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DETECTION OF COMPLEX VERTEBRAL MALFORMATION CARRIERS IN SLOVAK HOLSTEIN CATTLE BY HIGH RESOLUTION MELTING ANALYSIS

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The objective of this study was to apply high resolution melting analysis in the detection of complex vertebral malformation (CVM) carriers in Hosltein cattle. A total of 47 animals of Holstein cattle were included in this study. Genomic DNA was extracted from blood, hair follicles and sperm by commercial QIAamp® DNA Mini kit. The amplification and high resolution melting analysis (HRMA) was done by commercial SensiMix™ HRM kit. The confirmation of sensitivity of this method was done by PCR-PIRA method and sequencing. Four samples of heterozygous genotype GT for causal mutation in the bovine solute carrier family 35 member 3 gene (SLC35A3), which is responsible for CVM disease, were detected. Our results demostrated that the use of HRMA for genotyping of mutant allele T for SLC35A3 gene in Holstein cattle is an effective method for the selection of carriers of CVM disease.

Key words: complex vertebral malformation (CVM), high resolution melting analysis (HRMA), Holstein cattle, genomic DNA

INTRODUCTION

The complex vertebral malformation (CVM) is a hereditary lethal disorder which was for the first time described in a population of Holstein calves in Denmark (Agerholm et al., 2001). The typical symptoms of CVM are malformations of the cervical and thoracic segments of the vertebral column that lead to mild scoliosis, mild symmetric bilateral contractions of carpal joints and shortening of the neck and anterior limbs with medial rotation of the latter (Uffo and Acosta, 2009). CVM is inherited as a recessive autosomal trait (Bendixen et al., 2001; Agerholm et al., 2001) and calves with heterozygous genotype are phenotypically normal, without typical symptoms of CVM. The detection of carriers is very important because this disease has an impact on intrauterine mortality that decreases the success rate of artificial insemination, causing further economic losses (Agerholm et al., 2004, Uffo and Acosta, 2009). The causal mutation responsible for CVM was discovered in the bovine solute carrier family 35 member 3 (SLC35A3) located at chromosome 3 (Thomson et al., 2006). The

molecular base of this mutation is substitution of G to T at position 559 of the gene SLC35A3. The presence of mutant allele T can be identified with several molecular-genetic methods as PCR-PIRA method which is based on the introduction of a restriction site in the resulting amplicon, using allele-specific forward primers (Kanae et al., 2005), PCR-SSCP method based on the fact that the electrophoretic mobility of a single-stranded molecule of DNA depends on the conformation of analysed DNA fragments (Rusc and Kamiñski, 2007, Chu et al., 2008) or sequencing method (Meydan et al., 2010).

At the present time diagnostics of many heritable disorders such as CVM – complex vertebral malformation, BLAD – bovine leukocyte adhesion deficiency, FXID – factor XI deficiency, BC – bovine citrullinaemia or DUMPS – deficiency of uridine monophosphate synthase (Meydan et al., 2010,) and detection of any pathogens such as viruses and bacteria (Nišavić et al., 2010; Terzi et al., 2010; Lakicevic et al., 2011) is made by using a modification of polymerase chain reaction (PCR). One of the modification is a novel high resolution melting analysis (HRMA). The high-resolution melting analysis is a cost-effective and simple post-PCR technique that can be used for mutation discovery, SNP genotyping, detection of heterozygotes, analysis of microbial genetic differences or DNA methylation analysis. The HRMA is a mutation scanning technique that monitors the progressive change in fluorescence caused by the release of an intercalating DNA dye from a DNA duplex as it is denatured by a marginal increase in temperature (Wittwer et al., 2003). The advantage of this closed-tube screening method is that PCR amplification and melting curve analysis are performed within the same tube, without any post-PCR processing (White et al., 2007; Norambuena et al., 2009). Melting data is captured as a fluorescent signal from intercalating dyes that are evenly distributed within double-stranded DNA during PCR amplification, and then later on released from the amplicon during the melting phase (Wittwer et al., 2003; Erali et al., 2008).

