GMR Genetics and Molecular Research
Online Journal
ISSN:1676-5680

Effect of two single nucleotide polymorphisms on milk yield and composition

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Genet. Mol. Res. 12 (2): 995-1004 (2013)
Received April 24, 2012
Accepted September 25, 2012
Published April 2, 2013
DOI http://dx.doi.org/10.4238/2013.April.2.15

ABSTRACT. The bovine prolactin (*PRL*) and bovine growth hormone (*bGH*) genes exhibit several polymorphisms. Some of them can be detected by molecular techniques using restriction endonucleases, such as *Rsa*I for the *PRL* gene and *Msp*I for the *bGH* gene. We examined the relationship between the *PRL-Rsa*I and *bGH-Msp*I polymorphisms and some economically important characteristics of Holstein cows. Research was conducted on 315 Holstein cows from 5 municipalities in the Department of Antioquia, Colombia. Individuals were genotyped using PCR/RFLP. The statistical analysis was carried out using generalized linear models and a regression analysis. Polymorphism of the *bGH* gene was found to have a significant association with the percentage of protein in milk and milk yield. Genotype (-/-) was favorable for dairy yield, while genotype (+/+) was favorable for protein percentage. The *PRL* gene showed no significant association with any of the evaluated characteristics. The *bGH* gene appears to be a candidate for the implementation of marker-assisted selection programs. To determine the effect of the prolactin gene, research should

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be conducted with a larger sample size and a group of animals with more balanced genotypes.

Key words: Genetic association; Molecular markers; Holstein cows; Genetic polymorphism

INTRODUCTION

Genetic improvement is a strategy that uses tools from quantitative and molecular genetics to enhance milk yield in some populations. One such tool is the implementation of marker-assisted selection programs that, supported by classic methodologies, lead to developments in milk quantity and the quality of milk components. Achieving such development is faster through these programs than through the use of individual classic methodologies (Lagziel et al., 1999). The development of selection programs depends on the identification of polymorphic regions within genes associated with specific physiological processes. It also depends on the subsequent identification of the effects of polymorphisms on the production characteristics of interest (López et al., 2011).

Some potential candidate genes have been identified based on the relationship between physiological and biochemical processes and production parameters (Oprzadek et al., 2003). As a result, we have been able to identify hormones that are highly important for breast-feeding and pregnancy, thus affecting dairy yield and its quality. Two of the most important hormones for these processes are bovine growth hormone (bGH) and bovine prolactin (PRL) (López et al., 2011).

The *bGH* gene is located on chromosome 19 (19q26) and has 2800 bp, 5 exons, and 4 introns (Zakizadeh et al., 2006). It generates a single peptide of 190 or 191 amino acids with a molecular weight of 22 kDa. A single nucleotide polymorphism (SNP) occurs in intron 3 of the *bGH* gene, and it can be identified using the polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP) technique with the *Msp*I restriction endonuclease, which generates three genotypes: homozygote (+/+), heterozygote (+/-), and homozygote (-/-) (Dybus, 2002).

The *PRL* gene is located on chromosome 23. It has 5 exons and 4 introns and codes for a mature protein of 199 amino acids weighing approximately 23 kDa (Cabrera et al., 2000). This gene has several polymorphisms, among which one stands out: an SNP that can be recognized with the *Rsa*I restriction endonuclease using the PCR-RFLP technique (Dybus, 2002). This technique identifies two allelic variants (A and B), thus generating three distinct genotypes: homozygote (AA), heterozygote (AB), and homozygote (BB). These genotypes have been associated with economically important characteristics in milk production (Alipanah et al., 2007).

Several studies have found associations between polymorphisms in the *bGH* and *PRL* genes and production characteristics that are relevant for milk yield. However, the results have been inconsistent because the effect of the favorable genotypes shown in the studies varies depending on the geographic location and environmental conditions of the area in which the study is conducted (Zhou et al., 2005). Therefore, the goal of our study was to determine the association between the *bGH-Msp*I and *PRL-Rsa*I polymorphisms with economically relevant characteristics in Holstein cows from the Department of Antioquia (Colombia) to contribute to the implementation of marker-assisted selection programs.

