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Genetic polymorphism of the growth hormone (GH) gene and its effect on the incidence of lameness in dairy cows in Tunisia.

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Abstract- Podal diseases affects significantly dairy farms profitability. Multiple genetics program's addressed the subject of lameness resistance. This study adhere to this context. Four hundred and twelve Holstein cows were included in this study with the aim of identifying the effects of GH gene (G/C) allelic substitution's on the incidence of lameness in dairy cows. The genotypic frequencies associated with the GH / AluI polymorphism are respectively 0.80, 0.18 and 0.02 for the LL, LV and VV genotypes; hence the allelic frequencies of 0.89 and 0.11 respectively for the L and V allele. The effects of fixed environmental factors (calving year, calving season, and age at calving), genetic polymorphism of the GH / AluI gene and parity on the probability of lameness incidence were studied by logistic regression. The probability of observing lame cows in the herd in winter and autumn is higher than what is observed in summer and spring. The probability of lameness cases incidence for second and third parity cows is higher respectively of 35 % and 48.5 % than for first parity cows. Thus, miltipara are more threatened by developing podal diseases than primipara. The probability of the appearance of lameness to the heterozygous animals of genotype LV compared to the homozygous animals is less than 13 %. The genotype LV is a candidate genotype to the Holstein dairy cows in order to fight against lameness.

Key words: Polymorphism; Growth hormone (GH); lameness.

1. Introduction

lameness in ruminants was considered a trivial problem. Recognition of their importance is a recent matter, this recognition is due to economic reasons (Kossaibati and Esslemont, 1997) as for animal welfare reasons (pain and distress) (Whay and al., 2003). Lameness has become one of the serious diseases affecting dairy herds and fattening animals (more than 70% of lameness in feedlot animals comes from hoofs). In recent years, economic studies have shown considerable losses associated with lameness. These losses are due to: (i) to the decline of production (milk and ADG); (ii) to early culling due to lameness; (iv) to seizure applied to milk containing antibiotic residues; (vi) loss of time related to the handling of sick animals; and (vii)to mortality. The most common conditions are foot rot, laminitis (subclinical or chronic), digital dermatitis or Mortellaro's disease (Delacroix, 2000).

In Tunisia, despite the efforts made to minimize the losses caused by lameness, these pathologies remain widespread. Assessing the current status of this disease in a representative sample of bovine population in Tunisia can be a sure way for good management of genetic resources. Nationally no molecular studies have been done in this area. Nevertheless gene-assisted selection may be even more important for genetic improvement of reproductive or health traits (eg, mastitis or lameness), since they are of low heritability traits and sometimes difficult to record, and therefore their Genetic improvement with conventional breeding can be laborious and ineffective.

It should also be noted that the growth hormone influences reproductive functions (Spiteri and Neischlag, 1993) and assists the body's immune response, wound healing, and hematopoiesis (Golde et al., 1977). Given the positive effects of this hormone, the cattle hoof could benefit enormously (healing of wounds, help with the immune response, hematopoietic growth).

Wallis (1973) reported that Bovine somatotropin or bovine somatotrophin (abbreviated bST and BST), or bovine growth hormone (BGH)is a peptide with a molecular weight of about 22 kDa. Lingappa et al. (1977) and Wallis et al. (1973) reported, respectively, that it is composed of 190 or 191 amino acids, containing Alanine or Phenyl-Alinine. In addition, Leucine or Valine amino acid substitutions at residue 127 exist due to allelic polymorphism (Seavey et al., 1971). Gordon et al. (1983) and Woychick et al. (1982) reported that it consists of five exons separated by introns.

Several polymorphisms have been identified in the GH gene. Cowan et al.; 1989 and Hilbert et al. ; 1989 detected a polymorphic site for the endonuclease restriction enzyme Msp I, the polymorphism being localized in intron 3 of the GH gene at position 1547 (Zhang et al., 1993). Wang et al. (2003) detected a polymorphic site for the restriction endonuclease Apa I. Biswas et al. (2003) and Aruna Pal (2004) detected a polymorphic site for Alu I restriction endonuclease.

