High resolution melting as an alternative method to genotype diacylglycerol O-acyltransferase 1 (*DGAT1*) *K232A* polymorphism in cattle

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ABSTRACT: PCR-RFLP analysis is a common method for genotyping the *DGAT1 K232A* polymorphism in cattle. Our purpose was to develop a high resolution melting (HRM) assay in order to genotype the polymorphic alleles. Firstly, the PCR-RFLP method was used and the 411 bp products including the *DGAT1* polymorphism were digested by *Cfr*I enzyme. Direct sequencing was performed to confirm genotypes of the *K232A* polymorphism for 30 samples that presented different PCR-RFLP patterns. It was determined according to sequencing results that partial enzyme digestion had occurred for some samples. A 130 bp fragment including the polymorphism was amplified for real time PCR. Then, the HRM analysis was carried out using two fluorescent dyes, SYBR Green I and EvaGreen™. Although the HRM genotyping using SYBR Green I was contradicted by the sequencing results, three correct melting curves were obtained for the *K232A* polymorphism when EvaGreen™ was used. There were no false genotypes and all genotypes were in agreement with their sequencing results. The difference in the T_m between the two homozygous groups was about 0.5°C and the *AA* genotypes showed a higher T_m than the *KK* genotypes. The heterozygous genotypes showed a different pattern. Similar results were obtained from different concentrations of EvaGreen™ in the reactions. All 206 DNA samples were genotyped using this fluorescent dye with estimated allele frequencies of 0.66 and 0.34 for the *A* and *K* alleles, respectively. Our study showed that HRM analysis will be applicable for genotyping the *DGAT1 K232A* polymorphism in large populations of dairy cattle.

Keywords: HRM analysis; PCR-RFLP; *K232A* polymorphism

The *DGAT1* protein is an enzyme catalyzing the final step of triglyceride synthesis (Coleman et al., 2000). The bovine *DGAT1* gene was mapped to the centromeric end of BTA14 and completely sequenced (Grisart et al., 2002; Winter et al., 2002). A polymorphism in exon 8 of the gene was found (*K232A*) which results in the non-conservative substitution of amino acid 232 (Grisart et al., 2002; Winter et al., 2002). The *K232A* lysine allele is associated with an increase in fat and protein content, as well as fat yield, whereas the *K232A* alanine allele increases milk and protein yield (Grisart et al., 2002, 2004; Winter et al., 2002; Thaller et al., 2003; Gautier et al. 2007; Hradecká et al., 2008).

Detection of allelic variation at *K232A* polymorphism of the *DGAT1* gene in different breeds has been performed by diverse assays such as DNA pyrosequencing (Naslund et al., 2008), oligonucleotide ligation assay (OLA) (Grisart et al., 2002), TaqMan allelic discrimination (a labelled probe-based method) technology (Gautier et al., 2007; Schennink et al., 2007) and PCR-SSCP method (Ripoli et al., 2006; Nowacka-Woszuk et al., 2008). Winter et al. (2002) carried out a PCR-RFLP analysis by the *Cfr*I restriction enzyme for genotyping the *K232A* polymorphism that has been employed by most of other researchers in their studies on this polymorphism (Thaller et al, 2003; Kaupe et

al., 2004; Kuhn et al., 2004; Lacorte et al., 2006; Hradecká et al., 2008), in which an undigested fragment of 411 bp indicates the *K* allele, and two fragments of 203 and 208 bp indicate the *A* allele.

All these methods have their advantages and disadvantages in terms of simplicity, performance, sensitivity, turn-around time and cost (Krypuy et al., 2006). For example, DNA sequencing has been considered the "gold standard" technique because it can identify a specific mutation that may be present (Gonzalez-Cadavid et al., 1989). However, it has the disadvantage of a high relative cost for genotyping numerous samples. Pyrosequencing has the advantage of greater sensitivity and low cost (Ogino et al., 2005), but the expense of the equipment has limited its uptake. Fluorescently labelled probe-based methods such TaqMan may be used for mutation detection, but only for the bases covered by the probe, thus they are not amenable to mutation scanning (Vaughn and Elenitoba-Johnson, 2004). Methods such as PCR-RFLP or PCR-SSCP require the separation of PCR products on a gel or other matrix, which often takes hours to perform and increases the risk of contamination in future reactions.

