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Technical note: A new and cost-effective method for detection of the bovine acyl-CoA:diacylglycerol acyltransferase 1 K232A polymorphism in cattle

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ABSTRACT

A new, quick, and inexpensive method for detecting the bovine acyl-CoA:diacylglycerol acyltransferase1 (*DGAT1*) polymorphism (K232A) through tetra-primer amplification refractory mutation system by PCR (ARMS-PCR) was developed in the present investigation. The *DGAT1* gene was recently identified as underlying variation in milk production traits. To date, PCR techniques such as PCR-RFLP have been used for detecting the *DGAT1* K232A polymorphism, despite being expensive and laborious. The method proposed here, a tetra-primer ARMS-PCR, showed 100% sensitivity and specificity when compared with PCR-RFLP results obtained in a sample of 80 animals tested in a double-blind system. Our results suggest that the use of tetra-primer ARMS-PCR for *DGAT1* K232A polymorphism genotyping could greatly reduce costs providing information for both research purposes and for dairy cattle breeders who perform *DGAT1* genotyping for gene-assisted selection.
Key words: *DGAT1*, polymerase chain reaction-restriction fragment length polymorphism, tetra-primer amplification refractory mutation system-polymerase chain reaction, marker-assisted selection

The acyl-CoA:diacylglycerol acyltransferase 1 (***DGAT1***) gene encodes the diacylglycerol-O-transferase (EC 2.3.1.20) microsomal enzyme, which catalyzes the last and limiting step in the synthesis of triglyceride (Cases et al., 1998). In cattle, the QTL associated with milk production traits was mapped to the centromeric region of the bovine chromosome 14 (Riquet et al., 1999). One of the genes in the candidate interval is *DGAT1*. Therefore, *DGAT1* constitutes both a positional and a functional candidate gene that accounts for variation in milk yield and composition. Moreover, *DGAT1*−/− knockout mice have been reported that do not produce milk, providing additional evidence for the function of *DGAT1* in lactation (Smith et al., 2000). Studies have reported correlations between the *DGAT1* K232A polymorphism and milk production or composition in some *Bos taurus* breeds (Grisart et al., 2002; Winter et al., 2002; Kühn et al., 2004), such as the lysine-encoding allele (232K) that was associated with decreasing protein and milk yields and increasing fat yield. The alanine-encoding allele (232A) was associated with increasing protein and milk yields and decreasing fat yield. This polymorphism was also correlated with intramuscular fat deposition as reported for German Holstein and Charolais breeds, for which the lysine-encoding allele was associated with increased marbling score in the musculus semitendinosus (Thaller et al., 2003). Of note, *DGAT1* 232K was recently related to increased content of saturated fat in milk (Schennink et al., 2007, 2008). Increasing amounts of unsaturated fatty acids in milk is an important selection objective because milk is regarded as one of the major sources of saturated fat in the human diet in Europe (Hulshof et al., 1999). Therefore, selective breeding could make a significant contribution to changing the fat composition of cow's milk, which affects the human diet (Schennink et al., 2007).

Three methods have already been described for genotyping the *DGAT1* K232A polymorphism. The first is based on PCR-RFLP using the *Cfr*I restriction enzyme (Winter et al., 2002), which is an expensive and time-consuming method that requires a relatively large amount of the restriction enzyme to prevent partial digestion; the second is a laborious technique based on an oligonucleotide ligation assay (Grisart et al., 2002); and the third method is based on quantitative PCR (Schennink et al., 2007), and therefore, a real-time thermocycler is needed.

In the present study, a new, rapid, and inexpensive method for the detection of the *DGAT1* K232A polymorphism based on the tetra-primer amplification refractory mutation system-PCR (**ARMS-PCR**; Newton et al., 1989; Ye et al., 2001) is described. Usually, detection of SNP by tetra-primer ARMS-PCR relies on

