

*Full Length Research Paper*

# Effects of bovine prolactin gene polymorphism within exon 4 on milk related traits and genetic trends in Iranian Holstein bulls

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Accepted 10 August, 2009

**In this study, polymorphism of prolactin gene was analyzed as a candidate gene responsible for variation and genetic trends in milk yield and composition traits. Genomic DNAs were extracted from 268 semen samples belonged to Iranian Holstein bulls. Genotyping for the prolactin gene using PCR-RFLP technique and *RsaI* restriction endonuclease showed a mutation in 294-bp fragment located in exon 4. The frequencies of A and G alleles were 0.069 and 0.931, respectively. The allelic substitution effect was significant for milk and protein yield ( $p < 0.05$ ). The G allele was unfavorable for milk and protein yield. Genetic trends for all analyzed traits were significant ( $p < 0.01$ ) and that was progressive for milk, fat and protein yield, but diminishing for fat and protein percent. The effects of prolactin SNP on genetic trends and the difference between genetic trends produced by A and G alleles were not significant for all studied traits.**

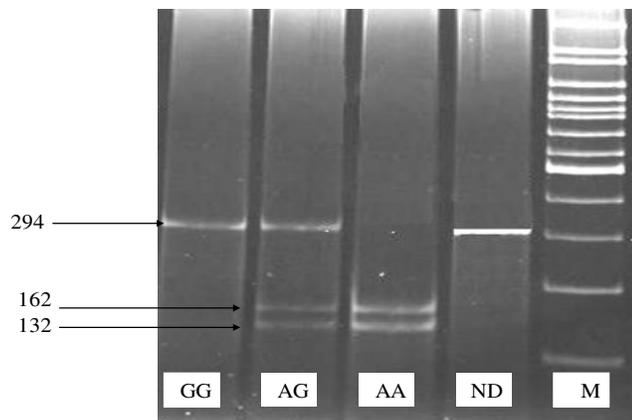
**Key words:** Holstein, prolactin, milk, genetic trend, SNP, PCR-RFLP.

## INTRODUCTION

Prolactin is a polypeptide hormone, synthesized and secreted mainly by the special cells (lactotrophs) in the anterior pituitary gland. Furthermore, it is produced by numerous other cells and tissues, including the mammary gland (Bole-Feysot et al., 1998). Prolactin gene disruption experiments proved its essential roles in mammary gland development (mamogenesis), lactogenesis, maintenance of milk secretion (galactopoiesis) and expression of milk protein genes (Horseman et al., 1997). It is also primarily responsible for synthesis of lactose, lipids and all other major components of milk (Le Provost et al., 1994). Therefore, the bovine prolactin gene (PRL) seems to be an excellent candidate for linkage analysis with quantitative trait loci (QTL) affecting milk production traits. The gene has been mapped on chromosome 23 (Hallerman

et al., 1988) and it is 10 kb in size, consisting of 5 exons (Camper et al., 1984) encoding a 229-amino-acid prolactin precursor (Cao et al., 2002). The signal peptide contains 30 amino acids; thus the mature bovin prolactin is composed of 199 amino acids (Cao et al., 2002). Extensive genetic polymorphism studies were carried out, finding several (more than 20) SNPs within the bovine PRL structure gene sequence, although all of them were silent mutations or located within introns (Sasavage et al., 1982; Brym, 2004; Brym et al., 2007). Nevertheless, one of them (recognizing by *RsaI* endonuclease) has become a popular genetic marker used for genetic characterization of cattle populations using PCR-RFLP (Mitra et al., 1995; Chrenek et al., 1998; Udina et al., 2001; Wojdak et al., 2008; Sacravarty et al., 2008). This marker was also previously used to study possible associations between prolactin gene variants and milk performance traits (Chung et al., 1996; Dybus, 2002; Khatami et al., 2005; Dybus et al., 2005; Brym et al., 2005; Alipanah et al., 2007).

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**Figure 1.** The 294 bp PCR product of PRL/exon4 was digested with *RsaI* on 8% acrilamid gel electrophoresis stained with ethidium bromide. GG = 294 bp fragment; AA = restriction fragment of 162 and 132 bp; AG = restriction fragment of 294, 162 and 132 bp; ND= undigested 294-bp PCR product. M = 100 bp ladder.

Genetic trend for each trait is affected by changes in allelic frequencies of genes contributing to the trait. It seems that candidate genes may have a noticeably effect on genetic trends, but, it has not up to now been studied.

