The Use of High Resolution Melting Analysis for Detection of the CAPN530 Polymorphism in Slovak Simmental Bulls

Michal Gábor, Anna Trakovická, Martina Miluchová, Nikola Kleknerová

Slovak University of Agriculture in Nitra, 949 76-Nitra Tr. A Hlinka 2, Slovakia

Abstract

The high-resolution melting analysis is a cost-effective and simple post-PCR technique that can be used for SNP genotyping. The objective of this study was to apply the high resolution melting analysis for detection of SNP polymorphism CAPN530 of bovine calpain gene (CAPN1) in Simmental bulls. A total 42 Simmental bulls were included in this study. Genomic DNA was extracted from sperm by commercial Nucleospin Tissue kit. The amplification and high resolution melting analysis (HRMA) was done by commercial SensiMix^{TM} HRM kit in the real-time thermocycler Rotor-Gene 6000. The confirmation of sensitivity of this method was done by PCR-RFLP method. In the analyzed population of Simmental bulls were detected the following frequencies of alleles and genotypes for the SNP CAPN530 of the CAPN1 gene. Frequencies of allele A and allele G were 0.3929 and 0.6071 and frequencies of genotypes were 0.1191 (genotype AA), 0.5476 (genotype AG) and 0.3333 (genotype GG).

Keywords: cattle, genomic DNA, high-resolution melting analysis (HRMA), SNP polymorphism CAPN530

1. Introduction

Calpain is a ubiquitous cytoplasmic cysteine protease, the activity of which is absolutely dependent on calcium [1]. Suzuki and Sorimachi [2] identified two isoforms of calpain. The μ - calpain (CAPN1) which requires for activity a micromolar concentration of calcium and the m-calpain (CAPN2) which need milimolar concentration of calcium for activity. The function of both calpains is to degrade the myofibrillar protein as actin and myosin [3]. The *CAPN1* gene that code protease μ-calpain *(EC 3.4.22.52)* is located on bovine chromosome 29 [4]. Page et al. [5] reported several SNPs by sequencing the 22 exons and 19 of the 21 introns of the bovine *CAPN1* gene (GenBank Accession number AF252504 and AF248054). Several SNPs in this gene have been reported to be associated with meat tenderness in beef cattle [5, 6]. The majority of the SNPs were found in introns or were synonymous substitutions, except one substitution in exon 9 (C/G) and another in exon 14 (G/A). Marker CAPN530 is an adenosine/guanosine (A/G) polymorphism in exon 14 of the CAPN1 gene (base 4558 of AF248054) that produces an amino acid substitution isoleucine (allele A) for valine (allele G)[5].

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 marginal increases in temperature [7]. The advantage of this closed-tube screening method is fact, that PCR amplification and melting curve analysis are performed within the same tube, without any post-PCR processing [8, 9].

The objective of this study was to apply the high resolution melting analysis for analysis of polymorphism for marker CAPN530 and to estimate the allelic and genotypic frequencies in group of Slovak Simmental bulls.

*Corresponding author: Michal Gábor, Tel: 00421-6414295, Email: misogabor@yahoo.com

2. Materials and methods

Samples were obtained from 42 bulls of Slovak Simmental cattle. Genomic DNA was isolated from semen using commercially Nucleospin Tissue kit (Macherey Nagel). The concentration and purity of DNA was measured using a spectrophotometer NanoPhotometer™ (Implen GmbH, Germany) and the samples with values of ratio A260 nm/A280 nm between 1.7 and 1.9 were diluted to a final concentration of 50 ng/µl.

PCR-RFLP: For PCR amplification of 87 bp fragment were used specific primers designed by software BatchPrimer3 v1.0 [10]. The sequences of primers were: forward primer 5′ GTTGACTGG CCCCTCTCTCT - 3′ and reverse primer 5′- CA GGGCACGTACCTCGTC - 3′. The reaction mixture in the total volume 10 µl containing 50 ng DNA, 1 U Taq polymerase (Fermentas), 1 x PCR buffer $(NH_4)_2SO_4$, 3 mM $MgCl_2$, 200 µM dNTP, 5 pM of each primer. The PCR reaction was optimized in the gradient thermocycler C1000^{TM} (Biorad, USA). The following amplification parameters were applied: 95^{o}C for 3 minutes followed by 30 cycles: 95°C for 10 seconds, 62°C for 20 seconds, 72°C for 20 seconds. The reaction was completed by the final synthesis: 72 °C for 5 minutes. The PCR products were digested by restriction endonuclease FastDigest *Sau*3AI (Fermentas). The digestion was performed with 7 µl of PCR product mixed with 1 µl of the restriction enzyme, 2 µl of 10 x FastDigest buffer in total volume 20 µl. The run conditions were 5 minutes at 37^{o}C. Digested fragments were visualized by electrophoresis on 3 % agarose gel (Invitrogen) containing GelRed dye (Biotium) at 180 V in 1 x sodium borate buffer for 15 minutes and the gel was analyzed by UV transilluminator and photographed with a documentation system Olympus C 7070.

