International Journal of Scientific Research in Biological Science
Research Paper Vol-1, Issue-1 E-ISSN: 2347-7520

Detection of Single Nucleotide Polymorphism by T-ARMS PCR of Cross Bred Cattle Karan Fries For A1, A2 Beta Casein Types

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Available online at www.isroset.org

Received: 19 July 2013 Revised: 08 August 2013 Accepted: 24 September 2013 Published: 28 February 2014

Abstract-"Detection of Single nucleotide Polymorphism by T-ARMS PCR of cross bred cattle Karan Fries for A1, A2 beta casein types" to distinguish between A1 and A2 type containing beta-casein and genotyping of hundred Karan Fries cross breed cattle. Casein is the main milk protein where it accounts for 80% of bovine milk protein and contains four fractions (alpha S1-CN, alpha S2-CN, beta-CN and k-CN). Beta casein contributes 25-35% of milk protein and many variants are reported (A1, A2, A3, B, C, D, E, F, G, H1, H2 and I) in different breeds of cattle. The beta casein variants A1 and A2 differs in the 67th amino acid position, the substitution of proline in A2 type with Histidine (in A1) is mainly due to a replacement of "C" nucleotide with "A" nucleotide in that corresponding nucleotide position. One hundred Karan Fries cross breed cattle were selected for genotyping of A1, A2 beta casein gene from the genomic DNA. The beta casein gene was amplified by Multiplex Tetra-Primer Amplification (T-ARMS-PCR). T-ARMS touchdown PCR and subsequent agarose gel electrophoresis could differentiate between the A1, A2 types of beta casein genes in these animals. The screening result showed three genotypes of animal in these 100 animals. The number of A2A2, A1A2 and A1A1 animals are 73, 19 and 8 respectively. The frequency of A2 and A1 alleles are 0.825 and 0.175 respectively.

Key words- Casein Genes, Karan Fries Cattle, Genetic Polymorphism, T-ARMS PCR, SNP's

I. INTRODUCTION

The total milk protein component of milk is composed of numerous specific proteins. Two major milk protein groups are caseins and whey proteins (ß-lactoglobulin and alpha-lactalbumin). Bovine milk contain four caseins (alpha s_1-CN 15-18%, alpha s_2-CN8-11%, beta-CN25-35% and k-CN 18-15%, Eigel, et al 1984; Roginsiki 2003). All other proteins found in milk are grouped together under the name of whey proteins. Bovine beta casein contains a total of 209 amino-acid residues. Bovine beta-casein gene is highly polymorphic. A total of 12 protein variants are known so far. Initially three variants of bovine beta casein were discovered and denoted as A, B and C. It was later found that A variants could be further resolved into A1, A2 and A3 by gel electrophoresis. The 12 genetic variants of bovine beta caseins are A1, A2, A3, B, C, D, E, F, H1, H2, I and G (Kaminiski, et al. 2007). Out of these, A1 and A2 variant forms are important in dairy cattle (Farrell et al., 2004). The nucleotide sequence change in 67th amino acid position of the beta-casein reading frame, from CCT to CAT, causes substitution of proline (A2) by histidine (A1, B) in the amino-acid sequence. This might cause a change of secondary conformation in the protein structure and affect the physical properties of casein micelle and vulnerability to enzymatic digestion. During this enzymatic process, beta-casein opioid peptide beta-casomorphin 7 is released exclusively from A1 and B variants (Hartwig, 1997, Jinsmaa and De Noni, 2008). The B-Casomorphin-7 (BCM-7), a bioactive seven-amino-acid peptide, can be released by digestion in the small intestine of A1 b-casein with pepsin, leucin amino peptidase and elastase. The term 'opioid' refers to chemical substances that have morphine-like activity in the body. These agents act by binding to opioid μ-receptors, which are found principally in the central nervous system and the gastrointestinal tract (Teschemacher 2003), BCM-7 has opioid and cytomodulatory properties. Synthetic BCM-7 can inhibit responses of lymphocytes to stimulants in vitro (Elliott et al, 1997). Elliott et al (1997) reported that NOD (Nonobese diabetic) mice fed with A1 b-casein did not develop diabetes if they were also given naloxone (the morphine antagonist). They suggested that appearance of diabetes in genetically susceptible NOD mice fed A1 b-

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casein not those fed with A2 b-casein might be due to release from A1 b-casein of the bioactive peptide, BCM-7 which had a strong inhibitory effect on immune function.

