Association of milk traits with SSCP polymorphisms at the growth hormone gene in the Serrana goat

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Abstract

The present study suggests the existence of an association between milk production traits and genetic polymorphisms at the growth hormone (GH) gene in the Portuguese indigenous Serrana goat. The DNA from 229 animals of two ecotypes (Jarmelista and Ribatejano) was analysed by polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) technique revealing a high degree of genetic polymorphism at the goat GH (gGH) gene. Two conformation patterns were detected in exons 1 and 2, 6 in exon 3, 10 in exon 4 and 5 in exon 5. The evaluation of an association effect between these SSCP patterns with milk, fat and protein yields and fat and protein percentages suggests a positive effect of pattern A/B of exon 4 for Ribatejano ecotype and of pattern A/B of exon 2 for Jarmelista ecotype with milk yield (P < 0.05) and of pattern A/B of exon 1 and pattern B/B of exon 2 with protein percentage (P < 0.05) for Ribatejano ecotype. The results support the hints suggested by previous studies of the importance of the gGH gene as a candidate gene for marker-assisted selection in goat breeds and suggest that exon 4 is a preferential target for further investigation on mutations that influence milk yield variation.

Keywords: Single strand conformation polymorphism; Genetic markers; Growth hormone; Goat; Milk traits

1. Introduction

Since its finding in the 1920s, it is well documented that growth hormone (GH) influences animal processes such as growth (Breier, 1999), lactation (Baldi, 1999), reproduction (Scaramuzzi et al., 1999) and metabolism (Bauman, 1999).

Less controversial than the exogenous administration of GH as a production stimulator, is the strategy based on the selection of animals with an endogenous GH secretion resulting in a superior quantitative trait. This productive advantage could be the result of a more active GH variant or of a more favourable upstream regulation directing its synthesis. The establishment of significant correlations between quantitative traits and molecular variation at the GH level or of factors regulating its secretion and action could lead
to the identification of markers useful for assisting selection programmes.

Genetic polymorphisms of GH have been reported in various domestic livestock, mainly in cattle, and several studies have related association effects between bovine GH (bGH) polymorphisms and milk yield traits. For example the \( MspI \) (−) allele of the \( MspI \) restriction fragment length polymorphism (RFLP) described by Zhang et al. (1993) was shown to be significantly associated with higher fat yield in three bovine breeds (Høj et al., 1993; Lee et al., 1994) and with higher protein yield and protein percentage (Lagziel et al., 1996).

Other polymorphic sites of bGH gene were described by Zhang et al. (1992) and by Unanian et al. (1994) who reported two polymerase chain reaction-restriction fragment length polymorphisms (PCR-RFLPs): an \( AluI \)-RFLP in exon 5 and an \( HaeIII \)-RFLP in the 3′ region of bGH. Investigating the association between bGH loci corresponding to the GH-\( MspI \), GH-\( AluI \) and GH-\( HaeIII \) polymorphisms and milk yield traits in Holstein cattle by means of pedigree analysis, Vukasinovic et al. (1999) concluded that these bGH loci are linked to a putative quantitative trait locus (QTL) affecting protein percentage and that they might be linked to a QTL affecting other milk traits.

Genetic polymorphisms at the GH gene have also been detected by single strand conformation polymorphism (SSCP) in cattle (Kirkpatrick, 1992; Lagziel et al., 1996; Yao et al., 1996), in sheep (Marques et al., 2001) and in goat (Malveiro et al., 2001). Most significant studies using the SSCP approach were accomplished in bovines by linkage analysis (Lagziel et al., 1996). In order to define intragenic GH haplotypes in Israeli Holstein cattle, Lagziel et al. (1996) analysed nine fragments of the bGH gene identifying eight SSCP haplotypes, one of which was associated with a high milk protein percentage. The SSCP haplotypes were later sequenced disclosing 12 sequence variants (Lagziel and Soller, 1999).

Marques et al. (2001) analysed the five ovine GH (oGH) exons by PCR-SSCP in 200 Portuguese Serra da Estrela ewes revealing that all exons but exon 1 are polymorphic. An increase of milk production was associated with a pattern found in oGH exon 4. In goats, a study on polymorphism at the goat GH (gGH) gene was described by Malveiro et al. (2001) who reported several SSCP patterns in the Portuguese Algarvia goat. Two of these patterns were positively associated with milk traits.

