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Optimization of Real-Time PCR-melting for detection of the Cholesterol-deficiency mutation in Holstein Friesian cattle

Optimización de PCR en tiempo real con curvas de disociación para la detección de la mutación causante de deficiencia de colesterol en bovinos Holando

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ABSTRACT

The purpose of this study was to optimize a real-time PCR-melting analysis for reliable and economical detection of the 7.5 Kb mutant insert of the *BoERVK* bovine transposable element in exon 5 of the Apolipoprotein B *(APOB)* gene, which causes cholesterol deficiency — *CD* — (*OMIA* 001965-9913). This technique was also used to perform a preliminary molecular screening to detect this mutation in a DNA sample of Holstein Friesian cows (HFc) of six commercial dairy farms from different regions of Uruguay. By amplifying the 170 and 146 bp PCR products, two genotypes were clearly identified: homozygote (wild type *wt/wt*) and heterozygote (carrier of the *CD* mutation: *MUT/wt*). The homozygous *wt/wt* genotype was detected in the representative sample of 103 HFc. It is concluded that Real-Time PCR-melting analysis is a fast, easily interpretable, low cost, and highly accurate technique for detecting this mutation, which can be implemented in genetic selection programs to prevent the spread of the disease in HFc.

Key words: Cholesterol deficiency; Holstein Friesian; real-time PCR-melting

RESUMEN

El objetivo de este estudio fue optimizar un análisis mediante *PCR* en tiempo real con curvas de disociación para la detección confiable y económica del inserto mutante de 7,5 Kb del elemento transponible bovino *BoERVK* en el exón 5 del gen de la Apolipoproteína B (*APOB*), determinante de la deficiencia de colesterol — *CD* — (*OMIA* 001965-9913). Asimismo, aplicando esta técnica se realizó un cribado molecular preliminar para determinar la presencia de esta mutación en una muestra de ADN de vacas Holando (H) pertenecientes a seis tambos o fincas comerciales de diferentes regiones del Uruguay. A partir de la amplificación de los productos de *PCR* de 170 y 146 pb se logró distinguir claramente dos genotipos: homocigota (tipo silvestre *wt/wt*) y heterocigota (portador de la mutación *CD*: *MUT/wt*). El genotipo homocigota *wt/wt* fue detectado en la muestra representativa de 103 vacas H. Se concluye que el análisis mediante *PCR* en tiempo real con curvas de disociación es una técnica rápida, fácilmente interpretable, de bajo costo y altamente precisa para la detección de esta mutación, el cual puede ser implementado en programas de selección genética para evitar la propagación de la enfermedad en bovinos H.

Palabras clave: Deficiencia de colesterol; Holando; *PCR* en tiempo real con curvas de disociación

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INTRODUCTION

Cholesterol deficiency — *CD* — (*OMIA* 001965-9913) [19] is caused by a loss-of-function mutation in the Apolipoprotein B (*APOB*) gene, which is necessary for liver lipid metabolism, steroid biosynthesis, and cholesterol absorption in the small intestine [17]. The *APOB* mutation influences cattle (*Bos taurus*) fertility, growth, and health [9]. *CD* disease is usually confused with other types of neonatal diarrhea [14]. The economic impact of *CD* is very important. A study in Germany calculated that 3,400 recessive homozygous calves were born per year, resulting in an annual economic loss of approximately € 1.3 million [13]. Furthermore, in the United States of America (USA), annual losses due to that disease were calculated at USA$ 1.7 million [3]. In addition to severe diarrhea, affected calves have hypocholesterolemia and usually die within the first weeks (wk) to 6 months (mon) of life [14]. Some heterozygous calves showed reduced blood cholesterol concentrations, whereas in recessive homozygous blood cholesterol levels and triglyceride concentrations were virtually zero [9, 14, 21]. Gross *et al.* [8] found that low cholesterol concentrations associated with the *APOB* mutation in carriers are not due to primary *CD* at the cellular level, as the term "CD" suggests, but a consequence of decreased cholesterol transport capacity in blood. These authors suggest that, despite the presence of the *APOB* mutation, cholesterol does not limit metabolic adaptation or yield in heterozygous Holstein Friesian cows (HFc) [8]. The origin of this disease was traced to the American sire *Maughlin Storm*, born in 1991 and widely used in the HF population worldwide [14, 23].

