hormone (bGH) gene in Sahiwal cattle

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ABSTRACT

The main objective of this study was to determine the association of production traits with genetic variants in exon 5 and intron 3 of bovine growth hormone (bGH) gene in Sahiwal cattle. The analyses were based on the detection of single nucleotide polymorphisms (SNPs) in GH-*Alu*I (exon 5) and GH-*Msp*I (intron 3) using the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) assay. The frequency of *Alu*I (L/V) and *Msp*I (T/C) alleles were 86.20/13.70 and 89.80/9.50, respectively. The distribution of the frequency of GH genotypes for LL, LV, and VV were 73.4, 25.5, and 1.0 and for TT, TC, and CC genotypes were 80.9, 17.7, and 13.0, respectively. Season of calving had none but period of calving had significant effect on the studied production traits. The phenotypic data was adjusted for non-genetic factors and regression analysis was done. There was detectable significant effect of the GH-*Alu*I and GH-*Msp*I on the analyzed production traits. The cows with LL genotype had significantly higher milk yield traits than the LV genotype. Similarly, the productive performance of the studied cows with TT genotype was also significantly higher than TC and CC genotypes. Results revealed that the Sahiwal cows with LL and TT genotypes of the bGH locus can be considered to be a favourable genotype for production traits, although these findings need to be confirmed by further research before SNPs can be used in a marker assisted selection program by the animal breeder.

Keywords: Bovine growth hormone, GH-*Alu*I, GH-*Msp*I, Production traits, Sahiwal cows

Functional traits are controlled by major and minor genetic loci therefore; linked candidate genes like growth hormone (GH) gene of the encoding loci are searched to optimize productive performance. GH is a major regulator of metabolism and postnatal growth in mammals, and it plays an important role in nutrient metabolism, tissue growth, milk production and reproduction of cows (Thidar *et al.* 2008). The metabolic and growth promoting actions of GH are primarily mediated by the insulin-like growth factor-1 (IGF-1) (Ramesha *et al.* 2015).

Many polymorphisms of the bovine GH (bGH) gene have been described and three polymorphic sites have been discovered within the fifth exon of this gene (Yao *et al.* 1996). Many researchers have studied the associations of GH gene polymorphisms with lactation traits in dairy cattle (Balogh *et al.* 2009a, Heidari *et al.* 2012) owing to the critical role of GH in growth, metabolism regulation, mammary gland development, and production (Mullen *et al.* 2010). A polymorphic site for GH-*Alu*I restriction endonuclease was identified due to cytosine to guanine transversion at position 2141. This transversion induced a change of leucine (L) amino acid into valine (V) amino acid sequence at position 127 of the bGH protein chain (Lucy *et al.* 1993). The genotype of GH-*Alu*I polymorphism was reported to be associated with milk yield and milk composition traits (Kovacs *et al.* 2006). The polymorphism which is digested by *Alu*I restriction endonuclease is located on the exon 5. However, GH-*Msp*I polymorphism is located in intron 3 of the GH gene at position 1547 (Zhang *et al.* 1993). As a consequence, two alleles occur, and *Msp*I contains a C–G transition at the C837 position and a T insertion at the C837 position (Lee *et al.* 1994). Until now, limited studies (Amiri *et al.* 2018) have been identified in association of GH-*Alu*I and GH-*Msp*I polymorphism with milk production traits. Based on the above mentioned evidence, the present investigation was carried out to identify GH-*Alu*I and GH-*Msp*I polymorphisms and uncover their association with productive traits in Sahiwal cattle.

MATERIALS AND METHODS

Experimental design: This study was conducted on the Sahiwal cows maintained at Livestock Research Centre (LRC) of National Dairy Research Institute (NDRI), Karnal, India. The data on first lactation production traits of 451 lactating Sahiwal cows over a period of 24 years (1992–

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2016) were recorded and used in this study. The data included in this study was collected from the history-cum-pedigree sheets and daily milk recording registers maintained at Animal Genetics and Breeding (AGB) Division. Information regarding animal number, date of calving, sire number, dam number, date of first calving, first lactation 305-day milk yield (FL305MY), first lactation total milk yield (FLTMY), and first lactation length (FLL) has been considered during data recording. The colostrum yield for the first 5 days after calving was not included in milk yield. The records of the cows with known pedigree and normal lactation were considered for this study. The cows with lesser than 500 kg of milk production and 100 days lactational length were excluded from this study. To ensure the normal distribution, the outliers were removed and data within the range of mean ± 2SD were only considered for this study.

