Association of SNP T125A on KiSS1 gene with reproduction hormone levels in Kaligesing goat

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ABSTRACT

The objective of this study was to assess the association of KiSS1 gene polymorphism with reproductive traits in Kaligesing goat. Genotypes of 48 ewes aged three years old were determined using PCR-RFLP and DNA sequencing. Sixteen out of 48 samples were monitored for the estrus cycle and determined their 17β-estradiol and progesterone levels using ELISA method. The results showed that KiSS1 gene in the studied population was polymorphic with one single nucleotide polymorphism (SNP T125A). Then SNP was used to analyze genotype all individuals by PCR-RFLP method using MboII enzyme. Three genotypes (TT, TA and AA) were identified. The allele frequencies of T and A were 29.79% and 70.21%, respectively. The genotype distribution for the SNP was deviated from Hardy-Weinberg equilibrium ($\chi^2=8.10; P<0.025$). Hormonal analysis showed that the levels of 17β-estradiol in the follicular and luteal phase were 50.12±61.26 and 42.35±42.43 pg/ml, respectively, while the levels of progesterone in the follicular and luteal phase were 7.87±10.59 and 12.39±15.93 ng/ml, respectively. Polymorphism of KiSS1 (SNP T125A) and their association with reproductive traits did not show a significant association in the Kaligesing population. The results of this study also increased the knowledge of genetic variation in the Kaligesing goat population.

Kata kunci: 17β-estradiol, gen KiSS1, kambing Kaligesing, progesteron, reproduksi
progesterone hormone in the follicular and luteal phase were 7.87±10.59 and 12.39±15.93 ng/ml. No significant associations of the polymorphism were observed for any hormonal levels. However, it was first report about polymorfism in KiSS1 gene of local Indonesian goat especially Kaligesing goat.

**Keywords:** 17β-estradiol, KiSS1 gene, Kaligesing goat, progesterone, reproduction

**INTRODUCTION**

The Kaligesing goat is dual-purpose goat breed for both meat and milk production. Kaligesing goats have been registered in the Minister of Agriculture Decree number: 2591 / Kpts / PD.400 / 7/2010. Kaligesing goats commonly have white bodies and black to brown around the head and neck, good adaptation, fertility 74 – 75%, birth rate 40 – 45%, carcass presentation 40 – 53%, milk production 0.5 – 3 liter per day. Body height 79±3 cm, body length 72±5 cm, chest circumference 84±7 cm, body weights 46±7 kg, ear length 31±4 cm; mane length 27±7 cm (Sanusi, 2013). In 2011, their population was estimated to be 1,75 million heads, which contributes to only 9.76% of the total goat population in Indonesia (Budiarsana and Sutama, 2006). In order to increase their utilization and contribution to the national goat production, identifying the Kaligesing goats with high reproductive ability is of a great interest. Reproduction traits of Kaligesing goat are kidding interval 221 – 253 day, litter size 1.2 – 1.5, conception rate 81 – 91%

Reproductive traits are under control of the hypothalamic-pituitary-gonadal (HPG) balance. Among the many genes controlling the reproductive traits, the KiSS1 gene plays a crucial role in the neuroendocrine regulation of GnRH secretion and puberty onset (Takei et al., 2016) in many species, like mice (Lapatto et al., 2007; Ieda et al., 2019), Bos taurus (Divya et al., 2017), Bos indicus (Divya et al., 2017), pig (Tomikawa et al., 2010; Basini et al., 2018) and mouse (Merhi et al., 2016). Tomikawa et al. (2010) reported that in porcine, KiSS1 gene was located in chromosome 9 and spans over 6 kb composed of two introns and three exons. This gene encodes a family of neuropeptides called kisspeptins, which activate G protein-coupled receptor-54 (GPR54) (Ohtaki et al., 2001; Yeo and Colledge, 2018; Harter et al., 2018). Kisspeptin and GPR54 are regulators for HPG axis (Acton, 2012). Kisspeptin activity in the reproductive system is controlled by sex steroid hormones. Sex steroid hormones provide feedback loops allowing the gonads to communicate with the hypothalamus and regulate the GnRH release. Estradiol and progesterone can stimulate KiSS1 in antero ventral periventricular nucleus (AVPV), which induces GnRH release, and in arcuate nucleus (ARC), which inhibites GnRH release (d'Anglemont de Tassigny and Colledge, 2010; Cao et al., 2019; Harter et al., 2018).

