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Research

A Preliminary Screening of Single Nucleotide Polymorphism (SNP) of A1 and A2 Beta-Casein Alleles in Dairy Cattle

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ABSTRACT

Milk containing A1 variants in the *beta-casein* gene is considered to be a risk factor for coronary heart disease (CHD), type I diabetes (DM-I), autism, and schizophrenia due to the release of beta-casomorphin 7 (BCM-7). The objectives of this study were to screen for polymorphism of beta-casein in dairy cattle and to examine the allelic and genotypic frequencies of A1 and A2 variants. A total of 290 cattle blood samples were collected from two states and the samples were extracted. This was followed by PCR amplification for exon 7 of the *beta-casein* gene (*CSN2*). The PCR products were then subjected to DNA sequencing. The sequencing results were aligned and the variants were screened. In Pahang, 125 samples are of the A2A2 genotype, followed by A1A2 (*n*=97) and A1A1 (*n*=28). While in Melaka, the highest genotype was A2A2 (*n*=20) as compared to A1A2 (*n*=18) and A1A1 (*n*=2). Meanwhile, the frequency of the A2 allele was higher than A1 in both states. The high genotypic frequencies of A2A2 in these cattle populations could be influenced by the cattle breed as well as the rate of crossbreeding on the farm. Thus, screening of dairy cattle for A1 and A2 beta-casein could be used in selecting the preferred animals for breeding.

Key words: Allelic frequencies, BCM-7, *CSN2*, genotypic frequencies, polymorphism

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INTRODUCTION

Bovine milk protein can be divided into two main components; casein and whey which account for 80% and 20% of total protein, respectively (Kumar *et al*., 2021). There are four main groups of casein, namely alpha s1-, alpha s2-, beta-, and kappa-casein, each contributes 38%, 10%, 36%, and 13% total milk protein (Sulimova *et al*., 2007). Out of the four caseins, beta-casein is the second most abundant and comprises 209 amino acids (Kumar *et al*., 2021). The gene that encodes for beta-casein is the *beta-casein gene* (*CSN2*), located at chromosome 6q31-33 (Sulimova *et al*., 2007). Attributable to the polymorphic characteristic of the *CSN2* gene, 15 variants have been reported in this gene thus far, specifically A1, A2, A3, B, C, D, E, F, G, H1, H2, I, J, K, and L (Pabitra *et al*., 2022). In dairy cattle, A1 and A2 are the most frequently observed genetic variants (Massella *et al*., 2017).

The type of amino acid at position 67 in the beta-casein chain plays an important role in determining A1 and A2 variants. It was observed that the amino acid at position 67 in the A1 variant is histidine, whereas the amino acid in the A2 variant is proline. Interestingly, a single substitution in the *CSN2* gene accounts for the variations in amino acids between the two variants. Substitution of the nucleotide cytosine (C) to adenine (A) has resulted in the production of amino acid histidine (CAT) in the A1 variant as compared to proline (CCT) in the A2 variant (Bhat *et al*., 2017). This single substitution, also known as single nucleotide polymorphism

(SNP) could contribute to changes in the genome either in the exon, intron, or intergenic region (van Dijk *et al*., 2014; Ahmad *et al*., 2018). SNPs in exons can affect the structure and function of proteins, gene expression level, and animal physiological metabolism (Lestari *et al*., 2020).

The occurrence of SNP in exon 7 of the *CSN2* gene has resulted in different nucleotide sequences and consequentially, producing A1 and A2 alleles. According to Woodford (2009), both A1 and A2 alleles are codominant, in which a heterozygote individual will produce both A1 and A2 proteins (A1A2) while a homozygote individual will either produce A1 protein (A1A1) or A2 protein (A2A2).

One of the bioactive peptides generated during the hydrolysis of milk proteins during digestion is beta-casomorphin 7 (BCM-7), an opioid peptide with properties akin to those of morphine (Chitra, 2021). In the A2 variant, proline prevents the enzymatic hydrolysis of peptide bonds between residues 66 and 67, which stops BCM-7 from being released (Sharma *et al*., 2013). Nevertheless, in A1 variations, histidine allows cleavage of the preceding seven amino acid residues at position 67, releasing BCM-7 through this process (Chitra, 2021). The level of BCM-7 in A1 milk was found to be four times higher as compared to A2 milk which trigger concern. BCM-7 in A1 milk has been associated with an increased risk of atherosclerosis, ischemic heart disease, diabetes mellitus type 1, autism, schizophrenia, and sudden infant death syndrome (SIDS) (Kamiński *et al*., 2007; Shashank *et al*., 2018; Raja *et al*., 2023).

