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Detection of bovine leukocyte adhesion deficiency (BLAD) carriers using a new PCR test

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Abstract. In this report we demonstrate a simple, effective and reliable diagnostic test of BLAD carrier detection based on specific PCR amplification of a 367 bp CD18 gene fragment and RFLP analysis using Taq I restriction enzyme. In a non-random population of 220 animals we found 48 BLAD carriers. Within the amplified PCR fragment an unknown intron sequence of 159 bp was identified.

Key words: cattle, BLAD, CD18, PCR.

Introduction

BLAD is an autosomal recessive disease characterised clinically by a severe persistant and recurrent bacterial infection associated with neutrophilia and early death (KEHRLI et al. 1990). Biochemically, it is recognized by a deficiency in the surface expression of the heterodimeric beta-2 integrin adhesion molecules on leukocytes. The beta-2 integrins are composed of identical beta subunits (CD18) and alfa subunits that vary in structure and are designated as CD11a, CD11b and CD11c (SPRINGER 1990).

So far, the genomic sequence of the entire gene encoding the bovine CD18 protein is unknown. A comparative study of DNA sequences of bovine cDNA encoding the CD18 gene with the human and murine sequences was performed by SHUSTER et al. (1992a). Two point mutations were identified within the gene coding bovine CD18 in Holstein cattle afflicted with leukocyte adhesion deficiency (LAD). One point mutation (adenine to guanine) causes an aspartic acid to glycine substitution at amino acid 128 (D128G) in the highly conserved

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exon IV coding a part of the extracellular region of the adhesion glycoprotein. Furthermore, a silent mutation was detected. The first mutation mentioned here has been prevalent among Holstein cattle throughout the world, placing this disorder among the most common genetic diseases known in breeding animals (SHUSTER et al. 1992b, WOMACK 1992, PAREEK, KAMIŃSKI 1996).

The combination of clinical signs, pedigree and laboratory findings provides compelling evidence for presumptive diagnosis in BLAD cases. Definitive diagnosis may be made by the demonstration of the mutant allele by molecular biology methods. The basic protocol for the identification of mutation in the bovine CD18 gene was described by SHUSTER et al. (1992b). This test, however, has some technical disadvantages, which forced us to develop a new method for BLAD genotyping.

Materials and methods

Blood samples were obtained from normal and BLAD carrier cows. These animals were chosen from the population of Polish Black-and-White cattle held in some herds located in the Żuławy district. DNA was isolated from blood by the Easy Genomic DNA Prep Plus Kit (DNA-Gdańsk).

Using the Dnasis computer program two primers were designed which flanked a 208 bp sequence located within the cDNA of the CD18 gene, position 327-534 (SHUSTER et al. 1992a). This sequence contained D128G. A PCR mix contained: 2.5 μl 10x PCR (Promega Co.), 1.5 μl $MgCl_2$ (25mM), 1.0 μl dNTP-mix (2 mmol each), 1.0 μl of primer BLAD-1 and BLAD-2 (100 ng/μl each), 0.5 u Taq polymerase (Promega), 200-400 ng of DNA template and H_20 to a volume of 25 μl. PCR program included the following steps: predenaturation – 3 min 94°C followed by 35 cycles of: 30 sec 94°C, 30 sec 61°C, 30 sec 72°C.
PCR primers were as follows: BLAD-1: 5AGGTCAGGCAGTTGCCTTCAA3, BLAD-2: 5 GGGGAGCACCGTCTTGTCCAC 3.

Seven μl of the PCR products were digested with Taq I restriction enzyme (Promega). The PCR products and restriction fragments were electrophorezed in 1.5% agarose/ethidium bromide gel. Gels were visualized and documented by the GDS 7500 system (UVP).

Results and discussion

The most frequently used PCR test for the detection of BLAD carriers was developed by SHUSTER et al. (1992b). This test is based on the amplification

of a very short gene fragment, namely 58 bp, which is then digested with Hae III or alternatively with Taq I. This method, however, has some technical disadvantages. Firstly, the PCR product and subsequently the restriction fragments are very small and therefore difficult to observe clearly on agarose gel due to their rapid diffusion. Secondly, the two PCR primers are not balanced in terms of length and annealing temperature. We also observed that they have an inherent tendency to create primer-dimer complexes running in the gel very close to specific PCR product, which disturbs the process of distinguishing genotypes. Additionally, these primers make the test less tolerant to critical samples or historic material. Taking this into account, we tried to make the test both more robust and convenient by using other primers, while retaining the simplicity of the agarose gel electrophoresis.

