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The identification of the kappa-casein genotype in Holstein dairy cattle using the polymerase chain reaction

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Summary. The polymerase chain reaction (PCR) was used to amplify a 99-bp region from the kappa-casein gene of Holstein dairy cattle which contains nucleotide substitutions that are diagnostic of the two major protein variants of kappa-casein. Identity of the amplified product was confirmed by direct sequencing. Digestion of the PCR product with MboII (A-variant specific) or TaqI (B-variant specific) allowed direct determination of the genotype of the animal (homozygous or heterozygous). A total of 58 lactating cows with known kappa-casein phenotype were tested using PCR. In all cases, the measured genotype confirmed the phenotype. We have also tested the genotype of 42 sires that were top ranked for milk yield by the CIAQ (Centre d'insemination artificielle du Quebec). The B-allele of kappa-casein which occurred at a frequency of 0.13 among the proven bulls is associated with superior milk for industrial applications. Identification of the kappa-casein genotype by PCR in bulls and calves would provide a means for rapidly changing the frequency of the B-allele in the breeding population by selection.

Key words: PCR – Kappa-casein – Genotype – Holstein

Introduction

Breeders of dairy cattle have long been interested in the composition of milk since variation in the protein and fat content affects the yield of further processed products. Studies on the manufacture of Parmesan (Morini et al. 1979) and cheddar cheese (Marziali and Ng-Kwai-Hang 1986) have shown that the B-variant of kappa-casein is associated with increased yields of cheese (8% and 4%, respectively), and it has been suggested that casein genotype represents an economicaly important selection criterion to improve industrial milk production (McLean et al. 1984; McLean 1987). Currently, however, the casein genotype is not included in the selection index since sires in artificial insemination programs can be assessed only at maturity by typing milk proteins of their progeny and dams. Earlier identification of the kappa-casein genotype could accelerate genetic selection.

Recently, cDNA coding for bovine kappa-casein (Kang and Richardson 1988) and genomic DNA spanning the bovine kappa-casein gene (Alexander et al. 1988) have been cloned and sequenced. These studies have confirmed that nucleotide substitutions characterize the two major genetic variants of K-casein, A and B. In the mature protein, the A-variant has threonine at position 136 and aspartate at position 148, whereas the B-variant has isoleucine and alanine at these positions, respectively (Mercier et al. 1973). The associated nucleotide substitutions generate restriction site polymorphisms that can be used to distinguish the allelic variant of an animal at the DNA level (Alexander et al. 1988; Rando et al. 1988).

Recently, the polymerase chain reaction (PCR) has been used in human genetics for prenatal and carrier testing of monogenic disorders such as sickle cell anemia (Saiki et al. 1986), hemophilia A (Kogan et al. 1987) and B (Tsang et al. 1988) and cystic fibrosis (Feldman et al. 1988) because linked variations in DNA can be detected by analysis of the PCR product. In this paper we have applied PCR to the determination of the kappa-casein genotype in Holstein cows and bulls. Since genotype can be rapidly and accurately assessed, this may prove useful for accelerating selection for specific milk production traits.

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An account of this work was presented at "Animal Health and Production in the 21st Century" (Sydney, Australia, 1989), and after submission of this manuscript, Medrano and Aguilar-Cordova (1990) have published a different methodology for the identification of kappa-casein genotype in cows using PCR.

Materials and methods

DNA was extracted from heparinized blood obtained from lactating Holstein cows maintained at Macdonald College according to Maniatis et al. (1982). For the isolation of DNA from bulls, semen straws were obtained from the Centre d'insemination artificielle du Quebec from 42 proven sires that were top ranked for milk yield. Briefly, cells from one to two insemination straws were pelleted by centrifugation in Eppendorf tubes, and the pellet was washed 4–5 times with phosphate buffered saline (PBS) to remove the diluent. Cells were then suspended in 100 µl PBS to which 400 µl of a solution containing 2% 2-mercaptoethanol, 10 m*M* TRIS, pH 8.0, 100 m*M* NaCl, 10 m*M* EDTA, pH 8.0, and 0.5% SDS was added, and the mixture was incubated at 50 °C for 30 min. Proteinase K was then added to a final concentration of 200 µg/ml, and the incubation was continued for 12–16 h. Following phenol and chloroform-isoamyl alcohol extractions, DNA was recovered by ethanol precipitation. The yield was in the range 20–40 µg DNA per insemination straw.