HRMA: Genomic DNA of 42 bulls with known genotypes for CAPN530 was used for optimizing of the high resolution melting analysis (HRMA). Optimization of the PCR conditions was carried out using the real-time thermocycler Rotor-Gene 6000® (Corbett Research). For HRMA were used the same primers as for PCR-RFLP method. The reaction mixture in total volume 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 50 ng DNA template. The PCR conditions were 95 °C for 10 min followed by 35 cycles of 95°C for 5 s, 62°C for 20 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 75°C to 90°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).

3. Results and discussion

PCR-RFLP: The digestion of 87 bp PCR product with restriction endonuclease *Sau*3AI differentiated alleles A and G for marker CAPN530 of the CAPN1 gene. The *Sau*3AI digestion of the PCR products produced two fragments for allele A (50 bp, 37 bp) and one fragment for allele G (87 bp). The PCR-RFLP method was used for genotyping of 42 bulls of Slovak Simmental cattle. In this group of animals were detected the all genotypes (AA, AG, GG) for marker CAPN530 (Figure 1).

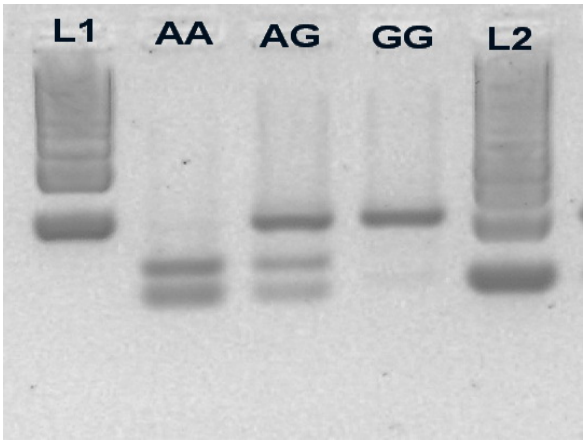

Figure 1. Representatively results of PCR-RFLP analysis by restriction enzyme *Sau*3AI on 3 % agarose gel

L1–ladder 100 bp (Fermentas), AA-genotype (50 bp, 37 bp), AG–genotype (87 bp, 50 bp, 37 bp), GG–genotype (87 bp), L2–ladder 50 bp (Fermentas)

HRMA: We used the all 42 samples with known genotypes for optimization of high resolution melting analysis. For HRM analysis we used the same primers as for PCR-RFLP method, which amplified the optimal fragment (87 bp) for HRM analysis described in our research. The HRM analysis was performed immediately after the pre-

amplification in the real-time thermocycler Rotor-Gene 6000 (Figure 2).

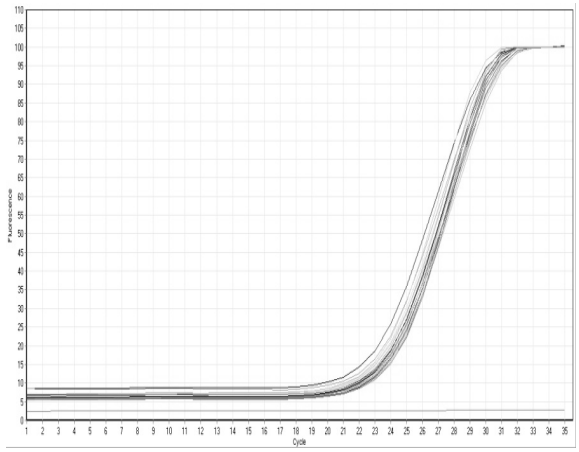

Figure 2. Amplification curves of 87 bp PCR products on the Rotor-Gene 6000

The HRM analysis consisted of one cycle with increase in the temperature of 75°C to 90°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 correcture of reaction quality by the comparative amplification function of the software Rotor-Gene 6000 Series Software version 1.7. 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. For the screening of SNP A/G for marker CAPN530 were used the normalized high resolution melt curves and the negative derivative of fluorescence to temperature (-dF/dT) dissociation curve (Figure 3 and 4). Normalization regions for the leading/trailing ranges were set at 75.7–76.4°C/88–88.7°C.

The HRMA is a novel screening technique for detection of 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 compare to convential mutation screening techniques such as SSCP, DGGE, DHPLC or pyrosequencing [8, 9, 11].

