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Research Article

Genetic polymorphisms at the leptin receptor gene in three beef cattle breeds

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Abstract

The genetic diversity of a single nucleotide polymorphism (SNP) at the exon 20 (T945M) of the leptin receptor gene (LEPR) and of three short tandem repeats (STRs BM7225, BMS694, and BMS2145) linked to LEPR was investigated in three beef cattle herds (Brangus Ibagé, Charolais, and Aberdeen Angus). A cheap and effective new method to analyze the T945M polymorphism in cattle populations was developed and the possible role of these polymorphisms in reproduction and weight gain of postpartum cows was evaluated. High levels of genetic diversity were observed with the average heterozygosity of STRs ranging from 0.71 to 0.81. No significant association was detected between LEPR markers and reproductive parameters or daily weight gain. These negative results suggest that the LEPR gene polymorphisms, at least those herein described, do not influence postpartum cows production.

Key words: molecular markers, genetic diversity, cattle production, LEPR gene polymorphisms.

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Introduction

Low bovine fertility rate is associated with suboptimal nutrition and is a major concern of livestock cattle production systems. Recently, much effort has been devoted to understand the role of the leptin protein and its receptor in regulating food intake and reproduction in ruminants (Chilliard *et al.*, 2005).

Leptin is secreted by adipose tissues and acts especially through its receptor on the hypothalamus, the center of energy homeostasis, as well as on ovarian follicular cells, on placenta and lactating mammary glands (Bartha *et al.*, 2005; Chilliard *et al.*, 2005). The leptin receptor (LEPR) is a member of the cytokine I family of receptors and signal transducers. Comparisons of the bovine LEPR DNA sequence with that of humans and mice indicated 81% and 75% of sequence identity, respectively (Pfister-Genskow *et al.*, 1997). In ruminants, LEPR expression seems to be affected by high and low nutrition levels (Chilliard *et al.*, 2005) and blood leptin concentrations seem to interfere in luteinizing hormone secretion (Kadokawa *et al.*, 2006) and to stimulate growth hormone release (Nonaka *et al.*, 2006).

Many studies concerning the relationship between LEPR gene polymorphisms and weight gain have been conducted in rodents and humans (Banks and Farrell, 2003, Park *et al.*, 2006), but few data are available for ruminants, which present different complexity levels concerning digestion and cerebral metabolic sensors (Chilliard *et al.*, 2005).

The LEPR gene is located on bovine chromosome 3q33 (Pfister-Genskow *et al.*, 1997) and several polymorphisms have been mapped in this chromosome, such as the short tandem repeats BM7225 at 101.7 cM, BMS694 at 94.6 cM, and BMS2145 at 93.8 (Kappes *et al.*, 1997). Inside the LEPR gene, Liefers *et al.* (2004) described a missense mutation T945M (Table1).

In this study the genetic variability of the polymorphisms described above was analyzed in three beef cattle herds, enabling the description of a new methodology to investigate the T945M polymorphism, and the evaluation of the possible role of these polymorphisms in reproduction and weight gain in these herds.

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Table 1 - Molecular markers mapped at BTA3 and analyzed in the present paper.

STRs	GenBank number	Observed allele sizes	Primers[1]	Annealing temperatures	References
BM7225	G18790	96-114	GGTGTTATGCATTCTCTAGGTGC AAGAGTTAGACATGACTGAGCACG	60 °C	Kappes *et al.,* 1997
BMS694	G18739	135-153	AAACTCTAGGTCCATCCAGGTT AGATGTGGAAGTGTCCATCAA	58 °C	Kappes *et al.,* 1997
BMS2145	G18925	148-164	ATGGAAGTGGCTTAAGTGTCC GCAAATAACCTCCATATTGCTG	58 °C	Kappes *et al.,* 1997
T495M	AJ580801	*T*: 67, 130 *M*: 37,67, 93	ACTACAGATGCTCTACTTTGG TGCTCCTCCTCAGTTT	56-50 °C[2]	This paper

[1]T495M primers are those employed in the present paper, and not those described by Liefers *et al.*, 2004; [2]Touchdown PCR.

Materials and Methods

Blood samples from three beef cattle herds [Aberdeen Angus (AA, n = 98), Charolais (C, n = 83), and Brangus Ibagé (n = 160)] were obtained from the jugular vein using acid-citrate-dextrose (ACD) as anticoagulant (Almeida *et al.*, 2003) and following the Principles of Veterinary Medical Ethics ("Código de Ética Profissional do Médico Veterinário") and the International Guiding Principles For Biomedical Research Involving Animals (1985).

