

Sequence Characterisation and Genotyping of Allelic Variants of Beta Casein Gene Establishes Native Cattle of Ladakh to be a Natural Resource for A2 Milk

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ABSTRACT

Bovine milk is regarded as nature's perfect food due to presence of vital nutrients. However some peptides are generated after proteolytic digestion of β -casein that have opioid properties and may increase the risk of chronic diseases. There are 13 genetic variants of bovine beta-casein; out of these A1 and A2 are the most common in dairy cattle breeds. The A1 and A2 variants differ only at position 67, which is histidine in A1 or proline in A2 milk. Earlier published reports have indicated that A1 β casein could be responsible for several health disorders like diabetes, coronary heart disease etc. while A2 β -casein is generally considered safe for human consumption. In the present study, an effort was made to sequence characterize β casein gene and identify allelic distribution of A1A2 alleles in native cattle of Ladakh region adapted to high altitude and low oxygen condition. The data showed 2 non-synonymous variations in coding region, while 5'UTR was completely conserved. The 3'UTR showed 2 more variations in Ladakhi samples. Further, the genotyping in 85 Ladakhi cattle for A1A2 alleles revealed that in Ladakhi cattle, A2 allele is predominantly present as reported for some of the other Indian breeds. The frequency of A2 allele was 0.90 and frequency of A2A2 genotype was found to be 0.79 in Ladakhi cattle. The present data strongly indicate that local cattle of Ladakh with higher frequency of A2 allele and A2A2 genotype is natural resource for A2 milk. Systematic efforts should be made for long term conservation and genetic improvement of this invaluable genetic resource of Ladakh.

Keywords: Ladakhi cattle; Beta casein; A1A2 allelic variants; Genotyping; Sequencing

1. INTRODUCTION

Cow's milk in general has been regarded as nature's perfect food, providing important source of nutrients including proteins, carbohydrates, essential minerals and vitamins. Cow's milk contains about 3.5 per cent proteins, of which approximately 80 per cent are caseins (Alpha S1, Alpha S2, Beta Casein and Kappa Casein) and 20 per cent are whey proteins (α -Lactalbumin and β -Lactoglobulin). In addition to serve as a source of amino acids, milk proteins have significant effects on health for example, lactoferrin has anti-microbial/anticancer properties; while osteopontin, and immunoglobulins have immuno-modulating effect¹⁻⁴. Among the caseins, beta casein (β -casein) is second most abundant protein and has excellent nutritional balance of amino acids.

Earlier, caseins in the milk were considered merely as a macronutrient source, but recent studies have revealed that casein derived bioactive peptides may have considerable impact on health. There are reports indicating the association of specific type of β -casein with certain human diseases. Specific bioactive peptides are released from beta casein obtained from animals

with different genetic variations. Different mutations in bovine β -casein gene have led to generation of 12 genetic variants: A1, A2, A3, B, C, D, E, F, H1, H2, I and G. Out of these variants, A1 and A2 are the most common. The A1 and A2 variants of bovine β -casein differ at amino acid position 67 with histidine in A1 and proline in A2. The amino acid change is the result of a single nucleotide change at 67th codon: CCT (A2, proline) and CAT (A1, histidine). The cows with A2A2 genotype give A2 type of milk (with amino acid proline) and A1A1 cows give A1 milk (with amino acid histidine). The polymorphism from proline to histidine leads to key conformational changes in the secondary structure of expressed β -casein protein^{5,6}. Due to presence of histidine, gastrointestinal proteolytic digestion of A1 β -casein (raw/processed milk) releases a 7 amino acid bioactive peptide 'opioid' called beta-casomorphin 7 (BCM-7) in small intestine, while proline in A2 milk at 67 position prevents the split at this particular site and generates nine amino acid peptide BCM-9^{7,8}. In past, some relationship between disease risk and consumption of a specific bovine β -casein fraction having milk with either A1 or A2 genetic variants has been identified. BCM7 is suggested to be associated as a risk factor for human health hazards^{5,6,9} as being small in size, BCM-7 can enter the blood stream, cross the blood brain

