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Polymorphisms at the five exons of the growth hormone gene in the algarvia goat: possible association with milk traits

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Abstract

The present preliminary study attempts to establish associations between milk production traits and genetic polymorphisms at the GH gene in the Algarvia goat. The DNA of 108 goats of the indigenous Portuguese Algarvia breed was evaluated. Single-strand conformation polymorphisms (SSCP) were identified at the five exons of the goat growth hormone (gGH) gene. Two conformational patterns were found in each of exons 1 and 2, four in exon 3, six in exon 4 and five in exon 5. An association between these SSCP patterns with milk, fat and protein production, and fat and protein content was examined. Patterns F/F of exon 4 and A/A of exon 5 were positively associated with milk production ($P < 0.05$). The results demonstrated that the gGH gene could be exploited as a candidate gene for marker-assisted selection in goat breeds.

Keywords: Polymorphism; Single-strand conformation polymorphism; Genetic markers; Growth hormone

1. Introduction

Milk production is a physiological function that is under the control of several genes. Genotyping animals for all the genes encoding a polygenic trait seems impractical and so it appears more realistic to focus on only a few genes having effects that account for a significant part of genetic variation in milk production traits. The growth hormone (GH) secreted by the pituitary gland plays an important role in lactation (Baldi, 1999). Some GH secretion parameters and peak frequency are associated with dairy animals of high genetic value (Reinecke et al., 1993). It has been demonstrated that those animals with high milk yield also have superior average GH levels when compared during peak lactation to those observed in animals with lower production (Vasilatos and Wangsness, 1981; Hart et al., 1980). Several authors have reported an association between some features of GH secretion and genetic values in cattle (Peel and Bauman, 1987; Reinecke et al., 1993). Furthermore, correlations between milk traits and polymorphisms at the GH gene have been observed. Genetic polymorphisms can be identified by several methods, and there are many studies in this area. The majority has made use of the

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restriction fragment length polymorphism (RFLP) technique. An allelic variation detected at amino acid position 127 of bovine GH was shown to be implicated in the variation of milk production (Lucy et al., 1993). A substitution of a cytosine (C) for a guanine (G) at position 2141 causes an amino acid change from Leu (L, codon CTG) to a Val (V, codon GTG) at the residue 127 (Zhang et al., 1992). This transversion destroys the restriction site recognised by the endonuclease *Alu*I enabling genotyping at this particular locus using this endonuclease. The *Alu*I (+/−) polymorphism is believed to be related to plasma levels of GH (Schlee et al., 1994) and the genotype LL was usually associated with higher circulating concentrations of GH when compared to genotype LV. Falaki et al. (1996, 1997) have reported a GH-*Taq*I polymorphism in Holstein and Simmental bulls associated with milk production. This polymorphism results from an insertion/deletion of ±1000 bp in the 3′-region, between the *Eco*RI and the *Taq*I excision sites (Hallerman et al., 1987; Høj et al., 1993b). In this study, Høj et al. (1993b) have shown that the polymorphism could affect the GH synthesis induced by thyrotropin-releasing hormone (TRH). Høj et al. (1993a) found a polymorphic insertion (I)/deletion (D) site in the GH gene of Red Danish cattle and observed that the frequency of the I/D genotype is higher in animals selected for high milk fat yield than in those selected for low milk fat yield. Finally, Lucy et al. (1993) have identified, using *Alu*I enzyme, two alleles at position 127, a valine/leucine variation. In contrast, Sabour et al. (1997) have not observed any association between the val/leuc genotype and milk yield in 100 Canadian Holstein bulls.