AA and C animals had been evaluated in a previous study which compared the efficiency of different hormonal treatments associated with 96-hour calf removal in relation to complete weaning of animals fed with different forages and analyzed the interaction between fertility and weight gain in postpartum (Terra *et al.*, 2008). The animals were adult cows (ages ranging from 4 to 6 years), with mean body condition of 3.0 (in a classification range from 1 - very thin – to 5 – very fat; Lowman *et al.*, 1973) at partum. Fifty to 70 days postpartum, the cows were sorted into six groups (A0, A2, A5, B0, B2, and B5) according to their body condition at partum. They were then submitted to different forage availabilities and hormonal treatments: A groups were managed on native pasture with 960 kg dry matter per hectare (DM ha^{-1}) and a stocking rate of 0.96 animal unit per hectare (au ha^{-1}; au = 400 kg live weight) at partum, and 400 kg DM ha^{-1} at weaning; B groups were also managed on native pasture but with 600 kg DM ha^{-1} and a stocking rate of 1.44 au ha^{-1} at partum and 240 kg DM ha^{-1} at weaning. The dry matter of the pasture was estimated by the double sample method (Wilm *et al.*, 1944). A0 and B0 were definitely separated from their calves at day 7 from the beginning of the experiment. A2, A5, B2, and B5 were submitted to estradiol benzoate [2 mg (A2, B2) or 5 mg (A5, B5)], plus a progesterone (P4) vaginal implant; six days later they received 1000 UI of equine chorionic gonadotropin and in the following day the vaginal implant was removed and the cows were separated from their calves for 96 h. All animals were weighed twice (at partum and at weaning). The cows that showed estrous between days 7 and 17 from the beginning of the treatment were artificially inseminated; they were then bred with a cow:bull ratio of 100:12 up to day 67; clinical and ultrasonic pregnancy diagnoses were performed on day 60 from the beginning of the experiment to calculate the proportion of cows that conceived in the first estrous after treatment, and on day 127 to estimate final pregnancy rate.

The Brangus Ibagé (BI) breed is a composite beef cattle herd (5/8 Aberdeen Angus x 3/8 Nelore) resulting from the crossing between Aberdeen Angus cows (*Bos primigenius taurus*) and Nelore bulls (*Bos primigenius indicus*) performed by the Brazilian Agricultural Research Corporation (EMBRAPA Pecuária Sul, Bagé, RS, Brazil). Breeding procedures include single sire mating in small paddocks, in groups of about 40 females for paternity identification purposes. The selection program began in 1945, with emphasis on body weight measurements at birth, at weaning adjusted to 205 days, and at 18 months of age, without any special selection for fertility. All animals have been exclusively managed on native pasture in an extensive livestock system (Oliveira *et al.*, 1998), with the mating season extending from November 15th to February 15th. Lifetime calving interval data (CI) were obtained for the females of the experimental herd as described by Oliveira *et al.* (2002). As an indicator of cow fertility, the weight at first calving (WFC) was computed as a predictor for growth potential of the heifers. From a total of 287 cows from this herd, samples were obtained from 160 animals for which there was available information about at least three calving intervals.

Genomic DNA was extracted from peripheral blood (Miller *et al.*, 1988). Three STRs [BM7225 (D3S75), BMS694 (D3S66), and BMS2145 (D3S64)] linked to the LEPR gene were PCR-amplified by standard methods with specific primers and annealing temperatures (Table 1). The amplicons were analyzed by vertical electrophoresis in 10% non-denaturing polyacrylamide gels (Sambrook and Russel, 2001).