barrier and then binds to $\mu/k/\delta$ - receptors located in the central nervous system, gastrointestinal tract, and some immune cells. Epidemiological evidences claim that consumption of beta-casein A1 milk is associated as a risk factor for type-1 diabetes, coronary heart disease, arteriosclerosis, sudden infant death syndrome, autism, schizophrenia¹⁰⁻¹⁵. Hence it is important to investigate the allelic status of beta casein gene in different indigenous cattle breeds.

India is bestowed with rich domestic livestock biodiversity. Amongst these, cattle is an important genetic resource supporting the agrarian economy of India by contributing milk, draught power, manure as well as by generating rural employment. These diverse cattle populations are the culmination of several complex and interactive factors like human needs, adaptability to specific agro-climatic conditions, selection and animal husbandry practices. In India, cattle population (190.90 million) constitutes about 37.28 per cent of total livestock, and has been categorised into 40 distinct breeds and many populations are still to be characterised. The cattle genetic resource developed over the years and adapted to specific ecological niche are still untapped and uncharacterised. One such population is native cattle of Ladakh region-situated roughly between 32 to 36 degree north latitude and 75 to 80 degree east longitude and altitude ranging from 2300 m to 5000 m above sea level. These local cattle can well survive under extreme conditions *viz.*, high altitude, huge barren land, thriving on poor quality roughage or assured water supply during lean winter periods. It is hypothesised that local native cattle or “Ladakhi cattle” might possess unique alleles or combinations of alleles to serve specific purpose(s) in particular agro-ecological zones.

In spite of extreme climatic conditions, subsistence on poor quality feed and low availability of water, it provides around 2.5 kg - 4.5 kg of milk and thus serves as an important animal protein source for local people, particularly during lean winter period. Ladakhi cattle along with local yak population is the major resources of milk for the local people hence it is important to know the A1/A2 allelic status of beta casein in this cattle population. The present study was therefore planned to characterize the beta casein gene and also delineate the A1A2 allelic pattern in Ladakhi cattle. The observations recorded will provide the basic information on the quality of milk of Ladakhi cattle that can form the basis for its value addition, propagation and conservation.

2. MATERIALS AND METHODS

2.1 Sampling and DNA Extraction

A total of 85 blood samples were collected from local Ladakhi cattle by visiting different villages/talukas of Ladakh region (Fig. 1). The blood samples were collected in EDTA vacutainer tubes by jugular vein puncture. The samples were transported to laboratory and were subsequently stored at -20 °C till DNA extraction. The study has been approved by the Institutional Animal Ethical Committee (IAEC). Genomic DNA was extracted using phenol chloroform method from whole blood as described by

Sambrook and Russel¹⁶ after SDS-proteinase K treatment at 56 °C overnight. After isolation and purification, DNA pellet was dissolved in TE buffer and was stored at -20 °C. The quality of isolated DNA was checked on 0.6 per cent agarose. The quantity of DNA was also checked at 260/ 280 nm using UV spectrophotometer (GE-NanoVue Plus). The samples having OD ratio between 1.8-1.9 were considered good and used for polymerase chain reaction.



Figure 1. Blood sampling sites of Ladakhi cattle covered in present study for A1/A2 genotyping.

