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Genotyping and protein profiling of milk β-casein variants (A1 and A2) in Ongole and Punganur breeds of milch cattle

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Abstract
The exotic and cross bred cattle milk contain A1 β-casein variant, whereas indigenous cattle milk contains A2 β-casein variant. They have structural diversity at 67th amino acid residue proline in A2 which is replaced by histidine in A1 during course of evolution. The histidine at 67th amino acid residue generates a proteolytic cleavage site in A1 leading to the production of betacasomorphin-7 (BCM-7) that is known to be a risk factor for several human diseases. The serious health concern associated with A1 milk consumption endures an urgent need to select cattle breeds with higher genotypic and allelic frequencies of A2 and conserve indigenous cattle breeds through breeding programmes. In this context, the present study was undertaken to screen thirtysix animals belonging to two indigenous cattle breeds of India viz., Ongole, Punganur and Holstein Friesan cross (twelve from each breed) to characterize β-casein variants (A1 and A2) geotypically by PCR-RFLP with TaqαI restriction enzyme and also to study milk β-casein variants (A1 and A2) by IEF.
PCR-RFLP with Taqα1 restriction enzyme yielded two genotypes as A1A2 (251 and 213 bp) and A2A2 (251 bp) in all indigenous cattle breeds studied, whereas only heterozygous (A1A2) genotype was found in the HF cross. The genotypic frequency of A1A2 heterozygous variant of exon 7 of CSN2 gene was the highest for HF cross (1.0) followed by Punganur (0.17) and Ongole (0.083). Whereas, for A2A2 homozygous variant was the highest for Ongole (0.92) followed by Punganur (0.83). The A1 allelic frequency of exon7 of CSN2 gene was the highest in HF cross (0.50) followed by Punganur (0.083) and Ongole (0.042), whereas the highest A2 allelic frequency was observed in Ongole (0.96) followed by Punganur (0.92) and HF cross (0.50).
The IEF gel electrophoresis pattern of milk β-casein protein showed two distinct bands corresponding to A1 and A2 β-casein protein variants at pI values 5.22 and 5.14 respectively and this polymorphism correlated positively with the results of PCR-RFLP with TaqαI restriction enzyme. The present study unveils the existing fact that there is incorporation of exotic germplasm in native cattle breeds to varying degrees in different breeds and emphasizes an immediate need for genotypic screening of bulls for β-casein gene variant A2 for documentation and further measures to improve the indigenous breeds with appropriate breeding strategies.

Keywords: A1A1, A1A2 and A2A2 genotypes, A1 and A2 allele, PCR, β-caseingene, PCR-RFLP, IEF, pI, β-casein protein

Introduction
Cattle milk is a rich source of nutrients, proteins and energy. Caseins and whey proteins are the two major types of milk proteins. Four different types of caseins are present in milk namely α-S1, α-S2, β and κ of which β-casein is more abundant and polymorphic with 13 known variants present (Farrel *et al*., 2004) [4]. The most frequently observed variants of β-casein in dairy cattle breeds are A1 and A2 of which A2 is the variant of ancestral origin from which A1 is derived during the course of evolution. When cows were domesticated few thousand years ago, spontaneous mutation in β-casein gene resulted in replacement of proline with histidine at 67th position (CCT to CAT) in European and American breeds (Malarmathi *et al*., 2014) [8]. Thus A1 variant of β-casein was established in European and American cattle population and was subsequently spread to other countries including India with breeding strategies.
The cross-breeding strategy resulted in rapid decline in indigenous breed population in India as reported by NBAGR in their project report on National Kamdhenu Breeding Programme (NKBP). Exotic and cross-bred cattle have become popularized all over India and consumption of their milk superceded that of indigenous cattle milk. The serious concern with the consumption of exotic and cross bred cattle milk is the presence of A1 β-casein variant.

A1 β-casein releases Beta casomorphin 7 (BCM7) when digested by the digestive enzymes of Gastro Intestinal Tract. BCM-7 is an important bioactive peptide with strong opioid like activity leading to adverse effects on health like neurological damage, susceptibility to type 1 diabetes etc (Kaminski *et al*., 2006; Mishra *et al*., 2009 and Pal *et al*., 2015) [6, 11, 13]. A2 β-casein does not release BCM-7 because of proline at 67th position, which renders the peptide linkage at that position inert to the action of digestive enzymes. This emphasizes the fact that milk of exotic and cross bred cattle is not good for human and animal health owing to the predominant A1 β-casein protein that is responsible for the production of BCM-7, which is the culprit of ill-health.

