

Research Article

Development of Allele-specific PCR for Detection and Differentiation of A1 and A2 Milk from Various Bovine Breeds

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A B S T R A C T

Bovine milk is considered as one of the nutritious foods. Beta-casein is the most variant protein, which constitutes around 30% in the milk. A1 and A2 variants of Beta-casein have received lot of attention in health as well as research sectors. A1 milk is known to release a peptide called Betacasomorphin-7 (BCM-7) during gastrointestinal digestion. BCM-7 plays key role in the development of many health issues like human ischemic heart disease, diabetes mellitus, atherosclerosis, schizophrenia, autism and Autistic Spectral Disorder (ASD). The present study has developed an allele specific PCR for detection and differentiation of A1 and A2 milk. DNA extraction from milk and allele-specific PCR amplification was standardized. Further, the developed assay was tested using various indigenous and exotic cow's milk samples and plausible results were obtained. Altogether, the assay will be an efficient way for screening and identifying A1, A2 homozygous and A1/A2 heterozygous milk.

Keywords: Beta-casein, Allele-specific PCR, Indigenous Breed**Introduction**

Bovine milk provides essential nutrients and is an important source of dietary energy, high-quality proteins and fats. Milk contains two kinds of proteins a) Whey and b) Casein protein constitute around 80% and 20%, respectively. Alpha S1-Casein, Alpha S2-Casein, Kappa-casein and Beta-casein are the different kinds of casein proteins.¹ Among them Beta-casein is the most polymorphic gene, considered to have health benefits over other variants of the casein proteins. Beta-casein has undergone single nucleotide polymorphism giving rise to 13 different genetic variants: A1, A2, A3, A4, B, C, D, E, F, G, H1, H2 and I.² The most frequently observed beta-casein genetic variants are A1, and A2 widely spread in the genetic pool of cattle. The A1 and A2 variants are differentiated by change of one nucleotide

at 202 position of CSN2 gene exon VII allele from cytosine (201-CCT-203) to adenine (201-CAT-203) results in amino acid change at 67th position from proline (A2) to histidine (A1) respectively. This single nucleotide polymorphism in beta-casein gene gave rise to three different genotypes such as CC (A2 homozygous), AC (A1/A2 heterozygous), AA (A1 homozygous).^{3,4} Numerous research studies concluded that consumption of A1 milk would rise to various health ailments. Proteolytic digestion of A1 beta-casein would release BCM-7 which is an important bioactive opioid peptide with strong opioid activity.⁵ Opioid peptides play key role in various biological processes, including respiration, analgesia, constipation and behaviour in humans. BCM-7, an opioid or narcotic have cytomodulatory properties can potentially affect numerous opioid receptors in the

nervous, endocrine and immune system leading to health hazards such as type-1 diabetes, heart disease, infant death, autism, atherosclerosis, schizophrenia.^{6,7} On the other hand, identification of indigenous A2 breeds is very much necessary for selective breeding and further preservation of the original Indian breeds. Therefore, in the current study, we have developed an allele-specific polymerase chain reaction method in order to identify the A1 and A2 homozygous as well as A1/A2 heterozygous milk. Further, the assay was validated using exotic and indigenous milk samples.

Materials and Methods

Materials

The primers used in this study were synthesized from integrated DNA technologies (IDT, USA). All the PCR reagents and chemicals used in this study were obtained from Sigma-Aldrich (India). All the chemicals were molecular grade and the buffer solutions were prepared in ultra-pure milli-Q water.

Collection of Samples

A total of number of 21 milk samples was collected from goshalas near Bangalore region in sterile conditions, with written consent from respective farmers and the collected vials were labelled. The vials have been transported to our laboratory facility in an ice box and DNA extraction was carried out within the same day.