2.2 PCR Amplification and Polymorphism Detection

The coding region as well as the untranslated region of β -casein gene were amplified in 25 DNA samples of Ladakhi cows and sequenced to find out variations. The primers used for amplification and sequencing are given in Table 1. PCR amplifications were performed in 25 μ l reactions containing 100-150 ng of genomic DNA, 5 pmol of each primer, 200 μ M dNTPs, 1.5 mM $MgCl_2$, and 1.0 U of *Taq DNA polymerase* (Invitrogen). The thermal cycle programme employed was 95 °C for 2 min, followed by 30 cycles at 95 °C for 60s, 59 °C for 60s and 72 °C for 1 min with a final extension at 72 °C for 10 min. The PCR products were purified and sequenced using forward and internal primers and ABI Prism® Big Dye™ Terminator Cycle Sequencing kit (Applied Biosystem, Foster City, CA). The contigs produced by the overlapping primers were assembled in consensus with *B. taurus* reference sequence

Table 1. Primer sequences used for amplification of exonic regions of β -casein gene

Abbreviation	Description/ Region Covered	Primer sequence (5'-3')	Amplicon size (bp)
β -casein-E1'F	Exon 1(1576-1983)	TTTGGGGACAGAAAATAGG	408
β -casein-E1'R		CCTGTAACTAGTCTCGTGG	
β -casein-E2-4'F	Exons 2 – 4 (3519-4870)	GATCTTGGTTCCTGATGAG	1352
β -casein-E2-4'R		TTCTCCAAGGCATGAAAGTG	
β -casein-E2-4in		ATCCCTTACTGTAGAGTGCT	
β -casein-E5'F	Exons 5-6 (6385-7005)	AGCCTTATTGAGAAGAGTG	621
β -casein-E5'R		GATCACAGTCCCTAGTCTGG	
β -casein-E7-9'F	Exon 7-9 (7818-10218)	GAGAAATCCTTCAGTGAGCA	2400
β -casein-E7-9'R		TAAGGACATATGCCGCATAG	
β -casein-E7in1	For sequencing	CACCACATGACTCATTTCAC	
β -casein-E7in2		TGATTCCAAGTAAGCCGATG	

to generate the complete sequence of β -casein gene. Sequences were compared to identify single nucleotide polymorphisms (SNPs) using Phrap and Gap4 integration softwares (<http://staden.sourceforge.net/phrap.html>) and further confirmed by manual inspection of individual chromatogram.

2.3 Genotyping of A1A2 Alleles of β -casein

Additionally, to understand the A1A2 allelic status of Ladakhi cattle, a total of 85 animals of this population were genotyped as per the procedure reported by Mishra¹⁷, *et al.* In brief, 251 bp fragment from exon 7 of the β -casein gene was amplified using primer set as earlier reported by Lien¹⁸, *et al.* The primer sequences were as follows: Forward primer (5'-GAGTCGACTGCAGATTTTCAAATCAGTGAGAGT CAG-3'); Reverse primer (5'-CCTGCAGAATTCTAGTC TATCCCTTCCCTGGGCCCATCG-3'). The PCR reaction of 25 μ l consisted of genomic DNA (150-200 ng), 4 pmol of each forward and reverse primers, 1X of PCR reaction buffer with 1.5 mM of MgCl₂, 200 μ M dNTPs and 1.0 unit of *Taq DNA Polymerase* (New England BioLabs). The reaction was carried out in Thermal Cycler (Eppendorf) with the following conditions: initial denaturation at 95 °C for 2.3 min, 35 cycles of denaturation at 94 °C for 45 sec, annealing temperature at 64 °C for 45 sec and extension at 72 °C for 45 sec followed by a final extension at 72 °C for 10 min. The PCR products were checked on 1.5 per cent agarose gel and subsequently digested using *Taq I* restriction enzyme (New England BioLabs) at 65 °C for 5 hours in a circulatory water bath. The restriction digestion reaction mix of 20 μ l volume consisted of 12 μ l PCR product, 2 μ l buffer 10X, 5 U (0.5 μ l) restriction enzyme and 5.5 μ l nuclease free water. After RE digestion, the fragments were separated on 2.5 per cent agarose gels. Allele/genotype frequencies were determined to understand the pattern of β -casein variants in Ladakhi cattle. Genotypes were determined by direct counting while the frequency of the specific allele was calculated as the sum of the frequency of the homozygous plus half the frequency of heterozygous genotypes as a proportion of the total animals.