The SNP T945M, which maps at the exon 20 of the LEPR sequence and corresponds to a mutation in the intracellular region of the functional protein, was also analyzed. This mutation was previously investigated by DNA sequencing (Liefers *et al.*, 2004). In order to screen this mutation, we used a method that introduces a point mutation into

one of the primers so that the PCR product contains a *Fok*I restriction site. Primers 5' ACTACAGATGCTCTACTT TGG 3' and 5' TGCTCCTCCTCAGTTT 3' (the underlined nucleotide corresponds to the mutation introduced) amplify a 197-base pair (bp) fragment. After digestion with *Fok*I, the MM animals presented 93-, 67-, and 37-bp fragments, while TT animals showed 130-, and 67-bp fragments (Figure 1). The PCR was performed with an annealing temperature of the reaction decreasing 1 °C from 56 °C every second cycle to a 'touchdown' at 50 °C, at which temperature 34 cycles were carried out. Each cycle consisted of 94 °C for 20 s, 15 s at the annealing temperature and 72 °C for 20 s followed by a final extension at 72 °C for 5 min. The cleavage products were analyzed by vertical electrophoresis in 10% non-denaturing polyacrylamide gels (Sambrook and Russel, 2001).

Allele and genotype frequencies were determined by direct counting. The expected heterozygosity (H) and average expected heterozygosity were both estimated according to Nei (1978). Association analyses were performed using the General Linear Models of SPSS® for WindowsTM software (SPSS Inc), version 10.0.5 (1999), according to the following models:

For A. Angus and Charolais:

$$Y_{ijklm} = \mu + \beta B_{ijklm} + F_j + H_k + G_l + F^*H_{jk} + F^*G_{jl} + H^*G_{kl} + F^*H^*G_{jkl} + e_{ijklm}$$

where Y_{ijklm} is the m^{th} ADG (in grams), P1, or P2 record of the i^{th} cow; μ is the effect of the population mean; βB_{ijklm} is the covariate effect of the body score condition; F_j is the effect of forage availability; H_k is the effect of hormone treatment; G_l is the effect of the marker genotype; and e_{ijklm} is the random error component.

For B. Ibagé:

$$Y_{ijklm} = \mu + \beta W_{ijklm} + S_j + Y_k + G_l + S^*Y_{jk} + S^*G_{jl} + Y^*G_{kl} + S^*Y^*G_{jkl} + e_{ijklm}$$

where Y_{ijklm} is the m^{th} CI (average of all CI information, in days) record of the i^{th} cow; μ is the effect of the population mean; βW_{ijklm} is the covariate effect of the weight at calving, S_j is the effect of calf sex, Y_k is the year of partum, G_l is the effect of the marker genotype, and e_{ijklm} is the random error component. And

$$Y_{ij} = \mu + A_i + e_{ij}$$

where Y_{ij} is the j^{th} WFC phenotype of the i^{th} individual; μ is the effect of the population mean; A_i is the effect of the ith genotype class; and e_{ij} is the random error component.

Descriptive statistics was carried out beforehand to verify the normality of the distribution of the productive parameters. Then, CI data were normalized by natural logarithmic transformation.

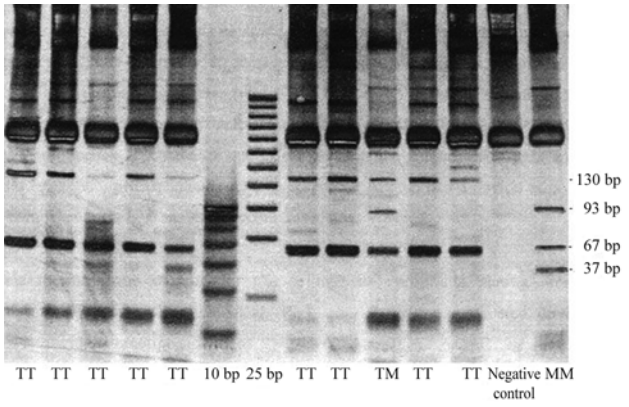

Figure 1 - Polyacrylamide gel electrophoresis showing the T945M mutation genotypes analyzed through the PCR/RFLP method.

Results

Gene frequencies varied among populations (Table 2), but the most frequent alleles were *BM7225*96*, *BMS694*145, BMS2145*154* and *T495M *T.* Some alleles were observed only in one population: *BM7225*98* in Charolais animals, *BMS694*149* and *BMS694*153* in Brangus Ibagé, and *BMS694*135* in Aberdeen Angus. The PCR-RFLP method employed to screening the T945M SNP permitted the identification of both alleles. The *M* allele was present at a low frequency and only one MM homozygous individual was observed (in the Charolais population). The expected heterozygosity values (H) were high in the STR systems, with a mean value ranging from 0.71 in AA to 0.81 in BI (Table 2).

