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Genotyping of HF Crossbred Breeding Bulls for A1/A2 Variant of β-Casein Gene

Trupti V. Kulkarni[1], Vikrant D. Pawar[1], Rahul S. Deshmukh[1], Mahadeo P. Sawane[1]

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ABSTRACT

Background: The variants of β-casein (CSN 2) gene *viz.* A1 and A2 are supposed to be related with human health. Hence, to know the status of breeding bulls used for AI services were screened for A1 and A2 variants. Total 47 blood samples of crossbred breeding bulls from Frozen Semen Laboratory, Pune and Aurangabad were used for present work.

Methods: The extraction of genomic DNA by phenol-chloroform method were carried out from the blood samples of 47 breeding bulls. Further, DNA based PCR-RFLP method was used to identify the A1, A2 variants. Pure DNA fragments of β-casein (CSN 2) gene were amplified by PCR using suitable pairs of primer to amplify 121 bp and 244 bp products. The primer used to amplify 244 bp was allele specific. The 121 bp fragment then subjected to restriction enzyme digestion. The restriction enzyme digestions resulted into two types of band pattern. The 244 bp fragment was used for validation of A1 and A2 allele of 121 bp.

Result: Two types of genotypes *i.e.* A1A2 and A2A2 were revealed in the experimental population of HF crossbred breeding bulls. The genotypic frequency of A1A2 and A2A2 was estimated 65 and 35 per cent, respectively. The present frequency status of A1 and A2 allele in the breeding bulls may be suggested for planning future breeding strategy in dairy cattle.

Key words: A1/A2 variant, β casein, Breeding bulls, Genotyping.

INTRODUCTION

The world is trending with the A2 milk brand which is the 'Original milk' by the dairy cows producing only A2-β-casein protein. Initially, there was existence of only A2 variant of β-casein A2A2 genotype. The occurrence of A1 was due to mutation occurred during the process of selective breeding for high milk production. The mutations resulted into 13 variants of β-casein gene as A1, A2, A3, A4, B, C, D, E, F, H1, H2, I and G (Roginsky, 2003). A1 and A2 variants of β-casein protein differ in structure, A1 has histidine at 67th position and A2 has proline at the same position (Sharma *et al.*, 2013). There is polymorphism at codon 67 of β-casein gene, CCT which codes for proline in A2 β-casein changes to CAT which codes histidine in A1 β-casein (Ganguly *et al.,* 2013a). The structural difference in A1 and A2 β-casein leads to differential digestion by gut mucosa. The digestion of A1 β-casein in the gut by the action of digestive enzymes (pepsin, pancreatic elastase *etc*.) results in the cleavage between histidine and adjacent amino acid and a bioactive peptide called beta casomorphin-7 (BCM-7) is produced (Stewart *et al.,* 1987; Lien *et al.*, 1992). BCM-7 has opioid like activity and binds to opioid receptors and neural cells. It is thought that β-casein variant A1 is to be considered as hazardous for human health. The hazardous health effects of A1 variant milk like arteriosclerosis, diabetes mellitus type-1 (Kaminski *et al.,* 2007), autism, coronary heart disease (McLachlan, 2001; Laugesen and Elliott, 2003), atherosclerosis (Tailford *et al.*, 2003) and sudden infant death syndrome (Sun *et al.*, 2003). Besides, neurological disorders such as schizophrenia and autism (Woodford, 2006).

[1]Department of Animal Genetics and Breeding, Mumbai Veterinary College, Mumbai-400 012, Maharashtra, India.

Corresponding Author: Mahadeo P. Sawane, Department of Animal Genetics and Breeding, Mumbai Veterinary College, Mumbai-400 012, Maharashtra, India.
Email: drmahadeo.ms@gmail.com

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A1 variant of β-casein gene is mostly found in the milk of European, USA, Australia and New Zealand cattle breeds. Holsteins and Ayrshires produces A1 milk as compared to other cattle breeds. In India, increased milk production has been targeted by crossing Indian cattle breeds with high milk yielding exotic cattle breeds. Therefore, there might be chances of introduction of A1 in Indian cattle population. There was remarkable increase in crossbred cattle population of India due to extensive use of cross breeding with HF and Jersey. Considering health hazards of A1 variant of β-casein there is urgent need to find out the genotypes of breeding bulls for securing human health. It is also important to know whether there is a definite health benefit to milk containing the A2 genetic variant and requires further investigation unlike harmful effects of A1 milk. The present study was aimed out to know the status of A1 and A2 allele in HF crossbred breeding bull population of Maharashtra state used at AI station.

