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IDENTIFICATION OF HOLSTEIN COWS CARRIERS OF COMPLEX VERTEBRAL MALFORMATION BY HIGH RESOLUTION MELTING CURVES (HRM)

(*Identificação de vacas holandesas portadoras da malformação vertebral complexa através de curvas de dissociação de alta resolução – HRM*)

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ABSTRACT: The objective of this study was the optimization and implementation of a reliable and economical molecular screening method for the detection of the mutant allele of CVM (complex vertebral malformation, c.559G>T, *SLC35A3*) by HRM analysis, as well as analyzing its existence in a representative sample of Holstein cows from the Milk Genomic DNA Bank of Uruguay. The optimization of the HRM methodology in the *RotorGene*™ 6000 equipment (Corbett Life Science, Australia) by amplification of the 79 bp PCR products clearly differentiated the two genotypes: homozygous, wild type: GG; and heterozygous, carrier for the mutation CVM: GT (c.559G>T; *SLC35A3*). In the analyzed sample, the frequency of the mutant allele (T) for CVM was high (q = 0.032), with a prevalence of carrier cows of 6.45%. It is concluded that the PCR-HRM analysis is a fast, easily interpretable, low cost, and highly accurate technique for the detection of this mutation in Holstein cattle, which may be implemented in genetic selection programs.
Keywords: CVM; high-resolution dissociation curve; Holstein.

RESUMO: O objetivo deste estudo foi otimizar e implementar um sistema fiável e econômico para a detecção molecular do alelo mutante CVM (malformação vertebral complexa; c.559G>T; *SLC35A3*) por meio da análise HRM, assim como analisar sua presença em uma amostra representativa de vacas Holandesas do Banco de ADN do Instituto Nacional de Pesquisa Agropecuária de Uruguai. A otimização da metodologia HRM no equipamento RotorGene™ 6000 (Corbett Life Science, Austrália) através da amplificação dos produtos de PCR de 79 pb permitiu diferenciar os dois genótipos: o homozigoto de tipo salvagem (GG) e o heterozigoto que presenta a mutação CVM (GT). Verificou-se que a frequência do alelo mutante (T) para CVM na amostra analisada foi alta, de q = 0,032, enquanto que a prevalência de vacas portadoras da mutação foi 6,45%. Concluiu-se que a análise por PCR-HRM é uma técnica rápida, facilmente interpretável, de baixo custo e alta precisão para a detecção dessa mutação no gado Holandês, que poderia ser implementada em programas de seleção genética.
Palavras-chave: Curvas de dissociação de alta resolução; CVM; Holandês.

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INTRODUCTION

Complex vertebral malformation (CVM, OMIA 001340-9913) is a recessive autosomal disease that causes miscarriages and perinatal problems. The single site mutation causing this disease is a substitution of Guanine (G) by Thymine (T) in the position 559 of exon 4 of gene *SCL35A3* (c.559G>T; Thomsen *et al.*, 2006), which plays an essential role in the development of the axial skeleton. This gene codes the UDP-N-acetylglucosamine transporter, and the mutation replaces a valine with a phenylalanine (V180F) at position 180 (Rusc and Kaminski, 2007). Thereof, the defective transporter molecule leads to vertebral malformations (Thomsen *et al.*, 2006). VanRaden *et al*. (2011) confirmed that this mutation is located in position 43.412.427 bp of chromosome 3.

This disease was reported by Danish scientists as from 2000 (Agerholm *et al*., 2001; 2004), and its origin could be traced to the elite American bull *Carlin-M Ivanhoe Bell*, who received the lethal recessive mutation of the CVM disease from his father, *Penstate Ivanhoe Star* (Revell, 2001; Chu *et al*., 2008). Due to the superior performance of his daughters at lactation, this bull was worldwide widely used for the raising of dairy cattle (Agerholm, 2007; Windsor and Agerholm, 2009; Agerholm *et al*., 2004). Among the bulls used for artificial insemination, CVM carrier animals were identified with a very high prevalence (10-30 %) in several countries (Kearney *et al*., 2005; Citek *et al.*, 2006; Thomsen *et al.*, 2006). Since then, the disease and several CVM carriers have been identified in other countries (Revell, 2001; Duncan *et al.*, 2001; Nagahata *et al*., 2002; Konersmann *et al*., 2003; Berglund *et al*., 2004; Rusc and Kaminski, 2007; Chu *et al*., 2008).

The high resolution melting curves analysis (HRM) is a simple an economic method, performed in closed tubes, for the analysis of PCR products (Wittwer *et al.*, 2003). This method is based in the melting behavior of PCR products (Reed *et al*., 2007). It shows a high sensitivity and specificity with PCR products smaller than 400 bp long (Reed *et al*., 2007). HRM has been widely used for genotyping and screening of mutations, both in human (Montgomery *et al*., 2004; Hung *et al*., 2008; Vossen *et al*., 2009; Vorkas *et al*., 2010) and animal health, particularly in cattle (Gabor *et al*., 2012a and 2012b; Santos *et al*., 2012; Federici *et al*., 2018).

