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Probing of alpha, beta and kappa-caseins polymorphic variants in Gangatiri cow milk with the use of polyacrylamide gel electrophoresis and HRAMS

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Research Article

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Abstract

This research communication aimed to probe the genetic polymorphisms of alpha, beta, and kappa caseins in Gangatiri cows (an indigenous Indian cattle). Detection of variants has received considerable research interest in the dairy industry and animal breeding in recent years as a source of good quality protein, but also of bioactive peptides that may be linked to health implications. The polymorphic nature of casein fractions and their association with milk production traits, composition, and quality also attracted several efforts in evaluating the allelic distribution of different casein locus as a potential dairy trait marker. Molecular techniques of polyacrylamide gel electrophoresis and high-resolution accurate mass-spectrometry have been applied to this probe. Sequence analysis of different casein types in the cows showed the presence of four specific variants.

Gangatiri is an elite breed of cattle of *Bos indicus* origin among the fifty well-defined indigenous breeds of cattle in India. This breed has been registered in ICAR-NBAGR with serial number thirty-nine with its accession number INDIA_CATTLE_2003_GANGATIRI_03039. Gangatiri has always been an important breed of cattle for small and marginal farmers because of its low input husbandry, high survival capacity and adaptability in harsh climatic conditions (Verma and Singh, 2018).

Casein exhibits polymorphic heterogeneity with four distinct gene products named α-casein (α-Cn, comprising α-S1 and α-S2 caseins), β-casein (β-Cn) and κ-casein (κ-Cn) that alter the size of casein micelles in the bovine milk (Vigolo *et al.*, 2022). Because of a significant correlation between milk casein genotypes and commercially relevant features in dairy animals, bovine protein variations have attrracted considerable research interest in recent years. Our aim was to isolate the α-Cn, β-Cn and κ-Cn fractions of Gangatiri cow's milk using polyacrylamide gel electrophoretic (PAGE) and then examine the identity of the separated α-Cn, β-Cn and κ-Cn phenotypes employing a high-resolution accurate mass spectrometry system (HRAMS).

Materials and methods

The chemicals and markers were purchased from Sigma and Himedia.

Milk sample collection

100 ml raw milk samples were drawn individually from twelve healthy native Gangatiri cows and one each of the Sahiwal, Jersey, and Holstein Friesian breeds. Gangatiri pooled raw milk samples were stored at −20°C until analysis (Nguyen *et al.*, 2020).

Whole casein fractionation and SDS-PAGE

As described by Hollar *et al.* (1991), a modified isoelectric precipitation method was used to fractionate whole casein. Isolated casein pellets were resuspended in distilled water and the pH was maintained at 7.5. The Lowry procedure was used to quantify protein concentration with bovine serum albumin as a control protein. The method of Andrews (1983) was used to perform the gel electrophoresis of Gangatiri milk casein with minor changes. On the first run, 5 to 10 µg of whole caseins from previously genotyped Jersey, Holstein Friesian, and Sahiwal cows along with Gangatiri casein were loaded into the fourth to seventh wells, respectively, for the comparative probing (optimization) of casein fractions from these breeds. Then in the second

experiment, 4 µg of casein protein extracted from different Gangatiri milk samples were uploaded into the second to ninth PAGE wells. The first well was loaded with 5 µl of prestained protein marker in both runs. The stacking gel solution contained 5% acrylamide and 10% SDS at pH 6.8, whereas the resolving gel was prepared with 15% acrylamide and 10% SDS at pH 8.8. The cell was filled with 5× running buffer and both the electrophoretic runs were completed at ambient temperature using the voltage stepped method up to 100 V. Gel staining was performed with Coomassie R-250 dye and followed by documentation.

High-resolution mass-spectrometry amino acid sequencing

After SDS PAGE casein gel fractions were excised from the gel, cut into small pieces and destained. The reduction process was conducted with 0.005 M tris(2-carboxyethyl)phosphine and further alkylation with 0.05 M indole acetic acid before being degraded for 16 h with trypsin (1 : 50, trypsin/lysate ratio) at 37°C. To exclude the salt, the digests were cleaned with a C-18 silica cartridge and dried (Chopra *et al.*, 2020). Then, the pellet was suspended in buffer A (containing acetonitrile and formic acid). A Thermo scientific Ultimate^{TM} 3000 RSLCnano system coupled with a QE Plus was employed for the mass spectrophotometric assay of the peptide mixture. 1µg sample was uploaded on a C-18, 50 cm, 3.0 µm easy-spray column at a flow rate of 300 nl/min, and peptides were eluted using a 0–40% gradient of buffer B. Chromatography gradients were run for 100 min. Mass spectra were captured at 70 K resolution in the Orbitrap.