The single-strand conformation polymorphism (SSCP) technique has also been used for the detection of genetic polymorphisms. It is a powerful method for identifying nucleotide sequence variation in amplified DNA, with the advantage over the RFLP technique in being able to detect single base substitutions besides enzymatic restriction sites. SSCP analysis of DNA has been used for detection of genetic mutations in humans (Orita et al., 1989), rats (Pravenec et al., 1992), cattle (Kirkpatrick, 1992; Neibergs et al., 1993) and in various bacteriological (Morohoshi et al., 1991) and viral (Fugita et al., 1992) systems. Most significant studies using the SSCP approach were accomplished on bovines in linkage analysis (Lagziel et al., 1996) and to define intragenic haplotypes for growth hormone. Lagziel et al. (1996) analysed nine fragments of the bovine GH gene, identified several SSCP haplotypes, and associated one of them with a high percentage of milk protein. Yao et al. (1996) have reported two polymorphisms, (T → C in intron 3 and A → C in exon 5) using the SSCP method in the GH gene in bovines which were associated with milk production traits.

The Algarvia goat is a Portuguese indigenous breed of unknown origin, whose main potential is in the production of milk for the manufacture of cheese. This breed is reared mainly in the Algarve region, where it is very well adapted to dry sylvan areas, and plays an important role as an economic resource to the rural population. The purpose of the present study was to investigate associations between milk production traits and polymorphisms at the GH gene in the Algarvia goat. To our knowledge, no polymorphisms at the goat GH (gGH) gene have been described. The search for single-strand conformation polymorphisms could lead to the identification of genetic markers useful for selection in goat populations.

As part of a programme of genetic selection aimed at the improvement of production traits, we have identified single-strand conformation polymorphisms at the five exons of the goat GH gene in the Algarvia breed by polymerase chain reaction (PCR)-SSCP analysis. This manuscript presents the preliminary results of works aimed at the establishment of an association of these polymorphisms with milk, fat and protein yield, and fat and protein content.

2. Materials and methods

2.1. Animals

The 108 Algarvia goats (2–10 years old) used in this study were selected from farmers of the northeast Algarve. The animals were randomly chosen from three different herds, provided by the breeder association "Associação Nacional de Criadores de Cabras da Raça Algarvia" (ANCCRAL).

The mean productive values were as follows: milk: 121 l; fat: 3.9 kg; protein: 4 kg. DNA was obtained from peripheral blood leukocytes using a DNA Isolation Kit from Puregene.

Table 1
SSCP fragments at the gGH gene and PCR analysis parameters

Exon	Fragment length and localisation (bp)	Primer sequence[a]	Annealing temperature (°C)	Primers (pmol)	DNA (ng)
1	112 360–471	5′-TAA TGG AGG GGA TTT TTG TG-3′ 5′-CAG AGA CCA ATT CCA GGA TC-3′	57	16	25
2	198 682–879	5′-TCT AGG ACA CAT CTC TGG GG-3′ 5′-CTC TCC CTA GGG CCC CGG AC-3′	65	16	50
3	157 1063–1219	5′-GTG TGT TCT CCC CCC AGG AG-3′ 5′-CTC GGT CCT AGG TGG CCA CT-3′	60	4	25
4	200 1416–1615	5′-GGA AGG GAC CCA ACA ATG CCA-3′ 5′-CTG CCA GCA GGA CTT GGA GC-3′	70	8	25
5	289 1854–2142	5′-AAA GGA CAG TGG GCA CTG GA-3′ 5′-CCC TTG GCA GGA GCT GGA AG-3′	67	16	50

[a] Primers were designed according to published gGH gene sequence (Kioka et al., 1989).

2.2. PCR amplification

The five exons of the gGH gene were amplified by PCR using the five primer pairs shown in Table 1. Five amplification fragments ranged from 112 to 289 bp. PCR reactions were performed in an UNOII thermocycler from Biometra using Ready to Go PCR beads from Amersham Pharmacia Biotech according to the following conditions: 25–50 ng of genomic DNA (Table 1); 1.5 U *Taq* DNA polymerase, 10 mM Tris–HCl, pH 9; 50 mM KCl; 1.5 mM $MgCl_2$; 200 μM of each dNTP; stabilisers, including BSA, for a final volume of 25 μl. The amplification included 30 cycles of denaturation at 95°C for 30 s, annealing at 57–70°C (Table 1) for 30 s and extension for 30 s at 72°C. The product of each amplification was analysed by electrophoresis on 2% agarose gel (5 V/cm), using ethidium bromide staining.

