Full Length Research Paper

Restriction fragment length polymorphism of bovine growth hormone gene intron 3 and its association with testis biometry traits in Iranian Holstein bull

Abolfazl Gorbani1, Rasoul Vaez Torshizi2, Mortaza Bonyadi3, 4 and Cyrus Amirinia5

1Department of Animal Science, Islamic Azad University- Science and Research Branch, Tehran, Iran.
2Department of Animal science, Faculty of agriculture, Tarbiat Modares University, Tehran, Iran.
3Center of Excellence for Biodiversity, Faculty of Natural Sciences, University of Tabriz, Tabriz, Iran.
4Liver and Gastrointestinal Disease Research Center, Tabriz University of Medical Sciences, Tabriz, Iran.
5Department of Biotechnology, Animal Science research Institute of Iran, Karaj, Iran.

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The association of bovine growth hormone gene polymorphism with testis biometry trait as average testis length (ATL), average testis width (ATW) and scrotum circumference (SC) in Iranian Holstein bulls. PCR-RFLP method with Msp-I restriction enzyme was used for genotyping. The frequency of the MspI(C) and MspI(D) alleles are 0.883 and 0.117 respectively. The genotype frequency for CC, CD and DD were 0.787, 0.191. The CD genotype was omitted of analysis of testis biometry trait. Mixed model analyses of considering genotype and environment as fixed effects and animal as a random effect suggested that sire was a significant source of variation (P < 0.001) in all traits. The CC genotype resulted in a significant increase in ATP (p < 0.0223), ATW (p < 0.0544) traits. These results indicate that new molecular markers associated with sperm quality traits can be used in marker-assisted selection in bulls.

Key words: Iranian Holstein bull, PCR-RFLP, bGH-Msp-I, polymorphism.

INTRODUCTION

Fertility is one of the most important economical traits in cattle production. Reproductive performance is controlled by the genetic make-up of the dam, bull and offspring, but in general it is largely affected by environment. Implementation of Artificial insemination (AI) from superior sires is a main tool for genetic improvement of the traits with economic importance in dairy cattle herds (Parmentier et al., 1999). The conception rate with AI depends on the quantity and quality of semen affected by environment, management, physiological status (especially hormones, e.g. FSH, LH and GH) and genetics factors (Mathevon et al., 1998). Sperm concentration, motility and testis biometry trait have usually been used as criteria for fertility semen quality evaluation in bulls. (Colenbrander et al., 1993). Molecular markers that reveal polymorphism at the DNA level are now key players in animal genetics. Recently, a number of potential candidate genes have been recognized. Allelic variation in the regulatory and structural regions of candidate genes may influence diversification of fertility (Mathevon et al., 1998; Rothschild et al., 1997; Linville et al., 2001; Parmentier et al., 1999). Hormone and hormone receptors are presumed to be good candidate genes for the reproductive traits because they modulate limiting steps in many reproductive pathways (Vincent et al., 1998).

Growth hormone (GH) is necessary for tissue growth, fat metabolism, and homeorhesis; thus, it has an important role in reproduction, lactation, and normal body growth (Burton et al., 1994; Ohlsson et al., 1998). Because of these important relationships, GH is a candidate gene for marker-assisted selection programs in cattle. Bovine growth hormone (bGH) is a single peptide with 190 or 191 amino acids and molecular weight equal to 22-KD (Walies et al., 1976; Lingappa et al., 1977; Dybus, 2002). bGH gene with 1800 bp length, five exons and four introns is a part of multiple gene family that contains prolactin and placental lactogens (Hediger et al.,

*Corresponding author. E-mail: abolfazlorbani@gmail.com.
Several polymorphic regions have been reported at different regions of bGH gene by SSCP and RFLP methods (Zakizadeh et al., 2006). The two most important polymorphisms are mutations at intron three (transition T to C) and exon five (transversion C to G (substitutes Leu by Val in protein)); which are detected by MspI and Alul restriction enzymes respectively (Lucy et al., 1993; Zhang et al.,1993; Yao et al., 1996).