All samples were analyzed, a file was created and Proteome Discoverer$^{(TM)}$ was used to examine it with the UniProt custom reference database of the proteome. The fragment and precursor mass limitations for Sequest search were set to 0.02 Da and 10 ppm, respectively. Due to the enzyme specificity for trypsin/P, protease was employed to produce peptides, as well as the maximum missed cleavage value for each was set. For the database, carbamidomethyl over cysteine is regarded as a permanent alteration, while methionine oxidation is considered a variable change. The peptide spectrum was matched, and the protein's false discovery rate was set to 0.01.

Results and discussion

We examined the genetic polymorphism of casein subtypes from the analysis of milk samples of Indian zebu (Gangatiri) cattle. For the method optimization, we ran the first comparative run of SDS-electrophoresis of Gangatiri casein and the whole caseins were isolated from the previously genotyped two international breeds, Jersey and Holstein Friesian, and one indigenous breed, Sahiwal (online Supplementary Fig. S1). All presented a similar type of separation of different casein fractions in all four wells. The separation of whole caseins into α-Cn, β-Cn and κ-Cn fractions showed uniformity in each lane of all four breeds of bovine with average Rf (Retention factor) values of α-Cn, β-Cn and κ-Cn as 0.42, 0.44 and 0.49, respectively.

In the second run, the eight distinct Gangatiri cow's caseins were loaded in the different wells of the electrophoresis unit after the comparative separation run of the Gangatiri casein with the previously genotyped breed caseins. Each eight-casein protein extracted from Gangatiri cow's milk was fractionated into α-Cn, β-Cn and κ-Cn (Fig. 1, representative gel: lanes 2–9), where κ-Cn showed a higher mean relative mobility followed by β-Cn and α-Cn. The mean Rf was 0.43 with a standard deviation of 0.4%. These results are similar to other reports (Kumar *et al.*, 2013). The bands of different casein fractions were reliably found in all three duplicate studies. Amino acid sequence results obtained from high-resolution HPLC-LTQ-MS of Gangatiri α-Cn, β-Cn and κ-Cn are shown in Table 1 and the detailed sequence database search result is given in online Supplementary Table S1.