This disease is caused by a 1,299 base pairs (bp) insertion of a long transposable element (*LTR_ERV2-1*) between nucleotides 24 and 25 of exon 5 of the *APOB* gene [17]. This insertion causes a shift in the reading frame of the *APOB* gene that leads to the truncation of 97 % of the bovine *APOB* protein [17]. These findings were independently confirmed by other authors [4, 22]. This result was confirmed by Charlier [4], albeit he estimated the size of the insertion of the bovine endogenous retroviral element in exon 5 of the *APOB* gene in 7.5 Kb (*BoERV*); this leads to transcriptional termination and loss of protein function. Due to this, the protein was synthetized to only 3 % of its normal size.

Although molecular methods such as Polymerase Chain Reaction (PCR) and its variants are currently applied to diagnose the *CD*-causing mutation [5, 12, 17, 22], there are no published studies in Uruguay on the application of these techniques for the accurate and effective detection of these transposable elements. The design of molecular diagnostic strategies for this mutation would be important for this Country, in order to achieve immediate results regarding the control of this disease, since Briano-Rodriguez *et al.* [2] reported a prevalence of *CD* carriers of 2.61 % in a population sample of HF calves using the GeneSeek Genomic Profiler — GGP — Bovine 50K genotyping panel. Hence, the purpose of this study was to optimize and implement a reliable and economical molecular screening procedure for the detection of the 7.5 Kb mutant insert (*BoERVK*) of the *APOB* gene through real-time PCR analysis with melting curve analysis (real-time PCR-melting), as well as to obtain preliminary results on its presence in a representative sample of HFc from the Dairy Cattle deoxyribonucleic acid (DNA) Genomic Bank of Uruguay.

MATERIALS AND METHODS

DNA samples and reference population

It worked with a representative sample of 103 second-lactation HFc of six commercial dairy farms from different Regions of Uruguay. Genomic deoxyribonucleic acid (gDNA) from these samples was stored in the Dairy Cattle DNA Genomic Bank of the Biotechnology Unit (INIA Las Brujas) as reference material for research projects (INML-UdelaR-INIA agreement). The extraction of these gDNA samples was initially performed from fresh blood samples at the Nuclear Techniques Laboratory (Facultad de Veterinaria, UdelaR) in 2008 using a digestion procedure with proteinase K and salting-out [18].

For optimization of the real-time PCR-melting, two gDNA samples were used as reference controls for comparison with the patterns of the melting curves to be analyzed. These control samples corresponded to: (1) gDNA of a bull (*ALTALeap* 011HO12336) diagnosed as a carrier of the *CD* mutation, and (2) gDNA of a bull (*ALTABolero* 011HO11572) free of the disease; both from *AltaGenetics* company (Montevideo, Uruguay). These gDNA samples were extracted from semen with the QIAamp DNA mini kit, according to the manufacturer's protocol #2.

gDNA was quantified in the NanoDrop equipment (NanoDrop 8000 Spectrophotometer, Thermo Fisher Scientific, USA), obtaining a range between 1.8 and 2.0 for the *OD260/OD280* ratio. The quality of the gDNA samples was assessed by 1 % agarose gel electrophoresis in TBE 0.5X buffer [7].