The debate about A1 and A2 milk types has been in the public arena for over a decade. There have been claims and counter claims about whether 'ordinary milk', which is a mixture of A1 and A2 milk, is linked to a range of disease conditions, and whether selecting cows that produce only A2 milk can avoid these problems. About 75% of the world's 300 million strong dairy herd, produces milk that contains the A1 beta casein. There is a somewhat controversial claim, backed by 16 years of research, that this milk, which is drunk by most people in the western world, could be a cause of diabetes, heart disease, autism and schizophrenia in people with immune deficiencies. It is also claimed that the protein beta casein A2 is benign in this respect. Cows in the well-known dairy breeds can produce either or both of the beta casein proteins. Genotyping has shown that about 80% of Indian (Bos indicus) cows produce only beta Casein A2 type.

II. MATERIAL AND METHOD

Experimental animals included in the study

Hundred cross bred NDRI Cow (Karan Fries) were included in the study animals maintained at Cattle Yard, National Dairy Research Institute, Karnal India. The blood sample (10 ml each) were collected from the jugular vein in sterile 10ml BD Vacutainer (BD Franklin Lakes NJ USA-8362817) under aseptic conditions and transported to laboratory in a box containing ice. The Isolation of genomic DNA from Blood by Phenol: Chloroform: Isoamylalcohol (PCI) method (De et al., 2009).and Quality check and Quantification of genomic DNA by using (Nano-drop) spectrophotometer. The optical density of the diluted DNA sample was taken at 260/280 nm using DNA storage solution (Mili-Q water) as blank. The conversion factor for DNA was taken as µg/µl per OD $_{260/280}$ units (Sambrook et al., 2001). Ratio of OD at 260 nm and 280 nm was 1.9-2.0; which indicated that the DNA was pure.

Primer designing for Tetra-primer ARMS PCR

This procedure adopts principles of the tetra-primer PCR method and the Amplification Refractory Mutation System (ARMS). Four primers are required to amplify a larger fragment from template DNA containing the SNP and two smaller fragments representing each of the two Allele specific (AS) products. Primers were designed in such a way that the allelic amplicons differ in size and can be resolved by agarose gel electrophoresis. The gene sequences of Beta-casein (CSN2) variant A2 gene, exon VII (CSN2 Bos indicus Accession no. AY366420.1, Bos taurus- BC111172.1,EF123100.1,Bos grunniens-HQ902899.1,JN655525.1, JN655525.1,EU310401,JN051276.1,JN051275.1 for Bos indicus species) were retrieved from GenBank (www.ncbi.nlm.nih.gov).The downloaded nucleic acid sequences were aligned using multiple alignment software Clustal W. Exon sequences for the above mentioned genes were also retrieved from the Ensemble genome browser. Tetra Primers for ARM-PCR were designed from the inter-exonic regions of respective genes by using the Primer3 software and were got custom synthesized from Sigma Pvt. Ltd. Bangalore. The sequences of primers used for amplification of CSN2 (Exon 7) by T-ARMS PCR:

Outer forward :

5'CCGTTAATGAGAAATCCTTCAGYGAGCA3'

Outer reverse :

5'TCTGGCTTTCAGTAAAGGGCTCAAACTGG3

Inner forward :

5'TAGTCTATCCCTTMCTGGGCCCATTCA 3'

Inner reverse : 5'

MGGGATGTTTGTGGGAGGCTSTCAG 3

Optimization of Annealing Temperature of T-ARMS PCR

Optimization of the primer pair was carried out by performing touchdown-PCR selecting different temperatures. The PCR mastermix was prepared in a 0.5ml tube by mixing nuclease free water,10XDream Taq buffer, dNTPs, primers and DreamTaq DNA Polymerase. Sufficient master mix for the number of reactions plus one extra for a no-template control was prepared. The master mix was aliquoted into individual 0.2ml PCR tubes. Then 1.5µl of genomic DNA was added to each aliquot of mastermix using a clean micropipette. The PCR reagents were mixed gently and spun down. The tubes were then placed onto the PCR machine.TARMS touchdown PCR program the PCR cycling conditions are listed in Table-1, was run after which it was stored at -4^{o}C

Table -1: Touchdown PCR cycling Parameters

Step	Temperature, ^{0}C	Time	Cycle number
Initial Denaturation	95	3 min	
Denaturation	95	30sec	
Annealing X5	59-54	30sec	Δ t -1^{0}c/ cycle
Extension	72	20 sec	
Denaturation	94	30sec	
Annealing	54	30sec	X 30
Extension	72	20sec	
Final Extension	72	4 min	

Agarose gel electrophoresis of PCR Product

The 3% agarose gel of high quality molecular biology grade agarose (Sigma Chem. Co., USA) was prepared by dissolving the agarose in 1X TAE buffer (pH 8.0) followed by heating in a microwave oven. Ethidium bromide stock solution was added directly to molten agarose solution at the rate of 0.5 µg/ml of gel volume before casting the gel. The surface of the gel casting tray was leveled before pouring the gel. After complete setting of the gel, the comb was removed carefully and the gel casting tray was placed in the electrophoresis tanks containing 1X TAE (pH 8.0) buffer. The amplified DNA samples were mixed with 5 µl of tracking dye and then loaded slowly into the wells of the submarine gel using micropipette. Electrophoresis was carried out at 150 V for half an hour. After completion of electrophoresis, the gel was examined under UV transilluminator /Gel documentation system (BioRad, Molecular Imager, GelDoc ^{TM} XR, Imaging System).

