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Full Length Research Paper

Polymorphism of the prolactin gene (PRL) and its relationship with milk production in American Swiss cattle

Edy Alfonso[1], Reyna Rojas[1], José G. Herrera[1], María E. Ortega[1], Clemente Lemus[2]*, César Cortez[1], Jaime Ruiz[3], René Pinto[4] and Heriberto Gómez[4]

[1]Colegio de Posgraduados. Recursos Genéticos y Productividad-Ganadería. Carretera México-Texcoco km 36.5. Montecillo, Estado de México. CP 56230.
[2]Unidad Acadèmica de Medicina Veterinaria y Zootecnia, Posgrado CBAP-UAN. Tepic, Nayarit, México.
[3]Facultad de Medicina Veterinaria y Zootecnia, UNACH. Tuxtla Gutiérrez, Chiapas, México.
[4]Facultad de Ciencias Agronómicas. UNACH. Villaflores, Chiapas, México.

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The modern dairy cattle breeding strategy in the Mexican tropic is to identify genes or allelic variants that can be incorporated into selection programs such as the prolactin gene (PRL) which is associated with milk production and quality. The aim of this study is to screen an American Swiss population in Chiapas, Mexico, in order to analyze the polymorphism of the prolactin gene as well as its relationship with milk production in blood samples of 417 American Swiss cattle. The genotypes were determined through the polymerase chain reaction-restriction fragments length polymorphism (PCR-RFLP) technique, using *Rsa*I restriction endonuclease, showing a 156 bp fragment located in exon 3. Allele frequencies in the studied breed were: A = 0.8765 and B = 0.1235. The genotype frequencies of AA, AB and BB were 0.776, 0.174 and 0.026, respectively. The Chi-square indicated that genotype distributions were not in the Hardy-Weinberg equilibrium (P<0.05). The results show that animals with genotype AA had a greater milk production during lactation than genotypes AB and BB (P<0.05), with genotype BB being the one that had the lowest production (P<0.05). It was concluded that the identification of the prolactin polymorphism in this population will allow the achievement of a better efficiency in the selection of breeding animals.

Key words: Brown Swiss, prolactin, polymorphism, milk, RFLP-*Rsa*I.

INTRODUCTION

American Swiss livestock has been the base of dual purpose cattle system in the Mexican tropics, as a pure breed or through crossbreeding with local genotypes, due to milk and reproductive efficiency and environment adaptation (Johnson and Vanjonak, 1976; Finch, 1986). In the Chiapas region, the introduction of sires to the local livestock populations is a common practice, being a breeding strategy. The contribution of molecular genetics to the selection procedures, identification of genes with important effects on characteristics such as milk production or its components and the results in the search of quantitative trait loci (QTLs) in several species have led to the development of selection methods assisted by molecular markers (MAS), currently considered as one of the most important tools in animal improvement (Georges et al., 1995; Grisart et al., 2002). In order to improve breeding efficiency of dual purpose cattle, nowadays farmers in Chiapas are developing programs of genes identification or allelic variants that can be incorporated into the selection programs, such as the prolactin gene (PRL), associated with milk production and quality (Brymet et al., 2005; Ghasemiet et al., 2009). Milk production is a complex phenomenon in which several

*Corresponding author. E-mail: drclemus@yahoo.com.mx or ealfonso@colpos.mx. Tel: 5951086161

Table 1. Distribution of samples of American Swiss cattle from six herds in Chiapas, Mexico.

Farm	Cow	Calve	Sire	Total
1	107	67		174
2	54	37		91
3	46	14		60
4	31	23	1	55
5	10	5		15
6	16	6		22
Total	264	152	1	417

genetic and hormonal factors interact. Hormones such as growth hormone, insulin, thyroxin and prolactin are involved (Collier et al., 1984), with prolactin being one of the most important in this process (Sacravarty et al., 2008).

PRL participates in multiple biological functions related with reproduction, osmoregulation, tegument growth and synergism with steroids (Barendse et al., 1997). It is necessary for the initiation and maintenance of lactation; it acts at the level of mammary alveoli, promoting synthesis and secretion of proteins, lactose, lipids, and other important components of milk (Leprovost et al., 1994). It also regulates immunological functions and participates in cell differentiation and growth (Loretz and Bern, 1982). Moreover, it is an immunomodulating molecule with relevant physiological effects, being considered as a cytosine. The PRL molecule can be linked to different groups: it can be glycolized, dimerized, polymerized or hydrolyzed to originate different variants (Méndez et al., 2005). PRL secretion does not differ between high and low milk production. However, some researchers have found that it increases its metabolism and distribution between days 30 and 150 of lactation (Collier et al., 1984).

