Effect of CYP2E1 gene polymorphisms on lamb odor and flavor in Indonesian sheep

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Abstract. The CYP2E1 gene (Cytochrome P450 2E1) plays an important role in the regulation of skatole and androstenone in liver tissue. The aim of this study was to investigate of the effect of CYP2E1 gene polymorphisms on lamb odor and flavour in Indonesian sheep. A total sample of 100 rams consisting of 20 Javanese fat-tailed (JFT), 20 Javanese thin-tailed (JTT), 20 compass Agrinak sheep (CAS), 20 Barbados cross sheep (BCS), and 20 Garut composite sheep (GCS) aged 10–12 months old were used. Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) was performed to identify the polymorphism of CYP2E1 genes. The association of the CYP2E1 genotypes with lamb odor and flavour were performed using T-test. The result showed that there were polymorphisms in CYP2E1 gene indicated by three genotypes namely GG (401 bp), GT (138, 263, and 401 bp), and TT (138 and 263 bp). Gene frequency of CYP2E1 (g.50657948 T>G) was in Hardy-Weinberg Equilibrium (HWE). The GT genotype was common in the population studied. Association of the CYP2E1 genotypes with lamb odor and flavour revealed a significant association (P<0.05) with the skatole (MI). The GG genotype had the highest skatole when compared to other genotypes. The SNP g.50657948 T>G of CYP2E1 gene might be a useful candidate marker for selecting sheep meat with desirable odor and flavour.

1. Introduction
Lamb usually has an undesirable odor and flavour that effect consumers choice to consume it. Lamb odor and flavour are influenced by several factors such as breed [1], age and nutrition [2], fatness [3], sex [4], different feeding systems [5], and chemical compounds [6,7]. The chemical compound of flavour is very complex and depends of interaction between volatile (aroma) and non-volatile (taste) compounds. A number of studies have been carried out to identify and define key volatile compounds associated with the flavour in cooked lamb [8,9,10]. The lamb odor and flavour are determined by two chemical compounds consisted of branched chain fatty acid and skatole (BCFA). The BCFA were consisted of 4-methyloctanoic acid (4-MeO) and 4-methylnonanoic acid (4-MeN), which dominated by fatty acids stearic and oleic were esterified in body fats, but released to an extent as free acids on cooking and thus contributing to flavour [6,7,11]. The skatole (3-methylindole) is a fecal-smelling decarboxylation product of dietary tryptophan and it is widely reported to be associated with boar taint [12–14]. Non-metabolized skatole is accumulated mainly in adipose tissue and to a lesser extent, also in muscle tissue [13,15,16]. Threshold average acceptance values is 0.2–0.25 μg/g fat for skatole [17].
Breeding is recommended as one of the most realistic approaches for reducing the levels of lamb odor and flavour. The molecular selection is likely the most effectively implemented for breeding programs [18]. The heritabilities of skatole level ranged of 0.41–0.55 indicates that the traits could be improved by doing selection [19,20]. From the result of our previous RNA seq analysis in liver tissue, the CYP2E1 genes showed higher expression level in the meat.

The CYP2E1 gene (Cytochrome P450 2E1) in sheep is located on chromosome 22. This gene plays an important role in the regulation of skatole and androstenedione in liver tissue in boar taint. Skatole is produced by bacterial fermentation of the amino acid L-tryptophan in the colon and is metabolized by liver enzymes which are responsible for oxidation and sulfation [16]. The high level of CYP2E1 gene expression in the protein causes low levels of skatole in liver tissue. SNP g.2414 C>T in the CYP2E1 gene promoter section has a significant effect on skatole of boar taint [14]. However, there was no study investigating the association CYP2E1 with odor and flavor in lamb. The other study reported an other Cytochrome family namely CYP2A6 gene was associated with odor and flavour traits in javanese fat-tailed [21]. Based on these findings, the present study was aimed to investigate the effect of CYP2E1 gene polymorphisms on lamb odor and flavour in Indonesian sheep.