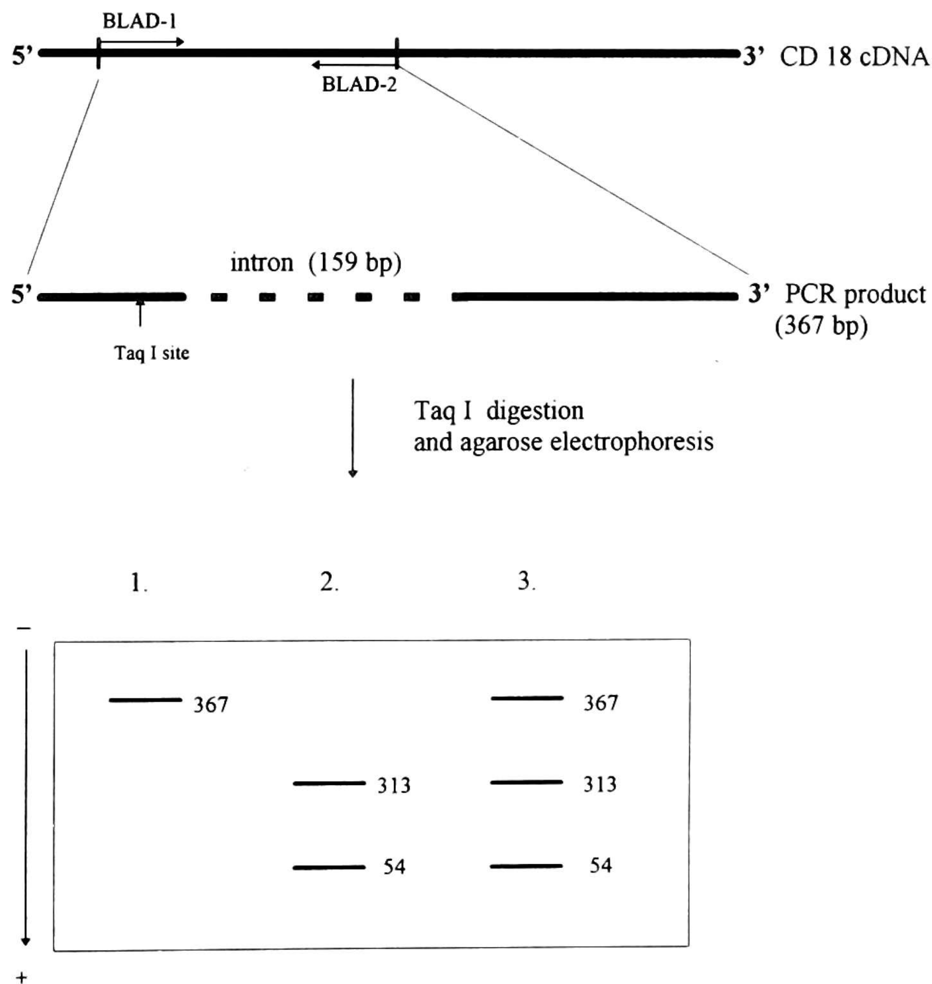

Fig. 1. General design of our BLAD test

First, we designed two primers flanking a 208 bp sequence of CD18. From the analysis of an alignment of the human CD18 gene (WEITZMANN et al. 1991) with the bovine cDNA of the CD18 gene (SHUSTER et al. 1992a) we expected to encompass the boundaries of the hypothetical fifth exon of the bovine CD18 gene. Indeed, the size of the amplified PCR product was not 208 bp,

