TaqMan allelic discrimination assay for *A1* and *A2* alleles of the bovine *CSN2* gene

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ABSTRACT: Alleles *A1* and *A2* of the *Bos taurus CSN2* gene are the most frequent in a number of dairy cattle breeds. In this study, a new allelic discrimination assay using TaqMan fluorogenic probes was developed to detect single nucleotide substitution characterizing the *A1*/*A2* alleles of the *CSN2* gene. The method was validated using DNA samples of known genotypes with different concentrations and the results were compared with those for the commonly used problematic ACRS-PCR. We found the TaqMan method to be more effective, 100% reliable and hundred times more sensitive for testing the *CSN2* genetic marker in cattle than the ACRS-PCR. As it enabled a rapid analysis of a large number of DNA samples in uniform format without previous DNA quality assessment and without the requirement for post-amplification manipulations, it presents an effective tool for the analysis of large-scale sample sets. The method was applied for testing on a sample of 120 Czech Holstein dairy cows. The observed relative genotype and allele frequencies were as follows: *A1A1*–0.20, *A1A2*–0.51, *A2A2*–0.29; *A1*–0.45, *A2*–0.55.

Keywords: allelic discrimination; *CSN2*; alleles *A1* and *A2*; cattle

A recent review of the bovine beta-casein (*CSN2*) gene has described its 13 alleles (Kamiński et al., 2007). Commercial analyses have however been done mostly on the best known *A1*, *A2* allele typing and according to the results of a large number of studies, cow's milk with the *A2A2* genotype possesses parameters with decidedly greater advantage to consumers than milk with the *A1A1* genotype. A number of authors have reported that the A2 type protein may have a positive impact on human health with a preventive function in the case of a number of diseases (McLachlan, 2001; Laugesen and Elliott, 2003; Sun et al., 2003; Tailford et al., 2003; Birgisdottir et al., 2006; Venn et al., 2006). The *CSN2* gene polymorphism has also been shown to be associated with specific milk performance parameters (Kučerová et al., 2006).

The MAS (Marker-Assisted Selection) process can play an important role in increasing the quality of milk/dairy products and in acquiring specific milk properties as indicated by results described by many authors (Hanuš et al., 1995; Michalcová and Krupová, 2007; Hradecká et al., 2008; Matějíček et al., 2008). The challenge for scientists remains the development of new, more powerful and more reliable methods which will give breeders new options. In this regard, TaqMan technology has found application in a number of studies in recent years as a basic and applied research tool (Leutenegger, 2001; Liu et al., 2006). Owing to its high sensitivity and accuracy, its universal application, simple reproducible implementation and finally its continuous development supported by commercial research, we can anticipate greater expansion of TaqMan assays in the future.

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To facilitate genotyping in a large number of samples, we sought to design a TaqMan probe assay to detect the single base-pair substitution in samples. Our new TaqMan allelic discrimination (AD) method for testing the SNP *rs43703011* (www.ncbi.nlm.nih.gov) of the *CSN2* gene could replace the less effective, less reliable and mostly used ACRS-PCR analysis.

MATERIAL AND METHODS

Animals

Samples from 120 Holstein dairy cows kept on two South Moravian farms, representing purebred animals (HA category), were tested.

DNA

The DNA used in TaqMan assay and ACRS analyses was isolated from somatic cells of cow's milk and bovine blood using the column method (Jetquick blood and cell culture DNA spin kit, Genomed^{SM}, St. Louis, MO, USA).