3. RESULTS AND DISCUSSION

3.1 Sequence Characterisation of Beta Casein Gene in Ladakhi Cattle

The present study was aimed to sequence characterised beta casein gene in high altitude adapted Ladakhi cattle to identify the variations and generate genotypic data with respect to A1A2 alleles of this particular gene. The sequence data generated for 25 animals of Ladakhi cattle revealed only 2 polymorphic sites in the coding region (-301, C>A, -467, C>G). Interestingly, both these SNPs in the coding region were nonsynonymous in nature. The C>A variation at -301 position was highly frequent with allele frequency of 0.96, while the C>G variation at -467 position showed allelic frequency of 0.90. Both these SNPs were identified as A2 and B alleles as per the nomenclature/information reported in previous studies. The sequence of 5' UTR showed no variation and was found to be completely conserved in Ladakhi cattle. On the other hand, 3'UTR region of β -casein showed 2 SNPs; G>A at position -778 and T>A at position -803. The variation G778A was

present in higher frequency as compared to T803A. Amongst the 4 variations, the noteworthy variation identified in Ladakhi cattle was A2/A1: C301A (Pro67His) which has been widely anticipated to be associated with human health. Some of the other known variant of beta casein gene *viz.*, A3, C, D, E, F, H1, H2, I and G reported for other breeds worldwide were not observed in animals of Ladakhi cattle. As A1/A2 variant holds importance with respect to human health, this locus was further genotyped in large number of Ladakhi cows.

3.2 Genotyping for A1/A2 Allelic Variants in Ladakhi Cattle

The amplicon of 251 bp region harboring A1/A2 variants from exon7 of beta casein gene was restriction digested with *Taq I* to yield three different banding pattern. In A2A2 genotype, only one fragment of 251 bp (no digestion) was observed while A1A1 and A1A2 genotypes were represented by fragments of 213 bp and 38 bp; 251 bp, 213 bp and 38 bp respectively (Fig. 2). The allelic frequency distribution data indicated predominance of A2 allele (0.90) across 82 animals of Ladakhi cattle screened. A1 allele was observed only in few animals with a frequency of 0.10. The observed genotypic frequency for homozygous A2A2 and heterozygous A1A2 genotype were 0.79 and 0.21, respectively (Table 2). None of the animal showed homozygous A1A1 genotype. The data clearly indicates the preponderance of A2 variant, which has not been implicated in human health issues, in Ladakhi cattle. Similar to the findings of the present study, the frequencies of A2 allele (0.95) and A2A2 genotype (0.90) have also been reported on higher side in some of the other native indicine cattle breeds of our country¹⁷. However, the frequency of A2 allele in Ladakhi cattle (0.90) is slightly on lower side than the average frequency of this particular allele across several Indian native cattle breeds (0.95). Similarly the frequency of A2A2 genotype was also on lower side (0.79) in comparison to other Indian native cattle breeds (0.90). Our previous study¹⁷ has shown that all the animals of major native milch breeds (Sahiwal, Rathi, Tharparkar and Red Sindhi) are of A2 type

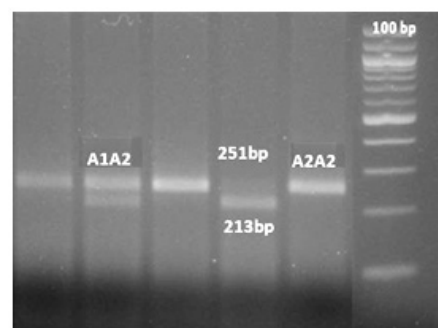


Figure 2. PCR-RFLP of β -casein/*TaqI* showing A2A2 and A1A2 genotypes in Ladakhi cattle.

