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Bovine Leukocyte Adhesion Deficiency in Danish Holstein-Friesian Cattle
I. PCR Screening and Allele Frequency Estimation

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Department of Animal Science and Animal Health, Division of Animal Genetics, Department of Anatomy and Physiology, Divison of Anatomy. The Royal Veterinary and Agricultural University, Copenhagen. Department of Pathology and Epidemiology, National Veterinary Laboratory, Copenhagen, and The Danish Agricultural Advisory Centre, Aarhus, Denmark.

Jørgensen, C. B., J. S. Agerholm, J. Pedersen and P. D. Thomsen: Bovine leukocyte adhesion deficiency in Danish Holstein-Friesian Cattle I. PCR screening and allele frequency estimation. Acta vet. Scand. 1993, 34, 231-236. – A screening program for bovine leukocyte adhesion deficiency (BLAD) in Danish Holstein-Friesian cattle has been initiated. During the first months 1611 animals were tested by a PCR based assay. Of these animals 1256, 346, and 8 were assigned normal, BLAD carriers, and BLAD affected animals, respectively. One bull, born as a co-twin, showed weak reaction for the BLAD allele on DNA isolated from leukocytes, but a normal genotype on DNA isolated from semen. Chromosome analysis showed that this bull was a blood chimaera. Estimation of the BLAD allele frequency upon the PCR test results showed that around 450 Danish calves born in 1991 might have been affected with the recessive disorder.

BLAD; hereditary disease.

Introduction

Bovine leukocyte adhesion deficiency (BLAD) is a recessive autosomal inherited disorder in Holstein-Friesian cattle caused by a deficiency in Mac-1 (CD11b/CD18), a leukocyte-surface glycoprotein involved in the defense against infections (*Hagemoser et al.* 1983, *Kehrli et al.* 1990, *Nagahata et al.* 1987, *Shuster et al.* 1992, *Takahashi et al.* 1987).

The molecular basis of BLAD is a single point mutation (adenine → guanine) at position 383 in the cDNA of the CD18 gene (*Shuster et al.* 1992). This mutation results in a substitution of a glycine for an aspartic acid at position 128 in the protein. Another difference (cytosine → thymine) between the normal and the BLAD allele has been discovered at position 775 in the cDNA, but unlike the 383 mutation, this mutation is silent, i.e. has no phenotypic effect. The point mutation at position 383 destroys a *Taq*I restriction site and creates a *Hae*III site. Thus restriction analysis of PCR-amplified DNA containing position 383 of the CD18 gene allows discrimination between normal, carrier and affected individuals.

Due to the import of semen from BLAD carriers to Denmark (*Nielsen et al.* 1992) a Danish BLAD screening program has been initiated. The aim of this program is to estimate the BLAD allele frequency and to eliminate the BLAD allele in the Danish Holstein-Friesian population by excluding BLAD carriers from the breeding schemes.

Materials and methods

DNA preparation

DNA was extracted from EDTA stabilized blood samples or semen straws. High molecular weight DNA was prepared from blood leukocytes (*Grimberg et al.* 1989) of animals used for breeding. From deceased breeding bulls DNA was prepared from semen as described by *Thomsen & Nielsen* (1991). Some of the important breeding bulls were tested both on semen and on blood samples. Quick preparation of DNA from EDTA blood was made from non-breeding animals by the following procedure: One hundred microliters of EDTA blood was vortexed in 1 ml H_2O, nuclei were recovered by 13000 x g centrifugation and washed in 1 ml 0.9 % NaCl. Lysis was performed by incubation in 400 µl 0.2 M NaOH at 99°C for 10 min. Before PCR the lysate was neutralized with 400 µl 0.2 M Tris-HCl giving an approximate final DNA concentration of 25 ng/µl.