ACRS analysis

The amplification created restriction site method (ACRS) was carried out according to McLachlan (2003) for confirming the results from the TaqMan assay. Briefly, a set of primers was used to amplify a 120 bp PCR fragment. The restriction site was created in the amplification step using a reverse primer with the nucleotide mismatch at the 3' end. A *Dde*I restriction site occurred when the nucleotides CT were present at nucleotide 200 and 201 (positions 2 and 3 of codon 67) of the *CSN2* gene. This specific digestion resulting in the occurrence of 86 bp and 35 bp fragments positively identified the presence of the CCT (proline) codon – the *A2* allele of the *CSN2*. PCR was carried out in 25 µl reaction volume with PTC-200 Peltier Thermal cycler (MJ Research, Waltham, MA, USA). PCR master mix with 50–100 ng DNA contained 10× PCR buffer; *Taq* DNA polymerase, 1U/25 µl (Fermentas, St. Leon-Rot, Germany); 2mM $MgCl_2$; 200mM dNTP; 200mM of each primer. The amplification was as follows: 94°C/10 min; 94°C/45 s; 60°C/30 s; 72°C/30 s; 40×; 72°C/10 min. Specific *Dde*I digestion of the PCR product was performed using 2U of restriction enzyme (New England Biolabs, Ipswich, MA, USA) per reaction in 1.5mM buffer, and incubated at 37°C overnight. The PCR-ACRS products were subjected to electrophoresis in 3.5% agarose gel and visualized by ethidium bromide staining.

TaqMan real-time PCR

Two primers were designed to amplify the 101 bp product involving SNP *rs43703011* (genomic DNA: *X14711* (http://www.ncbi.nih.gov); forward primer, 5´-CTTTGCCCAGACACAGTCTCTA-GT-3´; reverse primer, 5´-GCACCACCACAGGGG-TT-3´). Two fluorogenic TaqMan probes were designed with different fluorescent dye reporters to allow single-tube genotyping. The first probe was targeted to the wild type allele *A2* (5´-YAK-TGGACCCATCCCTAACAGCCTCCC-BBQ-3´) and the second one to the mutated allele *A1* (5´-FAM-CTGGACCCATCCATAACAGCCTCCCA-BBQ-3´) of the *CSN2* gene. The powerful BBQ quencher was linked to the 3´ end of both probes (BlackBerry quencher, Berry and Associates, Dexter, MI, USA). Primers and probes were designed using Primer Express software, version 2.0 (Applied Biosystems, Foster City, CA, USA) and were obtained from TibMolBiol (Berlin, Germany). The accuracy of the used sequence source was verified by comparison with sequences from the GenBank database using BLAST (http://www.ncbi.nlm.nih.gov/BLAST/). Real-time PCR was performed in 20 µl reactions with 10 µl of TaqMan universal PCR master mix containing AmpliTaq Gold DNA Polymerase (Applied Biosystems, Foster City, CA, USA), 300nM concentration of forward and reverse primer, 200nM of each probe, 0.2 µl UNG (uracyl-N glycosylase) and 1 µl (50–100 ng) of sample DNA. The PCR reaction was realised using the 7500 Real Time PCR System (Applied Biosystems). The time and temperature profile of the PCR reaction consisted of the following steps: 2 min at 50°C for UNG activation, 10 min at 95°C for starting AmpliTaq Gold activity, 40 cycles of 95°C for 15 s and 60°C for 1 min.

As a negative control, we used a sample without template. The negative control was helpful for measuring any false positive signal caused by contamination. An allelic discrimination experiment consisted of three steps: a pre-read run, an amplification run and a post-read run. After determin-

ing the baseline fluorescence at the first step, we started the PCR reaction and created the real-time PCR data. In the post-read run, the allele names were then assigned via the evaluation of baseline fluorescence that was determined at the pre-read run and at post-PCR fluorescence, respectively. Each sample was visually verified by analysing the generated PCR curves.

Analyses of amplification products were performed using SDS software, version 1.2. The efficiency of amplification (E) was calculated from the slope of the standard curves generated by individually screening each probe when testing diluted heterozygote samples. The following algorithm was used (Livak and Schmittgen, 2001):

$$E = 10^{-1/slope}$$

$$(\%)\ Efficiency = (E - 1) \times 100\%$$

The optimal slope of the standard curve (–3.32) or the E = 100% characterises absolute duplication of template in each cycle. The results of the TaqMan allelic discrimination (AD) assay were graphically interpreted as a function of ΔR_n

where:

R_n (normalised reporter) = ratio of the reporter dye fluorescence signal intensity to the fluorescence intensity of the passive reference dye signal