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MATERIAL AND METHODS

Animals

A total of 315 Holsteins were selected from 12 specialized dairy herds in different municipalities in the Department of Antioquia. The elevations at these locations ranged between 2000 and 2600 m with an average temperature between 13° and 17°C (Table 1). When selecting animals and herds, we took into account herds that had reliable information on pedigree and belonged to a recognized dairy yield control program.

Table 1. Distribution of animals by municipality and farm.

Municipality	No. of herds	No. of animals
San Pedro	4	75
La Unión	3	64
Bello	2	70
Medellín	2	51
Entrerrios	1	55
Total	12	315

Milk yield control

Three hundred and fifteen cows with information on pedigree, dairy yield (kg), fat (%), protein (%), and somatic cell count (SCC) were used. Information was collected by weighing herd production. Similarly, homogenized samples (5 to 10 mL) were taken from the total dairy yield per cow during milking. These samples were preserved using 2-bromo-2-nitropropane-1,3-diol to prevent decomposition during transportation to the laboratory. Once in the quality control laboratory, samples were evaluated using a MILKOSCAN FT 120™ (FOSS Analytical, Hillerod, Denmark) to determine the fat and protein percentages. The SCC was determined using a FOSSOMATIC 215™ (FOSS Analytical) automatic quantifier. The obtained SCC values were subsequently transformed into somatic cell score (SCS) using a mathematical approach described by Ali and Shook (1980) [LOG_2 (SCC / 100,000) + 3] to simulate statistical normality in the data. Based on the weights obtained during the periodic production control, cows that completed the lactation process were selected, and the total yield was calculated and adjusted for 305 days.

Genotyping

DNA was extracted from 315 peripheral blood samples using a salting out method modified by Miller et al. (1988). It was then stored in 1X TE buffer (Tris-ethylenediaminetetraacetic acid) at 4°C. Afterward, the purity and concentration of the genomic DNA were determined through the ratio of absorbance at two wavelengths (260/280 nm) using a Nanodrop 2000c (Thermo Scientific®, Wilmington, USA). Only genomic DNA with purity greater than 1.8 and less than 2 was used per recommendations for obtaining pure DNA.

Given the sequences for the *bGH* and *PRL* genes and taking into account reports by Dybus (2002) and Brym et al. (2005), we synthesized oligonucleotides that enabled the ampli-

fication of 329- and 294-bp fragments inside intron 3 and exon 4 of the *bGH* and *PRL* genes, respectively; these amplifications contained the restriction sites for the *Msp*I and *Rsa*I restriction endonucleases, respectively. The synthesized primers are shown in Table 2.

Table 2. Primers used for amplifying the 329-bp fragment in *bGH* and the 294-bp fragment in *PRL*.

	bGH primers	PRL primers
Forward	5'-CCCACGGGCAAGAATGAGGC-3'	5'-CCAAATCCACTGAATTATGCTT-3'
Reverse	5'-TGAGGAACTGCAGGGGCCCA-3'	5'-ACAGAAATCACCTCTCTCATTCA-3'

Each specific region was amplified individually. For the *bGH* gene, amplification proceeded via PCR with a final volume of 25 µL containing 30 to 60 ng genomic DNA and 2.5 µL 10X PCR buffer (1.0 to 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl, pH 8.3) in a final 1X concentration with 2 mM $MgCl_2$, 0.2 µM of each primer, 0.4 mM of each deoxyribonucleotide triphosphate, and 0.5 to 1 U Taq polymerase (Bioline®, Randolph, MA, USA). For the *PRL* gene, PCR was carried out in a final volume 25 µL, which contained 30 to 60 ng genomic DNA, 2.5 µL 10X PCR buffer, 1.5 mM $MgCl_2$, 0.15 µM of each primer, 0.5 mM of each deoxyribonucleotide triphosphate, and 0.5 to 1 U Taq polymerase.