Based on important role of prolactin gene in milk related traits and their genetic trends in dairy cattle, the aims of this study were, to screen Iranian Holstein bulls for mutation in the 4<sup>th</sup> exon of prolactin gene and to evaluate the mutation effect on milk performance and paternal path genetic trends.

## MATERIALS AND METHODS

### Samples

Semen samples were collected from 268 progeny-tested Holstein bulls born from 1990 to 2006. They were obtained from Animal Breeding Center of Iran (Karaj, Iran). Genomic DNA from semen samples were extracted as previously described by Zadworny and Kuhlenlein (1990).

### Genotyping

The PCR was carried out according to Brym et al. (2005). Briefly, in 25  $\mu$ L of a mix containing 2.5  $\mu$ L 10X PCR buffer; 0.5  $\mu$ L dNTP (2 mM each); 100 pmol of each primer: forward 5' CCAAATCCACTG AATTATGCTT 3', reverse 5' ACAGAAATCACCTCTCTCATTCA 3'; 1.2 mM MgCl<sub>2</sub>; 0.8 unit *TaqI* DNA polymerase; 100-600 ng of genomic DNA; and H<sub>2</sub>O up to 25  $\mu$ L. The PCR reaction was carried out in an Eppendorf thermocycler under the following conditions: initial denaturation (94°C/3 min), 35 cycles of denaturation (94°C/30 s), annealing (58.5°C/30 s) and extension (72°C/30 s) and final synthesis (72°C/5 min). In order to genotype each bull, 10  $\mu$ L of PCR product was digested with 10 unit of *RsaI* restriction endonuclease and analyzed by electrophoresis in 8% acrilamid gel with ethidium bromide.

## Statistical analysis

The allele frequencies were estimated by simple allele counting according to the Hardy-Weinberg equilibrium (Falconer and Mackay, 1996); the possible deviations of genotype frequencies from their expectations were analyzed using chi-square ( $\chi^2$ ) test.

The relations between the detected genotypes and estimated breeding values (EBV) for milk production traits namely, milk yield (kg), fat percent (%), fat yield (kg), protein yield (kg) and protein percent (%) were analyzed. Estimated breeding values were provided by Animal Breeding Center of Iran. The analysis was based on the following model:

$$EBV = b_0 + b_1x_i + e_{ij}$$

Where, EBV = the estimated breeding value for milk related traits;  $b_0$  = the intercept;  $b_1$  = the regression coefficients;  $x_i$  = birth year of  $i^{\text{th}}$  bull (for estimation of genetic trend) or PRL genotypes codes (for estimation of average allele substitution effect) and  $e_{ij}$  = random error. Codes of genotypes were as 0 (AA), 1 (AG) and 2 (GG) to represent the number of (G) alleles in PRL polymorphism. As described by Falconer and Mackay (1996), the regression coefficient estimates the average effect of allele substitution ( $\alpha$ ), or the average effect of replacing a (G) allele with (A) allele.

The estimated correlation coefficients between yearly EBVs means and yearly allelic frequency ratios (G/A) were used to investigate the SNP effect on genetic trends. In this manner, correlation coefficient between birth years of bulls and the above ratios was used for investigation of Iranian Holstein selection programs on change trend of allele A frequency during years.

The difference of genetic trends (regression curves) between allele A and G was tested by using a multiple regression model. The categorical variable (allele) was defined as a set of binary variables with assigned numerical values of 0 and 1 for A and G alleles, respectively. The multiple regression model was:

$$y_i = \beta_0 + \beta_1x_{1i} + \beta_2x_{2i} + \beta_3x_{1i}x_{2i} + \varepsilon_i$$

where,  $y_i$  = EBVs for milk related traits,  $\beta_0, \beta_1, \beta_2, \beta_3$  = regression parameters,  $x_{1i}$  = birth years of bulls,  $x_{2i}$  = assignment to allele (1 for allele G, 0 for allele A),  $x_{1i}x_{2i}$  = interaction of allele\*birth year,  $\varepsilon_i$  = random error. The estimated regression (genetic trend) for allele G was  $E(y_i) = (\beta_0 + \beta_2) + (\beta_1 + \beta_3)x_{1i}$  and that for allele A was  $E(y_i) = \beta_0 + \beta_1x_{1i}$ .

The GLM procedure of SAS (2002) was used for estimation of average effect of allele substitution and genetic trends and investigation of the SNP effects on paternal path genetic trends in Iranian Holsteins.