R_{n1} = pre-read baseline fluorescence of normalised reporters

R_{n2} = post-read PCR fluorescence of normalised reporters

$\Delta R_n = R_{n2} - R_{n1}$

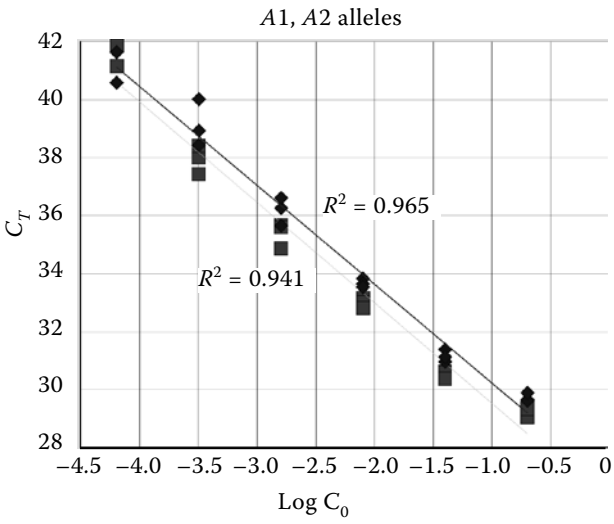

Figure 1. Real-time PCR standard curves for *A1* and *A2* AD assay; for more accurate results, the heterozygote samples were diluted; dilutions of standard samples ranging linearly from 3×10^{1} to 2×10^{-4} µg DNA per reaction were amplified by the standard real-time PCR protocol; the resulting C_T values were plotted as a function of the log DNA quantity; each dataset is represented as 3 sample reactions performed simultaneously; the linear ranges of detection were 6×10^{-1}–2×10^{-4} µg DNA per reaction for both probes; the value of the amplification efficiency (E) was estimated as 94.1% with FAM reporter dye detection and 96.5% with YAC reporter screening; the values of E obtained by measuring two different fluorescence dyes in diluted heterozygote samples were equal; this implies the identical binding efficiency of both probes

RESULTS

The SNP *rs43703011* of the bovine *CSN2* gene was chosen for the development of a TaqMan real-time PCR genotyping assay. To save universal TaqMan thermal cycling parameters, the primers were designed for 58–60°C annealing temperature, as calculated by Primer Express software. The probe design guidelines also included the condition of 10°C higher Tm (melting temperature) than the Tm of the primers. This ensures that the probe is fully hybridized during primer extension. Apropos the primer concentration, the TaqMan assay gives optimal performance by selecting the primer concentrations that provide the lowest C_T (cycle threshold) and highest ΔR_n values for a defined amount of target template. In our case, the optimal values of C_T and ΔR_n were reached using at least 300nM concentration of forward and reverse primer for a broad range of DNA quantity. Both *A1* and *A2* probes functioned correctly with the recommended 250nM concentration.

To assess the sensitivity and accuracy of the TaqMan assay, we used linear diluted DNA templates with heterozygote genotype. We tested these samples and in this way we estimated the efficiency of amplification (E) (Figure 1). The recording of amplification diluted heterozygotes revealed E = 94.1% through FAM reporter screening and E = 96.5% through YAC reporter detection. These results show the assay was robust and reproducible. Regarding the optimal amplification curve, the running of the TaqMan AD assay worked well with a DNA interval of $6 \times 10^{-1} - 2 \times 10^{-4}$ µg (Figures 2 and 3). The sensitivity of the TaqMan assay was also compared with the standard ACRS-PCR pro-

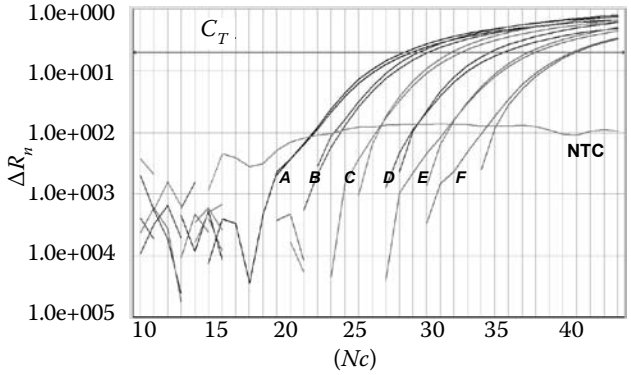

Figure 2. Real-time PCR amplification curves (ΔR_n = log N_C): comparison between the amount of used DNA and C_T values in our TaqMan AD assay; each curve originated as a ratio from 5 heterozygote sample curves; both FAM and YAC reporter fluorescence curves screening for each data set are depicted in the figure; (DNA amount (μg): A = 6×10^{-1}, B = 1.2×10^{-1}, C = 2.4×10^{-2}, D = 4.8×10^{-3}, E = 9.6×10^{-4}, F = 1.9×10^{-4}); ΔR_n – fluorescence variation, N_C – number of cycles, C_T – cycle threshold, NTC – no template control