The objective of this study was to apply high resolution melting analysis for the detection of heterozygous genotype GT of SLC35A3 gene. It was shown that HRMA assay represents a highly-effective and cost-effective scanning technique for detection of CVM carriers in Holstein cattle.

MATERIAL AND METHODS

Samples were obtained from 47 animals of Holstein cattle from Slovak republic. Genomic DNA was isolated from peripheral blood and hair follicles of 45 calves and semen of 2 bulls using commercially QIAamp® DNA Mini kit (Qiagen, Germany). The concentration and purity of DNA was measured using a spectrophotometer NanoPhotometer™ (Implen GmbH, Germany) and samples with values of the ratio A260 nm/A280 nm between 1.7 and 1.9 were diluted to a final concentration of 10 ng/μL.

PCR-PIRA (Polymerase Chain Reaction – Primer Introduced Restriction Analysis)

A 233 bp product of SLC35A3 gene was amplified using PCR protocol described by Kanae et al. (2005) with forward primer introducing a *Pst*I restriction site in the C1000^{TM} thermocycler (Bio-Rad, USA). The 25 μL reaction mixture contained 1.5 mM $MgCl_2$, 200 μM of each dNTP, 0.4 μM of each primer, 1×PCR buffer with $(NH_4)_2SO_4$, 1 IU of Taq DNA polymerase (Fermentas GmbH, Germany) and 50 ng of genomic DNA template. Thermal cycling conditions included: an initial denaturation step at 95°C for 3 min followed by 35 cycles of 95°C for 15 sec, 53°C for 35 sec, 72°C for 35 sec and a final extension for 5 min at 72°C. Digestion of the PCR products was performed 30 min with 1 μL of the FastDigest *Pst*I restriction enzyme (Fermentas GmbH, Germany) at 37°C. The identification of fragments after digest restriction was performed in 3% agarose gel (SERVA Electrophoresis GmbH, Germany) with an intercalation reagent GelRed^{TM} (Biotium Inc.,USA) in 1×SB buffer (Brody and Kern, 2004) at 180 V for 15 minutes.

DNA sequencing

The confirmation of the presence of homozygous and heterozygous genotypes for SNP polymorphism G/T in the SLC35A3 gene was performed by direct sequencing. The PCR products with size a 281 bp were amplified with the PCR primers described by Meydan et al. (2010) in the C1000^{TM} thermocycler (Bio-Rad, USA). The reaction mixture in a total volume of 20 μL contained 1×Phusion® High-Fidelity PCR Master Mix (Finnzymes, Finland), 0.4 μM of each primer and 30 ng of genomic DNA. After amplification the PCR products were purified using 1.5 IU FastAP^{TM} Thermosensitive Alkaline Phosphatase (Fermentas GmbH, Germany) and 10 IU Exonuclease I (Fermentas GmbH, Germany) according to the manufacturer´s protocol. The purified PCR products were sequencing in both directions by the GenomeLab^{TM} DTCS (Beckman Coulter, USA) sequencing kit. The sequencing analysis was performed on an eight-capillary sequencer GenomeLab GeXP^{TM} Genetic Analysis System (Beckman Coulter, USA).

HRMA (High Resolution Melting Analysis)

Genomic DNA of 47 animals with known genotypes for CVM disease was used for optimizing the high resolution melting analysis (HRMA). Optimization of the PCR conditions was carried out using the real-time thermocycler Rotor-Gene 6000® (Corbett Research, Australia). The PCR products 177 bp were amplified with primers desribed by Rusc and Kamiñski (2007). The reaction mixture in a total volume of 25 μL contained 1×SensiMix^{TM} HRM buffer (Bioline Reagents Ltd., UK), 1 μL of EvaGreen® qPCR dye (Biotium Inc., USA), 0.7 μM of each primer and 10 ng DNA template.The PCR conditions were 95°C for 10 min followed by 40 cycles of 95°C for 5 s, 59°C for 25 s (fluorescence acquisition on Green channel) and 72°C for 20 s. After real-time PCR, samples were heated to 95°C for 10 s, cooled to 45°C for 1 min, and melted from 72°C to 82°C, with the temperature increasing by 0.1°C increments with a 2-s hold at each step. HRM data were acquired by use of the HRM channel. HRM data were analyzed using Rotor-Gene 6000 Series Software version 1.7 (Corbett Research, Australia).

