Phenotypic and Genotypic Detection of *Staphylococcus aureus* and *Escherichia coli* in Subclinical Mastitis Goat Milk in Lumajang, Indonesia

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Abstract

This study aimed to identify the *Staphylococcus aureus* and *Escherichia coli* bacteria that cause subclinical mastitis in dairy goats in the Senduro sub-district of Lumajang regency, Indonesia. A total of 116 milk samples from 58 dairy goats were tested for the California Mastitis Test (CMT). CMT-positive samples were used for bacterial identification through phenotypic and genotypic analyses. Phenotypic analysis was conducted by culturing bacteria on natrium agar media and followed in selective media. The genotypic analysis employed a specific primer of *S. aureus* using the 23SrRNA gene and *E. coli* using the 16SrRNA gene as confirmation of the gold standart. The results of this study showed positive subclinical mastitis in dairy goat samples, which consisted of 21 samples of CMT 1 and a sample of CMT 2. Bacterial identification with phenotypic assays documented 16 samples having *S. aureus* and one sample having *E. coli*. The validated genotypic assays showed from 16 samples, validated 5 samples of *S. aureus* infection and one *E. coli* infection. In conclusion, bacterial identification of *S. aureus* and *E. coli* predominantly infects the SCM dairy goats in the Senduro sub-district as evidenced by the phenotypic analysis and followed by genotypic using the PCR method.

Keywords: bacterial infections, gram-negative bacteria, gram-positive bacteria, mastitis, SCM goat

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INTRODUCTION

Goat milk, which comes from the livestock sub-sector, is a valuable potential resource that can be further developed in the agro-industry sector. Dairy goat milk production in 2018 in Indonesia was recorded as being up to 3,805,296 kg (Direktorat Jenderal Peternakan dan Kesehatan Hewan Kementerian Pertanian, 2022). One of the goat milk producers in Indonesia is Lumajang Regency as mention in the Decree of the Minister of Agriculture of the Republic of Indonesia Number 472/Kpts/RC.040/4/2018 that Lumajang Regency is the Location of the Priority National Agricultural Area for Goat Farming. This proven by a high population of goats in Lumajang Regency, namely in 2019, there were 114,533 goats, and the number increased in 2020 to 121,591 goats (Dinas Ketahanan Pangan dan Pertanian, 2022). Senduro is one the sub-districts for dairy goat cultivation center. In 2018, the goat population in the Senduro sub-district reached 23,788, with a dairy goat population of 22,858 or 96% of the total dairy goat population in Lumajang (Dinas Ketahanan Pangan dan Pertanian, 2022). In order to produce milk that has good quantity and quality, it is necessary to carry out good farming practices to prevent livestock from being infected with diseases (Khasanah and Widianingrum, 2021). One of the common diseases in the dairy farming industry is mastitis (Khasanah *et al*., 2021[a]). Mastitis is generally divided into two categories, namely clinical and subclinical mastitis. The difference between

clinical and sub-clinical mastitis is when clinical mastitis shows symptoms when it infects livestock, while sub-clinical mastitis is asymptomatic. Subclinical mastitis can result in a decrease in the quality and quantity of goat milk (Silanikove *et al*., 2014). Subclinical mastitis is a common type of mastitis experienced by dairy goats, with a percentage of 5–45% (Zhao *et al*., 2015)(Suwito *et al*., 2022).

Bacterial pathogens such as *S. aureus* and *E. coli* are gram-positive and negative bacteria that are generally identified as causing clinical and subclinical mastitis in dairy cattle (Krishnamoorthy *et al*., 2021). Milk from cattle infected with subclinical mastitis containing more than 1×10^2 CFU/mL of *S. aureus* and more than 1×10^3 CFU/mL of *Enterobacteriaceae* are pathogenic, and it may endanger human health upon consumption(Suwito, 2010). Detection can be done to determine the presence of these two bacteria in two ways, namely phenotypic and genotypic. The research objective was to identify the *S. aureus* and *E. coli* bacteria that are caused in subclinical mastitis dairy goats phenotypically and genotypically validated.