Although several studies have addressed the association of bGH-MspI polymorphism with milk and meat production traits and inconsistent results have been reported (Falaki et al., 1997; Lee et al., 1996; Vukasinovic et al., 1999; Beauchemin et al., 2002; Pereira et al., 2005; Zhou et al., 2005; Thomas et al., 2007; Katoh et al., 2008). However, few studies have examined its effect on reproduction traits of bulls (e.g. sperm quality trait) (Lechniak et al., 1999, 2002; Unanian et al., 2002; Balogh et al., 2008).

Therefore, the present study was aimed to estimate the allelic frequencies at the bGH-MspI loci and examine its relationship with sperm quality and testis biometry traits of Iranian Holstein bulls.

MATERIAL AND METHODS

Animals

183 bulls of North West center (Tabriz, Iran) and Progeny Test center of Jahed Co (Karaj, Iran), were included in the study. The repeated measurements of sperm quality traits of bulls were available since 1991 to 2008 (41890 records).

Phenotypes

Testis biometry traits including average testis length (ATL), average testis width (ATW) and scrotum circumference (SC) were measured monthly for each bulls. The semen samples of bulls were collected with date and age.

Genotyping

Blood and semen samples were collected from the bulls. An anticoagulant (EDTA) was added to the blood samples and then stored at –20°C. Genomic DNA from whole blood was purified by standard protocol using proteinase K digestion as described by Miller et al. (1988), and from semen by DNA extraction kit (DNPM kit Cinnagen Co. Tehran, Iran). The quality of the DNA was checked on 0.5% agarose gel and the quantity was measured by UV spectrophotometry at A260/A280 nm.

Genotyping for bGH polymorphism was determined by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method. The PCR reaction conditions were approximately 100 ng of genomic DNA, 10 pmol of each primer, 0.2 mM of each dNTP, 1.5 mM of MgCl2, 1x PCR buffer [50 mM of KCl and Tris-HCl (pH 8.4)] and 0.4 U of Taq polymerase in a total volume of 25 µl. The PCR was conducted on Eppendorf Gradient thermalcycler, HotMasterMix (EPPENDORF, Germany) using a preliminary denaturation at 94°C for 1.5 min, 62°C for 1 min and 72°C for 1 min, followed by 48 cycles of a specific temperature regime. Each temperature regime consisted of 94°C for 30 s, 62°C for 1 min, 72°C for 30 s and a final extension at 72°C for 5 min. An 891 bp fragment of bGH consisting of the intron 2 (177 bp), exon III (117 bp), intron 3 (227 bp), exon IV (162 bp) and intron 4 (208 bp), was amplified using forward (5’ATCCACACCCCTCCACACAGT3’) and reverse (5’GATTTGCCACCTCCCCTACAG3’) primers (Unanian et al., 2002).

PCR products were digested with 4 U of MspI, using the supplied buffer and maintained at 37°C for overnight. The resulting fragments were separated by vertical electrophoresis (110 W 40 min) in 8% polyacrylamide gel, stained with ethidium bromide and were visualized under UV light. The C (MspI+) allele had fragment sizes of 526,193, 109 and 63 bp, whereas the D (MspI) allele had fragments of 635, 193 and 63 bp.

Statistical analysis

Allele and genotype frequencies

The bGH allele frequencies were calculated by simple allele counting (Falconer and Mackay, 1996). The possible deviations of allele and genotype frequencies from the Hardy–Weinberg equilibrium were examined with PopGene.S2 software by a Pearson’s Chi-square test.

Association analysis

Statistical analysis was performed using the MIXED procedure of SAS software (SAS System for Windows, release 8.02, 1999). The following linear model was used to examine the associations between bGH-MspI, polymorphisms and SV, SPOC, TS, TMSBF, TMSAF and TPP, ATL, ATW and SC traits:

\[ y_{ijklm} = \mu + a_i + YS_j + S_k + G_l + \sum b_m x_m + \epsilon_{ijklm} \]

where \( y_{ijklm} \) is the observation, \( \mu \) is overall mean, \( a_i \) is the random effect of the \( i^{th} \) animal, \( YS_j \) is fixed effect of the \( j^{th} \) year-season (\( j = 1 - 68 \)), \( S_k \) is fixed effect of the \( k^{th} \) station (\( k = 1 - 2 \)), \( G_l \) is fixed effect of the \( l^{th} \) bGH genotype (\( l = 1 - 3 \)), \( b_m \) is regression coefficient of \( m^{th} \) covariate (e.g. age), \( x_m \) is fixed effect of \( m^{th} \) covariate and \( \epsilon_{ijklm} \) is residual. Since FSM and PTSM traits were categorical variable, hence analyzed with logistic regression using GENMOD procedure by the following model:

\[ \eta_{ijklm} = \log[p_i/(1-p_i)] = m + a_i + YS_j + S_k + G_l + \sum b_m x_m + \epsilon_{ijklm} \]