2.3. SSCP analysis

For SSCP analysis, 5 μl of each amplification product was added to 15 or 20 μl (Table 2) 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, and chilled at 0°C, and loaded (20 or 25 μl of each) onto a 8–12% polyacrylamide/TBE gel. Gels were run at 25 W for 4–8 h at 15–20°C in a Dcode™ Universal Mutation Detection System, from BIO-RAD, coupled with a refrigerated system. After the run, the gel was removed from the apparatus and silver stained. The observed patterns and frequencies are shown in Table 2.

2.4. Statistical analysis

The data collected included milk, fat and protein production and fat and protein content adjusted to 145

Table 2
SSCP analysis parameters and results for the indicated exons

Exon	Number of patterns	Frequencies of patterns (%)
1	2	M (97.2); N (2.8)
2	2	A/B (75.9); B/B (24.1)
3	4	A/A (8.3); B/B (33.3); A/B (18.5); B/C (39.8)
4	6	A/A (13.9); B/B (27.8); C/C (35.2); D/D (5.6); E/E (14.8); F/F (2.8)
5	5	A/A (2.8); B/B (27.8); A/B (14.8); B/C (44.4); A/C (10.2)

days, measured in three consecutive years (1996–1998) in the same individuals. We considered data as repeated measures and the correlations between the measures in the same individual were considered in the statistical model.

A linear model, which included the fixed effects of type of parturition, exons 1–5, the random effect herds per year and considering repeated measures on the animal were used to determine associations between SSCP and milk production traits (SAS® System). The effect of the variable age was considered as a covariable (linear and quadratic effect), as well as classes (fixed effect). No influence of this variable was shown, so it was not considered in the final model. Since the relationships between animals were unknown, this information was not considered in the analysis.

The observations are repeated measures in the same subject (goat) and they are correlated and exhibit heterogeneous variability that makes inappropriate a standard ordinary least square analysis like ANOVA, because it assumes that the observations are uncorrelated and have constant variance. So a model was used that accounts for within-subject covariability.

The mixed effects model described as

$$\begin{bmatrix} y_1 \\ y_2 \\ \cdots \\ \cdots \\ y_n \end{bmatrix} = \begin{bmatrix} X_1 \\ X_2 \\ \cdots \\ \cdots \\ X_n \end{bmatrix} \beta + \begin{bmatrix} Z_1 & 0 & \cdots & \cdots & 0 \\ 0 & Z_2 & \cdots & \cdots & 0 \\ \cdots & \cdots & \cdots & \cdots & \cdots \\ \cdots & \cdots & \cdots & \cdots & \cdots \\ 0 & 0 & \cdots & \cdots & Z_n \end{bmatrix} \times \begin{bmatrix} v_1 \\ v_2 \\ \cdots \\ \cdots \\ v_n \end{bmatrix} + \begin{bmatrix} \varepsilon_1 \\ \varepsilon_2 \\ \cdots \\ \cdots \\ \varepsilon_n \end{bmatrix}$$

was used (Khattree and Naik, 1999), where y_i are the $p_i \times 1$ vector of repeated measures on the ith animal, X_i and Z_i the known matrices of orders $p_i \times q$ and $p_i \times r$, β the fixed $q \times 1$ vector of unknown (no-random type of parturition, exons 1–5) parameters, and v the $r \times 1$ vectors of random effects (herds per year). The ε_i are the $p_i \times 1$ vectors of random errors. For each sub-model, it was assumed:

$$E(v_i) = 0, \qquad \mathrm{var}(v_i) = \sigma^2 G_1$$
$$E(\varepsilon_i) = 0, \qquad \mathrm{var}(\varepsilon_i) = R_i$$
$$\mathrm{cov}(v_i, v_i') = 0, \qquad \mathrm{cov}(\varepsilon_i, \varepsilon_i') = 0,$$
$$\mathrm{cov}(v_i, \varepsilon_i') = 0, \quad \text{for all } i \neq i', \ \mathrm{cov}(v_i, \varepsilon_i) = 0$$

So the final model has the following assumptions:

$$E(v) = 0, \qquad E(\varepsilon) = 0$$
$$\mathrm{var}(v) = \sigma^2 I_n \otimes G_1 = \sigma^2 G$$
$$\mathrm{var}(\varepsilon) = \sigma^2 R$$
$$\mathrm{var}(y) = \sigma^2 [ZGZ' + R] = \sigma^2 V$$

The matrices G and R (or V) are estimated by REML under the assumption of multivariate normality of v and ε. In practice, certain structure is assumed, so V is a function of only a few unknown parameters.