The conditions for PCR were denaturation at 95°C for 6 min, denaturation at 95°C for 1 min, annealing for 30 s at 60°C for the *bGH* gene and at 57°C for the *PRL* gene, and extension for 40 s at 72°C. The process was repeated from step 2 for 39 cycles with a final 5-min extension at 72°C. PCR was performed in a thermocycler (Biometra®, Goettingen, Germany).

PCR products were resolved with electrophoresis on 2% agarose gel (Amresco®, Cochran Road, OH, USA) and stained with ethidium bromide (Promega®, Madison, WI, USA). Each well had 5 µL PCR product diluted in 2 µL 1X loading buffer (Fermentas®, Glen Burnie, MD, USA), and 2 µL marker with very low molecular weight was used (low range; Fermentas®). Gels were examined using gel photodocumentation equipment (Biometra®) to obtain photographs. In all cases, the PCR included a negative control without DNA and a positive control with previously amplified DNA.

For the digestions, 2 µL 10X Tango buffer was used to obtain a final 1X concentration and a reaction volume of 20 µL. Additionally, 5 U corresponding enzyme was added with ultrapure water until the mixture reached 15 µL. Then, 5 µL PCR product was used. Once the mix was complete, it was subjected to digestion for 3 h at 37°C. The enzyme in this reaction was used in excess to avoid the formation of false heterozygotes owing to partial digestion of the PCR product. All digestions included a positive control that had the restriction site on both alleles to avoid the generation of false positives or negatives.

Digestion products were resolved through electrophoresis on 2.5% agarose gel in 1X TBE buffer (0.05 M ethylenediaminetetraacetic acid, 0.089 M Tris-base, and 0.089 M boric acid) and stained with ethidium bromide. Each well contained a 15-µL digestion product diluted in 5 µL 1X loading buffer and 2 µL marker with very low molecular weight. Gels were examined under ultraviolet light using gel photodocumentation equipment and digitally photographed.

Statistical analysis

Evaluations for the *bGH* and *PRL* genes were carried out with 307 and 302 genotypes,

respectively, as genotyping some animals for both genes was impossible. The assumptions of normality and homogeneity of the variances were validated through various procedures using the SAS® software (SAS Institute Inc., Cary, NC, USA). Genotypes were represented as 0, 1, and 2 and corresponded to AA, AB, and BB, respectively, for the *PRL* gene. For the *bGH* gene, alleles were represented as 0, 1, and 2 and corresponded to +/+, +/-, and -/-, respectively. Statistical analysis was conducted using a generalized linear model for each of the dependent variables (dairy yield, fat percentage, protein percentage, and SCS). The difference between means was evaluated through the Duncan multiple range test. The general model used for this analysis was the following:

$$Y_{ijklmn} = \mu + NP_i + H_j + Gh_k + Gp_l + PL_m + e_{ijklmn}$$

where Y_{ijklmn} is the dependent variable (dairy yield through lactation, protein percentage, fat percentage, or SCS), μ is the mean for the characteristic, NP_i is the fixed effect of lactation number (l = 1 . . . 7), H_j is the fixed effect of herd m (m = 1 . . . 12), Gh_k is the fixed effect of the genotype for *bGH* (i = 0, 1, 2), Gp_l is the fixed effect of the genotype for *PRL* (j = 0, 1, 2), PL_m is the covariate of the effect of milk yield (only for fat percentage, protein percentage, and SCS), and e_{ijklmn} is the experimental error.

Effects of allele substitution

The second methodology used to establish the linkage between the markers and the quantitative trait loci was a regression analysis between each of the dependent characteristics corrected by herd, lactation number, and genotype. The genotypes were previously transformed into a quantitative variable to determine the additive effect for each of the alleles (+/+ = 0, +/- = 1, and -/- = 2). This effect was determined via the REG procedure of the SAS® software. Thus, using a regression analysis, we determined the effect of substituting each (+) allele for a (-) allele on the dependent characteristic. The following is the general model used for this analysis:

$$Y = \beta_0 + \beta_1 X_i$$

where Y is the dependent variable (dairy yield, fat percentage, protein percentage, or SCS), β_0 is interception, β_1 is the regression coefficient estimated for the genotype effect, and X_i is the genotype of the individual (0. . .2).