The Serrana goat is a Portuguese autochthonous breed with high production potential, high rusticity, and good productive and reproductive indexes in extensive systems, providing the rural population with two important economic resources: milk, for cheese production, and meat. This breed, presenting four ecotypes, is the most numerous in Portugal. The two Serrana ecotypes studied in this paper are reared in areas around the river Tejo, the Ribatejo region (Ribatejano ecotype) and in a climatically and topographically distinct region covering an interior area south of river Douro, the Beira Alta region (Jarmelista ecotype). Well adapted to its natural environment and showing good milk performances in poor feeding conditions, its preservation is nevertheless threatened by the introduction of foreign high milk yielding breeds. Genetic selection within this breed aimed at the improvement of production traits might be the best strategy to assure its preservation. Moreover, its preservation would contribute to biodiversity conservation.

As a first step towards these long-term objectives, we have analysed the five GH exons of the Serrana goat by PCR-SSCP and attempted to establish an association between detected polymorphisms and the milk, fat and protein yield and fat and protein percentage.

2. Materials and methods

2.1. Animals and DNA samples

Two hundred and twenty-nine Serrana goats (2–11 years old) were used in the present study: 111 females and 11 males of the Ribatejano ecotype, and 100 females and 7 males of the Jarmelista ecotype. The animals were part of eight randomly chosen flocks, four of each ecotype, belonging to associates of the following breeder associations: “Associação de Criadores de Reprodutores de Gado do Oeste” (ACRO), “Associação de Criadores de Caprinos e Ovinos do Ribatejo Oeste” (ACORO) and “Associação de Criadores da Guarda” (AGRICUALDA). The mean productive values were as follows: milk yield, 250 l; fat yield, 10 kg; protein yield, 9 kg; fat percentage, 3.5% and protein percentage, 3.3%.
DNA was extracted from peripheral blood leukocytes using a Puregene DNA Isolation Kit (Gentra Systems).

2.2. PCR amplification

The five exons of the gGH gene were amplified by PCR using the five primer pairs shown in Table 1. The resulting five amplification fragments ranged from 112 to 287 bp. PCR reactions were performed in an UNO II thermocycler (Biometra) using Ready-To-Go PCR Beads (Amersham Biosciences) according to the following conditions: 25–50 ng of genomic DNA; 4–16 pmol of each primer (Table 1); 1.5 U Taq DNA polymerase; 10 mM Tris–HCl, pH 9; 50 mM KCl; 1.5 mM MgCl2; 200 μM of each dNTP and stabilisers including BSA, for a final volume of 25 μl. The amplification included an initial denaturation at 95°C for 5 min, 30 cycles of denaturation at 95°C for 30 s, annealing at 57–62°C (Table 1) for 30 s and extension at 72°C for 30 s. Each amplification product was analysed by electrophoresis on a 2% agarose gel (5 V/cm), using ethidium bromide staining.

2.3. SSCP analysis

For SSCP analysis, 5 or 10 μl of each amplification product was added to, respectively, 15 or 10 μl of Stop Solution (95% formamide, 10 mM NaOH, 0.05% xylene cyanol and 0.05% bromophenol blue). The samples were heat-denatured at 95°C for 5 min, chilled at 0°C, and the total volume was loaded onto an 8–12% polyacrylamide/TBE gel, with a 2.5% crosslinking. Gels were run at 25 W for 4–8 h, at 15–20°C, in a DCode™ Universal Mutation Detection System (BIO-RAD), coupled to a refrigeration system. The SSCP analysis parameters were optimised for each fragment (Table 2). After the run, the gel was removed from the apparatus and silver stained using PlusOne DNA Silver Staining Kit (Amersham Biosciences).