Table 2. Allelic and genotypic frequencies of A1/A2 variant of β -caseingene in Ladakhi cattle

	Allelic Frequency		Genotypic Frequency		
	A1	A2	A1A1	A2A2	A1A2
Ladakhi cattle	0.10	0.90	0	0.79	0.21

while the frequency of A2 allele varied from 0.97 to 0.92 across dual and draft purpose breeds (Fig. 3).

The frequency of heterozygous A1A2 genotype was on higher side (0.21) in Ladakhi cattle in comparison to other Indian milch breeds (0.09). However, similar to other Indian cattle breeds, no A1A1 genotype was observed in Ladakhi cattle. Relatively higher frequency of A1 allele and A1A2 genotype in Ladakhi cattle as compared to other Indian cattle breeds might be attributed to the crossbreeding of local Ladakhi cattle with Jersey cattle in recent years for higher milk production. There are ample evidences to prove that exotic cattle including Holstein Frisian and Jersey possess high proportions of A1 allele (Fig. 4), and use of exotic germplasm in breeding program may increase the frequency of A1 allele in the native population. Here, we need to highlight the general notion that A2 is the original variant that probably mutated to A1 during intensive cattle breeding programme initiated many years ago in America and European countries. As A2 milk is considered safe for human health, milk of Ladakhi cattle could be used without much concern.

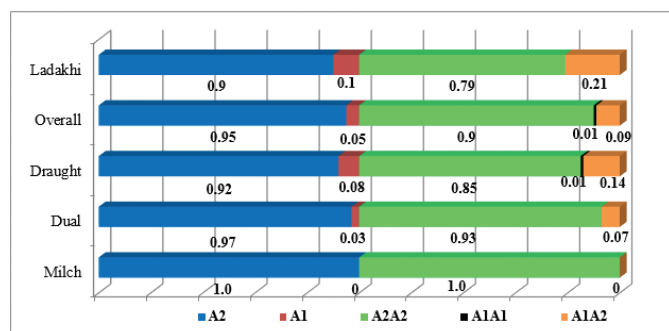


Figure 3. Allelic and genotypic frequency at β -casein (A1/A2 variant/*TaqI*) across different utility type indigenous cattle breeds vis a vis Ladakhi cattle.

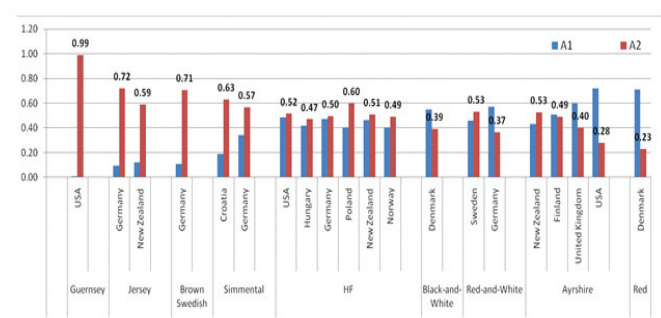


Figure 4. Frequency distribution of A1/A2 allele in cattle breeds of other countries

4. CONCLUSION

It is right time to generate awareness amongst the policy planners, field functionaries, farmers and all stake holders of the region to avoid mixing of local cattle with exotic Jersey cattle, as it may dilute the genetic makeup of Ladakhi cattle. This particular germplasm is unique with excellent adaptation potential to high altitude hypobaric stress and also rich resource for A2 milk. Hence, strategies should be made to conserve and improve this important cattle population so as to avoid

its genetic dilution. In specified areas, where crossbreeding with Jersey bull is essential like where ample feed and fodder is available, it would be reasonable to screen the breeding bulls to ascertain their A1A2 status in order to minimize the dissemination of A1 allele in our native germplasm.

Conflict of Interest: None

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ACKNOWLEDGEMENT

The authors duly acknowledge the financial support received from Indian Council of Agricultural Research, New Delhi to carry out this study under National Fellow and National Agricultural Science Fund projects.

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