Optimization of the genotyping of the BoERVK_APOB insertion with real-time PCR-melting

Real-time PCR reactions were performed in a RotorGene™ 6000 (Corbett Life Science, Australia) on a final volume of 25 microliters per sample containing 50 nanograms of genomic DNA, 1X NZY qPCR Green Master Mix (NZYTech Genes & Enzymes, Portugal), and 0.5 microMol of each primer. A combination of three allele-specific primers designed by Charlier *et al.* [5] was used. This combination of primers discriminates the wild type from the mutated sequence and corresponds to a forward primer (F1: 5′ AAG GAG GCT GCA AAG CCA CCT AG 3′) and two reverse primers (mutant R1: 5′ CCT TTG TCA CGA GTG GAA TGC CT 3′; and R2: 5′ CCT CTT GAT GTT GAG GAT GTG TT 3′).

Dip tubes without gDNA were used as a negative control to identify the possible contamination of reagents and the possible formation of primer dimers in each PCR reaction mix.

The cycling program consisted of a 5 minutes (min) pre-hold at 95°C; and 40 cycles of 45 seconds (s) at 95°C, 40s at 55°C, and 40s at 72°C; with a 5 min stop-hold at 72°C. The annealing temperature was adjusted to 55°C, with activation of fluorescence data in the green channel (excitation 470; detection 510 nanometers — nm—). The melting peak was adjusted using 1°C increments with a 5s retention for each increase from 75 to 95°C. Melting curve analyses were performed with the Rotor-Gene Q Series Software 2.3.1 (Build 49) of the RotorGene™ 6000 thermal cycler.

Electrophoresis was performed on a 3 % agarose gel in 0.5X TBE buffer [7] in order to assess primer function and specificity; upon completion of the PCR reaction, the PCR products had the expected fragment size. The expected fragment sizes for each amplicon are 170 bp for the wild-type allele, and 146 bp and 120 bp for mutant alleles A and B, respectively.

Confirmation of results by sequencing and multiple sequence alignment

To confirm the sequence identity of the amplicons identified by real-time PCR-melting, 23 PCR samples were selected and sent for sequencing (Humanizing Genomics Macrogen, Seoul, Korea). Sequencing was performed using the primers of Charlier *et al.* [5].

The obtained sequences were analyzed by multiple alignment with a reference sequence for the *APOB* bovine gene (GeneID 494004, GenBank), using BioEdit free software [11].

RESULTS AND DISCUSSION

The FIG. 1 shows the denaturation curve (*-dF/dT* vs. temperature) of the amplicons obtained by real-time PCR for the *BoERVK_APOB* insertion in the analyzed genetic materials of each genotype. A peak was observed at 86.8°C for the homozygous wild type (*wt/wt*), and at 88°C for the heterozygous control, carrier of the *CD* mutation (*MUT/wt*). The homozygous *wt/wt* genotype was detected in the representative sample of 103 HFc.

Specialized real-time PCR thermal cyclers, such as the RotorGene™ 6000 used in this study was programmed to produce the melting curve after the amplification cycles have been completed. As the temperature increases, the double-stranded gDNA denatures to single-stranded gDNA, and the fluorophore molecules that had intercalated within the double-stranded gDNA at the end of amplification begin to separate from it as the temperature increases, leading to a decrease in fluorescence. The melting curve is obtained by derivation of the denaturation curve (fluorescence vs. temperature), which shows an increase in absorbance, intensity, and hyperchromicity during heating. The shape of this curve is related to the guanine-cytosine (GC) base content and the fragment size of the amplicons. The temperature value at which 50 % of the gDNA is denatured is called melting temperature (Tm) [6]. The Rotor-Gene Q Series Software 2.3.1 (Build 49) plots the derivative of fluorescence as a function of fluorescence (ordinates) vs. temperature (abscissae). In the plot, it can be seen a maximum peak corresponding to the Tm for each of the samples. The negative derivative plot of melting curves (*-dF/dT*) represents the rate of fluorescence change during the gDNA melting process and allows the identification of the Tm value by means of the temperature peaks [1].