2.4. Statistical analyses

Of the 229 animals analysed by PCR-SSCP, only 210 animals were statistically analysed since 18 were males and one female had insufficient data. The data collected included cumulative milk yield, total milk yield, total milk yield adjusted for 150 days and mean of daily milk yield for both ecotypes—Jarmelista and Ribatejano—and fat and protein yield, fat and protein percentage adjusted to 150 days for the Ribatejano ecotype only. These variables were measured in seven consecutive years (1993–1999). Data was considered as repeated measures and the correlations between the measures in the same individual were considered in the statistical model. Linear mixed models were used to determine associations between SSCP patterns and milk traits (SAS® System). The fixed effects included were:
Table 2
Optimised conditions for SSCP analysis of the five GH exons of Serrana goat breed

<table>
<thead>
<tr>
<th>Exon</th>
<th>DNA (µl)</th>
<th>Stop Solution (µl)</th>
<th>T (%)</th>
<th>TBE buffer concentration</th>
<th>Running temperature (°C)</th>
<th>Running time (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10</td>
<td>10</td>
<td>12</td>
<td>1</td>
<td>15</td>
<td>8</td>
</tr>
<tr>
<td>2</td>
<td>5</td>
<td>15</td>
<td>10</td>
<td>0.5</td>
<td>15</td>
<td>4.30</td>
</tr>
<tr>
<td>3</td>
<td>5</td>
<td>15</td>
<td>10</td>
<td>0.5</td>
<td>15</td>
<td>7.30</td>
</tr>
<tr>
<td>4</td>
<td>5</td>
<td>15</td>
<td>10</td>
<td>0.5</td>
<td>15</td>
<td>6.15</td>
</tr>
<tr>
<td>5</td>
<td>10</td>
<td>10</td>
<td>8</td>
<td>0.5</td>
<td>20</td>
<td>4</td>
</tr>
</tbody>
</table>

T (%): polyacrylamide percentage.

The models were compared using Akaike’s Information Criterion and Schwarz’s Bayesian Criterion, in order to choose the model with the best fit. The nearest zero values were obtained with model (d) when ecotypes were analysed separately.

The F-values for fixed effects of exons 1–5 were not significant (P > 0.05) for all milk traits. Since not all SSCP patterns were observed in some exons in both ecotypes (Jarmelista’s had no A/B/D/E pattern in exon 4 and no A/B/C/E pattern in exon 5 and Ribatejano’s lack the A/C/D pattern in exon 3 and patterns A/C/F, A/B/E, C/F, A/A, A/B/F in exon 4; see Table 3), milk traits for each ecotype were analysed separately.

To determine associations between SSCP polymorphisms and milk traits for both ecotypes and fat and protein traits for Ribatejano ecotype the four linear mixed models described above were also used but only with the fixed effects of flock, type of parturition, linear and quadratic effect of the covariable age and exons 1–5 and considering repeated measures on the animal.

The effect of type of parturition was not significant (P > 0.05) for all traits and was removed from the models.

When F-test for the fixed effects was significant (P < 0.05) multiple comparison tests (least significant difference, LSD) of least squares means (LSMEANS) were used.

3. Results and discussion

3.1. SSCP polymorphisms

SSCP analysis of exons 1–5 of the gGH were performed on the fragments amplified by PCR using the primers described in Table 1, which showed the expected lengths. The 229 Serrana goats analysed showed several conformation patterns for each amplified fragment (Figs. 1 and 2, Table 3).
Table 3
SSCP analysis results of the five GH exons of Serrana goat breed

