The Pharma Innovation Journal 2023; SP-12(6): 268-271

www.ThePharmaJournal.com

The Pharma Innovation

ISSN (E): 2277-7695
ISSN (P): 2349-8242
NAAS Rating: 5.23
TPI 2023; SP-12(6): 268-271

www.thepharmajournal.com
Received: 20-04-2023
Accepted: 21-05-2023

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Status of A1 / A2 beta casein Gene and its association with Lactose, SNF% and density% of milk in Gir and HF crossbred, reared in Madhya Pradesh, India

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Abstract
The goal of work is identification of β - casein gene polymorphism along with its association with milk production traits in Gir and HF crossbred cows of Madhya Pradesh. The effect of genotypes was found significant (p<0.01) for Lactose per cent trait. The mean milk Lactose per cent ranged from 4.92±0.07 in A1A2 genotype of HF Crossbred to 4.97±0.09 in A2A2 genotype of HF crossbred cattle. Among A2A2 genotyped animals, the lactose per cent was significantly higher in Gir breed (5.93±0.07) as compared to HF crossbred cows. The effect of genotypes was found significant (P<0.01) for SNF per cent trait. The mean SNF (%) in A2A2 genotype of Gir was 8.66±0.12. The corresponding mean SNF (%) for A2A2 genotypes was 7.93±0.14 in HF crossbred cow. The mean SNF (%) of A2A2 genotype of Gir and HF crossbred differ significantly with each other. On the other hand there was no significant difference between A1A2 and A2A2 genotypes of HF crossbred cow was noticed. The effect of genotypes was found significant (P<0.01) for density (kg/L) trait. The mean milk density (kg/L) for A2A2 genotype of HF crossbred was 1.07±0.06 and for Gir cows was 1.04±0.05. Among both the genotypes, the higher milk density (kg/L) was noticed in A2A2 genotype of Gir breed of cow (1.07±0.06) cows whereas, it was significantly lower in A2A2 genotype of HF crossbred (1.02±0.04) cow. The frequencies of A1A1, A1A2 and A2A2 genotypes were found to be 0.00, 0.00 and 1.00 in Gir breed of cows and 0.00, 0.60 and 0.40 in HF crossbred of cow, respectively. The respective gene frequency for A1 and A2 alleles were found to be 0.00 and 1.00 in Gir breed and 0.30 and 0.70 in HF crossbred of cow.

Keywords: Lactose, SNF, density, HF cross breds, β casein

Introduction
Milk derivatives of cows are a major nutritional component and their consumption continues to increase worldwide. Presently a relationship between disease risk and consumption of a specific bovine β-casein fraction A1 genetic variants has been identified. Polymorphism at codon 67 of the beta-casein gene causes a substitution of amino acid Proline in A2 variant by histidine in A1 variant. The presence of histidine at codon 67 makes A1 variant. Milk with A1 variant reactive during gastrointestinal proteolysis and release bioactive peptide, beta-casomorphin-7 (BCM-7). The allele variants A1, B, and C differ from each other with respect to an amino acid in position 122 (serine in A1 and C, arginine in B) and one in position 37 (glutamic acid in the A1 and B variants and lysine in the C variant). The I and A3 variants were derived from a mutation of the A2 variant; specifically, the I variant has a leucine instead of a methionine in position 93 and the A3 variant has a glutamine instead of a histidine in position 106. In view of healthfulness of A2 milk as well as positive relationship of A2 allelic variant with milk performance traits in different cattle breeds, the existing variability of β-casein (A1/A2) locus in our indigenous breeds like Gir population may be exploited near future for genetic selection.

Material and Methods
The laboratory procedures *viz*., sample preservation, DNA extraction, polymerase chain reaction (PCR), quality and quantity check of DNA and restriction fragments length polymorphism (RFLP) were performed in the Department of Animal Genetics and Breeding and Central Laboratory, College of Veterinary Science and Animal Husbandry, Mhow.

Experimental animals
A total number of 100 animals were selected for the study 50 each of Gir and HF crossbred cows.