Kisspeptin as product of KiSS1 gene known to be involved in most reproductive process, such as GnRH secretion during estrous cycle, the onset of puberty, and initiation of breeding season (Clarke et al., 2015). KiSS1 gene and its receptor was found in ovaries of hamsters, pigs, goats, primates, and humans (Priyanka et al., 2018). Gottsch et al. (2004; Harter et al., 2018) reported that increasing mRNA level of KiSS1 gene was in correlation with the onset of puberty. Many reports also showed that exogenous administration of Kisspeptin can stimulate GnRH secretion by stimulating GnRH release (Harter et al., 2018) in sheep (Wang et al., 2012), bovines (Naniwa et al., 2013), and pigs (Lents et al., 2008) as well as synchronize of LH surge and ovulation in sheep (Caraty et al., 2007).

Although more research is needed to explain the detailed function of Kisspeptin in reproduction, Kisspeptin is a promising tool in the development of reproductive technology in livestock. As a product of the KiSS1 gene, increasing the KiSS1 gene mRNA level will increase the Kisspeptin level (Cao et al., 2019). The important role of KiSS1 in reproduction traits has been also proven by the mRNA expression level. Irwig et al. (2004) has reported that level expression of KiSS1 gene play an important role in feedback regulation of GnRH secretion in male rat. GnRH neurons was regulated by Kisspeptin while gonadal hormone regulated KiSS1 mRNA level. This provides a promising opportunity to use the KiSS1 expression level as a molecular marker for selection of animals with specific reproductive traits.

To improve the Indonesian local goat productivity through breeding programs, SNP markers significantly affecting the reproductive traits in goats are further used as a tool in selection programs, together with conventional methods. However, no information is available
regarding the KiSS1 polymorphism in Indonesian local goats, especially Kaligesing goats to date. Significant association between polymorphisms of the KiSS1 gene and 17β-estradiol and progesterone has been reported in Baladi, Damascus and Zaribi goats (El-Tarabany et al., 2017). According to Maitra et al. (2014), the polymorphisms in KiSS1 gene may associate with sexual puberty, year-round estrus phenotypes, and high prolificacy in the goats. The polymorphisms in goat KiSS1 was also found to be associated with litter size (An et al., 2013). According to the importance of KiSS1 as a candidate gene for reproductive traits, hence, the present study was conducted with the aim at assessing the association of KiSS1 gene polymorphism with reproductive traits in Kaligesing goat.

MATERIALS AND METHODS

Animals

Forty-eight ewes aged three years old, with body weight of approximately 39 kg, were sampled from Taman Ternak Kambing Kaligesing, Kaligesing District, Purworejo Regency, Central Java Province. The location is managed by Integrated Breeding Center and Livestock Production, belonging to the Ministry of Agriculture of Indonesia. All animals were kept in close pens during the experimental period and fed 2 times daily with forages mix (3.9 kg per animal per day) containing Calliandra calothyrsus, Brachiria mutica and pollard. Water was available at noon.

Estrus Detection, Blood Samples Collection and Hormonal Assay

Estrus phase was determined by the vaginal smear method. Vaginal epithelial tissue was collected using a cotton bud, smeared to the glass slide and washed using 70% alcohol for 10 min. The smears were stained with giemsa for 35 min, slide and washed using 70% alcohol for 10 min. The smears were stained with giemsa for 35 min, then observed using microscope. Epithelial cells were classified into superficial, intermediate and parabasal in order to determine the status of estrus phase. Blood samples, approximately 5 ml for each ewe, at both follicular and luteal phase, were collected from the jugular vein in vacutainer tubes containing anticoagulant. To obtain the serum, blood samples were centrifuged at about 3000 rpm for 15 min and the serums were stored at −20°C until used for further analysis. Serum 17β-estradiol and progesterone hormone levels were determined by using enzyme-linked immunosorbent assay (ELISA) method.

