PIT-1 GENE *HINF*I POLYMORPHISM DETECTION IN EIGHT PORTUGUESE BOVINE BREEDS

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ABSTRACT

Our aim was to reveal PIT-1-*Hinf*I polymorphism in eight major indigenous Portuguese cattle breeds. A total of 195 bulls of Portuguese beef cattle breeds (Alentejana, Arouquesa, Barrosã, Maronesa, Marinhoa, Mertolenga, Mirandesa and Preta) were genotyped for this polymorphism within the exon 6 of that gene by the PCR-RFLP method. Additionally, single-stranded conformation polymorphism (SSCP) was performed in order to detect possible additional polymorphisms in the fragment amplified. The 195 samples analysed by PCR-SSCP showed no discrepancies between the two methods and confirmed previous results. Double-stranded conformation polymorphism (DSCP) was shown to be a reliable and expedite method of detecting Pit-1-*Hinf*I polymorphism. The genotype and gene frequencies for each breed were determined.
Key words: Pit-1, polymorphism, Portuguese breeds

DETECÇÃO DO POLIMORFISMO *HINF*I NO GENE PIT-1 EM OITO RAÇAS BOVINAS PORTUGUESAS

RESUMO

O nosso objectivo foi detectar o polimorfismo *Hinf*I do gene Pit-1 nas oito principais raças bovinas autóctones portuguesas. O exão 6 deste gene foi analisado pelo método PCR-RFLP em 195 touros de raças portuguesas produtoras de carne (Alentejana, Arouquesa, Barrosã, Maronesa, Marinhoa, Mertolenga, Mirandesa and Preta). Foi também aplicado o método de análise "single-stranded conformation polymorphism" (SSCP) de modo a detectar a possível existência de outros polimorfismos na região amplificada do gene. As 195 amostras analisadas por PCR-SSCP não mostraram discrepâncias entre os dois métodos e confirmaram os resultados anteriores. O método "Double-stranded conformation polymorphism" (DSCP) revelou-se fiável e expedito de detecção do polimorfismo Pit-1-*Hinf*I. Foram determinadas as frequências genéticas e alélicas para cada raça.
Palavras-chave: Pit-1, polimorfismo, raças portuguesas

INTRODUCTION

Pit-1 is a pituitary-specific transcription factor responsible for growth hor-

mone (GH) expression in mammals that also regulates prolactin (PRL) and thyroid-stimulating hormone (TSH) genes. A *Hinf*I polymorphism was described at the bovine Pit-1 locus by Woollard *et al.* (1994). The loss of the *Hinf*I site is a silent mutation due to a transition polymorphism from guanine (G) to adenine (A) within exon 6 (Dierkes *et al.*, 1998). Mutations in the gene encoding Pit-1 have been found in growth disorders in mice (Li *et al.*, 1990) and humans (Cohen *et al.*, 1995) and is thus a candidate gene for improving growth efficiency in cattle. Recent studies suggest that the Pit-1 gene can act as a genetic marker to characterise specific traits of economical importance in animals. For instance, polymorphisms in this gene were significantly associated with growth and carcass traits in pigs (Yu *et al.*, 1995). Pit-1 gene *Hinf*I polymorphism was associated to yield traits in cattle by Renaville *et al.* (1997); Renaville and Portetelle (1998). The authors claim that it can be used to select superior traits in animals being the genotype BB (fully digested pattern) indicative of a trait for muscularity, whereas the genotypes AB and AA are associated with animals having superior milk production traits.

MATERIAL AND METHODS

Animals

A total of 195 bulls of the following indigenous breeds were included in this study: Alentejana (AL, n = 22), Arouquesa (AR, n = 24), Barrosã (BA, n = 23), Marinhoa (MO, n = 32), Maronesa (MA, n = 24), Mertolenga (ME, n = 22), Mirandesa (MI, n = 21) and Preta (PR, n = 27).

DNA amplification with Polymerase Chain Reaction (PCR)

DNA was extracted from peripheral blood leukocytes using DNA Isolation kit from Puregene. Based on the published nucleotide sequence of bovine PIT-1 DNA (Dierkes *et al.* 1998) a pair of oligonucleotide primers was designed to amplify a 239 base pair (bp) fragment that comprises the locus of the mutation, by the polymerase chain reaction (PCR). Twenty-five μl PCR were carried out in a Biometra UNO II 48 thermalcycler, using PCR beads Ready-To-Go (Amersham Pharmacia Biotec) with 50 ng of bovine genomic DNA, 16 pmol of each primer and 2.5 mM $MgCl_2$. The primers PITF (5´-CCTTCTTTCCTGCCAACT-3´) and PITR (5´-TCTGCATTCGAGATGCTC-3´) flanked a 239 bp fragment, consisting of 30 bp of the fifth intron and 209 bp from the exon 6. After a first denaturation step at 95ºC for 5 min, the samples were submitted to 30 cycles of amplification comprising the following steps: denaturation 95ºC x 30 s; primer annealing 60ºC x 30 s; primer

extension 72°C x 30 s. The amplification ended with a 5 min final extension step at 72°C.

