Detection of A1 and A2 genetic variants of β-casein in Indian crossbred cattle by PCR-ACRS

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Beta-casein A1 variant of milk protein has been found to be a source of β-casomorphins and is correlated with the etiology of various human disorders like diabetes, ischemic heart disease and sudden infant death syndrome. Therefore, from the applied perception, it is a prerequisite to screen animals for A1 and A2 like genetic variants of β-casein. DNA fragments containing A1/A2 polymorphic region were amplified and genotyped using the PCR-ACRS (Amplification Created Restriction Site). Indian crossbred, Karan Fries, showed an allelic frequency of A1- 0.208 and A2- 0.792 with genotypic frequency of A1A1-0.125, A1A2- 0.166 and A2A2- 0.709. The allelic frequency of A1 and A2 in Karan Swiss was found to be 0.107 and 0.893, respectively, with genotypic frequency of A1A1- 0.0, A1A2- 0.214 and A2A2- 0.786. These two cattle population were developed under a crossbreeding program with exotic breeds (Holstein Friesian and Brown Swiss) which may serve as carriers of the A1 allele because the indigenous cattle breeds (Sahiwal and Tharparkar) have no A1 variant.

Nachweis von A 1- und Art 2-Genvarianten des ß-Caseins bei indischen Kreuzungsrindern mit PCR-ACRS

Die ß-Casein A 1-Variante des Milchproteins ist eine Quelle von ß-Casomorphinen und ursächlich mit verschiedenen Erkrankungen des Menschen wie Diabetes, ischämischer Herzerkrankung und dem Syndrom des plötzlichen Kindestods korreliert. Daher ist es erforderlich, Rinder auf die genetischen Varianten A 1 und A 2 des ß-Caseins zu überprüfen. DNA-Fragmente mit der polymorphen A1/A2 Region wurden amplifiziert und mithilfe der PCR-ACRS (Amplification Created Restriction Site) genotypisiert. Indische Kreuzungskühe, Karan Fries, zeigten eine Allel-Häufigkeit von A1 - 0,208 und A2 - 0,792 mit einer genotypischen Häufigkeit von A1A1 - 0,125, A1A2 - 0,166 und A2A2 - 0,709. Die Allel-Häufigkeit von A1 und A2 bei Karan Swiss-Rindern betrug 0,107 bzw. 0,893 mit einer genoty- pischen Frequenz von A1A1 - 0,0, A1A2 - 0,214 und A2A2 - 0,786. Die beiden Rinderpopulationen wurden mit Kreuzungsprogrammen mit exotischen Rassen (Holstein-Friesian und Brown Swiss) entwickelt, die Träger des A1-Allels sein dürften, da die einheimischen Rinderrassen (Sahiwal und Tharparkar) keine A 1-Varianten aufweisen.

01 Cattle breeding (India, A1/A2-ß-casein variants) **01 Rinderzucht** (Indien, A1/A2-ß-Casein-Varianten)

1. Introduction

Milk is a complex oil-in-water emulsion containing protein, fat, lactose, vitamins and minerals, as well as biologic products such as enzymes, cells, hormones, and immunoglobulins. Cow's milk contains an average of 3.5 g/dl of total protein divided into two major classes - casein and whey proteins (1). Casein is a mixture of subclasses, α-casein (50-55%) containing several hydrophobic phosophoseryl residues, β-casein (30-35 %), κ-casein (15%) which contains the calcium-rich portion, and γ-casein (5%) which may arise from proteolysis of β-casein. β-casein is the most polymorphic milk protein gene and there are at least 12 variants of this protein that differ at different amino acid positions (2). The most common forms are A1- "like" and A2 "like" milk, on the basis of presence of amino acid (Histidine or Proline) at position 67 of β-casein. A1 "like" milk involves β-casein with A1, B, C, F and G alleles with common variant His67 (-Tyr60-Pro61-Phe62-Pro63-Gly64-Pro65-Ile66-His67-) but variants at other positions of amino acids. A2 "like" milk (-Tyr60-Pro61-Phe62-Pro63-Gly64-Pro65-Ile66-Pro67-) having β-casein with A2, A3, D, H1, H2 and I alleles with the common variant Pro67 but variants at other positions of amino acids (3, 4).

