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Short Communication

Bovine kappa-casein gene polymorphism and its association with milk production traits

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Abstract

Point mutations in exon IV of the bovine κ-casein (*CSN3*) gene determine two allelic variants, A and B. These variants were distinguished by polymerase chain reaction and restriction fragment length polymorphism (PCR-RFLP) analysis in the indigenous Sahiwal and Tharparkar cattle breeds. DNA samples (252 Sahiwal and 56 Tharparkar) were analyzed for allelic variants of the *CSN3* gene. Polymorphism was detected by digestion of PCR-amplified products with *Hind*III, *Hha*I and *Hae*III restriction enzymes, followed by separation on 3% agarose gels, and resolved by ethidium bromide staining. Allele A of the κ-casein gene occurred at a higher frequency than allele B, in both Sahiwal and Tharparkar breeds. The genotypic frequencies of AA, AB, and BB in the Sahiwal and Tharparkar breeds were 0.758, 0.230 and 0.012, and 0.0.732, 0.250 and 0.018, respectively. The frequencies of alleles A and B in the Sahiwal and Tharparkar breeds were 0.873 and 0.127, and 0.857 and 0.143, respectively. Genotype BB of the kappa-casein gene had more influence on the monthly milk yield, 305-days milk yield, monthly solids-not-fat (SNF) yield, and monthly protein yield, in the Sahiwal cattle.

Key words: Indian cattle, κ-casein, genetic polymorphism, PCR-RFLP.

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Milk protein genetic polymorphism has received considerable research interest in recent years because of possible associations between milk protein genotypes and economically important traits in dairy cattle. Many research reports have indicated that certain milk protein variants may be associated with milk production (Ng-Kwai-Hang *et al.*, 1984; Bech and Kristiansen, 1990), milk composition (Lunden *et al.*, 1997; Ng-Kwai-Hang, 1998; Robitaille *et al.*, 2002) and cheese production (van den Berg *et al.*, 1992; Lundén *et al.*, 1997; Ng-Kwai-Hang, 1998). Therefore, milk protein genes could be useful as genetic markers for additional selection criteria in dairy cattle breeding. The main components of the cow milk casein complex are αs1, αs2, β and κ-caseins, and each one of them is known to occur in the form of two or more variants, inherited according to a straight-forward Mendelian model. The κ-casein (CSN3) molecule is a single-chain polypeptide of 169 amino acids with a molecular weight of 19.2 KDa. CSN3 plays an important role in milk chemistry by providing colloidal stability to the casein micelle. In the micelle, κ-casein is mostly located at the periphery, with its hydrophilic C-terminal sequence protruding into the solvent. The κ-casein gene has been assigned to chromosome 6 (6q31) in cattle (Threadgill and Womack, 1990). Two κ-casein variants have been described in detail as κ-casein A and B. The difference between A and B lies in single amino acid substitutions at positions 136 and 148 (Eigel *et al.*, 1984). The present study aimed at genotyping Sahiwal and Tharparkar cattle breeds for the κ-casein gene and at finding out whether there is an association between *CSN3* polymorphic forms and production traits in Sahiwal cattle. Blood samples were collected in vacutainers (Becton Dickinson Vacutainer System) containing sodium EDTA as an anticoagulant from Sahiwal (252) and Tharparkar (52) cattle breeds maintained at the National Dairy Research Institute, India. Genomic DNA was extracted from 10 mL of whole blood by the phenol-chloroform method, as described by Sambrook *et al.* (1989), and from semen, as described by Lien *et al.* (1990). The quality of the DNA was checked on 0.6% agarose gel and the quantity was determined by UV spectrophotometry at A_{260}/A_{280} nm.

The sequence (GenBank Accession # AY380229) reported by Robitaille *et al.* (2005) was used for primer designing by using the Primer3 software (Rozen and Skaletsky, 2000) to amplify the 633 bp PCR product that consists of a part of intron III (4 bp), exon IV (516 bp) and intron IV (113 bp). The primer sequences were: 5'-CAG CGC TGT GAG AAA GAT GA -3' (forward) and 5'-CCC