In Uruguay, the disease has not been diagnosed, and the mutant allele has not been reported in the general cattle herd. Hence, the purpose of this study consisted in the optimization and implementation of a reliable and economic molecular screening method for detection of the mutant CVM allele (c.559G>T; *SLC35A3*) by HRM analysis, as well as analyzing its presence in a representative sample of Holstein cows of the Milk Genomic DNA Bank of Uruguay.

MATERIALS AND METHODS

Samples and reference material:

The analyzed cattle consisted in a representative sample of 279 second-lactation Holstein cows of 6 commercial dairy farms from different regions of Uruguay. The DNA of these samples is stored in the Milk Genomic DNA Bank of Uruguay of the Biotechnology Unit of the INIA Las Brujas as reference material for future investigation projects (INML-UdelaR-INIA agreement).

DNA extraction, concentration and quality

Genomic DNA extraction and purification from fresh blood samples

was performed in 2008 in the Laboratory of Nuclear Techniques (Faculty of Veterinary, UdelaR) according to the salting-out and proteinase K digestion protocol described by Miller *et al.* (1988). DNA concentration was assessed with nanodrop at 260 nm (*NanoDrop 8000 spectrophotometer, Thermo Scientific*), and DNA quality was assessed by means of the OD260/OD280 relationship, between 1.8 and 2.0.

Optimization of genotyping by real-time PCR-HRM and data analysis

In order to identify the mutant allele of the CVM disease (c.559G>T; *SLC35A3*), optimization of genotyping was performed by real-time PCR applying HRM in the *RotorGene™ 6000* (*Corbett Life Science*, Australia) equipment. 79 bp PCR products were amplified with the primers described by ZhiLing *et al*. (2015). PCR reactions were carried out on a final volume of 25 μl with 50 ng of genomic DNA, 1X *HRM-PCR Master Mix* (kit *Type-it® HRM-PCR, QIAGEN*, Hilden, Germany), and 0.7 μ of each primer (forward and reverse). The cycling program consisted in an initial denaturation of 10 min at 95 ºC, 40 cycles of 5 s at 95 ºC, 25 s at 60 ºC and 20 s at 72 ºC, and two holds of 10 s at 95 ºC and 1 min at 45 ºC, respectively. The annealing temperature was adjusted to 60 ºC with the activation of the fluorescence data in the green channel (excitation: 470 nm; detection: 510 nm). The HRM phase was adjusted with 0.1 ºC increments, with a 2 s retention in each increment from 74.5 to 84.5 ºC, with the acquisition of the fluorescence data in the green channel of HRM (excitation: 460 nm; detection: 510 nm). Analyses of the HRM curve were performed with the *Rotor-Gene Q* vers. 2.3.1 (Build 49) software and the provided HRM algorithm. The predetermined normalization regions for the CVM single site mutation (c.559G>T; *SLC35A3*) were applied. HRM data were viewed as normalized HRM curves and normalized difference graphs for the GG genotypes (homozygous, wild type) and GT (homozygous, carrier of the mutation). Genotypes of unknown samples were automatically assigned by the *Rotor-Gene* HRM software. The confidence threshold for accepting the genotype assigned by the software was 90%. In addition, in order to confirm the precision of genotypes by HRM, 25 DNA samples were selected at random and send to the Instituto de Genética Veterinaria of the Universidad Nacional de la Plata (IGEVET-CONICET, UNLP, La Plata, Argentina) to be genotyped with the low density panel microarray *ArBos1 50K* of the *Affymetryx* platform. Allelic and genotypic frequencies were calculated by direct counting according to the method of Nei (1989).

RESULTS AND DISCUSSION

Figures 1 and 2 show the results of the HRM analysis of gene *SLC35A3*. The *Rotor-Gene* HRM software automatically assigned the genotypes for the mutant CVM allele (c.559G>T; *SLC35A3*) according to the differences in the pattern of the melting curve and the melting temperature (*Tm*) of heterozygous and wild type homozygous. Figure 1 shows the different patterns of the single site mutation G for T, showing the two genotypes. Violet curves correspond to the wild type homozygous, and the green one to the heterozygous for the mutation (that is, a normal G allele, and a mutated T allele). The HRM methodology permitted to clearly differentiate the two genotypes of the mutant CVM allele (c.559G>T; *SLC35A3*). Figure 2 shows the difference curves obtained with the HRM analysis; these curves can be differentiated by comparing the shape of the difference curves based in the

genotypes with those of the CVM (c.559G>T; *SLC35A3*) carriers, which are taken as reference, clearly identifying the two genotypes: wild type homozygous (GG, violet), and heterozygous CVM carriers (GT, green). Figure 3 shows the melting curves of the negative derivatives (- *dF/dT*) generated from unprocessed data of the HRM analysis. The peak values were 78.75 °C for allele G, and 77.05 °C for allele T. The melting curves of the negative derivatives (- *dF/dT*) of the heterozygous genotype were identified by peaks with a value of 78.75 °C (Figure 3).