Where \( \eta_{ijklm} \) is MAF and MBF traits, \( m \) is overall mean, \( a_i \) is the random effect of the \( i^{th} \) animal, \( YS_j \) is fixed effect of the \( j^{th} \) year-season (\( j = 1 - 68 \)), \( S_k \) is fixed effect of the \( k^{th} \) station (\( k = 1 - 2 \)), \( G_l \) is fixed effect of the \( l^{th} \) bGH genotype (\( l = 1 - 3 \)), \( b_m \) is regression coefficient of \( m^{th} \) covariate (e.g. age), \( x_m \) is fixed effect of \( m^{th} \) covariate and \( \epsilon_{ijklm} \) is residual. Average effect of allele substitution was determined by coding genotype as 0(DD), 1(CD), 2(CC) to represent the number of C alleles present for the bGH polymorphism as described by Falconer and Mackay (1996). The regression coefficient estimates average effect of allele substitution.
Table 1. Gene and genotypic frequencies obtained at bGH-MspI loci in Iranian Holstein bulls.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Allele</th>
<th>Chi-square value</th>
<th>Pr &gt; ChiSq</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CC</td>
<td>CD</td>
<td>DD</td>
</tr>
<tr>
<td>Number</td>
<td>144</td>
<td>35</td>
<td>4</td>
</tr>
<tr>
<td>Frequency</td>
<td>0.787</td>
<td>0.191</td>
<td>0.022</td>
</tr>
</tbody>
</table>

RESULTS

Allele frequency

Data of 183 bulls were included in the final evaluation. The genotype and allele frequencies at bGH loci calculated by PopGene.S2 software, are shown in Table 1. Three genotype for bGH gene CC (526,193, 109 and 63 bp), CD635, 526, 193, 109 and 63 bp) and DD (635, 193 and 63 bp) was observed (Figure 1). The C allele was more frequent than D allele (0.883 vs. 0.117) and therefore (most of the bulls (78.7%) were homozygous for the C allele and only 19.1% were heterozygous. The DD genotype was found in only four animals and their results weren't reported. Pearson’s Chi-square test (P > 0.05) indicated that the genetic pool were in Hardy–Weinberg equilibrium.

Candidate gene effects

Least square means of testis biometry traits for bGH genotypes are presented in Tables 2. Analysis of variance indicated significant association of bGH genotypes with ATL (P < 0.0223) and ATW (p= 0.0544), but there was no significant association with ATW (P = 0.05). Moreover year-season and age had significant effects on some (P < 0.0001). In this population, bulls with CD genotype had average testis length, average testis width and scrotum circumference greater than bulls with CC genotype.

The allele substitution effects on testis biometry traits were estimated and shown in Table 3. The substitution effects of C to D in was not significant on Sc trait was observed. The substitution of C for D allele at GH locus resulted in a decrease of 0.884 cm (p<0.05) in average
Table 2. Least square means (±SD) of testis biometry traits for GH genotypes in Iranian Holstein bulls.

<table>
<thead>
<tr>
<th>trait</th>
<th>GH genotype</th>
<th></th>
<th></th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CC (n = 146)</td>
<td>CD (n = 37)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>average testis length</td>
<td>17.11 ± 1.7</td>
<td>17.99 ± 1.7</td>
<td>0.0223</td>
<td></td>
</tr>
<tr>
<td>average testis width</td>
<td>13.39 ± 4.22</td>
<td>14.54 ± 4.22</td>
<td>0.0544</td>
<td></td>
</tr>
<tr>
<td>scrotum circumference</td>
<td>38.78 ± 7.1</td>
<td>39.44 ± 7.1</td>
<td>0.3841</td>
<td></td>
</tr>
</tbody>
</table>

Table 3. Allele substitution effect of GH gene on sperm quality and testis biometry trait.

