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Association and Polymorphism of the APOA5 Gene Related to Carcass Characteristics and Lamb Quality in Sheep

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ABSTRACT

The apolipoprotein A-V (APOA5) gene is a candidate gene that plays an important role in carcass characteristics and lamb quality. This study aimed to association and polymorphism of the APOA5 gene with carcass characteristics and lamb quality in sheep. The breed of sheep were used 100 sheep consisting of Thin Tailed Sheep (TTS) (n=55), Compass Agrinac Sheep (CAS) (n=10), Garut Sheep (GS) (n=18), Garut Composite Sheep (GCS) (n=8), and Barbados cross sheep (BCS) (n=9). Identification of APOA5 gene polymorphism was performed using PCR-RFLP method and association study was performed using GLM analysis. The results showed that the APOA5 (g.26929941 C>T) gene polymorphism was polymorphic in TTS, CAS, GS, and GCS, while BCS was monomorphic. The APOA5 gene has three genotypes (CC, CT, and TT). Association showed that the APOA5 was a significantly ($P<0.05$) associated with carcass percentage and carcass length. The polymorphism of the APOA5 (g.26929941 C>T) was also significant ($P<0.05$) associated with lamb quality of the pH dan tenderness. The TT genotype showed greater carcass length and pH than the CC and CT genotypes. The CT genotype showed a greater percentage of carcass and tenderness than the CC and TT genotypes. The APOA5 gene helps improve sheep carcass characteristics and lamb quality.

Keywords: APOA5 gene, Carcass Characteristics, Lamb Quality, PCR-RFLP, Sheep

Introduction

Sheep are ruminants meat producing that have the potential to be developed in Indonesia for akikah, animal protein, traditional food such as sate, and others. The fulfillment of animal protein can come from lamb (Sunando *et al*., 2016). The Central Statistics Agency reported that sheep meat production in 2018-2020 was 82,274.30 tons, 70,072.93 tons, and 66,943.34 tons (BPS, 2022). The national demand for lamb meat has not been fullfilled, so Indonesia is still importing, which results in a high selling price of lamb meat. The high price makes people relatively prefer to consume other meats. In addition, low public consumption of lamb meat is influenced by a characteristic odor and high content of saturated fatty acids (Gunawan *et al*., 2018). Molecular genetic selection can increase the economic value and consumption of lamb meat by improving carcass characteristics and meat quality.

Carcass characteristics are influenced by several factors, including genetics, sex, body weight, age, and carcass performance (Dewantara *et al*., 2017). In addition, the quality of the meat also has a positive correlation with the fat in the carcass and meat (Gunawan *et al*., 2019). Meat quality can be viewed from several aspects the meat's chemical, microbiological, and physical quality. The physical qualities of the meat include pH, tenderness, water-holding capacity, cooking loss, and color. Processes before and after slaughter affected carcass characteristics and lamb quality. Factors before slaughter that affect the physical lamb quality are genetics, species, type of livestock, sex, age, feed, and stress conditions. After slaughter, meat pH, storage method, muscle type, and muscle location affect lamb quality and carcass (Sriyani *et al*., 2015).

Based on the Quantitative Trait Loci (QTL), the characteristics of carcass and fat have economic value, are controlled by many genes, and are located on chromosome 11, which is quite strategic (Gunawan *et al*., 2019). Apolipoprotein A-V (APOA5) is predicted as a candidate gene affected carcass characteristics and lamb quality. The APOA5 gene is associated with unsaturated fatty acid content (Gunawan *et al*., 2018). The content of unsaturated fatty acids can improve the quality of lamb meat and health. Consumption of

meat that contains high saturated fatty acids can cause an increase in plasma cholesterol which has implications for several diseases, including cardiovascular (Alvarengga *et al*., 2015). Cardiovascular disease associated with elevated plasma triglyceride (TG) levels has been recognized as an epidemiological risk for the coronary arteries. Apolipoprotein A-V (APOA5) gene is one of the key regulators of plasma triglyceride (TG) metabolism involved in coronary artery disease (Shou *et al*., 2014).

