Recebido em: 15/05/2024.
Aceito em: 22/06/2024.

Revista SODEBRAS – Volume 19
N° 221 – MAIO/ JUNHO - 2024

POLYMORPHISM OF THE BETA-CASEIN GENE IN HOLSTEIN-ZEBU COWS IN THE NORTH OF MINAS GERAIS, BRAZIL

POLIMORFISMO DO GENE BETA-CASEÍNA EM VACAS HOLANDÊS-ZEBU NO NORTE DE MINAS GERAIS, BRASIL

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Abstract – This study aimed to investigate A1 and A2 polymorphisms of the beta-casein gene in crossbred Holstein-Zebu (HZ) cows from four herds in Northern Minas Gerais, Brazil. Blood samples were collected from 112 randomly selected cows, of wich 66 were at ½ HZ, and 46 were at ¾ HZ. DNA extraction was conducted using the phenol-chloroform method, and polymorphisms were evaluated using the amplification refractory mutation system - polymerase chain reaction technique. The results demonstrated that 72/112 (64.3%) cows were heterozygous (A1A2), 33/112 (29.5%) were homozygous for A2A2, and 7/112 (6.2%) were A1A1 homozygous. The percentage of A2A2 cows ranged from 10.7 to 41.4% among the evaluated herds. The frequencies of the A1 and A2 alleles were 38.4 and 61.6%, respectively. The A2 allele was the most frequent in both breeds. The ½ HZ cows were 1.8 times more likely (Odds Ratio) to belong to the A1A1 genotype. The distribution of genotypes was in Hardy-Weinberg equilibrium in three of the herds; however, the total of samples and the stratified samples according to breeds were not in equilibrium considering α = 0.05.

Keywords: Bos Taurus. ARMS-PCR. Milk Yielding Animals. Single Nucleotide Polymorphism. Milk Production.

Resumo – Este estudo objetivou investigar o polimorfismo A1 e A2 do gene beta-caseína em vacas Holandês-Zebu (HZ) oriundas de quatro rebanhos no norte de Minas Gerais, Brasil. Amostras de sangue foram coletadas de 112 vacas, aleatoriamente selecionadas, sendo 66 delas ½ HZ e 46 foram ¾ HZ. A extração de DNA foi conduzida pelo método fenol-clorofórmio e os polimorfismos foram avaliados pela técnica de amplification refractory

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mutation system - polymerase chain reaction. Os resultados demonstraram que 72/112 (64.3%) das vacas foram heterozigotas (A1A2), 33/112 (29.5%) foram homozigotos A2A2 e 7/112 (6.2%) homozigotos A1A1. O percentual de vacas A2A2 variou de 10,7% a 41,4% entre os rebanhos analisados. As frequências dos alelos A1 e A2 foram de 38,4% e 61.6%, respectivamente. O alelo A2 foi mais frequente nas duas composições genéticas analisadas. As vacas ½ HZ apresentaram 1.8 vezes mais chance (Odds Ratio) de possuir o genótipo A1A1. A distribuição dos genótipos encontra-se em equilíbrio de Hardy-Weinberg em três das fazendas analisadas, no entanto, o total de amostras e as amostras estratificadas por composição genética não estão em equilíbrio considerando α = 0.05.

Palavras-chave: Bos Taurus. ARMS-PCR. Animal Produtor de Leite. Polimorfismo de Nucleotídeo Único. Produção Leiteira.

I. INTRODUCTION

Milk and dairy products are consumed by more than six billion people worldwide (Food and Agriculture Organization of the United Nations, 2023). Beta-casein is present in bovine milk at a concentration of 9.6 g per kg of proteins, equivalent to 45% of total caseins (Kaskous, 2020). What differentiates A2 beta-casein from A1 beta-casein is the replacement of a cytosine in the CCT triplet by an adenine, becoming CAT, thus encoding not a proline amino acid but a histidine at position 67 of the polypeptide chain (Kay *et al.*, 2021).

