



Original Scientific Article

**DETECTION OF RECESSIVE MUTATIONS (BLAD AND CVM)
IN HOLSTEIN-FRIESIAN CATTLE POPULATION IN
REPUBLIC OF MACEDONIA**Nikola Adamov¹, Dine Mitrov¹, Igor Esmerov¹, Peter Dovc²¹*Faculty of veterinary medicine, Ss. Cyril and Methodius University
in Skopje, Macedonia*²*Biotechnical faculty, University of Ljubljana, Slovenia*

Received 2 June 2013; Received in revised form 17 October 2013; Accepted 1 November 2013

ABSTRACT

BLAD and CVM are Holstein-specific recessive mutations which have had significant economic impact on dairy cattle breeding worldwide. The aim of this study was to optimize a diagnostic test for detection of these two disorders and to obtain a preliminary picture about their existence in Macedonian HF population. Blood samples were obtained from 84 HF cows from several different farms in Macedonia. Genomic DNA was extracted using DNeasy blood and tissue kit (Qiagen) which was also used for DNA extraction from deeply frozen sperm doses from 6 different HF bulls. Genotyping was performed by PCR-RFLP analysis with *TaqI* and *HaeIII* restriction endonucleases for BLAD and with *PstI* for CVM. The correctness of the genotyping was evaluated by direct sequencing of the amplified products. Among 90 HF cattle tested two cows were identified as carriers of the BLAD allele and one was carrier of the CVM allele. No bulls were found to be carriers of either of the two disorders. This study demonstrates that carriers of BLAD and CVM are present in Macedonia, although at low frequency. PCR followed by RFLP is efficient and inexpensive method for detection of the carriers and should be used as laboratory test in eradication programs. In order to establish a more accurate picture of the prevalence of these disorders, it is necessary to screen a larger number of animals. It is indispensable to test each bull before entering the A.I. program to prevent accidental transmission to a large number of offspring.

Key words: BLAD, CVM, bovine autosomal recessive mutations

INTRODUCTION

Bovine Leukocyte Adhesion Deficiency (BLAD) and Complex Vertebral Malformation (CVM) are recessively inherited diseases in Holstein cattle. They are both lethal in homozygous state while the heterozygous carriers show no clinical signs. When a bull intended for A.I. is a carrier of one or both of these recessive alleles, they can be transmitted to a large number of its offspring.

BLAD is inherited granulocytopeny syndrome that is caused by deficiency of the $\beta 2$ (CD11/CD18)

integrin receptor family expressed on leukocyte cell surface (20). These receptors have identical β (CD18) subunit but different α (CD11) subunit and have critical role for extravasation of the neutrophils and migration to the infected tissues. In 1992 Shuster et al. (40) reported that this disorder is caused by a point mutation (A383G) of the bovine CD18 gene on BTA1 that encodes the β (CD18) subunit of the $\beta 2$ integrin receptor family which causes D to G amino acid substitution at position 128 of the CD18 subunit (13, 18, 37). In healthy animals they are normally expressed on the polymorphonuclear neutrophils cell surface. Affected animals usually die during their first year of age since the absence of these receptors results in severe malfunction of the immune system or more precisely in inability of these cells to attach to the blood vessel endothelium and to migrate to the infected tissues. There are numerous published results that have identified this disorder in the Holstein cattle in different countries

Corresponding author: Nikola Adamov, MSc, DVM
E-mail address: adamovn@fvm.ukim.edu.mk;
Present address: Department of Animal Breeding and Genetics
Faculty of Veterinary Medicine - Skopje
Ss. Cyril and Methodius University in Skopje
Lazar Pop Trajkov 5-7, 1000 Skopje, Republic of Macedonia
tel: +389 2 324 0770 / fax: +389 2 311 4619

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Competing Interests: The authors have declared that no competing interests exist.

Available Online First: 15 November 2013
<http://dx.doi.org/10.14432/j.macvetrev.2013.11.005>

such as in Germany (42), Denmark (29, 3), the Netherlands (8), Austria (38), the United Kingdom (6), Japan (27), Switzerland (25), France (15), Taiwan (16, 17), Poland (28), Brasil (35), Korea (22), Iran (12), Turkey (4, 5, 24) and India (32, 33). It is internationally accepted to mark the pedigrees of the carrier animals with the code “BL” and those that are tested free with the code “TL”.