2. Material and method

2.1. Animals

A total 100 of rams that consisted of 20 Javanese fat-tailed (JFT), 20 Javanese thin-tailed (JTT), 20 compass Agrinak sheep (CAS), 20 Barbados cross sheep (BCS), and 20 Garut composite sheep (GCS) were used to investigated the polymorphism of the CYP2E1 genes. Local sheep were taken from Mitra Tani Farm, Ciampea, Bogor (Latitude/Longitude: 6º 35’ 06” S / 106º 42’ 23” E) were weaned and were given ad libitum fattening feed. A total 24 of rams consisted JFT (n=10) and JTT (n=14) with weight body between 25 to 30 kg and age between 10 to 12 months were slaughtered in a commercial abattoir for association study. Samples for association study with lamb odor and flavour were taken from loin muscle and liver tissues. The loin was taken as much as 500 g for odor and flavour analyses and 30 mg of loin for DNA extraction. The liver was taken as much as 30 mg for RNA extraction. All samples were put in an ice flask and were stored at a temperature of -20 °C prior to use.

2.2. DNA extraction and PCR-RFLP amplification

Genomic DNA was extracted from blood samples based on Sambrook and Russel protocols [22]. The extracted DNA sample was amplified by adding 1 μL of DNA to a 0.2 μL tube and adding 14 μL of premix solution consisting of 0.4 μL of forward and reverse primer mixture, 6.1 μL deionized water (DW), 7.5 μL My Red Taq. The CYP2E1 gene fragments amplification was conducted using the GeneAmp PCR system AB. Primers for the amplification were designed by using MEGA 7.0 (Table 1). The PCR process started with the pre denaturation step at 95°C for 60 s. Second step consisted of 35 cycles of denaturation step at 95 °C for 15 s, anneling step at 59°C for 15 s, and DNA extension at 72°C for 15 s. The final step was the primer extension at 72°C for 10 min. PCR-RFLP was used for genotyping SNP of CYP2E1 by NlaIII restriction enzymes with CATG cutting site. A total of 5 μL of amplicon was added to the mix consisting of 0.9 μL DW, 0.7 μL of tango buffer, and 0.4 μL of NlaIII enzyme restriction. The PCR-RFLP samples were incubated at 37 °C for 4 hours. The fragments were run using electrophoresis technique using 2% agarose 30 ml 0.5X TBE, 1 μL DNA/RNA dye peqGREEN, and calibrated with 100 bp ladder. The electrophoresis chamber was run on a 100 volt power supply for 37 minutes and then visualized by thermocycler UV transilluminator (Alpha Imager, Alpha Innotech, Santa Clara, USA). The primer sequences include the products of PCR-RFLP of the CYP2E1 genes is presented in table 1.
Table 1. Primer sequences and PCR-RFLP product.

| Gene (SNP) | Accession number | Primer | TM (°C) | Enzyme restriction | Digested fragments length (bp) |
|------------|------------------|--------|---------|--------------------|-------------------------------|
| CYP2E1     | NC_019479.2      | >F: 5'-CCC AGT CAT CAG AGT CAG TA -3 | 59       | NlaIII             | GG: 401                       |
|            |                  | >R: 5'-GCA TAC AGT GGT TTT CCT GG -3 |          | (CATG)             | GT: 138, 263, and 401         |

2.3. Lamb odor and flavour analysis

The phenotype of lamb odor and flavour for association study were refer to Listyarini et al. (7). A total 500 g of loin sample were carried out for odor and flavour analysis. The volatile odor and flavour compounds were extracted using LikensNicerson method which is a combination of distillation and extraction with solvent simultaneously using Gas Chromatography Mass Spectrometry (GC-MS) tool. The parameters measured were MP, MOA, MNA, MI or skatole, and EOA. The level of BCFA (MNA and MP) in fat lamb that greater than 215 μg/g were catagorized as low lamb odor and less than 229 μg/g were catagorized as high lamb odor [12]. While skatole level for the flavour less than 0.25 μg/g were categorized as low skatole and greater than 0.25 μg/g were catagorized as high skatole of fat lamb meat [23,24].

2.4. Data analysis

2.4.1. Statistical analysis for genotype and allele frequencies. The allele, genotype frequencies, and Hardy–Weinberg equilibrium in populations of sheep were calculated by the following formula:

1. Genotype and allele frequencies [25]:

   \[
   X_{ii} = \frac{n_{ii}}{N} \\
   X_i = \frac{2n_{ii} + \sum n_{ij}}{2N}
   \]

   Where :
   - \( X_{ii} \) = the frequency of \( ii \) genotype \( i \)
   - \( X_i \) = the frequency of \( i \) allele;
   - \( N_{ii} \) = the number of the sample of \( ij \) genotype;
   - \( N_{ij} \) = the number of the sample of \( ij \) genotype;
   - \( N \) = the population size.