From 100 animals 06 lactating cows of Gir and 06 of HF crossbred were selected on the basis of presence of A1 and A2 variants of Beta Casein gene. The selected animals were used for trial of its milk on mice.

Collection of Blood

About 5 ml of blood was collected from jugular veins in EDTA coated vaccutainers (Akuret) from each animal included in the study. The collected blood samples were brought to the laboratory by maintaining the cold chain and stored at 4℃ till further processing.

DNA extraction

The extraction of genomic DNA from collected blood samples was done by John's method (John *et al*., 1991) with minor modifications. The required solutions and reagents were prepared in the laboratory using molecular (Sigma) grade chemicals.

Required solutions and reagents

1. 1.solution-1 –Prepared in the lab.
2. Solution-2-–Prepared in the lab.
3. Phenol
4. Chloroform
5. Isoamyl alcohol
6. Sodium acetate (3M)
7. Isopropanol
8. Ethanol (70%)
9. Tris EDTA buffer

Procedure

1. 1.05 ml of blood was mixed with 5ml of solution-1 and 120µl of Nonidet P-40 to lyse the cells. It was mixed by inverting several times and centrifuged at 2000 rpm for 15 minutes at 24 ℃.
2. The supernatant was discarded and the pellet was gently resuspended in 800µl of solution-2 to lyse the nuclei and mixed well. Equal volume of saturated phenol (800µl) was added to the suspension and centrifuged at 11,000 rpm for 9 minutes at 4 ℃. Upper phase was transferred to the clean microcentrifuge tube and equal volume of saturated phenol: chloroform: isoamylalcohol (25:24:1) was added. 4. The tube was centrifuged at 11,000 rpm for 9 minutes, at 4℃ and the upper phase was transferred to another microcentrifuge.
3. Equal volume of chloroform: isoamylalcohol (24:1) was added and centrifuged at 11,000 rpm for 9 minutes, at 4℃. The upper phase was transferred to another tube.
4. The DNA was precipitated by adding $1/10^{th}$ volume of sodium acetate (3M) (82.03, molecular weight of sodium acetate X 3 = 246.09 g in 1000 ml) and equal volume of isopropanol. After gentle mixing the tube was centrifuged at 11,000 rpm for 9 minutes, at 4 to 8℃ to form DNA pellet.
5. The isopropanol was carefully discarded and the pellet was washed 3 times with 70% ethanol and the pellet was air dried.
6. Pellet was resuspended in 100 µl of 0.3X TE buffer and was incubated at 65℃
7. for 1 hour in a dry bath. The sample obtained was stored at -20℃ till further use.

Assessment of quantity and quality DNA

The concentration, purity and quality of DNA were checked by UV spectrophotometer and agarose gel electrophoresis.

Spectrophotometry

The concentration and purity of DNA were checked by UV – spectrophotometer. Optical density (OD) value at 260 nm and 280 nm were measured using UV-spectrophotometer. DNA samples with an OD 260/280 ratio of 1.7 to 1.9 were considered relatively pure.

Agarose gel electrophoresis

Furthermore, the DNA samples were subjected to 0.8% agarose gel electrophoresis.

PCR- Restriction fragment length polymorphism (PCR-RFLP)

Template DNA

The samples with higher concentration of DNA were diluted to 30 ng/µl using nuclease free water (Sigma). Three microlitre (3 µl) of DNA was used as template for PCR reaction.

Primers

The primers used for the amplification of β-casein gene were selected on the basis of the previous reports (Miluchova *et al.*, 2013) [2].

(F): 5´- CCT TCT TTC CAG GAT GAA CTCCAG G-3´

(R): 5´ - GAG TAA GAG GAG GGA TGT TTTGTG GGAGGC TCT- 3´

PCR reaction mixture

Amplification of the DNA samples extracted from the blood was done in a final volume of 25 µl reaction mixture in PCR tubes.

Setting of PCR reaction

The PCR tubes of 0.2 ml containing 25 µl reaction mixtures were kept in a preprogrammed thermo cycler (Applied Biosystems) and the standardized reaction program was set.

