Genotyping Single Nucleotide Polymorphism C4685T in 14. Intron of Bovine CAPN1 Gene by Rapid Tetra-Primer ARMS-PCR Method

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Abstract

Single nucleotide polymorphism (SNP) C4685T located in 14. intron of bovine CAPN1 gene have shown significant association with a higher lean share in valuable cuts for mutant genotype TT. The work was oriented to developed a sensitive single tube tetra-primer amplification refractory mutation system PCR (ARMS-PCR) method for detection of C4685T polymorphism in CAPNI gene and analysis of genotype structure in population of 130 animals of Slovak Pinzgau cattle. The genomic DNA was isolated from samples of blood and hairs of cattle. Design of primers for ARMS-PCR was realized by using program Tetra-Primer ARMS-PCR. The presence of wild allele C and mutant allele T on agarose gel was detected by one control 439 bp fragment for both alleles and one specific fragment for each allele C - 204 bp and T - 290 bp. For the checking of correct genotyping was used PCR-RFLP method with restriction endonuclease *Bse*GI. In the population of Slovak Pinzgau cattle we detected all genotypes. There were detected homozygote genotype CC with frequency 0.3308, heterozygote genotype CT with frequency 0.4 and homozygote genotype TT with frequency 0.2692. Frequency of alleles C and T for SNP C4685T of gene CAPN1 were 0.5308 and 0.4692.

Keywords: ARMS-PCR, bovine CAPN1 gene, SNP C4685T, Slovak Pinzgau cattle.

1. Introduction

Meat tenderness is an important issue in beef cattle production because it has a major impact on consumer satisfaction [1]. The calpain-calpastatin system is an endogenous, calcium - dependent proteinase system, theorized to mediate the proteolysis of key myofibrillar proteins during postmortem storage of carcass and cuts of meat at refrigerated temperatures [2]. Calpain is a ubiquitous cytoplasmic cysteine protease, the activity of which is absolutely dependent on calcium [3]. Suzuki and Sorimachi [4] 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 [5].

The *CAPN1* gene that code protease μ-calpain *(EC 3.4.22.52)* is located on bovine chromosome 29 [6]. Page et al., [7] discovered the SNP of CAPN1 gene located in exon 9. The base of this SNP is substitution C/G in position 5709 sequence *AF252504* which resulted in one amino acid substitution alanine/glycine in position 316 in sequence of amino acid chain and the marker was coded as CAPN316. The favourable allele C in exon 9 of the *CAPN1* gene is present in the most common *Bos taurus* beef breeds, but at low to intermediate frequencies [8].

Kubiak-Juzscuk et al., [9] identified new mutation in the 14. intron of bovine CAPN1. The basis of this mutation is substitution of T/C in position

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4685 sequence *AF248054*. The same authors described an association of the occurence allele T with a higher lean share in valuable cuts in cattle.
The next SNP polymorphism was discovered in CAPN1 introne [10]. The name of this SNP is CAPN4751 and a substance is substitution C/T in position 6545 sequence *AF248054*. The studies of CAPN4751 presence in cattle population showed that the increased frequency of allele C which is associated with the more tenderness meat, were detected in population Bos taurus cattle and the increased frequency allele T were detected in Bos indicus cattle.
The aim of this study was to optimalize fast and cost ARMS-PCR method for detection of SNP C4685T and to analyse the frequency of genotype TT and allele T in population of Slovak pinzgau cattle.

