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EVIDENCE OF SINGLE POINT MUTATION INDUCING BLAD DISEASE IN ROMANIAN HOLSTEIN-DERIVED CATTLE BREED**

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**Original scientific paper

Abstract: Bovine leukocyte adhesion deficiency (BLAD) is a genetic disease that affects the haematopoietic system via greatly reduced expression of the heterodimeric β_2 integrin, causing many defects in leukocyte function. It is known that BLAD is an autosomal recessive defect, caused by a point mutation in the gene encoding subunit CD18 for β_2 integrin adhesion molecule, which is lethal in the homozygous form. In this study, 80 cattle of the Romanian Black Spotted breed have been screened via the DNA test. We performed the PCR-RFLP test followed by sequencing to investigate the incidence of this genetic disease. A single point mutation was identified within the gene encoding bovine CD18 in two Romanian Black Spotted cattle affected with BLAD. The aim of our work was to optimize PCR (Polymerase Chain Reaction), RFLP (Restriction Fragment Length Polymorphism) and sequencing analysis as a diagnostic test to identify BLAD carrier cattle.

Key words: cattle, BLAD, single point mutation, PCR-RFLP, sequencing.

Introduction and Literature Review

Bovine Leukocyte Adhesion Deficiency (BLAD) is a lethal autosomal recessive disease in Holstein cattle characterized by a greatly reduced expression level of the β2 heterodimeric integrin (*Citek et al.*, 2004). The molecular basis of BLAD is a single point mutation (A→G) at position 383 in the cDNA of the CD18 gene. This mutation results in a substitution of a glycine for an aspartic acid at position 128 in the D128G protein (*Gerardi et al.*, 1996; *Jorgensen et al.*, 1993; *Meylan et al.*, 1997; *Rutten et al.*, 1996; *Shuster et al.*, 1992). DNA restriction with endonuclease *TaqI* or *HaeIII*

could detect differences between healthy and affected calves by elimination of the enzyme restriction site.

Viana et al. (1998) and *Shuster et al.* (1992) also described the existence of the silent point mutation (C→T) at position 775 in the cDNA without phenotypic manifestation. DNA studies with the use of restriction endonuclease *TaqI* or *HaeIII* have detected differences between healthy and affected calves. The defective leukocyte adherence leads to inadequate mucosal immunity and BLAD – affected cattle have severe and recurrent mucosal infections such as pneumonia, ulcerative gingivitis, periodontitis, papillomatosis, dermatophytosis, loss of teeth, delayed wound healing, and stunted growth (*Ackermann et al.*, 1996; *Ribeiro et al.*, 2000).

Materials and Methods

For this study, we have used blood samples from 80 Romanian Black Spotted cattle (ICDB Baloteşti farm). The isolation of genomic DNA was performed with the Wizard Genomic DNA Extraction Kit (Promega). The total amount of isolated DNA was resuspended in sterile distilled water and measured spectophotometrically.

PCR-RFLP Method

In order to characterize and to detect the mutation responsible for BLAD disease we performed a simple polymerase chain reaction (GeneAmp® PCR System 9700) followed by enzymatic restriction. DNA was amplified for 45 cycles (95°C for 30 sec; 57°C for 30 sec; 72°C for 1 min) in a 25µl reaction containing: PCR buffer, $MgCl_2$, dNTPs, AmpliTaq DNA Polymerase, sense primer (5'- CCT TCC GGA GGG CCA AGG GCT -3') and antisense primer (5'- CTC GGT GAT GCC ATT GAG GGC -3'). The first denaturation step was performed at 95ºC (10 minutes) and the last extension was 72°C (30 minutes).

PCR products were first detected by electrophoresis in 2% agarose gel stained with ethidium bromide and then digested with restriction endonuclease *Taq I* at 37°C for 3 h. Restricted products were analyzed by electrophoresis in 2% agarose high resolution gel stained with ethidium bromide.

PCR Amplification and Sequencing

For sequencing, we performed PCR in the same manner as above. The amplified fragments were purified with the Wizard PCR Preps DNA Purification System Kit (Promega).The purified fragments were amplified via PCR using the ABI Prism® BigDye Terminator Cycle Sequencing Ready

Reaction Kit and sequenced with ABI Prism 310 Genetic Analyzer. The sequences were processed with DNA Sequencing Analysis 5.1 Software (AppliedBiosytems). The nucleotide sequences were aligned with the Clustal X multiple alignment program and refined manually.