Recently, a mutation scanning method using high resolution melting (HRM) analysis has been described (Wittwer et al., 2003). HRM analysis is a rapid and closed-tube mutation scanning assay that detects sequence variation within the PCR products by use of a saturating double-stranded DNA (dsDNA) dye. This method does not require any post-PCR manipulation of samples, expensive labelled probes (Wittwer et al., 2003), restriction enzymes or gel separation (Vaughn and Elenitoba-Johnson, 2004). HRM analysis characterizes DNA samples according to their dissociation behaviour as they transition from double stranded DNA (dsDNA) to single stranded DNA (ssDNA) with increasing temperature by measuring the release of the dye.

An important advantage of HRM over many of the other methods is that it is an in-tube technology in which the analysis is performed immediately after the amplification, and thus it is particularly suitable for medium to high-throughput applications. Methods that require the amplified PCR product to be removed from the tube for analysis are inevitably more laborious and require stringent precautions to prevent crossover of PCR products. The purpose of this study was to develop an HRM assay in order to genotype the *K232A* polymorphism alleles in exon 8 of the *DGAT1* gene.

MATERIAL AND METHODS

Blood samples were obtained from 206 head of Holstein cattle belonging to a large commercial herd in Iran. Genomic DNA was isolated from the blood using the commercially available Master Pure™ DNA Purification Kit (Epicenter, USA) and the purity was assessed by spectrophotometer. Samples with an optical density (OD) ratio (260 nm/280 nm) between 1.7 and 1.9 were used for analysis.

PCR-RFLP assay

PCR amplification was performed to obtain the 411 bp product including the *DGAT1 K232A* polymorphism of exon 8, as described by Winter et al. (2002). The 25 µl reaction mixture contained 1.5mM $MgCl_2$, 200µM of each dNTP, 0.25µM of each primer, 1× PCR buffer, 1 IU *Taq* polymerase (MBI Fermentas, Germany) and 100 ng of genomic DNA template. Thermal cycling conditions included: an initial denaturation step at 94°C for 5 min followed by 35 cycles of 94°C, 60°C, and 72°C (1 min each) and a final extension for 10 min at 72°C. Digestion of the PCR products was performed overnight with 5 IU of the *Cfr*I restriction enzyme (MBI Fermentas, Germany) at 37°C. Uncut fragments (411 bp) for the *K* allele (lysine allele) and the two fragments of 203 bp and 208 bp for the *A* allele (alanine allele) were separated on 3% agarose gels (Figure 1).

DNA sequencing

Direct sequencing was performed to confirm genotypes of the *K232A* polymorphism for 30 samples that presented different PCR-RFLP patterns, including both homozygote and heterozygote genotypes (Figure 1). PCR products amplified with the PCR primers (Winter et al., 2002) were purified using the UltraClean™ PCR Clean-Up kit (MoBio Laboratories, Inc., Carlsbad, USA) according to the manufacturer's protocol. Sequencing was conducted in both directions using the Big Dye® Terminator Kit (Applied Bio Systems, USA). These sequences were then comparatively analyzed for the *K232A* polymorphism using Mac Vector™ 5.0.2 DNA Sequence Analysis Software. DNA of these 30 animals with known genotypes for the *K232A* polymorphism was used for optimizing the HRM analysis.