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ATT TCG CCT TCT CTG TA -3' (reverse). The PCR amplification reaction contained 100 ng DNA, 50 ng/µL of each primer 0.5 ug, 1.5 mM $MgCl_2$, 200 µM of dNTPs, PCR buffer (15 mM $MgCl_2$, 100 mM KCl, 20 mM Tris-HCl pH 8.0, 0.1 mM EDTA, 1 mM DTT, 0.5%Tween 20 (V/V), 0.5% Igepal, and 50% glycerol) and 0.9 units of Taq DNA polymerase. The fragment was amplified by hot-start PCR, in which 2 µL of genomic DNA (50 ng/µL) were overlaid with 1.5 µL of mineral oil in a PCR tube. PCR amplification was carried out in a programmable thermal cycler (MJ Research) as follows: denaturation at 95 °C for 5 min, then the temperature was lowered to 85 °C, immediately the PCR master mix was added at the top of the mineral oil. The PCR reaction then proceeded with: 2 min at 93 °C, 1 min at 94 °C, 30 s at 60 °C, 1.45 min at 72 °C, followed by 29 cycles of 1 min at 94 °C, 30 s at 60 °C and 1.45 min at 72 °C, and a final extension cycle of 15 min at 72 °C. The PCR products were loaded onto 1.5% agarose to confirm the amplification of the target region using a 100 bp marker. Restriction digestion of the PCR product was carried out with enzymes *Hind*III, *Hae*III, and *Hha*I, and the reaction was set up with 2.0 µL of ddH_20, 2.5 µL of restriction endonuclease buffer 2 (New England Biolabs), 0.6 units of restriction enzymes, and 20.0 µL of PCR product incubated at 37 °C for about 3 h. The restriction-digested fragments were separated on 3% agarose gels. The gels were stained with ethidium bromide and photographed by using a gel documentation system (Axygen Pvt. Ltd.), and the three genotypes were scored manually. Milk samples (80 mL) were collected three times a day (morning, afternoon, evening) from all lactating animals in a clean sterile 125-mL polypropylene container, taken to the laboratory and stored at 4 °C. The next morning, three milk samples from each animal were kept at room temperature for 1 h and mixed well, and about 100 mL of pooled samples were taken for fat (electronic milk tester method), total protein (Kjeldahl's method), and solids-not-fat (lactometer) analysis.

Data of the Sahiwal cattle of known pedigree and with normal lactation were included in the study. A lactation period of at least 100 days, in which the cow calved and dried under normal physiological conditions, was considered as normal lactation. Data were classified according to parity (lactation I, II, III IV, V and VI) and season [winter (Dec-March), summer (April-June), rainy (July-August), autumn (Sept-Nov)]. Four periods (set of bulls used for AI purpose over a period) and three stages of lactation [1st stage (7-90 days), 2nd stage (91-180 days), 3rd stage (181-305 days)] were also considered, to find out the effect of non-genetic factors on performance traits. The numbers of the Tharparkar animal herd were so small that there were no data available on production traits.

Statistical analysis was carried out at the Computer Centre, NDRI. In order to study the effect of season, parity, period and stage of lactation on various traits and to overcome the problem of non-orthogonality of effects due to unequal and disproportionate sub-class frequencies, least-squares analysis of fitting constants as suggested by Harvey (1987) was employed for data analysis. The following fixed linear model was used, with the assumptions that the different components fitted into the model were linear, independent and additive.

$$Y_{ijkl} = \mu + P_i + SE_j + PA_k + SL_l + e_{ijkl}$$

where Y_{ijkl} = observation of animal of i^{th} period of j^{th} season, k^{th} parity, l^{th} stage of lactation, µ = overall mean, $P_i = i^{th}$ effect of period (1...4), $SE_j = j^{th}$ effect of season (1.. 4), $PA_k = k^{th}$ effect of parity (1...6) and $SL_l = l^{th}$ effect of stage of lactation (1...3) and e_{ijkl} = random error.

Association between polymorphism patterns and performance traits (monthly and tri-monthly milk yield, 305-day milk yield, fat percentage and yield, protein percentage and yield, and SNF percentage and yield) was tested using category variables (genotypes) in adjusted data, as follows:

$$Y_i = \mu + G_i + e_i$$

where $Y_i = j^{th}$ production trait of animal in i^{th} genotype, µ = overall mean, G_i = effect of i^{th} genotype and e_i = random error.

The difference of means between monthly milk yield, tri-monthly milk yield, fat percentage and yield, protein percentage and yield and SNF percentage and yield within season, parity, period, stage of lactation and genotypes was tested for significance by applying Duncan's Multiple Range Test (DMRT) as modified by Kramer (1957).