PCR was used to amplify a 99-bp region of the kappa-casein gene, which contains the nucleotide substitutions diagnostic of the A- or B-allele. The structures of the primers and the amplified sequences are shown in Fig. 1. In brief, 200 ng of genomic DNA was used in the 100 µl standard reaction mix (GeneAmp™ DNA Amplification Reagent Kit, Perkin Elmer Cetus Corp) contaning 250 nM primers. Genomic DNA was either digested with restriction enzyme prior to amplification to disrupt the target sequence, or intact DNA was used. DNA was denatured for 5 min prior to the start of the temperature cycle and subsequently the temperature was cycled between 94° and 60 °C for 20 sec at each temperature for 30 cycles using a DNA Thermal Cycler (Perkin Elmer Cetus Corp). The PCR reaction products were recovered by ethanol precipitation and dissolved in 100 µl 10 m*M* TRIS, pH 7.5, 0.1 m*M* EDTA, pH 8.0. Aliquots of 10 µl were then separated in 7.5% PAGE following digestion with either no restriction enzyme, or 1 unit of MboII or TaqI. The gel was stained with ethidium bromide and visualized under UV light.

For sequencing reactions, DNA was amplified as above except that the concentrations of the two primers were 50 pmol and 0.5 pmol, respectively. The single-strand DNA generated was sequenced using the dideoxynucleotide chain-termination method and the limiting primer as the sequencing primer (Gyllensten and Erlich 1988). Gel-purified dsDNA resulting from the regular amplification reaction was also sequenced using the dideoxynucleotide chain-termination reaction with end-labelled PCR primers as sequencing primers (Wrischnik et al. 1987).

Milk protein phenotypes of the cows were determined by PAGE (Ng-Kwai-Hang and Kroeker 1984).

Results and discussion

Following 35 cycles of PCR, single-strand DNA amplified from cows which were either homozygous A or B was sequenced using the dideoxynucleotide chain-termination reactions. Both the forward and reverse limiting primers were used as sequence primers. In addition, amplified dsDNA which was gel purified was also sequenced. The molecular sequence confirmed that the PCR product contained the target sequence that was specific to either the A- or B-variant of kappa-casein (Alexander et al. 1988). In heterozygotes, both the A- and the B-allele were amplified by PCR to an equivalent extent, and subsequent extension from either sequencing primer occurred on both alleles. This resulted in superimposition of sequencing ladders derived from the two kappa-casein alleles. In all cases, the molecular sequence was of the expected size and confirmed the known kappa-casein phenotype of the animal.

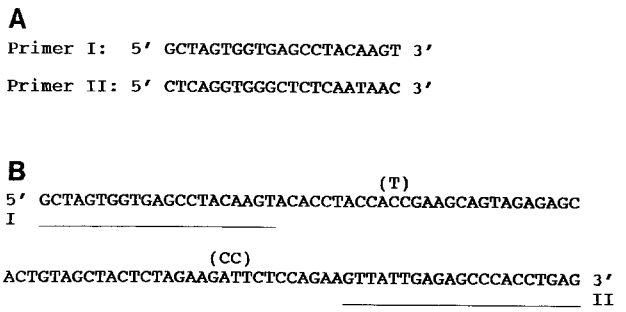

Fig. 1. Sequence of oligonucleotide primers (**A**) and their relation to the target region of the kappa-casein gene that is amplified (**B**). Primer I is complementary to the (−)-strand and primer II is complementary to the (+)-strand of kappa-casein. The sequence amplified is 99 bp in size, and the nucleotide substitution associated with the B-allele are indicated in *brackets*. The location of the primers (I and II) are *underlined*

The A- and B-alleles can be distinguished at the DNA level by restriction endonucleases (Alexander et al. 1988; Rando et al. 1988). For example, MboII and TaqI recognition sites are specific to the A- and B-alleles, respectively, and these sites are contained within the amplified target sequence. Digestion of genomic DNA prior to PCR with either MboII or TaqI would therefore be expected to interrupt the target sequence, and thus no PCR product would be obtained with DNA from homozygous animals digested with the appropriate enzyme. In heterozygous animals, about half the PCR product compared to the control would be expected since only one allele would be restricted by a given enzyme. This approach did differentiate the two alleles; however, incomplete digestions often resulted in ambiguous results (see Fig. 2; lane 1).