Agarose gel electrophoresis of PCR products

The PCR amplification was determined by 1.7% agarose gel electrophoresis. (1.7g) agarose in 0.5X TBE buffer (pH 8.0) was used. Agarose was melted in 0.5X TBE buffer.

Restriction digestion of the Polymerase chain reaction product

Restriction digestion of the PCR products was performed by using restriction enzyme (*DdeI)* which has its recognition site at G^AATTC.

Restriction endonuclease and its recognition site

S. No.	Restriction Endonuclease	Recognition site	Manufacturer
1.	*DdeI*	G^AATTC	Thermo Scientific, Lithuania

Determination of polymorphism by agarose gel electrophoresis

The polymorphism of β Casein gene was detected by 2% agarose gel electrophoresis.

Sequencing and analysis

Sequencing of amplicon will do for the confirmation of genotype of the cows. The sequences obtained from genotype

will align using Clustal W (Thompson *et al.*, 1994) [5] and analysed by using MEGA 6 software (Tamura *et al.*, 2013) [4].

Milk collection and milk records

Information on each animal included in study such as identification number and lactation yield were recorded. About 50 ml milk was collected. The milk samples were brought to the laboratory maintaining cold chain and were processed for determination of various milk composition traits by automatic milk analyzer (Ultra mb).

Statistical Analysis

Gene and genotype frequencies for different milk protein gene regions under study were estimated using Popgene 32(version1.32), Microsoft Windows-based freeware for population genetic analysis (Yeh *et al.*, 1999) [6]. The Chi-square test was use to test the populations of different breeds either in equilibrium at this locus or not.

Study the effect of various polymorphic variants of milk protein genes on milk yield, Fat % and Protein of the data were subjected to least squares analysis of variance employing linear model.

Testing Hardy-Weinberg (H-W) equilibrium

For testing the population in Hardy-Weinberg (H-W) equilibrium 50 animals from each cattle breed were taken and chi-square test was performed for calculating expected genotype frequencies and comparing them with the observed ones.

Result and Discussion

1.1 Association of β-casein (CSN2)/ gene polymorphic variants with Milk yield and milk composition traits

The polymorphic variants of β-casein gene (CSN2)/ *Ddel* in different breeds of lactating cows and their association with Lactose (%) SNF and Density (kg /L) have been studied as below:

1.1.1 Lactose (%) of different variants at β-casein (CSN2) gene of Gir and HF crossbred of cows

The results of analysis of variance have been presented in table no. 01. The effect of genotypes was found significant (*P*<0.01) for Lactose per cent trait.

Table 1: Least squares analysis of variance for Lactose (%) at β-casein (CSN2) gene of Gir and HF Crossbred cows

Source of Variance	DF	MS	F-Value
Genotypes	2	2.4093	10.12**
Error	98	0.2379	
Total	100		

****** Highly significant (*p*<0.01)

The mean Lactose (%) in Gir and HF crossbred cows has been presented in table 02. The mean milk Lactose per cent ranged from 4.92±0.07 in A1A2 genotype of HF Crossbred to 4.97±0.09 in A2A2 genotype of HF crossbred cattle. Among A2A2 genotyped animals, the lactose per cent was significantly higher in Gir breed (5.93±0.07) as compared to HF crossbred cows (Table 02).

Table 2: Means for Lactose (%) of different variants at β-casein (CSN2) gene of Gir and HF crossbred cows

Variants	Breeds	
	Gir	HF crossbred
A1A1	0.00±0.00	0.00±0.00
	(00)	(00)
A1A2	0.00±0.00	4.92^{a}±0.07
	(00)	(30)
A2A2	5.93^{b}±0.07	4.97^{a}±0.09
	(50)	(20)
Overall	5.93^{b}±0.07	4.94^{a}±0.08
	(50)	(50)

Means bearing the different superscript differ significantly (*p*<0.05), Number of animals are depicted in parenthesis.

1.1.2. SNF (%) of different variants of β-casein (CSN2) gene of Gir and HF crossbred cows

The results of analysis of variance have been presented in table no. 03. The effect of genotypes was found significant (*P*<0.01) for SNF per cent trait. The mean SNF (%) in Gir and HF crossbred cows has been presented in table no. 04.

