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PCR screening and allele frequency estimation of bovine leukocyte adhesion deficiency in Holstein and Gir cattle in Brazil

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Abstract

Bovine leukocyte adhesion deficiency (BLAD) is a disease known to affect the Holstein cattle breed throughout the world. Eighty-eight Holstein dairy cows and 88 Gir dairy bulls were genotyped by PCR for the CD18 BLAD alelle. The frequency of the BLAD mutant allele and the BLAD-carrier prevalence in Brazilian Holstein cows were 2.8 and 5.7%, respectively. No mutant allele was found in any of the 88 Gir animals. We conclude that the CD18 gene mutation is prevalent in Brazilian Holstein cattle and absent or present at a very low frequency in Gir cattle.

INTRODUCTION

Bovine leukocyte adhesion deficiency (BLAD) is a recessive autosomal disease that is caused by a deficiency in leukocyte surface glycoproteins known as integrins. These proteins are responsible for the cell-cell interactions necessary for neutrophils to adhere to vascular endothelium, enter the tissues, and destroy invading pathogens (Kishimoto *et al.*, 1987).

The integrin family includes leucocyte function-associated antigen (LFA-1), macrophage antigen (Mac-1) and p150,95. These three adhesion molecules are heterodimers, composed of a unique alpha subunit (CD11a, CD11b and CD11c, respectively) and a common beta subunit (CD18) (Springer, 1990). Since integrin expression requires intracellular association of both CD11 and CD18 subunits, CD18 defects prevent all integrin functions (Kishimoto *et al.*, 1987).

Cattle affected by BLAD have severe and recurrent bacterial infections such as pneumonia, gingivitis, impaired pus formation, delayed wound healing, stunted growth and persistent, marked neutrophilia (Kehrli *et al.*, 1990; Tajima *et al.*, 1993; Nagahata *et al.*, 1993; Gerardi, 1996). Blood samples analyzed 41 days after birth of suspect BLAD animals demonstrate progressive neutrophilia and excessive leukocytes (100,000 leukocytes/µl) (Kehrli *et al.*, 1994).

The relatively low mutant allele frequency rate found in Holstein cattle, as well as low expression of specific clinical signs suggest that most calves with BLAD die before diagnosis, probably before one year of age. Some cows are able to survive for more than two years; however their reproductive and milk performances are poor (Garcia *et al.*, 1996). Consequently, BLAD is an economically important disease, emphasizing the need for genetic screening for the elimination of the mutant allele from the population.

Two point mutations have been identified in the gene that encodes bovine CD18 in Holstein cattle affected with BLAD (Shuster *et al.*, 1992). One mutation replaces adenine with guanine at nucleotide 383, and the other replaces cytosine with thymine at nucleotide 775. The latter is a silent mutation. It causes no alteration in the amino acid sequence; consequently, it has no phenotypic effect. The mutation at nucleotide 383 substitutes glycine for aspartic acid at amino acid 128. This mutation eliminates a *Taq*I restriction site and creates a *Hae*III site, which allows the identification of normal, carrier and affected animals.

BLAD was first identified in Holstein-Friesian cattle at the beginning of the eighties (Gerardi, 1996), and no study has reported the occurrence and etiology of this disease in other breeds. The mutation found in the Holstein breed can be traced back to a heterozygote bull (Osborndale Ivanhoé), which due to its elevated genetic merit for milk production has been widely used in artificial insemination. This bull and its offspring (Penstate Ivanhow star - son and Carlin M Ivanhoe Bell - grandson) founded one of the main Holstein lineages. They are also responsible for spreading BLAD to several herds worldwide (Shuster *et al.*, 1992).

The occurrence of BLAD-affected animals throughout the world is probably due to the practice of artificial insemination with carrier bull semen. In Brazil, Holstein animals, semen and embryos are imported for dairy cattle breeding. Furthermore, Brazilian producers cross Holstein cattle with Gir cattle without controlling for BLAD, and thus there is a possibility that this mutation can be transferred to the Gir breed.

The Gir breed is considered, either as a pure breed or in crosses, as an alternative to Holsteins for milk production in tropical areas. It is responsible for close to 25% of the registered milk production in Brazil, being surpassed only by the Holstein breed. A breeding program that has

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accompanied the development of Gir herds reports an average of 2,766 kg of milk/291 lactating days for 7,944 cows (Martinez *et al.*, 1998). Nevertheless, calf mortality is still high, being 3% for animals under one year of age. Consequently, this study was conducted in order to determine the allelic frequency of the BLAD mutation in Brazilian Holstein animals and Gir breeding bulls.

MATERIAL AND METHODS

Animals

For this study, 88 not closely related Holstein cows were sampled. Twenty-eight animals from the Animal Production Department of ESALQ/USP, 30 from Empresa Brasileira de Pesquisa Agropecuária - Centro de Pesquisa de Pecuária do Sudeste (Embrapa/CPPSE) and 30 animals from a private property in the São Roque municipality in the State of São Paulo. Eighty-eight Gir bulls were sampled among 126 participants in Embrapa's dairy cattle progeny tests at the National Dairy Cattle Research Center (Embrapa-CNPGL).