The disadvantage of HRMA is the sensitivity to residues from different DNA extraction methods, which can make false positive signal. Therefore is needed to use the same DNA extraction method for all samples in one HRMA reaction.

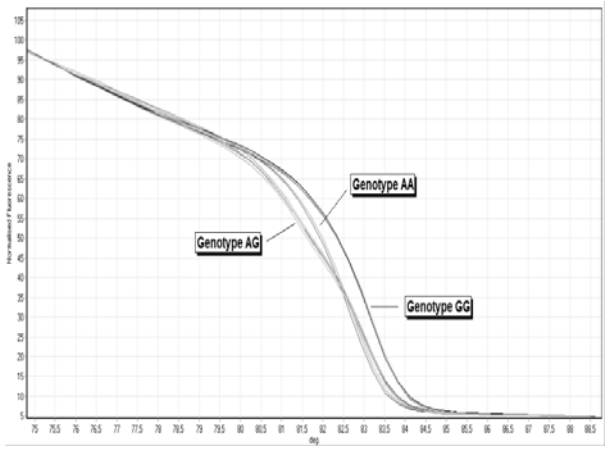

Figure 3. The high resolution normalized melting curves of a 87 bp PCR products for all three genotypes AA, AG, GG for marker CAPN530. The genotype assignments agreed with results of PCR-RFLP method

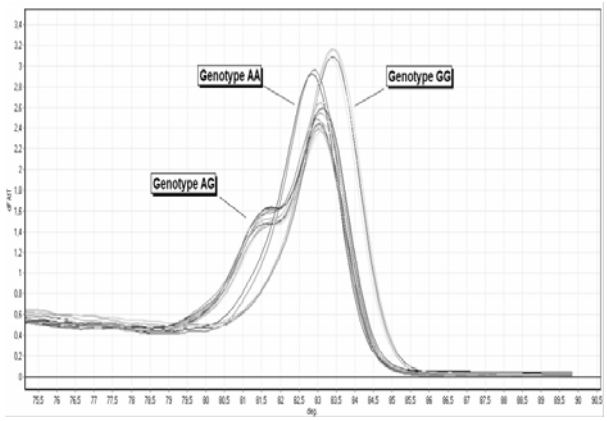

Figure 4. The negative derivative of fluorescence to temperature (- dF/dT) dissociation curve for all three genotypes AA, AG, GG of the marker CAPN530.

Genetic variants: By using PCR-RFLP was analyzed population of 42 Slovak Simmental bulls. It was detected all three genotypes AA (5 bulls), AG (23 bulls), GG (14 bulls) for marker CAPN530 in exon 14 of the CAPN1 gene. Frequencies of genotypes and alleles determined in the population were presented in Table 1.

The frequencies of minor allele A and major allele G for this groups of bulls were 0.3929 and 0.6071.

The frequencies of minor allele A and major allele G for this groups of bulls were 0.3929 and 0.6071.

The lower frequencies for allele A were detected in breed Charolais (0.24), Limousine (0.36) and Blonde d´Aguitine (0.36) [12]. Casas et al. [13] detected the similar results for frequencies of allele A (0.37) in population of 362 calves of

Table 1. Genotype and allele frequencies for marker CAPN530

BREED	n	GENOTYPES FREQUENCY			ALLELES FREQUENCY	
		AA	AG	GG	A	G
Slovak Simmental cattle	42	0.1191	0.5476	0.3333	0.3929	0.6071

American Simmental. White et al. [14] analyzed breeds as Angus, Red Angus, Simmental, Gelbvieh, Limousine, Charolais) and not found an association between marker CAPN530 and shear force. In contrast to the results from study of Corva et al. [15] which found the association between allele A and the lower shear force than allele G.

4. Conclusions

We have set up a rapid, simple and inexpensive high resulotion melting analysis for detection of the CAPN1 gene polymorphism in Slovak Simmental cattle. It may be concluded that the population of Slovak Simmental cattle is a polymorphic for marker CAPN530 situated in exon 14 of the CAPN1 gene. We detected the presence of the homozygous genotype AA (0.1191), the heterozygous genotype AG (0.5476) and the homozygous genotype GG (0.3333). The results proved the predominance of allele G (0.6071) before the allele A (0.3929).

Acknowledgements

This work has been supported by the grants: The Slovak Research and Development Agency under the contract No. LPP-0220-09 and the Excellence Center for Agrobiodiversity Conservation and Benefit project (ITMS: 26220120015) implemented under the Operational Programme Research and Development financed by the European Fund for Regional Development.