Post-HRMA sequencing

The positive HRM amplicons with heterozygous genotype GT were purified before sequencing using 1.5 IU FastAP™ Thermosensitive Alkaline Phosphatase (Fermentas GmbH, Germany) and 10 IU Exonuclease I (Fermentas GmbH, Germany) according to the manufacturer's protocol. Sequencing reaction was prepared with primers for HRMA using the GenomeLab™ DTCS kit (Beckman Coulter, USA) according to the manufacturer's manual and sequenced on an eight-capillary sequencer GenomeLab GeXP™ Genetic Analysis System (Beckman Coulter, USA).

RESULTS AND DISCUSION

Biological samples of 45 Holstein calves and 2 Holstein bulls were screened for the presence of the mutation in SLC35A3 gene by PCR-PIRA method. The PCR reaction ran at a total volume of 25 μL. Identification of the presence of mutant allele T was performed using restriction endonuclease *Pst*I and electrophoretic separation in 3% agarose gel. Using PCR-PIRA method with restriction endonuclease *Pst*I 4 calves with heterozygous genotype GT (233 bp, 212 bp, 21 bp) were dentified. The other 41 calves and 2 bulls had a normal homozygous genotype GG (212 bp, 21 bp). A UV transilluminator and the Olympus C-7070 system were used to visualize and record the results (Figure 1).

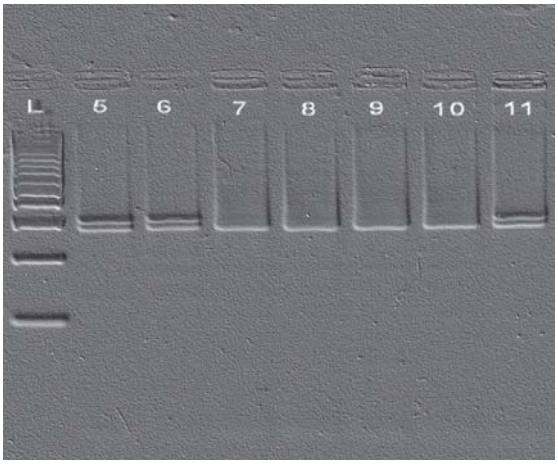

Figure 1. Representative results of PCR-PIRA method for 233 bp PCR product of SLC35A3 gene by using restriction endonuclease PstI on 3 % agarose gel. Lane L: 100 bp ladder (Fermentas); Lanes 5, 6 and 11: heterozygous genotype GT (233 bp, 212 bp, 21 bp – not visible); Lanes 7, 8, 9 and 10: normal homozygous genotype GG (212 bp, 21 bp – not visible)

The confirmation of the results from the PCR-PIRA method expecially for the heterozygous genotype GT was done by sequencing. The evaluation and data

processing were performed using GenomeLab^{TM} System software, version 10.2, which is part of the automatic genetic analyzer GenomeLab^{TM} GeXP Genetic Analysis System package (Beckman Coulter, USA) (Figure 2).