Isolation of DNA

DNA isolation from somatic cells of milk was carried out as per protocol developed by Pokorska and co-workers with certain modifications.⁸ Briefly, the milk sample was transferred to sterile eppendorf tube and centrifuged

repeatedly at 8000 rpm for 3 mins. Then, the supernatant was removed and pellet was washed with wash buffer. Further somatic cell lysis was carried out using lysis buffer with 10% SDS. The debris was removed by centrifugation at 8000 rpm for 3 mins. Further, 6M NaCl was added to the solution to precipitate the protein and centrifuge at 12,500 rpm for 10 mins then the supernatant was collected in to sterile eppendorf. Further, DNA was precipitated using ethanol followed by centrifugation at 12,500 rpm for 10 mins, and allowed to air-dry for 30 mins. Then, it is dissolved in milli-Q water and stored at -80°C refrigerator for later uses.

Designing of Primers

As the name indicates, allele-specific primers are designed in order to discriminate the single nucleotide polymorphisms by placing 3' terminal nucleotides correspond to an SNP, matching perfectly with one allele and having a 3' mismatch with other alleles⁹. A total of three primers are required to perform allele-specific PCR, two allele-specific primers and one common primer. If the allele-specific primers are forward, then common primer would be the reverse primer and vice-versa. The gene sequences of Beta-casein (CSN2), A2 variant are obtained from Gen bank (accession number: M55158.1). In case of beta-casein variants, the strategy of designing primers is represented in Figure 1, the forward allele-specific primers designed with adenine (A) at 3' terminal for A1 specific primer, corresponding to Thiamine (T), whereas Cytosine (C) at the 3' end of the A2 specific primer corresponding to Guanosine (G) and a common reverse primer was designed to amplify 840 bp and 838 bp products, respectively. The allele-specific primers designed are listed in the Table 1.

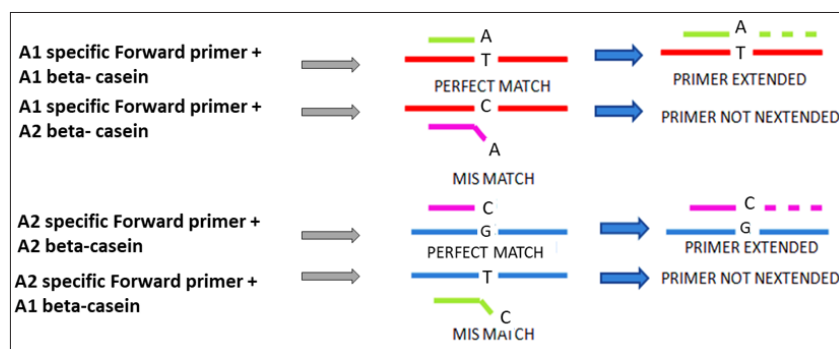


Figure 1. Strategy of primer designing with respect to A1 and A2 alleles

Table 1. Designed allele-specific PCR primers⁹

S. No.	Primer	Sequence (5' to 3')
1.	Forward A1 allele-specific primer	ATCCCTCCCTGGACCCATCCA
2.	Forward A2 allele-specific primer	CCCTCCCTGGACCCATCCC
3.	Common reverse primer	CCTTCTTAGGTTTGTATTCTTAGCC

Standardization of PCR

The allele-specific PCR was performed as per Rahimi and co-workers.⁹ The standardization of the allele-specific PCR was carried out by selecting various temperatures and conducted the PCR in combination with different MgCl₂ concentrations. Two different PCR master mixes were prepared with respect to the two allelic primers designed and aliquoted. A total of 20 µl volume for each reaction was prepared containing 2 µl of PCR buffer, 1.5 µl of 25 mM MgCl₂, 1 µl of dNTPs, 0.15 µl of *Taq polymerase*, 2 µl of extracted DNA, and nuclease free water to make up final 20 µl of volume. The standard parameters at which PCR was performed using Applied Biosystems Veriti Thermal Cycler are: 1 cycle at 94 °C for 5 mins of initial denaturation, 35 cycles by 94 °C for 1 min of denaturation, primer annealing at 58 °C for 1 min and extension at 72 °C for 1 min followed with final extension for 10 mins at 72 °C. The amplified products were electrophoresed using 2% agarose and visualized under Gel documentation system (Biobee, India) and result was analysed.

