

Technical Note: Detection of Bovine Kappa-Casein Variants A, B, C, and E by Means of Polymerase Chain Reaction-Single Strand Conformation Polymorphism (PCR-SSCP)¹

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ABSTRACT: We used polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) analysis to screen the most frequent variants (A, B, C, and E) found in the bovine kappa-casein gene. The PCR products (453 bp) were heat-denatured, loaded onto nondenaturing polyacrylamide gels, and silver-stained. Each variant yielded patterns clearly distinguishable from the others. Optimal conditions for the simultaneous detection of the four variants were 12% polyacrylamide gels (100:1 acryla-

mid-*bis*-acrylamide ratio) with 5% glycerol and a constant running temperature of 10°C. Eight reference samples initially used for this purpose and 40 anonymous samples of different cattle breeds diagnosed by PCR-RFLP and PCR-SSCP showed no discrepancies between the two methods and confirmed previous results. Because it is cost-effective, sensitive, and fast, PCR-SSCP is strongly recommended to routinely screen kappa-casein variants for industrial purposes or in cattle selection schemes.

Key Words: Cattle, Kappa-Casein, Genetic Polymorphism, PCR-SSCP

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Introduction

The kappa-casein (**CASK**) gene has been extensively studied in cattle for its stabilizing role of the casein micelles and, therefore, its influence on the manufacturing properties of milk. To date, six variants have been described: A, B, C, E, and the recently discovered F and G (see Kaminski, 1996 for an updated review). Many studies (reviewed by Grosclaude, 1988) demonstrate the influence of genetic variants of κ -casein on the manufacturing properties of milk, especially those of importance in cheese technology. Milk containing the B variant shows better lactodynamographic properties than milk containing A (Rahali and Ménard, 1991), E (Gravert et al., 1991), or C (Macheboeuf et al., 1993) variants. Point mutations distinguishing among variants have made possible the development of several PCR-RFLP diagnostic tests for A and B (Denicourt et al., 1990; Medrano and Aguilar-Córdova, 1990; Zadworny and Kuhnlein, 1990), C (Schlee and Rotmann, 1992) and E (Schlieben et al., 1991). Orita et al. (1989) published a simple method for detecting mutations in

DNA: the PCR-single strand conformation polymorphism (PCR-SSCP) method. The technique relies on the fact that denatured DNA molecules migrate across a nondenaturing polyacrylamide gel according to their size and their sequence. Hence, two PCR fragments differing by as little as a point mutation may display mobility shifts on the gel. We applied the PCR-SSCP method to detect κ -casein variants A, B, C, and E present in different European cattle breeds (Erhardt, 1989, 1993).

The aim of this work was to optimize the PCR-SSCP conditions to enable an easy and inexpensive genotyping for industrial purposes and application in cattle selection schemes.

Materials and Methods

DNA Samples. Eight reference DNA samples from cows, which were previously phenotyped at the protein level with the isoelectric focusing technique (IEF) (kindly provided by G. Erhardt), containing different combinations of the four variants (see Table 1 and Figure 1) were used to develop the optimal conditions of PCR-SSCP. We also collected blood from 40 animals of various breeds (Holstein Friesian, Brown Swiss, Limousin, and the local breeds Asturiana de los Valles and Asturiana de la Montaña). Total DNA was extracted according to Sambrook et al. (1989).