For the samples analyzed in this work, the observation of a single temperature peak in the melting curve of each sample confirmed the specificity of the primers chosen to amplify the 146 bp mutant allele with a peak at 88°C, and the 170 bp wild type at 86.8°C. In this study, the primers of the mutant and wild type alleles had a GC content between 40 and 60 %, which was an adequate one (43.48 and 56.52 %, respectively) since no nonspecific amplifications or primer dimers were observed in the negative reaction control. Differences in the biological origin of the gDNA samples (blood vs. semen, in this case) caused the use of different DNA extraction protocols. This means that there may be differences in the chemical solutions used during the extraction process for each protocol, as well as in the contaminating residues that could remain in the final gDNA solution. Since real-time PCR equipment is highly sensitive to these differences in the conditions of the gDNA samples, differences could potentially be observed in the melting curves of amplicons that, despite having the same genotype, could be detected as different because their gDNA was extracted following different protocols [24].

However, in this work, it was not detected any effect of the gDNA extraction procedure on the melting dynamics of the amplicons, since the melting peak pattern (*-dF/dT*) of the disease-free control bull matched that of wild-type homozygous cows (FIG. 1). Furthermore, at the end of the PCR reaction, i.e., when the reaction reached the plateau phase, an inflection point was observed in the denaturation curve of the amplified product of the heterozygous control bull when compared with the curves of the disease-free control bull and wild type homozygous cows. Obtaining small amplicons increases the difference in the signals at a given temperature between two sequences differing in their nucleotide position from the start site of the *BoERVK* insertion element. Differences between genotypes are more clearly visible when amplicons are smaller in size [10]. The temperature and fluorescence resolution of the RotorGene™ 6000 equipment used in this study demonstrated the accuracy of the melting curves and their ability to differentiate different genotypes.

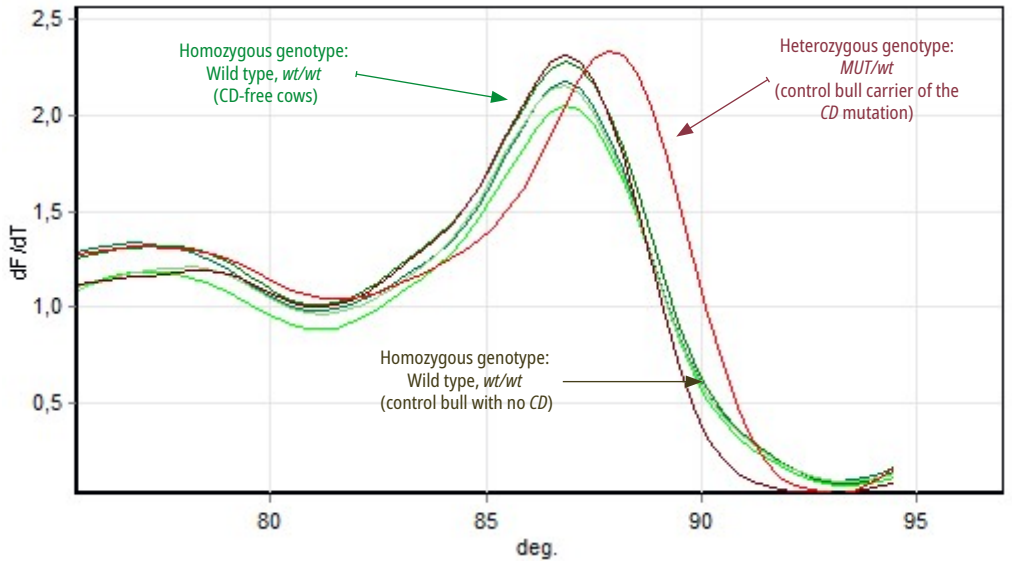

FIGURE 1. Negative derivative of the fluorescence melting curves at temperature (*-dF/dT*) for wild-type homozygous *wt/wt* and heterozygous *MUT/wt* genotypes. The graph shows the genetic materials of each genotype. Four homozygous *wt/wt* genotypes (four cows free of the disease –green curve–) were observed. Two controls were used, one heterozygous *MUT/wt* genotype (bull carrier of the CD mutation –red curve–) and one homozygous *wt/wt* genotype (bull free of the disease –black curve–).