The gene of bovine prolactin located in chromosome 23, is made up of five exons and four introns. There is a silent adenosine-guanine (A-G) mutation in the codon codifying amino acid 103 in exon 3 of the bovine prolactin gene. The restriction fragment length polymorphism (RFLP) technique is used to detect small alterations that happen naturally in the genome from changes due to deletions or insertions of one or more pairs of nucleotides (Lewin et al., 1992 and Skinkyté, 2005). To determine similarities among populations, the estimation of their genetic distances is necessary, referred as the difference between the gene frequencies for a specific characteristic (http://www.answer.com). A better way to represent these genetic distances is by using dendograms, tree-shaped data diagrams, which make possible a graphical overview of the relationship among the studied populations.

The aim of this study was to screen Mexican Brown Swiss population in Chiapas, Mexico, in order to analyze the polymorphism of the prolactin gene through RFLP, determine its allele and genotype frequencies, as well as its relationship with milk production.

MATERIALS AND METHODS

A total of 417 blood samples were taken from 264 cows, 152 calves, and a bull. All these animals were American Swiss cattle from six milking farms in the “Frailesca” region, Chiapas, Mexico (Table 1). The blood samples were taken from the caudal vein of the animals, using vaccutainer tubes and EDTA (2.5 mg/2.5 mL blood). Samples were refrigerated at 4°C and preserved until processing. DNA extraction was done using the technique by Miller et al. (1988) in the Physiology and Molecular Biology Laboratory of the Phytopathology Program of the Colegio de Postgraduados (COLPOS), Mexico.

To amplify the prolactin gene through polymerase chain reaction (PCR), a couple of specific primers were used: Forward (5'-CGA GTC CTT ATG AGC TTG ATT CTT-3') and reverse (5'-GCC TTC CAG AAG TCG TTT GTT TTC -3'). The PCR reaction mix was made up of: 11.25 µl dH_2O, 2.5 µl buffer 1X, 2.5 µl $MgCl_2$, 0.5 µl dNTPs, 0.25 µl Amplicase (Biogenic), 2.0 µl of each primer at 20 pmoles, and 4.0 µl DNA (50 ng aprox.) in a final volume of 25 µl. The reactions were run in a TECHNE TC-512 thermocycler at 30 cycles, denaturalized at 94°C/3 min, aligned at 55°C/30 s, extended at 72°C/1 min, and final extension was done at 72°C/3 min.

Once the reaction was finished, 5 µl PCR products were taken and placed in agarose gel at 1% with ethidium bromide. Electrophoresis was done at 80 V for one hour. After this time the gel was placed and observed in a UV transilluminator, Model Gel-Doc 2000, BIO RAD® and analyzed with the QuantityOne 4.0.3 software. Polymorphism was obtained once the presence of a 156 pb band corresponding to the molecular weight of the prolactin gene was verified. Digestion was carried out with the RsaI enzyme. To do this, 15 of the amplified product was taken from each processed sample and placed in a 0.5 ml tube with 2 µl dH2O, 2.5 µl enzyme buffer and 5 U or 0.5 µl, with a final volume of 20 µL. This mixture was digested in an incubator (Boekel Scientific Mod. 133000) at 37°C all night.

To verify digestion, 15 µl of the digestion product was taken and separated by electrophoresis in 3% agarose with SB 1X buffer (5 mM disodium borate decahydrate or 10 mM sodiun hydroxide, pH adjusted to 8.5 with boric acid) as run buffer, and ethidium bromide. The marker used was GeneRuller 50 bp DNA ladder from Fermentas®.

The determination of the prolactin genotypes was based on protocols described by Udina et al. (2001) and Mitra et al. (1995), with modifications.

