GENOTYPING OF KAPPA-CASEIN ALLELES IN HOLSTEIN DAIRY CATTLE USING PCR AND SSCP-PCR.

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SUMMARY

The polymerase chain reaction (PCR) was used to amplify a 432 bp region from the κ-casein gene of Holstein dairy cattle which contains the nucleotide substitutions diagnostic of the 2 major protein variants of κ-casein. Digestion of the PCR product with HinfI (A variant specific) or TaqI (B variant specific) allowed direct determination of the genotype of the animal (homozygous or heterozygous). The same samples were also analyzed using single strand conformation polymorphism analysis (SSCP) PCR. Full length (432 bp) amplicons and smaller fragments were analyzed in order to increase the probability of detecting DNA polymorphisms in addition to the 2 characterized above. Analysis of cows (n=123) and bull calves (n=102) revealed no difference in frequency of the A allele (.81 and .78, respectively), whereas selected sires had a frequency of .87 for this allele. Analysis of 60 animals (to date) using SSCP has revealed no additional changes in genetic structure of exon IV of κ-casein but did detect the DNA polymorphisms associated with the allelotypes. The major advantage over RFLP analysis is that SSCP-PCR has the potential to detect additional genetic variants in the population.

INTRODUCTION

Breeders of dairy cattle have long been interested in the composition of milk since variation in the protein and fat content affect the yield of further processed products. In Holstein dairy cows, for example, the B variant of κ-casein is associated with increased yields of cheese (Marziali et al., 1986) and it has been suggested that identification of casein genotypes would be an economically important selection criterion for dairy herds dedicated for industrial milk production (McLean et al., 1984). The homozygous B variant of κ-casein is present at a low frequency (.04) in the Holstein population (Ng-Kwai-Hang et al., 1984) and at present, sires in artificial insemination programs are assessed by typing milk proteins of their progeny and dams. Earlier identification of the κ-casein genotype would considerably accelerate genetic selection.

To accomplish this goal, a number of PCRs have been developed to amplify DNA from exon IV of the κ-casein gene which contains several nucleotide substitutions which give rise to the 2 major genetic variants of κ-casein, A and B (Medrano and Aguilar-Cordova, 1990; Zadworny and Kuhnlein, 1991; Sabour et al., 1991). In the mature protein, the A variant has threonine at amino acid position 136 and aspartate at position 148, whereas, the B variant has isoleucine and alanine at these positions, respectively (Mercier et al., 1973). All of these methods have relied on restriction endonucleases to detect the nucleotide substitutions.

Recently, a new method for detecting genetic variation has been developed (SSCP-PCR:Hayashi et al., 1989). This method is based on single stranded DNA forming secondary structures which are sequence dependent and result in altered mobilities in non-denaturing gels. Since even a single base change in primary sequence will alter the mobility of the confomer, this technique has found wide applications in DNA and RNA research (Hayashi, 1992). We have

used SSCP-PCR to analyze exon IV of the κ-casein gene and compared genotyping results with typical PCR and restriction analysis. Both methods are rapid and accurate, however, SSCP analysis has the added advantage of being able to detect genetic changes throughout the amplicon rather than only at specific restriction endonuclease sites. This may prove useful for identifying additional variants in the population which may be association with specific milk production traits.

MATERIALS AND METHODS

DNA was extracted from either blood or semen samples using established methods and PCR was used to amplify a 432 bp region of the κ-casein gene which contains the nucleotide substitutions diagnostic of the A or B allele. Structures of the primers and the amplified sequences are shown in Figure 1. In brief, 200 ng of genomic DNA was used in the 100 ul. standard reaction mix. DNA was denatured for 2 minutes prior to the start of the temperature cycle and subsequently the temperature was cycled between 92, 60 and 72 °C for 20, 30 and 90 seconds, respectively for 35 cycles. Ten ul of reaction mix were then separated in 7.5 % PAGE following digestion with no restriction enzyme, 1 unit of HinfI or TaqI. The gel was stained with ethidium bromide and visualized under uv light. For SSCP analysis, 1 ul. of the reaction mix was diluted with 29 ul of a solution containing 95 % formamide, 20 mM EDTA, 0.05 % bromophenol blue and 0.05 % xylene cyanol. Samples were heated at 95 °C for 5 minutes and then cooled on ice for 3 minutes prior to loading. Samples were applied to 5-20 % polyacrylamide gels (acrylamide/bis ratios 49:1) containing 50 mM Tris-borate (pH 8.3) and 5 mM EDTA. Electrophoresis was for 18 hours at 12.5 volts/cm at room temperature. Following electrophoresis gels were stained with silver.