2. Materials and methods

Sample Description

The study was conducted on a large commercial dairy farm (Agricultural Development Company) in northeastern Tunisia (37 ° 01'09.8 "N 9 ° 39'38.8" E), located at 36 m above sea level. The cows were housed in four free-stalls and were fed hay, greenery and concentrate supplementation to meet their energy and protein requirements. The milking was done twice a day (morning and evening). Samples of 5 ml blood were collected in EDTA tubes (anticoagulant) from 412 cows present on Mar 2013 until March 2017. These volumes of blood were preserved at -20 ° C. The sample contained lame cows, non-lame cows and healed lame cows. The average age of the cows is 4.25 ± 1.87 years old. the Tunisian Livestock and Grazing agency (l'office de l'élevage et du pâturage) provided us with a database containing 6575 milk control records. Each record contains the animal's identifier, lactation number, age at calving, calving month, and calving year, daily milk yield, fat content, protein content, and date of control.

Genotyping protocol

The DNA was extracted from a blood sample using the InnuPREP Blood DNA Kit Mini Kit (analytik jena, Germany) in accordance with the manufacturer's instructions.. The integrity of the DNA samples was examined by electrophoresis on a 1% Agarose gel.

For genotyping the gene GH (G / C), a fragment of 428 bp was amplified by PCR using the primer pair: 5 '-CCGTGTCTATGAGAAGC-3' and 5'-GTTCTTGAGCAGCGCGT-3 '(Lucy et al. (1993) and Oprządek et al., 2005).

The PCR amplification was carried out using approximately 200 ng of genomic DNA which corresponds to 4 µl of DNA, 2.5 µl of the PCR buffer, 2.5 µl for each primer (sense and antisense), 2.5 µl of dNTP, 0.2 µl of Taq DNA Polymerase Recombinant (SEGMA-ALDRICH) and 8.8 µl of autoclaved water in a total reaction volume of 23 µl.

The PCR conditions were 95 ° C for 5 minutes and then 35 cycles which contained a denaturation phase at 94 ° C for 40 seconds, a hybridization phase at 59 ° C for 40 seconds, and an elongation phase at 72 ° C for 40 seconds, and finally a final extension period at 72 ° C for 10 minutes. Pcr products were revealed by 1.5% Agarose gel electrophoresis.

Concerning the GH gene, the PCR products were digested with the AluI restriction enzymes in a reaction mixture which contains 12 µl of the PCR product, 0.3 µl of restriction enzyme (Alu I), 2.5 µl of buffer (Buffer), 0.2 BSA and 10 µl of autoclaved water. The reaction mixture was incubated at 37 ° C for 12 hours. Digestion products revealed clear and intense bands of (265 // 147 bp), (265 // 96 // 51 bp) and (265 // 147 // 96 // 51 bp) on the Agarose gel at 2% in the ultraviolet (UV) light.

Statistical analysis

Logistic regression (Agresti, 1990) was used to assess the importance of n independent factors included in the incidence of lameness in dairy cows. These factors represented fixed environmental factors (calving years from 2013, ..., 2017); calving season (fall, winter, spring or summer); age at calving; parity (1, 2, 3 or 4) and the genotype of the polymorphism of the GH / AluI gene (LL, LV et VV) on the probability of incidence of lameness. The search for significant explanatory variables was done with the logistic regression method, SAS (1989). The significance level of 5% was the upper limit for a factor to be considered important in explaining the incidence of lameness. Therefore there would be a binomial variable whose result is 1 if the animal is healthy and 0 if it is lame, and where Pi is the probability of success given a set of independent variables xi and (1 - Pi) is the probability of failure given the same set of xi.

$$Y_i = P_i(y_i = 1 \mid X = x_i) + e_i,$$

Yi is the binary response variable, and ei are independent of E (ei) = 0 and V (eijxi) = Pi (1 Pi). Then, logistic regression was used to study the effects of environmental factors, parity and the genetic

polymorphism of gene GH / AluI on the incidence of lameness in dairy cows. The link function between the probability of having a healthy animal and these various explanatory factors is:

$$\log\left(\frac{P_i(y_i = 1 \mid X = x_i)}{(1 - P_i(y_i = 1 \mid X = x_i))}\right) = \alpha + \sum_{i=1}^{n} \beta_i x_i,$$

Where α is the intercept, βi is the regression coefficient for xi, xi is the result for the ith explanatory variable and n is the number of explanatory variables.
Calving year, calving age and first milk day were treated as Covariates. The calving season, lactation number and genotype of GH / AluI gene were converted into regression variables 1 or 0.For example, fall, spring, and winter were given the value 0 if we assigned the value 1 for the summer, and so on.
The likelihood that a binary response variable leads to success can be described in terms of the chance of this event. In its simplest form, the probability is the probability of an event occurring divided by the probability that the same event does not occur. Frequently, the logistic regression model is expressed as a ratio of two odds ratio. Lameness Prevalence is exp (βi) times greater when the value of the explanatory variable is increased from xi to xi + 1. This ratio of two OR provides a measure of the association between the probability of occurrence of lameness incidence and the ith explanatory variable. OR is a number between 0 and infinity. OR> 1 indicates that the probability of observing podal pathologies is greater than the likelihood of having healthy animals. Independence, or lack of association, between the variable i and the incidence of lameness will have an OR = 1, which equals bi = 0. An OR <1 indicates that the probability of the incidence of lameness is lower than the probability of having healthy animals.