To establish the relationship between polymorphisms in the prolactin gene and milk production, the total production was estimated per cow through a periodic sampling with 14-day interval, at fixed monthly dates for 10 months. The dates were adjusted to 305 days, using multiplicative regional fit factors (Ochoa, 1991). Data were analyzed with SAS (2002), using the following model:

$$Y_{ijkl} = \mu + a_i + b_j + c_k + \xi_{ijkl}\ l = 1,2,3...t \qquad j = 1,2,3...r\ k = 1,2,3...l$$

Where, Y_{ijkl} = mean observed values of the characteristic; (milk production); μ = general mean;

a_i = effect of the i-th lactation year (l = 1,….,6); b_j = effect of the j-th lactation number (j = 1,…,6);

c_k = effect of the k-th Prl-RsaI genotype (k = AA, AB, and BB); ξ_{ijkl} = random error, $\xi_{ij} \sim N(0, \sigma^2)$.

To calculate the allele and genotype frequencies, Hardy Weinberg equilibrium, degree of heterozygocity, Shannon index, genetic distances among animal sub-populations, and construction of dendograms, the POPGENE version 1.31 software were used (Yeh et al., 1999).

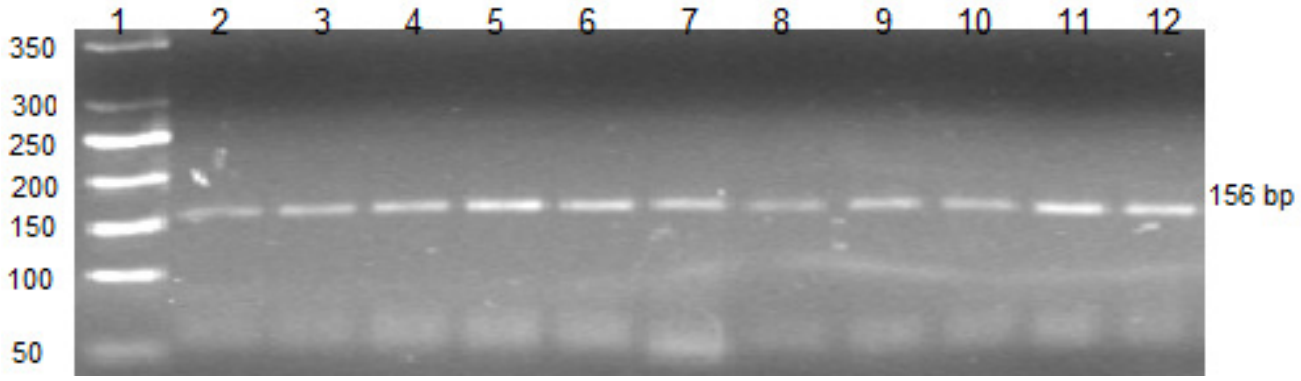

Figure 1. Amplification of prolactin gene by electrophoresis with agarose 1% and ethidium bromide. Lane 1, Marker of molecular weight of 50 bp; lanes 2 to 8, bands of 156 bp.

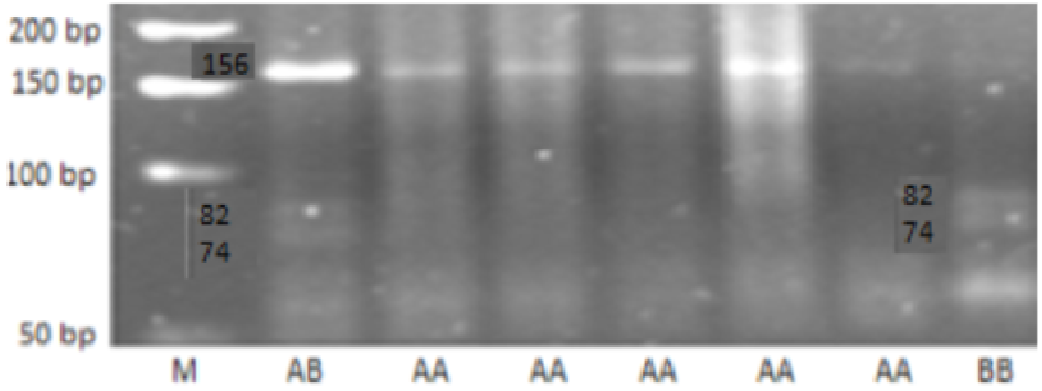

Figure 2. Polymorphism fragments of the gene prolactin obtained with the enzyme RsaI in agarose gel to 3% with ethidium bromide. Lane 1). Marker of molecular weight of 50 bp, 2). Lane 2, genotype AB, lanes 3 – 7, genotype AA and lane 8, genoype BB.