RESULTS

The 294- and 329-bp fragments for the *PRL* and *bGH* genes, respectively, were correctly amplified from the DNA samples. The amplified DNA fragments were properly digested for the *PRL* and *bGH* genes using the *Rsa*I and *Msp*I restriction enzymes, respectively. Given the aforementioned information, three genotypes were possible for *PRL*: an AA homozygote, which had only one fragment of 294 bp; an AB heterozygote, which had three fragments of 294, 162, and 132 bp; and a BB homozygote, which had two fragments of 162 and 132 bp (Figure 1).

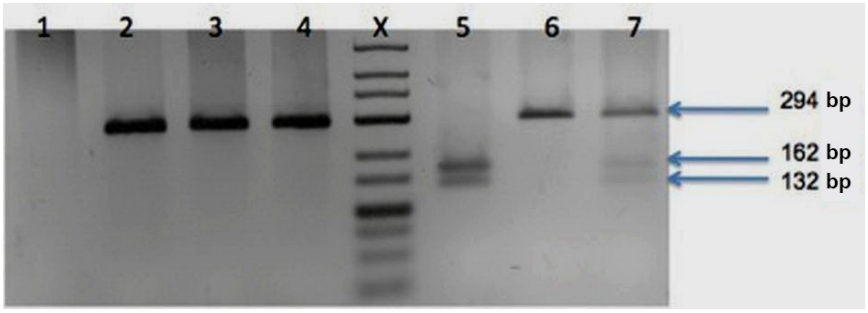

Figure 1. Amplified fragment and digestion of a region of the bovine prolactin gene (*PRL*) using the *Rsa*I restriction enzyme. *Lane X* = molecular weight marker; *lane 1* = negative control; *lanes 2*, *3*, and *4* = 3 samples of DNA amplified for the *PRL* gene; *lane 5* = restriction pattern for the BB homozygote genotype; *lane 6* = restriction pattern for the AA homozygote genotype; *lane 7* = restriction pattern for the AB heterozygote genotype.

Individuals exhibited three genotypes of the *bGH* gene - namely, (+/+), (+/-), and (-/-) - according to the generated banding pattern. Allele (-) showed no cleavage site and generated a fragment measuring 329 bp. Allele (+) generated two fragments of 224 and 105 bp (Figure 2).

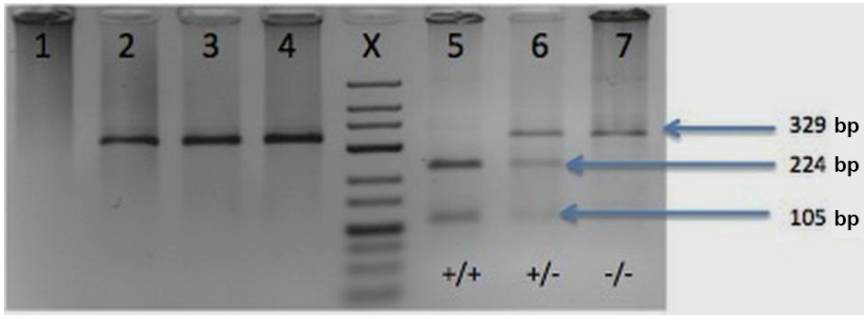

Figure 2. Amplified fragment and digestion of a region of the bovine growth hormone (*bGH*) gene using the *Msp*I restriction enzyme. *Lane X* = molecular weight marker; *lane 1* = negative control; *lanes 2*, *3*, and *4* = 3 samples of DNA amplified for the *bGH* gene; *lane 5* = restriction pattern for the (+/+) homozygote genotype; *lane 6* = restriction pattern for the (+/-) heterozygote genotype; *lane 7* = restriction pattern for the (-/-) homozygote genotype.

Table 3 shows the effects of *PRL* and *bGH* gene polymorphisms on each of the evaluated characteristics. Different letters indicate significant statistical difference ($P < 0.05$).