Gunawan *et al*., (2018) reported that the APOA5 gene is expressed by the liver and secreted into plasma at very low concentrations in sheep. The linkage of the APOA5 gene to several meat quality traits was obtained from the KEGG Pathway analysis (Sahadevan *et al*., 2014; Gunawan *et al*., 2017). The KEGG Pathway analysis shows that the APOA5 gene is involved in the PPAR pathway, which is important for observing carcass characteristics and meat quality in skeletal muscle and adipose tissue. The APOA5 gene binds to VLDL on the vascular endothelium by releasing fatty acid lipoprotein lipase (LPL) into skeletal muscle and adipose tissue. Muscles associated with bones are called skeletal muscles. Skeletal muscle is the main source of meat muscle tissue (Soeparno, 2005). The involvement of the APOA5 gene in fatty acids in skeletal muscle can be one of the reasons for identifying carcass characteristics and meat quality. In addition, there are Apolipoproteins associated with pork tenderness, namely the APOA5 and APOC3 genes are associated with several meat quality characteristics such as carcass percentage, water holding capacity, color, and pork tenderness (Hui *et al*., 2013: Zhu *et al.,* 2021). The association and molecular genetic selection on carcass characteristics and lamb quality have not been analyzed using the APOA5 gene. Therefore, the aim of this study was to analyze polymorphism and association study of the APOA5 gene with carcass characteristics and lamb quality in sheep.

Materials and Methods

Animals collected and ethical approval

This study used a sample consisting of 100 DNA samples from sheep. The sheep used consisted of barbados crossing sheep (BCS), compass agrinak sheep (CAS), garut composite sheep (GCS), thin tail sheep (TTS), and garut sheep (GS). DNA derived longissimus dorsi muscle from rams loin aged 10-12 months with an average weight of 25.35 kg. In this study, sheep were slaughtered in commercial abattoir PT Pramana Pangan Utama (PPU) Slaughter House by a halal butcher who met animal welfare standards. All procedures involving animals were approved by the Animal Ethics Commission of the IPB University (approval no.117-2018 IPB).

Analysis of carcass characteristics and meat quality

The sheep slaughtered must first be fasted for 17 hours to calm the livestock and reduce the amount of digesta in the digestive tract (Dagong *et al*., 2012). Sheep were slaughtered in three conduits is the jugular vein, esophagus, and trachea. The sheep blood that comes out is collected, weighed, and then hung so that the blood comes out perfectly. Slaughtered lamb is separated into carcass and non-carcass. All carcass and non-carcass were removed and weighed. In addition, carcass characteristics and lamb quality were measured. The warm carcasses were weighed and withered at 4°C for 24 hours. The weight of the cold carcass was recorded and then split in the vertebral column into two parts, left and right. The right carcass was divided into eight commercial cuts: neck, shoulder, rack, shank, leg, rib, loin, and flank, while the left carcass is stored. The commercial pieces are separated between meat, bone, and fat (subcutaneous and intermuscular fat), then each part is weighed to determine the weight and percentage of the part.

Carcass characteristics were observed by looking at the hot carcass, live weight, cold carcass, carcass percentage, and carcass length. The parameters used to measure lamb quality are water holding capacity, cooking loss, meat pH, and tenderness (Azizah *et al*., 2020). Cooking loss was measured by subtracting the samples initial weight and the weight of the samples after boiling in water at 80°C using a bimetallic thermometer. Meat tenderness was calculated after 24 hours postmortem (ultimate pH) of meat samples withering and measured by the magnitude of the strength (kg) indicated by the WBSF (*Warner Bratzler Shear Force*) needle for cutting meat cores. Meat pH measurements were performed using a pH meter after the meat had withered for 24 hours.

DNA extraction, PCR-RFLP amplification, and genotyping

The SNP (*Single Nucleotide Polymorphism*) of the APOA5 gene was at position g.26929941 C>T. This SNP is the result of RNA sequencing (Gunawan *et al*., 2021). Product length gene APOA5 of 258 bp forward primer (5'-CTG CAC AGG ATA GCT GGA GC -3') and reverse primer (5'-GAC CAG ACC CTG GGA TAA AG -3)' (Gunawan *et al*., 2018). The DNA was extracted from the longissimus dorsi muscle tissue of sheep using the phenol chloroform extraction (Sambrook and Russell, 2001). The collected DNA was amplified using PCR technology. The PCR was carried out in a total 15 µL mixture that contained 1 µL (50 ng) of DNA, 6.1 µL of ddH_2O, 0.2 µL of forward primer (5 pmol), 0.2 µL (5 pmol) of reverse primer, and 7.5 µL MyTaq Red Mix (Bioline, Meridian Bioscience, UK) which contains 0.4 µL dNTPs (0.16 mM), 1 µL $MgCl_2$ (1.5 mM), 6.05 µL of 1×buffer, 0.05 µL *Taq* polymerase enzyme (1 U).