Sequencing of PCR Product

The Amplified of PCR products 296,199,111 bp subjected to Automated DNA sequencer and the obtained sequences followed by multiple alignment of the three sequences 296,199, 111bp.by Clastal W.

III. Result and Discussion

3.1 PCR amplification of beta casein variants:

Primers were designed for amplification of bovine beta casein variants after analyzing the mutation point in the bovine beta casein gene .The important primer parameter like GC percent, Tm value, secondary structure and self-complementarity, were taken into consideration while designing the primer optimization of the concentration of PCR component and PCR cycling parameters were described in material method section .Touchdown PCR with 30 cycle at final annealing temperature of 55^{0}C. This optimum primer concentration and cycling parameter result in efficient amplification of desired fragment size of beta casein variants by PCR. The amplified PCR product for A1 allele (“A” allele) was 199bp and for A2 allele (“C” allele) i.e. wild type was 111bp shown in Fig- 1, based on the position of primer location and amplified specific bands.

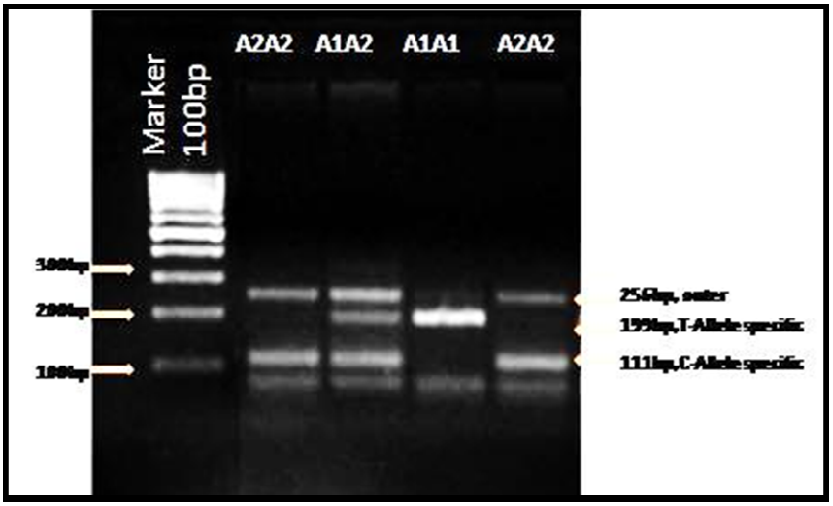

Fig-1:. Agarose gel electrophoresis (2%) of allele specific PCR product these main graph are applied in lane 2-5:-Lane 1=Marker DNA as in, Lane 2 and 5=A2A2 genotype specific PCR product, Lane 3=A1A2 genotype specific PCR product, Lane 4= A1A1 genotype specific PCR product.

Multiple alignments of A1 and A2 sequences:

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CLUSTAL W Result:  multiple sequence alignment of A1, A2 sequences

A               TATCCCTTCCCTGGGCCCATCCCTAACAGCCTCCCACAAAACATCCCTCC
A_1             TATCCCTTCCCTGGGCCCATCCCTAACAGCCTCCCACAAAACATCCCTCC
C               TATCCCTTCCCTGGGCCCATCCATAACAGCCTCCCACAAAACATCCCTCC
C_1             TATCCCTTCCCTGGGCCCATCCATAACAGCCTCCCACAAAACATCCCTCC
                ********************* **************************


A               TCTTACTCAAACCCCTGTGGTGGTGCCGCCTTTCCTTCAGCCTGAAGTAA
A_1             TCTTACTCAAACCCCTGTGGTGGTGCCGCCTTTCCTTCAGCCTGAAGTAA
C               TCTTACTCAAACCCCTGTGGTGGTGCCGCCTTTCCTTCAGCCTGAAGTAA
C_1             TCTTACTCAAACCCCTGTGGTGGTGCCGCCTTTCCTTCAGCCTGAAGTAA
                **************************************************