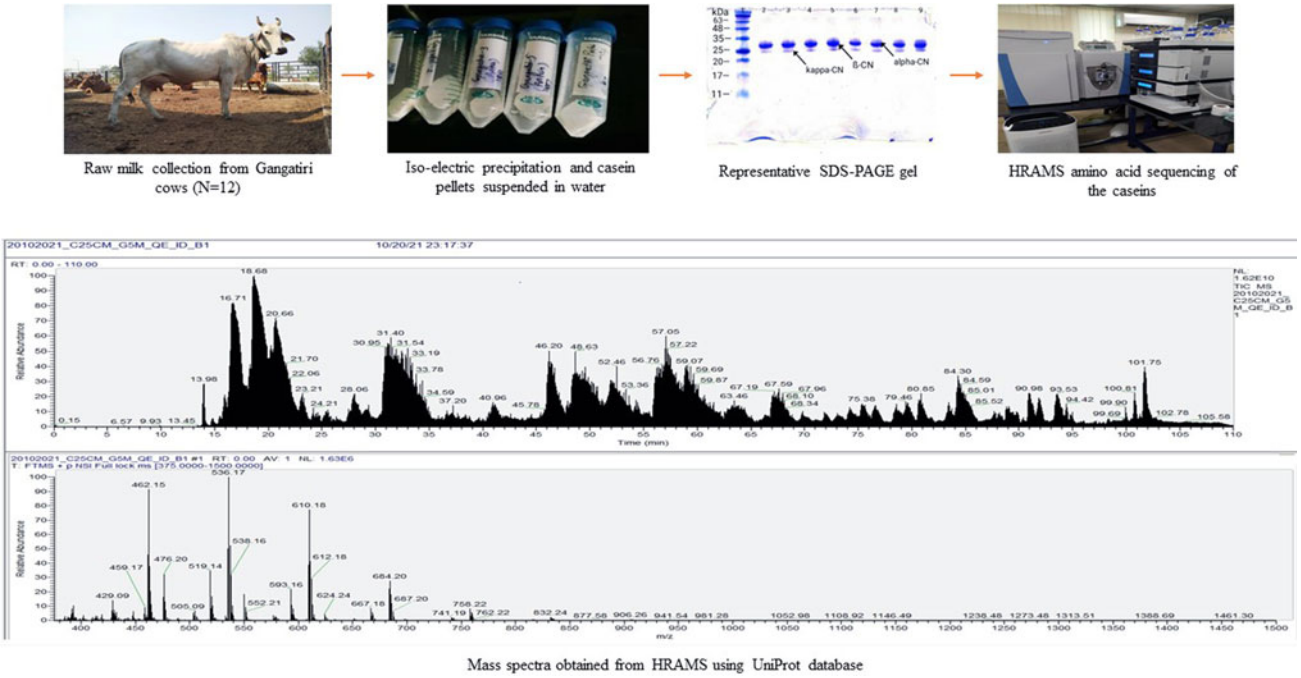

Fig. 1. Representative illustration of Gangatiri milk collection, casein isolated casein, and electrophoretic separation of whole casein fraction using tricine-SDS polyacrylamide gel followed by the mass spectra obtained from the HRAMS analysis using UniProt database.

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Alpha-casein

The sequence of alpha caseins obtained from mass spectrometry shows the Gangatiri αS1-Cn was showing homology with the variant B sequence given in the UniProt KB proteome database (online Supplementary Fig. S2). According to the NCBI database, variant B has UniProt KB ID number P02662. This has confirmed the B variant of αS1-Cn in Gangatiri milk. The Gangatiri αS2-Cn sequence was similar to the variant A sequence according to the NCBI proteome database (online Supplementary Fig. S3) with accession ID P02663. The Gangatiri alpha casein is coded as the CSN1 gene on chromosome number 6 (Gene ID α-s1 : 282208 and gene ID α-s2 : 282209).

The genetic polymorphisms that cause nine protein variations of αS1-Cn coded by the CSN1S1 gene in cattle have been identified and categorized from A to I (Nadugala *et al.*, 2022). The variants B and C are the two most prevalent variations, which differ in the exchange of glutamic acid positioned at 192 with glycine amino acid in mature proteins. The presence of alanine amino acid in the 26th and 53rd positions confirms variant B of this bovine casein. Furthermore, only the B and C alleles appear in zebu breeds. The present data show that the Gangatiri breed has a B variant, showing similarity with previous reports by Mir *et al.* (2014).

The Gangatiri αS2-Cn sequence obtained from LC-MS represents the allele A of the genetic polymorphism. The polypeptide chain αS2-Cn A is distinguishable from αS2-Cn D by deleting a quite acidic nonapeptide containing a cluster of three phosphoryl residues (Fang *et al.*, 2016). According to a genetic study, the clusters αS1-Cn, β-Cn and κ-Cn are all linked to the αS2-Cn genome. There is an important role of αS1-Cn in the potential of milk to transport calcium phosphate and in the creation of casocidin-I, where it inhibits *E.coli* and *S.carnosus* growth.

Beta-casein

The sequence of β-Cn obtained from mass spectrometry metabolomics shows the Gangatiri beta-casein was analogous to the BCA2 sequence as per the UniProt KB proteome database (online Supplementary Fig. S4). In the NCBI database, BCA2 has UniProt KB ID number P02666. That has confirmed the presence of the A2 variant of β-Cn in the Gangatiri cow's milk. Duarte-Vazquez *et al.* (2018) separated Holstein Friesian milk β-Cn variants by PAGE using an alkaline medium and liquid chromatography-mass spectrometry sequencing. Consuming dairy foods containing the A2 variant of β-Cn potentially results in less bloating and abdominal pain than the more common A1 variant. Variant A2 is desirable in bovine milk as it is said to induce protective activity through the release of glutathione in the blood, and it also appears to improve the digestibility of milk. Due to health concerns related to β-Cn A1 protein, especially gastrointestinal release of beta-casomorphins during digestion, the analysis of beta-casein variation is important in the bovine breeds (Lambers *et al.*, 2021).