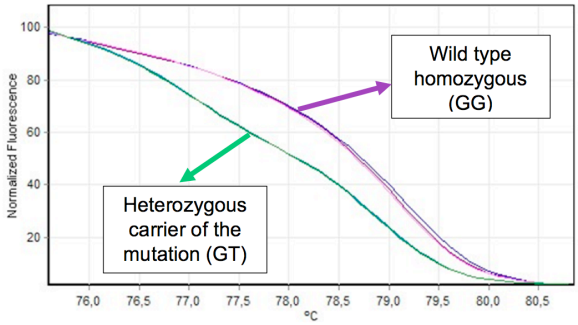

Figure 1. Graph with the normalization of the high resolution melting pattern: normalized denaturation curves obtained with the HRM analysis. Y-axis: normalized fluorescence; X-axis: temperature (ºC). The graph shows three genetic materials of each genotype.

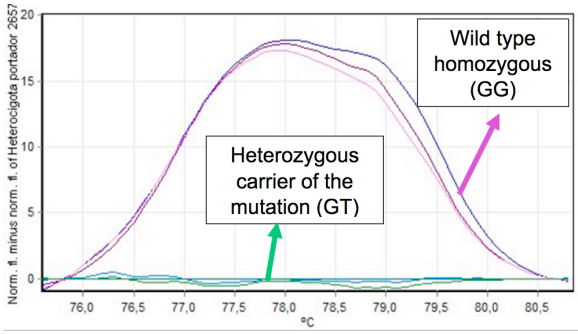

Figure 2. Graph of differences between genotypes: difference curves obtained with the HRM analysis. Y-axis: normalized fluorescence; X-axis: temperature (ºC). The graph shows three genetic materials of each genotype.

The HRM analysis has the disadvantage of its sensitivity to the residues of different DNA extraction methods, which may lead to melting curves with subtle differences that, in some cases, may cause an increase in the number of false positives (White and Potts, 2006). This was seen in the 6 groups from different dairy farms, maybe due to the fact that DNA was extracted by different operators or laboratories. Nevertheless, low quality samples can be easily identified and excluded from subsequent analyses if the PCR reaction is subjected to real time monitoring. Hence, for a HRM reaction, it is necessary to use the same bleeding (for fresh or coagulated blood) and DNA extraction procedures for all the samples.

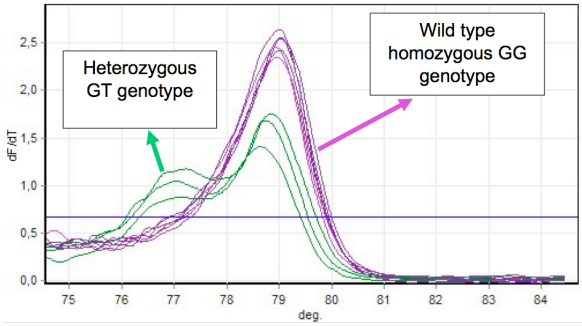

Figure 3. Negative derivative of the fluorescence melting curves at temperature (- *dF/dT*) for the homozygous wild type (GG) and heterozygous (GT) genotypes. The graph shows the material of three heterozygous (GT) and six homozygous (GG) genotypes.

The comparisons of the shapes and difference graphs of the melting curves of negative derivatives (- *dT/dF*) allowed the detection of two different CVM genotypes. CVM carriers (GT genotypes) were detected by two peaks for each G and T allele (78.75 and 77.05 °C respectively). In Gabor *et al*. (2012a), the melting point of the CVM carriers was lower than that of non-carrier animals (78.15 °C and 79.42 °C, respectively). HRM analysis permits to detect single base variations by comparing the melting curves of PCR products with the selection curve of a reference or control sample. The main advantages of HRM analysis when compared with gel-based methods are its lower cost, minimal labor, lesser time,

and reduction of the risk of contamination of the PCR reaction, the latter due to the fact that, unlike digestion with restriction enzymes or gel visualization, in HRM analysis it is not necessary to manipulate the sample after PCR (Tindall *et al*., 2009).

