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Detection of Melanocortin Receptor Type 4 (MC4R) Gene in Semen of Etawah Crossbreed and Senduro Goats

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Abstract

Etawah crossbreed and Senduro goats as superior local goats and biological genetic resources that must be preserved in Indonesia. Melanocortin receptor type 4 (MC4R) is the primary gene that regulates food intake and energy balance which can be used as a candidate marker for livestock selection genes. This study aimed to analyze the comparison of cement quality and detect the profile of the MC4R gene in Etawah crossbreed and Senduro goat semen. The samples of this study were Etawah crossbreed and Senduro goat semen. Macroscopic examination of cement includes the evaluation of volume, consistency, odor, color, and pH. Microscopic examination of semen includes motility, abnormalities, and spermatozoa concentration. Examination of motility was performed in the condition of fresh semen, after the addition of diluents, and post-thawing of frozen straw. The polymerase chain reaction (PCR) method was performed to amplify specific fragments of the MC4R gene. There was a decrease in spermatozoa motility before freezing and post-thawing frozen semen compared to fresh semen. The quality of frozen straw post-thawing Etawah crossbreed and Senduro goat was still worth using for artificial insemination and in vitro embryo production. The MC4R gene can be detected in liquid and frozen semen from Etawah crossbreed and Senduro goats using a PCR test in the form of a single band along 642 bp.

Keywords: genetic resources, goat, melanocortin receptor type 4, polymerase chain reaction, semen

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INTRODUCTION

Goat farming in Indonesia plays an important role in achieving food security through meat and milk production. In 2021, the goat population in Indonesia reported 18,9 million heads with meat production of 59.700 tons contributing to an export value of 708.000 USD (Ministry of Agriculture, 2022). The Etawah crossbreed and Senduro goats have been declared by the Indonesian Government as superior local goats and biological genetic resources that must be conserved (Almaida *et al*., 2020). Many people rear Etawah crossbreed and Senduro goats as dual-purpose goats whose meat and milk can be used (Mudawamah *et al*., 2019; Bahari *et al*., 2023). Breeding of Etawah crossbreed and Senduro goats is carried out through natural mating and artificial insemination (Ducha *et al*., 2021). Artificial insemination in goats can use liquid semen which can be stored for 15 days at 4–5°C (Wurlina *et al*., 2020) or frozen semen which can be stored longer in liquid nitrogen (Atmoko *et al*., 2021).

Molecular genetic detection and selection makes it possible to obtain goats with high productivity (Mucha *et al*., 2022). The melanocortin receptor type 4 (MC4R) gene plays an important role in nervous activity, adrenals, thyroid function and mediates the effects of leptin on energy balance (Namjou *et al*., 2021). The MC4R gene is associated with many growth traits such as fat thickness, live weight, carcass weight, and economic traits in animals (Prihandini and Maharani, 2019). The MC4R gene can be used as a candidate marker for superior candidate selection genes in livestock (Utomo *et al*., 2021). The location of the MC4R gene in goats is on chromosome number 24 and consists of 1 exon (Latifah *et al*., 2017). This study aimed to analyze

the semen quality of Etawah crossbreed and Senduro goats in fresh form and after freezing, as well as detecting the MC4R gene profile based on DNA fragments using the PCR method. The MC4R gene profile in Etawah crossbreed and Senduro goats can be used as a molecular marker candidate in breeding programs, improving genetic quality and preserving Indonesia's biological genetic resources.

MATERIALS AND METHODS

Samples

In this study, data collection procedures included storing semen samples, checking semen quality, DNA extraction, and PCR evaluation. This study has been declared ethically by the Animal Ethics Commission, Faculty of Veterinary Medicine, Universitas Airlangga with No: 1.KEH.085.07.2022. This study used goat semen samples. Goat semen samples were collected from healthy superior male goats aged 3–5 years in Singosari Artificial Insemination Center.

