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The Association of SNP g.49170107 G>T in the CYP2A6 Gene with Mineral Content in Sheep

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ABSTRACT

Mineral content plays a pivotal role in determining the quality of meat, which consumers prioritize when seeking healthy and high-quality lamb. Enhancing the genetic makeup of mineral content is therefore crucial to pique consumers' interest in lamb meat. Among the potential genes that could be used as markers for the nutritional quality, including the mineral content, of Indonesian lamb meat, the cytochrome P450, family 2, subfamily A, polypeptide 6 (CYP2A6) gene stand out. The main objective of this study was to identify the CYP2A6 gene polymorphism at SNP g.49170107 G>T and explore its association with mineral content in Indonesian sheep. For this purpose, 95 sheep samples were analyzed, consisting of 81 Javanese thin-tail sheep (JTTS) and 14 Jonggol sheep (JS). The researchers investigated the polymorphism through the PCR-RFLP (Polymerase Chain Reaction - Restriction Fragment Length Polymorphism) method. Subsequently, they analyzed the association between the CYP2A6 gene and mineral content in lamb meat using the GLM (General Linear Model) method. The study revealed that the CYP2A6 gene polymorphism was present in JTTS, while it remained monomorphic in JS. The identified genotypes in the polymorphism were GG and GT. Notably, the CYP2A6 gene exhibited a significant association (P<0.05) with Fe content. Specifically, the GT genotype displayed higher Fe content compared to the GG genotype. These findings suggest that the CYP2A6 gene holds promise as a potential candidate for identifying mineral content in lamb meat.

Keywords: CYP2A6 gene, mineral content, polymorphism, sheep

ABSTRAK

Kandungan mineral memainkan peran penting dalam menentukan kualitas daging, yang menjadi prioritas konsumen saat mencari daging domba yang sehat dan berkualitas tinggi. Oleh karena itu, meningkatkan susunan genetik pada kandungan mineral sangat penting untuk menarik minat konsumen terhadap daging domba. Di antara gen potensial yang dapat digunakan sebagai penanda kualitas nutrisi, termasuk kandungan mineral pada daging domba Indonesia, yakni gen cytochrome P450, family 2, subfamily A, polypeptide 6 (CYP2A6). Tujuan utama dari penelitian ini adalah untuk mengidentifikasi polimorfisme gen CYP2A6 pada SNP g.49170107 G>T dan mengeksplorasi hubungannya dengan kandungan mineral pada domba Indonesia. Sebanyak 95 sampel domba dianalisis, yang terdiri dari 81 ekor Domba Ekor Tipis (DET) dan 14 ekor domba Jonggol. Para peneliti menyelidiki polimorfisme tersebut melalui metode PCR-RFLP (Polymerase Chain Reaction - Restriction Fragment Length Polymorphism). Selanjutnya, dianalisis hubungan antara gen CYP2A6 dan kandungan mineral pada daging domba menggunakan metode GLM (General Linear Model). Penelitian ini menunjukkan bahwa polimorfisme gen CYP2A6 ditemukan pada domba DET, sementara pada domba Jonggol bersifat monomorfik. Genotipe yang teridentifikasi dalam polimorfisme tersebut adalah GG dan GT. Secara khusus, gen CYP2A6 menunjukkan hubungan yang signifikan (P<0.05) dengan kandungan zat besi (Fe). Genotipe GT menunjukkan kandungan Fe yang lebih tinggi dibandingkan dengan genotipe GG. Temuan ini menunjukkan bahwa gen CYP2A6 berpotensi sebagai kandidat potensial untuk mengidentifikasi kandungan mineral dalam daging domba.

Kata kunci: gen CYP2A6, kandungan mineral, polimorfisme, domba

INTRODUCTION

The consumption demand for lamb meat in Indonesia can be categorized as still low. So far, animal protein sources have been predominantly focused on beef and broiler chicken. According to data from the Livestock and Animal Health Statistics in 2022, based on average household consumption in Indonesia per capita, the consumption of lamb meat in 2017 was 0.0001 kg, while beef was 0.009 kg, and broiler chicken was 0.109 kg. One factor contributing to its popularity among consumers is the nutritional value of lamb meat. Nutritional value is indeed a significant factor in meeting human dietary needs. Addressing human nutritional requirements through this nutritional approach involves enhancing the quality of meat (Listyarini *et al*. 2018).