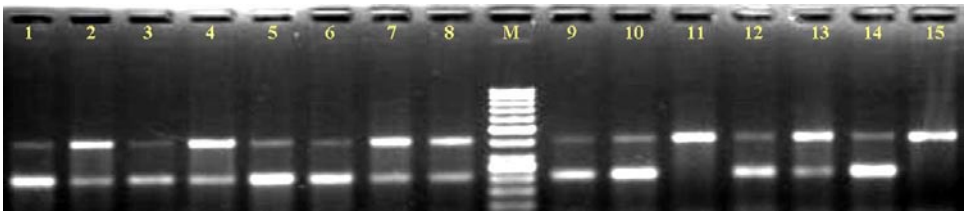

Figure 1. PCR-RFLP results of 411 bp fragment including the *K232A* polymorphism. Samples 11 and 15 showed uncut fragment (411 bp) and were homozygous *KK*. The remaining samples were apparently heterozygous because of the presence of 2 fragments on the gels. The *AA* genotype was not observed among samples based on this analysis. The sequencing results showed that partial digestion occurred using this method

RT-PCR amplification and HRM analysis

Optimization of the PCR conditions and monitoring of amplification efficiency in real time were carried out using the RotorGene™ 6000 (Corbett Life Science). Oligo 5.0 software was used to design a new pair of primers to amplify a 130 bp fragment including the *DGAT1 K232A* polymorphism. The forward and reverse primers were 5'-GGACCGGCAGGGGCTCG-3' and 5'-CCGCGGTAGGTCAGGTTGTCG-3', respectively. The reaction mixture contained 2.5mM $MgCl_2$, 125μM of each dNTP, 0.1μM of each primer, 1× PCR buffer, 1IU *Taq* polymerase (MBI Fermentas, Germany) and 50 ng of genomic DNA template in a total volume of 25 μl. The HRM analysis was carried out using two fluorescent dyes, SYBR Green I (1.5 or 3μM) and EvaGreen™ (1.5 or 3μM) (Quantace, UK). All samples were repeated in duplicate.

The amplification program consisted of an initial denaturation of 94°C for 5 min followed by 40 cycles of 94°C, 60°C, and 72°C (45 s each) and a final extension for 10 min at 72°C. After amplification, the melting analysis was immediately performed (without removing the samples from the machine) by slowly heating the reaction mixture from 75 to 90°C at a rate of 0.1°C/s. Amplification reactions were also routinely checked for the presence of non-specific products by agarose gel electrophoresis.

RESULTS

PCR-RFLP patterns

PCR-RFLP results of 15 samples are compared in Figure 1. Samples 11 and 15, which showed an uncut fragment (411 bp) on the agarose gel, were homozygous (*KK* genotype). The other samples were apparently heterozygous because of the presence of 2 fragments on the gel. The *AA* genotype for the *K232A* polymorphism was not observed among these samples based on the number of fragments.

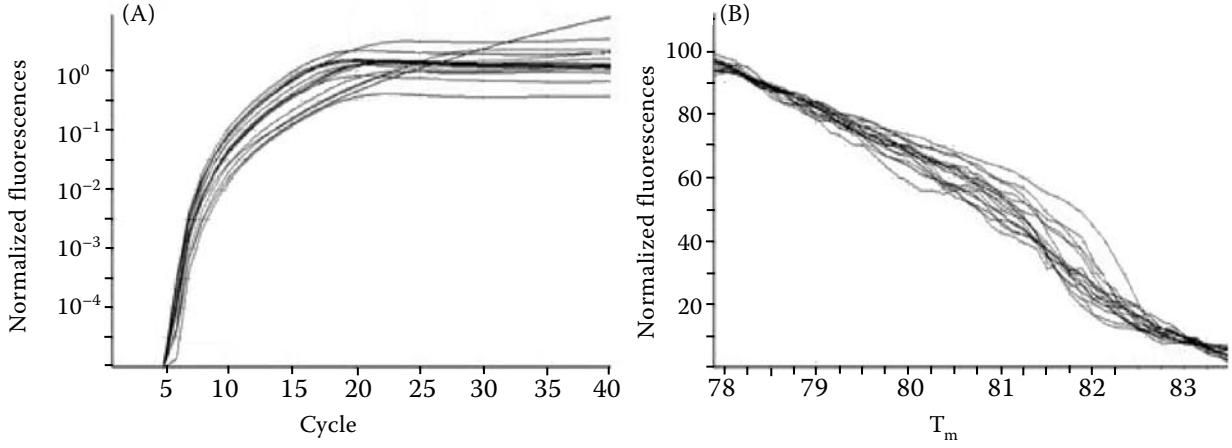

Figure 2. PCR products (A) and normalized high resolution melting curves (B) of a 130 bp fragment including the *DGAT1 K232A* polymorphism using SYBR Green I dye (3μM) in PCR reactions. There was inadequate amplification so that HRM analysis could not be used for genotyping