MATERIALS AND METHODS

The present research was carried out at 'Genetic Investigation Laboratory', Department of Animal Genetics and Breeding, Mumbai Veterinary College, Mumbai during the year 2018-2019. A total of 47 blood samples of HF crossbred breeding bulls were collected from Frozen Semen Laboratory, Kharki, Pune and FSL, Harsool, Aurangabad. The Genomic DNA was isolated by using phenol-chloroform method (Sambrook and Russell, 2006). Two different sets of primer were used to amplify 121 bp and 244 bp fragment of β-casein gene. Primer set used to amplify 121 bp fragment as: F- 5′ CCT TCT TTC CAG GAT GAA CTC CAG G 3′ and R- 5′ GAG TAAGAG GAG GGA TGT TTT GTG GGA GGC TCT 3′ (McLachlan, 2006). The 244bp fragments were amplified by using three primers set: F-(A) - 5′ CTT CCC TGG GCC CAT CCA 3′; F(C)-5′ CTT CCC TGG GCC CAT CCC 3′ and R- 5′ AGG GAA GGG CAT TTC TTT GT 3′ (Ganguly *et al.*, 2013a). Here, the combination of primer 'A' and common reverse primer 'R' resulted into A1 allele amplification and combination of primer 'C' and common reverse primer 'R' resulted A2 allele amplification. This set of primer was used to validate the results obtained for 121 bp fragment. The amplification of 121 bp was carried out at 58°C annealing temperature and the amplification parameters are shown in Table 1. Similarly, amplification parameters of 244 bp fragment are shown in Table 2.

The PCR products were visualized on 1.7 per cent agarose gel. Further, the PCR products of 121 bp were subjected to RE digestion with *Dde*I, Digestion was carried out in water bath at 37°C for 5 hours in total 20.00 µl reaction mixture comprising of RE *Dde*I :1.0 µl, 10Xk-buffer: 2.0 µl, PCR-product: 12.00 µl and nuclease free water: 5.00 µl.

Table 1: PCR thermal cycles for CNS 2 (121 bp) locus amplification.

Steps	Temperature	Time
Initial denaturation	95°C	5 min
Denaturation	94°C	40sec
Annealing	58°C	30 sec
Extension	72°C	1.5 min
	(step 2-4) 30 cycles	
Final extension	72°C	5 min

Table 2: PCR thermal cycles for CNS2 (244 bp) locus amplification.

Steps	Temperature	Time
Initial denaturation	94°C	5 min
Denaturation	94°C	30 sec
Annealing	66°C	30 sec
Extension	72°C	30 sec
	(step 2-4) 5 cycles	
Denaturation	94°C	30 sec
Annealing	64°C	30 sec
Extension	72°C	30 sec
	(step 2-4) 30 cycles	
Final extension	72°C	5 min

RE digested products were electrophoresed on 4 per cent agarose gel containing Ethidium bromide (@ 10 µl/100 ml) at constant voltage of 90 V for 60 minutes using 1X TAE buffer.

RESULTS AND DISCUSSION

Variants of β-casein gene has been studied by the various scientists. In the present investigation, 47 crossbred breeding bull from Frozen Semen laboratory, Pune and Aurangabad Maharashtra state were genotype for A1, A2 varients of β-casein. The findings of the present study have been presented and discussed as under. The good quality bands of DNA fragments 121 bp and 244 bp of β-casein gene were amplified successfully at annealing temperature 58, 66 and 64°C, respectively. The DNA fragment of 121 bp (Fig 1) of β-casein gene (exon 7) was successfully amplified with the primers as described by McLachlan (2006) and 244 bp (Fig 2) fragment of β-casein gene were successfully amplified by Allele Specific primers (Ganguly *et al.*, 2013a). *Dde*I/PCR-RFLP (Fig 4) revealed two restriction patterns

Table 3: Gene and genotypic frequencies (121 bp and 244 bp).