DNA Extraction and Genotyping

Genomic DNA was extracted from blood samples using gSYNC™DNA Extraction Kit (Geneaid, New Taipei City, Taiwan). In the present study, the specific primers goat KiSS1 gene was used to amplify genomic DNA of Kaligesing goats according to An et al. (2013) (forward: 5’ CCC GCT GTA ACT AGA GAA AG3’ and reverse 5’ CAT CCA GGG TGA GTG ATA CT3’). A 25 µL PCR mix containing 12.5 µL PCR Kit Bioline 2x MyTaq HS Red Mix (Bioline, United Kingdom), 2 µL genomic DNA (100 pmol), 0.5 µl of each primer (15 pmol), and 9.5 µL double distillate water was used. The PCR programmed conditions included a pre-denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30s, annealing at 61°C for 30 s and extension at 72°C for 1 min, and a final extension at 72°C for 5 min. PCR was performed in a Primus 25 advanced machine (Peqlab, Erlangen, Germany). An uniform fragment of 337 bp was visualized by electrophoresis with 1.5% agarose gel stained with ethidium bromide. Nine PCR products were sequenced forward and reverse then were sent to 1st BASE DNA. The DNA sequences were further analyzed with the BioEdit program ver. 7.00 (Tom Hall, Ibis Therapeutics, California, USA) and SNP were confirmed based on the electrophoregram results. The SNP in KiSS1 gene detected was used to genotype all animals by PCR-restriction fragment length polymorphism (PCR-RFLP) using MboII restriction enzyme. The digestion was performed in 15 µL reaction volumes containing 5 µl of PCR products, 1 µL 10x CutSmart NEBuffer, 0.5 µl of MboII restriction enzyme (Bio labs, New England) and 8.5 µL of double distilled water, and incubated at 37°C for 6 h. The digested products were run onto 3% agarose gels.

Statistical Analysis

The Hardy-Weinberg equilibrium was calculated using χ² test by SPSS program version 23.0 (SPSS, USA) in order to describe allele and genotype distribution in the population. The association between KiSS1 SNP and 17β-estradiol and progesterone levels were analyzed by independent samples T-test in the SPSS program version 23.0 (SPSS, USA).
RESULTS AND DISCUSSIONS

Hormonal Profile
Descriptive statistics for 17β-estradiol and progesterone hormone levels in the tested population are shown in Table 2. These reproductive traits were determined in both follicular and luteal phase. The level of 17β-estradiol in follicular phase was higher than that in luteal phase. In contrast, the level of progesterone in follicular phase was lower than that in luteal phase. The progesterone levels resulted from several studies revealed varying outcomes, the levels of progesterone tended to seem relatively similar each other, in which the progesterone levels in the follicular phase were extremely low and close to zero, while those in the luteal phase were high. The levels of progesterone correlated positively with the number of corpus luteum (Khanum et al., 2008; Mesen and Young, 2015). In the present study, the level of progesterone in follicular phase was quite high. This may be attributed to the following explanations: 1) there was an active corpus luteum that exceed its normal duration, or 2) the ewes experienced corpus luteum persistent (CL persistent). The CL persistent is mainly induced by the existence of intruterine liquid, which further prevents the regression of corpus luteum. The condition usually occurs due to any infection at postpartum (Risco et al., 2007). Abnormal progesterone level further affect on the levels of other reproductive hormones, including 17β-estradiol (Stevenson 2007; Kromrey et al., 2015).

Genotyping and Allele Distribution
The results revealed T/A nucleotide substitution at position 125 (SNP T125A) (Figure 2). The DNA sequencing was performed to validate the nucleotide substitution in the amplified fragments of KiSS1 gene in Kaligesing goats. Three genotypes were found in Kaligesing goat population, TT (114, 155, 97 and 11 bp), TA (211, 155, 114, 97, 11 bp) and AA (211, 155 and 11 bp) (Figure 1). To be noted that the fragment sizes under 100 bp to small to appear on the gel.