The presence of a faint band at 411 base pairs for some samples (e.g. 1, 3, 5, 6, 9, 10, 12, 14) is presumably due to only partial digestion of the PCR products by the *Cfr*I enzyme (Figure 1). To exclude any possibility of false genotyping, samples with ambiguous genotypes (especially the above-mentioned samples) were re-analyzed using different RFLP conditions (e.g. changing restriction enzyme, increasing restriction enzyme concentration, decreasing PCR product volumes in the digestion mix and even increasing digestion times), but the same results were obtained.

Sequencing results

Sequencing results for 30 samples re-confirmed the *KK* genotypes for samples 11 and 15. It was also confirmed that samples with a brighter band at ~400 base pairs and a fainter band at ~200 base pairs (Figure 1) were heterozygous *KA* genotypes. According to sequencing results, it was determined that ambiguous samples (e.g. 1, 3, 5, 6, 9, 10, 12, 14) were *AA* genotypes. Partial digestion by the enzyme is the most likely explanation of the faint band at ~400 base pairs for the *AA* genotypes.

RT-PCR and HRM analysis results

The results of HRM analysis for different concentrations of SYBR Green I dye in the reactions were compared (Figures 2 and 3). A high concentration of this fluorescent dye (3μM) prevented the amplification of 130 bp fragment so that melting curves were not obtained and samples could not be genotyped (Figure 2). Inhibition was confirmed by running the products on agarose gel.

However, the PCR amplification was successfully performed using lower concentrations (1.5μM) of SYBR Green I dye. Homozygous samples (*AA* and *KK*) could often be genotyped by an absolute change in T_m, while heterozygous samples (*KA*) could be identified through changes in the shape of the melting curves (Figure 3). The difference in the T_m between the two homozygous groups was about 0.5°C and the *AA* genotypes showed a higher T_m than the *KK* genotype because they had *GC* instead of *AA* dinucleotide at the *K232A* polymorphism. In addition, there were some samples showing a fourth pattern with a lower T_m than the *KK* genotypes but with melting curves parallel to two homozygous samples. These samples (15% of the total) were found to be false patterns as their sequencing results showed that they had the *AA* genotypes.

However, the three correct patterns of melting curves for the *K232A* polymorphism were obtained when the fluorescent dye was changed from SYBR Green I to EvaGreen™ (Figure 4). There were no false genotypes and all genotypes were in agreement with their sequencing results. The difference in the T_m between the two homozygous groups was similar to the *AA* genotypes using the SYBR Green I dye, with a higher T_m for the *KK* genotype and the heterozygous genotype showing a different pattern. Similar results were obtained from different concentrations of EvaGreen™ in the PCR