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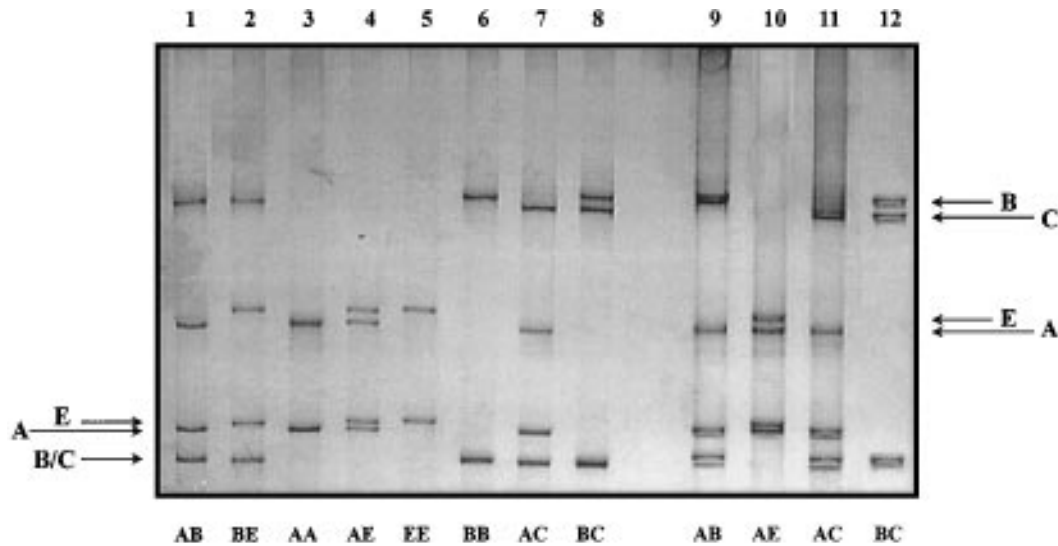


Figure 1. The PCR-SSCP patterns of alleles A, B, C, and E in different combinations. Lanes 1 through 8: purified PCR samples. Lanes 9 through 12: unpurified PCR samples (for detailed information, see text).

Polymerase Chain Reaction. A fragment of 453 bp was amplified using 300 ng of DNA in a PCR buffer solution containing 20 pmol of each primer (forward, 5'-TGT GCT GAG TAG GTA TCC TAG TTA TGG-3'; reverse, 5'-GCG TTG TCT TCT TTG ATG TCT CCT TAG-3'), 2 mM of $MgCl_2$, 200 μM of the dNTP mix, and 2 U of *Taq* polymerase in a total volume of 100 μL . Samples were denatured at 94°C for 5 min and then subjected to 35 cycles of 94°C for 1 min, 65°C for 1 min, and 72°C for 2 min with a final extension step of 72°C for 5 min.

Purification of PCR samples. For comparative results, the reference samples were purified using the High Pure PCR Product Purification Kit (Boehringer Mannheim GmbH, Mannheim, Germany) according to manufacturer's instructions.

PCR-RFLP. Fifteen microliters of the PCR products was digested with 5 U each of *HinfI* (recognition site, 5'-GANTC-3'), *HaeIII* (recognition site, 5'-GGCC-3') and *MaeII* (recognition site, 5'-ACGT-3') (Boehringer

Mannheim GmbH), in three separate reactions. Samples were incubated at 37°C for 3 h. The visualization of PCR fragments and digestion products was in 1.5% ethidium bromide-stained agarose gels following standard procedures (Sambrook et al., 1989).

PCR-SSCP. Two microliters of each PCR product was mixed with 8 μL of denaturing loading buffer (.05% xylene-cyanole, .05% bromophenol blue, 5.5 mM EDTA, pH 8.0, in formamide), denatured at 95°C for 5 min, and snap-chilled on ice for at least 2 min. Samples were then loaded onto a polyacrylamide gel containing .5 \times TBE (.045 M Tris-borate, .001 M EDTA, pH 8.0). After the electrophoresis run, bands were silver stained according to Bassam et al. (1991) with slight modifications: fixing was reduced from 20 to 5 min and staining was decreased from 30 to 20 min. We tested several factors known to affect the ability of SSCP to detect mutations: acrylamide concentration (8, 12, and 15%), glycerol concentration (0, 5, and 10%), bis ratio (19:1, 29:1, 49:1, and 100:

