PIT-1 GENE HNF1 POLYMORPHISM DETECTION IN EIGHT PORTUGUESE BOVINE BREEDS

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INTRODUCTION

Pit-1 is a pituitary-specific transcription factor responsible for growth hormone (GH) expression. In this study, we aimed to detect the Pit-1 HNF1 polymorphism in eight major indigenous Portuguese bovine breeds (Alentejana, Barrosa, Marinha, Murtilha, Minho, Béjar, and Estrela). A total of 195 bulls of each breed were genotyped using the conventional PCR-SSCP method. The results showed that the HNF1 polymorphism was present in all breeds, with the A allele being the most frequent. The detection of this polymorphism is crucial for genetic evaluation and selection programs in Portuguese bovine breeds.
mone (GH) expression in mammals that also regulates prolactin (PRL) and thy-
roid-stimulating hormone (TSH) genes. A *Hinfi* polymorphism was described at
the bovine Pit-1 locus by Woolard et al. (1994). The loss of the *Hinfi* 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. Re-
cent studies suggest that the Pit-1 gene can act as a genetic marker to character-
ise 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 *Hinfi* 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), Arroquesa (AR, n = 24), Barrosã (BA, n = 23),
Marinheira (MO, n = 32), Maronesa (MA, n = 24), Mertolenga (ME, n = 22), Mirandesas
(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 am-
plify 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
MgCl2. The primers PITF (5'-CTTGT TCCTCTTTCCT-GCCAAC-3') and PITR (5'-
TCTGCATTTGAGATGCTC-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 95°C
for 5 min, the samples were submitted to 30 cycles of amplification comprising the
following steps: denaturation 95°C x 30 s; primer annealing 60°C 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).
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 *Hinfl* 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 *Hinfl* 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-*Hinfl* 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 *Hinf* polymorphism at the Pit-1 locus by PCR-SSCP. Electrophoretic patterns of the PCR products of three animals showing the three Pit-1- *Hinf* 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- *Hinf* 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 Hinfl. 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>
<thead>
<tr>
<th>Breed</th>
<th>Genotype BB</th>
<th>Genotype AB</th>
<th>Genotype AA</th>
<th>Allele B</th>
<th>Allele A</th>
</tr>
</thead>
<tbody>
<tr>
<td>AL (n=22)</td>
<td>0.091</td>
<td>0.727</td>
<td>0.182</td>
<td>0.45</td>
<td>0.55</td>
</tr>
<tr>
<td>AR (n=24)</td>
<td>0.667</td>
<td>0.250</td>
<td>0.083</td>
<td>0.79</td>
<td>0.21</td>
</tr>
<tr>
<td>BA (n=23)</td>
<td>0.696</td>
<td>0.217</td>
<td>0.087</td>
<td>0.80</td>
<td>0.20</td>
</tr>
<tr>
<td>MO (n=32)</td>
<td>0.813</td>
<td>0.156</td>
<td>0.031</td>
<td>0.89</td>
<td>0.11</td>
</tr>
<tr>
<td>MA (n=24)</td>
<td>0.542</td>
<td>0.375</td>
<td>0.083</td>
<td>0.73</td>
<td>0.27</td>
</tr>
<tr>
<td>ME (n=22)</td>
<td>0.618</td>
<td>0.182</td>
<td>0.000</td>
<td>0.91</td>
<td>0.09</td>
</tr>
<tr>
<td>MI (n=21)</td>
<td>0.857</td>
<td>0.143</td>
<td>0.000</td>
<td>0.93</td>
<td>0.07</td>
</tr>
<tr>
<td>PR (n=27)</td>
<td>0.370</td>
<td>0.593</td>
<td>0.037</td>
<td>0.67</td>
<td>0.33</td>
</tr>
</tbody>
</table>

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), Hereford (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 Hinfl, 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- Hinfl genotypes on growth traits, will be of great interest in the breeds analysed in this study, as their main purpose is meat production.

Acknowledgements

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REFERENCES