A2A2 milk is produced from cattle that process the A2 homozygous genotype; therefore, it does not cause digestive discomfort due to its caseins (Diário Oficial da União, 2021). Conversely, milk or dairy products produced from animals that contain the A1 allele, including homozygous A1A1 or heterozygous A1A2, produce a stable peptide called beta-casomorphin-7 (BCM-7) when digested. This peptide can alter the gut microbiota and trigger an inflammatory response in sensitive consumers (Kay *et al.*, 2021). Common symptoms including bloating, flatulence, pain, and increased stool frequency (Sheng *et al.*, 2019; Kay *et al.*, 2021).

In several countries globally, A2A2 milk has good acceptance and added value (Bentivoglio *et al.*, 2020; Doval and Artega, 2021). This occurred in Brazil (Mendes *et al.*, 2019). Approximately 12 beta-casein gene (CSN2) variants are available, the most common of which are A1 and A2 (Marko *et al.*, 2020). Regarding the frequency, there were differences among breeds and herds. Antonopoulos *et al.* (2021) detected 52.2% of A2A2 in Holstein cows in Greece. In contrast, Dai *et al.* (2016) reported 28.5% of A2A2 in cows of the same breed in China. Rangel *et al.* (2017) detected 93.2% and 95.6% of A2A2 frequencies in the Guzerat and Gir Zebu herds, respectively. Beta-casein genotyping tests were conducted to separate females producing A2A2 milk and/or to select A2A2 animals for reproduction and artificial insemination.

The Holstein-Zebu (HZ) breeds play an imperative role in Brazilian dairy productivity, producing approximately 70% of the milk in the country (Verneque *et al.*, 2021). Vigolo *et al.* (2022) consider the Amplification Refractory Mutation System – PCR (ARMS-PCR) as an economical, fast, and user-friendly approach. Moreover, it requires only one PCR reaction, saves reagents, labor, and time, and generates less waste. Therefore, this study aims to detect A1 and A2 alleles polymorphisms by the ARMS-PCR method in 112 HZ cows from dairy herds in the north of Minas Gerais, Brazil.

II. METHODS

This research was approved by Animal Ethics Committee of the Federal

University of Minas Gerais under 16/2022 protocol. Samples were collected between April and May 2022 in Montes Claros, Francisco Sá and Manga, Minas Gerais, Brazil.

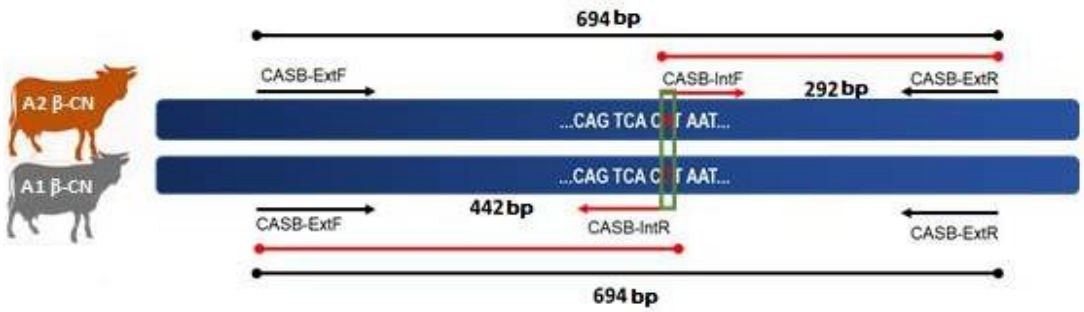
In total, 112 blood samples were collected from randomly selected Holstein-Zebu (HZ) cows, descendant of diverse bulls from four dairy herds. Antisepsis was conducted using 70% alcohol, and a sterile needle was used for each animal (Marko *et al.*, 2020). Five milliliters of blood was collected in Vacutainer tubes containing ethylenediaminetetraacetic acid (EDTA), and it was placed in a thermal box with ice and sent to the Laboratório de Sanidade Animal of Universidade Federal de Minas Gerais to be stored at -20°C. Post-freezing, the samples were transferred to the Universidade Estadual de Montes Claros at the Montes Claros campus. DNA extraction was conducted as described by Guha *et al.* (2018), with a few modifications. Thus, 300 µL of phosphate-buffered saline (PBS) was incorporated into 200 µL of previously thawed blood sample in a tube, it was homogenized, and 500 µL of lysis buffer (1 M Tris-HCl pH8.0; 0.04 M EDTA pH8.0; 0.02% SDS; 1 M NaCl) was incorporated. The tubes were homogenized and maintained in a water bath at 65°C for 60 min. Then, it was cooled to room temperature and 300 µL of chloroform: isoamyl alcohol (24:1) was incorporated. The mixture was homogenized and centrifuged at 10656 RCF for 10 minutes, and the supernatant was transferred to another tube. The chloroform:isoamyl alcohol step was repeated. Then, 300 µL of ice-cold absolute ethanol was incorporated into the supernatant, and the tube was kept at -20°C for 20 min. Then, it was centrifuged at 10656 RCF for 10 min, and after drying the pellet, 50 µL of nuclease-free water was incorporated. The tube was incubated at 42°C for 30 min, and after cooling down to room temperature, it was stored at -20°C.