Genomic DNA extraction, primers, PCR conditions and genotyping: Animal care procedures were approved and conducted under the established standard of the Institutional Animal Ethics Committee (IAEC). Out of 451 lactating Sahiwal cows, those Sahiwal cows crosses T6 (n=315) were used for genotyping. Among theses, genotypes of 226 Sahiwal cows were already present with Molecular Genetics Laboratory. Genotyping of rest Sahiwal cows were performed using isolation of genomic DNA and PCR-RFLP assay. Peripheral blood samples were collected by jugular venipuncture in the EDTA coated vacuutainer tubes (BD, Bioscience, India). Phenol-chloroform method, as guided by Sambrook and Russel (2001) with few modifications was used for DNA isolation. The quality and the quantity of DNA were checked by agarose gel electrophoresis (Maxi-Horizantal gel electrophoresis and power pack, GeNei, Bangalore, India) and nanodrop spectrophotometer (Bio-Rad Laboratories, India, Pvt. Ltd.), respectively. The ratio between OD_{260} and OD_{280} was calculated for each DNA sample. Sample with ratio of 1.8 or more was considered good and used for analysis.

In silico primer designing for target regions of bGH gene was carried out using Primer3 software available at NCBI database. Two sets of forward (GH-*Alu*I: GCTGCTCC-TGAGGGCCCTTCG and GH-*Msp*I: CCCACGGGC-AAGAATGAGGC) and reverse (GH-*Alu*I: GCGGCGG-CACTTCATGACCCT and GH-*Msp*I: TGAGGAACT-GCAGGGGCCCA) region specific oligonucleotide primers were designed. Digestion product size (bp) for GH-*Alu*I and GH-*Msp*I is LL 171, 52; LV 223, 171, 52; VV 223 and TT 224, 105; CT 329, 224, 105; CC 329, respectively. BLAST programme was used to check the specificity of the designed primers.

The used primer of *Alu*I *and Msp*I were designed to amplify a 223 and 329 bp fragments, respectively using the published DNA sequence of the bGH gene (Genbank Accession Number 280804, Hernandez *et al.* 2016). PCR reactions were performed in a total volume of 25 µL consisted of 0.50 µL forward primer, 0.50 µL reverse primer, 13.50 µL PCR master mix, 2.0 µL templates DNA (33.33 ng/µL), and 8.50 µL milli Q water. The PCR amplification was performed in programmed Thermal cycler (Bio-Rad PTC-200, India). The PCR cycling profile consisted of pre-denaturation at 95°C for 5 min, 40 cycles of denaturation at 94°C for 30 sec, annealing at 63°C (GH-*Alu*I) or 68.5°C (GH-*Msp*I) for 30 sec followed by a final expansion for 10 min at 72°C. The PCR amplification yielded 223 and 329 bp fragments that were revealed using electrophoresis on a 2% agarose gel.

RFLPs and SNP detection: The amplified PCR products were subjected to RFLP with a selected restriction endonuclease (*Alu*I and *Msp*I). PCR-RFLP reaction contained 10 µL of the PCR product, 2 µL of the reaction buffer, 0.3 µL of the restriction enzyme, 7.7 µL of autoclaved distilled water in a total volume of 20 µL incubated at 37°C for at least 16 h. Agarose gel (2.5%) electrophoresis with ethidium bromide was used to check the restricted PCR products. The agarose gels were photographed in the gel documentation system under the UV light for their respective genotypes according to band sizes. The forward and reverse sequences for each PCR fragments were assembled to form complete sequence for the respective region of bGH gene were visualized and edited using Bio Edit software. Each edited sequence with corresponding reference sequences were performed with Clustal W multiple sequence alignment programmes for DNA to identify SNPs (Larkin *et al.* 2007).

