Volume 1(2). Published February, 1, 2017
www.jnsciences.org
E-ISSN 2286-5314

Study of the genetic polymorphism of leptin in holstein dairy cows in Tunisia

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Abstract - Leptin is a glycoprotein which is involved in the defense mechanisms of the mammary gland of dairy cows. The level of this protein secretion rises significantly in response to viral or bacterial infections. This study aims to analyze the polymorphism of the gene responsible for the biosynthesis of leptin by PCR-RFLP technique. A total of 160 blood samples were collected from dairy Holstein breed cows situated in four Tunisian governorates: Bizerte, Nabeul, Kairouan, Kasserine. The genomic DNA extraction was realized by the salt extraction method. The regulatory region of the gene was amplified by a PCR reaction with the extension of specific primers. The amplification products were cleaved using a restriction enzyme Sau3AI. The results showed the presence of two alleles A and B and three genotypes AA, AB, BB with a dominance of the allele A. Results indicate that animals carrying the BB gene could contribute to a reduction of somatic cells in cattle. Based on this observation, they are more resistant to mastitis; this peptide can be considered as a candidate gene for udder health.

Keywords: dairy cattle, genetic polymorphism, leptin, PCR-RFLP.

1. Introduction

In Tunisia, dairy farming is a strategic sector of the agricultural policy. However, this sector is facing a major problem which is mastitis disease. 30% of dairy cows are culled because of the disease (Laajimi et al. 2003). This situation persists especially with the development of the battery farming (Lahsoumi et al. 2007). In order to cope with this problem, farmers usually take several measures; these measures are either preventative or antibiotic treatments which often affect milk quality. Another struggle can be seen: humoral. Leptin is a local growth factor which acts as a link between the adipocytes, epithelial cells of the mammary gland. This protein appears as a regulator in the epithelial cell. Thus, it could play an important role in the modulation of the mammary gland development. Like other cytokines, leptin is involved in the growth of various cell types (Fantauzzi et al. 2000) and in the responses of both innate and adaptive immune systems. It regulates the phagocytosis by macrophages or monocytes. It allows the elimination of bacterial infections over the E coli, Klebsiellapeunmoniae. In addition to the phagocytosis, leptin plays a role in the secretion of pro-inflammatory cytokines by macrophages (TNF-α, IL-6, IL-12), (Bonnet and al. 2002). In infectious and inflammatory processes, the rate of secretion of leptin increases considerably (Bernotiene and al. 2006). This protein enhances the immune system through the production of polymorphonuclear leukocytes. A study made by Liefers and al. (2002) shows that the polymorphism in the leptin gene is located in intron 2 (Figure 1). The objective of this study is to investigate the genetic polymorphism of leptin in Holstein dairy cows in Tunisia.

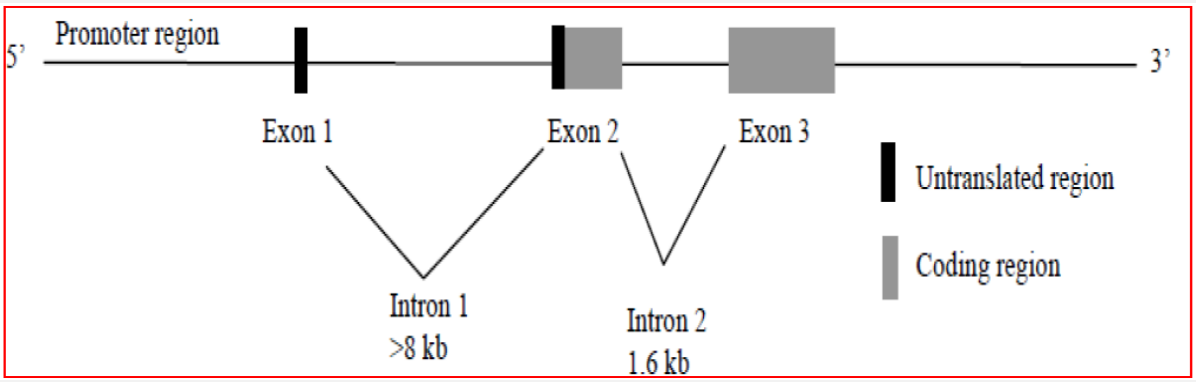

Figure 1. Structure of the gene coding for the leptin (Liefers et al. 2002).