In this work, we tested three structures for R indicated by Littell et al. (1996).

1. Compound symmetry structure with the following form:

$$\begin{bmatrix} \sigma^2 + \sigma_1 & \sigma_1 & \sigma_1 \\ & \sigma^2 + \sigma_1 & \sigma_1 \\ & & \sigma^2 + \sigma_1 \end{bmatrix}$$

2. Autoregressive of order 1 with the following form:

$$\sigma^2 \begin{bmatrix} 1 & \rho & \cdots & \rho^{p-1} \\ \rho & 1 & \cdots & \rho^{p-2} \\ \cdots & & & \\ \cdots & & & \\ \rho^{p-1} & \rho^{p-2} & \cdots & 1 \end{bmatrix}$$

3. Unstructured with the following form:

$$\begin{bmatrix} \sigma_{11} & \sigma_{12} & \sigma_{13} \\ & \sigma_{22} & \sigma_{23} \\ & & \sigma_{33} \end{bmatrix}$$

The models were compared using Akaike's Information Criterion and Scwarz's Bayesian Criterion in order to choose the model with the highest values in these criterions (Lagziel et al., 1996). The unstructured covariance model was the one that best fitted. The effect of type of parturition was significant for all studied exons.

3. Results and discussion

3.1. SSCP polymorphisms

We have chosen exons 1–5 of the GH gene for the SSCP analysis. The analysis of the amplified

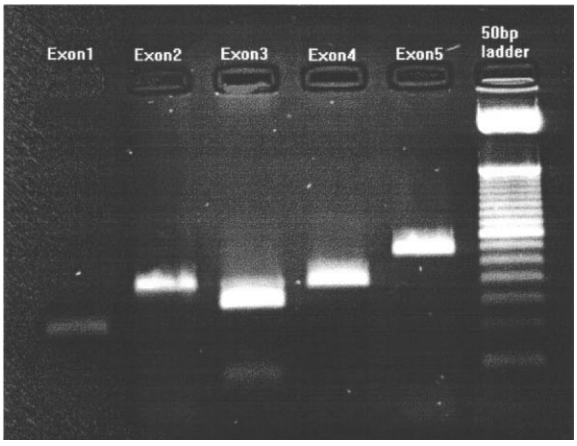

Fig. 1. Electrophoretic separation of the five gGH exons amplified by PCR in a 2% (w/v) (5 V/cm) agarose gel.

fragments by the PCR method, using the primers described in Table 1, is shown in Fig. 1. Their lengths correspond to those expected, according to the position of the primers, based on the described nucleotide sequence of the gGH gene from *Capra hircus* (Kioka et al., 1989). Considering the 108 Algarvia goats, we have observed several conformational patterns inside each of those fragments (Fig. 2 and Table 2). For exon 1, patterns M and N are heterozygous. The seven bands present in pattern M could be explained by the existence of a duplicate gene with two alleles per locus in these animals. This hypothesis requires investigations on more animals. One homozygous (B/B) and one heterozygous (A/B) conformational patterns were observed in exon 2. Two homozygous (A/A and B/B) and two heterozygous (A/B and B/C) were observed in exon 3. Two homozygous (A/A and B/B) and three heterozygous (A/B, A/C and B/C) were revealed in exon 5. Finally, the exon 4 is highly polymorphic. All patterns appear homozygous (A/A, B/B, C/C, D/D, E/E and F/F). However, the presence of numerous less intensive bands as well as the complexity of the observed polymorphism in exon 4 require more investigation and particularly the determination of the sequence of PCR products of these representative SSCP patterns. This part of the research is presently in investigation. The frequencies found for each pattern are reported in Table 2.

