Association of Cytochrome P450 2A6 (CYP2A6) Gene Polymorphisms with Fatty Acid Traits in Indonesian Native Sheep

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ABSTRACT

A member of the cytochrome P450 superfamily enzyme that play a role in microsomal fatty acids oxidation process encoded by cytochrome P450 2A6 (CYP2A6). This study aimed to examined the CYP2A6 gene polymorphisms and their association on fatty acid traits in Indonesian native sheep. A total of 299 rams aged 10-12 months with body weights between 20-30 kg used to identify the CYP2A6 gene polymorphism are consisted of 36 samples of barbados cross sheep (BCS), 35 samples of compass agninak sheep (CAS), 20 samples of javanese fat tailed sheep (JFT), 36 javanese thin tailed sheep (JTT), 20 samples of garut sheep (GS), 45 samples of garut composite sheep (GCS), meanwhile for the fatty acid analysis was carried out by using 107 of loin samples from the total sheep. Identification of the CYP2A6 gene polymorphisms were performed using PCR-RFLP (Polymorphism Chain Reaction-Restriction Fragment Length Polymorphism) with the BSmAI restriction enzymes. The amplification product was 286 bp. Polymorphism were found in JFT, JTT, GCS and JS with GG and GT genotypes, while BCS, CAS, and GS were monomorphic with TT genotype. The CYP2A6 BSmAI polymorphism was in Hardy-Weinberg Equilibrium for JFT, JTT and JS, while BCS, CAS, GS, GCS, and combined was deviated based on chi square. A SNP g.49170107 G>T of the CYP2A6 gene polymorphism was significantly associated (P <0.05) with only erucic acid (C22:1r9). The GT genotypes had a higher value than GG genotypes The CYP2A6 gene could be used as a selection marker to improve fatty acid traits in Indonesian native sheep.

Keywords: CYP2A6 gene, Fatty acid, PCR-RFLP, Sheep

Introduction

Sheep is one of the potential livestock to fulfill animal protein consumption. Lamb meat production in 2018 was 48,674 tons or only contributed 1.35% of the total national meat production in Indonesia (DPKH, 2018). Apart from the low national lamb meat production, the national lamb meat consumption rate is still low compared to other meats. The price of sheep which is relatively more expensive than other animal protein sources is one of the factors contributing to the low consumption of Indonesia. Gunawan et al. (2018a) reported that, apart from price, there is an assumption in Indonesian society that lamb meat contains cholesterol and high saturated fatty acid (SFA) content, the odor of meat which is less desirable and difficult to remove are factors that are thought to cause low national consumption of lamb meat. Consumption of SFA, and cholesterol correlates with an increase low density lipoprotein (LDL) in blood. High LDL content can trigger atherosclerosis which is the beginning of the onset of coronary heart disease (Ma’rufi and Rosita, 2014). A genomic selection-based on breeding program to produce lamb meat with high unsaturated fatty acid and low SFA content are potential to be implemented. Fatty acids composition is a quantitative trait that influenced by genetic factors and controlled by more than one gene (Gunawan et al., 2018a). Fatty acids trait in sheep has a heritability value ranging from 0.25-0.46 which indicates that increase in genetic quality through an effective selection program to be carried out (Rovadoscki et al., 2018).

Several genes were reported to be significantly associated with fatty acid content in sheep including APOA5 (Gunawan et al., 2018b), AHSG (Munyaneza et al., 2019a), BHMT (Munyaneza et al., 2019b), DGAT1 (Gunawan et al., 2019), and KIF12 (Gunawan et al., 2018a). Cytochrome P450 2A6 (CYP2A6) are genes thought to contribute in the fatty acid traits in sheep. The CYP2A6 gene is located on chromosome 14 in sheep and mainly expressed in the endoplasmic reticulum of the liver and olfactory mucosa (Listyarini et al., 2018; Kirby et
al., 2011). The CYP2A6 gene encodes a member of cytochrome P450 superfamily enzymes that was found to play a role in the microsomal monooxygenase system as the terminal oxidase and contribute to metabolizes several important substrates such as biosynthesis of cholesterol, convert cholesterol to bile acids, forming steroid hormones, metabolize vitamin D3 to the active 1,25-dihydroxyvitamin D3, fatty acids metabolism, and biotransformation of exogenous xenobiotics (McDonagh et al., 2012; Cederbaum, 2015; Wang et al., 2017). The CYP2A6 gene found known to be associated with class 1 obesity in humans (Wang et al., 2019), had a significant effect (P<0.05) to the off odor and flavor in sheep (Listyarini et al., 2018) and associated with C20:3n3 fatty acid (cis11,14,17-Eicosatrienoic acid) in Korean pigs (Roh et al., 2011). There was no study investigated CYP2A6 gene polymorphism effects on Indonesian native sheep fatty acid traits. Therefore, this study is needed to examined the CYP2A6 gene polymorphisms and their association on fatty acid traits in Indonesian native sheep.