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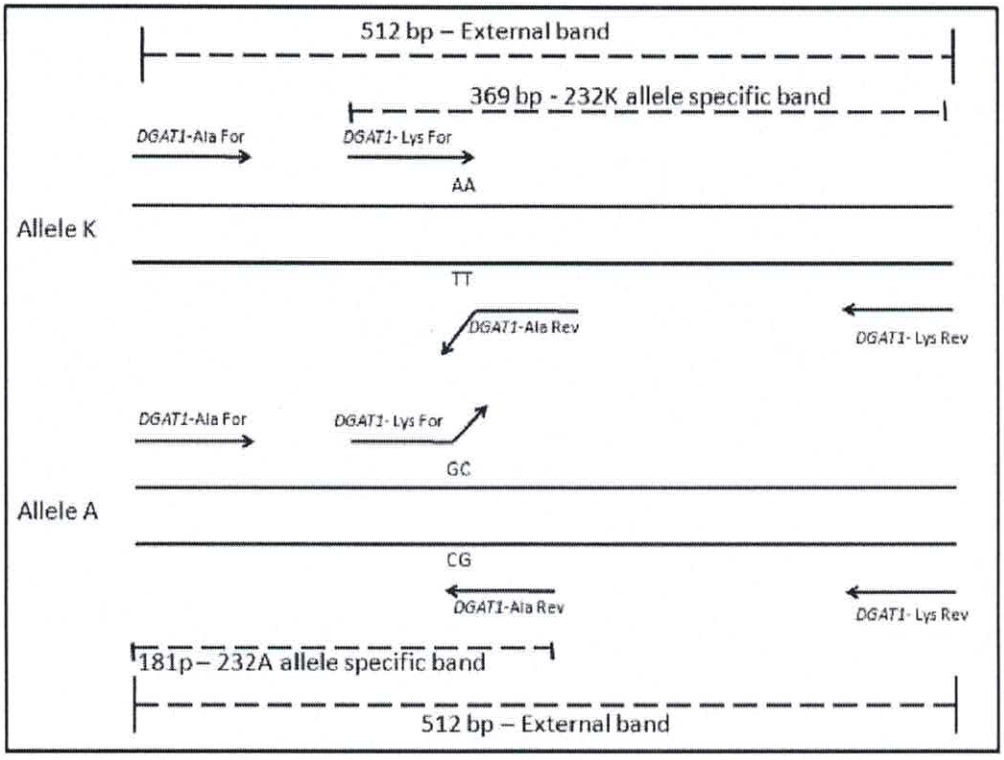

Figure 1. Amplification of the polymorphism *DGAT1* K232A by tetra-primer amplification refractory mutation system-PCR. Continuous lines represent the DNA fragment containing the *DGAT1* K232A polymorphism. Arrows indicate the primers used in the PCR reactions. Bent arrows show the annealing failure of the allele specific primers due to 3′ mismatches. Dashed lines indicate sizes of PCR products. Tetra-primer reactions produce 3 PCR products with 512 bp (external band), 369 bp (232K allele-specific band), and 181 bp (232A allele-specific band).

the pairing of the 3′ nucleotide of the primer with the SNP. To enhance the annealing specificity, a mismatch is introduced in 1 of the 3 positions previous to that base-paired with the SNP. The *DGAT1* K232A polymorphism is particularly suitable for the development of a tetra-primer ARMS-PCR system because it is produced by a double substitution (GC→AA). Therefore, the use of an additional mismatch is not necessary. This method is depicted in Figure 1.

Basically, the system is composedof 4 primers, 2 external (*DGAT1*-Ala-For and *DGAT1*-Lys-Rev) and 2 internal (*DGAT1*-Ala-Rev and *DGAT1*-Lys-For), each one of the latter annealing at its 3′ extremity to one of the alleles GC (232A) or AA (232K). Thus, both alleles are simultaneously amplified and subsequently identified by size after gel electrophoresis (Ye et al., 2001).

Genomic DNA was extracted by the proteinase-K:phenol-chloroform method (Sambrook and Russell, 2001) from blood or semen samples from 80 animals (33 of the Guzerat breed and 47 of the Holstein breed). Four primers were designed based on GenBank sequence AJ318490: *DGAT1*-Ala-For: 5′-gtc aac ctc tgg tgc cga gag-3′; *DGAT1*-Ala-Rev: 5′-agc tcc ccc gtt ggc cgc-3′; *DGAT1*-Lys-For: 5′-tcg tag ctt tgg cag gta aga a-3′; *DGAT1*-Lys-Rev: 5′-cac ctg gag ctg ggt gag gaa-3′. The PCR-amplified fragments were 512 bp (external band), 369 bp (232K allele), and 181 bp (232A allele). The external band functions as the internal PCR-positive control.

Tetra-primer ARMS-PCR amplifications were carried out in a final volume of 25 μL with IV-B PCR buffer (10 m*M* Tris, pH 8.4, 50 m*M* KCl, 0.1% Triton X-100, 1.75 m*M* $MgCl_2$), 0.2 m*M* each dNTP (Invitrogen, Carlsbad, CA), 8% dimethyl sulfoxide (J. T. Baker, Xalostoc, Edo. De Mexico, Mexico), 1 U of Taq DNA polymerase (Phoneutria Biotecnologia and Serviços, Belo Horizonte, MG, Brazil), 25 to 100 ng of genomic DNA, 0.2 μ*M* *DGAT1*-Ala-For and *DGAT1*-Lys-Rev primers, and 0.8 μ*M* *DGAT1*-Ala-Rev and *DGAT1*-Lys-For primers (Integrated DNA Technologies Inc., Coralville, IA). Additional primer proportions tested were 1:3 and 1:2 external to internal primers. The PCR cycling conditions were initial denaturation step for 5 min at 94°C, followed by 25 cycles at 94°C for 30 s, 65°C for 30 s, and 72°C for 30 s, and a final extension step for 5 min at 72°C. Reactions were undertaken using a PxE