<table>
<thead>
<tr>
<th>Exons</th>
<th>No. of patterns</th>
<th>Pattern frequencies (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ribatejano ecotype (n = 122)</td>
<td></td>
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</tr>
<tr>
<td>1</td>
<td>2</td>
<td>A/B (82.8); A/A (17.2)</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>A/B (91.2); B/B (8.8)</td>
</tr>
<tr>
<td>3</td>
<td>5</td>
<td>A/B (74.6); C/D (2.5); A/B/D (15.6); A/B/C/D (5.7); A/B/D/E (1.6)</td>
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<tr>
<td>4</td>
<td>5</td>
<td>A/B (68.9); A/C (9.8); A/B/C (13.1); A/B/D/E (1.6); A/B/C/F (6.6)</td>
</tr>
<tr>
<td>5</td>
<td>5</td>
<td>A/B (70.5); A/C/D (12.3); A/B/C/D (13.1); A/B/C/E (2.5); A/B/C/F (1.6)</td>
</tr>
<tr>
<td>Jarmelista ecotype (n = 107)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>2</td>
<td>A/B (84.1); A/A (15.9)</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>A/B (93.5); B/B (6.5)</td>
</tr>
<tr>
<td>3</td>
<td>6</td>
<td>A/B (72.0); C/D (0.9); A/B/D (0.9); A/B/C/D (9.4); A/B/D/E (5.6); A/C/D (5.6)</td>
</tr>
<tr>
<td>4</td>
<td>9</td>
<td>A/B (54.2); A/C (0.9); A/B/C (15.9); A/B/C/F (16.6); A/C/F (3.9); A/B/E (5.6); C/F (2.8); A/A (0.9); A/B/F (0.9)</td>
</tr>
<tr>
<td>5</td>
<td>4</td>
<td>A/B (57.0); A/C/D (2.8); A/B/C/D (34.6); A/B/C/F (5.6)</td>
</tr>
</tbody>
</table>

Fig. 1. SSCP patterns for exons 1–5 of Serrana goat GH gene separated by PAGE under non-denaturing conditions. Centre: schematic representation of gGH gene. Exons are represented by black boxes. For simplification, a single letter designation was assigned to the patterns, whose correspondence with the adopted designation is shown in Fig. 2.
Using the SSCP method, animal genotypes showing more than four bands in several patterns can be interpreted as the result of the GH gene duplication existing in goats. Indeed, RFLP studies have demonstrated that there are two alleles at the GH gene locus in sheep and goats (Valinsky et al., 1990). In sheep, the GH1 allele presents a single copy of the gene (GH1) while the GH2 allele presents two GH copies in tandem (GH2-N and GH2-Z). Individual animals homozygous for GH1 or GH2 alleles have two or four GH-like genes, respectively, while heterozygous animals with one copy of GH1 and one of GH2 (GH1/GH2), have three GH-like genes in their genomes. The SSCP method allows for the identification of these patterns, which can be used to infer the genetic makeup of the animals. The table and diagram below illustrate the SSCP patterns and their corresponding frequencies.

<table>
<thead>
<tr>
<th>SSCP pattern</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
<th>G</th>
<th>H</th>
<th>I</th>
<th>J</th>
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<td>Pattern</td>
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<tr>
<td>Exon 1</td>
<td>a</td>
<td>b</td>
<td>b</td>
<td>19(8.5)</td>
<td>38(6.4)</td>
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<td></td>
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<td>Pattern</td>
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<tr>
<td>Exon 2</td>
<td>b</td>
<td>b</td>
<td>b</td>
<td>21(8.1)</td>
<td>19(8.3)</td>
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<td></td>
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<td>Pattern</td>
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<td>Exon 3</td>
<td>a</td>
<td>a</td>
<td>a</td>
<td>a</td>
<td>a</td>
<td>a</td>
<td>a</td>
<td>a</td>
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<td>Exon 4</td>
<td>a</td>
<td>a</td>
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<td>a</td>
<td>a</td>
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<td>a</td>
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<tr>
<td>Exon 5</td>
<td>a</td>
<td>a</td>
<td>a</td>
<td>a</td>
<td>a</td>
<td>a</td>
<td>a</td>
<td>a</td>
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<td>a</td>
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</table>

Fig. 2. Schematic representation of SSCP and proposed patterns for each of the five GH exons of Serrana goat. Number of animals in which the pattern was found and respective frequencies (in brackets) are indicated.
genes (Wallis et al., 1998). Moreover, sequence differences between the sheep GH2-N and GH2-Z genes have been demonstrated (Ofir and Gootwine, 1997).