A1 and A2 beta-casein screening has been done worldwide in different populations of dairy cattle. Selecting the ideal animals for breeding could be made easier with the knowledge of the animals' genotypes. In Malaysia, however, no similar study has yet been carried out. Hence, this study was conducted to screen the population of dairy cattle from two states in Malaysia and to examine the frequencies of A1 and A2 alleles and genotypes in those cattle.

MATERIALS AND METHODS

Blood sample collection and DNA extraction

Dairy cattle blood samples were collected from two states in Malaysia, namely Pahang and Melaka. A total of 250 blood samples were collected from Australian Friesian Sahiwal (AFS) cattle in Pahang. While in Melaka, 40 samples from mixed-breed cattle were collected. Cattle selected for sampling were females with the age range between 2 to 7 years old. Approval for animal ethics was granted by the UKM Animal Ethical Committee (FST/2019/NADIATUR/27-NOV./1068-NOV.-2019-AUG.-2023). The process of blood drawn from the coccygeal vein was performed by veterinarians and certified technicians using BD Vacutainer Precision Glide™ Needle (18G x 1") (BD, USA), and the blood was transferred to the BD Vacutainer® EDTA 5 mL (BD, USA). Genomic DNA was isolated from the whole blood using DNeasy Blood and Tissue Kit (QIAGEN, Germany) according to the manufacturer's protocol and stored at -20°C for long-term storage. Before storage, the concentration of the extracted DNA was inspected using a NanoDrop ND-1000 Spectrophotometer (NanoDrop™, USA) and was standardized to a concentration of 10ng/µL.

PCR Amplification and DNA Sequencing

Amplification of the extracted DNA samples was then carried out via polymerase chain reaction (PCR) in a thermal cycler (miniPCR bio™, USA) for 35 cycles using the following protocol: initial denaturation of 95°C for 3 min; denaturation at 94°C for 15 sec; annealing of 56°C for 30 s; extension of 72°C for 10 s; final extension of 72°C for 10 min. PCR was performed using 30ng/µL of genomic DNA in a reaction volume of 25 mL containing 10 uM of each primer, I U of My Taq Red Mix (Bioline Pty Ltd, USA), and ddH20. To amplify a 202-bp fragment of exon 7 of the *CSN2* gene, DNA primers CSNex7F 5'-CCAGGATAAAATCCACCCCT-3' and CSNex7R 5'-AGGGAAGGGCATTTCTTTGT-3' (adapted from Bhat *et al*. 2017) were used. PCR products were then subjected to gel electrophoresis to evaluate the yield and specificity using 1% agarose gel and were examined using the Omega Fluor™ Gel Documentation System (Gel Company, Inc., USA). Following this, PCR products were sent to Apical Scientific Sdn. Bhd. (Malaysia) for DNA sequencing. Using Sequencher (Gene Codes Corporation, USA), MEGA7 (Molecular Evolutionary Genetic Analysis, USA), and BioEdit Sequence Alignment Edition 7.1.3.0 (Tom Hall, USA) software, sequencing results were aligned and screened for SNPs. The frequencies of alleles A1 and A2 as well as the genotypes were then calculated.

RESULTS

All 290 cattle blood samples from both states were successfully extracted and amplified to produce a PCR product of 202 bp. Figure 1 represents the result of gel electrophoresis of the PCR amplification products for five samples using 1% agarose gel with 100 bp DNA ladder as reference.