CVM is autosomal recessive inherited disorder in the Holstein breed which causes intra-uterine mortality leading to repeat breeding and culling of cows. It has been initially described in the Danish Holstein population in 1999 and shortly thereafter its presence was reported in the United States (11), the United Kingdom (34), the Netherlands (46), and in Japan (26). In 2006 Thomsen et al. (44) identified the molecular basis of this disorder and provided evidence that the missense mutation from G to T at nucleotide position 559 of the bovine solute carrier family 35 member 3 (SLC35A3) gene on BTA3 is responsible for this condition. This mutation results in V180F amino acid substitution of the Golgi-resident UDP-N-acetylglucosamine transporter enzyme which causes abnormal somitogenesis and impaired development of the fetal axial skeleton. The mutation in homozygous recessive state usually leads to death in utero although sometimes alive calves can be born with severe malformations such as hemivertebrae, misshaped vertebrae, ankylosis of mainly the cervico-thoracic vertebrae (fusion of 6th and 7th cervical vertebrae and distortion of the first three thoracic vertebrae), scoliosis, and symmetric arthrogryposis of the lower limb joints (1, 2, 30). In about 50% of the affected calves cardiac anomalies such as interventricular septal defects, right displacement of the aorta and hypertrophy of the right heart are observed. It is international standard to mark the pedigrees of the carrier animals with the code “CV” and those that are tested free with the code “TV”.

The genealogical analysis of the pedigrees of the affected calves allowed tracing the origin of the both disease-causing alleles back to common ancestors, an elite Holstein bulls *Carlin-M Ivanhoe Bell* (USA1667366 born in 1974) and his father *Pennstate Ivanhoe Star* (USA1441440 born in 1963) who have been used for A.I. in many countries throughout the world. Several molecular methods, including PCR-RFLP (9, 39, 41, 43), PCR-PIRA (19), AS-PCR (7, 14), CRS-PCR (45) and SSP (10, 36) have been developed and proved useful for identification of BLAD or CVM carriers in practice. More recently Zhang et al. (47) developed real-time PCR-based assay for discrimination of wild-type

and defective alleles which could be more useful for high-throughput sample testing.

The purpose of this study was to optimize and implement a reliable and cost-effective laboratory test for detection of these recessive alleles, as well as to obtain a preliminary results about their existence in the Macedonian HF population.

MATERIALS AND METHODS

Blood samples were obtained from 84 cattle of the HF breed raised on several different farms in Macedonia. Genomic DNA was extracted using DNeasy blood and tissue kit (Qiagen). This kit was also used for extraction of DNA from deeply frozen sperm doses from 6 different HF bulls that are used in Macedonia for A.I. Before extraction sperm samples (100 µl) were lysed with 2xSDS-Lysis buffer and incubated at 56 °C for 3-4 hours. The target sequences harboring the recessive mutations were amplified by PCR.

For BLAD allele amplification we used primers published previously (21) with the forward primer slightly modified since after checking the sequence for the bovine CD18 gene (GenBank Accession Number Y12672) we noticed that this primer lacks one C nucleotide. The amplifications were carried out in 25 µl reactions containing 1xPCR buffer (Biotools), 200 µM dNTP, 2.0 mM MgCl₂, 0.6 U DNA Polymerase (Biotools), 0.2 µM of BLAD-F and the same concentration of BLAD-R and 50-100 ng genomic DNA under following conditions: 95°C/3 min., 35 cycles of 94°C/1 min, 58°C/1 min, 72°C/1 min and final elongation 72°C/5 min. Amplified products were checked on 2% agarose gel. Identification of BLAD carriers was performed by digestion of the amplified products with *TaqI* and *HaeIII* restriction endonucleases and visualized on 2% agarose gel for *TaqI* or 3.5% agarose gel for *HaeIII* digestion.

For CVM genotyping we used primers previously published by Kanae et al. (19) in total volume of 25 ul reactions containing 1xPCR buffer (Biotools), 200 µM dNTP, 2.0 mM MgCl₂, 0.6 U DNA Polymerase (Biotools), 0.2 µM of CVM-PIRA-F and the same concentration of CVM-PIRA-R and 50-100 ng genomic DNA under following conditions: 94°C/5 min., 35 cycles of 94°C/1 min, 54°C/1 min, 72°C/35 sec. and final elongation 72°C/7 min. Identification of the CVM genotypes was performed by digestion of the amplified product with *PstI* endonuclease. The correctness of the genotyping for both disorders was evaluated by direct sequencing on ABI Prism 310 Genetic Analyzer (Applied Biosystems).