Evaluation of Developed Allele-specific PCR using Milk Sample

Isolated DNA from 21 collected samples were subjected for allele-specific PCR amplification in standardized conditions and compared with control (Table 2). After amplification, the DNA was loaded in 2% agarose gel and result was

interpreted using Gel documentation system (Biobee, India).

Result

Isolation of DNA

The extracted DNA from collected milk was electrophoresed in 1% agarose gel at 90V, observed under UV, containing single band indicates the purity of DNA without RNA or background noise represented in Figure 2. Evaluation of the purity and concentration of the extracted DNA was carried out by Nanodrop ND-1000 spectrophotometer (Thermo Scientific, India).

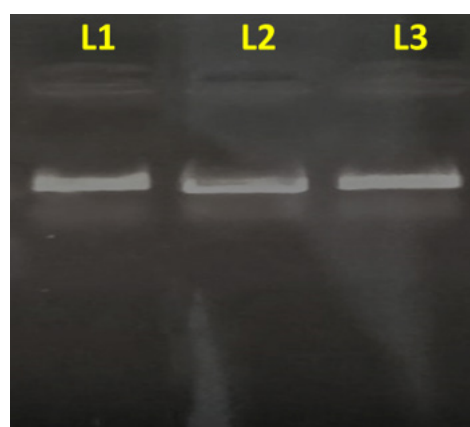


Figure 2. Isolated DNA observed under gel documentation to evaluate the purity of the DNA

Table 2. Table representing the genotype of the samples tested through allele-specific PCR

Sample. No.	A1 allele positive	A2 allele positive	Genotype
Control-1	+	-	A1A1 (homozygous)
Control-2	+	+	A1A2 (heterozygous)
Control-3	-	+	A2A2 (homozygous)
1	+	+	A1A2
2	+	+	A1A2
3	+	+	A1A2
4	+	+	A1A2
5	+	+	A1A2
6	+	+	A1A2
7	+	+	A1A2
8	+	+	A1A2
9	+	+	A1A2
10	+	+	A1A2
11	+	+	A1A2
12	+	+	A1A2
13	+	+	A1A2
14	+	+	A1A2

15	–	+	A2A2
16	+	+	A1A2
17	+	+	A1A2
18	+	–	A1A1
19	–	+	A2A2
20	+	+	A1A2
21	+	+	A1A2

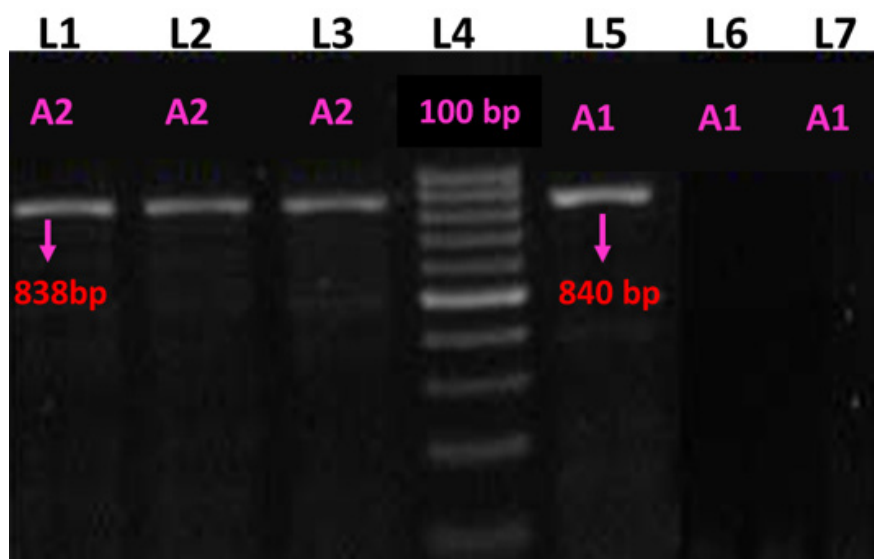


Figure 3. Image representing the allele-specific PCR amplified products in 2% agarose gel

L1: A2 amplified product of sample 1,
L2: A2 amplified product of sample 2,
L3: A2 amplified product of sample 3,
L4: 100bp ladder,

L5: A1 amplified product of sample 1,
L6: sample 2 not amplified with A1 primer,
L6: sample 3 not amplified with A1 primer.