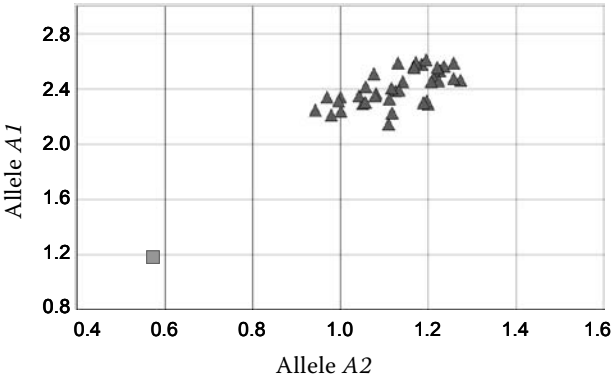

Figure 3. Testing of diluted samples – demonstration of the high sensitivity and universality of our TaqMan assay; regardless of the large differences in the quantity of used DNA (1×10^{1}–2×10^{-4} μg), all tested heterozygotes were placed accurately on the TaqMan AD plot; (▲) heterozygotes, (■) NTC – no template control

tocol by analysing diluted samples of known *CSN2* genotypes. Using the ACRS-PCR method, bands on the electrophoresis were visible down using on average 3×10^{-3} μg of template DNA, whereas the TaqMan assay detected down using 3×10^{-5} μg of template DNA per reaction. The TaqMan assay had thus an at least 100-fold higher sensitivity than the standard ACRS-PCR method.

The TaqMan assay and ACRS-PCR methods were used to genotype 120 individuals of the Holstein cattle breed. The results for the relative genotype frequencies were: *A1A1*–0.20, *A1A2*–0.51, *A2A2*–0.29, the relative allele frequencies were *A1*–0.45 and *A2*–0.55. The representative allelic discrimination plot used to assign the genotypes is displayed in Figure 4. The genotypes of the *CSN2* gene were easily discriminated by their distribution along the axis of the *A1* and *A2* probe. Samples that clustered with the NTC sample contained no template. The results from the TaqMan method corresponded with ACRS-PCR analyses exactly. However, when we used a DNA template of smaller quantity (3×10^{-2}–3×10^{-3} μg per reaction), the TaqMan assay results corresponded with those from ACRS-PCR analyses for only 80 of the total 120 tested samples.

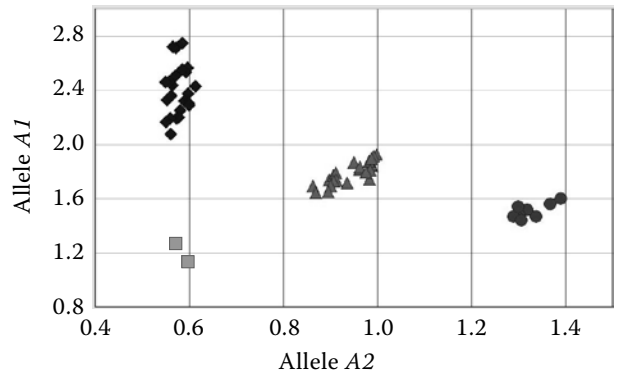

Figure 4. Testing of the bovine *CSN2* gene polymorphism in Holstein cattle with TaqMan AD assay; *A1* allele (♦), *A2* allele (●), heterozygotes (▲), no template control NTC (■); all samples with ACRS estimated genotypes were also correctly identified by the TaqMan method; data were plotted using ΔR_n (post-PCR fluorescence minus baseline fluorescence determined at the pre-read step) of the FAM and YAC reporter dye on the *x*, *y* axes of allele *A1* and *A2*