Polymerase Chain Reaction - Restriction Fragment Length Polymorphism (PCR-RFLP)

Amplification products (8.5 µl) were digested at 37°C for at least 14 h with 5 Units of the restriction enzyme *HinfI* [G|ANTC] (Gibco BRL, Life Technologies) and separated on a 3.5 % agarose gel containing 0.1 µg ml^{-1} ethidium bromide and photographed during exposure to UV light.

Single-Stranded Conformation Polymorphism (SSCP) and Double-Stranded Conformation Polymorphism (DSCP) analysis

The PCR samples were examined by electrophoresis on non-denaturing polyacrylamide gels in TBE buffer in a Bio-Rad DCode™ Universal Mutation Detection System electrophoresis unit. For SSCP analysis (Orita *et al.*, 1989) 5 µl of PCR product diluted in 16 µl of a denaturing solution. The mixture was then denatured at 95°C for 5 min, cooled in ice for 5 min and loaded on a non-denaturing polyacrylamide gel (T=9%, C=2.5) without glycerol. Electrophoresis was performed at 8 °C, constant wattage of 25 W, for a running time of VH=6000, (approximately 14 h). For DSCP analysis (Kirkpatrick *et al.*, 1993) 5 µl of PCR product was diluted in 15 µl of a non-denaturing solution and loaded on a nondenaturing polyacrylamide gel (T=10%, C=2.5) without glycerol and without previous denaturation. Electrophoresis was performed at 20 °C, constant wattage of 25 W, for a running time of VH=1650, (approximately 4 h). In both methods DNA was detected by silver staining using DNA Silver Staining kit Plusone (Pharmacia).

RESULTS AND DISCUSSION

1.Polymerase Chain Reaction - Restriction Fragment Length Polymorphism (PCR-RFLP)

A product of the expected size of 239 bp was obtained for all the 195 DNA samples. Digestion of 8.5 µl of the PCR product with 5 Units of *HinfI* revealed two alleles. These alleles generated three patterns after separation on a 3.5 % agarose gel. In homozygous animals either a unique band (239 bp, AA variants) or two bands (194 and 45 bp, BB variants) patterns were observed. Heterozygous animals gave a three-band (239, 194 and 45 bp) pattern (Figure 1).

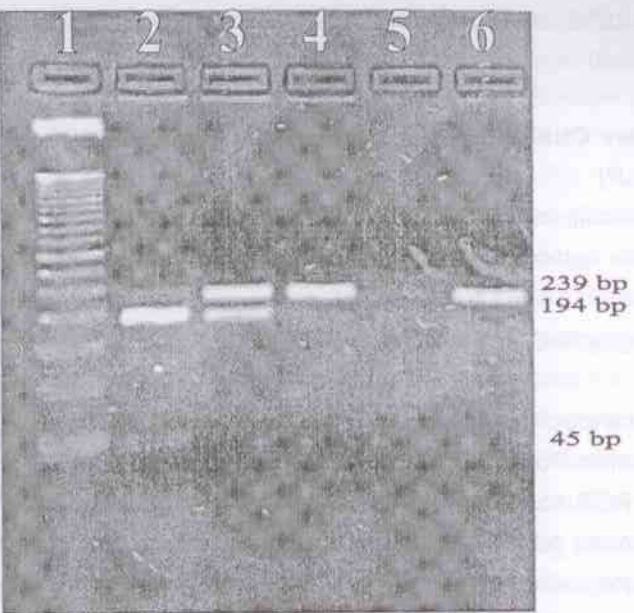

Figure 1. Analysis of the *HinfI* polymorphism at the Pit-1 locus by PCR-RFLP: Electrophoretic patterns of PCR products after digestion with *HinfI* of three animals showing the three different genotypes separated on a 3.5 % agarose gel. Lane 1: 50 bp DNA Ladder (Gibco BRL, Life Technologies); lane 2: BB genotype; lane 3: AB genotype; lane 4: AA genotype; lane 5: PCR control reaction (PCR reaction without template DNA); lane 6: Undigested PCR product.

2. Single-Stranded Conformation Polymorphism (SSCP)

For the analysis of the amplified fragment through SSCP, several electrophoretic parameters were tested. The optimal conditions established for the detection of the *HinfI* polymorphism were: constant 25 W, running time of VH=6000, (approximately 14 h) at 8°C in a non-denaturing polyacrylamide gel (PAGE) (T=9%, C=2.5) without glycerol. Besides the *HinfI* polymorphism no additional polymorphism was found in the amplified fragment. Results of PCR-SSCP are shown in Figure 2. The electrophoretic analysis of all the 195 animals analysed by PCR-SSCP exhibited different patterns, corresponding to different genotypes determined by PCR-RFLP.

3. Double-Stranded Conformation Polymorphism (DSCP)

The optimal electrophoretic conditions for the detection of the Pit-*HinfI* polymorphism by PCR-DSCP were: constant 25 W, running time of VH=1650, (approximately 4 h) at 20°C in a non-denaturing polyacrylamide gel (PAGE) (T=10%, C=2.5) without glycerol.