Standardization of PCR

The optimisation of the PCR conditions was carried out. The standard parameters are derived to be: 1 cycle at 94 °C for 5 mins, 35 cycles by 94 °C for 1 min, 58 °C for 1 min and 72 °C for 1 min followed with final extension for 10 mins at 72 °C at which the amplified beta-casein variant products are highly specific, and without non-specific amplification. The obtained PCR products are electrophoresed at 90 V in 2% high molecular grade agarose gel with 100 bp ladder. After electrophoresis, the gel was observed in Gel documentation system (Biobee, India). The amplified products are 838 bp and 840 bp with respect to the A2 and A1 allele-specific primers utilized in the reactions, represented in Figure 3.

Evaluation of Developed Allele-Specific PCR using Milk Sample

To evaluate the assay specificity, DNA isolated from 21 milk samples were employed in developed Allele-Specific PCR assay and compared with control. The assay was specifically able to detect and differentiate A1 and A2 alleles from all the samples. The obtained results are shown in Table 2;

18 samples were A1A2 heterozygous, 2 samples were A2 homozygous, and 1 sample was A1 homozygous. Hence, the assay can be used as a suitable tool for beta-casein genotyping detection of A1 and A2 milk.

Discussion

Bovine milk is the most consumed nutritious food throughout the world. Beta-casein is one of the major protein in milk. The single nucleotide polymorphism in Beta-casein gene raised to 13 different genetic variants, in which A1 and A2 are the most frequent genetic variants. A1 beta-casein would release BCM-7 which is considered as bioactive opioid peptide with strong opioid activity, during gastrointestinal digestion of A1 milk.⁵ Various studies indicated harmful effects like type-1 diabetes, heart disease, infant death, autism, atherosclerosis, schizophrenia caused by the consumption of A1 milk and its consequent release of opioid BCM-7.⁶ Numerous detection methods such as AS-PCR, ACRS-PCR, & SSCP-PCR, ELISA, HPLC, MS, IEF & UREA PAGE have been developed for A1, A2 milk detection. However these methods have their own limitations in terms

of accuracy and affordability. Hence, in the current study, the allele-specific PCR assay is developed for detection and differentiation of homozygous A1A1, A2A2 and heterozygous A1A2 breeds from milk. The developed assay have some unique aspects. Firstly, isolation of DNA from milk containing somatic cells was standardized.⁸ The allele-specific primers were designed according to the nucleotide present in the A1 and A2 genetic variants with corresponding nucleotide at 3' end of the respective primers.⁹ Further, using the DNA sample, optimization of the allele-specific assay was carried out using specific primers. The specific primers were able to detect and differentiate homozygous A1A1, A2A2 and heterozygous A1A2 specifically. Hence, the assay can be employed as a beta-casein genotyping tool. Further, validation of the developed assay was carried out using 21 collected milk samples from nearby goshalas. The assay able to differentiate all milk samples without any hindrance proving assay efficiency. Hence, the assay developed can be used as a suitable tool for A1 and A2 beta-casein genotyping tool for dairy industry.

Conclusion

The allele-specific PCR assay developed can be used for detection and differentiation of A1, A2 beta-casein from milk. The developed assay can be employed directly from blood samples for detection and differentiation of indigenous breeds and helps in preservation of such breeds. Altogether, the assay will be a suitable tool for dairy industries for differentiation of A1 an A2 milk for commercial aspect.

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Conflict of Interest: None

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