According to A_1/A_2 hypothesis, in A1 variant of β-casein, the histidine residue at 67^{th} position allows the enzymatic cleavage to release a seven-amino-acid bioactive peptide, β-casomorphin-7 (BCM-7), while as in A2 variant, the presence of proline instead of histidine resists the enzymatic cleavage and hence BCM-7 is not formed (5). Epidemiological data and investigations made on mice and rabbits have correlated BCM-7 with many diseases like, ischemic heart diseases (6, 7), insulin-dependent diabetes (5) and sudden infant death syndrome (8). The A2 Corporation was setup in New Zealand in the late 1990s to test cows and market A2 milk at premium price in several countries which appeared not to have the disadvantage and health issues associated with A_1 milk. But there are controversial reports available that show positive impact of these peptides also, for example, HONG *et al.* (9) have proposed that BCM-7 has a protective role against hyperglycemia and free radical-mediated oxidative stress and may have anti-diabetic effect (9). BCM-7 has a role in mucin expression; mucus secretion and mucus release, hence, may play a role in gut immunity (10). Also these peptides have been found to increase prolactin levels and aid in gastric motility by expression of gastrin (11, 12)

Beta-casein variants for various breeds of cattle and buffalo have been genetically characterized by different researchers, Guernsey, Jersey, Brown Swedish, Simmental, Holstein Friesian, Ayrshire Holstein, Sahiwal, Tharparkar, Rathi, buffalo (Murrah, Mehsana, Mainpur) (13-20). In this study, we report for the first time, the detection of A1 and A2 variants of β-casein in 2 high yielding crossbred cattle of India - Karan Fries (KF) and Karan Swiss (KS) by Polymerase Chain Reaction-Amplification created Restriction Site (PCR-ACRS).

Table 1: Showing allelic and genotypic frequency across Indian crossbred and indigenous cattle

Animal breed	Animal	No of animals	Allele frequency		Genotype frequency		
			A1	A2	A1A1	A1A2	A2A2
Karan Fries	Cattle	24	0.208	0.792	0.125	0.166	0.709
Karan Swiss	Cattle	14	0.107	0.893	0.000	0.214	0.786
Sahiwal	Cattle	15	0.000	1.000	0.000	0.000	1.000
Tharparkar	Cattle	14	0.000	1.000	0.000	0.000	1.000

2. Material and methods

2.1 Animals

The analysis involved 67 animals; 24 Karan Fries, 14 Karan Swiss, 15 Sahiwal, and 14 Tharparkar, kept in Cattle Yard, National Dairy Research Institute, Karnal, Haryana, India.

2.2 DNA

The genomic DNA used for analysis of A1 and A2 genetic variants of β-casein in different breeds of cattle and buffalo (Murrah) by PCR-ACRS was isolated from their blood by phenolchloro-phorm deproteinization and ethanol precipitation meth-od (21).