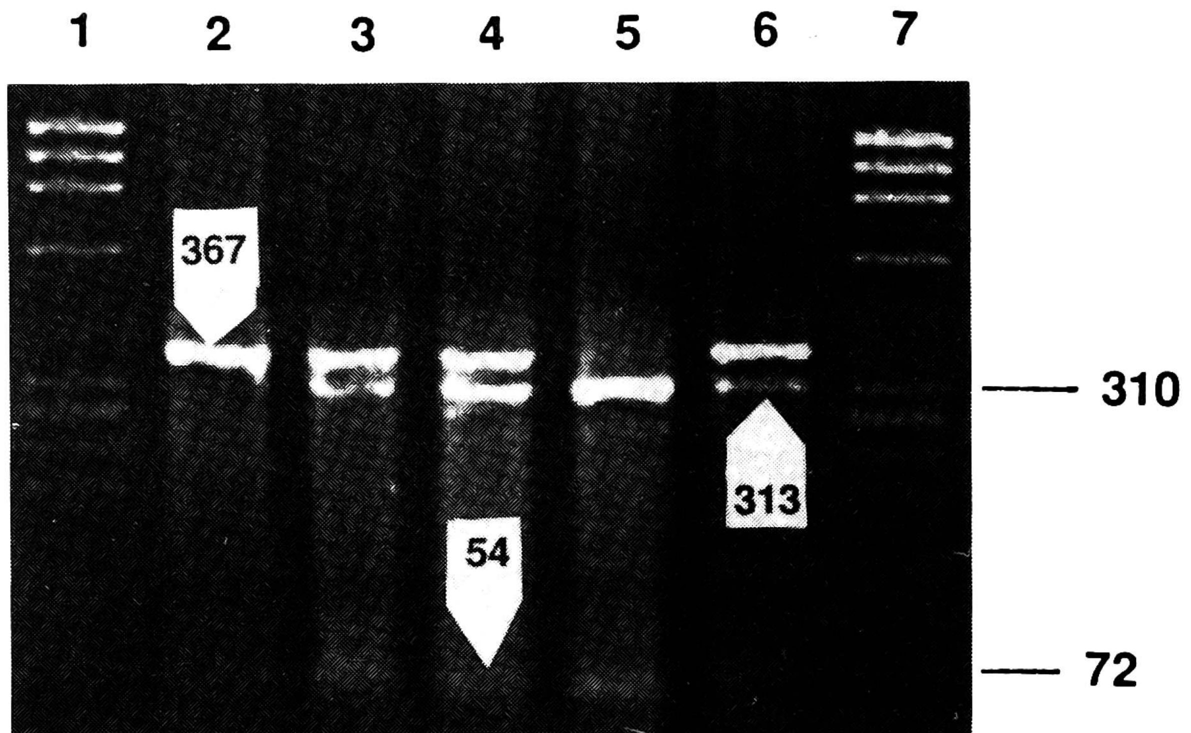

Fig. 2. 367 bp PCR-amplified CD18 gene fragment digested with Taq I
Paths 1 and 7: PhiX 174 Hae III-digest molecular marker. Path 2: PCR product not digested with Taq I (367 bp). Paths 3, 4, and 6: BLAD carrier (367, 313, 54 bp). Path 5: normal animal (313, 54 bp).

but approximately 360 bp. To check if we amplified a specific fragment of the CD18 gene we sequenced this PCR product (Lark Sequencing Technologies, Inc., U.K.). It turned out that the PCR product is specific and is the size of 367 bp. Moreover, we found that this PCR product contains a non-coding sequence (intron) of 159 bp. This PCR product was used for BLAD genotyping. The theoretical assumptions of the experiment are shown in Fig. 1. In the results we obtained two different DNA band patterns in the agarose gel: 313 bp + 54 bp (+/+ fragment completely cut, normal animal) and 367 bp + 313 bp + 54 bp (fragment partly digested, BLAD carrier) (Fig. 2). The computer analysis revealed no additional Taq I sites within the entire PCR product, including the intron sequence. In BLAD homozygous animals we should observe only the 367 bp PCR product. No BLAD homozygous animals were found.

Similar reasons have forced other authors to develop new methods of BLAD genotyping. BATT et al. (1994) developed a nonisotopic ligase chain reaction (LCR) assay. This technique does not require an electrophoretic step but needs 6 extra labelled oligonucleotides and a non-standard procedure. TAMMEN et al. (1994) developed a multiplex PCR for simultaneous sexing and BLAD genotyping of embryos. MIRCK et al. (1995) described a new PCR test in which a 159 bp fragment of the CD18 gene is amplified and digested with Taq I. Lately, ZSOLNAI and FESUS (1996) described a simultaneous genotyping

of kappa-casein and BLAD by multiplex PCR. These authors used primers generating a 134 bp fragment of the CD18 gene digested with Hae III. However, they do not discuss the situation where the tested animal has E allele of kappa-casein. This allele is cut by Hae III which multiplies the number of restriction fragments in the gel and may disturb clear genotyping of both loci.

By the use of our PCR-RFLP protocol we genotyped 220 animals. Among them, we found 48 BLAD carriers. It was not a random population, because some of the animals were the pedigree of known BLAD carriers.

Our PCR test is simple, effective and reliable and may be used for the screening of BLAD carriers. Moreover, the amplified PCR product is a good start point for the identification of other mutations of the CD18 gene.

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