Nucleic acids extracted from bovine blood samples were subjected to ARMS-PCR to detect exon 7 of the CSN2 beta-casein gene and the A1 and A2 variants. Hence, the following primers were simultaneously applied: CASB- ExtF:5'-AGTGGGTTAATGAGAAATCCTTC-3', CASB-ExtR:5'- TAATAGGGAAGGGTCC CCG-3', CASB-IntF: 5'-CCTTCCCTGGACCCATCCA-3', CASB-IntR:5'-TGTTTTGT

GGGAGGCTGTTAG-3' (GenOne-Brazil). These primers were designed based on Miluchová *et al.* (2013) and Chessa *et al.* (2013), with modifications based on GenBank (M55158.1) sequences. ARMS-PCR reactions were conducted in a final volume of 20 µL, comprising 10 µL Go Taq Green Mastermix 2x (Promega Corporation, USA), 10 µM CASB-ExtF primer, 10 µM CASB-ExtR primer, 5 µM CASB-IntF primer, 5 µM CASB-IntR primer, 1 µL bovine serum albumin (10 mg/mL), 3 µL nuclease-free water, and 50 ng DNA. The ARMS-PCR conditions were: initial denaturation at 95°C for 5 min; 40 cycles of 95°C for 30 s, 63°C for 45 s, and 72°C for 1 min and 30 s; and a final extension of 72°C for 10 min.

Amplicons were electrophoresed on a 1.3% agarose gel with ethidium bromide. The expected amplicon sizes for the A1 allele were 442 and 694 bp, and those for the A2 allele were 292 and 694 bp (Figure 1).

Figure 1 - Schematic representation of the partial exon 7 region of bovine beta-casein gene.



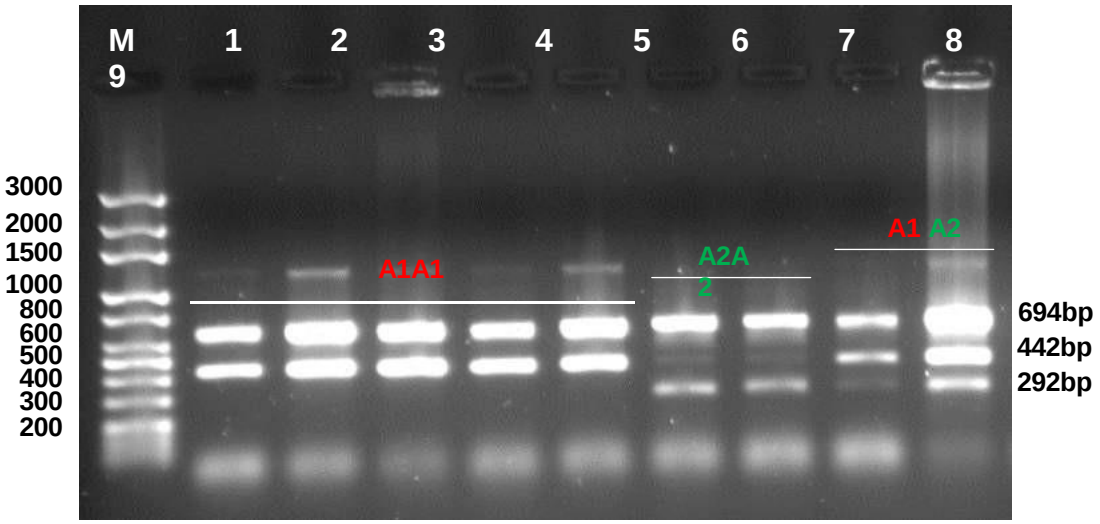
Autores, 2024.