The hypothesis that consumption of A1 β-casein increases the risk of several human diseases engenders the urgent need to select dairy cows with just A2 variant of β-casein. As indigenous breeds contain only A2 variant and cross-breeding strategies to enhance milk production have led to mixing up of indigenous germplasm with A1 variant, hence efforts to conserve native germ plasm of indigenous breeds requires serious attention.

The present study was taken up to characterize milk β-casein variants in Ongole, Punganur and H.F cross cattle breeds by genotypic and proteomic approaches with the following objectives. Genotypic characterization of β-casein variants (A1 and A2) in selected Ongole, Punganur breeds of Indian cattle by Polymerase Chain Reaction - Restriction Fragment Length Polymorphism (PCR-RFLP). To study milk β-casein protein variants (A1 and A2) in selected Ongole, Punganur breeds of Indian cattle by Isoelectric focusing (IEF).

Materials and Methods

For the present work, blood ssamples of 12 Ongole, 12 Punganur and 12 Holstein freiesian cross, were collected from different organized farms of Sri Venkateswara Veterinary university, Young Bull rearing center (YBRC), Nekerekal and livestock research station (LRS) Palamner, Chittoor district. Whole blood (8 ml) was collected asceptically from jugular vein of each second calving milch animal in morning times using 10 ml K3E (EDTA) BD Vacutainer tubes. Immediately after collection, the samples were labeled and transported to the laboratory in an ice packed container and stored at -20 ^{0}C until use. Isolation of DNA from blood sample was carried out by using a modified high salt method (Miller *et al*. 1988 and Aravindakshan *et al*. 1997) [9, 1]. The range of DNA concentration (ng/µl) and (OD) A260nm/A280nm ratio were 170.2 to 747.7 and 1.71 to 1.93 for Ongole; 280 to 800.5 and 1.78 to 1.94 for Punganur and 380 to 2000 and 1.78 to 1.93 for HF cross breeds cows. The quality and purity of DNA was analysed by using agarose gel electrophoresis (0.8%).

Primers and PCR-RFLP protocol that were employed to amplify the 251 bp fragment of exon 7 of β-casein gene and distinguish the A1/A2 variants is similar to that reported by Lien *et al.* (1992) [7].

Table 1: Sequence of primers used for PCR

Primer	Primer Sequence	TM	BP	Product (bp)
CASB67R	5'CCTGCAGAATTCTAGTCTATCCCTTCCCTGGGCCCATCG3'	71	39	251bp
CASB122L	5'GAGTCGACTGCAGATTTTCAACATCAGTGAGAGTCAGGCCCTG3'	70	43	

Table 2: Composition of PCR reaction mix

PCR Components	Volume
DNA Template	4.0µl
Primer Forward (10pmol/µl)	1.00µl
Primer Reverse (10pmol/µl)	1.00µl
2 x PCR MasterMix	12.5µl
Nuclease free water	6.5µl
Total	25 µl

Table 3: Cycling conditions for thermal cycler

Step No.	Step	Temperature/Time
1	Initial denaturation	95^{0}C / 60 sec
2	Denaturation	94 ^{0}C / 45 sec
3	Annealing	64^{0}C / 60 sec
4	Extension	72 ^{0}C / 40 sec
5	No. of cycles from step 2 to 4	35
6	Final Extension	72 ^{0}C / 10 min

Eight µl of each PCR amplicons were analyzed on 1.5% (w/v) agarose gel containing ethidium bromide (0.5 µg/ml) in 1X TAE buffer and it was visualized using UV transilluminator.

PCR-RFLP

Table 4: Digestion of PCR amplicons was performed by incubating 10 µl of PCR product with TaqαI enzyme for 5 hours at 65^{0}C as detailed in below.

1) Restriction enzyme (10 units/µl)	2.0 µl
2) Cutsmart buffer	5.0 µl
3) PCR product	10.0 µl
4) Nuclease free water	33.0 µl

For genotyping digested PCR products were run on 2.5 % agarose gel in 1X TAE buffer at 110V for 60 minutes. Gels were visualized under UV trans- illuminator. The sizes of PCR-RFLP products were compared with 100 bp DNA ladder. The photograph of the gel was obtained by a gel image system.