Results and Discussion

Our goal was to develop an easy and efficient method to be used for the accurate detection of the normal, carrier and affected cattle for BLAD disease. The identification of normal or carrier specimens was achieved via PCR amplification of genomic DNA with specific primers designed for a region of 136 bp followed by digestion with *Taq I* endonuclease.

Normal homozygote should show two bands of 108 and 28 bp, carrier heterozygote three bands of 136, 108 and 28 bp, and affected homozygote only one band of 136 bp. *Taq I* enzyme is used to identify the normal allele of CD18 at the BLAD locus through the digestion of the amplified fragment. Our results indicate that out of the tested animals, 78 are normal (no carrier) with two bands of 108 bp and 28 bp. The other two are heterozygous (carrier of one mutant allele) with three bands of 136, 108 and 28 bp (Fig. 1).

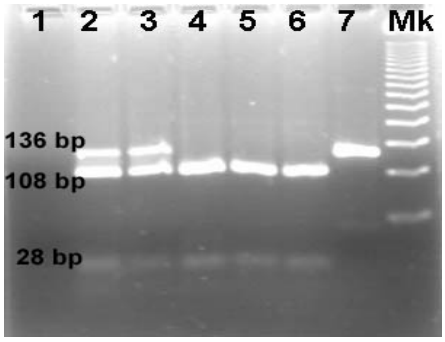

Figure 1. Electrophoresis pattern of BLAD locus after digestion with *Taq I* enzyme. Lane 1: negative control; lanes 2, 3: heterozygous genotype; lanes 4-6: homozygous normal cattle; lane 7: uncut PCR product; lane 8: molecular size marker (50 bp DNA Step Ladder).

For the two BLAD heterozygote carriers found in our samples we decided to sequence the PCR products. Sequencing was performed in order to confirm the sequences of amplified fragments from the normal CD18 gene but also to strengthen the diagnostic method. We identified and validated the

presence of the restriction site for *Taq I* enzyme in the CD18 gene. The profile of the region from the PCR product of a normal cattle comparing to the amplicons of the two BLAD heterozygote carriers that contain the point mutation are shown below (Fig. 2, 3).

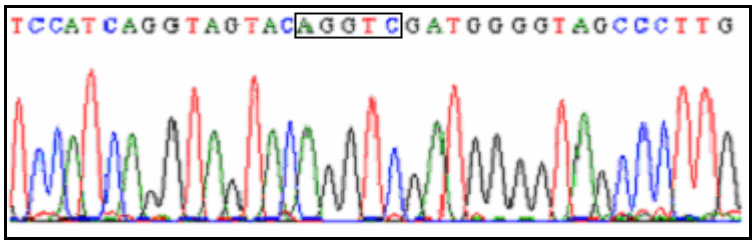

Figure 2. The reverse sequence of the PCR products for normal cattle.

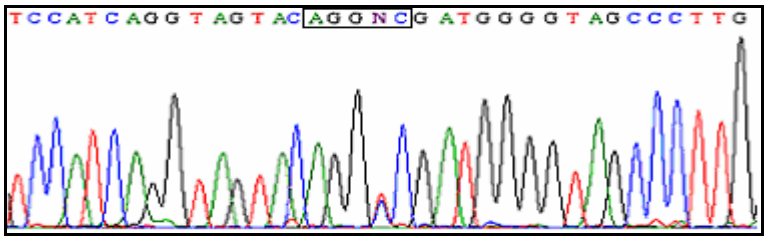

Figure 3. The reverse sequence of the PCR products for one carrier cattle.

The Clustal X alignment of a region from the CD18 gene comparing to the PCR product obtain from a normal bovine genotype and the amplicon of one heterozygous carrier cattle are shown in Figures 4 and 5.

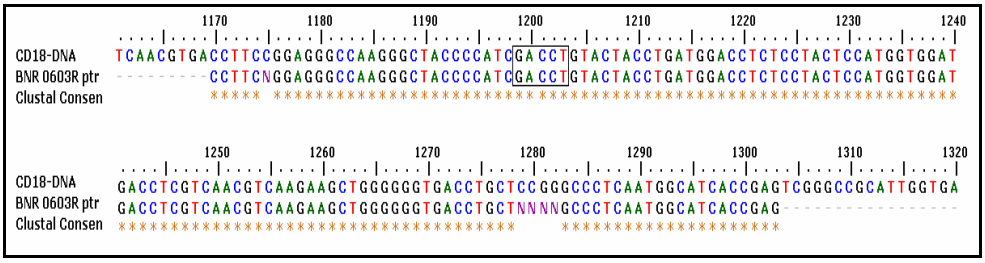

Figure 4. Clustal X reverse alignment between a Gene Bank fragment from the CD18 gene and our PCR product (normal cattle).