Table 1. Primer sequences, position in the reference sequence and the expected product sizes

Genetic defect	Primer sequence 5' to 3'	Position*	Expected product size*
BLAD	BLAD-F: CCTGCATCATATCCACCAG	1007 - 1025	341 bp
	BLAD-R: GTTTCAGGGGAAGATGGAG	1329 - 1347	
CVM	CVM-PIRA-F: CACAATTTGTAGGTCTCACTGCA	9848 - 9870	287 bp
	CVM-PIRA-R: CGATGAAAAAGGAACCAAAAGGG	10112 - 10134	
	SLC-F: GGTCTAAGAGTGGGCTCTAAAC	9722 - 9743	231 bp
	SLC-R: TCCACACTGATTGTTTGGTTTC	9931 - 9952	

* Annealing positions and expected product sizes were determined on basis of previously reported sequences of the CD18 gene for BLAD (GenBank Accession Number Y12672) and SLC35A3 gene for CVM (GenBank Accession Number AY160683)

For BLAD sequencing we used the same forward primer from the PCR-RFLP analysis, while for CVM sequencing we used either SLC-F or SLC-R that we designed with the Primer Premier software. All primers, annealing positions as well as the expected fragment sizes are listed in Table 1.

RESULTS

The results from the PCR - RFLP analysis for the detection of the BLAD allele carriers are shown in

Fig. 1 and Fig. 2. Digestion of the amplified product of 341 bp with *TaqI* yielded three fragments of 341, 191 and 150 bp when carrier DNA was cleaved and two fragments of 191 and 150 bp when non-carrier DNA was cleaved (Fig. 1). Digestion of carrier DNA with *HaeIII* resulted in six fragments of which the 83 bp one (arrow) was specific for this genotype (Fig. 2).

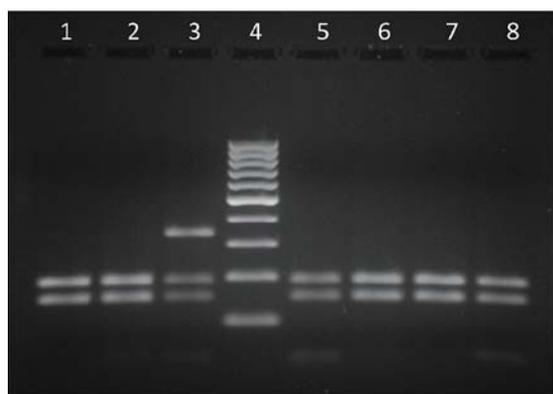


Figure 1. Lanes 1, 2, 5, 6, 7, 8 - BLAD free; lane 3 - BLAD carrier; lane 4 - GeneRuler 100 bp DNA Ladder (Thermo Scientific)

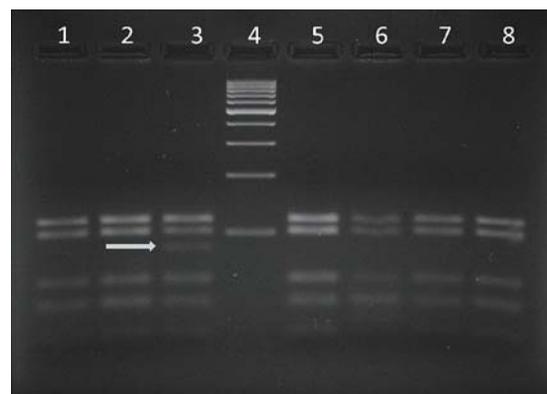


Figure 2. Lanes 1, 2, 5, 6, 7, 8 - BLAD free; lane 3 - BLAD carrier; lane 4 - GeneRuler 100 bp DNA Ladder (Thermo Scientific)

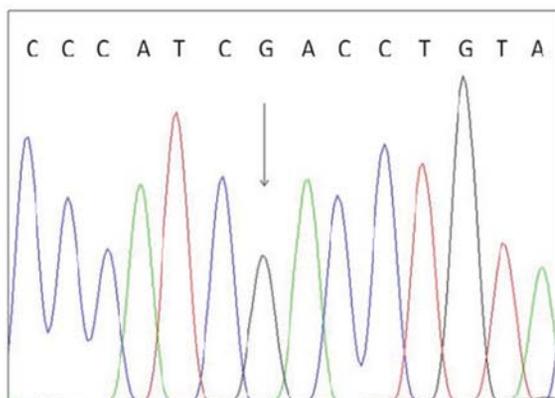


Figure 3. Electropherogram of BLAD free animal. The heterozygous position is indicated by an arrow.