MATERIALS AND METHODS

Ethical Approval

This research required no approval because there was a field study and did not perform any treatment on the animal.

Study Period and Location

The samples were collected from dairy goat farmers in Senduro Sub-district, Lumajang Regency. The isolation of bacteria was conducted in the Plant Protection Laboratory, Faculty of Agriculture, University of Jember, and the analysis of genotypic identification was performed in the Center for Development of Advanced Sciences and Technology (CDAST) Laboratory, University of Jember. Lumajang Regency is located at 112°-53' east longitude and 7°-54'-8°-23' south latitude. Based on the classification of Schmid and Ferguson (1951), Lumajang Regency has climate types C and D. Senduro District has an area of 288.68 km^2 so that 12.77% of the area of Lumajang Regency is Senduro District. Some areas in Lumajang Regency were classified as wet, moderate or dry. Senduro District is included in the area with a wet climate, which each year has an average number of dry months of 3 months. Data shows that in 2015, Lumajang Regency experienced rain for 57–175 days with an intensity of 919–2,566 mm^3, while the monthly solar radiation ranges from 126–261.1 hours with a percentage of 29.03–60.16% (BPS, 2020). Senduro District is located at an altitude of 500–700 meters above sea level (asl). According to Purwanto and Afton Atabany, (2016), comfortable conditions for dairy goats to produce milk are located at 400 m asl and optimal conditions at 600 m asl.

Sample Collection

A total of 116 goat milk from 58 goats was collected by purposive sampling from dairy goat farmers in Senduro District, Lumajang Regency, which had 5 to 20 goats that were lactating. Milk samples were taken from each nipple as much as 10 mL. The first and second streams of goat's milk were excluded. The milk sample taken was collected using a conical, which has been coded and the date of collection. The samples obtained were stored at 4^{o}C in a cool box and continued with further tests (Khasanah *et al*., 2021[a]).

California Mastitis Test (CMT)

The CMT test was carried out as a screening for samples infected with subclinical mastitis or not. The CMT test initiated with mixing the CMT reagent with dairy goat's milk from each teat with a ratio of 1:1. The mixture was homogenized in the paddle by moving it horizontally, and milk that tested positive for subclinical mastitis will experience curdling. The result was scored using a 5-point as 0 (negative, mixture stays liquid with no visible gel formation), trace (a slight precipitate which tends to fade with the persistent motion of the paddle. The results of the CMT test reaction, which experienced thickening, were assessed as positive 1+, 2+, and 3+. A positive 1 (+) was a mild reaction, seen from the mixture that has slightly thickened, positive 2 (++) was a moderate reaction seen from the mixture

thickening, while positive 3 (+++) was a strong reaction seen from the mixture thickening until it turns like gelatin and is difficult to move or a distinguishing gel that sticks to the base of the paddle formed (Khasanah *et al*., 2021[a]). All milk that was detected positive will be followed by the identification of *S. aureus* and *E. coli* bacteria.

Phenotypic Identification of Bacteria

The phenotypic test was carried out by culturing, identification and storage. We cultured all the bacteria from CMT positive (we only found CMT 1 and CMT 2) on nutrient agar (NA) media for 24 hours at 37°C, then identified cultured bacteria in selective agar of mannitol salt agar (MSA) for *S. aureus* and eosin methylene blue agar (EMBA) for *E. coli*. Both MSA and EMBA incubation were conducted for 24 hours at 37°C. Phenotypic testing was carried out in a sterile laminar airflow. Identification of *S. aureus* on MSA media was carried out according to standard procedures from Oxoid (Oxoid Ltd, Basingstoke, United Kingdom) with white colonies and yellow circles. Identification of *E. coli* growing according to the instructions of Oxoid (Oxoid Ltd, Basingstoke, United Kingdom), which was metallic green in color. Colonies of *S. aureus* and *E. coli* that grew were cultured, identified, and stored in glycerol media for further analysis.