In goats, a similar allelic polymorphism was observed revealing an allele, CgGH, with a single GH copy, gGH1, and the EgGH allele with the duplicated gene presenting also a tandem arrangement of the gGH2 and gGH3 genes (Yamano et al., 1991). The presence of this allelic polymorphism makes the identification of sequence polymorphism more difficult. One can thus expect to observe from 2 to 8 bands in SSCP analysis. Animals homozygous for GH1 can theoretically not give rise to more than four bands for each of the five exons of gGH. In the sample of 229 Serrana goats studied individual animals exhibiting two and/or four bands in each of the five exons could be homozygous for the GH1 allele. However, one can not exclude the possibility that they could be heterozygous, GH1/GH2, or homozygous GH2/GH2 for the same sequence variant of the GH2 allele (The GH2 alleles are homozygous but the gGH2 and gGH3 genes are heterozygous between each other). The letters used in this paper to designate the different patterns refer to sequence variants of any of the GH-like genes as it is impossible, with the present analysis, to establish which one is homogenous or heterozygous.

Analysing the SSCP results obtained (Fig. 2), one homozygous (A/A) and one heterozygous (A/B) conformation pattern were observed in exon 1 in both ecotypes. One homozygous (B/B) and one heterozygous (A/B) conformation pattern were observed in exon 2 in both ecotypes.

Exon 3 exhibited a high level of polymorphism. Six conformation patterns were observed in Jarmelista ecotype: patterns A/B, A/B/C/D, A/B/C/E and A/B/C/F. Pattern A/B/C/E was absent in the observed population of the Jarmelista ecotype.

Exon 4 appears to be the most polymorphic region in goat breeds. Sequencing of patterns corresponding, firstly to this region and then to the other exons is underway. The information thus obtained would allow the characterization of putative mutations of amino acid residues of the gGH, eventually involved in variations of milk yield and in gGH binding to its receptor.

3.2. Statistical analyses

For all studied exons, the analysis of fat and protein yield and fat percentage revealed no differences
between animals with different patterns. However, differences were found for milk yield in exon 2 (Jarmelista ecotype) and exon 4 (Ribatejano ecotype) and for protein percentage in exons 1 and 2 (Ribatejano ecotype).

Table 4 presents the LSMEANS obtained for two of the studied milk parameters. Jarmelista animals with pattern A/B in exon 2 have a superior milk yield ($P<0.05$) as do Ribatejano animals with pattern A/B in exon 4 ($P<0.05$). For cumulative milk yield (Table 4), similar results were observed for Jarmelista ecotype but for the Ribatejano ecotype the results are less clear due to the low frequency of some patterns. Ribatejano animals with pattern A/B in exon 1 and pattern B/B in exon 2 have a positive correlation with protein percentage (Table 5). In the analyses of the fat and protein percentage, the unspecified covariance matrix allowed the best fit.

The present results reinforce the idea that SSCP analysis is a valuable approach for the establishment of allelic variants in genes that express hormones associated with productive traits. In particular, the search of genetic markers located in the gGH gene is within the scope of this method. Indeed, SSCP polymorphisms that exert a positive influence on milk yield have previously been established at the GH gene from the caprine Algarvia breed (Malveiro et al., 2001) namely, polymorphisms in exons 4 and 5. The results presented here concerning Serrana goat, the most representative indigenous Portuguese caprine breed, were obtained with twice the number of animals than that of the Algarvia breed. These Serrana animals came from eight unrelated flocks. However, various degrees of relationship between some individuals do exist within each flock. The results could have been, to some extent, influenced by this fact.

4. Conclusions

SSCP polymorphisms can be rapidly and inexpensively detected in a population of dairy animals and their associations with milk traits studied. In spite of gene duplication, it is possible to genotype variants of the gGH gene using the PCR-SSCP technique. We have applied this approach to look for associations between polymorphisms observed in exons 1–5 of the GH gene from the autochthonous Portuguese caprine Serrana breed and milk traits. Milk yield appears to be under the influence of polymorphisms detected in exons 2 and 4 and protein percentage is apparently under the influence of polymorphisms detected in exons 1 and 2. The positive influence of exon 4 in milk yield established in this study confirms previous findings in caprine Algarvia breed suggesting that this region of the gGH gene is particularly important and deserves further studies.

Molecular and structural characterization of the GH genetic variants should allow the implementation
of fast analytic procedures based on specific PCR directed to mutations in exons where gGH genetic PCR-SSCP patterns are likely to be associated with milk traits. The application of this technique to larger populations should help us to determine whether the association found here is confirmed and, if so, help us in carrying out a more efficient selection programme.

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