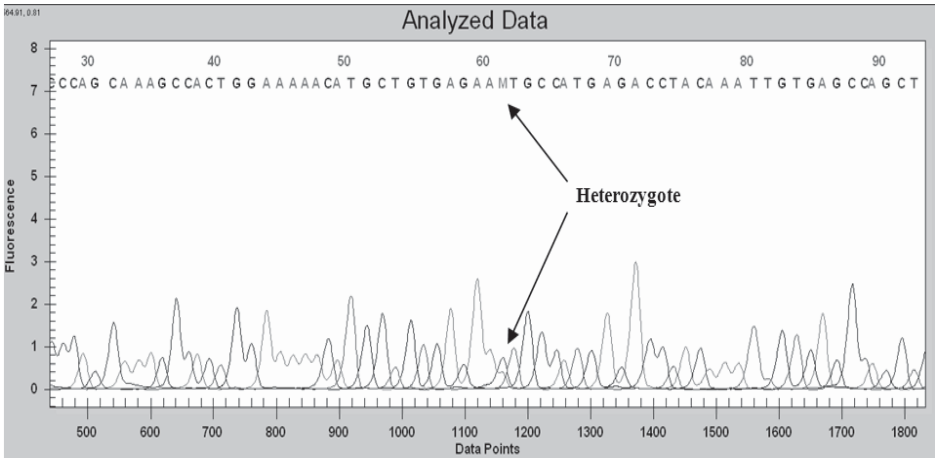

Figure 2. Sequencing analyse of heterozygous genotype GT with the equipment GeXP Genetic Analyser (Beckman Coulter, USA). Direct sequencing with reverse primer. The universal code M represent the combination of dideoxynucleotides C and A, which is comparative to nucleotide G and T in direct sequencing with forward primer

After verification of the restriction patterns of heterozygous genotype GT obtained from the PCR-PIRA method with direct sequencing method, we used all the 47 samples with known genotypes for optimization of high resolution melting analysis. For HRM analysis we used primers decribed by Rusc and Kamiński, (2007) which amplified the optimal fragment (177 bp) not only for SSCP method (Rusc and Kamiński, 2007; Rezaee et al., 2009), but also for HRM analysis described in our research. The HRM analysis was performed immediately after the pre-amplification in the real-time thermocycler. This analysis consisted of one cycle with increase in temperature from 72°C to 82°C, where the change in fluorescence was measured at each 0.2°C rise for 2 s. The data obtained from amplification plots were used for a first correction of reaction quality by the comparative amplification function of the software Rotor-Gene 6000 Series Software version 1.7 (Figure 3). Samples were considered to have failed if amplification had begun after 30 cycles. All samples were tested in duplicate to ensure the reproducibility of the melt curves.

The first amplification reaction on Rotor-Gene 6000 was also checked for the presence of nonspecific products by 2% agarose gel electrophoresis (data not shown) and by negative derivative of fluorescence to temperature (-dF/dT) dissociation curve. For the screening of heterozygous genotypes GT the normalised high-resolution melt curves (Figure 4) were used. Normalization regions for the leading/trailing ranges were set at 72.5–73.5°C/80.5–81.75°C.