DISCUSSION

The ACRS-PCR is one of the most widely used methods for genotyping the bovine *CSN2* gene. However, this method still requires on average two working days for complete analysis. Secondly, post-amplification manipulations increase the possibility of PCR product carryover leading to incorrect results. Nonspecific ACRS-PCR products occur as a result of an increased number of PCR cycles compensating low concentrations of the used DNA template. Another reason may be an excessive quantity of ACRS-PCR product for specific cleavage with restriction enzyme and this induces residual uncleaved bands on the electrophoresis gel. These drawbacks limit the standard ACRS-PCR method

for testing the *CSN2* gene polymorphism to narrow optimal reaction conditions (high quality of DNA, optimal composition of PCR master mix, number of PCR cycles), where an individual approach to each set of samples is required. In a commercial environment, this cannot be guaranteed owing to the time pressure.

The earlier published allelic discrimination method based on primer length technology (ADPL) or the allele-specific PCR, bi-directional PCR and ARMS-PCR system, have not resulted in massive expansion for *CSN2* genotyping (Lindersson et al., 1995; Rincón and Medrano, 2003). Who? Described the new MALDI-TOF MS analysis for screening *A1*, *A2* alleles of the *CSN2* gene apart from the above-mentioned ACRS method. The reliability and sensitivity of this method are undoubted but the higher financial and technical costs remain problematic.

On the other hand, the TaqMan system has been shown to be rapid, maximally sensitive and suitable for the detection of a range of targets. Recently, a large number of TaqMan assays for analysing SNP markers affecting specific performance parameters in livestock have been developed (Renaville, 2005; Barendse et al., 2008). TaqMan real-time PCR monitors the formation of PCR product by measurement of reporter dye fluorescence obviating post-amplification analyses of PCR product. To exclude the possibility of contamination, the AmpErase system (Applied Biosystems, Foster City, CA, USA) could be used. The present assay was designed to conform with standard real-time thermocycling conditions (40 cycles containing a 60°C annealing and extension step), allowing the assay to be performed simultaneously with other assays without additional steps. This benefit is critical for creating a multiplex TaqMan assay system capable of detecting multiple dyes with distinct emission wavelengths (Rudi et al., 2006). The use of one crucial reagent (TaqMan Universal PCR Master Mix) for all assays is another factor simplifying the process of assay implementation. Using a 96 or 386-well plate format, the AD analysis should genotype a large set of samples in less than two hours. Currently, non-invasive methods for sampling are preferred as they are trauma-free to the animals. Receiving of small milk samples could be an alternative. As the count of milk somatic cells is highly variable, the isolate has a broad range of DNA concentration. For this reason the advantages of the TaqMan assays could be maximally exploited.

The equally high levels of E obtained by screening via two specific probes with different reporter dyes for heterozygote samples refer to the identical binding efficiency of both probes. This feature as well as the constantly exponential profile of PCR amplification offers other possibilities for utilizing our assay. After preparing the correct mixture of DNA sample (pooled DNA), it is possible to quantify the frequency of both *CSN2* alleles there (Yu et al., 2006). In this case, with one TaqMan reaction, it should be possible to estimate the allele frequency of the *A1*, *A2* alleles for the whole sample set in a very short time. The condition of using a suitable quantity of DNA per reaction (1×10^{1}–2×10^{-4} μg in our study) is particularly important when gene expression or quantitative assay is to be carried out. However, the AD experiment was successfully completed using both higher (3.5 μg) and lower (1×10^{-4} μg) quantity of DNA.

The *CSN2* gene is assumed to be a perspective marker for cattle breeding. In conclusion, we have established a more effective and a more reliable method for testing known SNP of the *CSN2* gene in cattle than the commonly used ACRS-PCR method. The TaqMan assay had a 100% genotyping accuracy, rapid performance and a 100-times greater sensitivity than the standard ACRS-PCR method. These findings are also in agreement with results of Campsall et al. (2004). Losing post-amplification steps, possible mismatches and other technical problems are avoided. This makes the method ideal for commercial use. Our method could be exploited particularly in the MAS process of dairy cattle and it could also be used in the evaluation of breeding value or in the determination of genetic variability and breed distances (Čítek et al., 2006).

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