Indian J Dairy Sci 78(4): 389-392
https://doi.org/10.33785/IJDS.2025.v78i04.014

SHORT COMMUNICATION

Analysis of β-casein variants in Gir and crossbreed cows from Mumbai farms by Reverse phase high pressure liquid chromatography.

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Received: 11 February 2024 / Accepted: 15 July 2024 / Published online: 23 August 2025

Abstract: Beta casein polymorphism and its association with health implications on human health have attained crucial attention worldwide. In India, with the increasing population of exotic and crossbred cows, which produce milk containing A1 and B β-casein types, it is essential to screen the cows and bulls for their beta casein variant to avoid the health implications attributed to A1 and B β-casein cow milk. There are no available reports of milk protein analysis by HPLC method for beta casein variants from India. We have analysed 86 milk samples from Pure Gir (n=49), crossbred Holstein Frisian (n=19), crossbred Jersey (n=14) and crossbred Gir (n=4) cows by high-pressure liquid chromatography (HPLC) using a Photodiode array detector (PDA) to analyse A1 and A2 beta-casein proteins in milk samples. HPLC peaks for A1 and A2 β-casein in HPLC chromatogram were identified using milk samples of selected cows whose β-casein variant was conformed using Polymerase Chain reaction. All milk samples from pure Gir cows were shown A2 whereas a maximum number of crossbred Holstein Frisian and crossbred Jersey milk samples showed two peaks of A1 and A2.

Key words: β-casein, Polymorphism, HPLC, cow milk, A1 milk, A2 milk

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Cow milk contains multiple nutrients with various health benefits. It is a valuable source of proteins, fats, lactose and micronutrients. Milk proteins (casein and whey proteins) form 3.5% of cow milk, where caseins (α_{s1}, α_{s2}, β, and κ) account for 80% of the total protein. Among these, β casein is bovine milk's second most abundant protein. The genes for A1A2 β-casein alleles are located on the sixth chromosome of the bovine genome (Rijnkles 2002). It is a single polypeptide chain of 209 amino acids produced by the CSN-2 gene.

Around 1000 years ago mutation in this gene got introduced. Later, several such mutations resulted in the expression of 13 β casein variants in cattle of European origin. (Malarmathi et al. 2014). Among these, A1 β casein is the most common variant besides the wild-type A2 β casein. Replacement of proline by histidine at the 67th position of the amino acid chain in A1 beta casein makes it more susceptible to the cleavage of the preceding amino acids, releasing a heptapeptide β-casomorphin-7 (BCM-7) during intestinal digestion. BCM-7 is recognised to possess a potent opioid-like activity because of its high affinity for µ opioid receptors. -Activation of opioid receptors causes various health implications like ischemic heart condition (McLachlan, 2001), sudden infant death syndrome (Sun et al. 2003), schizophrenia, autism (Sokolov et al. 2014), type 1 diabetes (Elliott R et al. 1999; Thorsdottir et al. 2000) and lactose intolerance (Kaminski et al. 2006; Caroli et al. 2016).

The white revolution led to the crossbreeding of indigenous cows with European cows and the import of foreign hybrid breeds for a higher yield to meet the goal of mass production. The rampant crossbreeding led to rapid mixing of exotic gene pool with the genes of indigenous cows. This resulted into the replacement of the population of pure indigenous cows carrying gene for A2 β-casein by the crossbred cows carrying gene for A1 β-casein. Milk produced by these crossbred cows is hence either A1/A2 type or A1A1 type.– (De et al. 2015). Considering the reported association of consumption of A1 milk and various chronic diseases *(vide supra)*, it is important to preserve and propagate the indigenous cow breeds.

. There are a few chromatographic methods developed to identify beta casein subtypes. Hollar et al. (1991) identified and separated genetic variants of casein using the Fast protein liquid chromatography (FPLC) with cation exchange chromatography on a Mono S column. The method uses plasmin treatment for the proteolytic cleavage of β-casein to get a better separation than purified β-casein. Similarly, in a recent study a method was validated for β-casein quantification in infant formulas. It is a technique based on ultra-performance liquid chromatography high-resolution mass spectrometry (UPLC-HRMS) for fingerprinting of intact proteins~~.~~ (Fuerer et al. 2020). Identification and quantitation of β-casein variants in bulk milk samples is also facilitated by a rapid isoelectric focusing method (Caroli et al. 2016). A sophisticated method of HPLC was established with few modifications in various reported methods to analyse cow milk for its β- casein variants.

HPLC grade water, trifluoroacetic acid, and acetonitrile were obtained from Merck Life Science Pvt Limited, India. Bis-tris and sodium citrate monobasic was purchased from Sigma-Aldrich, USA. Guanidine hydrochloride was procured from MP Biomedicals, Illkirch, France. Dithiothreitol was purchased from Himedia Laboratories Pvt. Ltd., India.

Cow milk was collected from 3 farms in Mumbai, Bombay Gowrakshak mandali farm in Kandivali, Bombay Gowrakshak mandali farm in Boisar and Panjrapole farm in Kalbadevi. Samples were collected directly into sterile 50 ml tubes during the regular milking time ie. between 5 am to 7 am. Samples were transported to the lab in ice box. A total 90 samples were collected, out of which 49 were pure Gir, 19 were crossbred Holstein Frisian, 14 were crossbred Jersey and 4 were crossbred Gir.