Evaluation of Goat Semen

The quality of the semen obtained was performed macroscopically and microscopically. The macroscopic examination includes examination of volume, consistency, odor, color, and pH. Microscopic examination including motility, abnormalities, and concentration of spermatozoa was carried out with the Integrated Visual Optical System (IVOS II) Computer Assisted Sperm Analysis (CASA) system (IMV IVOS II, Ref. 024911, IMV Technologies, France). Motility examination was evaluated in fresh semen, before freezing, and post-thawing of frozen semen (Isnaini *et al*., 2020). Motility examination in fresh semen conditions, if the motility was more than or equal to 60% then continue with the addition of Andromed® diluent. Diluent was added to the semen to a concentration of 50 x 10^6 spermatozoa per straw and then put into mini straws (0,25 ml) using a sealing machine. Before freezing, the straw was equilibrated in cool storage (3–5°C) for 3–4 hours then the motility before freezing was observed. If the motility is more than or equal to 50% then freezing is continued. The process of freezing the cement at a temperature of -140°C with a freezer apparatus then the straw was stored in a container of -196°C containing liquid nitrogen. Frozen cement was thawed at 37°C for 30 seconds and then evaluated for post-thawing motility.

DNA Extraction

DNA samples were liquid semen before freezing and frozen semen from Etawah crossbreed and Senduro goats. The frozen semen was thawed at a temperature of 37°C for 30 seconds before extraction. DNA extraction from goat semen using Thermo K-182001 Invitrogen Purelink Genomic DNA Minikit, each 200 µl semen sample was put in a 1,5 ml tube and added with 20 µl proteinase K and 20 µl RNAse which were then mixed and incubated for 5 minutes at temperature 60°C. The solution that had been incubated was added with 200 µl of binding buffer, then homogenized using a vortex. The solution was incubated for 5 minutes at 60°C and homogenized every 2 minutes. The solution was added with 200 µl of absolute ethanol, and then the solution was homogenized again. The homogenized solution was transferred to the Purelink Spin Cloumn and centrifuged for 2 minutes at a speed of 10.000 rpm. The solution was centrifuged then discarded and the column replaced. Add 400 µl wash buffer (W1) and centrifuge for 30 seconds at 10.000 rpm. The liquid was discarded and 600 µl of wash buffer (W2) was added and centrifuged for 30 seconds at a speed of 10.000 rpm. The liquid was discarded and centrifuged again for 3 minutes at 10.000 rpm. The liquid was discarded and the column was replaced with a 1,5 ml tube and 100–200 µl of elution buffer was added and centrifuged for 30 seconds at 10.000 rpm. The DNA extraction results were put into a 1,5 ml tube that had been labeled and then stored in a refrigerator at -20°C for the next evaluation.

DNA Amplification using PCR Techniques

The PCR technique was used to amplify specific DNA fragments of the MC4R gene. The PCR technique was carried out with the MC4R

gene target in a total reaction volume of 20 µl consisting of 12,6 µl PCR mastermix, 1,6 µl dNTP, 2 µl 10x buffer, 0,2 µl Taq polymerase, forward and reverse primers 0,8 µl, and 2 µl of genomic DNA, respectively. The primers used were based on Latifah *et al*. (2017) (Table 1). PCR evaluation starts from denaturation, annealing, elongation, and final extension. In vitro amplification using a thermal cycler machine was carried out with initial denaturation conditions at 94°C for 5 minutes, 35 cycles consisting of denaturation at 94°C for 45 seconds, primer attachment at 57°C for 45 seconds, elongation of new DNA at 72°C for 1 minute and final elongation at 72°C for 5 minutes.

Electrophoresis Visualization

Visualization of DNA isolation results was carried out using the electrophoresis method using 1,5% agarose gel and SYBR dye. The visible results of electrophoresis were the formation of bands which were DNA fragments resulting from amplification and show the number of base pairs. Observation of electrophoresis resulted using Geldoc.