## RESULTS

The transition of G into A allele in the position 8398 of prolactin gene creates a restriction site for *RsaI* endonuclease. Digestion of the PCR product (294 bp) with *RsaI* enzyme resulted in two restriction fragments of 162 and 132 bp for AA homozygotes, one uncut original fragment for GG homozygotes and all three fragments for AG heterozygotes (Figure 1).

**Table 1.** Average allele substitution effects of PRL/exon4 polymorphism for milk related traits.

Traits	$\alpha$	SE	P value
Milk yield (kg)	-203.4924	94.687	0.0325
Fat yield (kg)	-4.0107	2.5198	0.112
Fat percent (%)	+0.027119	0.02376	0.2547
Protein yield (kg)	-4.3019	2.0935	0.0409
Protein percent (%)	+0.01755	0.00903	0.0530

$\alpha$  = Linear regression coefficient estimating average substitution effects of G allele, SE = Standard error of regression coefficient estimation.

Estimated allelic frequencies from 268 Iranian Holstein bulls were 0.069 and 0.931 for A and G alleles, respectively. The frequencies of AA, AG and GG genotypes were 0.007, 0.123 and 0.870, respectively. The chi-square test indicated that genotype distributions were in the Hardy-Weinberg equilibrium ( $p > 0.05$ )

Based on Table 1, the average allele substitution effect was negative and significant for milk and protein yield ( $p < 0.05$ ) that is, allele G was an unfavorable allele for milk and protein yield.

Genetic trends in the Iranian Holstein bulls were significant ( $p < 0.05$ ) for all analyzed traits and the trend was progressive for milk, fat and protein yields, but diminishing for fat and protein percent (Table 2). The positive and negative trends for the mentioned traits may be due to 2 reasons: more economic importance of milk in relation to fat and protein percent in the Iranian Holstein selection index and negative correlations between milk yield and fat/protein percent.

The correlation coefficient of birth year of bulls and their correspondence allelic frequency (A/G) ratios was not significantly strength ( $r = 0.1068$ ;  $p > 0.05$ ). This correlation showed that traditional selection programs did not affect the allelic frequency of the RsaI/PRL mutation. It was expected that number of allele A (favorable allele) would be increased during years, but the increasing rate was not significant. In the future, marker assisted selection based on major genes may increase the favorable allele frequency.

The polymorphism of RsaI/PRL showed no significant effect on genetic trends for all milk related traits (Table 3). Genetic trends for different traits (change in EBVs during years) occur in selected populations such as Iranian Holstein bulls and the rate of trend depend on selection strategies (combinations of traits in selection indices, the economic and breeding coefficient of each trait, accuracy of estimated breeding values, etc). It was expected that the effects of candidate genes or major genes on genetic trends may be more than others. In molecular level, genetic trends for each trait depend on allelic frequency variation (especially major genes) during years and the rate of independent effect of each gene on trait performance. The results of the present study indicated that

**Table 2.** Paternal path genetic trends for milk related traits in Iranian Holstein bulls.

Traits	b	SE	P value
Milk yield (kg)	+63.7608	5.0534	<0.0001
Fat yield (kg)	+1.5073	0.1419	<0.0001
Fat percent (%)	-0.0110	0.0014	<0.0001
Protein yield (kg)	+1.4181	0.1120	<0.0001
Protein percent (%)	-0.0057	0.0049	<0.0001

b = Regression coefficient representing the rate of genetic trend.

PRL polymorphism did not influence any genetic trend for milk related traits. This could most likely be due to negligible changes in the favorable allele frequency.

The null hypothesis for  $\beta_2$  and  $\beta_3$  in all studied traits were not rejected (Table 4) indicating that the regression slopes and curves (respectively) were identical for allele A and G. For example, the estimated regression for G allele in milk yield was  $E(y_i) = -4829.94514 + 63.40259x_{1i}$  and that for A allele was  $E(y_i) = -5436.662882 + 72.707274x_{1i}$ , but the estimated slopes and curves were not significantly different (Table 4). These results indicated that there was not any difference in genetic trends produced by A and G alleles for all studied traits.