Table 1. Sizes of fragments resulting from digestion of the reference PCR products with the restriction enzymes used

| Genotypes | Restriction enzyme | | | | | | | | | | |
|-----------|--------------------|-----|-----|---------------|-----|-----|-----|--------------|-----|-----|-----|
| | <i>HinfI</i> | | | <i>HaeIII</i> | | | | <i>MaeII</i> | | | |
| | bp | bp | bp | bp | bp | bp | bp | bp | bp | bp | |
| AA | — | 326 | 100 | 27 | 230 | 223 | — | — | — | 254 | 199 |
| AB | 426 | 326 | 100 | 27 | 230 | 223 | — | — | — | 254 | 199 |
| BB | 426 | — | — | 27 | 230 | 223 | — | — | — | 254 | 199 |
| AE | — | 326 | 100 | 27 | 230 | 223 | 145 | 78 | — | 254 | 199 |
| BE | 426 | 326 | 100 | 27 | 230 | 223 | 145 | 78 | — | 254 | 199 |
| EE | — | 326 | 100 | 27 | 230 | — | 145 | 78 | — | 254 | 199 |
| AC | 426 | 326 | 100 | 27 | 230 | 223 | — | — | 453 | 254 | 199 |
| BC | 426 | — | — | 27 | 230 | 223 | — | — | 453 | 254 | 199 |

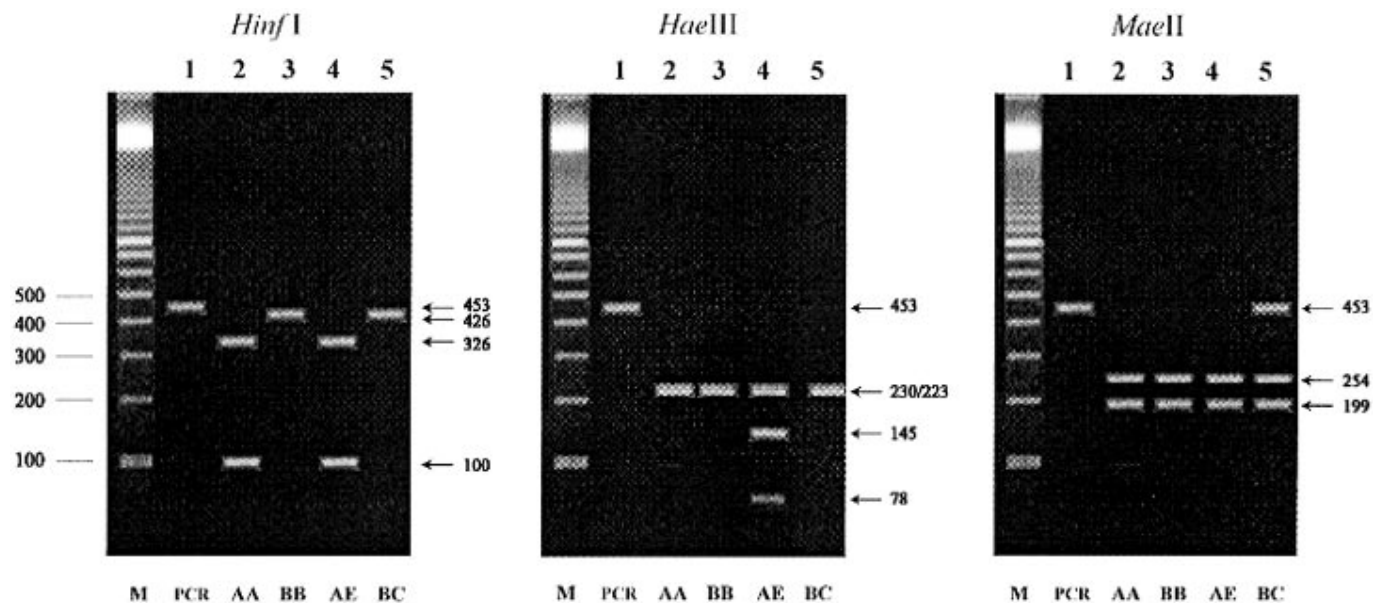


Figure 2. Lane 1: undigested PCR product. Lanes 2 through 5: PCR-RFLP patterns of four samples containing the alleles A, B, C, and E of the bovine κ -casein (CASK) gene. The M designates the 100-bp ladder (Pharmacia Biotech, Uppsala, Sweden). Notice that the unambiguous genotyping of the four alleles requires the combined information of the three enzymes.