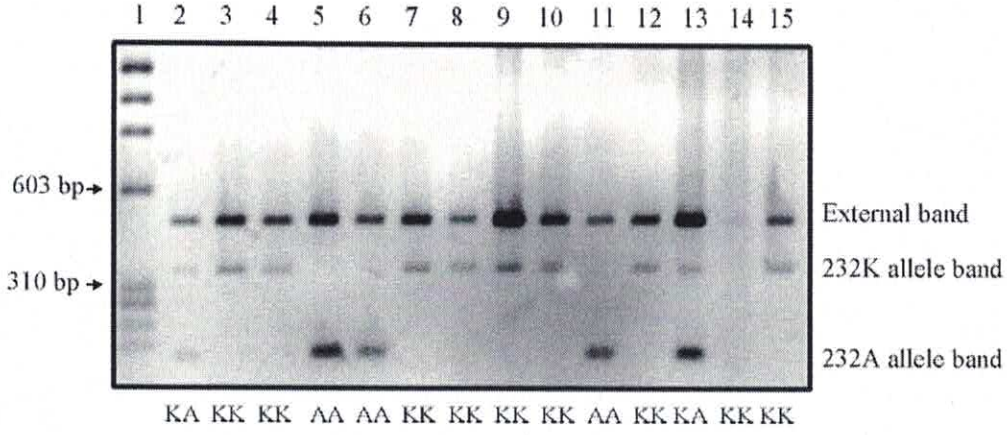

Figure 2. Ethidium bromide-stained (2%) agarose electrophoresis gel. Lane 1 = ΦX174 RF DNA/*Hae*III Fragments ladder (Invitrogen, Carlsbad, CA). Molecular size markers are indicated with arrows: 603 and 310 bp. Lanes 2 to 20 = tetra-primer amplification refractory mutation system (ARMS)-PCR products. Individuals in lanes 2 and 13 are heterozygous; those in lanes 3, 4, 7, 8, 9, 10, and 12 are homozygous 232K/232K; and those in lanes 5 and 6 are homozygous 232A/232A. *Amplification failure. The sizes of the tetra-primer ARMS-PCR fragments are 512 bp (external band), 369 bp (232K allele-specific band), and 181 bp (232A allele-specific band). The allele K band is frequently weaker than the other 2. To confirm genotypes in these cases, the reactions were repeated with larger amounts of DNA.

0.2 Thermal Cycler (Electron Corporation, Waltham, MA). The PCR products were run and visualized on 2% ethidium bromide-stained agarose gels or on 8% silver stained polyacrylamide gels. Allele sizes were estimated by comparison with a ΦX174 RF DNA/*Hae*III Fragments Ladder (Invitrogen). To confirm the identity and the size of amplified products, 3 independent PCR reactions were set up: 1) including the external primers only, 2) with the lysine-specific primers, and 3) including the alanine-specific primers. The sizes of the products obtained coincided with those observed in tetra-primer ARMS-PCR reactions. The products resulting from the 3 PCR reactions were sequenced using the Big Dye Terminator v3.1 Cycle Sequencing kit and a 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA). Sequence identities were confirmed by homology through a BLAST search (http://www.ncbi.nlm.nih.gov/blast/blast.cgi). Sensitivity and specificity of the method were evaluated in a double-blind experiment, for which 80 animals were simultaneously genotyped by both PCR-RFLP using the *Cfr*I restriction enzyme (Fermentas, Burlington, Ontario, Canada; Winter et al., 2002) and tetra-primer ARMS-PCR.

Tetra-primer ARMS-PCR amplifications yielded 3 bands as follows (Figure 2): a fragment with 512 bp, produced by the external primers, regarded as the internal control for PCR amplification, a 369-bp fragment (lysine-encoding allele), and a 181-bp fragment (alanine-encoding allele). Concordant results were obtained for all of the 160 alleles tested by PCR-RFLP with *Cfr*I restriction enzyme and tetra-primer ARMS-PCR, which showed 100% sensitivity and specificity. In the BLAST search, the 3 fragments sequenced showed homology with the bovine *DGAT1* gene, confirming the identity of the alleles.

The alanine-encoding allele has been suggested to be associated with a healthier milk, which means that it contains high protein levels and low fat, particularly low saturated fat. Large frequencies of this allele have been reported for many *Bos taurus* breeds. However, smaller frequencies of this allele were found in *Bos indicus*: 4% in Gyr and 2.5% in Red Sindhi, and null in Nellore and Guzerat animals (Lacorte et al., 2006). According to our recent results, alanine-encoding allele in *DGAT1* K232A polymorphism is found at frequencies of approximately 0.1% in Nellore (55 individuals) and 2% in Guzerat (352 animals, unpublished data). The 232A allele was not observed in a study including a small number of animals of 6 *Bos indicus* breeds (Sahiwal, Rathi, Deoni, Tharparkar, Red Kandhari, and Punganur; Tantia et al., 2006). Therefore, a large number of individuals should be tested to identify those bearing the advantageous allele for inclusion in marker-assisted selection programs. The costs of this procedure would be significantly reduced by the tetra-primer ARMS-PCR protocol reported here. Additionally, because of its high sensitivity and specificity, ease, and cost-effectiveness, this new method is appropriate for large-scale *DGAT1* K232A genotyping.

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