Descriptive statistics (mean ± standard error) for CI and WFC in BI, as well as ADG and pregnancy rates (in percent) on day 60 (P1) and 127 (P2) for AA and C cows (according to treatment groups) are shown in Table 3. No differences were observed in ADG, P1 or P2 among the several nutrition and hormone treatment groups in AA or C breeds. The association analyses performed between genotype classes and CI and WFC in Brangus Ibagé indicated no significant result. Also, no difference between ADG, P1 or P2 and genotypes was detected in Aberdeen Angus or in Charolais. The simultaneous comparison of these two herds also failed to reveal any significant association. For this last analysis, the mean ADG value was corrected considering herd weight to compensate the sharp differences in this parameter between Charolais (232.1 ± 2.5) and Aberdeen Angus (103.4 ± 2.0). No interaction between genotypes, hormone treatment or nutrition was observed.

Discussion

The STRs linked to the LEPR gene herein investigated presented high variability in the three populations. Although Charolais and Aberdeen Angus herds have a long history of artificial selection, these procedures have not reduced their genetic variability, at least for the polymorphisms investigated. The higher variability in STR systems observed in Brangus Ibagé as compared to the other two

Table 2 - Allele frequencies and heterozygosities (H) in Brangus Ibage (BG), Charolais (C), and Aberdeen Angus (AA) breeds.

Alleles	Markers		
	BM7225		
	BI (N = 156)	C (N = 81)	AA (N = 98)
**96*	0.18	0.24	0.36
**98*	-	0.03	-
**100*	0.01	0.04	-
**102*	0.13	0.10	0.27
**104*	0.16	0.11	0.15
**106*	0.10	0.16	0.07
**108*	0.08	0.13	0.10
**110*	0.13	0.05	0.01
**112*	0.16	0.05	0.02
**114*	0.05	0.09	0.02
H	0.86	0.87	0.77
	BMS694		
**135*	-	-	0.01
**141*	0.21	0.02	0.26
**143*	0.02	0.20	-
**145*	0.43	0.65	0.62
**147*	0.10	0.06	0.06
**149*	0.08	-	-
**151*	0.13	0.07	0.05
**153*	0.03	-	-
H	0.74	0.53	0.54
	BMS2145		
**148*	0.15	-	0.16
**150*	0.07	0.08	0.07
**152*	0.24	0.10	0.06
**154*	0.22	0.26	0.31
**156*	0.14	0.16	0.13
**158*	0.05	0.08	0.03
**160*	0.05	0.14	0.10
**162*	0.07	0.13	0.13
**164*	0.01	0.05	0.01
H	0.84	0.85	0.83
	T495M		
**T*	0.98	0.92	0.96
**M*	0.02	0.08	0.04
H	0.03	0.15	0.08
STR mean H	0.81	0.75	0.71
Mean H	0.62	0.60	0.57

N = sample size; genotype frequencies may be obtained with the authors, on request.

breeds probably results from its crossbreeding composition. High levels of STR genetic diversity in the BI herd has already been described (Almeida *et al.*, 2003, 2007; Duarte *et al.*, 2005; Oliveira *et al.*, 2005). The Charolais herd presented the highest variability concerning the T945M polymorphism.

The occurrence of some exclusive alleles is probably related to the founder effect associated with the origin of each population. Literature data about these STR markers are scarce and there is no information about *Bos primigenius indicus* samples. The presence of the *BMS694*149* and *BMS694*153* alleles only in the Brangus Ibagé population suggests that they possibly originated from *B. p. indicus* (Zebu), as these alleles have not been described for other *B. p. taurus* samples so far investigated.

The PCR-RFLP method that we used to analyze the T945M SNP was efficient to detect this mutation and allowed the detection of a low frequency of the *M* allele in the three populations. These data agree with those of Liefers *et al.* (2004), who verified a frequency of 0.07 in a population of 323 Holstein-Friesian cows and did not detect TT animals.

In cattle, as in other mammalian species, there is a positive relationship between circulating leptin and fat content (Murdoch *et al.*, 2005) and leptin also seems to play a role in reproduction (Kendall *et al.*, 2004; Kadokawa *et al.*, 2006). Leptin action is mediated by the leptin receptor protein and LEPR mRNA abundance is increased by acute food restriction (Murdoch *et al.*, 2005). Therefore, the analyses of the leptin receptor gene polymorphisms could be useful to understand the reproductive performance and weight gain variation in cattle. It is possible that there is an effect of STR polymorphisms on animal performance because these markers could affect gene regulation. Even being distant from the gene they regulate, they could alter the primary, secondary or tertiary DNA structure by binding to transcription factors, or by affecting RNA splicing or edition (Li *et al.*, 2004).