<table>
<thead>
<tr>
<th>trait</th>
<th>GH genotype</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>α</td>
<td>SD</td>
<td>p-value</td>
<td></td>
</tr>
<tr>
<td>average testis length</td>
<td>-0.884</td>
<td>0.377</td>
<td>0.0214</td>
<td></td>
</tr>
<tr>
<td>average testis width</td>
<td>-1.168</td>
<td>0.592</td>
<td>0.0520</td>
<td></td>
</tr>
<tr>
<td>scrotum circumference</td>
<td>-0.665</td>
<td>0.759</td>
<td>0.3828</td>
<td></td>
</tr>
</tbody>
</table>

testis length and 1.164 cm (p=0.05) in average testis width and in scrotum circumference (-0.665 cm), no significant effect of allele substitution was observed on these traits.

DISCUSSION

The results of the present study showed that the MspI* allele (C) was more frequent than the MspI (D) (0.883 vs. 0.117), so that most of the bulls (78.7%) were homozygous for the C allele, 19.1% were heterozygous and 2.2% were homozygous for the D allele. These findings were similar to those previously reported for Holstein dairy cattle (Zhang et al., 1993; Yao et al., 1996; Sabour et al., 1997; Vukasinovic et al., 1999; Zhou et al., 2005; Zakizadeh et al., 2006; Pawar et al., 2007). Comparison of the allelic frequency in different breeds showed that MspI (D) allele frequency is relatively low for breeds prevalent in most of European breeds, that is, zero for Herford cattle, 0.15 for Jersey and 0.14 for Angus cattle (Lagziel et al., 2000) and 0.13 for Polish Black and White cattle (Dybus et al., 2004). For the Eastern Europe or the Middle East cattle, these frequencies were reported to be moderate to high, that is, 0.26 and 0.39 for Ukraine Brown Carpathian and Limousine cattle (Lagziel et al., 2000); 0.45 for Iranian Sarabi cattle (Zakizadeh et al., 2006); 1.00 for Indian subcontinent and zebu breeds (Lagziel et al., 2000); 0.81 to 0.87 for Indian Zebu (Pawar et al., 2007) and 0.82 to 0.85 for Brazilian Nellore cattle(Unanian et al., 2002). These results suggested that breed of cattle is an important source of variation in allelic frequency of GH-MspI locus. Also, due to neutral and artificial selection, D and C alleles might be a characteristic of Bos indicus breeds (resistance to rough environmental condition) and Bos taurus breeds (high (high production), respectively.

The bovine testis has been shown to be a site of GH action; it influences the steroidogenesis, gametogenesis and gonadal differentiation as well as gonadotropin secretion and responsiveness (Kerry et al., 2000). In the present study, bulls with CC genotype had lower average testis length, average testis width and scrotum circumference, compare to CD by 5% (P < 0.0223), 8% (P < 0.054) and 1.7% (P < 0.3841).

Although there are several studies regarding the association of bGH-MspI polymorphism with different traits, to date few have examined the reproduction traits. Only one study assessed the effect of bGH-MspI polymorphism on sperm quality trait in bulls. The results didn't show any significant association of bGH-MspI polymorphism with fresh sperm motility, sperm concentration and minor and major defects (Unanian et al., 2002) which was in contrast with our findings. It was may be related to breed differences. [Brazilian Nellore (Bos indicus) vs Holstein (Bos taurus)].

Moreover in consistent with our findings, the study of Unanian et al. (2002) indicated that bGH-MspI polymorphism had significant effect on scrotal circumference and testicular growth after puberty. The results of Rocha et al. (1992) demonstrated a significant association of the bGH-Msp-I polymorphism with body weight gain and scrotum circumference.

The study of Lechniak et al. (1999, 2002) indicated that AluI polymorphism of bGH gene had no effect on the sperm quality traits, non-return rate, number of oocytes (collected from donor ovaries) suitable for in vitro maturation, the number of matured oocytes, mean oocyte diameter and number of embryos produced.

In conclusion, the results of the present study showed that including the bGH-MspI polymorphism in breeding program will improve the sperm quality traits in AI bulls. But it is currently unknown how this mutation alters the...
structure and conformation of growth hormone. However, further studies are required to test the biochemical effects of bGH’s various isoforms, resulting from this polymorphism on reproduction traits.

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REFERENCE


