Technical Note: Use of PCR-Single-Strand Conformation Polymorphism Analysis for Detection of Bovine β-Casein Variants A^1, A^2, A^3, and B

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ABSTRACT: We have optimized the polymerase chain reaction-single-strand conformation polymorphism (PCR-SSCP) technique to screen the most frequent variants (A^1, A^2, A^3, and B) of the bovine β-casein gene. Five partly overlapping PCR products (233, 234, 265, 466, and 498 bp) of Exon VII of the β-casein gene that encompass the target point mutations were heat-denatured, separated on nondenaturing polyacrylamide gels, and silver-stained. Simultaneous detection of all variants in reference samples of known genotypes (A^1A^2, A^2A^2, A^1A^3, A^1B, and A^2B) was best achieved on 17% polyacrylamide (100:1 acrylamide:*bis*-acrylamide ratio) gels with the PCR product of 234 bp. These results were confirmed by sequencing the allele-specific SSCP bands directly excised from polyacrylamide gels. A population of 65 anonymous samples belonging to various breeds was then analyzed twice, without discrepancies in a blind trial. Routine β-casein genotyping using PCR-SSCP is proposed as a cost-effective, fast, and sensitive technique.

Key Words: Cattle, β-Casein, Genetic Polymorphism, Polymerase Chain Reaction

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Introduction

Genetic polymorphism of the six major lactoproteins of cattle (α_{s1}, α_{s2}, β and κ caseins, α-lactalbumin, and β-lactoglobulin) is well documented (Grosclaude, 1988). The B alleles of β-casein, κ-casein, and β-lactoglobulin have been associated with higher protein (Ng-Kwai-Hang et al., 1986; van Eenennaam and Medrano, 1991), fat yields (McLean et al., 1984; Ng-Kwai-Hang et al., 1986), or better cheese-making properties (Ng-Kwai-Hang et al., 1984; Rampilli et al., 1988). To date, a minimum of 10 genetic variants have been described for bovine β-casein (reviewed in Mercier and Grosclaude, 1993): A^1, A^2, A^3, B, C, D, E, A′, $A^{3Mongolie}$, and B_2. However, only A^1, A^2, A^3, and B are universally distributed in nearly all *Bos taurus* and *Bos indicus* populations. Amino acid differences among these four variants are located in Exon VII of the gene, which encodes for 80% of the mature protein (Bonsing et al., 1988). The mutation characterizing the C variant, which often has a frequency of less than .05 (Ng-Kwai-Hang and Grosclaude, 1992), is situated in Exon VI and does not lie in the region analyzed. The remaining alleles have never been detected in European bovine breeds. At the DNA level, amplification created restriction sites (**ACRS**) (Lien et al., 1992) and allele discrimination by primer length (**ADPL**) (Lindersson et al., 1995) diagnostic tests for A^1, A^2, A^3, and B have been developed. The sensitivity of these tests is restricted to detection of the target point mutations differing among the alleles. By contrast, the single-strand conformation polymorphism (**SSCP**) technique is a simple and efficient means to detect any small alteration in PCR-amplified products. It is based on the assumption that subtle nucleic acid changes affect the migration of single-stranded DNA fragments and, therefore, result in visible mobility shifts across a nondenaturing polyacrylamide gel (Orita et al., 1989). The objective of this work was to develop an inexpensive, fast, and sensitive method for screening large numbers of cattle.

Materials and Methods

Animals. Five reference samples previously phenotyped at the protein level (A^1A^2, A^2A^2, A^1A^3, A^1B, and A^2B) using the ultra thin layer-isoelectric focusing (UTL-IEF) technique (Seibert et al., 1985) were initially used to achieve optimum electrophoretic separations among variants using PCR-SSCP. We also collected blood from 65 cows of different breeds (Holstein Friesian, Brown Swiss, Limousin, and the local Spanish

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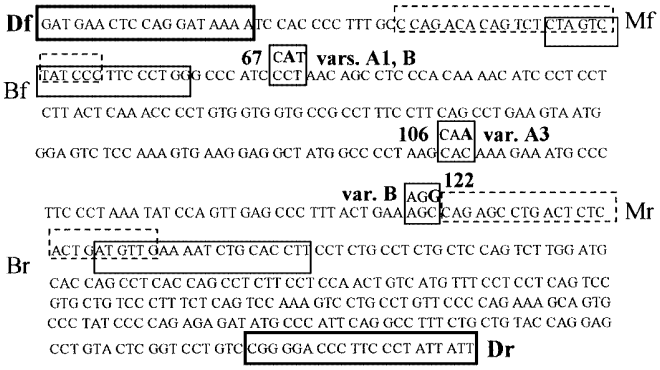