Calculations and statistical analysis: The significance of non-genetic factors was identified by least squares analysis (Harvey 1990). The effect of period of calving and season of calving was studied using following model and data were adjusted for significant non-genetic factors.

$$Y_{ijk} = \mu + S_i + P_j + e_{ijk}$$

where, Y_{ijk} is the observation on k^{th} individual belonging j^{th} period and i^{th} season of calving, μ is the overall population mean, S_i is the fixed effect of i^{th} season of calving, P_j is the fixed effect of j^{th} period of calving, and $e_{ijk}$is the random error associated with each observation which is assumed to be normally and independently distributed with mean zero and variance σ^2e. The pairwise comparison of means was carried out using "Tukey's honestly significant difference (HSD) test." Significance was determined at $P<0.05$ and the values are presented in the tables.

The seasons of calving were classified into S_1 (winter season, December to March), S_2 (summer season, April to June), S_3 (rainy season, July to September), and S_4 (autumn season, October to November). The total span of 24 years was divided into eight periods (P_1 to P_8). P_1 and P_8 have four consecutive years and P_2–P_7 have three consecutive years. Genotypic and allelic frequencies were calculated by using gene counting method (Falconer and Mackay 1996).

Association of bGH SNP genotype with first lactation traits in Sahiwal cow was analyzed using Fixed Model Least Squares Analysis (Harvey 1990). The significant effect of

SNP variants on first lactation traits were analyzed using the following model:

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

where, Y_{ij}, j^{th} observation on lactation traits of Sahiwal cow having i^{th} SNPs genotypes; μ, overall mean; G_i, effect of i^{th} genotype of SNP and e_{ij}, random error associated with Y_{ij} observation, and assumed to be NID (0, σ^2e).

RESULTS AND DISCUSSION

Gene and genotypic frequency: In the present study, 223 and 329 bp fragments from the bGH gene were characterized and successfully amplified from the DNA of each sample. This indicated a strong conservation of the DNA sequence existing in cattle. The findings of this study showed that the L allele was significantly ($P<0.05$) more frequent than the V allele (Table 1).

Table 1. Genotypic and allelic frequency of different genotype

Restriction endo-nuclease	Locus	Geno-type	Animal no.	Geno-typic frequency	Allele	Allelic frequency
*Alu*I	Exon 5	LL (216)	294	0.734	L	0.862
		LV (75)		0.255	V	0.137
		VV (3)		0.010		
*Msp*I	Intron 3	CC (4)	305	0.013	C	0.095
		CT (54)		0.177		
		TT (247)		0.809	T	0.898

The GH-*Msp*I frequency distribution showed that the occurrence of *Msp*I (TT) genotype was the most abundantly followed by *Msp*I (CT). The least frequently found genotype was *Msp*I (CC). The *Msp*1 (T) allele was more frequent than the *Msp*I (C) allele.

The PCR-RFLP results of the present study revealed the polymorphism in exon 5 of bGH gene with 2 alleles (L and V) and in intron 3 of bGH gene with 2 alleles (C and T). Similarly, 2 alleles at GH loci were reported by Pawar *et al.* (2007). However, Ferraz *et al.* (2006) identified 3 alleles at bGH-*Alu*I locus. In the present study, frequency found for allele L (0.862) was higher than that of allele V (0.137), which was in close agreement to the results of Ozkan *et al.* (2005) who found 0.84 and 0.16 allelic frequencies of allele L and V, respectively in Holstein Friesian cows. Results of Dybus (2002) and Vasconcellos *et al.* (2003) also observed similar L and V allele frequency that support the GH allele frequency data observed in our study. Allele and genotype frequencies for the GH-*Alu*I polymorphisms in the present study were lesser than those reported in previous studies (Amiri *et al.* 2018, Ozdemir *et al.* 2018).

