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HIGH- RESOLUTION MELTING (HRM) CURVE ANALYSIS: NEW APPROACH USED TO DETECT BLAD AND DUMPS IN URUGUAYAN HOLSTEIN BREED

(*Análise da curva de alta resolucao (HRM): nova abordagem usada para detectar BLAD e DUMPS em bovinos da raça Holandesa Uruguaia*)

María Teresa Federici[1]*, Andrea Branda Sica[1]*, Rody Artigas[2], Carolina Briano[3], Fernando Dutra[3], Silvia Llambí[2]

**in similar contribution*

[1]Instituto Nacional de Investigación Agropecuaria (INIA). INIA Las Brujas. Unidad de Biotecnología Ruta 48 Km 10. 90.200-Rincón del Colorado, Canelones, Uruguay; [2]Universidad de La República. Facultad de Veterinaria. Sección Genética. Alberto Lasplaces 1550/1620 - 11600-Montevideo, Uruguay; [3]División Laboratorios Veterinarios. Ministerio de Ganadería, Agricultura y Pesca. Avelino Miranda 2045. Treinta y Tres, Lavalleja, Uruguay.

[1]Corresponding author: maritefe@gmail.com

ABSTRACT: The widespread use of artificial insemination has allowed the expansion of genetic progress. However, it also brought consequences such as the expansion of lethal hereditary diseases and the increase in inbreeding. The object of this study was to establish a fast and sensitive molecular assay to detect bovine leukocyte adhesion deficiency (BLAD) and deficiency of uridine monophosphate synthase (DUMPS) carriers in Uruguayan Holstein cattle by means of high resolution melting (HRM) curve analysis. By testing previously confirmed carrier and non-carrier animals, we set up a rapid, simple, and inexpensive diagnostic test using PCR followed by HRM curve analysis. The PCR-HRM genotyping method was effective for the discrimination of BLAD and DUMPS homozygous genotypes, and the BLAD heterozygous genotype. We conclude that the PCR-HRM assay is a robust, reliable, and economical tool for the detection of these mutations in the Holstein breed, which may be implemented in genetic selection programs.

Keywords: BLAD; DUMPS; heifer; Holstein; high-resolution dissociation curve.

RESUMO: O uso generalizado de inseminação artificial, permitiu expandir o progresso genético, no entanto, também trouxe consequências na expansão de doenças hereditárias letais e aumento da endogamia. O objetivo deste estudo foi estabelecer um ensaio molecular rápido e sensível para detectar animais portadores da deficiência de adesão leucocitária bovina (BLAD) e da deficiência da uridina monofosfato sintetase (DUMPS) usando a análise de curva de alta resolução (HRM) em bovinos da raça Holandesa Uruguaia. Através do uso de animais portadores e não portadores previamente confirmados, montamos um teste de diagnóstico rápido, simples e de baixo custo usando PCR seguido de análise de curva HRM. O método de genotipagem da PCR-HRM foi efetivo para a discriminação dos genótipos homozigotos BLAD e DUMPS e do genótipo heterozigoto BLAD. Concluímos que o ensaio PCR-HRM é uma ferramenta poderosa, confiável e econômica para a detecção destas mutações na raça Holandesa, o que poderia ser implementado em programas de seleção genética.

Palavras-chave: BLAD; DUMPS; novilha; Holandesa; curvas de dissociação de alta resolução.