Figure 1. Nucleic acid sequence of Exon VII of the bovine β-casein gene, variant A^2. Target point mutations that differentiate alleles A^1 (codon 67), A^3 (codon 106), and B (codons 67 and 122) are indicated, as well as annealing sequences of the three primer pairs used (see Materials and Methods): Damiani.for (Df) – Damiani.rev (Dr) (thick lines), Medrano.for (Mf) – Medrano.rev (Mr) (dashed lines), and Beta.for (Bf) – Beta.rev (Br) (thin lines). Note that Exon VII is entirely amplified with primer combination Df-Dr.

breeds Asturiana de los Valles and Asturiana de la Montaña). Total DNA was extracted following standard methods (Sambrook et al., 1989).

Primer Design and PCR Amplifications. Five partly overlapping fragments of different lengths (233, 234, 265, 466, and 498 bp) were amplified with PCR using different combinations of three primer pairs (Figure 1): Medrano.for (**Mf**) (5′-CCA GAC ACA GTC TCT AGT CTA TCC C-3′) and Medrano.rev (**Mr**) (5′-CAA CAT CAG TGA GAG TCA GGC TCT G-3′) (Medrano and Sharrow, 1991); Damiani.for (**Df**) (5′-GAT GAA CTC CAG GAT AAA ATC-3′) and Damiani.rev (**Dr**) (5′-AAT AAT AGG GAA GGG TCC CCG-3′) (Damiani et al., 1992); and Beta.for (**Bf**) (5′-CTA GTC TAT CCC TTC CCT GG-3′) and Beta.rev (**Br**) (5′-AAG GTG CAG ATT TTC AAC AT-3′). These primers were designed using Primer 0.5 software (1991, MIT Center for Genome Research and Whitehead Institute for Biomedical Research, MA). For all the amplification reactions, 100 ng of DNA was used in a PCR buffer containing 200 μM of the dNTP mix and 400 nM of each of the pertinent primers, overlain with a thin layer of mineral oil. Samples were denatured at 94°C for 5 min and then subjected to 30 cycles of 94°C for 30 s, the specific annealing temperature for 1 min at 62°C for primer combination Mf-Mr (233 bp), 54°C for Bf-Br (234 bp), and 59°C for Df-Mr (265 bp), Mf-Dr (466 bp), and Df-Dr (498 bp), and 72°C for 1 min, with a final extension step of 5 min at 72°C in a MiniCycler (MJ Research, Watertown, MA).

PCR Purifications. The amplification products of the reference samples and SSCP reamplified bands (see below) were purified using the High Pure PCR Product Purification kit (Boehringer Mannheim GmbH, Mannheim, Germany) following the manufacturer's instructions.

PCR-SSCP Analysis. Three microliters of each PCR sample was mixed with 10 μL of denaturing loading buffer (.05% xylene-cyanole, .05% bromophenol blue, 5.5 mM EDTA, pH 8.0, in deionized formamide), heat-denatured at 95°C for 5 min, and snap-chilled on ice. Samples were then loaded onto nondenaturing polyacrylamide gels using the Penguin Dual-Gel Water-Cooled Electrophoresis System (OWL Scientific, Woburn, MA). A cooling device (Cooline Plus 8-30e, Heto-Holten A/S, 3450 Allerød, Denmark) kept a constant temperature during the electrophoresis. We tested several variables known to affect the mutation detection ability of SSCP:acrylamide concentration (8, 10, 12, 14, 15, 16, 17, and 18%), *bis* ratio (19:1, 29:1, 39:1, 49:1, and 100:1), glycerol effect (0 vs 5%), running temperature (20, 18, 16, 15, 14, 13, 12°C, and no control), and buffer conditions (1× and .5×). Bands were silver-stained following Bassam et al. (1991) with minor modifications of the protocol previously reported (Barroso et al., 1998).