One of the nutritional requirements needed by humans is minerals. Minerals are essential for the body and are important components of food. Lamb meat is a high-quality source of bioavailable iron, zinc, and selenium. In regions with high deficiency prevalence and where lamb is culturally acceptable and available. While lamb's nutritional profile is highly relevant to the deficiency burdens (iron, zinc), its current public health significance is limited due to very low consumption levels, high cost, and dietary habits (USDA Foreign Agricultural Service 2023). The functions of minerals are diverse, including bone growth, muscle and nerve function, regulation of body water balance, reproduction, and other functions (Weyh *et al.* 2022). Numerous minerals engage in interactions both among themselves and with vitamins. For instance, maintaining optimal bone health necessitates a harmonious interplay between vitamin D, calcium (Ca), potassium (K), phosphorus (P), magnesium (Mg), selenium (Se), zinc (Zn), iron (Fe), chlorine (Cl), manganese (Mn), copper (Cu), and sulfur (S). Inadequacy of any of these elements can impact the health of the skeletal system (Shankar 2020). Iron (Fe) is a vital micromineral required by the body. Inadequate levels of Fe can result in malnutrition, anemia, chronic kidney disease, and inflammatory bowel disorders among human individuals (Kumar *et al.* 2022). Iron in livestock has been proven to affect immunity, antioxidants, organ development, and growth (Crilly and Plate 2022).

One of the genes suspected to correlate with mineral content is cytochrome P450, family 2, subfamily A, polypeptide 6 (CYP2A6). In cellular systems, cytochrome P450 functions as an iron and sulfur-containing ferredoxin protein. Positional and functional candidate gene by mapping a QTL for liver iron concentration to its genomic location, providing evidence of its co-regulation with iron status, and proposing a plausible biological link through oxidative stress and metabolic pathways (Munro *et al.* 2007). Aside from medications, foreign substances, such as flavonoids, can potentially engage with cytochrome P450. Flavonoids are considered vital compounds akin to vitamins, which are crucial in managing oxidative stress and serving as antioxidants. Consequently, flavonoids offer numerous advantageous impacts on health, including anti-allergy, anti-inflammatory, antioxidative, antimicrobial, anti-tumor, and anti-mutagenic effects, thereby acting to prevent conditions like cancer, heart ailments, loss of bone density, and a range of other illnesses (Bojić *et al.* 2019). CYP2A6 stands out as a high-priority candidate the influence of mineral cofactors or structural ions (iron, magnesium, zinc) on its function and stability for several key reasons. High genetic variability and clinical importance of CYP2A6 (Tanner and Tyndale 2017). The foundational role of heme (iron) and metal ions in CYP structure or function (Zhang and Wu 2015). Zhang *et al.* (2018) to support the specific hypothesis that other metal ions (Mg^{2+}, Zn^{2+}) can influence CYP folding, stability, and function, making them credible candidates for explaining variable activity in a polymorphic enzyme like CYP2A6. Previously, research related to this gene had yet to be carried out. Gunawan *et al.* (2018) performed an initial screening to establish polymorphism frequencies for several cytochrome P450 genes, including CYP2A6, in lamb, while Listyarini *et al.* (2018) examined the association and expression of the CYP2A6 gene in relation to flavor and odor compounds. Together, these studies provide a essential genotypic and phenotypic baseline. The present work is designed to bridge these datasets by investigating the specific mechanistic relationship between CYP2A6 variants and mineral metabolism, a novel pathway with implications for meat quality and nutritional science. Therefore research related to the association between the CYP2A6 gene and mineral content in sheep still needs to be completed. Thus, there is a need for studies concerning the relationship between the CYP2A6 gene and mineral content in Indonesian sheep. The objective of this study is to detect variations in the CYP2A6 gene and explore its relationship with mineral content in sheep meat.

MATERIALS AND METHODS

Sample Collection

The study involved a group of 95 Indonesian rams, consisting of 81 Javanese thin-tail sheep (JTTS) and 14 Jonggol sheep (JS) to identify polymorphism. DNA samples were gathered from the longissimus dorsi (LD) muscle of the sheep samples. All the sheep were males aged between 10 to 12 months. Their body weights fell within the range of 20 to 35 kg. The sheep were selected from farmers who maintained high standards of care, including regular cleaning of sheep manure, proper disposal of waste and left-over feed, and ensuring clean bedding, among other practices. During the study period, the sheep were fed a diet consisting of Pennisetum purpureum and concentrate. They were processed in a slaughterhouse for commercial reasons. The Animal Ethics Commission of the IPB University granted approval for all animal-related procedures (approval no. 117-2018 IPB).