Total individu of each genotype and allele frequencies of the KiSS1 gene in Kaligesing goats are presented in Table 1. Only two genotypes were used in the allele calculation (TA and AA), while TT genotype was not because it was only found in 1 sample of the entire sample used. Genotype frequencies of TA and AA were 59.57 and 40.43%, respectively. The allele T and A frequencies were 29.79 and 70.21%, respectively. A alleles is dominan in Kaligesing goats. The genotype distribution for the SNP was deviated from Hardy-Weinberg equilibrium (HWE) ($\chi^2 = 8.10; P<0.025$). HWE deviation is explained by the existence of nonrandom mating, migration, mutation, selection, undetected “silent” or deleted alleles in heterozygotes, and a mixture of

Figure 1. Genotyping of the SNP T125A of KiSS1 Gene by PCR-RFLP, number 1: TT genotype; number 2, 4 and 5: TA genotype; number 3: AA genotype
subpopulations that do not completely interbred (Graffelman et al., 2017). In the previous study El-Tarabany et al. (2017) reported SNP T121A in Damascus, Zaribi, and Baladi goats having linked to reproductive trait including hormonal profile and litter size. Two genotypes (TA and TT) were identified. The genotype of TA was higher than TT, except in Zaribi. The highest TA genotype frequency was reported in Baladi goats (82%) followed by Damascus (75%) and Zaribi (48%). The T allele frequency was higher than A allele in all breed. Zaribi goats have 76% of T allele followed by Damascus (63%) and Baladi (59%). Damascus and Zaribi goats were in a state of HWE but Baladi Population was in disequilibrium.

**Polymorphism Effect of Gene KiSS1**

In the present study, mutation in intron 1 KiSS1 gene has no significant associations with level of 17β-estradiol and progesterone either in follicular or luteal phase (P>0.05). The genotype TA and AA found in KiSS1 gene of Kaligesing goats may not lead to different level of both hormone. Descriptive statistics for 17β-estradiol and progesterone levels in Kaligesing goats are performed in Table 2 and the effects of different genotype on the levels of 17β-estradiol and progesterone are presented in Table 3 and Table 4. The insignificant results was due to the number of samples is not large enough so that it did not represent the condition of the population well. The uneven distribution of hormone level data also indicates the need for estrous synchronization in goats. Zheng et al. (2018) who compared the

Table 1. Frequency of Genotypes and Alleles of KiSS1 Gene in Kaligesing Goat

<table>
<thead>
<tr>
<th>Totals</th>
<th>Genotype</th>
<th>Allele frequency</th>
<th>( \chi^2 ) stat</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TA</td>
<td>AA</td>
<td>T</td>
</tr>
<tr>
<td>Observed</td>
<td>28</td>
<td>19</td>
<td>29.79</td>
</tr>
<tr>
<td>Expected</td>
<td>19.87</td>
<td>23.06</td>
<td></td>
</tr>
</tbody>
</table>

Figure 2. Genotyping of the SNP T125A of KiSS1 Gene by Sequencing indicating TT in Homozygous Animal (a) and T/A Substitution in Heterozygous Animal (b).

Table 2. Means ± SE of 17β-estradiol and Progesterone Levels according to Estrus Phase in Kaligesing Goat

<table>
<thead>
<tr>
<th>Hormone</th>
<th>Estrus Phase</th>
<th>Level</th>
</tr>
</thead>
<tbody>
<tr>
<td>17β-estradiol (pg/mL)</td>
<td>Follicular</td>
<td>50.12±61.26</td>
</tr>
<tr>
<td></td>
<td>Luteal</td>
<td>42.35±42.43</td>
</tr>
<tr>
<td>Progesterone (ng/mL)</td>
<td>Follicular</td>
<td>7.87±10.59</td>
</tr>
<tr>
<td></td>
<td>Luteal</td>
<td>12.39±15.93</td>
</tr>
</tbody>
</table>
expression of the KiSS-1 gene in goats with high and low prolificacy have synchronized estrous in the studied goats. However, estrous synchronization in research related to the effect of the KiSS-1 gene on livestock reproductive traits does not always go through the estrous synchronization stage first (El-Tarabany et al., 2017). The insignificant result could also be due to the fact that the KiSS-1 gene polymorphisms were associated with levels of other hormones (not estradiol and progesterone) or other reproductive traits.