Restriction digestion of the 633 bp PCR product with enzyme *Hind*III revealed three genotypes AA (633 bp uncut), AB (633, 423 and 210 bp) and BB (423 and 210 bp). The frequencies of genotypes AA, AB, and BB were, respectively, 0.758, 0.230 and 0.012 in Sahiwal, and 0.732, 0.250 and 0.018 in Tharparkar cattle. The gene frequencies were calculated based on Mendelian inheritance, and the frequencies of genes A and B were found to be, respectively, 0.873 and 0.127 in Sahiwal, and 0.857 and 0.143 in Tharparkar. The frequency of genotype AA was higher than that of BB in both breeds studied. These results also show that the chosen primers were adequate for amplifying the κ-casein gene exon IV sequence in *Bos indicus* cattle. Restriction digestion of the 633 bp PCR product with enzymes *Hae*III and *Hha*I revealed 610 and 23 bp for *Hha*I and 343 and 290 bp fragments for *Hae*III in both breeds studied. This indicates that digestion with these enzymes did not reveal any polymorphism in any of the breeds studied.

The frequency found for allele A was higher than that of allele B, which was in close agreement to the results of earlier studies performed in *Bos taurus* (Ng-Kwai-Hang *et al.*, 1984; Pinder *et al.*, 1994; Kemenes *et al.*, 1999). The genotyping results of Sahiwal and Tharparkar breeds found are similar to those reported for Korean native cattle, Japa-

nese brown, Angus, Hereford, Charolais and Holstein cows by Chung *et al.* (1995, 1998). Similar observations were reported by Malik *et al.* (2000) in fourth-generation crossbred cattle (50% Holstein, 25% Jersey, 25% Zebu, *i.e.*, Haryana and Sahiwal cattle).

The present study was the first attempt to associate the genotype with phenotypic data in Sahiwal cattle. The significance of various non-genetic factors influencing production traits is summarized in Table 1, and data were adjusted for significance effects. For the association studies, the adjusted data were used.

The least-squares means of monthly milk yield, tri-monthly milk yield and 305-days milk yield, monthly milk fat percentage and yield, monthly milk protein percentage and yield, monthly milk SNF percentage and yield for different genotypes are presented in Table 2. The mean monthly and 305-days milk yield in different κ-casein variants were, respectively, 206 ± 1.62 kg, 188 ± 2.84 kg and 239 ± 9.92 kg, and 1548.71 ± 42.41 kg, 1336.65 ± 2.04 kg and 2284.74 ± 292.12 kg in genotypes AA, AB and BB. The animals with genotype BB had a higher monthly and 305-days milk yield than those with genotypes AA and AB. A similar effect had been noticed by Lin *et al.* (1986*)*, who reported that the BB genotype had a higher average milk yield than AA and AB, and the lactational yield values were 4244, 4280 and 4465 kg for genotypes AA, AB and BB, respectively. Alison Van Eenennaam and Medrano (1991) compared κ-casein AA and BB genotypes and showed that the BB genotype showed first lactational yields increased by 296 kg with regard to AA. In contrast, Gonyon *et al.* (1987) and Curi *et al.* (2005) reported that the κ-casein genotype AA was associated with higher milk production than BB, with the heterozygous AB being intermediate, whereas Ng-Kwai-Hang *et al.* (1986) and S Kim, M.Sc. Thesis, McGill University, Montreal, QC, 1994) showed that κ-casein AB animals were better milk producers than either of the homozygous animals, in Ayrshire, Holstein, Jersey, brown Swiss, Canadienne and Guernsey cattle breeds. This is indicative of variations among species, environments and management practices adopted at different farms.

The mean monthly fat percentage and fat yield in different κ-casein variants were 4.92 ± 0.11, 4.79 ± 0.19 and 4.91 ± 0.68 kg, and 10.31 ± 0.32, 9.10 ± 0.56 and 11.94 ± 1.93 kg in genotypes AA, AB and BB, respectively. BB animals had a higher monthly fat yield than those with genotypes AA and AB. However, the results were not statistically significant. The results of the present study are in concurrence with those of Ng-Kwai-Hang *et al.* (1986), Horne *et al.* (1997) and Strzalkowska *et al.* (2002), who also reported that the κ-casein variant BB genotypes were associated with increased milk fat yield. The mean monthly protein percentage and yield in the AA, AB and BB κ-casein genotype variants were 3.61 ± 0.018, 3.58 ± 0.030 and 3.67 ± 0.136 kg, and 22.91 ± 0.49, 20.96 ± 0.80 and 23.28 ± 3.59 kg, respectively. The AB animals had a lower monthly protein yield compared to those with genotypes AA and BB, but no significant difference was observed in protein percentage among the genotypes. Similar results were reported by Bovenhuis *et al.* (1992), Van den Berg *et al.* (1992), Ron *et al.* (1994) and Ikonen *et al.* (1996), whereas Kim (1994) and Strzalkowska *et al.* (2002) found no difference in the milk protein content between the two κ-casein variants in Ayrshire, Jersey, brown Swiss, Canadienne, Guernsey and Polish Black-and-White cattle. The mean monthly milk SNF percentage and yield in the different κ-casein variants were 8.88 ± 0.003, 8.87 ± 0.005 and 8.90 ± 0.016 kg, and 18.32 ± 0.14, 16.77 ± 0.25 and 21.32 ± 0.88 kg in genotypes AA, AB and BB, respectively. The BB animals had higher monthly SNF percentage and yield than those with genotypes AA and AB. The results of the present study are in concurrence with those of Strzalkowska *et al.* (2002).