An alternative approach was to digest the amplified product after PCR with the A- and B-variant specific restriction enzyme. MboII specifically cuts the A variant, producing bands of 76 and 23 bp, while digestion with TaqI produces bands of 67 and 32 bp. In heterozygotes

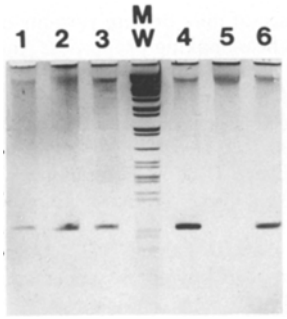

Fig. 2. Separation of PCR product in 7.5% PAGE. Genomic DNA was digested with MboII (*lanes 1–3*) or TaqI (*lanes 4–6*) prior to PCR. Cows were AA (*lanes 1* and *4*), BB (*lanes 2* and *5*) and AB (*lanes 3* and *6*) kappa-casein. Molecular weight markers were a PstI digest of lambda DNA, and size in bp is indicated. The gel was stained with ethidium bromide

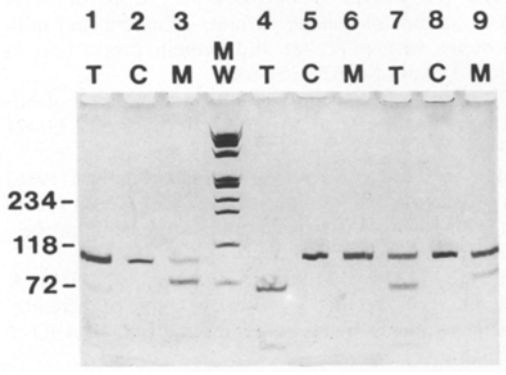

Fig. 3. Separation of PCR products in 7.5% PAGE following digestion with TaqI (*T*), no enzyme (*C*) or MboII (*M*). Amplified DNA was from homozygous A (*lanes 1–3*), B (*lanes 4–6*) or heterozygous AB (*lanes 7–9*) kappa-casein cows. Molecular weight markers were an HaeIII digest of phiX 174 DNA, and size in bp is indicated. The gel was stained with ethidium bromide

only about half of the PCR product is digested by either restriction enzyme, thus the 99-bp region is also present (Fig. 3). The use of these two enzymes allowed clear discrimination between the kappa-casein alleles of an animal (Fig. 3). Other restriction enzymes which are specific to the A-allele are EcoR1' and HinfI, whereas the B-allele is cut by MnlI (data not shown). Although genetic assignments can be made on the basis of a single restriction enzyme (A- or B-allele specific), the amplified DNA was digested by restriction enzymes specific to each allele since use of a single enzyme often failed to differentiate between homozygotes and heterozygotes due to incomplete digestion of the PCR product (Fig. 3; lane 3).

Given the redundancy in the genetic code, there are two codons for alanine, three for isoleucine and four each for both threonine and aspartic acid. For example, the neutral substitution of the codon ATC by either ATT or ATA would result in the loss of the TaqI recognition sequence, and thus the B-allele could not be differentiated from the A-allele on the basis of TaqI digestion alone. We do not know how frequently such neutral substitutions occur within the population, but it is notable that for all 58 cows that were tested, the genotype confirmed the known phenotype (Table 1). We have also assessed the genotype of 42 bulls, and sequencing of the PCR product has confirmed the genotype assigned on the basis of restriction maps. Thus, this methodology appears to provide a rapid and accurate method of genotype determination that obviates the necessity of indirectly assigning a kappa-casein genotype to bulls by analyzing the milk of the female progeny. This is important for the artificial insemination industry since the B-allele is associated with superior milk for cheese production (Morini et al. 1979; Marziali and Ng-Kwai-Hang 1986).

Table 1. Kappa-casein genotype in Holstein cows and bulls assessed using PCR. The genotypic variant of kappa-casein was confirmed in cows by assessing the milk phenotype using PAGE

	N	K-casein genotype		
		AA	AB	BB
Cows	58	34	23	1
Bulls	42	31	11	0

Genetic typing of 42 proven sires used by the artificial insemination industry yielded frequencies of 0.87 and 0.13 for the A- and B-alleles, respectively (SE 0.04), whereas in the Macdonald College dairy herd cows the respective frequencies were 0.78 and 0.22 ($\pm$0.04; Table 1). These allelic frequencies are not significantly different. However, in a survey of 2,045 cows sampled from 63 dairy herds in Quebec, Ng-Kwai-Hang et al. (1984) estimated the frequencies of the A- and B-alleles as 0.74 and 0.26, respectively ($\pm$0.007), which differ significantly from those in the proven bull population ($P<0.05$). This observation indicates that the selection criteria used to prove bulls discriminate against the B-allele. Why this should be so is not clear since extensive analyses revealed no unequivocal correlations between kappa-casein variants and milk yield (McLean et al. 1984; Ng-Kwai-Hang et al. 1984).

Since proven bulls are extensively used for artificial insemination and will produce many thousands of progeny, discrimination against the B-allele of kappa-casein would lead to a reduction of this allele in the breeding population. Due to the economic importance of this allele for industrial milk production it is therefore mandatory to substantiate our initial observation by analyzing larger bull populations, including bulls which were rejected as a result of the proving program.

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