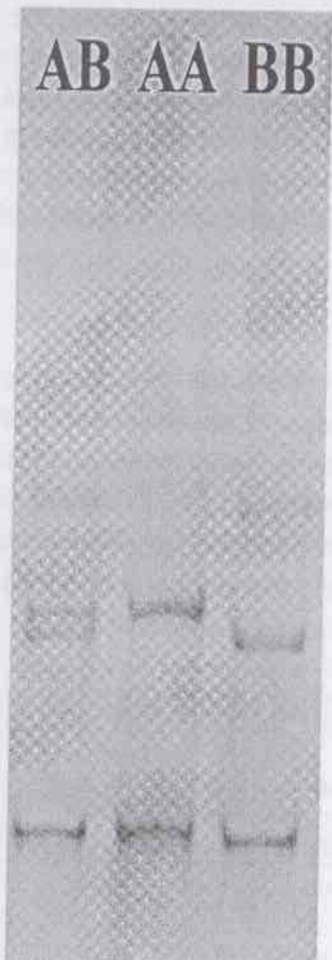

Figure 2. Analysis of the *HinfI* polymorphism at the Pit-1 locus by PCR-SSCP. Electrophoretic patterns of the PCR products of three animals showing the three Pit-1- *HinfI* genotypes. Lane 1: AB genotype; lane 2: AA genotype; lane 3: BB genotype.

Figure 3. Electrophoretic patterns PCR products of three animals showing the three Pit-1- *HinfI* genotypes detected by PCR-DSCP. Lane 1: AB genotype; lane 2: AA genotype; lane 3: BB genotype.

PCR-DSCP provides an accurate mean of detecting this polymorphism using the primers and conditions of electrophoresis described. This method is as reliable as the other two methods mentioned avoiding the digestion step and the use of the restriction enzyme when compared to the PCR-RFLP approach. On the other hand, it is much faster than PCR-SSCP in terms of electrophoresis running time with no need for the denaturing step of the PCR products. Results of PCR-DSCP are presented in Figure 3.

4. GENOTYPIC AND ALLELIC FREQUENCIES

In this report we consider allele A to be the allele which is not digested by the restriction enzyme *HinfI*. In other words, A is the allele in which an adenine (A) is present instead of a guanine (G). We use this assumption because it is the most widely accepted in the literature (but not universally; *vide infra*). In general, allele A was less frequent than allele B in the populations analysed. Only the Alentejana breed constituted an exception showing a frequency for allele of 0.55 (genotypic and allelic frequencies are shown in Table I).

Table I - Genotypic and allelic frequencies of the Pit-1 *HinfI* polymorphism in the populations analysed. AL, Alentejana; AR, Arouquesa; BA, Barrosão; MO Marinhoa; MA, Maronesa; ME, Mertolenga; MI, Mirandesa; PR, Preta.

Breed	Genotype BB	Genotype AB	Genotype AA	Allele B	Allele A
AL (n=22)	0.091	0.727	0.182	0.45	0.55
AR (n= 24)	0.667	0.250	0.083	0.79	0.21
BA (n= 23)	0.696	0.217	0.087	0.80	0.20
MO (n= 32)	0.813	0.156	0.031	0.89	0.11
MA (n= 24)	0.542	0.375	0.083	0.73	0.27
ME (n= 22)	0.818	0.182	0.000	0.91	0.09
MI (n= 21)	0.857	0.143	0.000	0.93	0.07
PR (n= 27)	0.370	0.593	0.037	0.67	0.33

These observations should be considered as preliminary results due to the small sample size and high degree of inbreeding in the bulls of the breeds Alentejana, Maronesa and Preta. So far higher frequencies of allele B have always been observed for several breeds by other authors. Frequencies of 0.15 for allele A and 0.85 for allele B were reported for a population of 214 animals of the Holstein breed (Wollard *et al.* 1994). Moody *et al.* (1995) determined allelic frequencies for allele A of 0.45, 0.26, 0.21, 0.18 and 0.10 for Angus (n = 19), Holstein

(n = 17), Herefold (n = 45), Gelbvieh (n = 17) and Brahman (n = 5) breeds, respectively, with an overall frequency of 0.25. For Italian Holstein-Friesian bulls (n = 89) a frequency of 0.188 was found for allele A and 0.812 for allele B (Renaville *et al.*, 1997, Renaville and Portetelle, 1998). Dierkes *et al.* (1998) found frequencies of 0.68 and 0.32 for allele A and allele B, respectively, although in this case the authors regard allele A as the one which is cut by *Hinf*I, corresponding therefore to what we consider to be allele B. So, again the allele B was more common.

CONCLUSIONS

Genotyping bovine Pit-1 gene transition polymorphism in exon 6 was possible using the three methods. This substitution of A for G affects double-strand DNA conformation and electrophoretic mobility which enables the distinction between the three possible genotypes in the conditions described. Because it is cost-effective, sensitive, and fast, PCR-DSCP is strongly recommended to routinely screen Pit-1 variants in cattle breeding programs. A study of the effects of Pit-1- *Hinf*I genotypes on growth traits, will be of great interest in the breeds analysed in this study, as their main purpose is meat production.

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