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Polymorphism of κ-Casein in Italian Goat Breeds: A New ACRS-PCR Designed DNA Test for Discrimination of A and B Alleles

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Summary

The objective of this study was to develop a DNA test for rapid characterisation of goat κ-casein (κ-CN) A and B variants and to study this polymorphism in Italian goat breeds. Genetic polymorphism of κ-CN gene was, in addition to isoelectric focusing, analysed according to a new technique designated as amplification created restriction site. Two alleles that differ in one nucleotide mutation (G ∅ A) in exon 4 were characterised. The 167-bp PCR product surrounding the nucleotide mutation was amplified from genomic DNA and the PCR product was digested with *Mae*III. After digestion the A allele gives three fragments of 77, 65 and 25 bp in comparison with the B allele which gives two fragments of 90 and 77 bp. The analysis of allele frequency distribution at κ-CN locus, based on 401 individual samples, revealed significant differences among three goat breeds from the north of Italy (Nera di Verzasca, Frontalasca and Alpine) with frequency of κ-CN B allele around 0.3, versus two goat breeds from the south of Italy (Maltese and Sarda) with frequency of κ-CN B allele around 0.5. While two goat breeds (Maltese and Nera di Verzasca) did not show significant deviations from the Hardy-Weinberg equilibrium, a highly significant excess of heterozygote genotype (AB) was observed in Alpine, Frontalasca and Sarda goats. Here the developed DNA method and observed relatively high frequency of κ-CN B allele give a prerequisite for the assessment of research related to the simultaneous estimation of the effects of composite α_{s1}/κ-CN genotypes on milk production and cheese-making properties.

Key words: ACRS-PCR, genetic polymorphism, goat, κ-casein

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Introduction

The detection of milk proteins polymorphism (*1*) initiated considerable research activities on milk protein variants in all domestic dairy animals (cow, sheep and goat). From the beginning, the research was primarily focused on cows and was oriented towards establishment of the associations between certain genetic variants with milk production and cheese-making abilities. Although in many cases the results from different studies were not consistent, the association between kappa casein (κ-CN) and cheese-making is generally accepted. Thus, in cow milk, κ-CN BB genotype is associated with high contents of protein (*2–4*), high cheese yield and with shorter coagulation time and better curd firmness (*4–7*). Connection between genotype and cheese yield could be demonstrated in some cheese types but not in others (*8*).

On the other side, in goats the main focus of research was related to the polymorphism of alpha-s_1-casein (α_{s1}-CN), particularly since, with respect to α_{s1}-CN content, the existence of »strong« (A, B, C), »medium« (E), »low« (F, D) and »null« (O) alleles have been established (*9*). Today, there is a large number of studies related to α_{s1}-CN polymorphism and its effects on the dairy performances, physico-chemical and cheese-making properties of milk. Also the use of α_{s1}-CN polymorphism as a selection tool has already been considered (*10*). Much less attention has been given to the polymorphism of the goat κ-CN. Probable reasons for this were low observed polymorphism and low resolution between A and B variants. Thus, two phenotypic κ -CN variants, (named A and B), observed in the N-terminal region of para-κ-CN (*11*) showed a different electrophoretic mobility essayed by polyacrylamide gel electrophoresis (PAGE). Other authors (*12,13*) remarked on the presence of κ-CN polymorphism by protein analysis. At DNA level, Coll *et al.* (*14*) determined the mRNA nucleotide sequence.

The aim of this work was to establish a DNA test that would give the possibility for characterisation of the polymorphism of goat κ-CN variants A and B, determined by isoelectric focusing (IEF). Further, we studied the allele distribution of κ-CN in two goat breeds from the south of Italy (Maltese, Sarda) and three goat breeds from the north of Italy (Nera di Verzasca, Frontalasca and Alpine goat breeds).

Materials and Methods

Sample collection

Individual milk samples (401) were collected from 99 Maltese and 58 Sarda goats located in southern Italy (Sardinia), as well as 95 Nera di Verzasca, 74 Frontalasca and 75 Alpine goats located in northern Italy (Lombardia). After collection, the milk samples were stored at –20 °C without any preservative.