Table 3
Least squares means (±S.E.) for milk yield associated with patterns of exon 4

Pattern	Exon 4
A/A	140.3 ab ± 15.41
B/B	134.3 ab ± 13.42
C/C	136.8 ab ± 12.45
D/D	78.6 a ± 27.81
E/E	85.6 a ± 28.30
F/F	165.8 b ± 32.82

a,b: means with like lower cases do not differ ($P > 0.05$).

3.2. Statistical analysis

The analysis of fat and protein production and fat and protein content reveals no differences between production values of animals with different patterns in all studied exons. However, we found differences for milk production in exons 4 and 5.

Tables 3 and 4 present the least squares means obtained for milk yield. Animals with pattern A/A for exon 5 are superior milk producers ($P < 0.05$). These same animals have also the pattern F/F for exon 4 corresponding to the highest milk production value. Milk yield varied significantly between exons 4 and 5. Since the number of animals with pattern A/A in exon 5 and F/F in exon 4 was so small, these were eliminated from the model. However, no differences between milk yield in the exons 4 and 5 were observed. It seems that animals with patterns A/A and F/F in exons 5 and 4, respectively, have higher milk yield, though it will be necessary to analyse a larger sample to infer with more precision.

In the analysis of the fat and protein content, the unstructured covariance matrix allowed the best fit. However, no differences between patterns were

Table 4
Least squares means (±S.E.) for milk yield associated with exon 5 patterns

Pattern	Exon 5
A/A	213.0 b ± 22.67
A/B	121.5 a ± 13.64
B/B	124.5 a ± 12.88
B/C	134.7 a ± 12.65
A/C	133.5 a ± 13.95

a,b: means with like lower cases do not differ ($P > 0.05$).