RESULTS AND DISCUSSION

Based on the data in Table 2, there are differences in volume, color, and pH in the semen of Etawah crossbreed and Senduro goats. The semen volume of Etawah crossbreed goats from this study was 1,5 ml, which was less than in the study by Mokoagow *et al*. (2021) as much as 2,47 ± 1,00 ml in the summer season, while 2,30 ± 0,22 ml in the rainy season. Semen volume and ejaculate frequency in Etawah crossbreed goats are influenced by season. The results of the macroscopic examination of Etawah crossbreed goats were yellowish-white, soupy in consistency, and had a typical normal smell of cement in accordance with a previous study (Mokoagow *et al*., 2021).

The semen production of Senduro goats in this study was 1,2 ml and was not much different from the observations of Ducha *et al*. (2021) as much as 1,24 ± 0,22 ml. Senduro goat semen volume is also influenced by season and humidity, in the summer season the semen volume is 1,94 ± 0,06 ml and 1,80 ± 0,06 ml in the rainy season (Isnaini *et al*., 2020). The results of the macroscopic examination of fresh Senduro goat semen collected in an artificial vagina were milky white in color, soupy in consistency, and the typical normal smell of the semen was in accordance with a previous study (Ducha *et al*., 2021). The degree of acidity (pH) of normal goat semen ranges from 5,9–7,3 (Hafez, 2004) therefore Senduro's pH was 6,6, while Etawah crossbred goats' pH was 6,4, all of which are regarded normal. The quality of fresh semen from Etawah crossbreed and Senduro goats is macroscopically suitable for use in artificial insemination.

Microscopic examination of the semen quality of Etawah crossbreed and Senduro goats includes motility, abnormalities, and spermatozoa concentration. Motility examination is seen in the condition of fresh semen, before freezing, and post-thawing frozen semen. The results of the microscopic examination of the semen quality of Etawah crossbreed and Senduro goats were presented in Table 3.

Senduro goats have a higher spermatozoa concentration than Etawah crossbreed goats. The concentration of spermatozoa in Etawah crossbreed goats is directly proportional to scrotal circumference (Hendri *et al*., 2017). Senduro goat spermatozoa concentration is also influenced by season and humidity, semen concentration in the rainy season is higher than the summer season (Isnaini *et al*., 2020). Semen abnormalities are considered normal if the number of abnormal spermatozoa is no more than 20% of the total semen counted (Hafez, 2004; Wijayanti *et al*., 2023).

Based on SNI 4869.3 2014, only fresh semen with sperm motility of more than 70% considers the requirements for freezing, so the fresh semen of Etawah crossbreed and Senduro goats in this study suitable the requirements. There was a decrease in spermatozoa motility before freezing and post-thawing frozen semen compared to fresh semen. The quality of cement will decrease if it is not used immediately after collection. The quality of fresh semen obtained during storage process

Table 1. Primer sequences for detection of the MC4R gene in goats

Target Genes	Primer	Base sequencing primers	Annealing temperature
MC4R	Forward	5'-TCGGGCGTCTTGTTCATCAT-3'	57°C
	Reverse	5'-CAAGACTGGGCACTGCTTCA-3'	

Table 2. Evaluation of the semen quality of Etawah crossbreed and Senduro goats macroscopically

Breed	Volume	Consistency	Odor	Color	pH
Etawah crossbreed	1,5 ml	Soupy	Normal	Yellowish white	6,4
Senduro	1,2 ml	Soupy	Normal	Milky white	6,6

Table 3. Evaluation of the semen quality of Etawah crossbreed and Senduro goats microscopically

Breed	Concentration	Abnormality	Fresh semen	Before thawing	Post-thawing
Etawah crossbreed	5.040 x 10^6	0,4%	89,4%	56%	40%
Senduro	5.910 x 10^6	3,8%	82,5%	55%	40%