Mineral Content Analysis

The analysis of mineral content in longissimus dorsi (LD) muscle is carried out using the Atomic Absorption Spectrophotometry (AAS) method. AAS involves an analytical technique employed to measure the amounts of metallic elements by observing the radiation absorption caused by unbound gas-phase atoms. The mineral content of the meat to be analyzed in this research includes potassium

(K), iron (Fe), zinc (Zn), and selenium (Se). A 2.5 g meat sample was placed into an Erlenmeyer flask, followed by adding 25 mL of concentrated HNO3 and subsequently subjected to boiling for 30-35 minutes. The solution was cooled, and 10 mL of 70-72% HClO4 was added. The solution is slowly boiled until it becomes colorless. The solution was cooled, 50 mL of H2O was added, then boiled until all NO2 gas was released. The solution was cooled and filtered into a 100 mL measuring flask. The solution was then analyzed using an Atomic Absorption Spectrophotometer (AAS) to determine the K, Fe, Zn, and Se minerals content in lamb meat.

DNA Extraction and PCR Amplification

The extraction of DNA from the Longissimus dorsi muscle samples were carried out utilizing the Geneaid gSYNC DNA Extraction Kit. The SNP g. 49170107 G>T observed in this study is consistent with the discovery made by Gunawan *et al.* (2018) in the context of the CYP2A6 gene (Gunawan *et al.* 2018). A pair of primers (Forward: 5' -CTT TCT GGT CCT CAT CTT TG- '3 and Reverse: 5' -GGT ATT GAT GAG GAA TGG TG- '3) was employed to amplify the CYP2A6 gene. The primer and PCR product and obtained, which covered a length of 286 base pairs, matched the dimensions specified in the study (Listyarini *et al.* 2018). The PCR amplification was performed using a 16 µl reaction mixture, which included 2 µl of DNA sample, 0.4 µl of forward and reverse primers, 7.5 µl of MyTaq Red Mix, and 6.1 µl of distilled water. PCR amplification was conducted using an ESCO thermal cycler, commencing with an initial denaturation step at 95 °C for 1 minute. Subsequently, 35 amplification cycles were performed, involving denaturation at 95 °C for 10 seconds, annealing at 55 °C for 15 seconds, extension at 72 °C for 10 seconds, a final extension at 72 °C for 10 seconds, and concluding with an incubation at 25 °C for 5 minutes. The resulting PCR products were then visualized through electrophoresis on a 1.5% agarose gel.

Genotype Determination Using PCR-RFLP

The genotyping process was carried out using the PCR-RFLP technique, employing the BsmAI restriction enzyme. A mixture containing 0.9 µl of distilled water, 0.7 µl of Tango buffer, and 0.4 µl of BsmAI restriction enzyme (Thermo Fisher Scientific, USA) was combined with five µl of the PCR product, followed by an incubation at 37 °C for 4 hours. The G>T substitution directly abolishes the core "GTCTC" recognition sequence for BsmAI. This differential cutting of PCR amplicons based on the allele present forms the basis for a robust. Subsequently, the PCR-RFLP results were subjected to electrophoresis on a 2.5% agarose gel. For reference, a 100 bp DNA marker was used to compare DNA fragments. The size of these fragments facilitated the identification of CYP2A6 genotypes: GG corresponded to 286 bp, GT to 286, 217, and 69 bp, and TT to 217 and 69 bp.

Data Analysis

The determination of allele frequency and genotype frequency was expounded upon following the approach outlined by Nei and Kumar (2022). To explore the genotype effects, PROC GLM techniques within SAS 9.4 software were employed to ascertain the links between the phenotype and the CYP2A6 gene polymorphism (g. 49170107 G>T). Examining the link between the CYP2A6 gene and mineral content followed the methodology outlined in the study conducted by Khasanah *et al.* (2016), employing the subsequent formula:

$$Yij = \mu + genotypei + eij \quad (1)$$

Here, Yij represents the mineral content performance of each lamb; µ denotes the mean mineral content; genotypei stands for the fixed effect associated with the i-th genotype; and eij represents the random error.