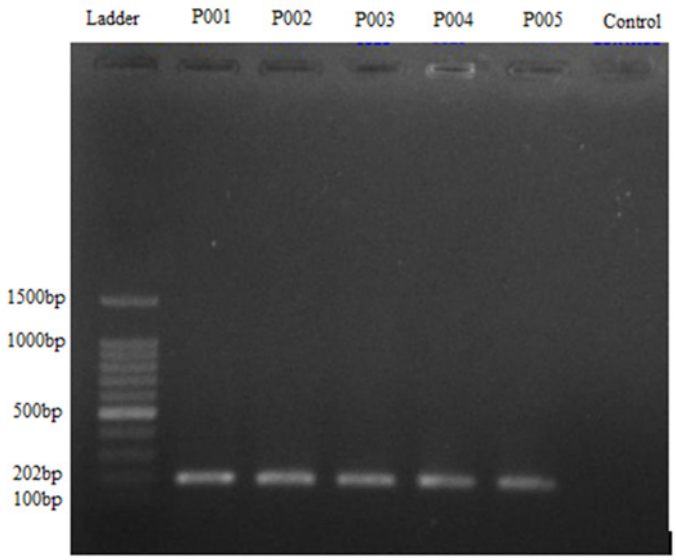

Fig. 1. Gel electrophoresis for PCR products using 1% agarose gel. Well 1 is the 100 bp ladder, well 2-6 are samples P001 – P005, and well 7 is the negative control.

Following PCR amplification, all samples were subjected to DNA sequencing and the sequencing results were aligned using MEGA7 (Molecular Evolutionary Genetic Analysis, USA) (Figure 2). Screening for SNPs using only the aligned sequences could not distinguish if there were heterozygotes in the samples. Thus, screening of SNPs using a chromatogram is crucial to check each of the peaks present at the specific location. This was done using the Sequencher software (Gene Codes Corporation, USA).

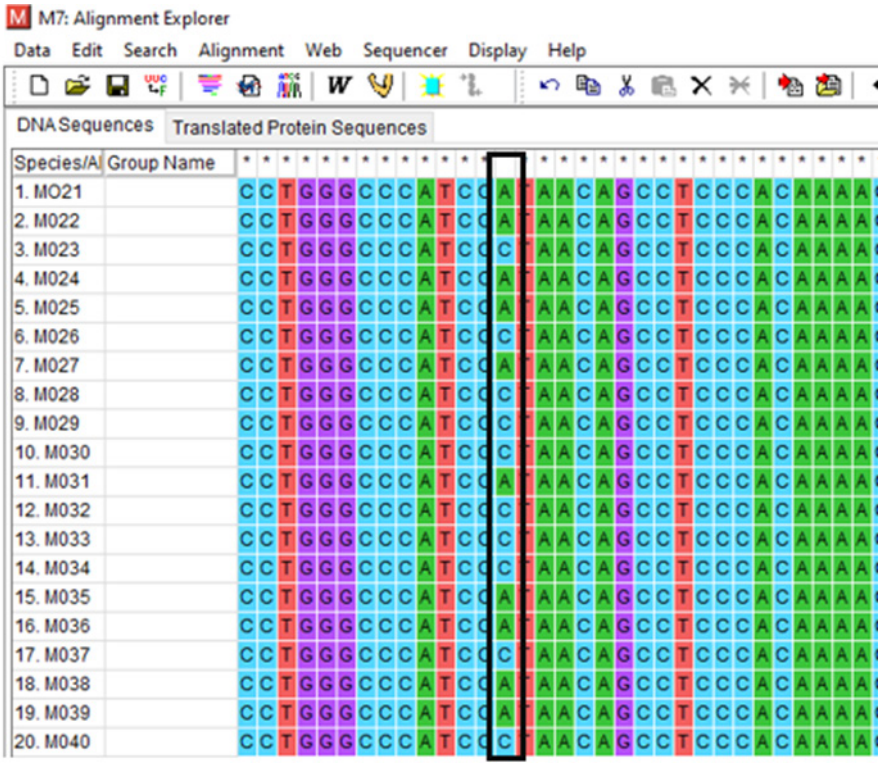

Fig. 2. Multiple sequence alignment of 20 samples using Mega7 Software.