Detail	Genotypic frequency			Allele frequency	
	A1A1	A1A2	A2A2	A1	A2
Exon-7 (121 bp)	0.00	0.65	0.35	0.335	0.665
Exon-7 (244 bp)	0.00	0.73	0.27	0.36	0.64

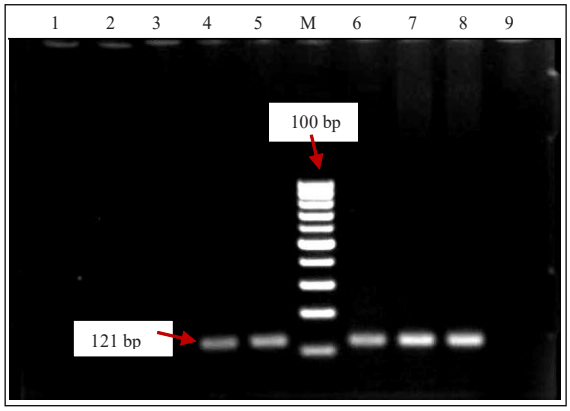


Fig 1: PCR amplicon of 121 bp fragment of β casein.

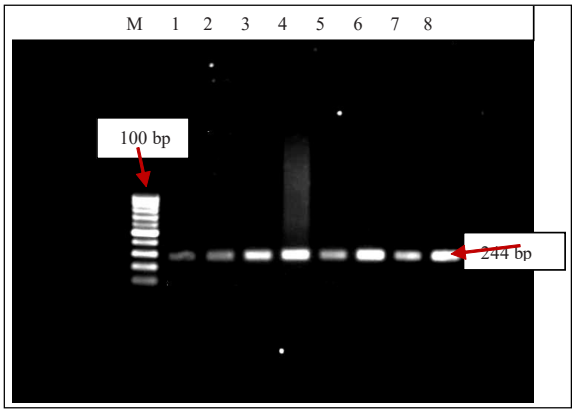


Fig 2: PCR amplicon of 244 bp fragment of β casein gene.

and were designated as A1A2 and A2A2 genotypes. The heterozygote A1A2 pattern had three bands of size 121 bp, 86 bp and 35 bp and A2A2 pattern had two bands of size 86 bp and 35 bp. The band of 35 bp was not visible in agarose gel. The estimated allelic frequency was found to be 0.335 and 0.665 for A1 and A2 allele (Table 3), respectively. The observed genotypic frequency for the genotypes A1A2 and A2A2 was, 0.65 and 0.35 (Table 3), respectively.

Present findings are partially in agreement with Shende *et al.* (2017) who reported only two genotypes A1A1 and A1A2 with frequencies of 0.28 and 0.72 in HF crossbred cows by PCR-RFLP analysis of 121 bp fragment. Kumar *et al.* (2019a) observed all three genotypes in Vrindavani crossbred cattle with genotypic frequencies of 0.11 (A1A1), 0.47 (A1A2) and 0.42 (A2A2) and allelic frequencies of A1 and A2 were 0.35 and 0.65, respectively in Vrindavani cows. Many researchers reported that all indigenous cattle breeds and buffaloes are of A2 type. Whereas, the presence of A1A1 genotype in Vrindavani population indicates that these animals may belong to F_2 generation and backcrossed with exotic bulls resulting in A1A1 genotype. However, in Sahiwal cows, only two types of genotypes were observed with frequency of 0.13 (A1A2) and 0.87 (A2A2). The allelic frequencies for A1 and A2 were 0.06 and 0.94, respectively in Sahiwal cows. Predominance of A2 allele was confirmed in indigenous and crossbred cattle, supporting the present findings. Further, Kumar *et al.* (2020) reported two genotypes in Tharparkar cattle *i.e.* A2A2 and A1A2, with A1 allele frequency as 0.04, indicating very high frequency of A2 variants. They found all three genotypes in Frieswal cattle, the genotype frequencies of A1A1, A1A2 and A2A2 were found to be 0.17, 0.39 and 0.44, respectively. The Frieswal cattle showed 0.37 for A1 and 0.63 for A2 allele frequencies which was in accordance with present findings indicating predominance of A2 allele in crossbred cattle.

Kumar *et al.* (2019b) analyzed association between β-Casein genotypes and different production and reproduction traits in Frieswal cattle. They found that A1A1 genotype cows had significantly lower TMY, MY300, PY and DP as compared to A1A2 and A2A2 genotypes. A1A2 genotype had highest TMY, MY300, PY and DP; however, A2A2 and A1A2 genotypes were not significantly different from each other. The results pointed towards the superiority of A2 β-casein variant for several production traits in crossbreds.