RESULTS AND DISCUSSION

Polymorphism in The CYP2A6 Gene

The CYP2A6 gene PCR, amplification of the G>T mutation, was effectively carried out utilizing primers crafted based on recommendations furnished by an accessible online tool (https://primer3.ut.ee/), and its validity was confirmed using Primer Stats (286 bp) as shown in Figure 1 of this study. Primer3 and Primer Stats are checked against important parameters, such as melting temperature (Tm), secondary structure, GC content, and amplicon length (verifying the expected product size is suitable for the intended downstream analysis (gel electrophoresis). The PCR products were subsequently subjected to PCR-RFLP amplification using the BsmAI restriction enzyme. The primers were specifically designed for the CYP2A6 gene SNP g. 49170107 G>T were utilized to perform gene amplification. PCR-RFLP analysis unveiled two CYP2A6 genotypes: GG (286 bp) and GT (286, 217, and 69 bp). The findings in this study confirm the results presented by Listyarini et al. (2018). Listyarini *et al*. (2018) study identified two genotypes, GG and GT, corresponding to G and T allele combinations for the CYP2A6 gene. The GG homozygous genotype manifests as a single 286 bp band, while the GT heterozygous genotype is characterized by three bands at 286, 217, and 69 bp, as depicted in Figure 2 of this study.

Genetic polymorphism analysis in the CYP2A6 gene (SNP g. 49170107 G>T) was analyzed as presented in Table 1. Observed genotype frequencies display variability, with the GG genotype frequency reaching 0.96 and the GT genotype frequency amounting to 0.04. Genetic polymorphism was solely evident in the JTTS populations. In this study, the population displayed conformity to the Hardy-Weinberg equilibrium, showing a value of 0.044.

The study results indicate that the G allele is the dominant allele in all sheep populations, with a frequency ranging from 98%. In contrast, the T allele's occurrence is limited, constituting around 2% of the population. This implies that the heterozygous allele only comprises 2% of the total. The rarity of specific alleles can be attributed to factors such as small population sizes, isolated breeding systems, genetic abnormalities, and non-random mating behaviors (Asmare *et al*. 2023). Conversely, the allele and

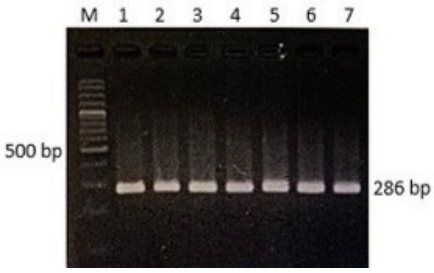

Figure 1. The outcomes of the CYP2A6 gene amplification displayed on a 1.5% agarose gel. M= 100 bp marker; 1-7= samples from sheep

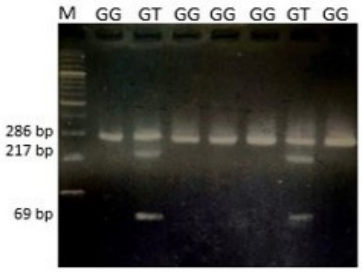

Figure 2. PCR-RFLP outcomes for the CYP2A6 gene utilizing the BsmAI enzyme, visualized on a 2.5% agarose gel. M=100 bp marker

Table 1. Genotype and Allele Frequencies of The CYP2A6 Gene and Chi-square Analysis

Sheep	N	Genotype frequency			Allele frequency		Chi-square (x^2)
		GG	GT	TT	G	T	
JTTS	81	0.95 (77)	0.05 (4)	-	0.98	0.02	0.05
JS	14	1.00 (14)	0.00 (0)	-	1	0	-
Total	95	0.96 (91)	0.04 (4)	-	0.98	0.02	0.044

N= the sample size; (..)= indicates the count of samples with GG. GT. and TT genotypes; x^2 table 0.05= 3.84

genotype frequencies 1.00 signify that the CYP2A6 gene is monomorphic in the JS populations. Moreover, the utilization of the Hardy-Weinberg equilibrium presupposes the absence of influences stemming from natural selection, migration, mutation, and consistent genetic fluctuations from one generation to the next within the population.

CYP2A6 Gene Association with Mineral Content

A significant association (P<0.05) between the CYP2A6 gene and mineral content, particularly iron (Fe), has been identified. The highest Fe content was found in sheep with the GT genotype. Sheep have the GT genotype display elevated Fe levels compared to those with the GG genotype (Table 2). Minerals consist of macro minerals (Na, Ca, P, K, S, Mg) and micro minerals (Zn, I, Fe, Mn, Cu, Cr, Se, F, Co). The average daily requirement for macro minerals in humans is more than 100 mg/day (Tangkilisan *et al.* 2021). The World Health Organization recommends that adults consume a minimum of 3510 mg of potassium daily (WHO 2012). As for micro minerals, less than 100 mg/day is required, with varying daily needs for each micro mineral (Tangkilisan *et al.* 2021). Commonly, zinc (Zn) consumption by humans intake within the range of 14 to 30 mg/kg (Roohani *et al.* 2013). The European Food Safety Authority (EFSA) suggests a daily selenium (Se) intake of 55 g (Kieliszek *et al.* 2022). Excessive selenium consumption can lead to selenosis, a harmful state where the body becomes excessively exposed to selenium (Stoffaneller and Morse 2015).