The absence of an association between the molecular markers analyzed and CI, WFC (in BI) or between ADG and pregnancy rates (in AA and C) suggests that there is no effect of these polymorphisms on these cattle production measurements in the postpartum period. However, the parameters here investigated are indirect measurements of reproductive performance and fat depots.

Liefers *et al.* (2004) verified an association between the T945M mutation with circulating leptin concentrations during late pregnancy, but not during lactation. As the cows analyzed in this study were in lactation period, the negative results do not exclude possible effects of these polymorphisms on cows weight gain or reproduction during other life periods. In species such as mouse and human, mutations at LEPR seem to be associated with obesity (Clément and Ferré, 2003; Zhang *et al.*, 1994).

In AA and C samples the objective was to verify the joint effect of molecular markers, nutrition, weight gain, and hormone treatment on the reproductive performance of postpartum cows. The pregnancy rates were dependent on weight gain and on hormone treatment (Terra *et al.*, 2008), but the molecular markers analyzed did not seem to influence weight gain or reproduction.

Table 3 - Descriptive statistics for CI and WFC in Brangus Ibagé, for ADG, P1 and P2 in Aberdeen Angus and Charolais populations, in relation to hormone treatment and nutrition groups.

Breeds							
Brangus Ibagé							
		Mean	se	Minimum	Maximum		
	CI[a]	549.71	41.72	352.0	933.2		
	WFC[b]	351.56	4.35	235.1	510.4		
Aberdeen Angus							
Groups[d]	Number	ADG[c]				Pregnancy (%)	
		Mean	se	Minimum	Maximum	P1	P2
A0	26	113.65	37.60	-266.80	544.00	77	96
A2	20	63.44	41.86	-190.90	425.90	85	95
A5	7	169.07	46.44	-054.70	324.70	71	86
B0	15	102.65	49.44	-151.30	560.00	73	100
B2	13	170.28	58.47	-242.70	463.20	69	92
B5	17	57.11	58.55	-296.80	575.30	59	82
Total	98	103.38	20.03	-296.80	575.30	73	94
Charolais							
Group[d]	Number	ADG[c]				Pregnancy (%)	
		Mean	se	Minimum	Maximum	P1	P2
A0	14	219.07	61.33	-97.00	832.00	50	93
A2	11	224.45	97.66	-244.00	637.00	64	91
A5	15	308.60	63.16	-255.00	812.00	43	93
B0	19	261.47	36.99	-85.00	486.00	79	100
B2	12	228.92	59.45	-52.00	575.00	67	75
B5	12	115.51	51.35	-219.00	442.00	67	67
Total	83	232.12	24.77	-278.00	832.00	60	88

[a]CI = calving interval in days; [b]WFC = weight at first calving in kg; [c]ADG = average daily weight gain in grams; [d]A groups: 960 DM ha^{-1}; B groups: 400 kg DM ha^{-1}; A0 and B0 definitive weaning; A2, B2: 2 mg of estradiol benzoate treatment; A5, B5: 5 mg of estradiol benzoate treatment; se: standard error: P1: pregnancy frequency at day 60; P2: pregnancy frequency at day 127.

Many genetic and environmental factors influence reproductive performance and weight gain, with each individual gene having a small effect. As a matter of fact, mutations in other genes, mainly in leptin, are being described as affecting cattle weight gain and reproduction (Almeida *et al.*, 2003, 2007; Liefers *et al.*, 2002, 2003, 2005).

Conclusions

The analysis of three STRs and one SNP at the LEPR gene indicated a high variability of the beef cattle populations herein investigated, suggesting that the artificial selection applied to the breeds has not reduced their diversity, at least in these systems. Two alleles (*BMS694*149* and *BMS694*153*) were exclusive to Brangus Ibagé, suggesting a likely *B. p. indicus* (Zebu) origin. No association between these markers and CI or WFC in Brangus Ibagé, and ADG and pregnancy rates in Aberdeen Angus and Charolais animals was detected. These negative results suggest that the LEPR gene polymorphisms, at least those herein described, do not influence postpartum cows production. This paper describes a cheap and effective new method to analyze T945M polymorphisms in cattle populations.

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