Rapid and Sensitive Detection of BLAD in Cattle Population

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Abstract
Bovine leukocyte adhesion deficiency (BLAD) is an autosomal recessive disorder with negative impact on dairy cattle breeding. The molecular basis of BLAD is a single point mutation (A→G), resulting in a single amino acid substitution (aspartic acid → glycine) at amino acid 128 in the adhesion molecule CD18. The object of this study was to establish a fast and sensitive molecular genotyping assay to detect BLAD carriers using high-resolution melting (HRM) curve analysis. We tested animals with known genotypes for BLAD that were previously confirmed by PCR-RFLP method, and then examined the sensitivity of mutation detection using PCR followed by HRM curve analysis. BLAD carriers were readily detectable using HRM assay. Thus, the PCR-HRM genotyping method is a rapid, easily interpretable, reliable and cost-effective assay for BLAD mutant allele detection. This assay can be useful in cattle genotyping and genetic selection.

Keywords: BLAD, CD18, genotyping, high-resolution melting.

1. Introduction

Bovine leukocyte adhesion deficiency (BLAD) is a lethal autosomal recessive disease that affects the Holstein cattle breed and is characterized by greatly reduced expression of the heterodimeric β_2 integrin adhesion molecules on leukocytes, resulting in multiple defects in leukocyte function [1-3]. The β_2 integrin subfamily consists of four distinct α chains (CD11a, CD11b, CD11c, and CD11d) and a common β chain (CD18) [4]. Since integrin expression requires intracellular association of both CD11 and CD18 subunits, CD18 defects prevent all integrin functions [5].
The impaired leukocyte adherence causes deficient mucosal immunity and BLAD-affected animals have severe and recurrent mucosal infections including gingivitis, periodontitis, pneumonia, loss of teeth, papillomatosis, dermatophytosis, delayed wound healing, stunted growth as well as death before sexual maturity [1, 6, 7]. The body weight of affected cows may be 50-60% of the expected weight. The diseased animals die either before reaching one year of age or survive for more than two years. However, their reproduction and milk performances are poor.
The molecular basis of BLAD is a single point mutation (A→G), at nucleotide 383, resulting in a single amino acid change at aminoacid 128 in the β subunit (CD18) of the β_2 integrin [4]. This mutation removes a *Taq*I restriction site and creates a *Hae*III site, which allows the identification of normal, carrier and affected animals [8]. An additional silent mutation of CD18 gene involving replacement of cytosine to thymine (C→T) at nucleotide 775, known as SNP775 (C→T), was also noted [1].
The risk of BLAD prevalence has been reduced, and, currently, carriers among Holstein population are less frequently reported [9-12].
Nevertheless, BLAD is still a prevalent disease in cattle, with significant economic effect. Therefore,

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genetic screening is of utmost importance in order to obtain disease-free animals and to eliminate the mutant allele from the population [13].

The diagnosis of BLAD is usually based on the genotyping of animals using PCR-RFLP assay. The new approach reported here is based on a high-resolution melting (HRM) analysis. The HRM analysis is a closed-tube system and a cost-effective and simple post-PCR assay that allows analysis of genetic mutations or variance without the need of separation steps.

High-resolution genotyping by amplicon melting curve analysis requires unlabeled primers and a double-stranded DNA-binding dye and does not require the use of fluorescent-labeled probes. This method is based on the properties of double-stranded DNA dissociation (melting), which is subsequently examined by melting curve analysis. Different melting profiles are obtained from the transition of double-stranded DNA to single-stranded DNA due to a gradual increase in temperature after the PCR procedure [14].

HRM is considered the simplest method for genotyping and mutation detection because it is done in the same tube and immediately after the PCR procedure [15].

The aim of the present study was to establish a rapid and sensitive assay for genotyping the point mutation of CD18 gene using HRM analysis.

2. Materials and methods

Sampling and DNA extraction

Samples were collected from a total of 50 cattle from different farms located in Timiş, Arad and Bihor counties. Genomic DNA was extracted from blood samples using the Wizard Genomic DNA Purification Kit (Promega, Madison, USA), according to manufacturers' instructions. The DNA concentration was assessed spectro-photometrically, with NanoDrop-2000 (Thermo Fisher Scientific Inc., USA).

PCR and HRM assays

Initially, DNA samples were processed for BLAD screening using PCR-RFLP technique. For the PCR-RFLP analysis, a 357 bp fragment was amplified. The sequence of primers has been published elsewhere [12]. The amplicons were then digested for two hours at 65°C with the *TaqI* restriction enzyme. The digested DNA fragments were visualized on a 3% agarose gel and stained with ethidium bromide. Images of the gels were taken using Vilber Loumart Print II Systems.

For the PCR-HRM analysis we used a set of primers [16] that amplified a shorter fragment of 134 bp. The PCR-HRM assay was performed on a LightCycler Nano Real-Time PCR System (Roche Diagnostics GmbH, Mannheim, Germany), in 8-tube strips, in 20 µl volume reactions containing 20 ng of genomic DNA template, 5 pmoles of each primer, 2.5 mM of $MgCl_2$ and 1x LightCycler 480 High Resolution Melting Master (Roche Diagnostics GmbH, Mannheim, Germany) consisting of FastStart Taq DNA Polymerase, dNTP mix, reaction buffer and high resolution melting dye. The amplification conditions included an initial denaturation at 95°C for 10 min, followed by 45 cycles of denaturation at 95°C for 10 s, annealing at 62°C for 25 s and extension at 72°C for 15 s. After amplification, the melting analysis was immediately performed. Samples were denatured at 95°C for 1 minute and cooled to 40°C for 1 minute to allow heteroduplex formation. The final HRM step was performed from 65°C to 95°C with 0.05°C increase/ second.