Samples

Blood samples

Whole blood (500 µl) collected with EDTA from Holstein cattle, was lysed and washed three times with 1 ml of the lysing solution (0.32 M sucrose, 10 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$ and 1% Triton X-100). The leukocyte pellet was then resuspended in 0.5 ml of 50 mM KCl, 10 mM Tris-HCl, pH 8.3, 1.5 mM $MgCl_2$, 1% Triton X-100 and 60 ng/µl proteinase K. The solution was incubated at 50ºC for 1 h, and subsequently at 95ºC for 15 min, causing proteinase K to be heat inactivated. DNA was stored at -20ºC until use.

Semen samples

Approximately 0.5 ml of Gir cattle semen was centrifuged (10,000 *g*, 10 min) and washed four times with 1 ml PBS. The final pellet was resuspended in 100 µl PBS and 400 µl of lysing solution (2% β-mercaptoethanol, 10 mM Tris-HCl, pH 8.0, 100 mM NaCl, 10 mM EDTA, pH 8.0, and 0.5% SDS). After incubating semen at 50ºC for 30 min, 200 ng/µl proteinase K was added and the semen incubated at 52ºC for 16 h. Following this treatment, the sample was heated to 95ºC for 15 min to inactivate proteinase K. DNA was stored at -20ºC until use.

PCR assay

Amplification reactions of the DNA samples extracted from the blood were prepared in a final volume of 25 µl containing: PCR buffer (20 mM Tris-HCl, pH 8.4, and 50 mM KCl), 0.2 mM dNTPs, 0.5 units Taq DNA polymerase, 1.5 mM $MgCl_2$, 200 nM each, of forward primer, 5'CCCTGCCAGTCCAGCTGGACACC3', reverse primer, 5'CCACGCCCATCATTCTGGGGCAG3' and 100 ng of genomic DNA. Amplification was performed in 35 cycles of 15 s at 94ºC and 20 s at 69ºC. Aliquots of the amplified products (10 µl) were digested separately with four units of restriction enzymes *Taq*I and *Hae*III, followed by incubation for 1.5 h at 65ºC or 37ºC, respectively. Digested products were analyzed in 4% agarose gel and stained with ethidium bromide. DNA samples extracted from semen were amplified in a similar way to those extracted from blood; however, the PCR program included an initial denaturation of 3 min at 94ºC, followed by 35 cycles of 30 s at 94ºC, 40 s at 65ºC and 40 seconds at 72ºC and a final extension of 5 min at 72ºC. The amplified product was precipitated to concentrate the DNA for digestion. The pellet was resuspended in 10 µl MilliQ H_2O, followed by digestion with four units of the restriction enzyme *Taq*I for 3 h at 65ºC. The digested product was analyzed in a 3% agarose gel and stained with ethidium bromide.

Allelic frequency estimate

The gene frequency of the CD18 locus was calculated based on the Hardy-Weinberg law as follows:

$$p = \frac{2(AA) + (Aa)}{2N} \qquad q = 1 - p$$

where p = normal allele frequency, q = mutant allele frequency, N = total number of animals tested, AA = number of BLAD-free cattle, and Aa = number of BLAD carriers.

The estimate of the occurrence of BLAD-affected animals was based on the genotypic frequency of BLAD carriers.

RESULTS AND DISCUSSION

The primers used were designed to amplify a DNA fragment of 58 bp. The restriction enzyme *Taq*I digested the PCR product into two fragments of 26 and 32 bp in normal homozygote animals (Figure 1, all lanes, except 5, 7, 18 and 22, and Figure 2, lanes 1, 2, 3, 4 and 5). Heterozygote animals gave three fragments of 58, 26 and 32 bp (Figure 1, lanes 5, 7, 18 and 22).

In order to confirm the occurrence of the CD18 mutation, samples were digested with the *Hae*III enzyme. This enzyme digests the PCR product into two fragments of 9 and 49 bp in normal homozygote individuals and into three fragments of 9, 19 and 30 bp in affected animals. Heterozygote animals had four fragments of 9, 19, 30 and 49 bp.