RESULTS

The primers used allowed PCR amplification of a 156 bp fragment, corresponding to the prolactin gene (Figure 1). The digestion of the amplified fragment with the RsaI restriction enzyme showed the presence of three genotypes: AA, which had no digestion, obtaining the 156 bp fragment; AB, with three fragments, 156, 82, and 74 bp; and BB, with two fragments, 82 and 74 bp (Figure 2).

The most abundant genotype was AA with 0.776, AB 0.174, and BB 0.026 (Table 2). The allele with the highest frequency was A with 0.8765 and B 0.1235. The degree of heterozygocity was 0.196. The Shannon index was 0.3762; thus rejecting the null hypothesis of the existence of the Hardy Weinberg equilibrium (X2, $P < 0.05$). This could be attributed to the characteristics of management in the studied herds, because in these herds breeding is done with semen and studs brought from outside the herds, and to the frequent inclusion of young cows and embryos to improve the genetic quality of the herds. This causes an increase in the probability for mutations to happen, as a result of constant gene combinations, besides the environment effect.

Only if the population is product of a random breeding generation of the individuals of the original population, it will be in HW equilibrium for each specific locus.

The genetic distances between herds 2, 3 and 4, 6 are closely related, which indicates a great similarity between the populations. Therefore the behavior observed in the prolactin gene in this study was the one expected. On the other hand, a notable genetic distance was observed between herds 1 and 5, with a value of 0.0273, and a genetic identity of 0.9731 (Table 3 and Figure 3). The relationships between herds 4 and 6 were closer at 0.9999 and 0.0001 genetic distance, and the relationship between herds 2, 3, and 6 had a value of 0.9998 genetic identity, and 0.0002 and 0.0007 genetic distance.

Herd one is different from the rest of the herds since it showed a higher frequency of genotypes AB and BB. This could be a consequence of the type of crosses, or the selection done in this farm. The structure of the populations was determined with the Chi-square test,

Table 2. Allelic and genotypic frequencies of PRL in American Swiss cattle in Chiapas, Mexico.

Locus	Genotypic	American Swiss cattle in Chiapas, México				Heterozygocity (Ho)
		Frec. genotypic	Frec. allelic	Shannon Index	Chi-square test HW test	
PRL RFLP-RSal	AA	0.776	(A) 0.8765	0.3762	4.230563	0.1966
	AB	0.174	(B) 0.1235		Degree of freedom: 1	
	BB	0.026			Probability: 0.039702	

Table 3. Genetic distance between herds of American Swiss cattle in Chiapas, México.

Farm	1	2	3	4	5	6
1	****	0.9926	0.9900	0.9848	0.9731	0.9873
2	0.0074	****	0.9998	0.9986	0.9939	0.9993
3	0.0101	0.0002	****	0.9995	0.9959	0.9998
4	0.0154	0.0014	0.0005	****	0.9983	0.9999
5	0.0273	0.0061	0.0041	0.0017	****	0.9973
6	0.0128	0.0007	0.0002	0.0001	0.0027	****

Nei's identity genetics (upper diagonal) and distance genetics (lower diagonal).

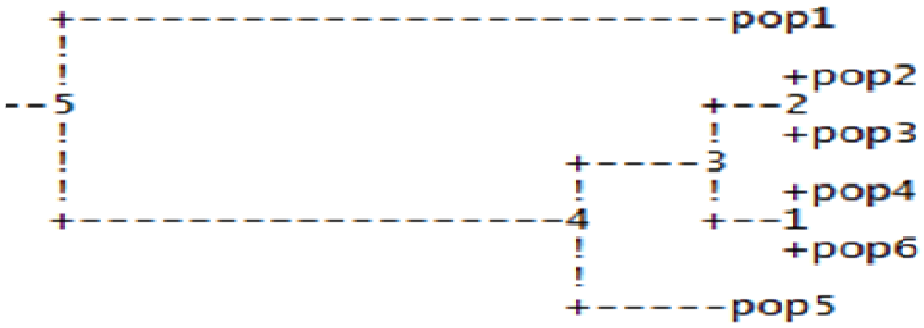

Figure 3. Dendrogram of studied herds of American Swiss cattle in Chiapas, México.

Table 4. Effect prolactin genotypic on milk production in American Swiss cows in Chiapas, México.