Isoelectric focusing of milk samples

IEF was carried out over 2.5–6.5 pH gradient obtained by mixing 2.5–5.0 and 4.0–6.5 Pharmalyte ampholytes (Pharmacia Biotech) in a volume ratio 1:1.5 according to Enne *et al.* (*15*) and 2.5–8 pH gradient obtained by mixing 2.5–5.0 and 5–8 Pharmalyte ampholytes in a volume ratio 1:1. Proteins were electrophoresed using Multiphor II Electrophoresis Cell (Pharmacia Biotech) cooled at 4 °C with a refrigerating thermostat and connected to an Electrophoresis Power Supply EPS 3500 (Pharmacia Biotech). Gel solutions were prepared according to Krause and Belitz (*16*) with a final concentration of carrier ampholytes of 3 % (volume fraction). Protein separation was performed in $124 \times 258 \times 0.4$ mm polyacrylamide gel (30 %T/2.6 %C). Defatted milk (100 μL) was denatured in 150 μL of denaturing solution (*16*). For each sample, 20 μL/lane were loaded by sample application pieces (Pharmacia Biotech). Gels were stained overnight with Coomassie Brilliant Blue G-250. The apparent isoelectric point of κ-CN variants was determined by reference samples for the genotypes that were determined by DNA analysis.

DNA extraction from milk somatic cells

After thawing at room temperature the milk samples were centrifuged at 800 rpm for 10 min at 4 °C. The supernatant was removed and the pellets were suspended in 1 mL lysis buffer (2 M Tris-HCl, pH=7.5, 40 mM EDTA, 3 M NaCl, 10 % SDS), proteinase K (Sigma) was added to the final concentration of 1 mg/mL. The solutions were shaken overnight at 42 °C and mixed with 100 μL of RNase I (stock solution: 10 mg/mL), (Roche Diagnostics) for 1 h at room temperature. DNA was extracted by phenol and chloroform (1:1 v/v). DNA was precipitated using 3 M sodium acetate, pH=5.2, absolute ethanol (1:3.5 volume fraction) and washed with 70 % ethanol. For each sample total DNA was pelleted by a speed vacuum and suspended in distilled water at 37 °C overnight.

DNA purification

DNA isolated from milk somatic cells was further purified and concentrated by DNA filtration in micro--spin columns (Centricon 10, Millipore). The DNA (100 μL) was centrifuged in 1.5 mL tube containing the specific hydrophilic YM membrane that captures DNA and eliminates PCR-inhibiting salts. The DNA was removed from the centricon membrane by centrifugation and measured by spectrophotometer (Pharmacia Biotech).

Amplification conditions for amplification created restriction site-polymerase chain reaction (ACRS-PCR) and restriction enzyme analysis

Sequence surrounding the nucleotide mutation of κ-CN region (exon 4) was amplified by using a partially mismatched PCR primer. The primer K2d and K2r sequences were:

forward 5'-CCA AAC TCT TCA ATG GCA AGT-3'

reverse 5'-TAG CAA TGG TAT TGA TGG CAG TGA-3'

The reaction mix (50 μL) comprised: 100 ng of genomic DNA, 150 nM of each primer, 1.25 U *Pwo* DNA Polymerase (Roche Diagnostics), 50 mM KCl, 10 mM Tris-HCl pH=8.3, 2.5 mM $MgCl_2$, dNTPs each at 200 μM. Amplification was carried out using a 9600 DNA

Thermal Cycler (Perkin Elmer, Norwalk, USA). Thermocycling was performed by 35 cycles for 30 s at 93 °C (denaturation), 30 s at 53 °C (annealing) and 30 s at 72 °C (extension). Final extension was 10 min at 72 °C. The identity of the PCR products was confirmed by sequencing (GeneBank Accession no. AF434987 and AF434988). The amplified sequence contained a *Mae*III created restriction site for A allele. Mix digestion was prepared with 9.7 μL DNA purified by phenol/chloroform, 0.6 μL *Mae*III (Roche Diagnostics), 1.5 μL incubation buffer for *Mae*III, (Roche Diagnostics) and 3.5 μL of double distillated water. The digestion was performed at 55 °C for 2 h.

PAGE analysis

The digestion result was analysed by 8 % continuous PAGE (*17*). Gels were cast using Mini Protean II vertical apparatus (Bio-Rad). The samples (5 μL of digested DNA and 2 μL of loading buffer) were run at constant 150 V for 1.5 h at room temperature. The rapid silver staining procedure (Pharmacia Biotech) was performed to visualise genetic markers.