Six aliquots of 2 ml fat milk were prepared and stored at -80°C for each sample until further HPLC analysis. The samples were processed using the method given by Bobe et al. (1998). Briefly, to 500 ul aliquot of cow's milk, an equal volume of sample buffer containing 0.1M Bis-tris, 5.37 mM sodium citrate, 19.5 mM dithiothreitol, and 6 M guanidine hydrochloride were added. The solution was allowed to stand at room temperature for 1hr. After the incubation, the solution was centrifuged at 4°C at 14,400 rpm for 5 min~~s~~ to remove the fat layer. Further, 0.5 ml of the obtained supernatant was added to 0.5 ml of 4.5 M guanidine hydrochloride. The mixture was filtered using a 0.4-micron filter and 5 μl of the solution was injected into the system. The β-casein obtained from Sigma Aldrich USA was processed the same way and gave an A1/A2 pattern in our method and was used as a standard.

HPLC analyses were performed with a Waters India Pvt. Ltd. Model 515 binary system equipped with a Waters Pump Control Module II and Waters Model 2998 photodiode array detector. Temperature control was done using the HPLC column oven (PCi Analytics Pvt. Ltd., Mumbai, India). The equipment was controlled by the Empowers 2 software that controlled the solvent gradient, data acquisition and data processing.

Separations were performed on a reversed-phase analytical C4 column (Jupiter®, Phenomenex, USA, with a silica-based packing (5μm, 300 Å, 250 X 2mm I.D.)~~,~~ was used. Gradient elution was carried out with a mixture of two solvents. Solvent A consisted of 0.1% trifluoroacetic acid (TFA) in water and solvent B is 0.1% TFA in acetonitrile.

The method utilizes hydrophobic interaction between the protein and stationary-phase made of porous silica in low ionic aqueous phase. The protein is eluted by increasing the content of the 0.1% trifluoroacetic acid (TFA) in acetonitrile thus increasing its hydrophobicity.

For the gradient, the following modification was done in the method reported by Givens et al.~~,~~ (2013)~~.~~. A linear gradient starting from 33% to 35% B in 10 min~~s~~, from 35 to 37% B in 9 min~~s~~, from 37 to 40% B in 5 min~~s~~, from 40 to 41% B in 6 min~~s~~, from 41 to 43% B in 15 min~~s~~ and 43 to 75% B in 5 min~~s~~ and return linearly to starting condition in 5 mins. Before injecting the next sample, the column was re-equilibrated under the starting conditions for 15 mins. Therefore, the total analysis time per sample was 70 min~~s~~.

The flow rate was 0.2 ml/min, the column temperature was kept at 40°C and the detection was made at a wavelength of 214 nm. The injection volume consisted of 5μl. Fig. 1 shows, typical chromatograms of the β-casein.

HPLC analysis of each cow milk sample was correlated with results of genotyping of blood sample of the same cow in blood done by Polymerase Chain reaction-restriction fragment length polymorphism (PCR-RFLP) method established in our lab (Paradkar et al. 2021).

Percentage of A1A1, A2A2 and A1A2 genotypes from each breed of cows were calculated.

β-casein analysis of various milk samples was carried out by employing a modified HPLC method. The aim of this study was to validate an HPLC method for identification of β-casein subtypes in milk and to evaluate the distribution of β-casein variants in indigenous pure breed and crossbred cows in a small cattle population of Mumbai city. There are no reports available on milk analysis by HPLC method for β-casein subtypes from India.

The analysis of milk samples revealed that the pure breed Gir cows produced A2A2 β-casein milk whereas crossbred Gir cows had A1A2 as the most predominant β-casein type. Jersey and Holstein Frisian cow samples analyzed in the present study were collected from the crossbred cows. Jersey cows had A2A2 as the most common β-casein type (57.14%) followed by A1A2 (28.57%) and A1A1 (14.28%). Majority of the Holstein Frisian cows produced A1A2 β-casein type (68.42%) followed by A2A2 (15.78%) and A1A1 (15.78%). Although a small number but crossbred Gir had A1A2 as the most common beta casein variant.

Table 1: The percentage of A1A1, A1A2 and A2A2 β-casein variants in four breeds of cow

Cow Breed	No. Of cows	A1A1(%)	A1A2(%)	A2A2(%)
Gir	49	0	0	100
Crossbred Jersey	14	14.2857	28.5714	57.1429
Crossbred HF	19	15.7895	68.4211	15.7895
Crossbred Gir	4	25	50	25
Total no. of cows	86			