2. Materials and Methods

2.1. Animal material

A total of 160 blood samples were taken from the jugular vein of Holstein dairy cows. Subjects were randomly taken from four geographically separated farms and all belong to the State Lands Office (OTD), these farms belong to four different governorates: Nabeul, Kairouan, Bizerte, Kasserine (Table 1). Blood quantity collected is 5 ml, the blood was placed in evacuated tubes containing K3EDTA and stored at -20 ° C for the genomic DNA extraction.

Table 1. Sample Collection Sites

Governorate	Nabeul	Kairouan	Bizerte	Kasserine
Farm	Khayem	Elalam	Ras elein	Oued Darib
Number of samples	40	40	40	40

2.2. Extraction of the genomic DNA

The method for the extraction of genomic DNA is the one developed by Sambrook et al. (1989) based on the saline extraction.

2.3. Estimation of the DNA quantity and quality

The estimation of the DNA amount was made using the optical density measure at a wavelength of 260 nm with a spectrophotometer. As for the verification of the quality of DNA, it was performed by electrophoresis on agarose gel [0.8% (W / V)].

2.4. Dilution of the DNA

This step allows us to have equal concentrations of DNA. The protocol followed during dilution is the protocol of Jemmali et al. (2013). The final chosen concentration is 50 ng/µl.

2.5. Development of PCR-RFLP technology

2.5.1. Development of the PCR reaction (Seyfert and Kuhm et al., 1994)

Table 2. Primers used for the amplification process

Gene	Primers	Size of the amplified fragment
Leptin	F: 5´-TGGAGTGGCTTGTTATTTTCTTCT-3´	442 pb
	R: 5´-GTCCCCGCTTCTGGCTACCTAACT-3´	Joanna (2011)

2.5.2. Preparation of primers

The lyophilized primers (Invitrogen) were dissolved in dilution buffer to give a final concentration of 100 pmol /µl.

2.5.3. Preparation of the reaction mixture

The reaction mixture consists of two primers, dNTPs, MgCl2, H2O, Taq polymerase and buffer. PCR is performed with a final volume of 25µl. Reagents of the amplification solution are presented in Table 3.

Table 3. Reagents used for the PCR reaction of leptin

Solution	Concentration of the stock solution	Volume (µl)
PCR buffer	5X	5
$MgCl_2$	25mM	2.5
dNTP	25mM	2.5
Sense Primer	10pmol/µl	4
Antisense Primer	10pmol/µl	4
Taq DNA polymerase	5U/µl	0.25
Genomic DNA	50ng/µl	1
Double distilled water autoclaved	----	5.75
total volume	----	25

2.5.4. Implementation of a cyclic amplification

After some tests, a suitable hybridization temperature was found. The initial denaturation was performed for 5 min at 95 ° C. 39 amplification cycles, each cycle consists of denaturation for 30s at 95 ° C, followed by primer annealing for 30 s at 61.5 ° C, elongation for 45s at 72 ° C, and then a final extension for 10min at 72 ° C.

2.5.5. Control amplification

The specificity and the performance of an amplification reaction are verified by migration on a 2% agarose gel with a voltage of 120 volts during 60min.

2.6. Application of RFLP

2.6.1. Development of RFLP

After amplification, 8µl of the PCR product were digested by the enzyme in a reaction volume of 25µl. The reagents of the enzyme solution and their concentrations are described in Table 4.

Table 4. Parameters of the digestion process

Gene	Restriction enzyme	Digestion time	Quantity	Digestion temperature
Leptin	*Sau3AI*	24 heures	10U	37°C

2.6.2. Control of enzymatic digestion

This step is crucial, it is done by a migration on a 2% agarose gel. The power voltage is 70 volts during 40min. 12µl digestion product were placed in each well. Each series typically includes an undigested PCR product made in the first well. As for the last well it contains the size marker in order to know the size of the digested products.

2.7. Statistical analysis

Let N diploid

2 alleles: A1, A2 autosomal codominant

Genotypes	A1A1	A1A2	A2A2
Number	n1	n2	n3
Genotype Frequencies	n1/N	n2/N	n3/N

The total number of A1 and A2 allele in a population of diploid N is 2N.

Allele Frequencies: F(A1) = (2n1 + n2)/2N = p

F(A2) = (2n 3 + n2)/2N = q

Genotype frequencies:

A1A1 = p2x N A1A2 = 2 pqxN A2A2= q2xN

The frequency of the A1 allele p is the proportion of the A allele among all genes of the population that may occupy the locus (A1, A2). Similarly, q is the proportion of alleles A2, since the locus (A1, A2) is necessarily occupied by either A1 or A2.