Population genetic analysis

Allele frequencies and related standard errors were derived from the genotypic frequencies. Significance of the allele frequencies among the studs (pairwise comparisons) were carried out with Genepop program version 3.2a (*18*). As ten pairwaise comparisons were performed, P values were adjusted according to Hochberg's step-up Bonferroni method (*19*) with the SAS statistical package (*20*). To know whether the deviations from Hardy-Weinberg equilibrium (HWE) were in direction of heterozygote excess or deficit, HWE tests for each goat breed were performed by an exact test according to the Markov chain Monte Carlo algorithm (*21*) with Genepop program version 3.2a (*18*). The exact test was chosen because the frequency of BB κ-CN genotype was very low in Alpine and Frontalasca breeds.

Results and Discussion

ACRS-PCR designed DNA test for the characterisation of A and B allele

The first identification of phenotypic κ-CN variants was performed by IEF (pH gradient 2.5–6.5). The method was based on studies using denatured milk proteins and offers a good resolution for all caseins (Fig. 1). For optimal separation and definition of κ-CN variants, a special pH gradient obtained by mixing Pharmalyte pH 2.5–5 and 5–8 was required (Fig. 2). A and B variants were found in the Italian goat breeds analysed. The κ-CN A variant travelled to the cathode with an apparent pI more acid than κ-CN B variant. To confirm the genotypes at κ-CN locus, we analysed the DNA from milks by a new and fast method based on an amplification created restriction site (ACRS-PCR). By PCR using primer K2r we identified an restriction site specific for A allele. Digestion with *Mae*III of the 167 bp amplified DNA, allowed us to distinguish A and B alleles (Fig. 3). Samples from homozygous κ-CN goats show fragments (κ-CN AA: 77, 65 and 25 bp; κ-CN BB: 90 and 77 bp);

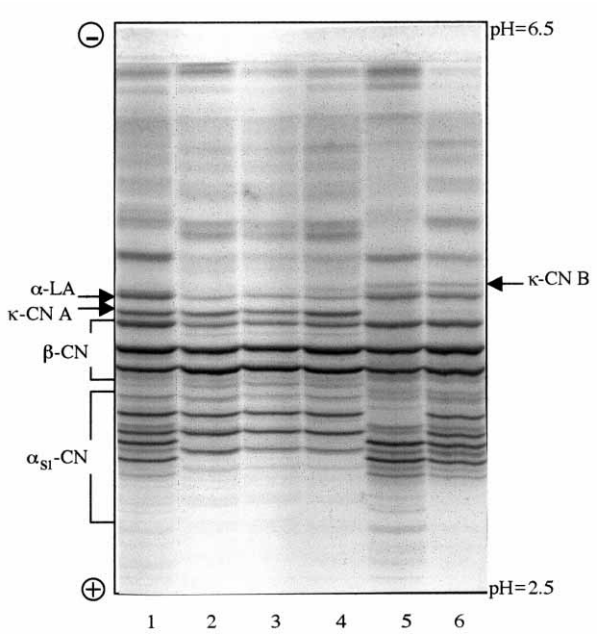

Fig. 1. Electrophoretic profiles of individual milk samples from Sarda and Maltese goat breeds showing different phenotypes at κ-CN locus. Lanes 1, 2 and 3: κ-CN AA; lane 4: κ-CN AB; lanes 5 and 6: κ-CN BB.

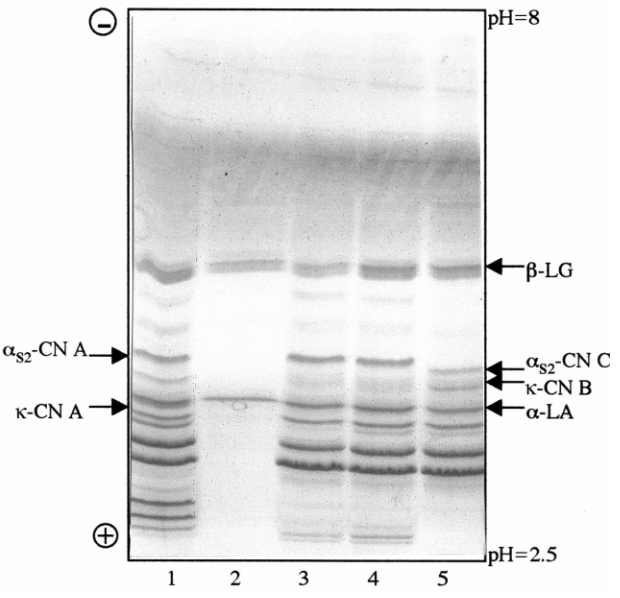

Fig. 2. IEF analysis (pH gradient 2.5–8) of four individual milk samples from Verzaschese goat breed. Lanes 1 and 5: κ-CN AB; lanes 3 and 4 κ-CN AA. Lane 2: whey from goat milk.