Table 2. Genotype and Association of The Candidate Gene's Link with Mineral Content

Parameters (mg/100 g)	Genotype of CYP2A6 ($\bar{x}$ ± SE Mean)	
	GG (91)	GT (4)
Fe	17.67 ± 0.62b	26.37 ± 6.72a
Zn	25.19 ± 0.79	27.84 ± 2.10
K	2743.56 ± 89.90	2663.08 ± 539.00
Se	6.20 ± 0.32	6.89 ± 1.19

a and b exhibit significant differences at the P<0.05 level; SE is Standard Error Mean

Iron deficiency is linked to decreased physical work capacity, diminished mood and cognitive function, and adverse pregnancy outcomes. Consequently, individuals with iron deficiency are more susceptible to developing iron deficiency anemia (Beck *et al.* 2014). The role of iron in regulating the immune system is notably intricate, involving various mechanisms that highlight the balance between its function and the advantages of maintaining healthy iron levels (Weyh *et al.* 2022). For example, iron deficiency has been found to hinder B-cell growth, T-lymphocyte activity, and adaptive antibody responses, all of which seem to be effectively improved with iron supplementation (Jiang *et al.* 2019). The regulation of iron balance primarily occurs through its absorption in the intestines, given that iron lacks an active elimination mechanism in the human body. The interactions between nutrients might contribute to the absorption of dietary iron in the intestines. Compounds found in foods, such as calcium, phytate, polyphenols, and substances like ascorbic acid and protein, predominantly impact iron availability (Priskin *et al*. 2022).

The structure of CYP enzymes includes a hemoprotein component, consisting of around 400-500 amino acids and a single heme prosthetic group that plays a crucial role in the active site (Zhao *et al.* 2021). The proximity to the heme is directly correlated with structural conservation, particularly in helices I and L, which are connected to the heme. The most conserved features of CYPs are centered around the heme-thiolate oxygen activation chemistry, including the β-bulge segment that houses the Cys ligand. Another highly conserved region involved in O2 activation is the portion of helix I near the heme. A notable structural characteristic of

CYPs is their ability to accommodate substrates of varying sizes and shapes. Our understanding of CYP-substrate interactions is largely derived from highly specific CYPs that bind tightly to their respective substrates. The size and shape of CYP substrates are relatively diverse. Substrates typically enter the active site near the junction of the F and G helices, which serves as the primary entry point for substrates in many CYPs. Structural changes in regions including the F and G helices may be responsible for the requirement for substrate specificity (Cryle and Schlicting 2008). According to Lee *et al*. (2011), three CYP2A6, two CYP4A11, and two CYP2D6 variants showed expression in COS-7 cells, but with distinct differences in expression levels. The reasons for these differences in expression between P450 gene alleles in COS-7 cells are unclear. However, it is possible that variations in mRNA stability, resulting from a single base change, may contribute to these differences. Similar findings have been reported for CYP1B1 variants. Another possibility is that differences in protein stability may also contribute to these variations, as a single amino acid change can impact the stability of P450 gene allele proteins.

The identification of the rare CYP2A6 T allele as a candidate marker for enhanced meat iron content holds scientific promise, its low allele frequency limits direct utility in breeding; therefore, strategies such as Genomic Selection to capture polygenic architecture, targeted introgression, or expanded population screening are recommended, with the finding ultimately prioritizing the CYP2A6 pathway for further functional validation to inform genetic improvement of nutritional traits. The outcomes from this genetic association study involving the CYP2A6 gene and iron content in sheep can be a genetic marker for selecting mineral content, particularly in sheep carrying the GT genotype. For further research, a more in-depth investigation based on this study is needed, involving a larger sample size and diverse types of sheep.

CONCLUSION

The CYP2A6 gene displayed polymorphism within JTTS but remained monomorphic within JS sheep. The dominant genotype in Indonesian sheep continued to be GG. There was an association between iron (Fe) and the variation in the CYP2A6 gene. Specifically, the GT genotype within the CYP2A6 gene showed statistical significance ($P<0.05$) about elevated iron (Fe) levels. Considering the noteworthy mineral content disparity among Indonesian sheep, the CYP2A6 gene holds promise as a prospective genetic marker for sheep breeding selection.

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