Genotypic Validation of Bacteria

The genotypic test was carried out by isolating the total genome of *S. aureus* and *E. coli* bacteria from the colony using the Geneaid extraction kit (Taiwan, Ltd) according to the manufacturer's procedures (Geneaid, 2017). The isolated DNA was then used for amplification using primers (Table 1). Amplification using PCR Master Mix (Bioline, Meridian Bioscience). The amplification used a mixture consisting of 2 µl DNA template, 2 µl forward primer, 2 µl reverse primer, 10 µl master mix and 4 µl Water Nuclease-Free. The PCR mixture was run on a PCR machine with denaturation conditions of 94ºC for 20 seconds, primary annealing at 56ºC for 20 seconds, primary extension at 72ºC for 30 seconds for 35 cycles and final extension at 72ºC for 5 minutes. The amplification results were visualized using electrophoresis on 1.5% agarose and Redsafe staining as DNA fluorescence. Electrophoresis was run at 100 volts for 30 minutes. Electrophoresis results were visualized using a transilluminator. We used positive control of *S. aureus* from the previous study (Widianingrum *et al*., 2022) and *E. coli* BL21 from the Agrotechnology Laboratory, Faculty of Agriculture, University of Jember, Indonesia.

Data Analysis

The results of this study are presented descriptively in the form of figures and tables.

Table 1. Primers used for genotypic identification of *S. aureus* and *E. coli*

Bacteria	Gen	Sequences	Base pairs	Ref.
S. aureus	23SrRNA (F) 23SrRNA (R)	ACG GAG TTA CAA AGG ACG AC AGC TCA GCC TTA ACG AGT AC	1250	(Straub *et al*., 1999), (Akineden *et al*., 2001), (Salasia *et al*., 2011)
E. coli	16SrRNA (F) 16SrRNA (R)	GGG AGT AAA GTT AAT ACC TTT GCT C TTC CCG AAG GCA CAT TCT	584	(Lin and Tsen, 1999)

RESULTS AND DISCUSSION

Phenotypic Test

A phenotypic test was conducted on CMT-positive samples. We collected 116 milk samples, and screening using the CMT test resulted in 1 sample positive CMT 2 and 21 samples positive CMT 1 were obtained (Table 2). Total positive CMT in our study, about 22 samples contained 21 samples positive CMT 1+ and 1 sample positive CMT 2+. Then, all 22 samples were tested for phenotypic and genotypic bacterial detection. The result of cultured bacteria on selective media is shown in Figure 1. *S. aureus* was the predominant bacteria detected in 16 samples, accounting for 72% of the total isolate. *E. coli* was only detected in 1 of 22 samples, accounting for 4.5% of the isolate, as summarized in Table 3. The 5 samples were attributed to other bacterial species and not specifically named in the data.

Table 2. The summarized CMT test results from dairy goat milk in Senduro Sub-district

CMT	Samples	Percentage (%)
Negative	94	81.03
Trace	0	0.00
Positive 1+	21	20.19
Positive 2+	1	0.96
Positive 3+	0	0.00
Total	116	100.00

Table 3. Identification of bacteria in each milk sample positive for subclinical mastitis

Bacteria	Samples	Percentage (%)
S. aureus	16/22	72.73
E. coli	1/22	4.55
Other bacteria	5/22	22.73
Total	22	100

The results of the study by Birhanu *et al.* (Birhanu *et al*., 2017) showed that 89.5% of the samples obtained a single pattern and 10.5% mixed in the milk samples of sub-clinically infected cows in Ethiopia. In Egypt, 49.69% of goats with subclinical mastitis were recorded as single infected, while 23.9% were mixed (Hussein *et al*., 2020). Cow's milk in Bogor, Indonesia, was detected to be 25% infected with a mixture of bacteria (Tasso Pinheiro Machado *et al*., 2018).

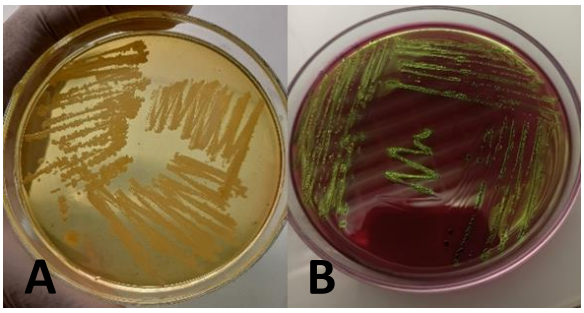

Figure 1. (A) *S. aureus* on MSA and (B) *E. coli* on EMBA after 24 hours incubation at 37°C.