PCR assay

Primers (*Shuster et al.* 1992), which gave rise to a 58 bp PCR product containing the position 383 nucleotide were used (see Fig. 1). PCR was carried out in a total volume of 25 µl on 100 ng genomic DNA. The conditions were as follows: 50 mM KCl, 10 mM Tris-HCl, pH 8.4, 1.5 mM $MgCl_2$, 0.4µM primers, 90 µM of each dNTP (Pharmacia) and 0.5 units Taq DNA polymerase (Perkin Elmer). Hot-start was used, and therefore the addition of dNTP's was postponed until after 3 min initial denaturation at 94°C. Hereafter, 35 cycles at 94°C for 45 s and 67°C for 1 min were performed. The PCR product was divided into 3 aliquots before restriction analysis: 1) undigested, 2) *Hae*III digested and 3) *Taq*I di-

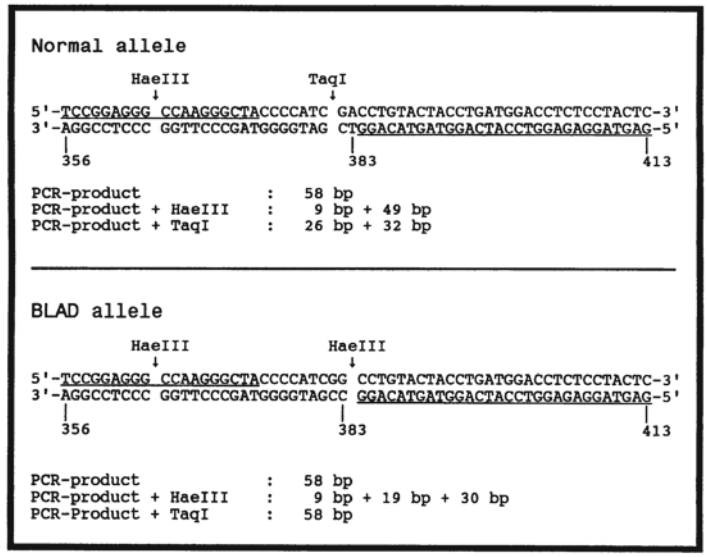

Figure 1. Schematic presentation of the PCR assay for BLAD. The primers are underlined, and the HaeIII and TaqI restrictions sites are indicated. The sizes of the restriction fragments are shown below each of the 2 alleles.

gested. Digestions were done simply by adding 3 units of the appropriate enzyme directly to the PCR sample and then incubating at least 4 h at the temperature recommended by the supplier. The PCR product and the digestions were analyzed on a 4% NuSieve GTG agarose gel containing 0.2 µg/ml ethidium bromide. Four controls were included: 1) a blank, 2) DNA from a normal animal, 3) DNA from a BLAD carrier animal, and 4) DNA from a BLAD affected animal. The interpretations of the electrophoresis band patterns are shown in Fig. 1.

Allele frequency estimation

Estimations of the BLAD allele frequency among calves born in 1991 could be made after the initial screening of the breeding bulls. Only offspring, which had a registered sire and a registered maternal grandsire were included in the estimations and the status of the maternal granddam was always considered as non-carrier of BLAD. The BLAD allele frequency b and the genotype frequencies BB, Bb and bb among calves born in 1991 were calculated as follows:

BLAD allele frequency (b): $\sum_i \sum_j (1/2 \times b_s + 1/4 \times b_{gs})/N$

BLAD free (BB): $\sum_i \sum_j ((1 - b_s) \times (1 - 1/2 \times b_{gs}))/N$

BLAD carrier (Bb): $\sum_i \sum_j ((1 - b_s) \times (1/2 \times b_{gs}) + b_s \times (1 - 1/2 \times b_{gs}))/N$

BLAD affected (bb): $\sum_i \sum_j (b_s \times 1/2 \times b_{gs})/N,$

where both b_s and b_{gs} equal ½ or 0, i.e. the probability that sires(*s*) and grandsires (*gs*) transmit a BLAD allele to their first-generation offspring. The variables i and j represent the number of sires and the number of grandsires, respectively, and N is the total number of calves included in the calculation.