2. Materials and methods

In this study were collected bloods samples from 130 animals of Slovak pinzgau breed. Bovine genomic DNA was isolated by fenol-chlorophorm deproteinization and ethanol precipitation and by using commercial kit Nucleospin Blood (Macherey Nagel) too.
PCR-RFLP: The PCR amplification of specific 670 bp fragment DNA included SNP in 14. intron which is characterize substitution of C/T in position 4685 of the bovine genomic *CAPN1* gene sequence GenBank: AF248054. For PCR amplification were used specific primers [9]. The sequences of primers were: forward primer 5´-TTC AGG CCA ATC TCC CCG ACG - 3´ and reverse primer 5´ - GAT GTT GAA CTC CAC CAG GCC CAG - 3´. The reaction mixture in the total volume 25 µl containing 100 ng DNA, 1.25 U Taq polymerase (Fermentas), 1 x PCR buffer KCl, 1.75 mM $MgCl_2$, 200 µM dNTP, 5 pM of each primer. The PCR reaction was optimized in the gradient thermocycler MJ Mini™ (Biorad, USA). The following amplification parameters were applied: 94 °C for 5 minutes followed by 30 cycles: 95 °C for 30 seconds, 62 °C for 45 seconds, 72 °C for 45 seconds. The reaction was completed by the final synthesis: 72 °C for 10 minutes. The PCR products were digested by restriction endonuclease FastDigest *Bse*GI (Fermentas). The digestion was performed with 10 µl of PCR product mixed with 1 µl of the restriction enzyme, 2 µl of 10 x FastDigest buffer and ultra pure H_2O in total volume 25 µl. The run conditions were 5 minutes at 37 °C. Digested fragments were visualized by electrophoresis on 2 % agarose gel (Invitrogen) containing GelRed dye (Biotium) at 200 V in 1 x sodium borate buffer for 15 minutes and the gel was analyzed by UV rays .
ARMS-PCR: The PCR amplification of allele specific fragments which representing specific alleles C and T of SNP C4685T was analyzed ARMS-PCR method. The sequence of using PCR primers were designed by program Tetra-Primer ARMS-PCR [11] from sequence *AF248054*. The primers sequences were: CAPN4685 FOR outer primer : 5´GAAGAGCGCAGGGACCCAGTGAGTAGA – 3´, CAPN4685 REV outer primer : 5´-CCTCTGAGAGGAGAGACGGTGACAGCA – 3´, CAPN4685 FOR inner primer: 5´-AGCCCCTCCTGGCAGGAGCCATA<u>A</u>AT - 3´, CAPN4685 REV inner primer : 5´-ACTGTAAAGTGCTTGGCAGAGTAGGG<u>C</u>TG – 3, where the underlying bases are mismatch base. The reaction mixture in the total volume 25 µl containing 100 ng DNA, 1 U Taq polymerase (Fermentas), 1 x PCR buffer $(NH_4)_2SO_2$, 2.5mM $MgCl_2$, 800 µM dNTP, 5 pM of each outer primers and 15 pM of each inner primers. The PCR reaction was optimized in the gradient thermocycler C1000™ Thermal Cycler (Biorad). For the specific annealing of primers was used for first nine cycles touchdown from 65°C to 56 °C with time 30 seconds. The following amplification parameters were applied: 94 °C for 5 minutes followed by 25 cycles: 94 °C for 30 seconds, 56 °C for 30 seconds, 72 °C for 30 seconds. The reaction was completed by the final synthesis: 72 °C for 10 minutes. Allelic specific fragments were loaded on 2.5 % agarose gel (Invitrogen) containing GelRed dye (Biotium) at 200 V in 1x sodium borate buffer for 15 minutes and the gel was analyzed in the UV rays.

3. Results and discussion

PCR-RFLP: The digestion of 670 bp PCR product for SNP C4685T of CAPN1 gene with restriction endonuclease *BseGI*I (Fermentas) differentiated alleles C and T. The *Hha*I digestion

of the PCR products produced two fragments for allele C (530 bp, 140 bp) and one fragment for allele T (670 bp). The PCR-RFLP method was used for genotyping of 56 steers of Slovak pinzgau cattle. In this group of animals were detected the all genotypes – CC, CT and TT (Figure 1).

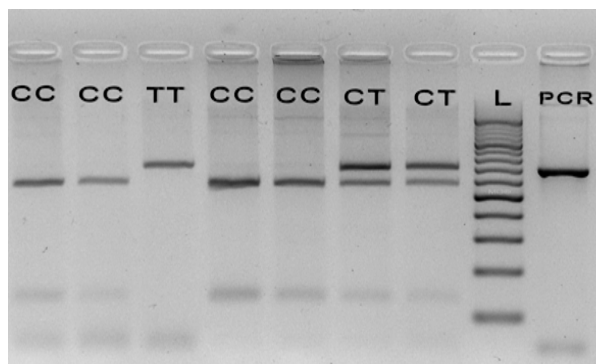

Figure 1. Representatively results of analysis PCR-RFLP for CAPN1 gene (670 bp) by restriction enzyme *Bse*GI on 2 % agarose gel
Homozygote genotype CC (530 bp, 140 bp), homozygote genotype TT (670 bp), heterozygote genotype CT (670 bp, 530 bp, 140 bp), L – ladder 100 bp (Fermentas), PCR – product of PCR (670 bp).