1), running temperature (20, 16, 14, 12, and 10°C), and TBE concentration (1 \times and .5 \times) using a Penguin™ Dual-Gel Water-Cooled Electrophoresis System (OWL Scientific Inc., Woburn, MA). A COOLINE PLuS 8-30e cooling unit kept a constant temperature during electrophoresis.

Results and Discussion

All samples were evaluated with the PCR-RFLP technique using *HinfI*, *HaeIII*, and *MaeII* and with the PCR-SSCP technique. Table 1 shows the expected sizes of the bands for reference samples with the three restriction enzymes. The *HinfI* misidentified alleles A and E, and B and C; *HaeIII* distinguished A and E alleles but still misidentified B and C; and with *MaeII* it was possible to differentiate the C allele from the others because it was the only one that remained undigested. Thus, it was necessary to use a minimum of three enzymes for an unambiguous genotyping of the four alleles by PCR-RFLP, making the technique time-consuming and expensive, and not practical for routine use. Figure 2 shows the restriction patterns of four individuals containing all the alleles after digestion with the restriction enzymes mentioned above. The 27-bp band was not distinguishable when digesting with *HinfI*, and 230 and 223 bp formed a single band in the gel for *HaeIII*, although this did not prevent correct genotyping.

To optimize SSCP conditions, we used eight reference samples previously phenotyped at the protein

level by IEF. Best results were obtained using 12% PAGE gels with a ratio of acrylamide to N,N'-methylene-*bis*-acrylamide of 100:1, 5% glycerol, and run for 14 h at 200V in .5 \times TBE at 10°C.

In SSCP gels, DNA migrates in a single-stranded form, which usually yields two bands for homozygotes and four for heterozygotes. This is confirmed in Figure 1 (showing the SSCP patterns of eight purified and four unpurified samples) except in the case of individuals BC, whose lower bands did not separate. Samples were tested with and without PCR purification, because previous work reported that excess nucleotides and primers cause the appearance of SSCP artifacts or dramatic effects on the sensitivity of the technique (e.g., Cai and Touitou, 1994). In both cases, mobility shifts of the four variants were clear enough to distinguish among them, even though unpurified samples rendered "double" bands for some strands. Therefore, PCR purification was not necessary. No discrepancies between PCR-RFLP and PCR-SSCP patterns were detected for any of the animals tested.

The influence of temperature, percentage of acrylamide, ionic strength of the electrophoretic buffer, *bis* ratio, and levels of glycerol on the ability of SSCP to detect point mutations was crucial. The *bis* ratio and running temperature were the most critical factors affecting resolution power.

Because the PCR-SSCP approach does not require expensive restriction enzymes, and because the purification step is not necessary, the cost of genotyping is drastically reduced, compared with PCR-RFLP,

despite the initial investment in a cooling unit. The use of PCR-RFLP to detect new mutations is only feasible if such mutations create or destroy a restriction target for the enzymes used. The SSCP does not seem to be affected by the type of nucleotide substitution (Sheffield et al., 1993), and it allows the detection of new mutations as aberrant mobility shifts throughout the fragment amplified.

Implications

The results presented here support the consideration of single strand conformation polymorphism (SSCP) as the method of choice for large-scale screening of κ -casein variants in cattle selection schemes and industrial purposes. The SSCP method allows the genotyping of known variants A, B, C, and E in a faster and easier way and still retains its ability to detect new mutations.

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