Analysis of the Holstein breed revealed that of the 88 Holstein cows genotyped, 83 were normal homozygotes and five were heterozygotes for the disease. The frequency of the BLAD mutant allele and the BLAD-carrier preva-

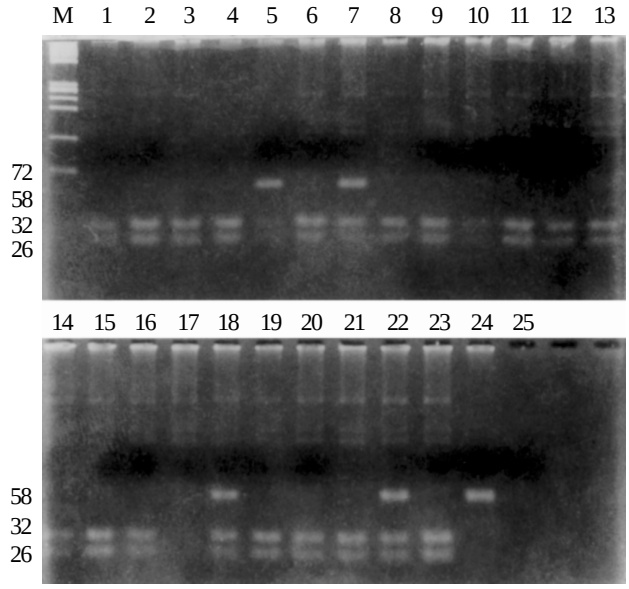

Figure 1 - Agarose gel electrophoresis of amplified DNA from BLAD-free and BLAD-carrier cows following restriction digestion with *Taq*I. Lanes 5, 7, 18 and 22 correspond to BLAD-carrier animals; lane 24 corresponds to undigested PCR-product and the remainding lanes correspond to DNA from BLAD-free cows.

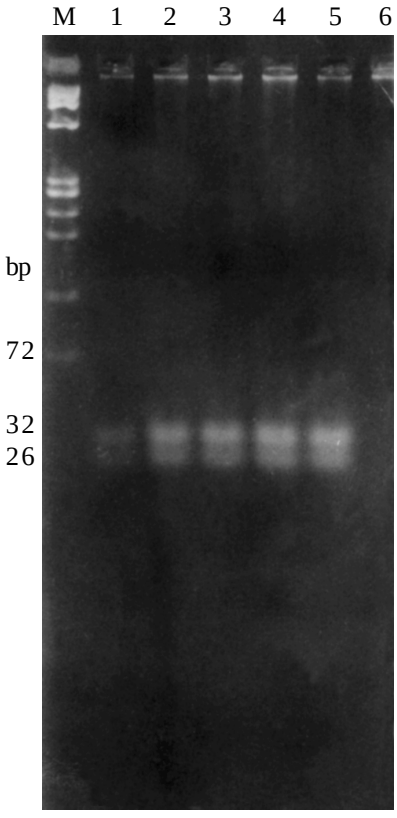

Figure 2 - Agarose gel electrophoresis of amplified DNA from Gir bulls following restriction digestion with *Taq*I. All animals were identified as normal homozygotes, i.e, BLAD-free animals.

lence in Brazilian Holstein cows were 2.8 and 5.7%, respectively. Of the Gir bulls tested, all were homozygous for the normal allele. We used cows in this test because the Holstein bulls that are used in artificial insemination are usually imported and are currently tested for BLAD.

The frequency of the heterozygote for the CD18 gene was similar to that found by Shuster *et al.* (1992) in American Holsteins, in which the observed heterozygote frequency was approximately 14.1% for bulls and 5.8% for cows. Holstein herds have been genotyped for this mutation in several countries. Frequencies are 3.5% in Argentinean Holstein cows (Poli *et al.*, 1996), 13.4% in Danish Holstein-Friesian cattle (Jorgensen *et al.*, 1993) and 8.1% in Japanese Holstein animals (Nagahata *et al.*, 1997), indicating that this mutation is prevalent among Holstein cattle throughout the world.

We found that the genetic mutation responsible for BLAD in Holstein cattle was absent in all the Gir bulls analyzed from the Embrapa's dairy cattle progeny test. This result could be explained by two factors: either the CD18 gene mutation does not exist in Gir cattle or the frequency of this allele is extremely small and the size of the sample was not large enough for detection. New studies analyzing calves with characteristic BLAD symptoms could be conducted in order to further verify if this mutation occurs in Gir. In Holsteins, however, the incidence of the mutation was similar to that observed in herds in North America. Since BLAD is an economically important disease, Brazilian dairy farmers should avoid the use of semen from carrier bulls. This would prevent the introduction of the BLAD mutation in the Gir breed and the death of Holstein calves from BLAD.

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RESUMO

Oitenta e oito vacas da raça Holandesa e 88 touros da raça Gir foram genotipados através da PCR para o gene CD18 da deficiência de adesão de leucócitos em bovinos (BLAD). As freqüências do alelo mutante BLAD e de vacas heterozigotas da raça Holandesa foram 2,8 e 5,7%, respectivamente. Por outro lado, todos animais Gir foram identificados como homozigotos normais, ou seja, nenhum alelo mutante BLAD foi encontrado. Estes resultados sugerem que a mutação no gene CD18 é prevalente em bovinos brasileiros da raça Holandesa e ausente ou presente numa freqüência muito baixa em animais Gir.

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