ARMS-PCR: The principle of ARMS-PCR method is using two outer primers which produce outer control fragment of PCR reaction and two inner primers. The primers are designed such that the two primer pairs overlap at a SNP location but each match perfectly to only one of the possible allele. The allele specific primers differentiated alleles C and T. Genotype CC was detected by specific 204 bp fragment for allele C and one control fragment with size 439 bp. The genotype TT was detected on the base of presence specific 290 bp fragment for allele T and one 439 bp control fragment. The heterozygous genotype CT was detected by specific 204 bp and 290 bp fragments and one control fragment with size 439 bp (Figure 2). We analysed others 76 cows from population of Slovak pingau cattle with ARMS-PCR method.

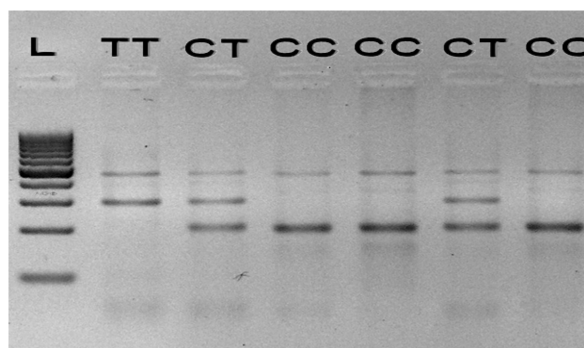

Figure 2. Representatively results of analysis ARMS-PCR for SNP C4685T of CAPN1 gene on 2.5 % agarose gel
L – ladder 100 bp (Fermentas), TT genotype (439 bp, 290 bp), CT genotype (439 bp, 290 bp, 204 bp), CC genotype (439 bp, 204 bp).

Genetic variants: By using PCR-RFLP and ARMS-PCR method was analysed 130 animals Slovak pinzgau cattle. We detected all three genotypes CC, CT, TT for SNP C4685T in 14. intron of CAPN1 gene. Frequencies of genotypes and alleles determined in the population are presented in Table 1. The frequency of occurrence for homozygous genotype CC was 0.33. The frequency of heterozygous genotype CT was 0.4 and homozygous genotype TT was 0.27. The frequency of allele T was 0.47 and allele C was 0.53. Our results showed that SNP C4685T had approximately similar value for frequency mutant C and wild T allele for population of Slovak pinzgau cattle. The increased frequency of occurence allele T were detected in population of charolais breed (0.7) and limousine (0.65) [4]. In contrast to population of polish red and black-and-white cattle they detected low frequency of allele T – 0.17 and 0.27. The similar low frequency for allele T (0.27) was detected in population of friesen bulls [12].
In population of simental and hereford were detected frequency of favorite allele T with frequencies 0.45 and 0.39 [4].

Table 1. Frequency of genotypes and alleles of *CAPN1* gene for SNP C4685T in the population of Slovak pinzgau cattle in Slovak Republic

SNP C4685T		Alleles		Genotypes		
Slovak Pinzgau cattle	n	C	T	C/C	C/T	T/T
Total	**130**	**0.5308**	**0.4692**	**0.3308**	**0.4**	**0.2692**

4. Conclusions

We detected the genotype CC with frequency 0.33 and a predominance of mutant allele C (0.53) and the preferable homozygous genotype TT with frequency 0.27 and frequency of allele T – 0.47. The frequency of heterozygous genotype CT was 0.4. It may be concluded that ARMS-PCR method presented the cheapest and quickest method for detection of SNP C4685T and can be use for fast detection with the other marker as CAPN316 and CAPN4751 of CAPN1 gene for which were ARMS-PCR developed [13].

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