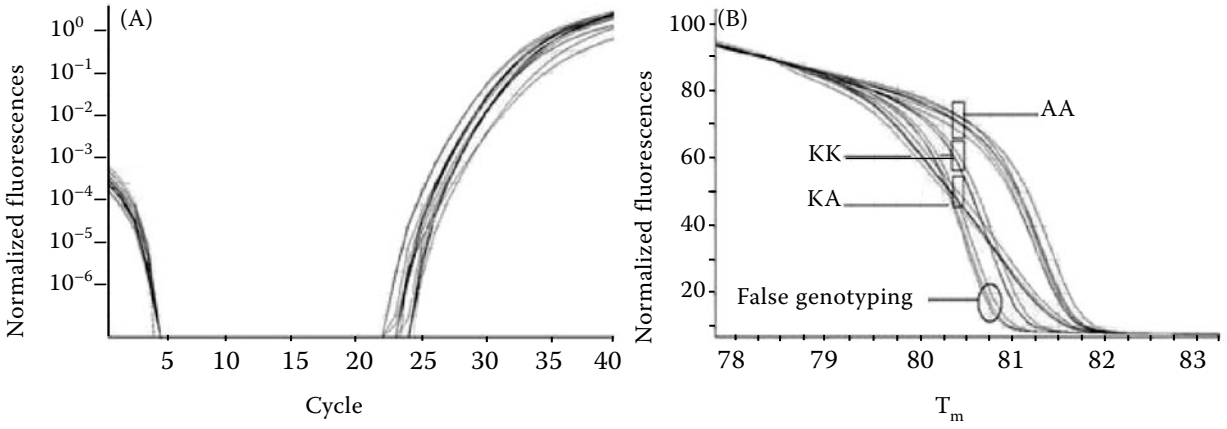

Figure 3. PCR products (A) and normalized high resolution melting curves (B) of a 130 bp fragment including the *DGAT1 K232A* polymorphism using SYBR Green I dye (1.5μM) in PCR reaction. Although the low concentration of SYBR Green I allowed amplification, there were four patterns of HRM curves whereas three patterns for different genotypes of the *K232A* polymorphism were expected. The fourth new pattern gave false genotypes in 15% of the samples

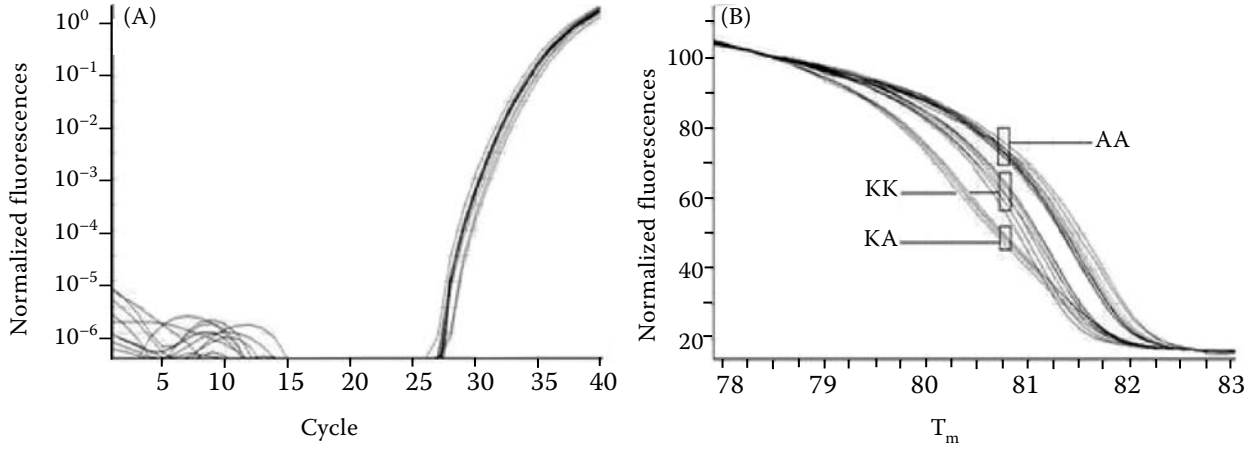

Figure 4. PCR products (A) and normalized high resolution melting curves (B) of a 130 bp fragment including the *DGAT1 K232A* polymorphism using EvaGreen™ dye in PCR reactions. Results for different concentrations of this dye (1.5 or 3μM) were the same. Three patterns were obtained for HRM curves and the genotype assignments agreed with sequencing results

and the confidence of the genotype assignment was more than 90% (this criterion is calculated by the RotorGene™ 6000 based on the similarity of melting curves to control samples for each genotype). All 206 DNA samples were genotyped using this fluorescent dye and the estimated allele frequencies were 0.66 and 0.34 for the *A* and *K* alleles, respectively.

DISCUSSION

Partial digestion is one of the main problems for genotyping SNPs using PCR-RFLP. It is more important when there is only one restriction site so that an undigested PCR product indicates the presence of an allele (like the *K232A* polymorphism of *DGAT1* gene). To solve this problem, some researchers used different primers to amplify a fragment including the *K232A* polymorphism and digested with the *Bgl*I enzyme so that two and three fragments were obtained for the allele *K* and *A*, respectively (Szyda and Komisarek, 2007; Komisarek and Michalak, 2008).