2.3 PCR-ACRS analysis

The amplification created restriction site method (ACRS) was carried out using two sets of primers (22, 23). One set of primers, Casein4 (Forward): 5´C CTTCTTTCCAGGATGAACTCCAGG-3´and Casein Dde2 (Reverse): 5´GAGTAAGAGGAGGGATGTTTT GTGGGAGGCTCT-3´ was used to amplify 121bp product, the mismatch at the penultimate nucleotide (cytosine underlined) at 3'end of Casein Dde2 primer (Reverse) created a restriction site for DdeI enzyme. The specific digestion resulted in the occurrence of 86bp and 35bp fragments due to the presence of the CCT (proline) codon on 1.5% Agarose gel i.e., the A2 allele of β-casein. The results were confirmed by amplifying 321bp product with another set of primers, CASB (Forward): 5´GCAGAA TTCTAGTCTATCCC TTCCCTGGACCCATGC-3'and CASB (Reverse): 5'ACGGACTGAGGAGGAAACAT GACAGTTGGAG-GAAG-3', but in this case CASB forward primer had a mismatch at penultimate base (guanine, underlined) of the 3'end that creates restriction site for MPH1103I. The specific digestion resulted in the occurrence of 284bp and 37bp fragments due to the presence of the histidine codon on 2.5% agarose gel i.e., the A1 allele of β-casein. Both the PCR conditions were performed in 25µl volume contained 150-300ng genomic DNA, 10×PCR buffer; Taq DNA polymerase, 0.75U/25µl (Fermentas), 2mM $MgCl_2$; 200µM dNTP; 0.2µM of each primer. The PCR program for amplification was as follows: an initial denaturation (94℃/3 min), 3 5 cycles of denaturation (94℃/30s), annealing (60℃/30 s) and elongation (72℃/30s), and final synthesis (72℃/10 min). Specific digestion of the PCR produc ts were performed using 1U of DdeI and Mph1103 restriction enzymes (Fermentas), 10µl of PCR-product, 18µl of nuclease free water and 2µl of Buffer Tango (Fermentas), the mixture was mixed gently, spin down for few seconds and incubated at 37℃ overnight.

3. Results

PCR-amplification with Casein 4 and Dde2 primers yielded the specific product of expected size-121bp. Restriction digestion with DdeI (HpyF3I) enzyme showed three genotypes: A2/A2 (86 and 35bp), A1/A1 (121bp, uncut product) and A1/A2 (121, 86 and 35bp) as shown in Fig. 1. (band for 35bp is not visible). Karan Fries cattle showed an allelic frequency of A1-0.208 and A2-0.792 and genotypic frequency of A1/A1-0.125, A1/A2-0.166 and A2/A2-0.709. Karan Swiss cattle showed allelic frequency of A1-0.107 and

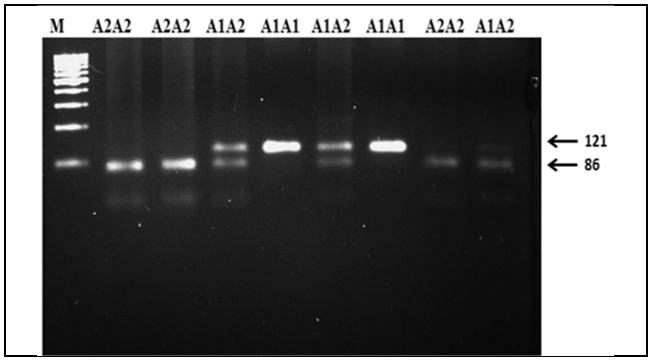

Fig 1: Example image of 2.5% agarose gel after electrophoresis, showing 3 genotypes of A1/A2 β-casein gene polymorphism, obtained by PCR-ACRS method (DdeI enzyme). M-100bp Molecular Marker

A2- 0.893 and genotypic frequency: A1/A1- 0.0, A1/A2-0.214 and A2/A2- 0.786. In Sahiwal and Tharparkar only A2 allele was observed, therefore, showing only A2/A2 genotype as shown in Table 1.

The above results were confirmed by PCR-ACRS using CASB-forward and CASB-reverse primers, PCR amplification yielded the specific product of 321bp. Restriction digestion with MPH1103I (NsiI) enzyme showed 3 genotypes: A1/A1 (284 and 37bp), A2/A2 (321bp, uncut product) and A1/A2 (321, 284 and 37bp) - see Fig. 2 (37bp not visible). Allelic and genotypic frequencies of all the breeds of cattle (KF, KS, Sahiwal and Tharparkar) were found same as with PCR-ACRS using DdeI (HpyF3I) restriction enzyme.