Genotypic Test

The genotypic test results (Figure 2) validated the presence of *S. aureus* and *E. coli* bacteria with the results of 6 of 16 samples of *S. aureus* (40%) and 1 of 1 sample of *E. coli* (100%). Based on this validation, the total *S. aureus* that infected milk contaminated with bacteria was 27% and *E. coli* 4.5% of the total sample. The genotypic method was reported to have higher accuracy than the phenotypic method in staphylococcus infection (Kartikasari *et al*., 2019; Muñoz-Gamito *et al*., 2020). The result of this study is in line with Suwito *et al.* (Suwito, 2010) that gram-positive bacteria are more dominant in contaminating milk as a cause of subclinical mastitis than gram-negative bacteria. Previous studies revealed that the bacteria that caused subclinical mastitis originated from *Staphylococcus spp*., with 59.1% in Selangor, Malaysia (Marimuthu *et al*., 2014), 73.2% in Kota Bahru, Kelantan (Omar and Mat-Kamir, 2018), and 36% in Kulon Progo, Yogjakarta, Indonesia (Artdita *et al*., 2020). *E. coli* was reported to be the cause of subclinical mastitis in goats with a low percentage, namely 11.43% in China (Zhao *et al*., 2015) and 2% in Kebo Sleman (Suwito *et al*., 2019). Identifying *S. aureus* and *E. coli* confirms their association with subclinical mastitis in goat. The PCR-based detection method confirmed sensitivity specifically of the bacterial infection, and it is suggested that its method is a

reliable tool for diagnosing subclinical mastitis etiology.

Phenotypically identification using Mannitol salt Agar, a selective media for Staphylococcus, is based on the principal of high tolerance. In addition, Baired parker agar can also be used as a discriminatory medium between *S. aureus* and others. Morphology of *S. aureus* on non-selective media is varied, the colony size can reach up to the dimensions of about 4–6 mm in diameter and may show variation in colours due to the production of pigments, the colour ranging from orange to greyish or greyish-white (Pal *et al*., 2023). On the selective medium of MSA, to confirm fermentation of mannitol, the growth of yellow colonies appear and turn mediam become yellowish (Oxoid Ltd, Basingstoke, United Kingdom).