Based on the screening of the sequencing results, the SNP of interest in exon 7 of the *CSN2* gene was observed. The peaks in the chromatogram clearly distinguished the specific bases, either a cytosine (C), an adenine (A), or an overlap of A and C as can be seen in Figure 3. When reading the bases as a codon, a sample with a cytosine will be read as CCT and represents the amino acid Proline. Whereas

the amino acid Histidine will be produced when the codon is read as CAT from the substitution of base C to A. Consequently, the substitution of these bases leads to three different genotypes, specifically A1A1, A1A2, and A2A2. The findings from the screening of SNP in all samples are shown in Table 1.

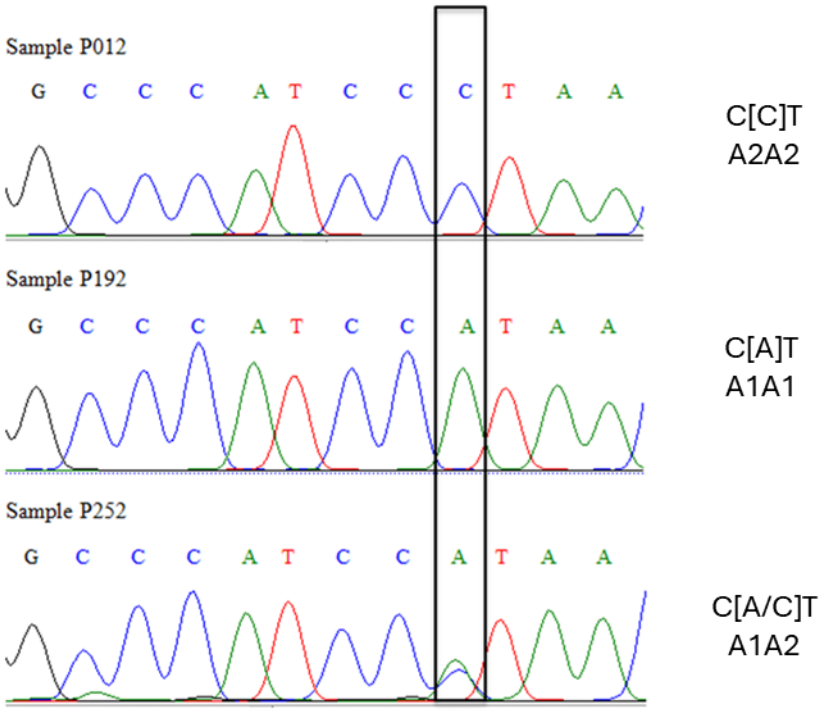

Fig. 3. SNP screening using chromatogram for three samples with three different genotypes.

Table 1. Screening of SNPs of A1 and A2 beta-casein alleles of all samples in Pahang and Melaka

State	A1A1	A1A2	A2A2	Total
Pahang	28	97	125	250
Melaka	2	18	20	40

Allelic and genotypic frequencies were calculated and shown in Table 2. The genotypic frequencies for A2A2 were the highest and the same in both states with 0.50. Both states recorded the lowest frequencies for genotype A1A1, Pahang (0.11), and Melaka (0.05). From the genotypic frequencies, it can be anticipated that the A2 allele would have a higher frequency. The A2 allelic frequencies in Pahang were 0.69 and 0.72 in Melaka as compared to the A1 allelic frequencies of 0.31 and 0.28, respectively.

Table 2. Genotypic and allelic frequencies of all samples in Pahang and Melaka

		Genotypic Frequencies			Allelic Frequencies	
State	Total	A1A1	A1A2	A2A2	A1	A2
Pahang	250	0.112	0.388	0.500	0.306	0.694
Melaka	40	0.050	0.450	0.500	0.275	0.725

DISCUSSION

The issue of A1 and A2 milk has long been debated in a few countries especially after it was highlighted by Woodford (2009). Since then, most countries across the globe have taken the initiative to screen their cattle population for the A1 and A2 variants of beta-casein. In Malaysia, such screening has yet to be done, thus this study aimed to conduct a preliminary screening of A1 and A2 variants in the cattle population of this country.

In this study, 250 samples were collected in Pahang from a farm raising Australian Friesian Sahiwal cattle. The frequency for the A2A2 genotype (0.50) which accounts for half of the population was the highest among the three genotypes observed. This was followed by A1A2 (0.388) and A1A1 (0.122).