Amplification started with a 5 min pre-denaturation step at 95°C. The second stage consists of 35 cycles. Each cycle consisted of denaturation at 95°C for 10 s, DNA extension at 72°C for 30 s, and primer annealing at 60°C for 20 s. The final step is primer extension at 72°C for 5 min. Amplified samples were electrophoresed on a 1.5% agarose gel. The five µL of PCR product was digested in 1 µL of ddH_2O, 0.7 µL of buffer enzyme, and 0.3 µL (4 unit) of *BssSI* restriction enzyme in a final volume of 7 µL, followed by an incubation at 37°C for 4 hours. After genotyping, the DNA was electrophoresed on a 2% agarose gel. Electrophoretic DNA samples were then visualized under UV light. DNA fragments generated by electrophoresis are compared to markers to determine fragment length and genotype. The resulting DNA migration positions were identified as alleles during genotyping based on the product length of each sample. The genotypes and product lengths of PCR-RFLP results for the APOA5 gene were CC (159 and 99) bp, CT (258, 159 and 99) bp, and TT (258) bp.

Analysis data

Genotype and allele frequency study. Genotype and allele frequency analysis was performed on the sheep population. Genotype frequency compares the number of genotypes with the number of populations. The formula for calculating genotype and allele frequency (Nei and Kumar, 2000).

$$Xii = \frac{\sum_{i=1}^{n} nii}{N} \text{ dan } Xi = \frac{(2nii+\sum_{j\neq 1} nij)}{(2N)}$$

Description:
Xi = frequency of allele i;
Xii = frequency of genotype ii;
nij = number of individuals with genotype ij;
ni = number of individuals of genotype ii; and
N = total sample.

Hardy-Weinberg equilibrium. The Hardy-Weinberg equilibrium was determined using the Hartl and Clark, (1997) method. Hardy-Weinberg equilibrium is the value of the equilibrium between genotype frequency and allele frequency calculated by the following formula:

$$X^2 = \sum \frac{(O-E)^2}{E}$$

Description:
X^2 = chi-square;
O = total the number of observations of the i-th genotype; and
E = total number of expected of the i-th genotype.

Association of APOA5 gene with meat quality and carcass characteristics. Analysis of the association of the APOA5 gene with carcass characteristics and lamb quality was carried out by ANOVA using the PROC General Linear Model (GLM) method and SAS 9.4 software. Significantly different ($P<0.05$), then a Tukey test is performed. The mathematical model follows Listyarini *et al*. (2018):

$$Yij = \mu + Gi + Eij$$

Description:
Yij = The composition of carcass characteristics and lamb quality is the overall average
µ = Overall populations average of carcass characteristics and lamb quality;
Gi = The i-th genotype CC, CT, and TT;
Eij = The residual error.

Results and Discussion

Amplification and genotyping APOA5 gene

The length of the PCR product produced in the APOA5 gene was 258 bp. The polymorphism of the APOA5 gene was identified by the PCR-RFLP method. The APOA5 gene was cut using the *BssSI* enzyme and *BssSI* buffer. *BssSI* enzyme is a restriction enzyme that can form fragments of different sizes.

The analysis of the APOA5 gene using the PCR-RFLP method resulted in a genotype consisting of two combinations of alleles, i.e C and T. The CC genotype had a product length of 159 and 99 bp, the CT genotype had a product length of 258,159, and 99 bp, while the TT genotype had a product length of 258 bp (Figure 1).

Polymorphism of APOA5 gene

The APOA5 polymorphism (g.26929941 C>T) was analyzed in TTS, CAS, GS, GCS, and BCS sheep (Table 1). Hardy Weinberg Equilibrium that the frequency of dominant and recessive genes in a large enough population does not change from generation to generation if there is no selection, mutation, migration, or genetic drift (Noor, 2010). The genotypes produced by the APOA5 gene consisted of three genotypes, CC, CT, and TT, with respective frequencies of 0.53, 0.30 and 0.17. The C and T had allele frequencies were 0.68 and 0.32, respectively. The frequency of the C allele is the dominant allele compared to the frequency of the T allele.