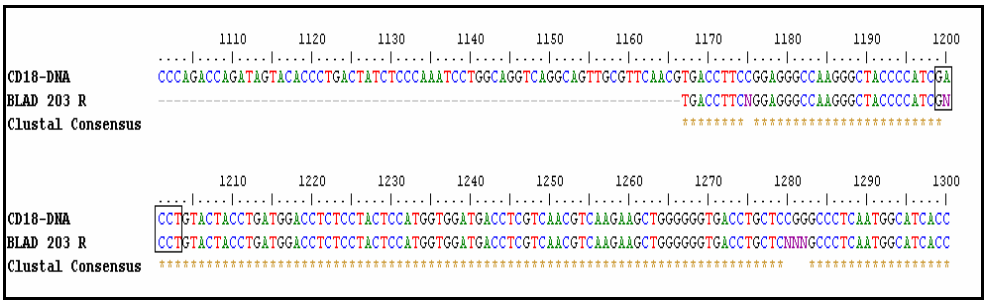

Figure 5. Clustal X reverse alignment between a Gene Bank fragment from the CD18 gene and our PCR product (carrier cattle).

Conclusions

The major focus of this study has been to identify the normal homozygous, heterozygous carriers and affected homozygous cattle for BLAD syndrome in Romanian cattle breeds and to implement a useful diagnosis methodology in order to assist veterinarians and breeders in controlling the disease.

The primers used in our study for detection of normal and mutant BLAD alleles, successfully amplified the BLAD sequence with 136 bp lengths. *Taq I* enzyme makes possible the identification of both BLAD alleles by digestion of the amplified samples, showing fragments with similar size as literature data indicates. Sequencing analysis used in the diagnosis method confirmed the sequences of amplified fragments from the normal CD18 gene.

The diagnosis method based on PCR – RFLP test and sequencing analysis is a powerful tool for detecting the presence of the BLAD disease, allowing a good and rapid identification of carrier cattle.

DOKAZ O POJAVI BLAD OBOLJENJA IZAZVANOG MUTACIJOM U JEDNOJ TAČKI KOD RUMUNSKIH GOVEDA KOJA POTIČU OD HOLŠTAJN RASE

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Rezime

BLAD je nasledna bolest goveda mlečne holštajn rase. Defektivni alel CD18 gena, koji je odgovoran za ovu bolest, ima recesivnu naslednost. Recesivna homozigotna forma je smrtonosna i pošto su životinje-domaćini/nosioci sposobne za život, učestalost BLAD oboljenja se povećava korišćenjem bikova-nosilaca u veštačkom osemenjavanju. BLAD nosioci mogu biti jednostavno otkriveni pomoću PCR i restriktivnom analizom amplikona.

Populacija rumunskih crno-belih goveda je korišćena za istraživanje pojave ovog genetskog oboljenja u najznačajnijoj mlečnoj rasi goveda u Rumuniji. Za dijagnozu BLAD, korišćen je PCR-RFLP metod. Set prajmera je određen za povećanje/pojačavanje regiona 136 bp CD18 gena koji sadrži mutaciju u jednoj tački (A→G). Uslovi za PCR su odabrani na način da dva prajmera mogu da pojačaju/povećaju DNK od normalnih goveda, goveda nosilaca/domaćina i goveda obolelih od BLAD bolesti. Proizvodi digestije su su filtrirani pomoću elektroforeze na 2% agaroznom gelu visoke rezolucije i posmatrani nakon bojenja etidijumbromidom. Naši rezultati ukazuju da su dve životinje od svih testiranih bile nosioci bolesti, pokazujući abnormalne heterozigotne genotipove. Prema tome, uradili smo sekcneciranje proizvoda PCR, čime smo dobili prisustvo nizova pojačanih fragmenata iz normalnog CD18 gena i ustanovili dijagnostički metod.

Razvijanje genetskih testova za ovo oboljenje će omogućiti odgajivačima da identifikuju nosioce BLAD, i budu usredsređeni na odgajivačke programe. Takođe, bilo bi korisno i interesantno istraživati pomoću genetskih testova mogućnost pojave pogođenog gena u populacijama goveda u drugim zemljama i različitim rasama.

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