Milk samples Ongole, Punganur and H.F cross (twelve from each breed) from organized farms of the University and Young Bull Rearing center (YBRC), Nekerekal, livestock research station (LRS) Palamner, Chittoor district were screened for mastitis using CMT. The samples which were negative for mastitis were only included in the present study.

Iso Electric Focusing analysis of milk samples

Isoelectric focusing (IEF) is a technique for separating different molecules based on their isoelectric point. The individual milk samples were analyzed by IEF to study main variants of β-casein. Iso Electric Focusing was carried out as per the procedures of Bollag *et al.* (1996) [2] with slight modifications using protocols of Caroli *et al.* (2016) [3].

Table 5: Protocol for Iso Electro Focussing Ultrathin polyacrylamide gel (10 x 12 mm)

Solution A (30% acrylamide and 0.8% bisacrylamide)	2ml
Urea (8M) 10%solution	6 g
Ampholyte (pH ~3.5-9.5) 100%	48 µl
Ampholyte (pH 4-6) 40%	600 µl
Ammonium per sulphate (APS)	25 µl
TEMED	20 µl
Total volume	6 ml

Table 6: Protocol for Gel loading buffer for IEF (2X) - 5 ml

Urea	2.4 gram
Ampholyte solution (pH ~3.5-9.5) 100X	20 µl
Ampholyte solution (pH 4-6) 40X	250 µl
Triton X-100 (20%)	500 µl
2-Mercaptoethanol	50 µl
Distilled water	1.7 ml
50% Glycerol	2.5 ml
1% Bromophenol blue	200 µl

The gel cassette was fixed in vertical electrophoresis unit, catholyte (20mM NaOH) was added to the upper buffer chamber, anolyte (10mM orthophosphoric acid) was added to the lower buffer chamber. Samples were prepared by adding whole milk and gel loading buffer in 1:1 ratio. After the IEF run, the gels were fixed in Trichloroacetic acid (TCA) to visualize β-casein variants instead of staining with coomasie brilliant blue (Caroli *et al*., 2016) [3]. β-casein variants are very sensitive to acid precipitation with TCA contrary to β-lactoglobulin which is very resistant. As pI values of A2 β-casein and β-lactoglobulin are the same, coomasie brilliant blue staining reveals overlapping bands of A2 β-casein with β-lactoglobulin. The different behavior of β-casein and β-lactoglobulin in the presence of TCA was exploited to characterize β-casein variants in the milk samples. The pH gradient in the gel was confirmed by cutting a small strip from the edge of the gel before staining. The strip was cut into 20 slices of 0.5 cm each, the slices were suspended in 1ml of 10 mM KCl for 30 minutes and pH was determined using a sensitive pH meter.

Results and Discussion

Digestion of PCR amplicons with TaqαI restriction enzyme yielded fragments in three combinations: A2A2 genotype showed the product size of 251 bp, while A1A2 genotype showed the product size of 251 bp and 213 bp. The A1A1 genotype, which is expected to show the product size of 213 and 38 bp, were absent in the present study. Genetic variants of β-casein observed in Ongole, Punganur and H.F cross cattle shown in fig: 1, 2 and 3. Genotypic frequency and Gene frequency of A1 and A2 variants among Ongole, Punganur and H.F cross cattle are presented in Table 1.