Table 1 - Least-squares variance analysis (mean squares) of lactation traits of Sahiwal cattle**.**

Trait	Season	Parity	Period	Stage of lactation	Error
MMY	218640.88**	303288.22**	2681852.0**	3525942.5**	9247.58
TMMY	341527.84*	542197.94*	5731153.50*	3497162.50*	82531.32
MFP	13.22^{NS}	35.14^{NS}	29.64^{NS}	26.62^{NS}	39.99
MFY	645.98^{NS}	1152.51**	7567.20**	8346.93**	317.89
MPP	0.45*	0.15^{NS}	0.72**	0.44^{NS}	0.14
MPY	535.15**	709.40**	6767.37**	3926.51**	110.59
MSNFP	0.05^{NS}	0.47**	1.67**	0.05^{NS}	0.02
MSNFY	1746.99**	2334.86**	21982.59**	27760.11**	72.80

**Significant at 1% level.

*Significant at 5% level.

MMY: monthly milk yield; TMMY: tri-monthly milk yield; 305 MY: 305-days milk yield; MFP: monthly fat percentage; MFY: monthly fat yield; MPP: monthly protein percentage; MPY: monthly protein yield; MSNFP: monthly SNF percentage; MSNFY: monthly SNF yield.

Table 2 - Least-squares means for κ-casein (*CSN3*) genotypes of Sahiwal cattle.

Trait/genotype	AA	AB	BB
Monthly milk yield (kg)	206.22 ± 1.62[a] (3213)	188.92 ± 2.84[b] (1045)	239.10 ± 9.92[c] (86)
Tri-monthly milk yield (kg)	635.46 ± 13.26[a] (439)	584.55 ± 22.03[b] (159)	620.23 ± 98.22[ab] (8)
305-days milk yield (kg)	1548.71 ± 42.41[a] (429)	1336.65 ± 2.04[b] (148)	2284.74 ± 292.12[c] (9)
Monthly fat percentage (kg)	4.92 ± 0.11 (3264)	4.79 ± 0.19 (1054)	4.91 ± 0.68 (86)
Monthly fat yield (kg)	10.31 ± 0.32 (3205)	9.10 ± 0.56 (1039)	11.94 ± 1.93 (86)
Monthly SNF percentage (kg)	8.88 ± 0.003[a] (3264)	8.87 ± 0.005[b] (1054)	8.90 ± 0.016a[b] (86)
Monthly SNF yield (kg)	18.32 ± 0.14[a] (3209)	16.77 ± 0.25[b] (1045)	21.32 ± 0.88[c] (86)
Monthly protein percentage (kg)	3.61 ± 0.018 (446)	3.58 ± 0.030 (159)	3.67 ± 0.136 (8)
Monthly protein yield (kg)	22.91 ± 0.49[a] (437)	20.96 ± 0.80[b] (159)	23.28 ± 3.59[a] (8)

In parentheses: number of observations. a, b, c: values with same superscript do not differ significantly.

We concluded that the genotype frequency of AA was higher than that of BB in the Sahiwal and Tharparkar populations studied, and the frequency of gene A was also higher. At the κ-casein exon IV locus, *Hind*III enzyme revealed polymorphism, but *Hae*III and *Hha*I did not. These results further confirm that in *Bos indicus* cattle the predominant genotype is *CSN3* AA, as in European breeds. The kappa-casein gene BB genotype showed a greater influence on monthly milk yield, 305-days milk yield, monthly SNF yield and monthly protein yield. The present study was an aid in the selection of superior animals, i. e, those with genotype BB, in order to increase the production of milk and its constituents without increasing the herd size in India.

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