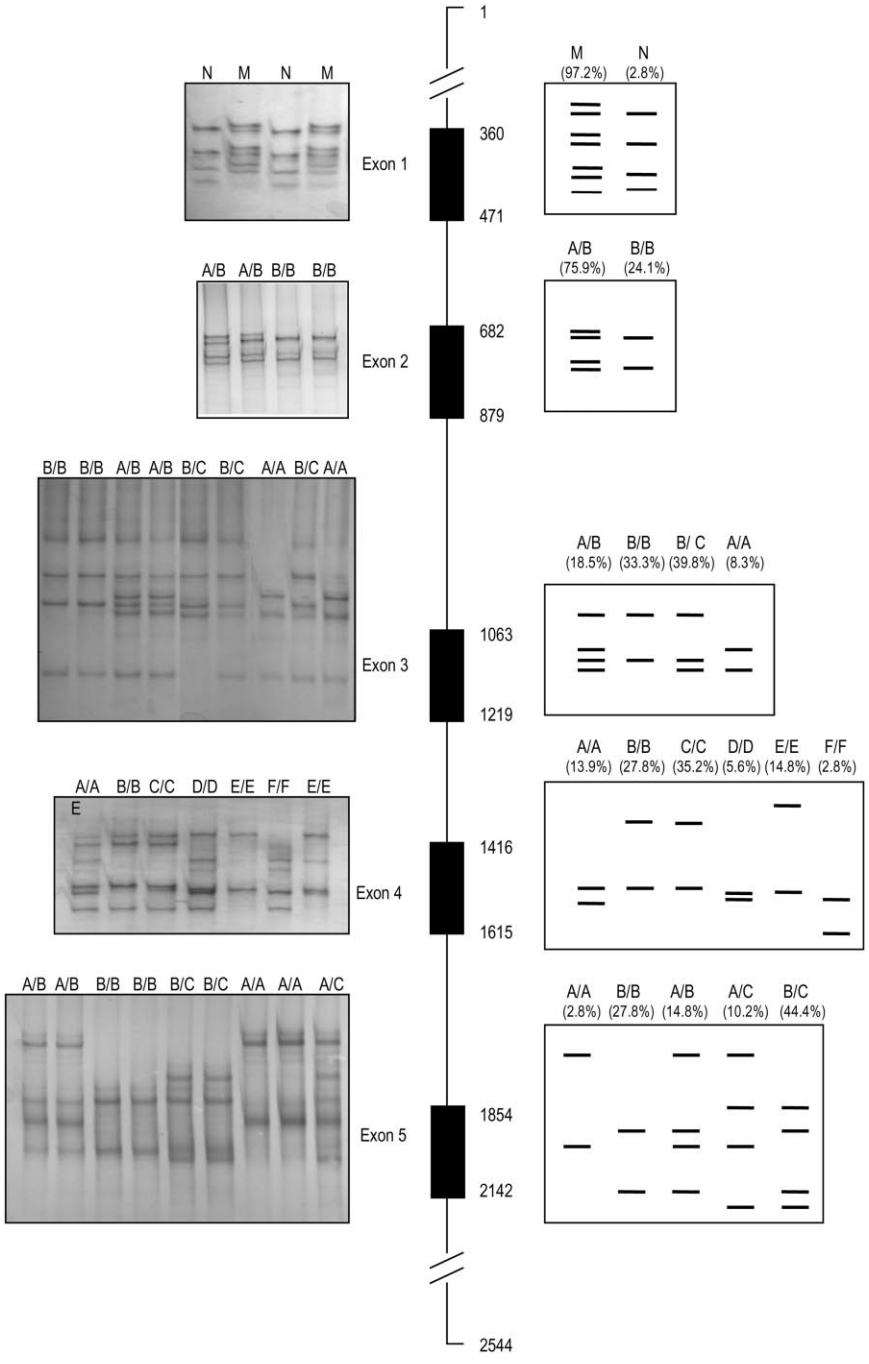

Fig. 2. SSCP patterns for exons 1–5 of goat GH gene. The SSCP patterns in a non-denatured PAGE are shown on the left, while the schematic goat GH gene with exons represented by black boxes and the schematic representation of SSCP patterns for each exon and respective frequencies are illustrated on the centre and the right column, respectively. Finally, the numbers indicate localisation of amplified fragments.

detected. Type of parturition was eliminated from the models, since its effect was not significant.

The SSCP analysis of genes, whose product is associated with production traits, could be a valuable alternative approach for the establishment of allelic variants useful as markers to aid selection. We have applied this technique to exons 1–5 of the GH gene from the indigenous Portuguese caprine Algarvia breed. The SSCP polymorphisms we have found in the gGH gene coding for a hormone that exerts a positive influence in milk production, hint at the possibility of exploring this approach for the search of genetic markers located in this gene. The SSCP polymorphic variation makes it a potential candidate for the establishment of associations with quantitative traits. If specific haplotypes can be defined at this candidate gene that could be associated with milk yield, protein and fat content, it would be a valuable genetic resource for improvement of this caprine breed. The associations between the polymorphisms observed in exons 4 and 5 and milk production exist. However, it will be necessary to increase the number of animal observations in order to determine haplotypes for the GH gene, and to look for their association with milk yield, protein and fat yield and content.

Exons 4 and 5 have to be sequenced for each pattern, and genotypes determined in order to characterise putative mutations in gGH, eventually implicated in variations of milk yield. It would be interesting to determine if one of the polymorphisms observed in exon 5 is related to the *Alu*I (+/−) polymorphism in bovines and it would be important to corroborate these results for other goat breeds.

4. Conclusions

The PCR-SSCP technique allows the genotyping of variants of a gene. In this preliminary study, we have applied this technique to the exons 1–5 of GH gene from the indigenous Portuguese caprine Algarvia breed. The SSCP polymorphisms, particularly for exons 4 and 5, were detected in the goat GH gene coding for a hormone that exerts a positive influence in milk production. However, before defining a specific haplotype as a candidate gene, it is necessary to expand the study with more animals to establish highly significant association between observed polymorphism and milk traits. Further studies are being continued to verify the above findings.

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