Whereas, Miluchová *et al.* (2013) studied same fragment of beta casein gene and reported three genotypes in HF crossbred cattle as A1A1, A1A2 and A2A2 with genotypic frequencies of 0.14, 0.46 and 0.40, respectively and allelic frequency for A1 and A2 to be 0.30 and 0.70, respectively. Mishra *et al.* (2009) genotyped 618 cattle (15 zebu cattle breeds), 231 buffaloes (8 river buffalo breeds) for β-casein gene polymorphism and reported the predominance of A1 in *Bos taurus* and A2 in zebu cattle breeds, while river buffaloes having only A2 variant. Hanusova *et al.* (2010) amplified 121 bp of β-casein gene in 92 cows and 5 bulls and reported frequencies of A1 and A2 allele of CSN2 in cows to be 0.54 and 0.46, respectively. CSN2 genotypic frequencies in cows were A1A1 (0.13), A1A2 (0.83) and A2A2 (0.04). Only A1A1 and A1A2 genotypes with frequencies 0.20 and 0.80 were found in bulls. High proportion of heterozygote individuals and genetic disequilibrium were recorded. Sodhi *et al.* (2012) reported predominance of the desirable A2 allele across all cattle types studied with a mean frequency of 0.645.

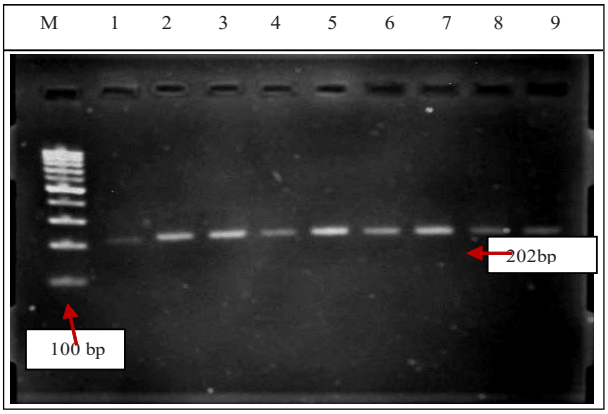
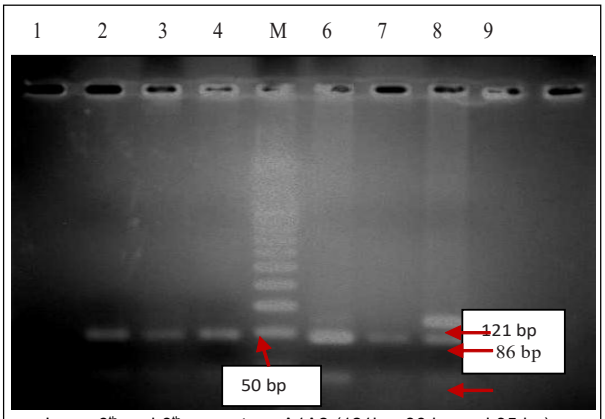
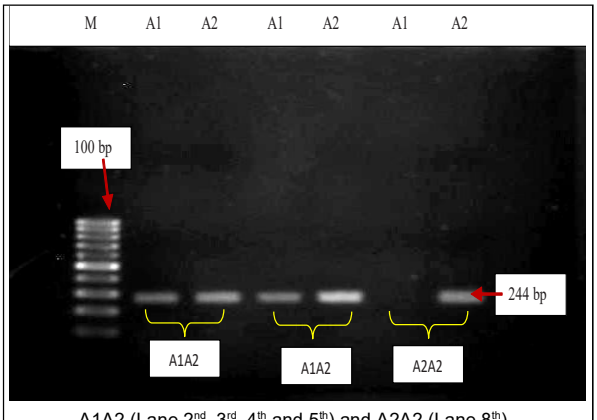


Fig 3: PCR amplicon of 202 bp fragment of β-casein gene.



Lane: 6th and 8th - genotype A1A2 (121bp, 86 bp and 35 bp);
Lane: 2nd, 3rd, 4th and 7th - genotype A2A2 (86 bp and 35 bp).

Fig 4: PCR-RFLP of 121 bp fragment of β-casein gene.



A1A2 (Lane 2nd, 3rd, 4th and 5th) and A2A2 (Lane 8th).

Fig 5: AS-PCR analysis of β-casein gene (244 bp).