For some bulls BLAD status had to be deduced from their relation to known BLAD carriers, because the bulls were in private ownership and therefore inaccessible to BLAD genotyping. These private bulls are included as the group designated "suspected".

Results

PCR assay

Fig. 2 shows agarose gel electrophoresis of the 3 BLAD genotypes after *Hae*III and *Taq*I digest of the PCR product.

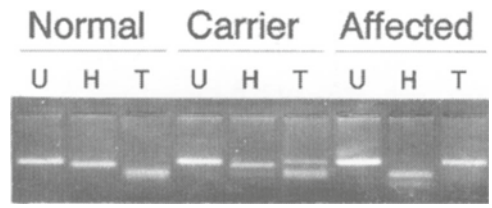

Figure 2. Agarose gel electrophoresis of amplified DNA from a normal animal, a BLAD carrier and a BLAD affected animal: U, PCR-product undigested; H, PCR-product *Hae*III-digested; T, PCR-product *Taq*I-digested. The normal animal shows a 58 bp PCR-product, which generates a 49 bp plus a 9 bp fragment after *Hae*III-digest and a 26 bp plus a 32 bp fragments after *Taq*I-digest. The affected animal shows a 58 bp PCR-product, which is digested into a 9 bp, a 19 bp and a 30 bp fragment by *Hae*III, while the PCR-product remains undigested after incubation with *Taq*I. DNA from the carrier animal shows all fragments from both the normal and affected animal.

Table 1. Results of the BLAD screening. The animals have been divided into 3 groups; breeding bulls, other males, and females. All totals are shown initalics.

	Normal	Carriers	Affected	Chimaera	Total
Breeding bulls	297	108	0	0	*405*
Other males	846	144	3	1	*994*
Females	113	94	5	0	*212*
Total	*1256*	*346*	*8*	*1*	*1611*

A total of 1611 Danish Holstein-Friesians were tested for BLAD during the first months of the screening program and 1256, 346, and 8 animals were assigned normal, BLAD carriers and BLAD affected animals, respectively.

One bull was suspected to be a blood chimaera due to a normal genotype from the semen sample and a weak BLAD allele band pattern from the blood sample. This bull was born with a twin sister. The freemartin diagnosis was established by chromosome analysis of 100 metaphase spreads from blood lymphocytes, which revealed 7 cells with a 60,XX (female) and 93 cells with a 60,XY (male) chromosome complement (*Christensen,* unpublished results).

The results of the BLAD screening are shown in Table 1.

Allele frequency estimation

In Table 2 the BLAD allele frequency in Danish Holstein-Friesian calves, who were the offspring of registered sires and maternal grandsires in 1991, is estimated to 6.9%. Of a total of 276,887 calves born, 234,953 had sires and maternal grandsires of known CD18 genotype. Of these calves 1183 had both a sire and a maternal grandsire, who were BLAD carriers. Theoretically, 1/8 or 12.5 % of these calves will be BLAD affected, which leads to an estimate of 148 BLAD affected calves born in 1991. If the suspected sires and suspected maternal grandsire are included, the theoretical estimate will be around 450 BLAD affected animals born in Denmark.

Discussion

We have established a DNA-bank of the most important Danish Holstein-Friesian breeding animals, performed PCR screening for bovine leukocyte adhesion deficiency and described the spread of this disorder in Denmark.

Initially the PCR based assay caused difficulty due to unspecific amplification of an approximately 50 bp fragment. Fortunately, this problem could be solved by using Hot-start PCR. After Hot-start was introduced, the analysis gave a clear genotype interpretation in all cases except for the blood chimaera. The latter case emphasizes that special precautions have to be taken when dealing with blood genotyping in cattle where most twin foetuses shows vascular anastomoses and are thus blood chimeric. It is therefore advisable to use semen typing whenever this is possible. Young bulls could initially be screened on DNA isolated from blood for convenience, but a twin diagnosed as a carrier should be re-tested on DNA isolated from semen before this bull is excluded from breeding.