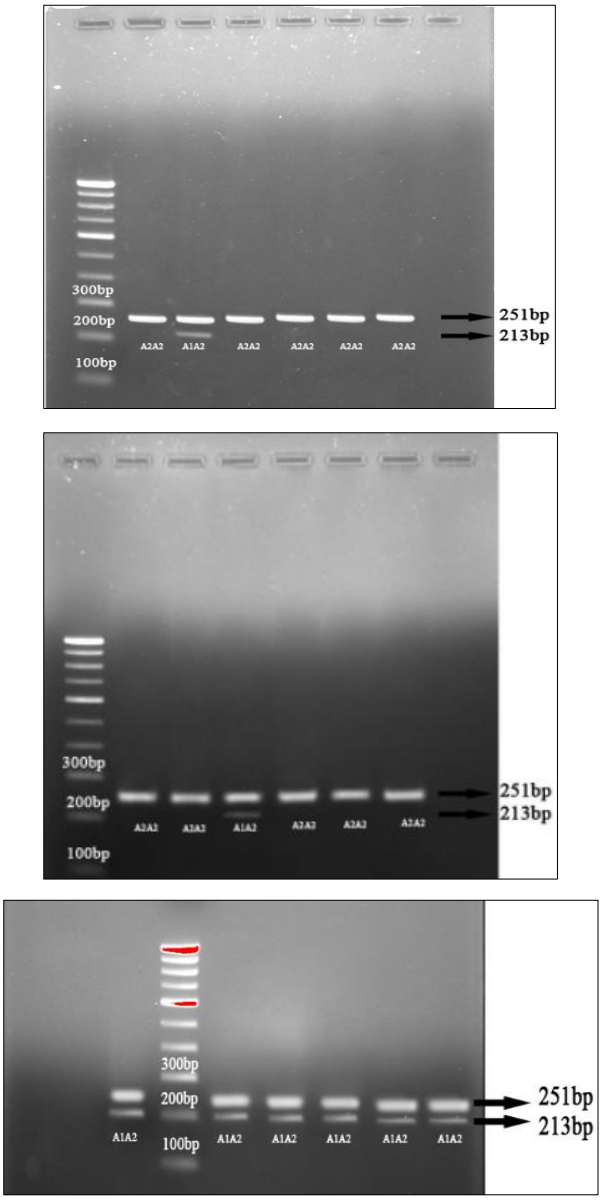


Fig 1-3: PCR-RFLP pattern of β-casein gene of Ongole, Punganur and H.F cross, repspectively with TaqαI restriction enzyme on 2.5% agarose gel, M: 100 bp DNA ladder

Table 1: Gene and Genotype frequencies of A1 and A2 variants of β-casein gene

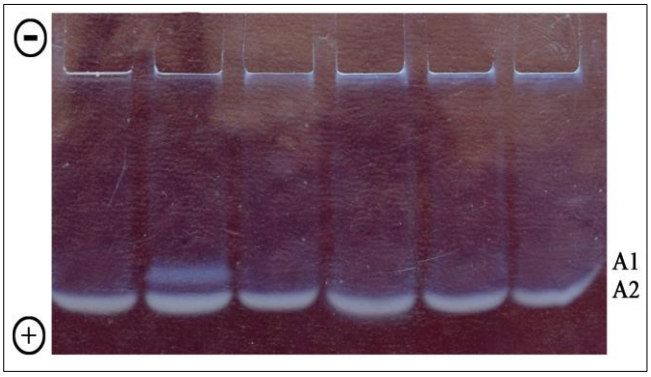
Breeds	Number of animals	Gene frequency		Genotype frequency			χ 2 value d.f=1
		A1	A2	A1A1	A1A2	A2A2	
Ongole	12	0.040	0.96	0	0.080	0.92	8.33**
Punganur	12	0.080	0.92	0	0.17	0.83	5.33NS
H.F cross	12	0.50	0.50	0	1.0	0	12.0**

**P<0.01 NS: Non significant

Chi-square test revealed significant deviation from genetic equilibrium in Ongole and H.F cross, but not in Punganur breed.

Isoelectric focusing (IEF) electrophoresis analysis of milk samples

The pattern of Trichloroacetic acid fixed IEF gels of milk samples revealed single band at pH of 5.14 representing A2 β-casein (pI-5.14) and two bands at pH values of 5.22 and 5.14 corresponding to A1 (pI-5.22) and A2 (pI-5.14) β-caseins respectively. IEF pattern of Milk samples from Ongole, Punganur and H.F cross Cattle shown in fig: 4, 5 and 6.