3. Results and discussion

Fragments containing the gene coding for the leptin were amplified by the PCR technique. The optimization of the PCR reaction has concerned parameters related to the quantity of DNA, Taq polymerase and the quantity of MgCl2.

The thermal program was modified slightly which showed a much improved efficiency of the amplification. Proving that, the optimum temperature at which the amplification profiles are clearer is 61.5 ° C.

The amplification results of genomic DNA were positive for all tested cows, which show the existence of the coding sequence for leptin. Indeed, the electrophoretic analysis of PCR amplification products generated bands of 422 bp in individuals analyzed. These results are similar to studies which allows the use of this protocol as a standard. Figure (2) shows the profile of the bands generated after PCR amplification using appropriate primers delimiting the coding sequence for leptin.

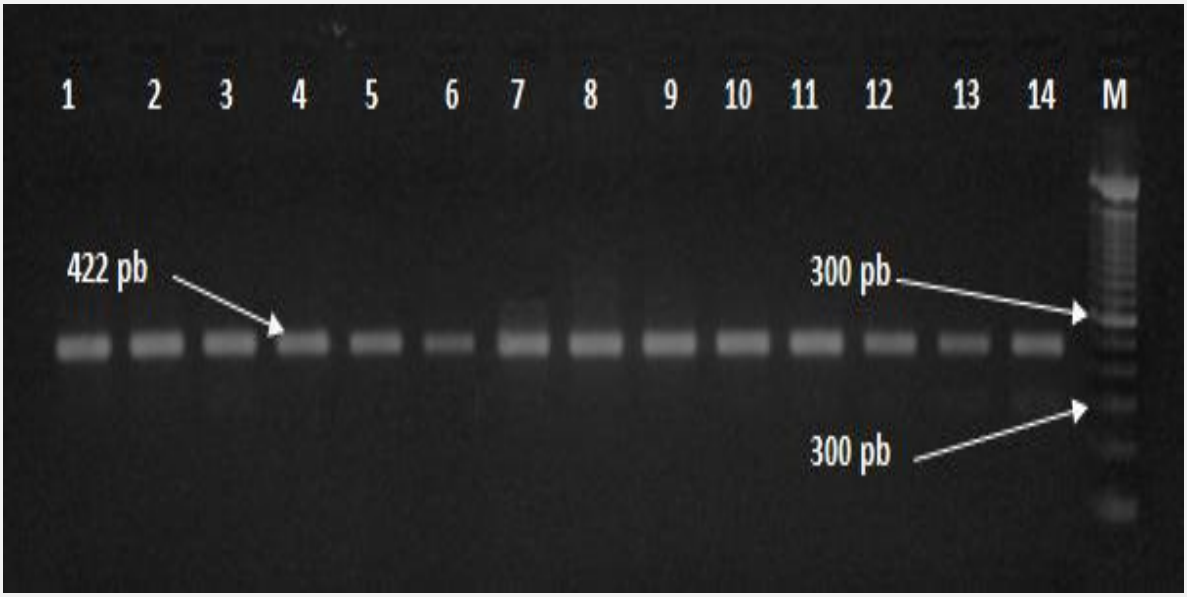

Figure 2. electrophoretic profile obtained by amplification of the gene coding for the leptin after migration on agarose gel (2%).

PCR-RFLP was used to identify polymorphic sites of the gene coding of leptin in dairy cattle. For the fragment of 422 bp three profiles were detected indicating the presence of the following genotypes: AA (390 and 32 bp), AB (390, 303, 88 bp) and BB (303, 88, 32 bp) (Figure 3). These results are in agreement with those of Arash and al. (2005) Kaygisiz and al. (2009).

The variation in the restriction profile shows the existence of a mutation, which is similar to the result found by Trakovicka and al. (2012) and suggests that the polymorphism of the gene coding for leptin is under the effect of a substitution mutation at position 1180 (C:T).