Another genotypes, TT and TA has been reported to have significant effect on 17β-estradiol and progesterone level of Damascus and Zaribi goats (El-Tarabany et al., 2017). SNP among individuals may found either in exon or intron, they may be synonymous or nonsynonymous. This is likely to occur because SNP are found in the intron 1 position which is a part of the gene that is not expressed. Intron is actually copied into RNA through a transcription process and becomes DNA through a replication process, but the intron sequence does not play a role in sequences that encode proteins and their functions (Jo and Choi, 2015). Further studies are needed to identify other SNP in different location of KiSS1 gene in Kaligesing goats that correlate either with 17β-estradiol and progesterone hormone level or other reproductive traits.

The KiSS1 gene has essential role in controlling reproductive functions in different species, especially at the hypothalamic-pituitary-gonadal (HPG) balance (Navarro et al., 2005; Yeo and Colledge, 2018). In a previous study, the polymorphism in goat KISS1 gene was detected to have a significant effect on the reproductive traits. As reported by El-Tarabany et al. (2017), KISS gene polymorphism with TT genotype in Damascus and Zaribi goats had superior 17β-estradiol level at estrus phase and high progesterone level at metestrus and diestrus phases of the estrous cycle. Moreover, a significant association between KISS1 gene polymorphism and litter size was observed in Guangzhong, Saneen and Xinong goats (An et al., 2013). The litter size in those goats was associated with SNP G384A, T2489C, G2510A, and C2540T. Cao et al. (2010) revealed that SNP G296C in Jining goat showed association with litter size. Genotype CC had kids more than genotype GC or

In cattle, Divya et al. (2017) found that SNP T153C and insertion G at 291_292 bp in KiSS 1 gene in bulls were correlated with acrosome integrity in fresh semen. Cao et al. (2019) and Lapatto et al. (2007) reported that the secretion of GnRH-dependent luteinizing hormone (LH) and follicle stimulating hormone (FSH) in a variety of mammalian species are triggered by administration of KiSS1 gene. Both LH and FSH bind to receptors in the gonad and regulate gonadal function by enhancing sex steroid production like 17β-estradiol and progesterone.

### Table 3. Means ± SE of 17β-estradiol (pg/mL) at Different Phases of Estrus Cycle according to Genotypes at the SNP T125A of KiSS1 Gene in Kaligesing Goat

<table>
<thead>
<tr>
<th>Phase</th>
<th>TA</th>
<th>AA</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Follicular</td>
<td>65.94±63.57</td>
<td>28.79±51.85</td>
<td>0.280</td>
</tr>
<tr>
<td>Luteal</td>
<td>54.52±51.71</td>
<td>27.91±35.40</td>
<td>0.323</td>
</tr>
</tbody>
</table>

### Table 4. Means ± SE of Progesterone (ng/ml) at Different Phases of Estrus Cycle according to Genotypes at the SNP T125A of KiSS1 Gene in Kaligesing Goat

<table>
<thead>
<tr>
<th>Phase</th>
<th>TA</th>
<th>AA</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Follicular</td>
<td>11.28±13.54</td>
<td>5.93±4.72</td>
<td>0.414</td>
</tr>
<tr>
<td>Luteal</td>
<td>16.97±18.51</td>
<td>5.70±5.50</td>
<td>0.213</td>
</tr>
</tbody>
</table>
(Heffner and Schust, 2010; Radovick et al., 2012; Das and Kumar, 2018). That study appropriate with the hypothesis that the polymorphisms of the KiSS1 gene have some correlations with reproduction traits in small ruminants.

**CONCLUSION**

The study findings highlight the genetic variant of KiSS1 gene in Indonesian local goat, and although SNP T125A was detected in Kaligesing goats, neither 17β-estradiol nor progesterone levels were significantly affected.

**ACKNOWLEDGEMENTS**

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