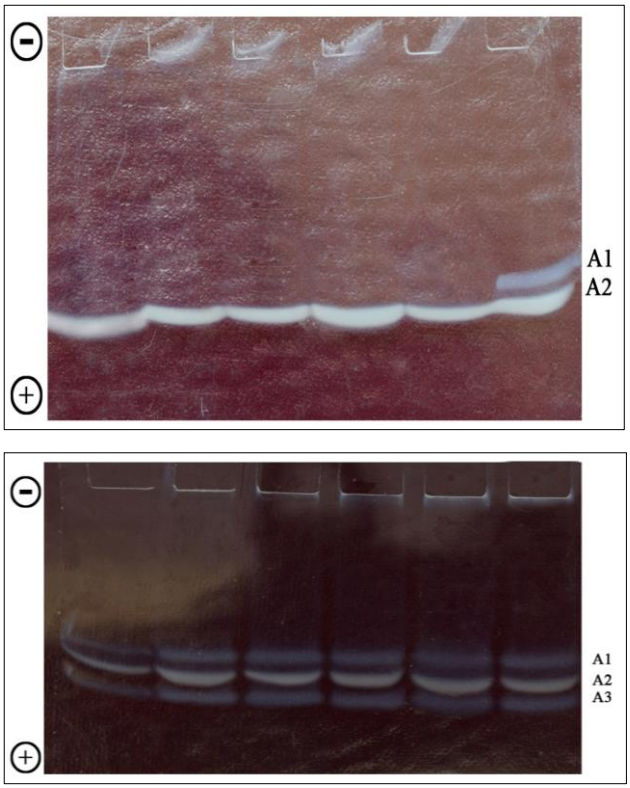


Fig 4-6: IEF analysis of individual milk samples from Ongole, Punganur and H.F cross cows, respectively.

The genotypic frequencies of Ongole cattle obtained in the present study were 0.92 (A2A2) and 0.083 (A1A2) and allelic frequencies were 0.042 (A1) and 0.96 (A2). Ganguly *et al.* (2012) [5] made similar findings in Ongole cattle with genotypic frequencies of 0.11 (A1A2) and 0.89 (A2A2) and allelic frequency of 0.94 (A2) using allele-specific AS-PCR. The mean genotypic frequencies reported by Mishra *et al.* (2009) [11] were 0.974 (A2A2) and 0.026 (A1A2) and allelic frequencies of 0.013 (A1) and 0.987 (A2) in all *Bos indicus* cattle breeds.

Analysis of Punganur cattle germplasm revealed genotypic frequencies of 0.17 (A1A2) and 0.83 (A2A2) and allelic frequencies of 0.083 (A1) and 0.92 (A2). There are no reports on the β-casein polymorphism in Punganur cattle breeds, however the findings are suggestive of greater number native Punganur with low cross breeds.

Holstein Friesian cross-bred cattle had high prevalence of A1A2 with genotypic frequency of 1.0 and allelic frequencies of 0.5 in A1 and A2. However, Shende *et al.* (2017) [14] identified the presence of A1A1 genotype with a frequency of 0.28 and A1A2 genotype with a frequency of 0.72 in HF cross-bred cattle and allelic frequencies reported were 0.6383 (A1) and 0.3617 (A2). The percentage of A1 genotype in HF crossbred cattle studied by Shende *et al.* (2017) [14] was more when compared to those of the present study and homozygous A1A1 genotype is completely absent. Our findings are in accordance with the reports of Sodhi *et al.* (2012) [15], who reported the genotypic frequency of 0.451(A1A2) in HF and 0.506 (A2A2) in cross bred cattle using PCR-RFLP with Taqα1 restriction enzyme.

The nearly equal genotypic frequencies of A1A2 and A2A2 as 0.4598 and 0.4023 was observed by Miluchova *et al.* (2014) [10]. Olenski *et al.* (2010) [12] reported as 4.41 (A1A2) and 4.31 (A2A2) were in contrary to our findings, where in the genotypic frequency was 1.0 (A1A2) with complete absence of A1A1 and A2A2 homozygous variants. Both the teams observed the presence of A1A1 genotype of 0.1379 (A1A1) and 1.28 (A1A1) in HF cattle, which was completely absent in our study. Polish HF bulls as reported by Kaminski *et al.* (2006) [6] had preponderance of A1A2 (5.804) genotype similar to our findings when compared to A2A2 (3.077), which was completely absent in our study.

Conclusion

The indigenous breeds analyzed in the present study for polymorphism of exon 7 of CSN2 gene revealed the highest genotypic frequency of A1A2 for HF cross followed by Punganur and Ongole. Whereas, A2A2 homozygous variant was the highest for Ongole followed by Punganur and HF cross. The allelic frequency of A1 was the highest for HF cross followed by Punganur and Ongole and A2 allelic frequency was the highest for Ongole followed by Punganur and HF cross.

Considering the increasing trends of crossbred cattle population for higher milk production and positive effects of A2 β-casein variant over A1, there is an immediate need to study the association of A2 allele with milk production and also to screen the indigenous cattle population for A2 allele. Further, there is a need for genotypic screening of bulls for β-casein gene variant A2 and to adopt breeding strategy with higher genotypic and allelic frequencies of A2 to enhance A2A2 population.

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