The analysis of the restriction fragments using the *Msp*I enzyme originated two restriction patterns, viz. T and C alleles. The occurrence of TT genotype was more frequent as compared to the CC genotype hence; the presence of allele T was more frequent than the allele C. Genotypic and gene frequencies obtained in this study agree with those reported by Gorbani *et al.* (2009), who, in a population of 183 Holsteins, found frequencies of 0.787, 0.191, and 0.022 for TT, CT, and CC genotypes and 0.883 and 0.117 for T and C alleles, respectively. Arango *et al.* (2014) reported 0.91 and 0.09 allelic frequencies for respective T and C alleles and the genotype frequencies were 0.77, 0.20, and 0.03 for TT, CT, and CC genotypes, respectively in Holstein cows.

Non-genetic factors affecting production traits: Season of calving had none whereas, period of calving had significant ($P<0.05$) effect on the studied production traits (Table 2).

Trend followed by production traits during the different periods can be generalized as phases where first they increased then decreased and then again increased. However, the increase in later periods was lesser than that of the earlier period. Results revealed that FL305DMY, FLTMY, and FLL were found highest ($P<0.05$) during third period.

The findings of the present study revealed that least square mean of FL305DMY and FLTMY was similar to the earlier report of Verma *et al.* (2016). Contrary to the

Table 2. Least squares mean and standard error of production traits in Sahiwal cattle

Code	No. of observations	FL305DMY (kg)	FLTMY (kg)	FLL (days)
Overall mean				
μ	451	1756.61± 48.89	1873.05± 58.54	290.77± 5.42
Season of calving				
S_1	208	1754.50± 48.23	1864.75± 57.75	290.24± 5.34
S_2	163	1729.57± 54.85	1851.88± 65.67	284.93± 6.08
S_3	60	1775.31± 89.27	1892.34± 106.89	300.59± 9.90
S_4	20	1767.07± 158.44	1883.25± 189.71	287.31± 17.57
Period of calving				
P_1	55	1968.95^{e}± 100.63	2140.43^{e}± 120.49	315.86^{d}± 11.61
P_2	45	1977.33^{e}± 110.70	2048.31^{d}± 132.55	271.76^{b}± 12.28
P_3	45	2164.57^{f}± 109.13	2372.81^{f}± 130.66	320.41^{d}± 12.10
P_4	44	1594.30^{b}± 110.70	1704.54^{b}± 132.54	288.79^{c}± 12.27
P_5	67	1160.81^{a}± 95.86	1194.30^{a}± 114.78	253.58^{a}± 10.63
P_6	71	1678.59^{c}± 95.86	1814.82^{c}± 105.97	290.85^{c}± 9.81
P_7	49	1682.77^{c}± 99.12	1766.10^{b}± 118.68	293.19^{c}± 10.99
P_8	75	1825.57^{d}± 87.98	1943.11^{d}± 105.35	291.69^{c}± 9.75

Means bearing different superscript in a column differs significantly ($P<0.05$).

findings of the present study, FL305DMY was higher in study that reported by Ratwan *et al.* (2019). In the present study, FLTMY was lower as compared to that reported by Dongre *et al.* (2013) and it was higher than that reported by Ratwan *et al.* (2019). The overall least square mean of FLL in our study was closely similar to the findings of Manoj *et al.* (2012). On the contrary, FLL was lower than that reported by Dahlin *et al.* (1998) and higher than that reported by Singh and Singh (2016).

Season of calving had non-significant effect on FL305DMY as reported by Pandey *et al.* (2019), and Ratwan *et al.* (2019). FLTMY was also found to be non significant and according to the findings reported by Singh and Singh (2016) and Ratwan *et al.* (2019). The effect of season of calving on FLL was found to be non significant. Similar observation was reported by Singh and Singh (2016), and Ratwan *et al.* (2019) but significant by Dhawan *et al.* (2015). The non significant influence of season on the production traits may be due to better management practices throughout the year in all seasons and also because adaptability of Sahiwal cattle is better under local climatic conditions.