References

1. Sorimachi, H., Ishiura, S., Suzuki, K., Structure and physiological function of calpains, Biochemical Journal, 1997, 328, 721–732
2. Suzuki, K., Sorimachi, H., A novel aspect of calpain activation, FEBS Lett, 1998, 433, 1–4
3. Beltran, J.A., Jaime, I., Santolaria, P., Sanudo, C., Alberti, P., Roncales, P., Effects of stress-induced high postmortem pH on protease activity and tenderness of beef, Meat Science, 1997, 45, 201–207
4. Smith, T.P.L., Casas, E., Rexroad III, C.E., Kappes, S.M., Keele, J.W., Bovine CAPN1 maps to a region of BTA29 containing a quantitative trait locus for meat tenderness, Journal of Animal Science, 2000, 78, 2589–2594
5. Page, B.T., Casas, E., Heaton, M.P., Cullen, N.G., Hyndman, D.L., Morris, C. A., Crawford, A.M., Wheeler, T.L., Koohmaraie, M., Keele, J.W., Smith, T.P.L., Evaluation of single-nucleotide polymorphism in CAPN1 for association with meat tenderness in cattle, Journal of Animal Science, 2000, 80, 3077-3085
6. Page, B.T., Casas, E., Quaas, R.L., Thallman, R. M., Wheeler, T.L., Shackleford, S. D., Koohmaraie, M., White, S. N., Bennett, G.L., Keele, J. W., Dikeman, M., E., Smith, T. P. L., Association of markers in the bovine CAPN1 gene with meat tenderness in large crossbred populations that sample influential industry sires, Journal of Animal Science, 2004, 82, 3474-3481
7. Wittwer, C.T., Reed, G.H, Gundry, C.N., Vandersteen, J.G., Pryor R.J., High-resolution genotyping by amplicon melting analysis using LCGreen, Clin Chem, 2003, 49, 853-860
8. White, H.E., Hall, V.J., Cross, N.C., Methylation-sensitive high resolution melting-curve analysis of the SNRPN gene as a diagnostic screen for Prader-Willi and Angelman syndromes, Clin. Chem, 2007, 53, 1960-1962
9. Norambuena, P.A., Copeland, J.A., Křenková, P., Štamberková, A., Macek, M., Diagnostic method validation: High resolution melting (HRM) of small amplicons genotyping for the most common variants in the MTHFR gene, Clin Biochem, 2009, doi:10.1016/j.clinbiochem.2009.04.015.
10. Frank, M.Y., Naxin, H., Yong, Q.G., Ming-cheng, L., Yaqin, M., Dave, H., Gerard, R. L., Jan, D., Olin D, A. BatchPrimer3: a high throughput web application for PCR and sequencing primer design., BMC Bioinformatics, 2008, doi:10.1186/1471-2105-9-253
11. Poláková, K.M., Lopotová, T., Klamová, H., Moravcová, J., High-resolution melt curve analysis: Initial screening for mutations in BCR-ABL kinase domain, Leuk Res, 2008, 32, 1236-1243
12. Allais, S., Journaux, L., Leveziel, H., Payet-Duprat, N., Hocquette, F.J., Lepetit, J., Rousset, S., Denoyelle,C., Bernard-Capel, C., Renand, G., Effects of polymorphisms in the calpastatin and μ-calpain genes on meat tenderness in 3 French beef breeds, Journal of Animal Science, 2011, 89, doi: 10.2527/jas.2010-3063.
13. Casas, E., White, S. N., Wheeler, T. L., Shackelford, S. D., Koohmaraie, M., Riley, D. G.,

Chase, Jr C. C., Johnson, D. D., Smith, T. P. L., Effects of calpastatin and μ-calpain markers in beef cattle on tenderness traits, Journal of Animal Science, 2006, 84, 520-525

14. White, S. N., Casas, E., Wheeler, T. L., Shackelford, S. D., Koohmaraie, M., Riley, D. G., Chase, C. C. Jr, Johnson, D. D., Keele, J. W., Smith, T. P., A new single nucleotide polymorphism in *CAPN1* extends the current tenderness marker test to include cattle of *Bos indicus*, *Bos taurus*, and crossbred descent, Journal of Animal Science, 2005, 83, 2001-2008

15. Corva, P. M., Soria, L., Villarreal, E.L., Schor, A., Perez, Cenci, M., Motter, M., Mezzadra, C., Melucci, L. M., Paván, E., Depetris, G., Miquel, M. C., Santini, F. J., Grigera, Naón, J.J., Association of polymorphisms on the *CAPN1* and *CAST* genes with meat tenderness in beef cattle of Argentina, Genet. and Molecular Biology, 2007, 4, 1064-1069.