Amplicon analyses were conducted and tabulated using Excel (2013). Allele and genotypic frequencies were computed using Excel (2013), according to Teixeira *et al.* (2021) and Oliveira *et al.* (2021). Hardy-Weinberg equilibrium was computed using specific software (Cog-genomics, 2020), and the other analyses were conducted using Select Statistical Services (2023). The difference between the occurrence of alleles or genotypes and non-occurrence was computed using *Odds Ratio* (OR). Statistical confidence was considered at 0.5 as a significance level.

III. RESULTS AND DISCUSSION

In this study, Figure 2 depicts the results of ARMS-PCR with the detection of genotypes A1A1, A1A2, and A2A2 in samples. Containers 1–5, 6–7, 8, and 9 demonstrated the respective genotypes A1A1, A2A2, and A1A2 found in samples from the four rural herds included in this study.

Figure 2 – Results of ARMS-PCR standardization to detect exon 7 of the CSN2 beta-casein gene and A1 and A2 variants in cows from four rural herds in Northern Minas Gerais, Brazil.



M = mid-range DNA ladder (Cellco Biotechnology).
Authors, 2024.

ARMS-PCR is a routine technique for genotyping beta-casein alleles (Chessa *et al.*, 2013). However, Vigolo *et al.* (2022) obtained the amplification results for allele fragments without control fragment, making it impossible to verify their identification. The choice of primers and standardized specific conditions are crucial for a successful PCR. Herein, control fragments (694 bp) were amplified, and all searched genotypes were identified.

The results obtained from ARMS-PCR for the A1A1, A1A2, and A2A2 genotypes were subjected to statistical analysis to determine the frequencies of the A1 and A2 alleles of CSN2 (Table 1).

Table 1 – Allelic and genotypic frequencies of the CSN2 gene in cows from four herds located north of Minas Gerais, Brazil.

Herds	Beta-casein allele frequencies N (%)		Genotype frequencies N (%)			P-value (>0,05)
	A1	A2	A1A1	A1A2	A2A2	
A	22 (39,3)	34 (60,7)	3 (10,7)	16 (57,2)	9 (32,1)	0,3365
B	27 (48,2)	29 (51,8)	2 (7,1)	23 (82,2)	3 (10,7)	0,0012
C	18 (31,0)	40 (69,0)	1 (3,4)	16 (55,2)	12 (41,4)	0,1413
D	19 (35,2)	35 (64,8)	1 (3,7)	17 (63,0)	9 (33,3)	0,0629
Total	86 (38,4)	138 (61,6)	7 (6,2)	72 (64,3)	33 (29,5)	0,0001

The A2 allele frequency was higher than the A1 allele frequency in all the herds evaluated. Herd B presented percentages of both alleles close to 50%. In contrast, in herd C, there was a large difference between the percentages of the A1 (31%) and A2 (69%) alleles (Table 1). The results obtained in this study agree with the findings of Oliveira *et al.* (2021) for Araguaína – Tocantins, which were measured in crossbred herds, and the frequency of the A1 allele was lower than that of the A2 allele (28.3% and 71.7%, respectively). Antonopoulos *et al.* (2021) obtained allele frequencies from Holstein cows in Greece ranging from 74.4 to 25.6% for the A2 and A1 alleles, respectively; Dai *et al.* (2016) detected 50.8 versus 49.2% frequencies for the same alleles and breeds in herds from China. The results of Sebastiani *et al.* (2020) in Italy and Ivankovic *et al.* (2021) in Croatia agree with the findings of Antonopoulos *et al.* (2021), with 63.9% of the A2 allele versus 36.1% of the A1 allele and 65.0% versus 35.0%, respectively.