Figure 1. Sequence of oligonucleotide primers and their relation to the target region of the κ-casein gene which is amplified. Primer I is complementary to the (-)-strand and primer II is complementary to the (+)-strand of κ-casein. The sequence amplified is 432 bp in size and the known nucleotide substitutions associated with the B allele are indicated in brackets.

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5′ CCAATTCAGTATGTGCTGAGTAGGCATCCTAGTTATGGACTCAATTACTACCAACAG
AAACCAGTTGCACTAATTAATAATCAATTTCTGCCATACCCATATTATGCAAAGCCAGCT
GCAGTTAGGTCACCTGCCCAAATTCTTCAATGGCAAGTTTTGTCAAATACTGTGCCTGCC
AAGTCCTGCCAAGCCCAGCCAACTACCATGGCACGTCACCCACACCCACATTTATCATTT
ATGGCCATTCCACCAAAGAAAAATCAGGATAAAACAGAAATCCCTACCATCAATACCATT
GCTAGTGGTGAGCCTACAAGTACACCTACCAC(T in B-allele)CGAAGCAGTAGAG
AGCACTGTAGCTACTCTAGAAGAT(CC in B-allele)TCTCCAGAAGTTATTGAGAG
CCCACCTGAGATCAACACAGTCCAAGTTACTTCAACTGCAGTCTAA  3′
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RESULTS

Digestion of DNA amplified from A-variant animals with HinfI (A allele specific) produced bands of 326, 68 and 38 bp, whereas, digestion of DNA from B-variant animals produced bands of 394 and 38 bp. Only DNA amplified from B-variant animals was restricted with TaqI producing bands of 325 and 107 bp. In heterozygous animals only about half of the PCR product was digested by either of the restriction enzymes, thus producing all fragments. The use of 2 enzymes allowed unequivocal assignment of genotypes (Fig. 2A) and about 300 animals

were genotyped using this methodology (Table 1).

In the case of analysis using SSCP-PCR, the amplicon was denatured then electrophoresed in a 20 % non-denaturing gel. Following silver staining, the position of the single stranded DNA molecules was clearly different between DNA amplified from homozygous type A or B animals (Fig. 2B) and heterozygous animals could easily be distinguished by the presence of the composite banding pattern. Electrophoretic conditions (including gel composition, Vcm^{-1}, DNA concentration, temperature etc.) markedly affected the separation. In the analysis of 60 individuals, genotypes assigned on the basis of SSCP analysis were identical to those assigned using restriction analysis. No other differences in nucleotide sequence in these individuals were detected. The 432 bp fragment was also cleaved using HaeIII to yield 2 bands of 232 and 200 bp which were analyzed by SSCP but no additional nucleotide differences were detected.

Figure 2. A). Separation of PCR products by 7.5 % PAGE following digestion with TaqI (T) and HinfI (H). Amplified DNA was from AA (lane 1-2), AB (lanes 3-4) and BB (lanes 5-6) Holsteins. Approximate molecular sizes are indicated. B). Separation of single stranded confomers by 20 % PAGE. Amplified DNA was from AB (lane 1,2, and 4), BB (lane 3) and AA (lane 5-6) Holsteins.