A               TGGGAGTCTCCAAAGTGAAGGAGGCTATGGCTCCTAAGCACA
A_1             TGGGAGTCTCCAAAGTGAAGGAGGCTATGGCTCCTAAGCACA
C               TGGGAGTCTCCAAAGTGAAGGAGGCTATGGCTCCTAAGCACA
C_1             TGGGAGTCTCCAAAGTGAAGGAGGCTATGGCTCCTAAGCACA
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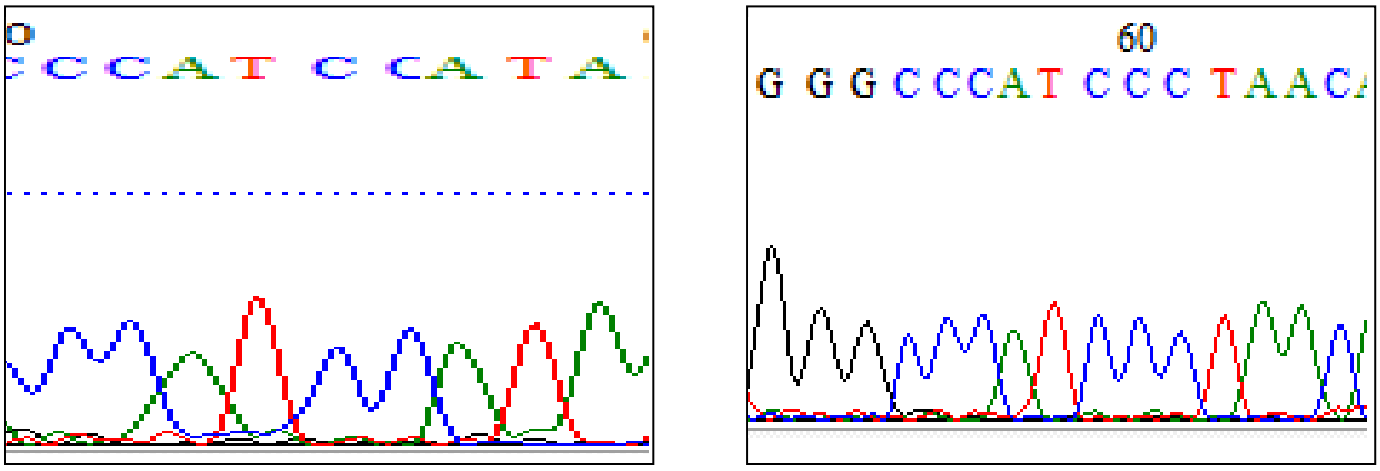

A B

Fig-A: Sequencing result for A1 (CSN2) and Fig-B: A2 (CSN2)beta casein.

The multiple alignment of A1 and A2 sequences result and fig.: A , B clearly showing that base the A1 in (CAT) and in A2 (CCT) means in the A1A1 the prolein replaced by Histidine and A2A2 no changes found.

4.4 Gene and Genotypic frequency

In Karan Fries beta casein gene, three genotype were revealed namely, CC, AC, and AA .At this particular SNP location two nucleotide present namely A and C. The genotype and allele frequency for both these allele

were calculated and presented in Table-13.The homogenous genotype CC was found to be the predominant genotype in Karan fries breed .Overall frequencies of CC,AC,AA genotypes were 0.73,0.19, and 0.08 respectively. In Karan Fries cattle the wild type allele i.e. "C" nucleotide is prevalent.

Out of 100 animal tested, 73animals (73%) showed A2A2 genotype containing "C" nucleotide (wild type allele; A2 allele). Nineteen animals were having A1A2 genotype containing "C" and "A" nucleotide (Both A1 and A2 allele respectively) in the same animals. While eight animals (8%) showed "A" nucleotide). The Table -13shows the inheritance pattern of variant allele (A2 allele containing "A" nucleotide) was present in the both heterozygous and homozygous conditions. Only eight animals were found homozygous for A1A1genotype containing only "A" allele (A1 allele).

Table-2: A1, A2 genotypes and their frequencies in Indian Cross breed cattle (Karan Fries) in NDRI Genotype and allele frequency of bovine beta casein variants in Karan fries cattle.

A1,A2 Genotypes	Variant Number	Genotype Frequencies	Allele Frequencies	
A1A1	8	0.8	A2	82.5
A2A2	73	0.73		
A1A2	19	0.19	A1	17.5

IV. CONCLUSION

From the above result we can say that frequency of undesirable "A" nucleotide is 0.175 which is supposed to be responsible for production of A1 milk .This frequency of undesirable "A" nucleotide allele may be attributed to the fact that Karan fries breed is evolved from European Holstein breed with higher A1A1 genotype .Thus, we can say that Indian cattle breed are still on the safer side that allele responsible for A2 milk production is comparatively high of "C" nucleotide containing wild type allele .

ACKNOWLEDGEMENTS

This work was supported by NAIP Project (ICAR, Pusha, New Delhi) and by National Dairy Research Institute, karnal-132001(Haryana).

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