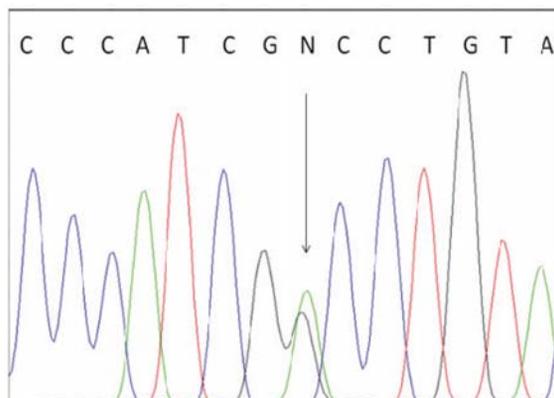


Figure 4. Electropherogram of BLAD carrier. The heterozygous position is indicated by an arrow.

To confirm the correctness of these results we performed direct sequencing of one BLAD-free and two BLAD-carrier PCR products. The results are shown in Fig. 3 and Fig. 4.

CVM carriers were detected by PCR-PIRA followed by digestion with *Pst*I restriction

endonuclease and confirmed by direct sequencing of the heterozygous region. The amplification product size obtained with CVM-PIRA-F and CVM-PIRA-R primers is shown in Fig. 5. The restriction pattern for discriminating between non-carrier and heterozygous carrier animals is shown in Fig. 6.

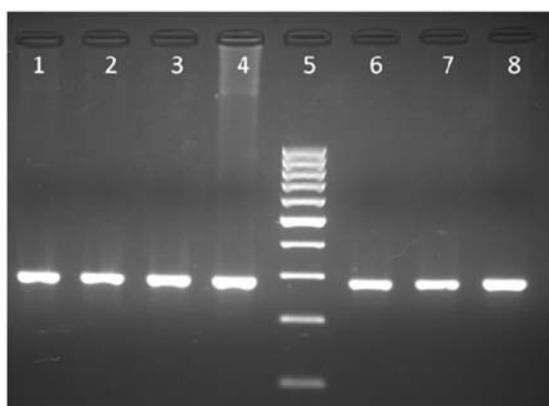


Figure 5. Lanes 1, 2, 3, 4, 6, 7, 8 - PCR product of 287 bp obtained with CVM-PIRA-F and CVM-PIRA-R primers; lane 5 - GeneRuler 100 bp DNA Ladder (Thermo Scientific)

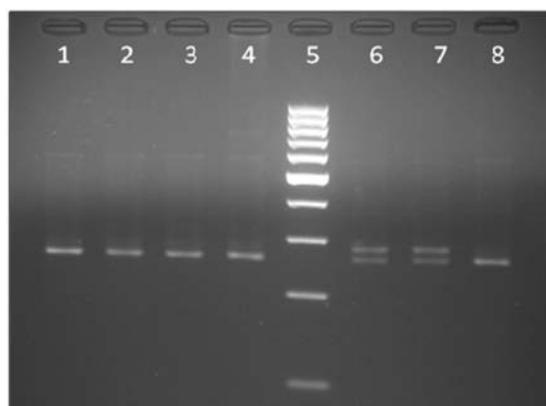


Figure 6. Lanes 1, 2, 3, 4, 8 - CVM free; lanes 6 and 7 - CVM carriers; lane 5 - GeneRuler 100 bp DNA Ladder (Thermo Scientific)

These results were confirmed by direct sequencing of the PCR product with one of the newly designed primers either SLC-F or SLC-R (Table 1). The results of the sequence analysis are shown in Fig.7 and Fig. 8.

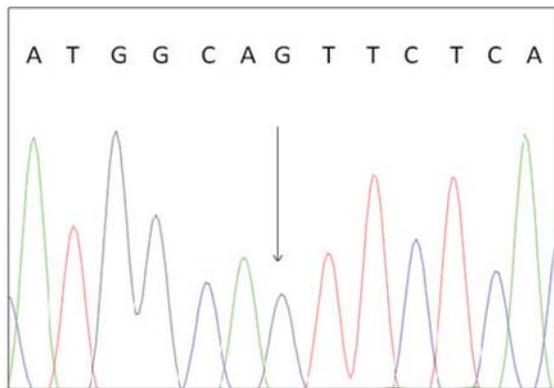


Figure 7. Electropherogram of CVM free animal. The heterozygous position is indicated by an arrow.