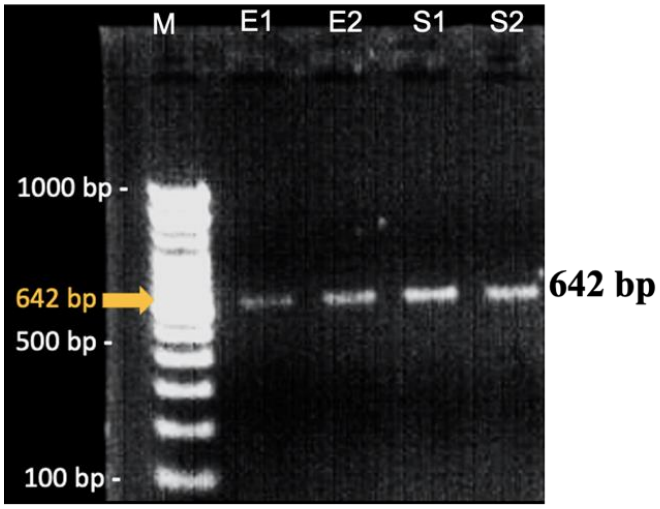

Figure 1. MC4R gene DNA fragmentation in Etawah crossbreed and Senduro goat semen. M= Marker, E1= Etawah crossbreed semen, E2= Etawah crossbreed frozen semen, S1= Senduro semen, S2= Senduro frozen semen.

greatly determines the quality of the frozen semen produced (Prayogo *et al*., 2022; Saputro *et al*., 2022). Extreme temperature changes during the semen freezing process cause the formation of ice crystals and changes in electrolyte concentration, causing damage to spermatozoa cells (Pini *et al*., 2018; Purnama *et al*., 2019). Thawing frozen semen at 37°C for 30 seconds had the best motility compared to the thawing protocol at 35°C for 40 seconds (Goshme *et al*., 2021). The decrease in the motility of frozen goat post-thawing semen is directly proportional to the decrease in membrane viability and integrity (Pamungkas *et al*., 2014). The motility of post-thawing frozen semen of goats according to SNI 4869.3 2014 is at least 40% so that the quality of post-thawing frozen semen of Etawah crossbreed and Senduro goats is still suitable for use for artificial insemination and in vitro embryo production.

PCR evaluation consists of amplifying specific DNA fragments that have specific band lengths for each species (Guan *et al*., 2018). Detection of the MC4R gene in spermatozoa was successfully identified by Seong *et al*. (2012) on Hanwoo Cattle. This study used the DNA sequence of the MC4R gene based on Genbank Accession number data. NM_001285591.1 from the National Center of Biotechnology Information (NCBI) website. Determination of primers based on references by Latifah *et al*. (2017). The target sequence of the MC4R gene is at positions 924–1565 bp with a total of 642 bp. The results of the PCR test in the form of amplification of the

MC4R gene in Etawah crossbreed and Senduro goats are presented in Figure 1.

The MC4R gene was detected in all semen samples of Etawah crossbreed (E1–E2) and Senduro (S1–S2) goats which were characterized by the presence of a single band with a length of 642 bp. In samples from Etawah crossbreed and Senduro goats, no negative results were found. PCR results are declared negative if no single band is found with a length of 642 bp. The research results are in line with research by Latifah *et al*. (2017) which showed amplification of the DNA fragment of the MC4R gene in Bligon goats in the form of a single band of 642 bp. The Etawah crossbreed and Senduro goats are similar to Capra hircus species therefore the PCR results obtained are homologous to Latifah *et al*. (2017). The detection percentage and sensitivity of PCR depend on the primers used, which are determined by the number of copies and the level of primer homology with the target (Gonzales *et al*., 2006). Homology PCR results show high genetic similarity between goat types. The genetic similarity is directly proportional to the phenotypic similarity between Etawah crossbreed and Senduro goats, so it is necessary to continue with PCR-RFLP for genotype frequency analysis. These two types of local goats are large in size, have ideal body proportions, have beards, long hair, have high adaptability, and are very suitable for Indonesia's tropical environment (Kusminanto *et al*., 2020).

CONCLUSION

In conclusion, there was a decrease in spermatozoa motility before freezing and post-thawing frozen semen compared to fresh semen. The quality of frozen semen after thawing of Etawah crossbreed and Senduro goats is still suitable for use for artificial insemination and in vitro embryo production. The MC4R gene profile can be detected in liquid and frozen semen from Etawah crossbreed and Senduro goats. There was homology to the PCR results of the MC4R gene of Etawah crossbreed and Senduro goats with the presence of a single band 642 bp.

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