The allele specific primers for the fragment of 244 bp was used to validate the genotypes produced through *Dde*I/ PCR-RFLP of 121 bp. The AS-PCR amplification of 244 bp (Fig 5) shown no amplification for A1 allele by specific forward primer and common reverse primer. Whereas, amplification of 244 bp product with A2 allele specific forward primer with one common reverse primer revealed one band of 244 bp in 13 HF crossbred bulls. Amplification of 244bp fragment with both the allele specific (A1 and A2) forward primer and common reverse primer yielded two bands of 244 bp representing A1 and A2 allele in 34 breeding bulls. Thus, the present study confirms that crossbred breeding bull population belongs to two genotypes *viz.* A1A2 and A2A2 and A1A1 genotype was found to be absent in all breeding bulls under investigation. The frequencies of A1 and A2 allele were found to be 0.36 and 0.64 respectively. The observed genotypic frequencies were as A1A1 (00), A1A2 (0.73) and A2A2 (0.27). The sequence analysis of 202 bp (Fig 3) fragment validated the genotypes by AS-PCR of 244 bp fragment.

Five genotypes of β-casein gene *i.e.* A1, A2, A3, A5 and B reported by using allele specific PCR (Lindersson *et al*., 1995). Similarly, differences in β-casein variant were evaluated in bovine breeds by using AS-PCR and relevance to BCM. Further, differentiation of A1 and A2 alleles was carried out by AS-PCR and observed genotypic frequencies of A1/A1 about 21.4 per cent, A2/A2 about 30.9 and A1/A2 about 47.6 (Keating *et al*., 2008). Slovak Pinzgau cattle population was studied for β-casein gene polymorphism and analyzed genotype structure. They reported heterozygous A1/A2 cattle with frequency of 0.52 while homozygous A2/ A2 cattle with frequency of 0.02. This suggests a slight superiority of allele A1 (Miluchova *et al*., 2009).

The 321 bp fragment of β casein gene was amplified in 478 bulls to screen the A1/A2 allele status by using PCR-ACRS and then digested with the *Mph*I enzyme resulted into 3 genotypes A1A1 (12.8%), A1A2 (44.1%) and A2A2 (43.1%). The estimated allele frequency for A1 and A2 allele was 0.35 and 0.65 respectively (Olenski *et al*., 2010). The 362bp of exon 7 of beta casein gene was amplified and subjected for sequencing to study the A1/A2 polymorphism in Gir (68) and Guzerá. Allele frequencies were 98 and 97 per cent for the A2 allele and 0.96 and 0.93 per cent for the genotype A2A2 for Gir and Guzerá, respectively (Rangel *et al*., 2017). Ramesha *et al.* (2016) studied polymorphism of β casein gene in breeding bulls of seven cattle breeds and two buffalo breeds by using PCR-ACRS amplification of 251 bp fragment with *Taq*I enzyme. They observed only A2 allele in Deoni and Khillar cattle and also in Murrah and Surti buffaloes. In the remaining cattle breeds they observed two genotypes A1A2 and A2A2. The frequency of A1 allele was very low in MalnadGidda (0.014), Kasargod variety (0.042) and Jersey (0.077), while the frequency of A1 allele in Holstein Friesian and Holstein Friesian crossbred males was 0.169 and 0.294, respectively.

The present result for AS-PCR was similar with (Rahman *et al*., 2016); they observed two genotypes in the studied population of cattle. But the frequency of A1 allele in the studied population was less than the present findings, approximately 0.20 (local breed) and 0.10 (crossbred). The 244 bp fragment of beta casein gene was amplified by using AS-PCR. A1 and A2 allele specific primers were designed to determine A1/A2 polymorphism in the Indian Zebu cattle breed Ongole and crossbred cattle Frieswal (HF × Sahiwal). They observed three (A1A1, A1A2 and A2A2) genotypes in the Frieswal and two A1A2 and A2A2 in Ongole (Ganguly *et al*., 2013b).

Similarly, 244 bp product was amplified by using AS-PCR in Kangeyam and Holstein Frisian crossbred cattle to study polymorphism of A1/A2 variants of beat casein gene. HF cross cattle breed showed three genotypes (A1A1, A1A2 and A2A2) and Kangeyam cattle showed only A2A2 genotype. Frequency of A2 allele was found higher than A1 in HF crossbred animals as 0.595 (Malarmathi *et al*., 2014).

Conclusion

Considering the health hazards and importance of A1 variant of beta casein gene there is urgent need to screen the breeding population (breeding bulls and bull mothers) of cattle for A1A2 allele and the said data may be utilized in formulating suitable breeding plans in order to minimize undesired A1 allele in future generations.

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Conflict of interest

All authors declare that they have no conflict of interest.

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