The period of calving had significant effect on the studied productive traits. The significant effect of period of calving on FL305DMY was also reported Singh and Singh (2016), and Pandey *et al.* (2019) in dairy cows. Ratwan *et al.* (2019) also observed significant effect of period of calving on FLTMY in Sahiwal cows. However, non significant effect of period of calving on FLTMY was reported by Singh and Singh (2016). Alike to the present study, Singh and Singh (2016), and Ratwan *et al.* (2019) also noted a significant effect of period of calving on FLL in Sahiwal cows. The decrease in the performance in the middle periods might be due to increased focus given to cross breeding as compared to the improvement of indigenous cattle. The significant effect indicated the environmental changes and the change in managemental practices during these periods which caused the variations in production traits.

Association of bGH gene with production traits: As shown in Table 3, GH-*Alu*I had significant (P<0.05) effect on FL305DMY, FLTMY, and FLL.

Table 3. Association of bGH-*Msp*I and bGH-*Alu*I polymorphism with lactation traits

Locus	Geno-type	No. of observ-ations	First Lactation Traits		
			FL305 MY	FLT MY	FLL
bGH-*Alu*I	LL	155	$1622.48^{b} \pm 67.97$	$1819.62^{b} \pm 80.37$	$265.15^{b} \pm 9.64$
	LV	62	$1380.08^{a} \pm 107.47$	$1469.52^{a} \pm 127.08$	$227.96^{a} \pm 15.25$
bGH-*Msp*I	TT	43	$1964.32^{b} \pm 128.74$	$2230.23^{b} \pm 154.51$	$313.23^{b} \pm 18.31$
	TC	42	$1418.95^{a} \pm 130.26$	$1582.53^{a} \pm 156.34$	$242.56^{a} \pm 18.52$
	CC	136	$1457.36^{a} \pm 72.39$	$1600.12^{a} \pm 86.88$	$238.58^{a} \pm 10.29$

The replacement of the V allele by the L allele was found to be responsible for an increase in the productive performance. Therefore, cows with the LL genotype had the highest FL305DMY, FLTMY, and FLL compared with cows having LV genotypes. VV was genotype not considered in this study due to their low incidence (n=3) and higher error variance. Few studies have investigated the relationship between GH-*Alu*I and GH-*Msp*I polymorphisms and productive traits in dairy cattle. Similarly, Dybus (2002) in Black and White cattle also suggested an additive effect of the L allele on production traits with LL genotype cows yielding more milk than LV and VV genotype. Contrary to the above mentioned studies and to us, Zwierzchowski *et al.* (2001) observed that V allele had no effect on milk yield in Holstein cattle. Sonmez *et al.* (2018) also noted non significant relationship between the mean lactational milk yield, peak daily yield, 305 days milk yield, and lactation length in different genotypes Holstein cows.

There was detectable significant (P<0.05) effect of the GH-*Msp*I on the production traits analyzed. The replacement of the C allele by the T allele was found to lead to an increase in the FL305DMY, FLTMY, and FLL. The productive performance of the cows with TT genotype was significantly higher (P<0.05) compared with similar values of the TC and CC genotypes.

In a study conducted by Hoj *et al.* (1993) it was observed that cows with T allele showed higher milk production compared to cows with C allele. Accordingly, Yao *et al.* (1996) in Holstein cows also found better milk yield in T allele than C allele. *Msp*I cows with TT genotype are considered to yield more milk, protein and fat than TC genotype (Zhou *et al.* 2006). However, a non-significant association was reported between GH-*Msp*I and 305-day milk yields in Egyptian Baladi cows (El-Nahas *et al.* 2018).

In conclusion, *Alu*I and *Msp*I polymorphisms of the bGH gene were identified in a population of Sahiwal cows which displayed L and T allele dominance. Cows with LL and TT genotypes showed better productive performance than cows with other genotypes. Although the confirmation of our findings needs further analysis on other herds, the association of genotypes with better milk production is a very interesting finding and could be used in marker assisted selection to improve the animals which will ultimately lead to higher milk production.

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