heterozygous κ-CN AB goats show four fragments (90, 77, 65 and 25 bp). Due to the size of the fragment, which is identical to the primer size, the fragment of 25 bp was not visible on the gel. According to Caroli *et al.* (22) sequencing of κ-CN B allele revealed four transitions at position 247, 309, 471 and 591 compared to κ-CN A allele and only the 44 Gln ♦ Arg is responsible for allele

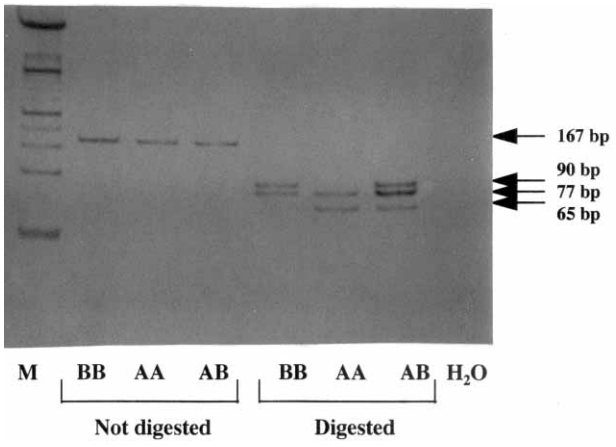

Fig. 3. Polyacrylamide gel (8 %) showing the amplification of a 167 bp DNA fragment of the nucleotide mutation region (G φ A). The genetic markers of κ-CN AA (77, 65 and 25 bp), BB (90 and 77 bp), AB (90, 77, 65 and 25 bp) genotypes after digestion are reported. (Due to the size of the fragment, which is identical to the primer size, the fragment of 25 bp was not visible on the gel). M: MWM 50 bp ladder (Boehringher Mannheim).

discrimination by IEF. Therefore it is possible that linkage between polymorphic sites is not necessarily complete and these four sites hypothetically offer the substrate for more than two alleles.

Population genetics

Allele frequencies of the κ-CN B with related standard errors, for five goat breeds from Italy, are shown in Table 1. Allele frequencies of κ-CN B (around 0.3) in three goat breeds from the north of Italy were different in comparison with the allele frequencies of κ-CN B (around 0.5) in two goat breeds from the south of Italy. This fact was also confirmed by statistical analysis where all pairwise comparisons between northern versus southern goat breeds were highly significant, even after Hochberg's step-up Bonferroni correction for multiple comparisons was applied (Table 1). In our previous study (*15*) it was shown that »null« α_{s1}-CN O and »weak« α_{s1}--CN F alleles predominate in goat breeds from the north of Italy (Orobica, Bionda, Nera di Verzasca, Alpine), while »strong« α_{s1}-CN A and α_{s1}-CN B alleles predominate in goat breeds from the south of Italy and North Africa (Sarda, Maltese, Maroccan, Tunisian). Thus, the obtained results suggest linkage disequilibrium between α_{s1}-CN and κ-CN locus, *i.e.* that κ-CN B allele is positively correlated with »strong« α_{s1}-CN A and α_{s1}-CN B alleles, and the opposite, that κ-CN A allele is positively correlated with the presence of »null« α_{s1}-CN O and »weak« α_{s1}-CN F alleles. If this is true, any single gene (genotype) estimation, for example, the estimation of association between genetic variants with milk production and cheese-making abilities of α_{s1}-CN locus without considering the effects of κ-CN locus might lead to incorrect results and conclusions.

Two goat breeds (Maltese and Nera di Verzasca) did not show significant deviations from the Hardy--Weinberg equilibrium. In contrast, there was highly significant excess of heterozygote genotype (AB) in other three goat breeds (Alpine, Frontalasca and Sarda). Selection for heterozygote AB genotypes might be one potential reason for the observed heterozygote excess in Alpine, Frontalasca and Sarda goats. However, as κ-CN locus is closely linked to other casein loci, for example, to α_{s1}-CN locus, the presence of associative overdominance cannot be excluded either.