3. Results

Genotypic and allelic frequencies

Digestion of the PCR products (428 bp) of the gene GH by the method PCR-RFLP was performed by an endonuclease enzyme AluI. It allowed to identify three genotypes: LL, LV and VV whose sizes are respectively: 265 // 96 // 51bp, 265 // 147 // 96 // 51bp, 265 // 147bp and (Figure 1).

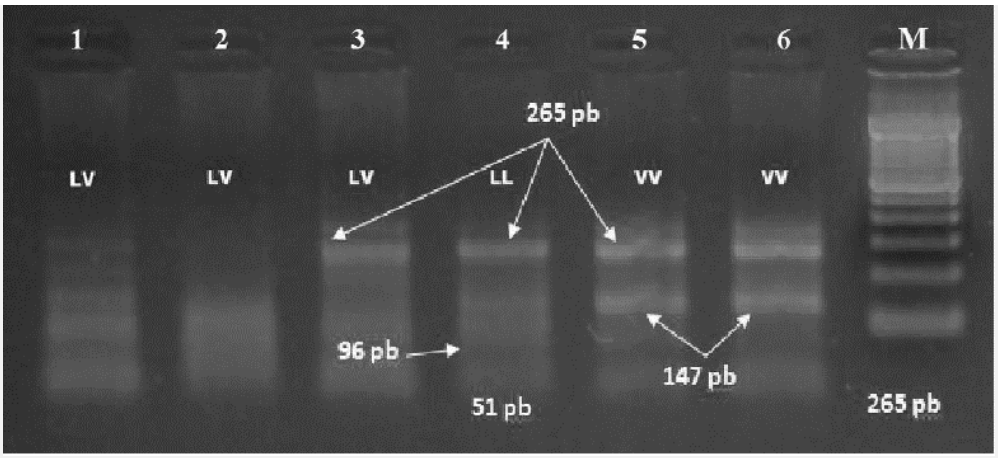

Figure 1. Electrophoresis profile of GH / AluI gene amplification products. M: 100 bp size marker (Biomatik); 1-2-3-4-5-6: samples analyzed.

The genotypic frequencies associated with the GH / AluI polymorphism calculated in the present work are 0.80, 0.18 and 0.02 respectively for the LL, LV and VV genotypes; hence the allele frequencies of 0.89 and 0.11 respectively for the L and V allele (Table 1). Note that the LL genotype is the most dominant compared to LV and VV genotypes.

Table 1. Genotypic and allelic frequencies (%) in the studied gene loci.

Gene locus	Polymorphism	Allele		Genotypic frequencies (%)			Allelic frequencies (%)	
		L	V	LL	LV	VV	L	V
GH		G	C	0.80	0.18	0.02	0.89	0.11

Factors affecting the incidence of lameness in Holstein cows

In this work, the incidence of lame cows is 67.08%. This result recorded on this farm does not agree with that of Bouraoui et al. (2014) which showed that podal pathologies have an incidence equal to 38.71% (score> 2) within the Chergui farm.

All factors included in the step-by-step logistic regression model (lactation number, calving season, age at calving, GH / AluI gene polymorphism, and calving year) significantly affect the incidence of lameness in Holstein cows (Table 2)

The probability of having lame cows in the herd in winter is 11% higher than the incidence of lameness in the fall season. While the probability of lameness occurring in heterozygous animals of genotype LV is less than 13% compared to homozygous animals. Similarly, the probability of the incidence of lameness in cows at second and third parity is 35% and 48.5% respectively higher than first-parity cows. The incidence of lameness in the herd increases by 3.2% of year from a calving to another (Table 2).

Table 2. Analysis of Maximum Likelihood Estimates and odds ratio estimates.