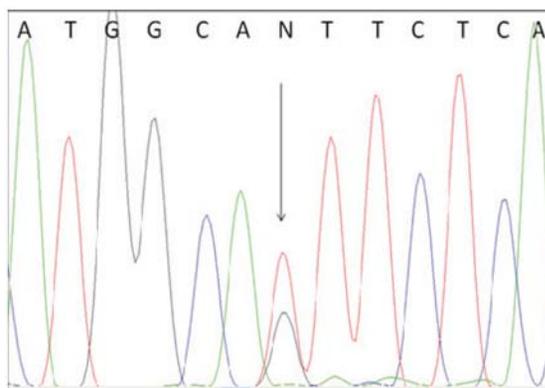


Figure 8. Electropherogram of CVM carrier. Heterozygous position is indicated by an arrow.

DISCUSSION

PCR followed by RFLP showed to be a reliable method for detection of these two single nucleotide polymorphisms since digestibility by restriction endonucleases strictly depends on the nucleotide sequence of the amplified product. Since *TaqI* cleaves only the wild type allele while the mutation destroys the recognition site, the restriction pattern of the amplified DNA product of 341 bp for detection of CD18 genotype with this enzyme revealed two fragments of 191 and 150 bp when non-carrier DNA was cleaved and three fragments of 341, 191 and 150 bp when carrier DNA was cleaved. Affected animals would show only one undigested fragment of 341 bp, but were not found in this study. Although our findings revealed slightly different fragment sizes than the ones previously reported with the same primer pair (5, 21, 31), the restriction pattern for identifying carrier animals after digestion of the amplified product with *TaqI* is in accordance with these authors.

Detection of the BLAD carriers with the *HaeIII* enzyme was done in the following manner: the fragment amplified from a non-carrier animals contained four recognition sites for this enzyme, thus this genotype showed five fragments of 25, 39, 54, 102 and 121 bp; since the point mutation creates another recognition site affected animals,

although not found in this study, would yield six different fragments of 19, 25, 39, 54, 83 and 121 bp. Following this, heterozygous genotypes showed six fragments (19 bp fragment was not visible) of which the 83 bp fragment (arrow) was specific for

this genotype. Thus, the restriction fragment pattern obtained with *HaeIII* in this study is in accordance with previously published reports (23, 35, 41) in which this enzyme was used to detect heterozygous BLAD carriers.

To confirm the results of the PCR-RFLP analysis we performed direct sequencing of one BLAD-free and two BLAD-carrier PCR products. The electropherogram of a wild type homozygous animal indicated only nucleotide A at the position 383 of the bovine CD18 gene, while the carriers of the mutant allele revealed presence of two nucleotides (A and G) at the same position.

CVM carriers were detected by PCR-PIRA (Primer Introduced Restriction Analysis), a method initially described by Kanae et al. (19) and confirmed by direct sequencing of the heterozygous region. Since the G559T mutation of the SLC35A3 gene neither creates nor destroys recognition site for any restriction enzyme, the forward primer is designed to have two mismatched nucleotides in order to create, in the wild-type allele, a recognition sequence for the *PstI* enzyme while the mutant allele remains undigested. But unlike Kanae et al. (19) who reported amplification product of 233 bp, our results showed a longer PCR product which, after comparing with the sequence submitted by Thomsen et al. (44) with GenBank Accession Number AY160683, we calculated to be 287 bp long. Since

the mismatched forward primer creates a recognition site for *PstI* only in the wild-type allele, non-carriers yielded two fragments of 264 and 23 bp of which the latter was not visible on 2.5% agarose gel and heterozygous carriers showed two fragments of 287 and 264 bp (Fig. 6). Affected animals would show one fragment of 287 bp, but were not found in this study.

The electropherogram of the non-carriers showed only nucleotide G at the position 559 of the SLC35A3 gene, while heterozygous carriers revealed presence of two nucleotides (G and T) at the same position.

CONCLUSION

The detection method based on PCR - RFLP analysis is a powerful tool for identification of both BLAD and CVM carriers. The primers that we used for identification of the BLAD genotype successfully amplify a region of 341 bp and digestion with either *TaqI* or *HaeIII* enzymes makes possible easily to distinguish DNA samples of non-carriers from heterozygote carrier animals. Detection of CVM carriers can easily be accomplished with PCR-PIRA amplification with mismatched forward primer followed by RFLP analysis with *PstI* endonuclease.

This is the first published report on existence of BLAD and CVM carriers in the HF cattle population in Republic of Macedonia. Although they were found at low frequency, this study demonstrates a need for further examination of more animals in this population in order to determine more accurately the extent of dispersion of these deleterious alleles, but also it emphasizes the need for obligatory testing of each candidate bull before entering the A.I. program to prevent their accidental spread to a large number of offspring.

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