Table 1. Allele frequencies and statistical inference of differences in allele frequencies among Italian goat breeds at κ-CN locus

Origin of samples	Goat breed	Sample size	Allele frequency			Statistical inference[1]
			A	B	SE	
Lombardia	Alpine	75	0.673	0.327	0.038	A1, B1
Lombardia	Frontalasca	74	0.676	0.324	0.038	A2, B2
Lombardia	Nera di Verzasca	95	0.689	0.311	0.034	B3, C1
Sardinia	Maltese	99	0.500	0.500	0.036	A1, A2, B3[1]
Sardinia	Sarda	58	0.448	0.552	0.046	B1, B2, C1

[1]Goat Breeds with the same symbols are significantly different (A1, A2: P<0.01; B1, B2, B3: P<0.005 and C1: P<0.001) after the adjustment of single test P values (pairwise comparisons) by Hochberg's step-up Bonferroni method.

SE = standard error.

Table 2. Hardy-Weinberg equilibrium inference at κ-CN locus in Italian goat breeds

Goat breed	Number of observed genotypes			Heterozygote deficit[1]	Heterozygote excess[1]
	AA	AB	BB		
Alpine	27	47	1	1.000	0.000
Frontalasca	29	42	3	1.000	0.010
Nera di Verzasca	46	39	10	0.416	0.763
Maltese	25	49	25	0.522	0.636[1]
Sarda	4	44	10	1.000	0.000

[1]P – values obtained by the exact tests according to the Markov chain Monte Carlo algorithm

Prospects

The developed DNA method and the observed relatively high frequency of κ-CN B allele give a prerequisite for the assessment of research related to the simultaneous estimation of α_{s1}-CN and κ-CN gene (genotype) effects on milk production and cheese-making properties. This would be of particular interest with respect to the large number of studies finding significant single gene (α_{s1}-CN) effects on casein contents and cheese-making traits and suggesting the incorporation of certain α_{s1}-CN alleles in goat breeding schemes. Further, as the obtained results indicate that different κ-CN variant might be distributed differently depending on the geographic region (south versus north) it would be interesting to study linkage disequilibrium between those two genes in different populations.

Conclusions

The polymorphism of goat κ-CN determined by IEF (variants A and B) can be confirmed on a DNA level by ACRS-PCR designed DNA test that is described in this paper. With this method (ACRS-PCR) on a DNA level, it is possible to identify A and B alleles for non-lactating goats (kids, rams) as well as from other sources (blood, hair, sperm, *etc.*), which can be important information for future selection. In comparison with other goat breeds, high frequency of κ-CN B allele (0.5) was observed in the goats from Sardinia (Maltese and Sarda). As those breeds are known for the predominant presence of »strong« α_{s1}-CN alleles (A and B), the obtained results suggest that κ-CN B might be positively correlated with »strong« α_{s1}-CN alleles. Since casein loci are tightly linked, the developed method described will further enable and stimulate research related to the simultaneous estimation of the effects of composite α_{s1}/κ-CN genotypes on milk production and cheese-making properties.

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Polimorfizam κ-kazeina talijanskih pasmina koza: novi ACRS-PCR test za razlikovanje A i B alela

Sažetak

Svrha je bila razviti DNA test za brzo određivanje genetičkih varijanti κ-kazeina u talijanskih pasmina koza. Genetički je polimorfizam istraživan izoelektričnim fokusiranjem (IEF) i amplifikacijom nastalim restrikcijskim mjestom na genomu (ACRS). PCR produkt na ekzonu 4, dug 167-bp koji okružuje nukleotidnu mutaciju (G ∅ A), amplificiran s genomske DNA i razgrađen je s *Mae*III enzimom. Identificirana su dva alela koja se razlikuju u jednoj nukleotidnoj mutaciji (G ∅ A). Dobiveni su fragmenti 77 i 65 bp za alel A te 90 i 77 bp za alel B. Signifikantna razlika za frekvenciju alela B ustanovljena je za tri pasmine

koza sa sjevera Italije (Nera di Verzasca, Frontalasca i Alpine) s frekvencijom κ-kazeina B oko 0,3, u usporedbi s dvjema pasminama s juga Italije (Maltese i Sarda), s frekvencijom κ-kazeina B otprilike 0,5. Pasmine Maltese i Nera di Verzasca nisu pokazale signifikantno odstupanje od Hardy-Weinbergove ravnoteže, za razliku od pasmina Alpine, Frontalasca i Sarda u kojima je uočeno veliko signifikantno odstupanje. Prikazana DNA metoda i promatrana relativno velika frekvencija κ-kazeina B alela omogućavaju daljnja istraživanja zajedničkog utjecaja α_{s1}-kazeina i κ-kazeina na proizvodnju mlijeka i sira.