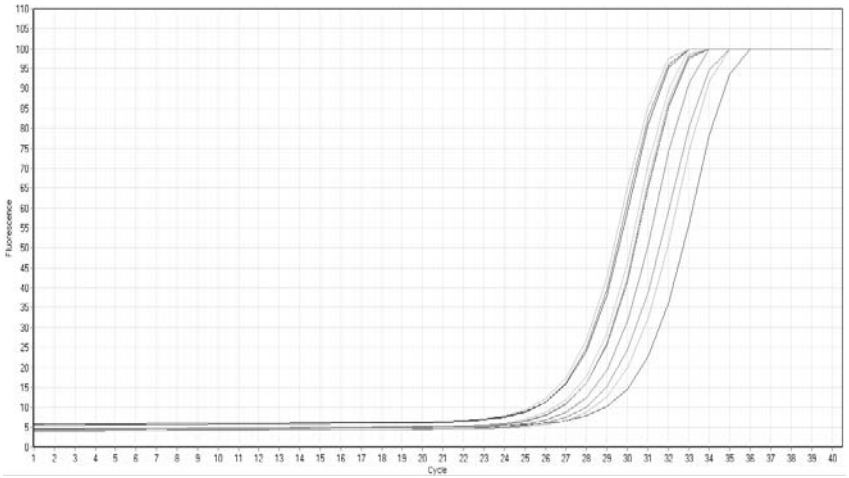

Figure 3. Amplification curves of 177 bp PCR products on the Rotor-Gene 6000

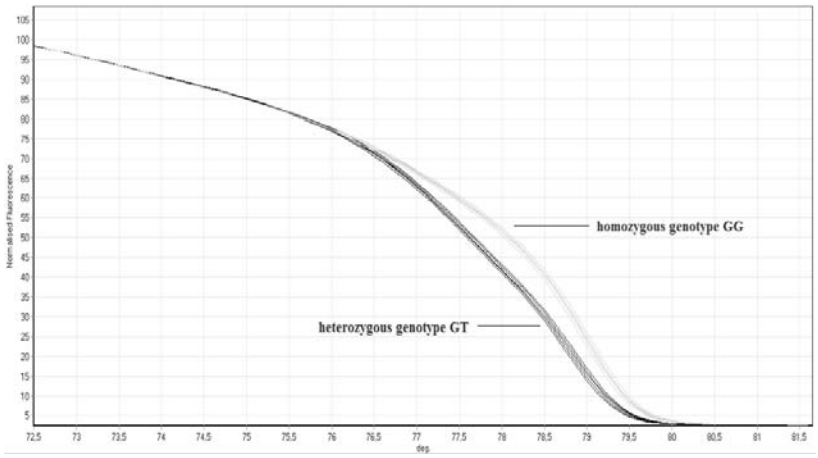

Figure 4. The high resolution normalized melting curves of a 177 bp PCR products for normal homozygous genotype GG (shadow) and heterozygous genotype GT (black). The genotype assignments agreed with sequencing results

The negative dF/dT melt curves were generated from raw data of high resolution melting analysis. The negative dF/dT melt curves of heterozygous genotypes were identified by two peaks for each alelles G and T (Figure 5). The value of peaks for the mutant alelle T was 78.15°C and for the normal alelle G was 79.42°C.

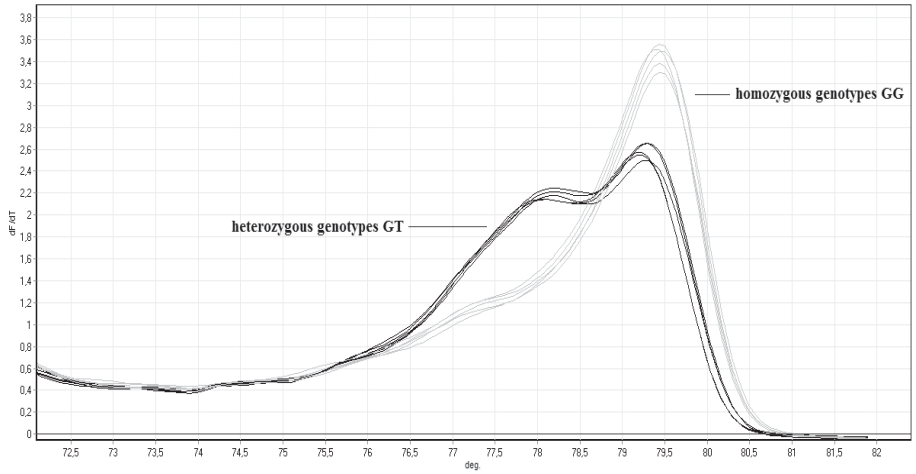

Figure 5. The negative derivative of fluorescence to temperature (- dF/dT) dissociation curve for normal homozygous genotyp GG (shadow; one melt domain: allele G – 79.42°C) and heterozygous genotypes GT (black; two melt domains: allele T – 78.15°C and allele G – 79.42°C)