LightCycler Nano Software (version 1.0; Roche) was used for the analysis of melting curves. Data was analyzed by automated quantification, Tm calling (melt temperature analysis) and HRM (high resolution melt analysis and genotyping). For sample analysis, melting curves were normalized, temperature adjusted and, finally, difference plots (curves) were generated, then automatically clustered into separate groups according to their melting profiles.

The melting curves data was normalized by manual adjustment of linear regions before and after the major fluorescence decrease representing the melting of the amplicons. This algorithm allows the direct comparison of the samples that have different starting fluorescence levels [17].

One control (i.e., DNA from the normal homozygous animal) was used to normalize melting profiles of the other samples against this pre-defined horizontal baseline.

3. Results and discussion

We applied a method based on post-PCR melting curve analysis under high-resolution conditions for discrimination of normal and mutant alleles of BLAD. The point mutation located in CD18 gene

affect the melting behavior of PCR products and therefore generates different melting curves for BLAD carriers (heterozygous) allowing discrimination and identification of BLAD genotypes by HRM analysis.

As described previously by others [18,19] HRM scanning accuracy depends on amplicon length, PCR optimization and high quality PCR product. Presence of non-specific products or primer dimers may change the melt curve characteristics, thus generating false positive results. Therefore, HRM analysis optimization was previously achieved by using different concentrations of Mg^{+2} and also a positive and a negative control (DNA from wild type animals and BLAD carriers). The optimum Mg^{+2} concentration was 2.5mM, which significantly enhanced the amplification.

The point mutation located in CD18 gene detected previously by PCR-RFLP was clearly detected by HRM with LightCycler Nano Real-Time PCR System. To achieve the best results for BLAD genotyping we determined the quantity and quality of DNA template by spectrophotometry.

The results of the high-resolution melting analyses of CD18 gene are shown in Figures 1 and 2, respectively. Figure 1 shows the normalized, temperature-shifted melting curves produced by the HRM analysis.

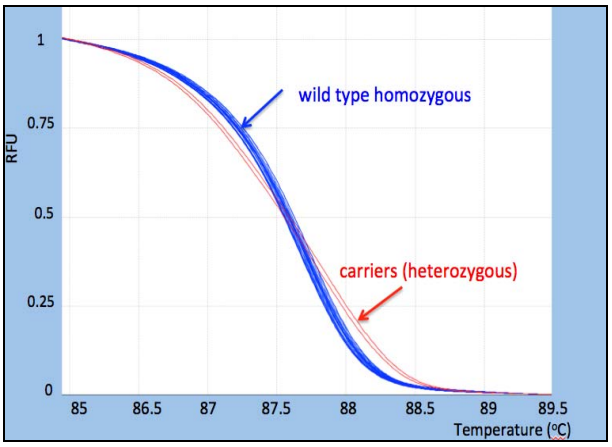

Figure 1. Normalized HRM plots related to the CD18 amplicons

To validate the assay we used genomic DNA samples of known genotypes for BLAD. The difference curves in the HRM assay shown in Figure 2 clearly separate samples according to their genotype, which are easily distinguished based on shape of the melting curves.

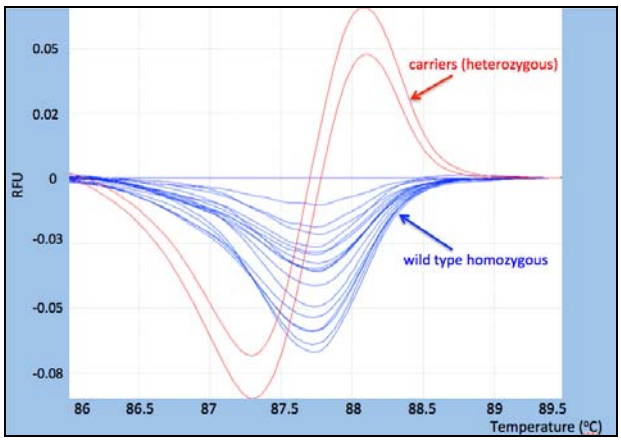

Figure 2. Difference curve of HRM analyses of the CD18 amplicons

We only genotyped variants of CD18 occurring in the investigated population and shown that HRM is a closed tube, homogenous genotyping assay, applicable for fast detection of BLAD in cattle.

HRM analysis allows clear discrimination between homozygous (wild type) and heterozygous (BLAD carriers). When HRM genotyping accuracy was compared to PCR-RFLP assay no discordant results were observed.

Our result showed that the commonly used PCR–RFLP technique was laborious and time-consuming comparing with PCR-HRM where we obtained the results faster and with less labor.

4. Conclusions

In our study we intended to improve diagnostic tools for BLAD detection by using a high-throughput genotyping technology named high-resolution melting analysis, and applied this assay to analyzing CD18 gene in cattle.

HRM is a closed tube system that increases sample throughput because there is no requirement for separation of DNA molecules after PCR. Moreover, HRM offers significant savings in cost and time when compared with other genotyping methods.

Our results show that the HRM technique is a rapid, easily interpretable, low-cost and highly efficient assay for detection of genomic variants in CD18 gene allowing genotyping of bovine for BLAD.

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