PCR Extraction and Reamplification of SSCP Bands of the 234-bp Fragment. Silver-stained allele-specific SSCP bands were excised from the gel, placed in 1.5-mL Eppendorf tubes containing 100 μL of distilled water, and covered with 30 μL of mineral oil to prevent evaporation. Samples were then heated to 95°C for 15 min, subjected to two freeze (–70°C) and thaw cycles to fragment the $AgNO_3$ "coat," and spun at 10,000 × g for 2 min. Five microliters of this solution was used in subsequent reamplification 50-μL reactions using primers Bf and Br and 50 cycles in the amplification program.

Automatic Sequencing of PCR-Enriched SSCP Alleles. The PCR-reamplified allele-specific SSCP bands were purified and sequenced in an ABI 310 DNA Sequencer using the ABI Prism Dye Terminator Cycle Sequencing Ready Reaction kit (both from Perkin Elmer Cetus Corp., Foster City, CA) according to the manufacturer's instructions.

Results and Discussion

Starting from purified samples, initial SSCP trials were aimed to determine the optimum length of the fragment to be screened. Different levels of acrylamide:*bis*-acrylamide ratios and acrylamide concentrations tested led us to reject 466- and 498-bp fragments for further optimization, because their electrophoretic mobilities were very slow, and the separation between forward and reverse strands was not satisfactory (not shown).

We then focused on 233-, 234-, and 265-bp PCR products. In theory, the smallest fragment should be the most adequate, due to an inverse correlation between product length and discrimination power that most authors report for SSCP (e.g., Beier, 1993). However, location of the mutation within the fragment also may play

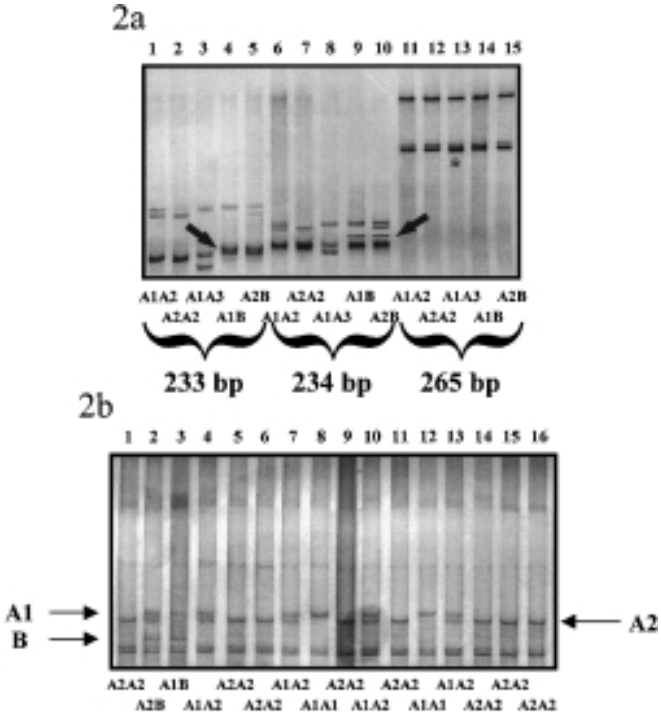

Figure 2. (a) The single-strand conformation polymorphisms (SSCP) patterns of the purified reference samples for the 233- (lanes 1–5), 234- (6–10), and 265-bp (11–15) PCR fragments analyzed under optimized conditions. Alleles A^1, A^2, and A^3 are clearly distinguishable in 233- and 234-bp amplification products, whereas the B allele (indicated by arrows) is much more evident for the 234-bp fragment (lanes 9, 10). The asterisk denotes a subtle shift of allele A^3 in the 265-bp product. (b) Genotyping of bovine β-casein alleles A^1, A^2, A^3, and B of 16 anonymous samples using PCR amplification of a 234-bp fragment with primer combination Beta.for (Bf) – Beta.rev (Br) at concentrations of 50 n*M*. The polyacrylamide gel shows unpurified SSCP patterns of alleles A^1, A^2, and B. Allele A^3 could not be detected in the population analyzed.

a key role (e.g., Sekiya, 1993; Sheffield et al., 1993). Those mutations toward the end of the amplicon seem much more difficult to detect. This should be considered with the 233- and 265-bp fragments, because the C to G transversion at position 122 that characterizes allele B is situated adjacent to the 3′ terminus of the reverse primer Mr (Figure 1).