This study optimized the HRM analysis protocol as a simple, fast, and reliable genotyping method for CVM disease in Holstein cattle. The first step consisted in finding the control samples for the CVM mutation in the SLC35A3 gene. A preliminary HRM analysis with 25 DNA samples detected 3 control samples carriers of the mutation for the corresponding gene. As there were no CVM positive controls (sick homozygous), we used the ArBos1 genotyping service for identification and isolation of control samples carriers of the CVM mutation, these samples with known genotypes were used as controls for the HRM analysis. The need of using an alternative genotyping method before HRM is one of the main limitations of the PCR-HRM technique. In addition, primers were selected from those amplicons with the smallest possible size. For this study we used the primers used in ZhiLing et al. (2015) for real-time PCR with TaqMan probes: the 79 bp long amplicon amplified for the HRM analysis described in this study. The GC content of the forward and reverse primers used in this study ranged between 40 and 60 %; this was a proper content (45.5 and 47.8 %, respectively), as neither non-specific amplifications nor primer dimers were observed; thus validating the primer design of ZhiLing et al. (2015) for HRM analysis. The reduction of amplicon size increases the difference of the signal emitted at a given temperature between two sequences differing in just one nucleotide position. Genotypic differences are more clearly visible with smaller amplicons (Gundry et al., 2003). Heterozygotes can be identified by a modification in the shape of the melting curve, while homozygotes are identified by a modification in the melting temperature (Tm) (Liew et al., 2004).

Both the 3 DNAs of the heterozygous genotype (carrier of the mutation) and the 22 DNAs of the wild type homozygous genotype were confirmed by genotyping with the ArBos1 50K SNP panel; these were used as controls for carriers of the mutant allele for assignation of genotypes in HRM analyses with the *Rotor-Gene Q* program. Both the genotyping data of ArBos1 and the HRM analyses of the SLC35A3 gene confirmed the assigned genotype for all samples. The single site mutation of gene SLC35A3 influences the melting pattern (Tm) of PCR products, generating different high resolution melting curves that permit genotype differentiation and identification by HRM analysis.

In 18 cows of the analyzed sample, it was detected a mutant CVM allele (T) (c.559G>T; SLC35A3), with a confidence interval greater than 90 %. These results obtained are shown in Table 1. As a first report for molecular diagnosis of CVM, this study found a high frequency (q = 0.032) of the mutant CVM allele (c.559G>T; SLC35A3) in the analyzed sample, with a prevalence of 6.45 % of carrier cows. Both the frequency of the mutant allele and the prevalence of CVM carriers of this study were high when compared with those of the cows of Turkey (q = 0.01 and 3.4%, respectively, Meydan et al., 2010). Nevertheless, the prevalence was low when compared with other countries, such as Denmark (31.0%, Thomsen et al. 2006), Poland (24.8%, Rusc and Kaminski, 2007), Japan (32.5%, Nagahata et al., 2002), Sweden (23.0%, Berglund et al., 2004), and Germany (13.2%, Konersmann et al., 2003). The

reason for these differences may be due to the fact that there are samples from different populations, that is, cattle from different regions, although it may also be influenced by the use, over time, of semen from bulls carriers of the mutation. Approximately since the turn of the century, most countries have developed improvement programs to decrease the frequency of CVM carriers in the cattle population (Rusc and Kaminski, 2007). Nevertheless, In Japan, Ghanem et al. (2008) found 26 CVM carriers in 200 Holstein cows (13.0 %). Hence, the frequency of CVM carriers seems to continue to be high in some Holstein populations.

Table 1 - Number of carriers, distributions of mutant allele frequency, and prevalence of complex vertebral malformation carriers in the six sampled farms of different regions of Uruguay.

Farm	Sample size (*call rate* >0.90)	Number of carriers	Prevalence of carriers (%)	Mutant allele frequency (q)
“A”	56	7	12.5	0,063
“V”	63	7	11.11	0,056
"L"	63	1	1.59	0,008
"G"	16	2	12.5	0,063
"M"	28	0	0	0,000
"B"	53	1	1.89	0,009
TOTAL	**279**	**18**	**6.45**	**0,032**

In this study, the first report on CVM in Uruguay, it was possible to clearly identify two different genotypes for the CVM mutation (c.559G>T; *SLC35A3*) with the PCR-HRM procedure, thus validating it for genotyping in cattle.

CONCLUSIONS

The PCR-HRM analysis here in described provides an alternative approach for genotyping of mutant alleles in cattle. The HRM application is a fast, easily interpretable, low-cost, and highly precise procedure for the detection of the mutant allele (T) of gene *SLC35A3*, allowing the genotyping of great volumes of cattle for the CVM disease. The high prevalence of CVM carriers observed in the reference population of Uruguayan Holstein cattle justifies the need of implementing strategies for a gradual elimination of the mutant disease in dairy herd.

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