The *Msp*I polymorphism of intron 3 of the *bGH* gene had a significant effect on dairy yield and protein percentage ($P < 0.05$). Genotype (-/-) is better for dairy yield, with a measured mean of 6603.3 ± 1619.5 kg. In addition, genotype (+/+) was superior to genotype (-/-) in terms of the percentage of milk protein, with means of 3.02 ± 0.2538 and 2.82 ± 0.1203, respectively. No significant difference ($P < 0.05$) was found between genotypes (+/+) and (+/-); therefore, allele (+) is favorable for percentage of milk protein, and its effect is more relevant when the allele is a homozygote. The genetic polymorphism evaluated in the *bGH* gene showed no significant association with either the percentage of fat or SCS. However,

individuals with genotype (-/-) displayed the lowest means, suggesting that allele (+) tends to increase the amount of solids, whereas allele (-) tends to favor dairy yield.

Table 3. Effect of the polymorphisms of the *PRL* and *bGH* genes on dairy yield and composition.

Gene	Genotype	N	Milk yield (kg)	% Protein	% Fat	SCS
PRL	AA	229	5247.5 ± 1466.4[a]	3.00 ± 0.2380[a]	3.83 ± 0.4515[a]	3.84 ± 0.8691[a]
	AB	71	5214.5 ± 1560.7[a]	2.98 ± 0.2432[a]	3.73 ± 0.4388[a]	3.90 ± 0.9171[a]
	BB	7	4657.1 ± 1824.5[a]	3.09 ± 0.3474[a]	3.91 ± 0.3320[a]	3.39 ± 0.7290[a]
bGH	+/+	219	5260.8 ± 1597.0[a]	3.02 ± 0.2538[a]	3.81 ± 0.4536[a]	3.82 ± 0.8864[a]
	+/-	79	5165.1 ± 1329.1[a]	2.94 ± 0.2220[ab]	3.84 ± 0.4583[a]	3.90 ± 0.8627[a]
	-/-	4	6603.3 ± 1619.5[b]	2 .82 ± 0.1203[b]	3.57 ± 0.3693[a]	3.93 ± 1.295[a]

Data are reported as means ± SD. Means followed by different superscript letters are sigficant at $P < 0.05$ (Duncan multiple range test). SCS = somatic cell score.

The evaluated *PRL* gene polymorphism had no significant effect on any of the evaluated characteristics ($P > 0.05$). Nevertheless, the effect of this polymorphism nearly reached significance in terms of its association with protein percentage and dairy yield, showing a favorable trend for the protein percentage of the BB genotype with a mean of 3.91 ± 0.3320. The most important genotype in terms of dairy yield was genotype AA, which had a mean of 5247.5 ± 1466.4 kg.

Effects of allele substitution

The β value for milk yield was +254 kg, i.e., every time a (+) allele is changed into a (-) allele, new individuals could be expected to produce 256 kg milk above β_0 (Table 4). In addition, the value of β for the protein percentage was -0.078%; therefore, the substitution of one (+) allele is expected to result in new offspring whose milk has -0.078% less protein than β_0 (see Table 4).

Table 4. Effect of substituting a (+) allele for a (-) allele in the *bGH* gene on dairy yield and protein percentage.

Characteristic	% Protein	Milk yield (kg)
Intercept (β_0)	3.014	5237.75
Standard error	0.029	103.30
Effect (β)	-0.078	254.52
Standard error	0.016	94.95

The allelic substitution effect was not determined for *PRL*, as none of the evaluated genotypes showed a significant effect on the production characteristics in question (milk yield, fat percentage, protein percentage, and SCS).

DISCUSSION

The study of candidate genes is one of the most effective methods for determining whether specific genes are related to economically important parameters for agricultural production. Two of these genes are *bGH* and *PRL*, which are promising not only because of their key role in mammary gland development and lactation but also because they have some polymorphisms that are associated with economically important traits for milk yield.