The HRMA is a novel screening technique for detection of new SNP mutation. The great advantages of HRMA are a short time of analysis and detection of individual genotypes in the same tube that was used for PCR amplification in comparison to convential mutation screening techniques such as SSCP, DGGE, DHPLC or pyrosequencing (White et al., 2007; Poláková et al., 2008; Norambuena et al., 2009). The disadvantage of HRMA is the sensitivity to residues from different DNA extractio aan methods, which can make a false positive signal. Therefore, is needed to use the same DNA extraction method for all samples in one HRMA reaction. In our methods we used for detection of different genotypes not only normalized melt curves but the negative derivative melt curves (-dT/dF), too. On the basis of shape comparison and difference plots of negative derivate melt curves (-dT/dF) were detected two different genotypes for CVM. The presence of CVM carries (genotypes GT) were detected by the presence of two peaks for each allele T and G (78.15°C and 79.42°C). The significant difference between the peaks represented the divergence approximately 1°C allowed the simple identification of heterozygous genotype GT and homozygous genotype GG analysed by HRMA method from different biological materials such as blood, hair follicles or semen. The results of the real-time PCR amplification and the HRM analysis were confirmed by post-HRM direct sequencinq without any interference of EvaGreen® qPCR dye during sequencing of HRM product. The same results from post-HRM direct sequencing without any interference of the intercalating dye LC Green I® (Idaho Technology Inc., USA) described Poláková et al. (2008). The sequence analysis used bilateral sequencing with the primers for HRMA. The sequences of homozygous and

heterozygous genotypes (Figure 6) were comparatively analyzed together with nucleotide sequences of normal allele (*GenBank sequence HM183012*) and mutant allele (*GenBank sequence HM183013*) using software Seaview version 4.2.12 (Gouy et al., 2010).

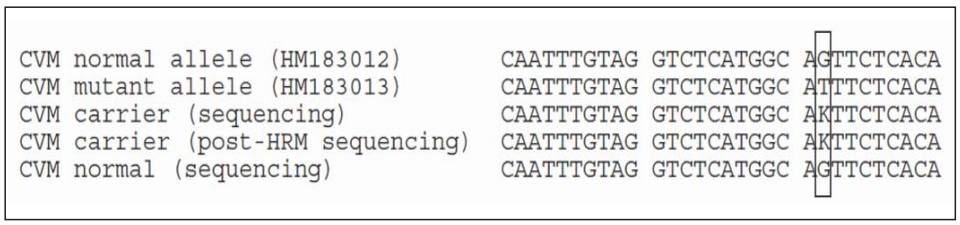

Figure 6. Sequence alignment of the bovine SLC35A3 gene with the GenBank sequence HM183012 (normal allelle G) and HM183013 (mutant allele T) and results of direct sequencing and post-HRM sequencing. The SNP is shown in box. The presence of heterozygous genotype GT is shown by universal code K (G↔T)

CONCLUSION

The detection of carriers for complex vertebral malformation disease is very important because this syndrome has a significant economic impact on breeding of Holstein cattle in the world. We have set up a rapid, simple and inexpensive scanning method for detection of CVM carriers in Holstein cattle. In our study we detected four carriers of mutant allele T for complex vertebral malformation in calves. The optimization of a high resolution melting analyses for detection of heterozygous genotypes GT of SLC35A3 gene, which is responsible for CVM disease, seems to be suitable for rapid screening of carriers in cows of Holstein cattle.

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carriers in Slovak Holstein cattle by high resolution melting analysis

OTKRIVANJE NOSILACA KOMPLEKSNE VERTEBRALNE MALFORMACIJE KOD SLOVAČKIH HOLŠTAJN GOVEDA PUTEM MELTING ANALIZE VISOKE REZOLUCIJE

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SADRŽAJ

Cilj ovih ispitivanja je bio da se kod Holštajn goveda, primenom melting analize visoke rezolucije, otkriju nosioci gena kompleksne vertebralne malformacije (CVM). Ispitivanjima je bilo obuhvaćeno ukupno 47 jedinki. Genomska DNK je izdvajana iz krvi, dlačnih folikula i sperme komercijalnim QIAamp® DNK Mini kitom. Amplifikacija i melting analiza visoke rezolucije (HRMA) su obavljene komercijalnim SensiMix™ HRM kitom. Potvrda senzitivnosti ove metode je obavljena PCR-PIRA metodom i sekvencioniranjem. Registrovane su četiri jedinke heterozigotnog genotipa GT sa uzročno-posledičnom mutacijom na alelu SLC35A3, koji je odgovoran za bolest CVM. Naši rezultati ukazuju da je primena HRMA metode za genotipizaciju mutiranog T alela, gena SLC35A3 kod Holštajn goveda efikasan metod za selekciju nosilaca bolesti CVM.