Table 3: Least squares analysis of variance for SNF (%) at β-casein (CSN2) gene of Gir and HF Crossbred cows

Source of Variance	DF	MS	F-Value
Genotypes	2	3.7963	5.93**
Error	98	0.6393	
Total	100		

****** Highly significant (*p*<0.01)

As shown in table 04, the mean SNF (%) in A2A2 genotype of Gir was 8.66±0.12. The corresponding mean SNF (%) for A2A2 genotypes was 7.93±0.14 in HF crossbred cow. The mean SNF (%) of A2A2 genotype of Gir and HF crossbred differ significantly with each other. On the other hand there was no significant difference between A1A2 and A2A2 genotypes of HF crossbred cow was noticed (Table no. 04).

Table 4: Means for SNF (%) of different variants at β-casein (CSN2) gene of Gir and HF Crossbred cows

Variants	Breeds	
	Gir	HF crossbred
A1A1	0.00±0.00	0.00±0.00
	(00)	(00)
A1A2	0.00±0.00	8.05±0.13
	(00)	(30)
A2A2	$8.66^{b\pm}$0.12	$7.93^{a\pm}$0.14
	(50)	(20)
Overall	8.66^{b}±0.12	7.99^{a}±0.13
	(50)	(50)

Means bearing the different superscript differ significantly (*p*<0.05), Number of animals are depicted in parenthesis.

1.1.3. Density (kg/L) of different variants at β-casein (CSN2) gene of Gir and HF crossbred cows

The results of analysis of variance have been presented in table no. 05. The effect of genotypes was found significant (*P*<0.01) for density (kg/L) trait. The mean density (kg/L) in Gir and HF crossbred has been presented in table no. 06.

Table 5: Least squares analysis of variance for Density (kg/L) at β-casein (CSN2) gene of Gir and HF Crossbred cows

Source of Variance	DF	MS	F-Value
Genotypes	2	158.93	14.50**
Error	98	10.96	
Total	100		

****** Highly significant (p<0.01)

The mean milk density (kg/L) for A2A2 genotype of HF crossbred was 1.07±0.06 and for Gir cows was 1.04±0.05. Among both the genotypes, the higher milk density (kg/L) was noticed in A2A2 genotype of Gir breed of cow (1.07±0.06) cows whereas, it was significantly lower in A2A2 genotype of HF crossbred (1.02±0.04) cow (Table 06). No relevant reference found for above findings. Similar trend noticed by Pandet *et al*. (2021) Among Malvi Nimari, Sahiwal and HF Crossbred, the higher milk density (kg/L) was noticed in A2A2 genotype of Nimari (1.05±0.06) cattle.

Table 6: Means for milk density (Kg/L) of different variants at β-casein (CSN2) gene of Gir and HF Crossbred cows

Variants	Breeds	
	Gir	HF crossbred
A1A1	0.00±0.00	0.00±0.00
	(00)	(00)
A1A2	0.00±0.00	1.05±0.05
	(00)	(30)
A2A2	1.07^{a}±0.06	1.02^{b}±0.04
	(50)	(20)
Overall	1.07^{a}±0.06	1.04^{b}±0.05
	(50)	(50)

Conclusions

1. Association study of genotype and milk production traits revealed that the A1A2 genotype of HF crossbred showed higher milk yield on other hand A2A2 genotype of Gir breed of cows showed that higher Fat % and Protein %
2. The effect of genotypes was found significant (P<0.01) for Lactose per cent trait. Among A2A2 genotyped animals, the lactose per cent was significantly higher in Gir breed as compared to HF crossbred cows.
3. The effect of genotypes was found significant (P<0.01) for SNF per cent trait. The mean SNF (%) in A2A2 genotype of Gir was significantly higher than the corresponding mean SNF (%) of both genotypes. On the other hand there was no significant difference between A1A2 and A2A2 genotypes of HF crossbred cow was noticed.
4. The effect of genotypes was found significant (P<0.01) for density (kg/L) trait. Among both the genotypes, the higher milk density (kg/L) was noticed in A2A2 genotype of Gir breed of cow (1.07±0.06) cows.

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