## DISCUSSION

The polymorphism of RsaI/PRL was studied in various cattle breeds and populations. The G allele (RsaI) frequency in previous studies on Holstein cows had different estimates including 0.73 (Chung et al., 1996), 0.78 (Golijow et al., 1999), 0.9 (Ratna-Kumari et al., 2008) and 0.95 (Chrenek et al., 1998). In this study, the estimated frequency of G allele (0.93) was similar to results of Chrenek et al. (1998) and Ratna-Kumari (2008). This variation of allele frequency within various Holstein populations may be created by the different sample sizes and/or various selection programs in any population.

The polymorphism of RsaI/PRL was studied in other breeds such as Polish Black-and-White (Dybus, 2002; Dybus et al., 2005), German Black-and-White (Mitra et al., 1995; Khatami et al., 2005), Swiss Brown (Mitra et al., 1995; Chrenek et al., 1999), Ayrshire, Gorbатов-Red and Black-Pied (Udina et al., 2001); Bos Indicus Sahiwal (Mitra et al., 1995), Indian Kankerj (Sacravarty et al., 2008), Montebeliard (Ghasemi et al., 2009), Argentina Creol (Golijow et al., 1999) and Jersey cows (Dybus et al., 2005; Ratna-Kumari et al., 2008). The range of G allele frequency variation in these breeds was from 0.29 in Jersey cows (Brym et al., 2005) to 0.95 in Argentina Creole cows (Golijow et al., 1999). This can be explained

**Table 3.** Coefficients of correlations between yearly EBV means of milk related traits and yearly ratio of allele A to G frequencies.

Parameter	Milk yield	Fat yield	Fat percent	Protein yield	Protein percent
A/ G Ratio	0.03005	0.10826	0.13671	0.02542	-0.04965
p-value	0.9058	0.6689	0.5886	0.9202	0.8449

**Table 4.** The multiple regression parameter estimates with their related p-values<sup>1</sup> for investigation of genetic trends by allele A and G.

Trait parameter <sup>2</sup>	Milk yield	Fat yield	Fat percent	Protein yield	Protein percent
$\beta_0$	-4829.945145 (<0.0001)	-110.9034861 (<0.0001)	0.8836787525 (<0.0001)	-106.152162 (<0.0001)	0.4480163436 (<0.0001)
$\beta_1$	63.402590 (<0.0001)	1.4853886 (<0.0001)	-0.011268393 (<0.0001)	1.41111708 (<0.0001)	-0.00567098 (<0.0001)
$\beta_2$	-606.717738 (0.5880)	-27.9840657 (0.3722)	-0.243370611 (0.4429)	-12.4559469 (0.6134)	0.0525191205 (0.6345)
$\beta_3$	9.304684 (0.5151)	0.3837736 (0.3377)	0.002940018 (0.4677)	0.19110822 (0.5437)	-0.000790577 (0.5751)

<sup>1</sup>The p-values were shown in parenthesis.

<sup>2</sup> $\beta_0$  = intercept,  $\beta_1$  = regression parameter for birth year,  $\beta_2$  = regression parameter for PRL allele codes (0 for allele A and 1 for allele G),  $\beta_3$  = regression parameter for interaction of birth year\*allele code.

by different history of the breeds, long-term geographical isolation and selection towards high fat and protein percents of milk in some breeds such as Jersey cows. It also indicated that RsaI/PRL polymorphism may be a marker of a linked SNP or locus involved in variation of milk composition.

Association of RsaI/PRL variants with milk related traits was confirmed by different studies in various breeds. Studies of Brym et al. (2005) on Jersey cows and Alipanah et al. (2008) on Russian Red Pied cows showed that RsaI/PRL had a significant effect on milk yield that was concurred with result of present study. The associations of RsaI/PRL mutation with milk fat yield and fat percent were reported by Khatami et al. (2005), Dybus et al. (2005), Brym et al. (2005) and Alipanah et al. (2007), but our results for fat percent and fat yield were not similar to their outputs. Also, the significant association of the polymorphism with milk protein yield in this study was not previously reported. Although, the A to G mutation in RsaI recognition site in prolactin gene is a silent mutation and its amino acid product (amino acid 102) for both of bases is valin. However, the observed relations probably resulted from linkage of the RsaI/PRL mutation and causative polymorphism in PRL gene regulatory sequences (Brym et al., 2007).

The results of this study confirmed that PRL may be a strong candidate gene for application in marker assisted selection. This study did not prove a significant effect of PRL polymorphism on paternal path genetic trends for milk production traits in Iranian Holsteins. Effects of the

SNP on selection indices or other traits, especially conformation traits and semen related traits of bulls should be the subject of future research.

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