The genotype and allele frequencies obtained in TTS, CAS, GS, and GCS show that the APOA5 gene is polymorphic, whereas, in BCS, it is monomorphic. Allele and genotype frequency values equal to or less than 0.99 indicate polymorphism in the population (Gunawan *et al.,* 2017). The genotype frequencies of TTS and GCS samples were 0.18 and 0.04, indicating Hardy–Weinberg Equilibrium, while CAS, GS, and BCS samples showed were not in Hardy–Weinberg Equilibrium. The CAS, GS, and BCS were not in Hardy-Weinberg Equilibrium probably due to a non-random mating system or because of direct selection. The Hardy-Weinberg equilibrium is affected by non-random mating, selection, mutation, and genetic migration shift (Allendorf *et al*., 2013; Khasanah *et al*., 2016). The chi-square(X^2) test was performed to determine if the population is in Hardy-Weinberg equilibrium. The population is said to be balance if the value is $X^2_{count} < X^2_{table}$ (Allendorf and Luikart, 2006). Hardy Weinberg Equilibrium that the frequency of dominant and recessive genes in a large enough population does not change from generation to

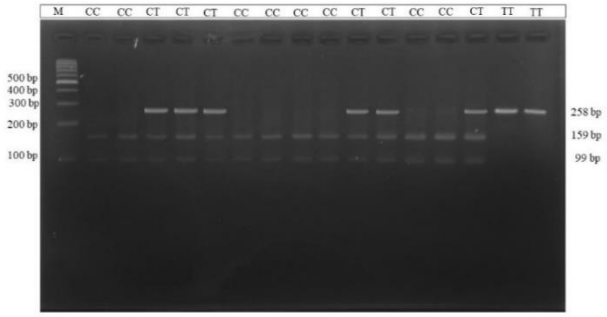

Figure 1. APOA5 gene amplification results in *BssSI* on 2.0% agarose gel.

Table 1. Genotype frequency, allele frequency, and Hardy-Weinberg Equilibrium

Sheep breed	N	Genotype frequency			Allele frequency		Hardy-Weinberg equilibrium	
		CC(n=53)	CT(n=30)	TT(n=17)	C	T	X^2_{count}	X^2_{table}
TTS	55	0.49 (27)	0.43 (24)	0.08 (4)	0.71	0.29	0.18	3.84
CAS	10	0.60 (6)	0.00 (0)	0.40 (4)	0.60	0.40	10.00	3.84
GS	18	0.39 (7)	0.11 (2)	0.50 (9)	0.44	0.56	10.81	3.84
GCS	8	0.88 (7)	0.12 (1)	0.00 (0)	0.94	0.06	0.04	3.84
BCS	9	1.00 (9)	0,00 (0)	0.00 (0)	1.00	0.00	10.00	3.84
TOTAL	100	0.53 (53)	0.30 (30)	0.17 (17)	0.68	0.32		

N= number of samples, TTS=thin tail sheep, CAS=composs agrinak sheep, GS= garut sheep, GCS = garut composit sheep, BCS= barbadoss cross sheep.

generation if there is no selection, mutation, migration, or genetic drift (Noor, 2010).

Association of APOA5 gene with carcass characteristics

The association analyzed of the APOA5 gene with carcass characteristics showed that significant differences (P<0.05) with carcass percentage and length (Table 2). Meat is the main component of the carcass which has high economic value. According to Soeparno (2005), carcasses comprise fat, adipose tissue, bone, cartilage, connective tissue, and tendons. The APOA5 gene has an important role in carcass characteristics because APOA5 gene is secreted in other tissues, such as the intestine or adipose tissue (Meriem *et al*., 2020). Data analysis showed that the APOA5 gene was significantly (P<0.05) correlated with weight-related traits carcass percentage and carcass length. The positive genetic correlation among traits for body weight suggests that genetic improvement in either trait may be affected through indirect selection in the future (Hasan *et al*., 2014).

The factor determining carcass value are weight, amount of meat produced, sex, age, and intramuscular fat (Soeparno, 2005). In addition, genetic and environmental factors affect the growth rate and body composition, including weight distribution and chemical composition of carcass components. Sheep with CC and TT genotype had a lower carcass percentage than the CT genotypes. The CT genotype shows dominance, so its efficiency exceeds the CC and TT genotypes (Noor, 2008). CT genotype can be used to select the desired amount of meat. According to Kulsum *et al*. (2017), carcass percentage is affected by slaughter weight. The growth rate influences the increase in slaughter weight. In addition, the increase in carcass percentage was also influenced by increasing age and live weight (Nuraini *et al*., 2018). According to Martinus (2018), carcass percentage and slaughter weight related to carcass length. Livestock body size will affect carcass length. The research data showed that the carcass length of the TT genotype was longer than that of the CT and CC genotypes. The shows a negative correlation between carcass length and carcass percentage. Differences in the carcass length of livestock are influenced by age, sex, temperature, and environment.