2. Hardy weinberg equilibrium (HWE) [26]:

   \[
   \chi^2 = \sum [(O - E)^2 / E]
   \]

   where :
   - \( \chi^2 \) = chi-square value;
   - \( O \) = the observed values of \( i \) genotype;
   - \( E \) = The expected values fo the \( i \) genotype.

2.4.2. Statistical analysis for associsiation of CYP2E1 genes related to lamb odor and flavour. The association between the SNP of the CYP2E1 gene and lamb odor and flavour were performed using the following T-test :

   \[
   t = \frac{(X_1 - X_2)}{\sqrt{\frac{\delta^2}{n_1} + \frac{\delta^2}{n_2}}}
   \]

   Where :
   - \( X_1 \) and \( X_2 \) = the average of genotype 1 and 2
   - \( n_1 \) and \( n_2 \) = a total of genotypes individual 1 and 2
   - \( \delta^2 \) = variants
3. Results and discussion

3.1. The allele and genotypes of a SNP of the CYP2E1 gene

The PCR amplification of CYP2E1 produces a genotype called GG with product length 401 base pair (Figure 1). There is a mutation in Thymine (T) to Guanine (G) at SNP g.50657948 in the CYP2E1 genes. The electrophoresis of PCR-RFLP showed that there were polymorphisms in CYP2E1 gene indicated by three genotypes namely GG (401 bp), GT (138, 263, and 401 bp), and TT (138 and 263 bp) (Figure 2).

![Figure 1. The amplicon for primer sequences of the CYP2E1 gene.](image)

![Figure 2. The polymorphism of genotypes in the CYP2E1 gene.](image)

Overall, about 50 the tested sheep were genotyped as heterozygous (GT). The allele frequencies of thymine was common in the population studied. The distributions of genotype in the CYP2E1 genes was in Hardy-Weinberg Equilibrium (HWE). Polymorphisms of genotyped in the CYP2E1 gene in this study different from the result found by Listyarini et al. [7]. They found that the family of cytochrome P450 (the CYP2A6 genes) had two genotypes in Indonesian sheep with TT as dominant genotypes while GG genotypes was not found. The genotype frequency, allele frequency, and Hardy Weinberg Equilibrium of CYP2E1 (g.50657948 T>G) polymorphism of CYP2E1 gene in Indonesia sheep are showed in table 2.
Table 2. The number of animals per genotype and allele frequency of CYP2E1.

| Polymorphism | n  | Genotype frequency | Allele frequency | $\chi^2$ |
|--------------|----|--------------------|------------------|----------|
| CYP2E1       | 100| 0.23 (23)          | 0.50 (50)        | 0.27 (27) | 0.48 | 0.52 | 0.00 |

Note: n= number of samples, (..) = number of sample which GG, GT, TT genotype, $\chi^2$ table = 3.84.

3.2. Effect of CYP2E1 gene polymorphisms on lamb odor and flavour

The CYP2E1 gene (g.50657948 T>G) was associated with MI or skatole. Association analysis of SNP in the CYP2E1 gene with lamb odor and flavour is presented in Table 3. MI is one of the major contributors to boar taint and flavour and odor [27,7]. The lower skatole values were determined in genotype GT whereas GG has the higher levels of skatole. The skatole levels in this study were ranged from 0.128–0.870 μg/g. Similarly, Listyarini et al. [7] has reported the CYP2A6 gene was associated with the skatole levels and showed the skatole values of each genotypes ranged from 0.215–0.825 μg/g. The association of the CYP2E1 gene with skatole levels has been widely reported in boar taint [14,16,28,29]. Morlein et al. [14] was found TT genotypes had low skatole levels in backfat of boars. Conversely, Zadinova et al. [13] found that SNPs in the CYP2E1 genes were similar in the skatole levels in different genotypes in the boar taint. Level of skatole found by Neuhoff et al. [30] were in the range of 4.63–5.13 μg/g.