The influence of the studied *bGH* gene polymorphism on milk yield was significant,

and cows with the (-/-) genotypes were more productive than those with the other genotypes. These results differ from those obtained by Echeverri et al. (2010a), who studied a population of Colombian Holstein cows and found no association with milk yield. Their study, however, was conducted on a smaller sample. Our results are consistent with those of Lagziel et al. (1999), who found that allele (+) has a negative effect on milk yield. Nevertheless, the authors disagree in general with most accounts from different parts of the world that attribute a positive effect of allele (+) on milk yield; the authors consider the genotype (+/+) to be superior (Zhou et al., 2005; Pawar et al., 2007).

The *bGH-Msp*I polymorphism also had a significant effect on milk protein percentage, and allele (+) was favorable; therefore, milk from individuals with genotype (+/+) had the highest protein percentage. Nonetheless, no significant differences were found between this genotype and genotype (+/-). Similar results have been reported in dairy cattle (Dybus, 2002; Zhou et al., 2005). However, some authors have found allele (-) to be superior for protein percentage in milk. Consequently, milk from individuals with allele (-/-) is superior in terms of protein percentage (Falaki et al., 1996; Lagziel et al., 1999). Echeverri et al. (2010a) have reported no significant association between genotype and protein percentage in a Holstein cow population in Antioquia, but they did find a trend favoring allele (+).

No significant differences were found for the association of the *bGH* gene polymorphism and fat percentage. Other authors have obtained similar results (Echeverri et al., 2010a), but most research has found significant associations (Mattos et al., 2004; Zhou et al., 2005). No significant association was found between the *bGH* gene polymorphism and SCS. This result is consistent with accounts provided by Yardibi et al. (2009), who found no significant effect of the *Msp*I polymorphism on SCS in milk. Conversely, Lagziel et al. (1999) found a significant effect ($\alpha = 0.1$) of this polymorphism, which reduced SCC in individuals with allele (-).

The influence of the *Rsa*I polymorphism in the *PRL* gene on the evaluated characteristics was not significant. Although some trends were observed, the sample did not make it possible to show any significant difference between the various genotypes. Similar results have been obtained elsewhere under different circumstances (Lu et al., 2010; Echeverri et al., 2010b), but most studies have found a significant association, and the superior genotype for each economically important characteristic varies (Brym et al., 2005; Alipanah et al., 2007; Mehmannavaz et al., 2009).

The allele substitution effect in the *bGH* gene, i.e., substituting a (+) allele for a (-) allele was +254 kg for dairy yield and -0.078% for protein content. These values are highly variable and depend on the breed and the region and its productive characteristics evaluated.

This study on the *PRL* polymorphism was conducted in 307 genotypes, and the sample size may have been inadequate for proper identification of the association between genotypes and production characteristics. Therefore, we recommend further evaluation using a larger set of data to obtain a more reliable assessment of the effect of this polymorphism on other factors. Our results suggested a trend that neared significance in favor of a particular allele, mainly regarding dairy yield and protein percentage. Furthermore, reports show that most of the association analyses that have been conducted in several places throughout the world using the bovine *PRL* gene have found a significant effect of the different genotypes on at least one economically important characteristic for dairy yield (Brym et al., 2005). Despite these findings, reports exist in which the association and effect of allele substitution were not significant (Chrenek et al., 1999).

In conclusion, most of the Holstein cows in Antioquia had genotype (+/+) for the *bGH* gene, which is associated with higher protein levels. The presence of this genotype is likely a result of indirect selection processes that have been conducted over several years in the specialized dairy industry in the region. Additionally, individuals with allele (-/-) were superior in terms of dairy yield, but they had a lower proportion overall within the population. Current results postulate that the *bGH* gene polymorphism is an interesting target for marker-assisted selection programs and for inclusion in the genetic evaluation of bulls and cows. The association of the *Rsa*I polymorphism of the *PRL* gene shows a trend close to statistical significance for dairy yield ($P < 0.11$) and protein percentage ($P < 0.1$). Hence, it is reasonable to suppose that if sample size were increased, the polymorphism would show a real effect on these characteristics, thus favoring implementation in genetic evaluation programs for Holstein cattle. Evaluation of these molecular markers is an effort to understand the association between polymorphisms and some economically important characteristics and constitutes the first step toward understanding each population to identify and promote the use of molecular markers for the genetic improvement of animals.

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