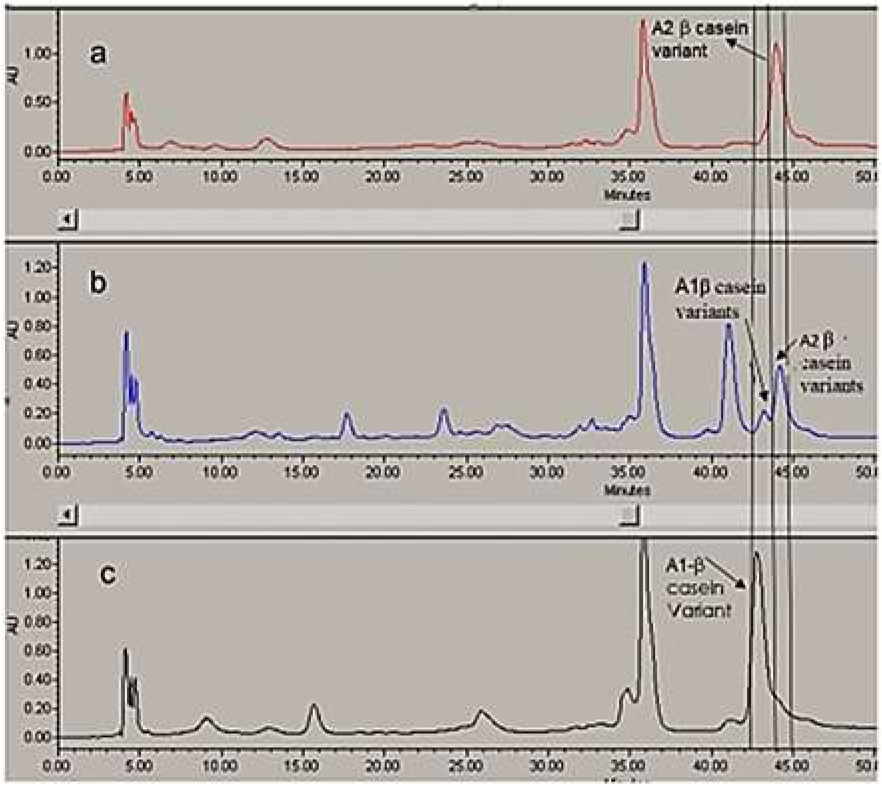

Fig 1: The RP-HPLC Chromatogram of cow milk samples (a) A2A2 sample (b) A1A2 sample (c) A1A1 sample

The results of this study are in concurrence with the results of a similar study conducted by Patel et al (2019) where they investigated three breeds of cows, two pure breeds i.e. Gir, Kankrej and ~~1~~ one hybrid breed Holstein ~~and~~ Frisian, where they reported the dominance of the A2 allele in Gir cow (84%) and Kankrej (92%). Joshi, (2011) conducted similar studies where they investigated indigenous cow breed and observed that there was 100% dominance of A2 allele gene in Gir breed. ~~(~~Mishra et al. (2009) studied the frequency of A2 allele in 15 indigenous breeds of cows and reported the presence of only A2 β-casein variant in Gir breed. The results of this study stating the high percentage of A1 allele type in Holstein Frisian agrees with the results observed by Hanusova et al. (2010), Kaminski et al. (2006) and NgKwai-Hang et al. (1990).

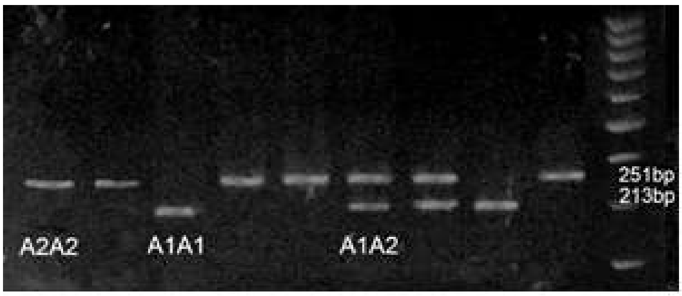

Fig. 2 PCR/RFLP pattern of the blood samples collected from different breeds of -cows

The population of exotic cow breeds has increased drastically in the last decade and gene pool of indigenous cow has been contaminated with the gene of the exotic breed as observed in our study. Though the hypothesis of A1 β-casein variant milk implication in various diseases is still not confirmed, screening of cows for the A1 and A2 β-casein polymorphism would be considered a precautionary step to protect the gene pool of indigenous cows.

Conclusion

The study highlights the significance of β-casein variants, particularly the A1 and A2 types, in cow milk and their potential health implications. The findings confirm that indigenous Gir cows predominantly produce A2A2 milk, while crossbred cows, especially those from exotic breeds like Holstein-Frisian and Jersey, exhibit a higher frequency of A1A2 and A1A1 variants.

Although the health risks associated with A1 β-casein are not conclusively established, the potential connection to various chronic diseases warrants caution. Therefore, the study underscores the importance of preserving the genetic integrity of indigenous cow breeds, such as the Gir, which produce A2A2 milk. screening of cows for A1/A2 β-casein polymorphism as a precautionary measure to protect the gene pool of indigenous cattle and potentially reduce health risks associated with A1 milk Should be carried out.

The study advocates for further research on the health effects of A1 versus A2 milk and emphasizes the need to prioritize the conservation of indigenous cattle breeds to maintain a healthier milk supply and safeguard public health.

Acknowledgement

We are grateful to Bombay Gow Rakshak Trust, Mumbai for supporting the research project.

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