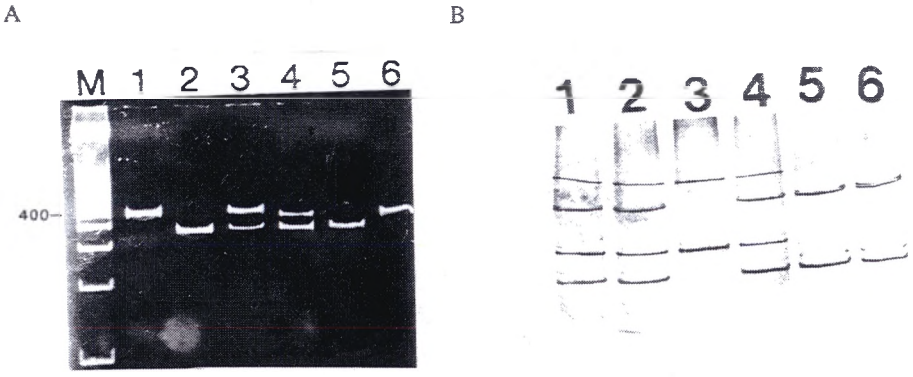

Table 1. The Genotypes and Allelic Frequencies of κ-CN in Holsteins. Cows and bull calves represent random samples from the population, whereas bulls are (>1980) or were (<1960) used by the artificial insemination industry.

	No.Typed	Genotypes			Allelic Frequencies	
		AA	AB	BB	A	B
Cows	123	79	42	2	.81	.19
Bull Calves	102	62	37	3	.78	.22
Bulls(>1980)	68	51	16	1	.87	.13
Bulls(<1960)	27	8	18	1	.63	.37

DISCUSSION

Both PCR methods were rapid and accurate in assessing the genotype of Holsteins. However, the SSCP method has an advantage in that not only are the 3 nucleotide substitutions examined but the entire sequence is scanned for polymorphisms which are not detectable by RFLP analysis. This may be important in cases where alternative codon usage results in ablation of a restriction endonuclease recognition sequence which could in turn lead to mistyping an animals. The latter would appear to occur only rarely since our analysis of 60 individuals by SSCP did not detect any nucleotide substitutions. Never-the-less, SSCP is a powerful method for detecting additional variants in the population.

The frequency of the κ-CN A-allele was dominant over the B-allele in the populations measured (Table 1). This result is in close agreement with other workers. There were no significant differences ($P > 0.05$) in allelic frequency between random sampled male calves and cows, or between AI bulls and a random sample of male calves. However, the frequency of the B-allele was found to be much higher in the sires used by AI centres before 1960 than the more modern bulls (0.37 vs 0.13). This difference was statistically significant ($P < 0.01$) and suggests that the selection criteria used to evaluate the breeding value of bulls may discriminate against the B-allele. Since the major selection pressure is applied to sires and not dams and sires are used extensively in AI programs, a reduction of the frequency of the B-allele in the sire population would be expected to precede a decrease in the dam population. Unfortunately, DNA from earlier generations in the dam population are not available for analysis. However, a comparison between our data on calves and cows ($n=225$) genotyped in 1990-91 and data on cows ($n=2,045$) phenotyped between 1979 and 1982 (Ng-Kwai-Hang *et al.* 1984) indicate that the frequency of the B-allele may also be decreasing. During this interval, the frequency has decreased from 0.26 to 0.20. We do not know the reasons for this apparent decrease in the frequency of the B-allele in the population. The focus of sire selection in North America has been on milk yield and fat content and the value of a bull to a breeding program is estimated on the average performance of its test offspring. The association of κ-CN genetic variants with either of these selection parameters is controversial.

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REFERENCES

Hayashi, K. 1992. GATA 9:73-79,

Marziali, A.S. and Ng-Kwai-Hang, K.F. 1986. J. Dairy Sci. 69:2533-2542.

McLean, D.M., Graham, E.R.B., Ponzoni, R.W. and McKenzie, H.A. 1984. J. Dairy Res. 51:531-546.

Mercier, J.C., Brignon, G. and Rimbadeau-Dumas, B. 1973. Eur. J. Biochem. 23:41-45.

Ng-Kwai-Hang, K.F., Hays, J.F., Moxley, J.E. and Monardes, H.G. 1984. J. Dairy Sci. 67:835-840.