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INTRODUCTION

The widespread use of artificial insemination has allowed the expansion of genetic progress. However, it also led to consequences such as the expansion of lethal hereditary diseases, and the increase in inbreeding (Kelly et al., 2012). In animal breeding, genetic disorders in cattle are one of the most critical issues, and their impact on the populations needs to be controlled. Known inherited disorders in cattle are mostly caused by autosomal recessively inherited genes. The characteristic feature of autosomal recessive genes is that they are expressed as a diseased phenotype only if both alleles are present. Therefore, unrecognized dissemination of such defective genes is possible, and autosomal recessively inherited disorders are of greater concern in cattle breeding than disorders with dominant inheritance as the latter are easily recognized (Angerholm et al., 2007; Meydan et al., 2010). Bovine leukocyte adhesion deficiency (BLAD) (OMIA 000595-9913) is a lethal autosomal recessive disease that affects the Holstein cattle caused by a single point mutation (A/G) at nucleotide 383 of CD18 gene of the chromosome 1 (Angerholm et al., 2007, OMIA 000595-9913); this results in a single amino acid change at aminoacid 128 in the B subunit (CD18) of the (β) integrin (Radi et al., 2001) that leads to a greatly reduced expression of heterodimeric (β2) integrin adhesion molecules on leukocytes. This mutation, that causes inadequate mucosal immunity and death before one year of age due to severe and recurrent mucosal infections, has been detected in many countries such as Germany, Denmark, Netherlands, Austria, United Kingdom, Japan, Switzerland, France, Taiwan, Poland, Brazil, Korea, Iran, Turkey, India (Adamov et al., 2014), and Uruguay (Kelly et al., 2010, Branda Sica et al., 2016, Branda Sica et al., 2018). The deficiency of uridine monophosphate synthase (DUMPS) (OMIA 000595-9913) is another recessive genetic disorder of Holstein cattle characterized by decreased blood activity of the uridine monophosphate synthase (UMPS) enzyme, which leads to embryonic death during the first two months of gestation, more services per calving, and longer than normal calving intervals are required. DUMPS is caused by an autosomal-recessive mutation in the UMPS gene (C/T), which is necessary for de novo synthesis of pyrimidine nucleotides, constituents of DNA and RNA. No carrier animals were found in Poland, Iran, India, and Turkey (Kumar et al., 2010); however, the mutant allele was detected in other studies carried out in the USA, Europe, Argentine and Taiwan (Kumar et al., 2010, Patel et al., 2006)

The development of molecular genetics has enabled efficient and rapid identification of heterozygous animals by genomic analysis. In Uruguayan Holstein cattle, the BLAD allele was previously reported using PCR- RFLP and amplicon sequencing (Llambí et al., 2003; Kelly et al., 2010; Branda Sica et al., 2016). Afterwards, with the advances in molecular genomics, genetic diseases tests were included on microarray platforms like the *GGP-LD 26K Dairy Cattle* panel (*GeneSeek*, California, USA). This matrix was also used to screen for BLAD and DUMPS alleles validating BLAD previous detection while no DUMPS-carrier animals were found (Branda Sica et al., 2018).

PCR- HRM is a novel screening technique for detection of SNP mutation based on PCR melting behavior coupled with melting curve analysis. Different melting profiles were obtained from the double-stranded to single-stranded DNA transition through a gradual increase in temperature after the PCR procedure

(Ilie et al., 2013). This technique was introduced by Wittwer et al. (2009) to detect both known (Hung et al., 2008) and unknown gene mutations (Rapado et al., 2009). PCR followed by high resolution melting analysis (HRM or HRMA) has been proved to be an efficient method to detect genetic diseases of Holstein cattle, like BLAD in Romania (Ilie et al., 2014) and the complex vertebral malformation (CVM) in Slovakia (Gabór et al., 2012a). However, it has not been reported for detection of DUMPS.

The objective of this study was to optimize the PCR-HRM technique for the screening of both BLAD and DUMPS alleles in Uruguayan Holstein cattle, comparing its advantages and disadvantages with those of other genotyping platforms: PCR-RFLP, amplicon sequencing and low-density microarray panels.

MATERIAL AND METHODS

Sampling and DNA extraction

The samples used in this study were collected from 181 heifers of a representative population of the southeast region of Uruguay. Genomic DNA was extracted from blood samples at the Genomic DNA Bank located in the Unit of Biotechnology of INIA Las Brujas following a modified protocol of Green and Sambroock (2012). DNA concentration was assessed with the NanoDrop® ND-1000 spectrophotometer.