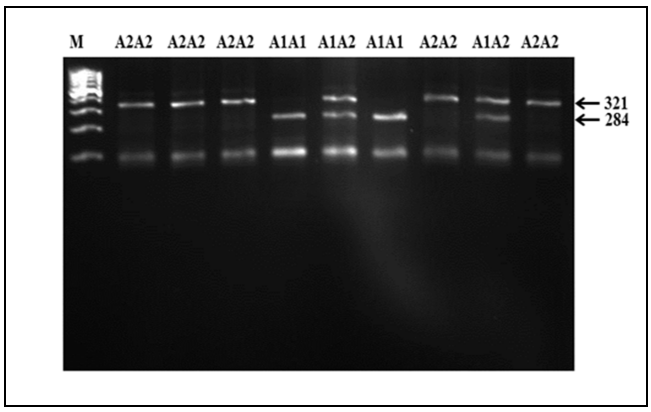

Fig. 2: Example image of 1.5% agarose gel after electrophoresis, showing 3 genotypes of A1/A2 β-casein gene polymorphism, obtained by PCR-ACRS (MPH 1103I ; NsiI enzyme). M-100bp Molecular Marker

4. Discussion

As a result of point mutation on exon VII of bovine β-casein gene on sixth chromosome, a conversion from cytosine to adenine leads to replacement of proline (CCT) by histidine (CAT) at position 67 (24). After observation of the new variant at 67th amino acid position, β-casein was classified into A1 and A2 β-casein. Since this amino acid change leads to the production of bioactive peptides i.e. β-casomor-phins, from the A1 β-casein, further, these peptides have been linked with many diseases. Therefore, it is prerequisite to screen the animals for these alleles. The point mutation that discriminates A1-like β-casein from A2- like β-casein is believed to have occurred about 5000 years ago in European taurine (*Bos taurus*) cattle probably somewhere close to Anatolia on the way to Europe from the domestication center (25, 26). The A1 allele frequency in different breeds varies between 0.06 (Guernsey), 0.3–0.4 (Holstein) and 0.72 (Danish Red) (3). Till date no report is available that shows the A1 and A2 allele frequency in Karan Fries and Karan Swiss (crossbred cattle).

Karan Fries was developed under crossbreeding programme in 1971, by using Tharparkar as zebu and three exotic breeds, namely Holstein Friesian, Brown Swiss and Jersey (27). In the present study, the observed A1 and A2 allele frequency in Karan Fries was found to be 0.208 and 0.792. OLENSKI *et al*. (23) have found the allele frequency of A1-0.35 and A2-0.65 in Holstein Friesian (23), HANUSOVÁ *et al*.(18) have found frequency of A1 and A2 alleles of β-casein in Holstein cows as 0.54 and 0.46 while in bulls it was found to be 0.60 and 0.40, respectively. MANGA and DVOØÁK (19) have also found the A1 and A2 allele frequency of 0.45 and 0.55 in Czech Holstein dairy cows. Karan Swiss is a cross between American Brown Swiss and Sahiwal. These cows are expected to have 50% level of exotic inheritance (27). Present study revealed the allele frequency of A1 and A2 in Karan Swiss 0.107 and 0.893, respectively. The frequency of A1 allele in Brown Swiss (Canada) was found to be 0.32 (28) and Brown Swiss, USA 0.14-0.18 (29, 30), respectively. MISHRA *et al*. (20) reported absence of A1 allele in Indian Zebu cattle Tharparkar (N=44) and Sahiwal (N=47) that supports our findings. Therefore, it is apparent that Holstein breed and Brown Swiss may act as a carrier of A1 allele to Karan Fries and Karan Swiss, respectively.

For the first time, we report the β-casein allele frequency data in Indian crossbred Karan Swiss and Karan Fries, which indicates the presence of A1 allele that is quite low as compared to exotic breeds. More importantly, the native milch breeds investigated in the present study, viz. Sahiwal and Tharparkar (cattle) indicated presence of only A2 variant which is the favored β-casein variant in cow milk.

Although A1/A2 hypothesis is still controversial, but is intriguing and potentially very important for human health, if it is proved correct. Therefore, a deeper research with release of casomorphins from respective milks during digestion is needed to explore this hypothesis to unravel its mechanism for further confirmation of the animal experiments and epidemiological data.

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