Materials and Methods

Animals and samples
Identification of CYP2A6 gene polymorphisms sample were obtained from blood and liver of 299 rams from seven breeds, while fatty acid analysis were obtained from loin sample of 110 rams (Figure 1 and Table 1). All sheep had age between 10-12 months old, body weights between 20-30 kg, and slaughtered in a commercial abattoir. The blood and liver sample were taken 100 µl for identification of CYP2A6 gene polymorphisms and the loin samples were taken 100 mg for fatty acids analysis. The samples were put in an ice flask and stored at a temperature of -20°C.

Fatty acid analysis
A total of 100 mg loin sample were used for fatty acid analysis. The fatty acid analysis was conducted according to the protocol of Association of Official Analytical Chemists (AOAC, 2012). These measurements including fat content, saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA) and fatty acid total.

DNA isolation and PCR-RFLP amplification
The DNA isolation refers to phenol-chloroform standard (Sambrook and Russel, 2001). Previous research from Listyarini et al. (2018) was described a single nucleotide polymorphism (SNP) of CYP2A6 g.49170107 (G>T) gene that used in this study. A pair of primers used for amplification of the 286 bp (base pair) target DNA refers to Listyarini et al. (2018) were F: 5'-CTT TCT GGT CCT CAT CTT TG-3' and R: 5'-GTT ATT GAT GAG GAA TGG TG-3'. DNA amplification was initiated by the pre-denaturation stage for 1 minute at a temperature of 95°C. Then, there is 35 cycles consisted of 15 seconds at 95°C denaturation, 15 seconds at 55°C primary annealing, 10 seconds at 72°C DNA extension and 1 minute at 72°C final primer elongation. The PCR product then visualized by electrophoresis on 1.5% agarose gel. The PCR product then digested using the RFLP technique using BsmAI enzyme. The digested product then visualized on 2% agarose gel using UV Transilluminator.

Statistical analysis
Gene frequency including genotype and allele were calculated according to the populations of BCS, CAS, JFT, JTT, GS, GCS and JS genotyping data by the formula of Nei and Kumar (2000). Genotype frequency:

\[ X_{ii} = \frac{n_{ii}}{N} \]

Allele frequencies:

\[ X_i = \frac{2(n_{i} + \sum_{|i|} n_{ij})}{2N} \]

Description:
- \( X_{ii} \) = frequency of ii genotype,
- \( X_i \) = frequency of i allele,
- \( n_{ii} \) = number of the ii-genotype sample,
- \( n_{ij} \) = number of the ij-genotype sample,
- \( N \) = total samples.

Hardy-Weinberg equilibrium (Nei and Kumar, 2000):

\[ X^2 = \sum (o-e)^2 / e \]

Description:
- \( X^2 \) = chi squared,
- \( O \) = total of observations genotype
- \( E \) = total of genotype to expectations.

Association analysis
The CYP2A6 gene polymorphism association with fatty acid traits was conducted according to the GLM procedure (SAS Inst., Inc., Cary, NC). The mathematics model was:

\[ Y_{ij} = \mu + G + E_{ij} \]

Description:
- \( Y_{ij} \) = dependent variable for trait measured in the population,
- \( \mu \) = populations mean for traits,
- \( G \) = genotype fixed effects,
- \( E \) = random error.

Differences was considered statically significant if p-value <0.05. Pair wise differences between the effects of genotype was tested by performing Duncan Multiple Range Test (DMRT).

Results and Discussion

CYP2A6 gene polymorphism in Indonesian native sheep
The CYP2A6 gene (g.49170107 G>T) was amplified with product length 286 bp (Figure 2). The polymorphism of the CYP2A6 gene (Figure 3) were detected and define as GG (286 bp), GT (286 bp, 217 bp, and 69 bp) and TT (217 bp, and 69 bp) which are a combination of T and G alleles.
The genotype frequencies of GG and GT were 0.900 and 0.100 for JFT, 0.889 and 0.111 for JTT, and 0.991 and 0.009 for JS, respectively. The GG and TT genotype frequencies were 0.978 and 0.022 for GCS, respectively. Samples of BCS, CAS, and GS only has GG genotype. Total combined sample showed that GG genotype was the most frequent (97.3%) followed by GT (2.4%) and TT (0.3%). Statistical result of the CYP2A6 gene analysis results is presented in Table 2. The G allele was dominant in all sheep breeds. Gene polymorphism only occurred in JFT, JTT, GCS, and JS while BCS, CAS, and GS did not vary because the values of genotype frequency and allele frequency were equal to 1.00. The CYP2A6 BSmiI polymorphism is in Hardy-Weinberg Equilibrium for JFT, JTT and JS, while BCS, CAS, GS, GCS and combined is deviated. Polymorphisms of genotyped in this study more polymorphic than Listyarini et al. (2018) that found two genotypes in the CYP2A6 gene that is GT and TT genotypes. Listyarini et al. (2018) found TT genotypes as dominant genotypes than others in JFT sheep. With a larger population sample compared to Listyarini et al. (2018), we found the GG genotypes were common in seven breed of Indonesian native sheep. Beside CYP2A6 gene, there are other families cytochrome that also associated with fatty acid in sheep. Study conducted by Harahap et al. (2020) reported that CYP2E1 gene was found three genotypes in Indonesian native sheep is GG, GT, and TT. The mutation in the genotype of guanine to thymine in the CYP2A6 gene is categorized a translation mutation.