Levels of CD18 on the surface of the neutrophil granulocytes were recorded on a BLAD affected animal by flow cytometry with FITC-conjugated anti human CD18 (Code 839, DAKO). This BLAD affected an-

imal showed around 1% of the normal CD18 level, which supported our PCR genotyping (*Aasted,* unpublished results). Additionally, 3 of the calves that were diagnosed BLAD affected at PCR genotyping, were examined clinically, haematologically and patho-anatomically and showed changes consistent with the BLAD syndrome (*Agerholm et al.* 1993).

Some of our first results showed that the most commonly used Danish Holstein-Friesian breeding bull, NJY Hubert, was a BLAD carrier. NJY Hubert was the sire of 17% of all registered calves born in 1991 and he was involved in 820 of the 1,183 carrier x carrier matings in Table 2. NJY Hubert therefore holds the main responsibility of the quick spreading of BLAD in Denmark.

There are still some suspected bulls remaining in the BLAD screening program, and most of these bulls will be genotyped soon. This will allow the final estimate of the BLAD allele frequency in the Danish Holstein-Friesian population.

The BLAD genotyping program and the previously reported screening program for citrullinaemia (*Thomsen & Nielsen* 1992) illustrate the importance of storing DNA from influential breeding animals in order to trace back new disorders and minimize their spread in the future. With the fast expanding knowledge of the genomes of domestic animals and the more intensive usage of the genetic resources, screening for genetic disorders and more or less beneficial genotypes will become increasingly important.

Acknowledgment

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Table 2. Estimation of the BLAD allele frequency (b) in percent in the 1991 population of registered newborn calves. The percentage of BLAD affected animals and the percentage of BLAD carriers are also estimated. Sires and maternal grandsires of known genotype are indicated in bold-faced type, and all totals are shown in italics.

Sire genotype	Maternal grandsire genotype	Calves no.	BLAD allele freq. (b) (%)	BLAD affected (%)	BLAD carriers (%)
Normal	Normal	172634	0.0	0.0	0.0
Normal	Suspected	19573	5.4	0.0	10.7
Suspected	Normal	14989	8.9	0.0	17.9
Normal	Carrier	7235	12.5	0.0	25.0
Carrier	Normal	53901	25.0	0.0	50.0
Without BLAD risk		*268332*	*6.2*	*0.0*	*12.5*
Suspected	Suspected	1996	13.9	1.8	24.2
Suspected	Carrier	474	20.9	4.2	33.4
Carrier	Suspected	4902	30.0	5.0	50.0
Carrier	Carrier	1183	37.5	12.5	50.0
With Blad risk		*8555*	*26.8*	*5.3*	*43.1*
Total		*276887*	*6.9*	*0.2*	*13.4*

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Sammendrag

Bovine leukocyte adhesion deficiency hos Sortbroget Dansk Makerace.
I. PCR screening og allel frekvens estimering.

Et screeningsprogram for bovine leukocyte adhesion deficiency (BLAD) i Sortbroget Dansk Malkerace er påbegyndt. I løbet af de første måneder er 1611 dyr blevet testet ved hjælp af en PCR baseret metode. Af disse dyr var 1256 normale, 346 var bærere af BLAD, mens 8 dyr var BLAD afficerede. En tyr var 60,XY/XX blodkimære ved kromosomundersøgelse, og den gav en svag reaktion som bærer ved PCR genotypning af DNA fra blodceller, men viste normal genotype ved undersøgelse af DNA isoleret fra sæd. På basis af en estimeret BLAD allelfrekvens på 6.9% er det beregnet, at der blev født omkring 450 BLAD afficerede kalve i 1991.

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