The high frequency of A2A2 could be influenced by the breed available in Pahang, which was the Australian Friesian Sahiwal (AFS). AFS is a cross between Sahiwal and Holstein-Friesian. Sahiwal, a dairy breed originated from Pakistan is a breed from the *Bos indicus* species. Most *Bos indicus* cattle originated from Africa and Asia and can be distinguished by the distinct character of having a hump on their body, a dewlap, smaller size, and mostly brown. These breeds are normally tolerant to heat and humidity, however, the milk production is lesser than *Bos taurus* (Pal *et al*., 2015). Interestingly, most *Bos indicus* breeds naturally carry the A2 allele in the *CSN2* gene (Mukesh & Sodhi, 2013). This can be seen in a study conducted in India using 306 Sahiwal cattle which reported that more than 94% of the cows had the A2 beta-casein variant (Kumar *et al*., 2019). Additionally, in another study in India, the same results were shown where 28 Sahiwal cattle carried the A2 allele out of 30 studied (Saran *et al*., 2023).

On the contrary, *Bos taurus* cattle are better known for producing high milk yield and better milk quality such as in Holstein and Friesian breeds, which makes these breeds the most preferred dairy cattle breeds across the world. However, these breeds have a low tolerance to heat and humidity in most Asian and African countries and most of these breeds naturally carry the A1 allele in the *CSN2* gene. Therefore, to overcome this problem, crossing between breeds from these two species was seen to be a good idea in producing a better breed. For instance, the Australian Friesian Sahiwal breed was first introduced in 1961 to accommodate the needs of farmers to have a high milk yield with better tolerance to heat and humidity (Tierney, 1992). Since then, numerous crossbreeds involving Holstein and Friesian have been conducted such as Frieswal, Mafriwal, Jamaica Hope, and Karan Swiss (Rehman *et al*., 2014). A study conducted by Ganguly *et al*. (2013) showed a high frequency of A2A2 genotypes in a population of Frieswal cattle, a hybrid of Holstein Friesian and Sahiwal cattle which involved a total of 124 female cattle. It was discovered that the frequency of the A2A2 genotype was the highest with a value of 0.48. The findings of Ganguly *et al*. (2013) aligned with other studies (Kumar *et al*., 2020; Mumtaz *et al*., 2021) which reported that the frequency of the A2 allele was higher than the A1 allele in the Frieswal cattle population. Thus, the higher frequency of the A2A2 genotype in the Australian Friesian Sahiwal cattle population in this study is not unexpected.

In Melaka, a total of 40 blood samples were collected from mixed breeds of cattle. The setback with small farms was the absence of pedigree data and trait measurements. Breeds found in the farms range from Gir, Jersey, Friesian, and Local Indian Breed, and most of them are unknown. Thus, it was difficult to deduce and interpret the frequencies. However, the frequencies of genotypes in Melaka were the highest for A2A2, followed by A1A2 and A1A1. This could be due to the use of breeds mostly from the *Bos indicus* breeds, sometimes known as the Zebu cattle which is known to naturally carry the A2 allele. This is proven by a study conducted on 618 cattle from 15 different Indian Zebu breeds. The results of the study found that there were only A2A2 and A1A2 genotypes, while none of the cattle carried the A1A1 genotype (Mishra *et al*., 2009). In a study using 38 Ongole breed cattle (Zebu India), it was discovered that the A2 allele has the highest frequency with 0.68 and the highest genotypic frequency was 0.89 for the A2A2 genotype (Ganguly *et al*., 2013). In a study in India, it was found that 356 Gir (Zebu) breeds that had been screened had the highest A2 allele frequency of 0.96 (Khan *et al*., 2023).

CONCLUSION

The preliminary screening of the *CSN2* gene in these dairy cattle populations has shown that there was presence of SNPs which contributed to different alleles and genotypes. The frequencies of alleles and genotypes were different in both states and are most probably influenced by cattle breeds. Since the sample size is considered small, there results do not represent each state nor the country. For a more accurate analysis, more samples, more breeds, pedigree data, and trait measurements should be considered in future studies.

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ETHICAL STATEMENT

This study was approved by the UKM Animal Ethical Committee, approval number UKMAEC FST/2019/NADIATUR/27-NOV./1068-NOV.-2019-AUG.-2023.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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