![Figure 1. Phenotypic of Indonesian native sheep.](image)

![Table 1. The samples of study](table)

| Samples       | N   | DNA extraction sample | Fatty acid analysis sample |
|---------------|-----|-----------------------|---------------------------|
| BCS           | 36  | 36                    | 10                        |
| CAS           | 35  | 35                    | 10                        |
| JFT           | 20  | 20                    | 20                        |
| JTT           | 36  | 36                    | 33                        |
| GS            | 20  | 20                    | -                         |
| GCS           | 45  | 45                    | 10                        |
| Jonggol sheep (JS) | 107 | 107                  | 27                        |
| **Totals**    | 299 | 299                   | 110                       |

N= number of samples
Association of CYP2A6 gene polymorphism and fatty acid traits

The SNP of CYP2A6 (g.49170107 G>T) gene study showed that there is association between CYP2A6 gene polymorphism with fatty acids traits in Indonesian sheep. The CYP2A6 polymorphism is associated (P<0.05) with monounsaturated fatty acid (MUFA), especially erucic acid (C22:1n9). Sheep with the GT genotype contained higher erucic acid (C22:1n9) than the GG genotype. The effect of the CYP2A6 genotype on fatty acid traits of Indonesian sheep is presented in Table 3. Cis-13-docosenoic acid or 22:1Δ13 was commonly known as erucic acid. Erucic acid is an unbranched long chain monounsaturated fatty acid that formed naturally in the body and metabolized to oleic acid. It contains 22 carbons and a cis-configurated double bond on C-13. Erucic acid also a member of omega 9 fatty acid. Long chain monounsaturated fatty acid is thought to have cardiotoxic effect as the study reported that there is association between high circulating of erucic acid content in the body with congestive heart failure (Imamura et al., 2013). High content erucic acid food consumption has negative impact on human health as they classified as natural toxicant (Abbott et al., 2003). Begin with myocardial lipidosis (accumulation of lipid in the heart muscle fibers) as the effect of poor mitochondrial beta-oxidation of erucic acid in the heart, high accumulation of triacylglycerol will lead into reducing heart contractility before finally damage the tissue (Bremer and Norum, 1982). European Food Safety Authority (EFSA) recommends erucic acid Tolerable Daily Intake (TDI) are 7 mg per kg human body weight per day (EFSA, 2016). Overconsumption of SFA, trans MUFA, and
cholesterol and has an overweight body (obesity) prone to fall on cardiovascular disease, atherosclerosis, and other diseases (USDA, 2010). This research result indicated that the CYP2A6 gene might plays important role in fatty acid metabolism. The family of cytochrome known as a gene that plays a role in meat quality, such us flavour and odour (Harahap et al., 2020; Listyarini et al., 2018; and Gunawan et al., 2013), pH, meat tenderness, and fatty acid composition (Harahap, 2021). Besides that, constitutive regulation of CYP2A6 (CYP2A5 in mice) expression in the liver was influenced by hepatic nuclear factor 4 alpha (HNF-4a) (Ulvila et al., 2004). HNF-4 transcription factors contribute in the activation genes involved in fatty acid oxidation (Chen et al., 2020). Genes contribute to lipid metabolism and transport such as ApoA-I (Malik and Karathanasis, 1996), ApoA-II (Ribeiro et al., 1999), ApoA-IV (Kistitaki et al., 1994), ApoE (Dang et al., 1995), MCAD (Carter et al., 1994), microsomal triglyceride transfer protein (MTP) (Hagan et al., 1994), and cholesterol 7a-hydroxylase (CYP7A1) (Stroup and Chiang, 2000) were reported to be activated by HNF-4a. The disappearance of HNF-4a expression led to the accumulation of lipid in the liver, decrease serum cholesterol and triglyceride level, and excess concentration of serum bile acid (Hayhurst et al., 2001).

Conclusions

The CYP2A6 gene was polymorphic in JFT, JTT, GCS, and JS, but monomorphic in BCS, CAS, and GS. The analysis showed that the CYP2A6 gene was significantly associated with erucic acid. The research indicates that SNP g.49170107 G>T of CYP2A6 gene potential to be used as a genetic marker for selection of fatty acid traits in sheep quality.

Acknowledgments

This work was financially supported by PTUPT project from Indonesian Ministry of Research, Technology and Higher Education Fiscal Year 2021 Number: 1/E1/KP.PTNBH/2021 dated 08 March 2021.

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