Kappa-casein

The sequence of κ-Cn obtained from LC-MS shows the Gangatiri κ-Cn was homologous to the variant A sequence according to the UniProt KB proteome database (online Supplementary Fig. S5). In the NCBI database, κ-Cn has UniProt KB ID number P02668. This has confirmed the presence of A variant of kappa casein in Gangatiri cow's milk. κ-Cn is especially interesting because it correlates with milk quality attributes and

Table 1. Depiction of the amino acid sequences of the different casein fractions of the Gangatiri cow milk obtained after HRAMS using UniProt reference proteome database

Accession	Description	Sequence	Coverage %	Peptides	PSM	AAs	MW (kDa)	Calc. pI	Score MS Amanda 2.0	Score sequest HT	Gene symbol
P02662	Alpha-S1-casein	MKLLILTCLVAVALARPKHPIKHQGLPQEVLNENLLRFFV**A** PFPEVFGKEKVNELSKDIGSESTEDQ**A**MEDIKQMEAESISSSEEIVPNSVEQKHI QKEDVPSERYLGYLEQLLRLKKYKVPQLEIVPNSAEERLHSMKEGIHAQQKEPMI GVNQELAYFYPELFRQFYQLDAYPSGAWYYVPLGTQYTDAPSFSDIPNPIGSENS**E**KTTMPLW	59	20	504	214	24.5	5.02	78 106.07	864.9	CSN1S1
P02663	Alpha-S2-casein	MKFFIFTCLLAVALAKNTMEHVSSSEESIISQETYKQEKNMAINPSKENLCSTFCK EVVRNANEEEYSIGSSSEESAEVATEEVKITVDDKHYQKALNEINQFYQKFPQY LQYLYQGPIVLNPWDQVKRNAVPITPTLNREQLSTSEENSKKTVDMESTEVFTK KTKLTEEEKNRLNFLKKISQRYQKFALPQYLKTVYQHQKAMKPWIQPKTKVIPYVRYL	53	13	95	222	26	8.43	10 445.27	111.17	CSN1S2
P02666	Beta-casein	MKVLILACLVALALARELEELNVPGEIVESLSSSEESITRINKKIEKFQSEEQQQTE DELQDKIHPFAQTQSLVYPFPGPI**P**NSLPQNIPPLTQTPVVPPFLQPEVMGVSK VKEAMAPKHKEMPFPKYPVEPFTESQSLTLTDVENLHLPLPLLQSWMHQPHQP LPPTVMFPPQSVLSLSQSKVLPVPQKAVPYPQRDMPIQAFLLYQEPVLGPVRGPFPIIV	33	13	302	224	25.1	5.35	34 586.7	298.86	CSN2
P02668	Kappa-casein	MMKSFFLVVTILALTLPFLGAQEQNQEQPIRCEKDERFFSDKIAKYIPIQYVLSRYP SYGLNYYQQKPVALINNQFLPYPYYAKPAAVRSPAQILQWQVLSNTVPAKSCQA QPTTMARHPHPHLSFMAIPPKKNQDKTEIPTINTIASGEPTSTPTT**E**AVESTVATL EDSPEVIESPPEINTVQVTSTAV	25	4	18	190	21.3	6.77	3146.90	31.75	CSN3

The different amino acid variations are highlighted in red and scores of the two used search engines (MS Amanda 2.0 and Sequest HT) are listed above.

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compositional parameters. The length of the κ-Cn gene is 13 kilobases in chromosome 6q31, which is divided into V exons. The two point mutation at exons IV is the reason for polymorphism resulting in allelic variation of variants A and B, which have dissimilarity in amino acids at positions 136 and 148 (Pishchan and Sylychenko, 2021).

In conclusion, our study of casein genetic variants is the first to be done in Gangatiri zebu cattle. Although studies were conducted on genetic polymorphism of milk proteins using electrophoresis and mass spectrometry, there is a lack of its utilization in the zebu breeds. After our probe, we have come to the conclusion that the Gangatiri breed of cows produces milk that contains the A2 beta-casein variant rather than the A1 variant, similar to many Indian indigenous breeds of cattle. This analysis has also determined the presence of genetic variants B, A, and A of alpha-S1, alpha-S2 and κ-casein, respectively, in our breed of interest.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S0022029923000079.

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