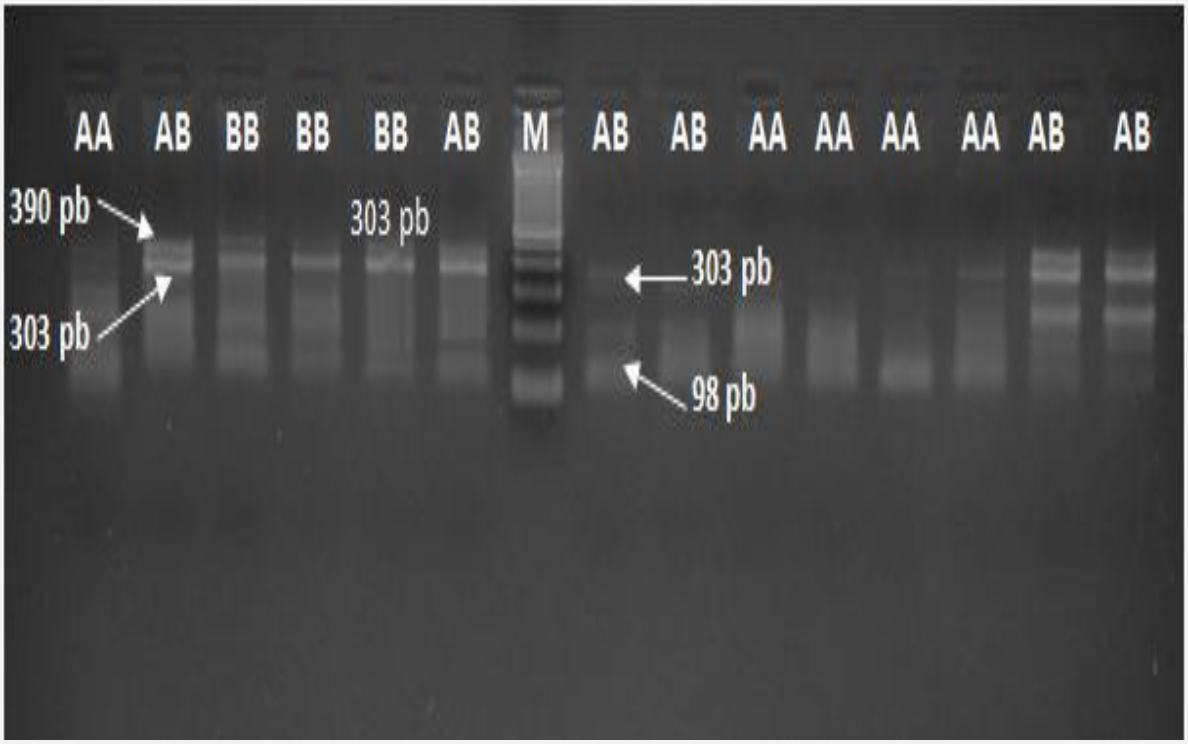

Figure 3. Analysis by PCR-RFLP of the gene coding for leptin with the endonuclease Sau3AI made with agarose gel (2%). The line M is a molecular weight marker (100 bp), wells 3, 4, 5 correspond to genotypes BB (303, 88 and 32 bp), the wells: 2, 6, 8, 9, 14, 15. the genotype AB (bp 390, 303, 88 and 32) and the wells: 1, 10, 11, 12,13 AA genotype (390 and 32 bp).

Table 5. Genotypic frequencies of the gene encoding the leptin.

Livestock	Locus du gène de la Leptine				
	Genotypefrequencies			Allelefrequencies	
160	AA	AB	BB	A	B
	0.46	0.29	0.25	0.677	0.323

Table 5 shows the frequencies and sizes of alleles of different populations. At the locus of the gene coding for leptin louse which has two alleles (A / B), the dominance of the A allele is quite remarkable and its frequency is 0.677. The results correspond to those obtained by Buchanan and al. (2003) and Hanna and al. (2010).

Buchanan and al. (2003) and Hanna et al. (2010) showed significant associations between the polymorphism of the gene encoding the leptin and somatic cells. BB genotype animals might contribute to a low concentration of somatic cells, compared to other animals with AB and AA genotypes.

4. Conclusion

The results obtained in this work show that all individuals have the sequence of the gene coding for leptin. The polymorphism of the gene encoding the leptin is under the effect of a substitution mutation at position 1180 (C:T).

It should be noted that changes in the restriction pattern of the gene encoding leptin were observed, and this variability is due to a mutation that could lead to changes in the expression of the gene coding for leptin. The leptin receptors are expressed in a variety of cells and tissues, including those of the immune system. This facilitates the adjustment of phagocytes' cells.

Several studies have reported the highest circulating leptin in infectious and inflammatory processes of the mammary gland. The results indicate that selection for animals with BB genotype, could contribute

to a reduction of somatic cells in cattle. And therefore, they are more resistant to mastitis, this peptide can be considered as a candidate gene for the health of the udder.

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