Variable	coefficient	Odds Ratio	95 % wald Confidance limits		P-value
Parity					
1	***Referent***	-	-	-	-
2	0.2991	1.349	1.115	1.631	0.01
3	0.3955	1.485	1.097	2.022	0.01
Season					
Autumn	***Referent***	-	-	-	-
Winter	0.1058	1.112	0.933	1.324	0.01
Spring	-0.4508	0.637	0.518	0.783	0.01
Summer	-0.1984	0.820	0.690	0.974	0.09
Genotypic					
LL	***Referent***	-	-	-	-
LV	-2.0238	0.132	0.112	0.156	0.01
VV	0.0169	1.017	0.586	1.764	0.01
Age at calving	0.00083	1.001	1.000	1.001	0.01
test-day milk yields	-0.0015	0.998	0.998	0.999	0.01
Calving year	0.0318	1.032	1.029	1.036	0.01

4. Discussion

In this work, we have studied the genetic polymorphism of the GH / AluI growth hormone gene on the incidence of lameness in dairy cows (Holstein). Since this mutation was associated with the litter weight of Holstein-Friesian cows (Biswas et al, 2003), milk production and its components in the Italian Jersy breed (Dario et al, 2008) and the Holstein-Friesian anguish breed (kovacs et al, 2006), this polymorphism was considered important for determining its effect on the studied parameter.

The calculated genotypic frequencies of the GH gene polymorphism agree with those found by Kovacs et al. (2006) which are of the order of 87.1, 12.4 and 0.6 respectively for LL, LV and VV genotypes in Holstein-Friesian cows Angroisse. Aruna et al. (2004) who had reported frequencies of 0.115 and 0.884 respectively for LV and LL genotypes in Karan bulls (In Karan Friesbulls). The allelic frequencies found in our work are slightly elevated compared to those of Grochowska et al. (1999) which are of the order of 0.69 and 0.31 respectively for the L and V allele.

In this study, environmental and genetic factors seem to affect significantly the incidence of lameness in dairy cows ($p < 0.01$).

The calving season, one of the most important environmental factors, affects the incidence of podal pathologies. According to Rowlands et al. (1983) and Cook (2003), the frequency of podal pathologies is higher in winter and autumn than in summer and spring. This is due to the high soil moisture that will soften the horn and decrease wear resistance (Wells et al, 1999). According to Fayer (1986), the appearance of lameness in stabling is higher than in pasture. During the winter period the feeding in green is sometimes unavailable due to the state of the pasturelands. Clément (2005) mentioned that the incidence of lameness can be explained by an ill-adapted diet, as a ration richer in concentrate, richer in starch and lower in fiber causes more severe lameness, particularly laminitis.

The effect of parity on the lameness incidence is significant ($P < 0.01$). Foot lesions are more numerous in multiparous cows. These results agree with those of Enevoldsen et al. (1991) and Alban (1995). The highest scores are recorded in cows in second and third parity. This is consistent with the studies of Bergsten (1994) and Manske et al., (2002)

The association of the genetic polymorphism of the GH / AluI gene and the incidence of lameness significantly affect the statistical model ($p < 0.01$)

The chance of having lameness is lower in animals of genotype LV. In several genetic polymorphism studies of the GH / AluI gene, the frequency of the VV genotype is still low. And several authors have mentioned that cows with LL genotype produce more milk than cows with LV genotypes. Lucy et al. (1993) showed that Holstein-Friesian cows homozygous for Leu-127 bGH produced more milk than LV

animals. Lee et al. (1996) determined that the genetic value decreases with the presence of the GH gene allele Val-127. Similarly, Dybus (2002) found statistical differences between individuals of different genotypes with respect to milk yield, amount of fat and protein material. Cows of the LL genotype produced more milk (+225 kg), fat (+7 kg) and protein (+7 kg) than the LV individuals ($p \leq 0.01$). This is relevant to our results, since if the LV genotype is less productive then it is more resistant to podal pathologies.

The calving year seems affecting the lameness incidence and its probability is of the order of 3.2% from one year to another. This could be explained by the degradation of the breeding barn, soil, the exercise and sleeping area and the cow's hooves condition.

To conclude, the genetic polymorphism of GH / AluI genes is associated to the lameness incidence in dairy cows ($p < 0.01$). V alleles appear to affect the incidence of lameness. The LV genotype is the most resistant. Further studies must be done to confirm these results.

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The calculated genotypic frequencies of the GH gene polymorphism agree with those found by Kovacs et al. (2006) which are of the order of 87.1, 12.4 and 0.6

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