Control samples of this population were previously genotyped through PCR-RFLP and amplicon sequencing for BLAD detection, and with the *GGP-LD 26K Dairy Cattle* panel to screen both BLAD and DUMPS mutant alleles (Branda Sica et al., 2016, Branda Sica et al., 2018). Samples with both wild-type and recessive homozygous genotypes, as well as samples of BLAD carrier animals, were used to optimize PCR-HRM conditions. DUMPS and BLAD homozygous recessive controls were provided by the Laboratory of Genetics (Veterinary Faculty, UDELAR).

To identify both CD18 D128G and UMPS mutant alleles we performed HRM analysis in *Rotor-Gene Q* (Corbett) in a mix using EvaGreen fluorescent dye after the PCR reaction and the *Type-it*® *HRM PCR* kit (QIAGEN, Hilden, Germany), with 50 ng of DNA and 0.7 µM of BLAD (Mirk et al, 1995) or DUMPS (Shwenger et al, 1993) primers, respectively. PCR reaction was performed in a final 25 µl volume, and the cycling program consisted in denaturation at 95°C for 5 min, 40 cycles of denaturation at 95°C, annealing at 55°C for 30 s, and extension at 72°C for 10 s, followed by a final extension of 10 min at 72°C. Annealing temperature was adjusted at 55°C for both BLAD and DUMPS PCR reactions. HRM and melting analysis were performed in *Rotor-Gene* version 2.1.0 software immediately after amplification by increasing the temperature from 65 to 95°C in 0.1°C during 2s. After that, curves were normalized, and different plots were generated for the samples, which were automatically assigned to the corresponding genotype according to their curve profile. Melting analysis was also performed using the mentioned above software to confirm PCR amplification.

RESULTS AND DISCUSSION

We optimized and applied PCR-HRM for identification of BLAD and DUMPS carrier animals in Uruguayan Holstein cattle. This technique resulted in a very efficient and sensitive tool to detect BLAD heterozygous carriers from homozygous control genotypes (Figure 1). It also resulted very efficient to discriminate the DUMPS recessive genotype control sample from wild-type homozygous genotypes (Figures 2 and 3). HRM is considered the most straightforward method for genotyping

and mutation detection, as it is done in the same tube and immediately after PCR procedure (Montgomery et al., 2004; Ilie et al., 2014). The procedure is performed in a closed-tube system that uses minimal labor, has a rapid turnaround time and requires only a PCR reaction, a DNA dye, and melting instrumentation (Witter et al., 2009).

HRM scanning accuracy depends on amplicon length, PCR optimization, and a high-quality PCR product (Ilie et al., 2013; Santos et al., 2012; Ilie et al., 2014). PCR reaction was previously optimized using different amounts of DNA. Finally, we used 50 ng of high-quality DNA determined by spectrometry. The A260/ A280 value for all the samples used in this study was between 1.7 and 2.0 to ensure reliable genotyping results. The dependence of scanning accuracy on PCR length has been studied on the HR-1 (Reed and Witter, 2004) and Light Scanner® instruments (McKinney et al., 2009), revealing more errors as the length increases above 400 bp (Witter, 2009). The small fragments of PCR product (300 bp or less) were correctly assigned without error, while the sensitivity and specificity for PCR product sizes between 400 and 1000 bp with the mutation near the center was 96.1% and 99.4%, respectively (Reed and Witter, 2004; Taweechue et al., 2015). Krenkova et al. (2009) also reported similar sensitivity and specificity for *wild-type* and mutant analysis. In our study, we amplified PCR products smaller than 400 bp, suitable for HRM analysis, using real-time PCR in Rotor-Gene 6000 (Corbett Research). PCR products have a length of 159 and 108 bp, corresponding to CD18 and UMPS genes, respectively. We used the same primers previously employed for PCR-RFLP and amplicon sequencing (Branda Sica et al., 2016).