Association of APOA5 gene with lamb quality

Polymorphism APOA5 gene (g.26929941 C>T) was significantly associated (P<0.05) with lamb quality tenderness and pH in sheep populations (Table 3). The APOA5 gene encodes a modifiable apolipoprotein expressed in human liver tissue. The APOA5 gene on chromosome 15 in sheep is an important regulator of triglyceride-rich lipoprotein (TLR) metabolism (Gunawan *et al*., 2018). The APOA5 gene plays an important role in controlling meat quality due to the association between the APOA5 gene and pork quality, such as color, pH, tenderness, water holding capacity, and cooking loss (Hui *et al.*, 2013).

The pH values for the CC, CT, and TT genotypes were 6.13, 5.77, and 7.04, respectively. Nurwantoro *et al*., (2012) reported that the pH of meat is between 5.46 to 6.29. The meat pH for the CC and CT genotypes was in the normal pH range, except for the abnormal pH values for the TT genotype. The TT genotype has a high pH value. A high pH value can be influenced in the process before slaughter, namely the process of resting livestock to suppress stress. Stress levels will affect the amount of glycogen in the muscle used as muscle energy reserves during the rigor mortise process. The low amount of glycogen in the muscle can reduce the metabolic process after slaughter so that the pH of the meat is higher than usual (Hidayat *et al*., 2015). In addition, in livestock in a

Table 2. Association the APOA5 gene traits carcass characteristics

Carcass characteristics	Genotype (μ±SD)			P value
	CC (n=69)	CT (n=27)	TT (n=4)	
Live weight (kg)	24.65±5.49	24.16±3.44	25.47±2.66	0.86
Hot carcass (kg)	9.86±3.31	9.81±1.69	8.71±1.27	0.80
Carcass percentage (%)	40.38±5.9[a]	42.28±3.5[ab]	34.07±1.59[b]	0.02[*]
Carcass length (cm)	73.04±17.82[a]	65.00±6.04[b]	116.00±4.58[b]	0.00[*]
Cold carcass (kg)	9.51±3.31	9.76±1.68	8.14±1.043	0.65

n= number of samples, *= significant association was found, (P<0.05).

Table 3. Association the APOA5 gene traits lamb quality

Meat quality	Genotype (μ±SD)			P value
	CC (n=63)	CT (n=26)	TT (n=11)	
pH	6.13 ± 0.59[a]	5.77±0.27[b]	7.04±0.64[c]	0.000[*]
Tenderness (kg/cm^2)	3.62±0.83[a]	3.94±0.67[ab]	3.05±0.71[b]	0.007[*]
Cooking loss (%)	47.76±7.36	44.23±9.46	48.06±5.08	0.135
WHC (water holding capacity) (%)	81.44±9.33	84.77±10.96	81.93±5.22	0.321

*= significant association was found (P<0.05), n= number of samples.

low glycogen state, there is not much lactic acid which causes the meat to look darker, known as DFD (Dark Firm Dry) because the myoglobin vein pigment cannot combine with oxygen to produce a bright red color (Sriyani *et al.,* 2015).

Several factors, such as species, temperature, cutting process, and storage time, can affect the pH of the meat. The CT genotype showed that low pH. The low pH of meat is caused by the anaerobic breakdown of muscle glycogen by glycolytic enzymes into lactic acid. The value of lamb meat tenderness for genotypes CC, CT, and TT in this study showed that the meat was very tender. According to Komariah *et al*. (2009), criteria based on trained panelists stated that very tender meat <4.15 kg/cm^2, tender meat 4.15-5.86 kg/ cm^2, moderately tender meat, 5.86-7 .56 kg/cm^2, slightly tough meat 7.56-9.27 kg/cm^2, tough meat 9.27-10.97 kg/ cm^2, and very tough meat >10.97 kg /cm^2. Meat tenderness is related to the meat's pH value and water-holding capacity. Meat with high acidity or pH and high water holding capacity has a higher tenderness score than meat with low pH and water holding capacity (Hambakodu and Enawati, 2019).

Conclusions

The APOA5 gene polymorphisms in TTS, CAS, GS, and GCS are polymorphic and monomorphic in BCS. The APOA5 gene was significantly associated with lamb quality tenderness and pH, while carcass characteristics are carcass percentage and length. The APOA5 gene has the potential as a genetic marker in sheep selection, especially with sheep carcass characteristics and lamb quality.

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