Table 3. Genotype and association analysis of CYP2E1 genes with odor and flavour compounds.

| Flavour and odor compound (μg/g) | Genotype (μ ± Std Dev) | GG (n=3) | GT (n=8) | TT (n=13) |
|---------------------------------|------------------------|---------|---------|---------|
| MOA                             |                        | 0.037 ± 0.006 | 0.099 ± 0.126 | 0.195 ± 0.442 |
| MNA                             |                        | 0.530 ± 0.579 | 0.416 ± 0.399 | 0.325 ± 0.772 |
| MI                              |                        | 0.870 ± 0.465 | 0.128 ± 0.070 | 0.212 ± 0.212 |
| MP                              |                        | 30.607 ± 15.642 | 24.961 ± 15.093 | 20.840 ± 14.494 |
| EOA                             |                        | 0.203 ± 0.352 | 0.531 ± 0.625 | 0.303 ± 0.313 |

Note: n= Number of samples; MOA= 4-methyloctanoic; MNA= 4-methylnonanoic; MI= 3-methylindeole; MP= 4-methylphenol; EOAt= 4-ethyloctanoic.

The CYP2E1 gene is the main enzyme involved in phase I of skatole and indole metabolism [30]. The activity of CYP2E1 in the liver significantly influences skatole concentrations in fat. Skatole and indole are reported to be strongly correlated in fat 0.70 of pig [31]. In mature uncastrated male pigs, MI causes a problem of boar taint, where high levels of the compound present in the adipose tissue release an unpleasant odor when the meat of boars is heated. The skatole levels in the fat of boars increase during puberty and were correlated with the fat androstenone levels [32]. Consequently, male pigs were routinely castrated for meat production. Down-regulated expression of CYP2E1 was regarded as a major factor in skatole accumulation [33]. Previous study using sheeps conducted by Listyarini et al. [7] indicated that high expression of the cytochrome P450 family (CYP2A6 genes) occurred in the liver had low levels of skatole in fat.

Skatole concentrations in adipose tissue result from a complex process, which includes the availability of tryptophan and the presence of specialized bacteria in the gut in need of tryptophan for energy production, as well as absorption, transport and accumulation of skatole in adipose tissue. The concentrations of skatole in portal blood, peripheral blood and feces is highly correlated within individuals, suggesting that the amount of skatole absorbed is proportional to the amount produced [34,35]. Skatole and indole are transported by the portal vein (V. porta) to the liver, where most of the indole derivatives are metabolized by specific enzymes. A small amount of indoles, which is absorbed in the distal colon or rectum, it can pass through the liver and be transferred via the vena cava caudalis.
directly into the peripheral bloodstream [15,34]. The liver metabolism was highly effective and skatole concentrations of the portal vein may be reduced severely in the liver (up to 90%) [36,37]. In general the hepatic degradation of indoles can be divided in two distinct steps: an oxidative step, phase 1 metabolism, and a conjugative step, phase 2 metabolism. Responsible enzymes are various cytochrome P450 isozymes, which are known to play a predominant role in drug and xenobiotic metabolism [38].

Two specific enzymes were CYP2E1 and CYP2A identified as major enzymes of the phase 1 metabolism of skatole [39,40]. Gunawan et al. (29) have identified several polymorphisms in cytochrome family CYP2A5, CYP4A24, and CYP4B24 but a variant in CYP4A25 (ANCg.152197351) failed to be associated with skatole level. Selection for the CYP2E1 genes to reduce of skatole levels could reduce lamb odor and flavour because the skatole is more volatile and has low detection and rejection thresholds [14]. MI is a well known acute pneumotoxin for cattle and it has important implications for pork production. Uncastrated boar are usually used for meat production in several countries, due to a better feed conversion, improved carcass leanness, and a better composition of fatty acids compared to castrated pigs [27]. The SNP g.50657948 T>G of CYP2E1 that associated with the skatole compounds indicate that this gene has an important roles for reducing the unwanted lamb odor and flavour.

4. Conclusion
The SNP g.50657948 T>G of CYP2E1 was polymorphic in Indonesian sheep. The polymorphism showed by three genotypes i.e. GG, GT, and TT. The GT genotype was the most common in the population. Polymorphisms of CYP2E1 genes associated (P<0.05) with skatole or MI (3-methylnindole) where the GT genotype had the lowest skatole level. While the highest skatole level found at the GG genotype. The SNP g.50657948 T>G of CYP2E1 gene might be a useful candidate marker for selecting sheep meat with desirable odor and flavour.

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