Prolactin genotypic	Sample	Production mean $305_{d.}$ (kg)
AA	175	3251.57
AB	32	2789.91
BB	3	2603.79

showing differences among groups as a consequence of the identification of different genotypes in the studied populations. The effect of the prolactin gene in milk production showed the best mean value for the genotype AA, with 3251. 57 kg L^{-1}, followed by AB with 2789.91, and BB with 2603.79 kgL^{-1} (Table 4). There was a significant difference between genotype AA and the other two, thus determining the influence of the A allele in milk production, which could be due to the similarity in management of the genetic improvement programs focused mainly on milk production.

DISCUSSION

From the prolactin genotype variants obtained through the RFLP-PCR technique with the RsaI endonuclease, the genotype AA had the greatest frequency, which is similar to data obtained by several researchers in different regions of the world, with different breeds, and sample sizes, who had reported genotype frequencies from 0.47 to 0.96 in the breeds: Black and White, Red Pied, Jersey, Gorbatov Red, Ayrshire, Black Pied, Montebeliard, Sahiwal and Holstein Friesian (Kalashnikova et al., 2009; Ghasemi et al., 2009; Kumari et al., 2008; Brym et al., 2005; Khatami, 2005; Dybus et

al., 2005; Alipanah et al., 2007; Skinkyté, 2005; Ripoli et al., 2003; and Udina et al., 2001). However, other authors have reported lower frequencies for genotype AA, and higher frequencies for genotype AB: Jersey (0.65), Kankrej (0.62), Gyr (0.49), and Red Sindhi (0.62) (Kumari et al., 2008). In the case of Black and White cattle, Khatami (2005) found frequencies of 0.47 which is similar to genotype AA.

When analyzing the genotypes favorable for milk production, it was found that the genotype AA had the best average with 3251.57 kg^{-1}. This result is similar to that reported by Brym et al., (2005) for Black and White cattle, and Ghasemi et al. (2009) with Montebeliard cattle, who reported a production of 5805 L. Dybus et al. (2005) found that the genotype AA was favorable for the second and third lactations, while the genotype AB was in the first lactation in Jersey cattle, and both genotypes AA and AB were favorable in Black and White cattle.

Other authors (Alipanah et al., 2008) indicated that the genotype AB in Black Pied cattle affected milk, fat and protein production, while in the case of Red Pied cattle, the genotype BB was favorable. Sacravarty et al. (2008) reported that the best genotype for milk production was BB in cows from the second to fourth lactation in Kankrej cattle from India. Chrenek et al. (1999) examined the influence of polymorphism of PRL-RsaI in Brown Swiss cattle, and found no significant differences among cows with diverse PRL genotypes.

Another important characteristic related with the PRL genotypes reported by other authors is somatic cell count (SSC), related to the presentation of sub-clinic mastitis, being the genotype BB more favorable. However, it came out negative for fat content in milk in Yaroslavl cattle (Brym et al., 2005).

With regard to the heterozygocity found in this study with American Swiss cattle, it was 0.196, and the population was not in Hardy Weinberg equilibrium. This heterozygocity is similar to that estimated by Skinkyté (2005), who found a heterozygocity value of 0.33 and 0.23 in Black and White and Red cattle. Ghasemi et al. (2009) reported 0.15 in Montebeliard cattle. Kalashnikova et al. (2009) observed values of 0.40 in Black Pied cattle. Brym et al. (2005) found 0.038 in Black and White, and 0.33 in Jersey cattle. Alipanah et al. (2007) reported 0.39 in Russian Red Pied cattle. Dybus et al. (2005) found 0.28 in Black and White and 0.43 in Jersey.

The genetic distances found in this study, related with the effect of the gene, are notorious, mainly in populations 1 and 5, proven by the presence of genotype BB in population 1, which was absent in population 5. To this regard, Plastow et al. (2003) determined the genetic distances in pigs using RFLP through the polymorphism found in the bands, considering this technique as appropriate for this end.

The differences found in genotype frequencies in different studies, related with polymorphism of the prolactin gene, can be attributed to differences in the breeds and the reduced number of analyzed samples ($n < 50$), which does not allow the genotypes to be efficiently represented (Brym et al., 2005). This is not the case of this study, where the sample size was greater ($n > 400$).

According to the results obtained in this study, it is concluded that the structure of the studied populations show similarities with regard to productive characteristics, as a consequence of the use of genetic material from the same origin.

On the other hand, the determination of prolactin genotypes at an early animal's age represents an advantage, considering that this hormone is a good candidate to be considered in programs of marker assisted selection, since it shortens the interval between generations. Finally, allele A of prolactin can be considered as a good indicator for milk production in the American Swiss breed.

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