Therefore, the primer design of Charlier *et al.* [5] was validated for the detection of the 7.5 Kb *CD* mutant insert (*BoERVK*) in exon 5 of the *APOB* gene by real-time PCR-melting on the RotorGene™ 6000.

By analyzing the electropherogram and multiple alignments of the sequences obtained with the reference sequence, the location of the insertion start point was determined at position 468 (26593) of the *APOB-UP1-F1* sequence of the HF bull control carrier of the *CD* mutation (*MUT/wt* or *indel_BoERVK_APOB*) after the GTTCC**TGAAGG** fragment on the left and before CAAGCAAGTTC, as reported by Charlier *et al.* [5]. The mutant *BoERVK APOB* insert was not found in the sequences of HFc and, therefore, those DNA samples corresponded to cows free of the *CD* disease and with a wild-type homozygous genotype (*wt/wt*, FIG. 2).

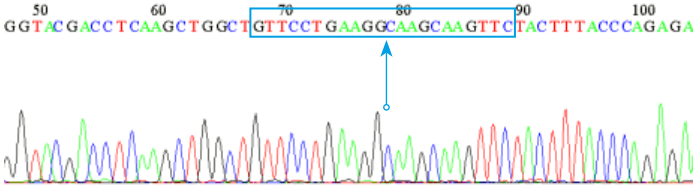

FIGURE 2. Electropherogram of the forward sequence (*APOB-UP1-F1*) of a *CD* disease-free cow showing the absent insertion start site

Based on these results, no cows carrying the *CD* mutation were found in the six dairy farms sampled from different regions of Uruguay. However, as reported by Briano-Rodriguez *et al.* [2] a prevalence of 2.61 % of *CD* carriers was found in a population sample of HF calves from different Regions of Uruguay. These results were similar to those observed for India (1.67 %, [15]) and the USA (2.6 %, [3]), but lower than those reported for other Countries. For example, in Germany, Kipp *et al.* [13] calculated the frequency of HF calves born homozygous for the *CD* haplotype in approximately 8.7 % of 3,400 calves born each year. However, Kipp *et al.* [14] reported a maximum carrier frequency of 12.25 % in the German livestock population in 2012, which was reduced to 7.87 % in 2016 due to the genetic identification strategies applied. In another study in Germany, Schütz *et al.* [22] found 12.5 % of carriers among HF bulls born between 2012 and 2015. Kamiński and Ruoeæ [12] reported a much higher *CD* carrier frequency (33.33 %) in Polish HFc. In Chinese HFc, a carrier prevalence of 5.07 and 1.11 % was found for bulls and cows, respectively [16]. Pozovnikova *et al.* [20] reported *CD* carrier frequencies in two groups of Russian HFc, where 23.26 % were found in the offspring born between 2016 and 2019 of *CD* carrier cows, and 8.09 % in the offspring born between 2010 and 2017 of *CD* carrier bulls.

Therefore, before taking mating decisions (to produce disease-free calves), it is recommended to perform strict monitoring and control by means of testing for the detection of the *CD* mutation, thus preventing the spread of this disease in Uruguayan dairy farms. It is also recommended the introduction of disease-free bulls into the HF genetic improvement and semen production programs.

In this study, real-time PCR-melting made possible the clear identification of two different genotypes, wild type homozygote *wt/wt*; and carrier *MUT/wt* (or *InDel_BoERVK_APOB*), for the *CD* mutation using *CD* disease-free controls and confirmed carriers for the *CD* disease mutation, validating the use of this technique for genotyping of HFc.

CONCLUSIONS

The real-time PCR-melting analysis herein described provides an alternative approach for genotyping of mutant alleles in cattle. Real-time PCR testing with the melting application is a fast, easily interpretable, low cost, and highly accurate technique for the detection of the *BoERVK* mutant insert of the *APOB* gene, allowing the genotyping of great volumes of HFc for the *CD* disease.

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