The PCR-RFLP method was used to genotype the *K232A* polymorphism initially, but partial digestion by the enzyme was observed (even after changing the conditions such as enzyme concentration, PCR product volume and digestion time) and genotyping a large number of samples would be difficult. Therefore, an HRM method was developed for genotyping this important polymorphism in dairy cattle.

HRM analysis is a pre-screening approach with improved turn-around time, which together with the reduced technical expertise required compared to gel-based methods such as PCR-RFLP, makes this method an ideal tool. HRM allows melt profiles of 96 or 384 different PCR products to be achieved in minutes, compared to at least 12 h for most gel-based methods (Tindall et al., 2009). HRM analysis has primarily been used for the discovery and genotyping of single nucleotide polymorphisms (SNPs) (Graham et al., 2005), but it has also been used for precise amplicon verification (Erali et al., 2006), sequence matching applications such as HLA identity (Zhou et al., 2004) and generating STS markers in linkage mapping studies (Croxford et al., 2008).

In our study, high concentrations of SYBR Green I dye in PCR reactions prevented amplification. In addition, 15% of the samples showed a fourth pattern using low concentrations of this dye and providing false genotyping results based on known sequences. Problems with SYBR Green I melting curve analysis have been well described with the preference of the dye for intercalating into *GC*-rich regions of amplicon and its tendency to translocation during melting phases (Monis et al., 2005).

The use of non-saturating dsDNA binding dyes (second generation dyes), such as SYBR Green I, may inhibit PCR when used at a concentration sufficient to saturate the number of dsDNA molecules generated during amplification. This can result in dye jumping during melting, which decreases the sensitivity

of mutation detection. In contrast to SYBR Green I, saturating dsDNA binding dyes (third generation dyes), such as SYTO®9, LC Green® and EvaGreen™, can be used at saturating concentrations without inhibiting PCR, thereby increasing the sensitivity and specificity of mutation detection (Wittwer et al., 2003; Herrmann et al., 2006). Our results also showed that EvaGreen™ dye is a suitable dye for genotyping the *K232A* polymorphism in dairy cattle in comparison with SYBR Green I dye. High concentrations of EvaGreen™ dye did not inhibit amplification. Moreover, no false genotyping results were observed using this dye and all genotypes were in agreement with the sequencing results.

The allele frequency estimated herein for the *K* allele (0.34) of the *DGAT1* gene is in agreement with a value almost identical to the 0.34 frequency reported by Winter et al. (2002) and Hradecká et al. (2008) in German Holstein. Also, Gautier et al. (2007) found *K* variant frequencies equal to 0.37 in French Holstein.

Although HRM analysis has been tested and shown to be successful in a number of research laboratories for clinical use, there are some main limitations for this approach. Firstly, it has been suggested that *GC*-rich regions and multiple melting domains should be avoided for HRM analysis (Krypuy et al., 2006). However, there are other reports (Reed and Wittwer, 2004) documenting that the *GC* content has no effect on HRM analysis, and that biphasic melting curves may be easier to analyze than those with a single melt domain. Secondly, HRM analysis requires careful consideration of primer design for amplification of small fragments (Krypuy et al., 2006). Also, good knowledge of the respective locus and its close vicinity in terms of polymorphisms is required. Thirdly, the different genotypes of each SNP must be found as controls for optimizing the HRM analysis and confirmed by sequencing. Furthermore, the costs of the dye have to be considered as well as the real-time PCR machine enabling the analysis of melting curves and fluorescent detection.

In conclusion, a quick alternative technique to the common PCR-RFLP method was developed for genotyping the *DGAT1 K232A* polymorphism in dairy cattle using HRM analysis. This approach will be useful for genotyping this polymorphism in large populations of dairy cattle. In addition, HRM seems to be suitable for improving efficiency and cost in QTL mapping studies and marker assisted selection programs.

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