To detect BLAD mutant alleles, we performed a normalization of the fluorescence values using Rotor- Gene version 2.1.0 software which assigned the corresponding genotype to each sample calculating a confidence value (%). The confidence value was above 95% for all the analyzed samples. As temperature increased, the double strand DNA was unwound and fluorescence was released. Normalization allows the elimination of those differences due to the fluorescence background, increasing the software capacity for detection of small differences in the melting profile. Through previously performed melting analysis, we detected one peak for the PCR product of 159 bp at the corresponding melting temperature and defined a baseline to avoid unspecific amplification. The stability of DNA duplexes was monitored by the fluorescence as the solution temperature was increased. In Figure 1 we can appreciate the normalized, temperature-shifted melting curves produced in the HRM analysis corresponding to the heterozygous genotype (BLAD-carrier) and homozygous genotypes (wild-type and the BLAD-recessive, respectively). We could discriminate the homozygous GG (wild-type), homozygous AA (homozygous recessive), and the heterozygous AG (BLAD-carrier) genotypes as different plot patterns of the high resolution normalized melting curves. The heterozygous genotype is easily distinguished as it follows a different curve pattern because heteroduplexes alter the shape of the melting curves.

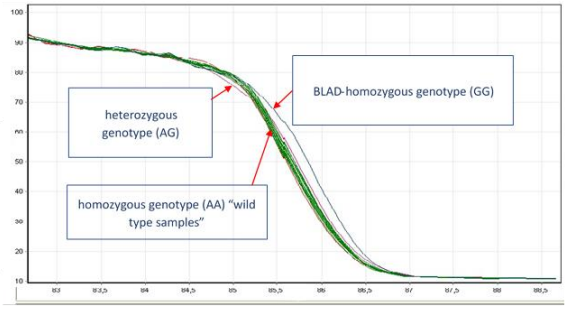

Figure 1- Normalized melting curves of CD18 gene amplicons. X: Temperature (°C), Y: Normalized fluorescence.

In our study, the DUMPS mutation (C/T) in the UMPS gene was also easily detected using PCR-HRM. The 108 bp product surrounding the mutation was amplified from genomic DNA, and fluorescence lectures were normalized with *Rotor-Gene* version 2.1.0 software to identify the melting curves and assign the corresponding genotype (Figure 2). To adjust PCR-HRM analysis, we compared the wild-type homozygous control samples genotyped in Branda Sica et al., 2016 with the DUMPS recessive genotype sample, provided by the Genetics Laboratory of the Veterinary Faculty (UDELAR). In Figure 2 we can appreciate the normalized, temperature-shifted melting curves produced in the HRM analysis, that correspond to the recessive homozygous and the "wild-type" genotype control samples. Using the melting analysis, we previously detected the peak corresponding to the PCR amplicon of 108 bp and defined a baseline to prevent unspecific amplification. When the fluorescence of samples was normalized against the control sample, the software classified most of the samples as "wild-type" with more than 95% of confidence; however, other samples were classified as "variation". These melting curves were visually compared with the curve of the control sample and manually assigned to the corresponding genotype. The two types of homozygous sample patterns were very different between each other, so they could be easily differentiated. If available, DUMPS carrier heterozygous genotype would be placed between the curves of both homozygous genotypes, following a different pattern as we observed for the BLAD heterozygous genotype in Figure 1.

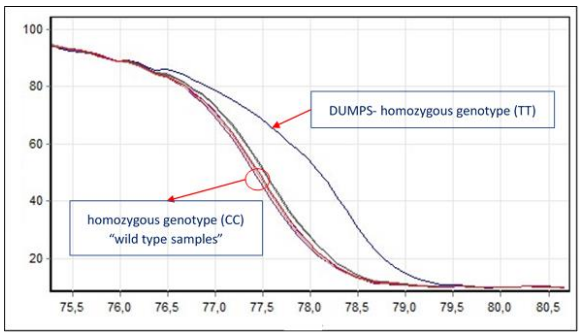

Figure 2- Normalized melting curves of UMPS gene amplicons. X: Temperature (°C), Y: Normalized Fluorescence.

For better visualization, we also performed the negative derivative of the fluorescence to temperature (-dF/dT) dissociation curve. In this graph, wild-type homozygous genotypes (CC) were clearly differentiated from the homozygous recessive control genotype (TT) (Figure 3).