Figure 2a shows SSCP profiles obtained for 233-, 234-, and 265-bp purified fragments under the best conditions: 17% polyacrylamide gels with a ratio of acrylamide to N,N′-methylene-*bis*-acrylamide of 100:1, 5% glycerol, run at 200 V overnight in .5× TBE (.045 *M* Tris-borate, .001 *M* EDTA, pH 8.0) at 15°C. Simultaneous differentiation of the four alleles was only possible for the 234-bp fragment, which confirms that mutation detection in this region is both length- and position-dependent. Indeed, the impact of length is amplified for the 265-bp fragment, for which only the mutation of allele A^3 (Figure 2a, lane 13) can be distinguished. The mobility shift of the B allele for 233- and 234-bp products (designated by arrows in Figure 2a, lanes 4, 5 vs 9, 10) is more evident for the latter, which clearly illustrates the influence of mutation position. The upper band in lane 10 (A^2B genotype), common to alleles A^1 and B, does not preclude the correct genotyping of the sample. Further evidence of the correct assignment of genotypes was supplied by direct sequencing of SSCP allele-diagnostic bands and confirmed the presence of the expected point mutations that characterize alleles A^1 (Baev et al., 1987), A^2 (Stewart et al., 1987), A^3 (Lien et al., 1992), and B (Damiani et al., 1992).

Having determined the SSCP conditions and the size of the fragment, we tested the role of purification, which eliminates excess PCR primers, on SSCP patterns. We found that unpurified samples give rise to additional bands in more retarded positions that reduce the relative intensity of those of importance for allele discrimination (not shown). This phenomenon, due to interactions between residual PCR primers and single strands (Cai and Touitou, 1994), is minimized by reducing primer concentrations in amplification reactions from 400 to 50 n*M*. Thus, the purification step before SSCP analysis can be avoided.

To assess the validity of the technique, a population of 65 anonymous samples was independently genotyped twice, and no discrepancies were found. Three of the four alleles (A^1, A^2, and B) could be detected in this population (Figure 2b), with relative frequencies of 23.1, 64.6, and 12.3%, respectively. The absence of variant A^3 was not surprising, considering its low frequency in all breeds analyzed: less than 4% whenever present (Ng-Kwai-Hang and Grosclaude, 1992).

We propose this SSCP protocol to perform simultaneous genotyping of A^1, A^2, A^3, and B, the most frequent alleles of bovine β-casein in all bovine breeds studied so far (Ng-Kwai-Hang and Grosclaude, 1992), as an alternative strategy to methods already available, both at the protein (UTL-IEF) and at the DNA levels (ACRS and ADPL). Due to well-known advantages of methods using DNA (versatility of source, no limitations of sex, age, or physiological status) over those using proteins and for the particular structure of this gene with a large exon encoding most of the mature protein (Bonsing et al., 1988), allele detection methods analyzing DNA are particularly recommended in this case.

It is important to keep in mind that allelic mutations studied in this work do not alter restriction targets for any commercially available endonuclease. Therefore, use of PCR-RFLP is not feasible as a reference technique. Two main approaches have been proposed to overcome this limitation: ACRS and ADPL. The ACRS technique is based on the design of primers that create allele-specific restriction sites in the PCR product (Lien et al., 1992). The ADPL method relies on allele discrimination through length-specific amplifications (Li et al., 1990). Both methods are more expensive and cumbersome than SSCP. Indeed, ACRS requires the combined

information of three restriction enzymes, use of highly concentrated low-melting-point agaroses, and the purchase of long allele-specific primers. Although ADPL avoids restriction enyzmes, allele-specific primers of different lengths and a very sensitive automated DNA sizing technique are needed to assign each variant (Lindersson et al., 1995). Finally, both techniques, although of diagnostic value, do not allow detection of variants other than those specifically screened, which is feasible with screening methods such as SSCP.

Implications

The single-strand conformation polymorphism (SSCP) protocol developed here may be the tool of choice for genotyping the bovine β-casein locus in large-scale industrial or breeding projects directed toward improved cheese making properties of milk. The capability to detect any mutation occurring throughout the fragment analyzed is a considerable advantage of the SSCP technique over other molecular methods currently available for detecting allelic variation in this protein.

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