Considering that the evaluated cows were Holstein Gir breeds, it is viable to compare the results of this research to those of Zebu breeds. The findings of Rangel *et al.* (2017) for allele frequencies in Brazilian herds were 97.8% for the A2 allele versus 2.2% for the A1 allele in the Gir breed and 96.6% for the A2 allele versus 3.4% for the A1 allele in the Guzerat breed. Therefore, it was observed that the HZ breeds demonstrated a reduction in the A2 allele frequency (61.8%) (Table 1) compared to the Gir breed (97.8%) (Rangel et. al., 2017). Alternatively, it was similar to the average frequency of the Holstein breed (63.0%) described by Antonopoulos *et al.* (2021), Dai *et al.* (2016), and Ivankovic *et al.* (2021).

Because there were A2A2 females in all herds sampled in this study, an alternative to increasing the number of A2A2 animals would be to directly reproduce with males that contain the same A2A2 genotype. Herds B and C demonstrated discrepancies ranging from 10.7 and 41.4%, respectively, for cows producing A2A2 milk.

According to Kay *et al.* (2021), the A2 allele is the oldest and a mutation in it causes a difference between these two beta-casein variants. This may elucidate the existence of herds with more A2 alleles than A1 ones and/or herds that are not in Hardy-Weinberg equilibrium yet (p-value below significance level $\alpha = 0.05$). The results obtained by Firouzamandi *et al.* (2018), even with frequencies close to 50% in both alleles, were not in Hardy-Weinberg equilibrium in any of the three evaluated breeds: Holstein, Sarabi and Gaja. Gaja were 100% heterozygous genotypes (Firouzamandi *et al.*, 2018). Alternatively, a search by Cieślińska *et al.* (2019) provides the distribution of the

SODEBRAS, v. 19 – n. 221 – Maio/Junho - 2024. ISSN 1809-3957

A1 and A2 alleles of the CSN2 beta-casein gene in equilibrium.

Regarding breed, the results demonstrated differences between the A1 and A2 allele distributions (Table 2). The OR computed suggests that A1 allele occurrence is 1.2 (95% confidence interval = 0.69–2.08) times more among ½ HZ cows. For the A2A2 homozygous genotype, the OR value was 0.8 (95% CI = 0.34–1.76), indicating a lower frequency in ½ HZ cows. Regarding the A1A1 genotype, the OR indicates 1.8 (95% CI = 0.33–9.72) times more occurrence in ½ HZ cows than in ¾ HZ cows.

Table 2 – Frequencies of CSN2 gene stratified by cow breed in four herds situated north of Minas Gerais, Brazil.

Breeds	Allele frequencies N (%)		Genetic frequencies N (%)			P-value
	A1	A2	A1A1	A1A2	A2A2	
½ HZ	53 (40,2)	79 (59,8)	5 (7,6)	43 (65,1)	18 (27,3)	0,005
¾ HZ	33(35,9)	59 (64,1)	2 (4,4)	29 (63,0)	15 (32,6)	0,017

Marko *et al.* (2020) described 54.72% of the evaluated samples as heterozygous genotypes in Holstein cattle, 33.02% as A2A2 genotypes, and 12.26% as A1A1 genotypes. In contrast to the results expected for Hollstein cattle compared to Gir in previous studies (Antonopoulos *et al.*, 2021; Sebastiani *et al.*, 2020; Ivankovic *et al.*, 2021; Rangel *et al.*, 2017), this study demonstrated that the A2A2genotype was more likely in ¾ HZ cows than in ½ HZ cows. Although the findings of Antonopoulos *et al.* (2021) presented the A2A2 genotype as the most common genotype in Holstein cows, at 52.2%, compared to Rangel *et al.* (2017) in Zebu cows, the percentage of the A2A2 genotype exceeded 90% in both Gir (95.6%) and Guzerat (93.2%) cows.

IV. CONCLUSION

The A1 and A2 alleles of the CSN2 beta-casein gene can be differentiated by ARMS PCR, making this approach promising for screening and genotyping these two alleles. This study demonstrates that the A1 allele and A1A1 genotype are more frequent in ½ HZ than in ¾ HZ studied cows. The frequency of the A2 allele is higher than that of the A1 allele in the ½ and ¾ HZ cows.

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VI. ACKNOWLEDGEMENTS

To Programa de Pos-Graduação em Biotecnologia of Universidade Estadual de Montes Claros (Brazil) for the support and to Instituto de Ciências Agrárias of Universidade Federal de Minas Gerais (Brazil) for the partnership.

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