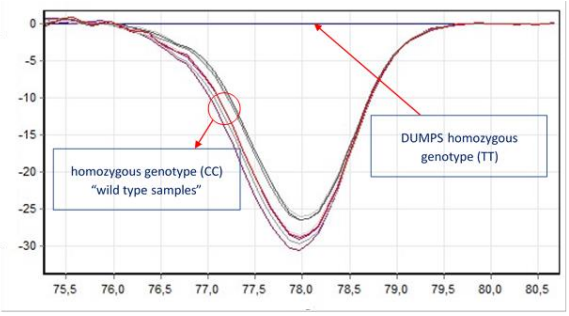

Figure 3- Different plots of genotypes fluorescence normalized to DUMPS-homozygous genotype. X: Temperature (°C), Y: Relative signal difference (Δfluorescence).

We have evaluated the sensitivity of the PCR-HRM technique for the detection of CD18 and UMPS mutated alleles in Uruguayan Holstein cattle. Normalized high-resolution melting curves and negative derivative of fluorescence to temperature (-dF/dT) dissociation curves were used for the

screening of BLAD-carriers by comparing against both control samples: BLAD-carrier heterozygous genotype and BLAD homozygous genotype. Similarly, DUMPS-carriers were screened with PCR-HRM using both homozygous genotypes as control samples. Therefore, once adjusted, PCR-HRM allowed the identification of BLAD and DUMPS-carrier animals by comparing them against wild-type genotype control samples. However, if available, it would be advisable to include in the analysis the BLAD or DUMPS-heterozygous carriers, as well as the recessive homozygous genotypes, for a better visualization and and discrimination of genotypes.

HRM is a simple post-PCR procedure, performed immediately after the PCR reaction, which allows the detection of mutations and genetic variability without the need of separation steps, preventing contamination of PCR products and increasing the sensitivity of the assay (Montgomery et al., 2004). The main significant advantages of PCR-HRM, when compared to conventional mutation screening techniques such as SSCP, DGGE, DHPLC or pyrosequencing, are the short time of analysis (approximately 1 hour), and the detection of individual genotypes in the same tube that was used for PCR amplification (White et al., 2007; Norambuena et al., 2009; Polákova et al., 2008). The disadvantage is the sensitivity to residues from different DNA extraction methods, which can provide a false positive signal. For that reason, it is recommended to use the same DNA extraction method for all the samples of one HRM reaction (Gabór et al., 2012b). PCR-HRM requires DNA of high quality and homogeneous among the compared samples. In our study, all DNA samples, except for homozygous recessive control samples, have been extracted using the same protocol, thus allowing an easy differentiation of genotypes. Compared with the previously applied PCR-RFLP genotyping platforms, amplicon sequencing, and *GGP-LD 26K Dairy Cattle* panel (*GeneSeek,* California, USA), PCR-HRM has great advantages for the screening of BLAD and DUMPS mutated alleles. PCR-RFLP is a time consuming and laborious technique, while PCR-sequencing or SNP low-density microarray panels are still expensive to use as a routine diagnostic technique (Branda Sica et al., 2018), especially for the detection of only few genetic diseases. On the other hand, PCR- HRM is cheaper, rapid, more sensitive and, if properly optimized, can be used as a routine diagnostic tool to monitor genetic diseases in Uruguayan dairy cattle. The cost per sample of high throughput platforms like the microarray SNP panels varies between 40 to 60 U$, while the cost per sample of the PCR-HRM analysis is 2,5 UDS per sample. In addition, BLAD detection through PCR-RFLP could take more than 6 hours to complete the whole process comprising PCR reaction, enzyme digestion, gel electrophoresis and digitalization of the image, compared to 1.5 hours for PCR-HRM in Rotor-Gene 6000 (Corbett Research).

CONCLUSION

We have set up a rapid, simple and inexpensive high-resolution melting analysis to detect genomic variants in CD18 and UMPS genes for the screening of BLAD and DUMPS, respectively. We show that this method provides significant savings in cost and time compared to other genotyping techniques; therefore, it could be implemented for routine molecular screening of both genetic disorders in Holstein cattle.

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