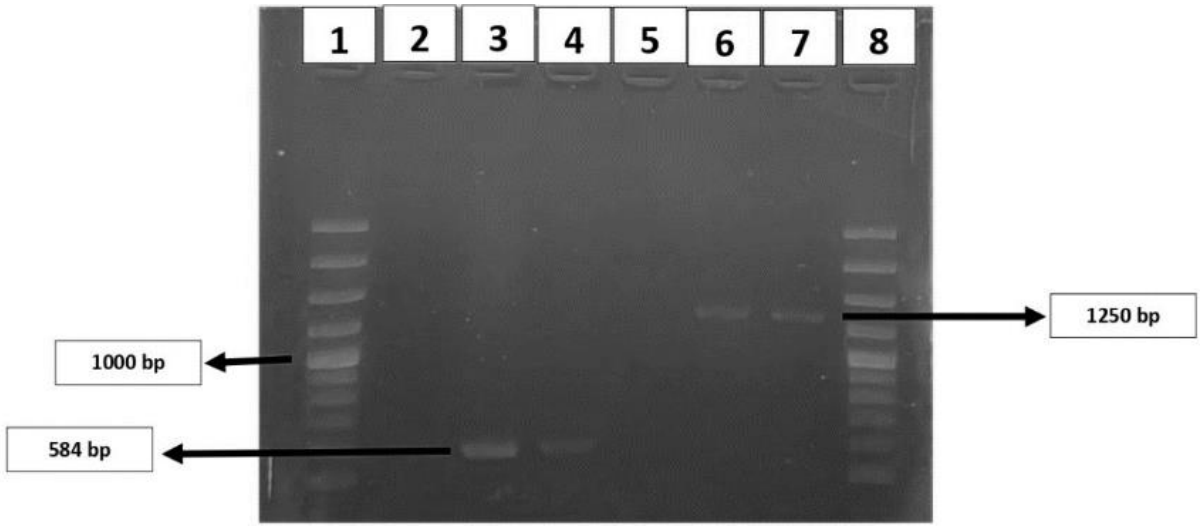

Figure 2. Electrophoresis of bacteria causing subclinical mastitis; lines 1 and 8 were markers; lines 2 and 5 were negative control; line 3 was positive *E. coli* samples; line 4 was positive control of *E. coli*; line 6 was positive samples of *S. aureus* and line 7 positive control of *S. aureus.*

Of the 16 samples that were phenotypically *S. aureus* positive, only 6 samples were genotypically detected *S. aureus* using this gene marker. Based on the research by Salasia *et al.*, (2011), this 23SrRNA gene marker has been proven to be 99% able to detect *S. aureus* bacteria based on sequencing data. Although mannitol salt agar is reported as a selective medium capable of distinguishing *S. aureus* and others, with the large number of genera under staphylococcus it is possible that this medium is overgrown by other staphylococcus that have similar characteristics to *S. aureus* resulting in false positives detection. Becker *et al.*, (2014) reported that the Staphylococcus genus divided into more than 47 species and 23 sub-species. Kateete *et al.*, (2010) also explained that there is no single test that is accurate and reliable to detect *S. aureus* so it is recommended to use test sequences such as growing on MSA, DNAse test, and tube coagulase.

S. aureus contaminating the milk was probably caused by the bacteria carried by the farmer's hands while milking the udder of one cow to others. The hands of udder milkers may be the cause of the spread of *S. aureus* in livestock (Pradika *et al*., 2019; Khairullah *et al*., 2022). In our research, the milk collection was done by farmers who sterilize their hands first before milking to mitigate the contamination. Milking performed by different milkers increases the chances of spreading *S. aureus*, causing subclinical mastitis in livestock. *E. coli* attacks livestock in general due to the udder interacting with the floor of the cage, especially after milking when the sphincter muscles are still open. *E. coli* attacks more in traditional farms than in modern farms (Suwito *et al*., 2019).

We observed the condition of the cleanliness of the goat farm pens in Senduro sub-district, Lumajang Regency, which is quite clean. The floor of the cage, where to feed and drink, is

cleaned once a day. The cage used is the back cage, and the dirt that has accumulated below is cleaned regularly. Mastitis disease will be prone to attack traditional farms if farmers do not pay attention to the cleanliness of the cage environment (Khasanah *et al*., 2021[b]). Milking is done using the traditional way, and before milking the udder and milkers' palms are washed with warm water, as well as afterwards. Subclinical mastitis that appeared in PE goat farms which were washed with warm water was 29% while disinfectants were 21%, but 79% of farms that were embarrassed to use disinfectants did not contract subclinical mastitis compared to farms that did not do dipping (Suwito *et al*., 2022). Preventing mastitis can be done by improving the management of the cleanliness of the stables and maintaining the health of the livestock by controlling the health of the livestock on a regular basis and then separating the livestock infected with mastitis and dipping the nipples with an antiseptic such as 70% alcohol.

CONCLUSION

S. aureus predominantly infects the SCM dairy goats in the Senduro sub-district. Double infection of S. *aureus* and *E. coli* present in one sample. The phenotypic identification using selective media should be followed by genotypic using PCR for more accurate results. Although CMT 1 is not considered subclinical mastitis, *S. aureus* was found in the CMT 1 milk sample, so that farmers are highly advised to take serious measures to address pathogenic infection in goat milk.

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AUTHORS' CONTRIBUTIONS

HK and DCW conceptualization, resources, supervision, writing, editing and validation, NGW: investigation, sample collection, data analysis, visualization, NTH: supervision and review, RY and NW: methodology and data curation, NP and JFDC writing and review. All the authors have reviewed and approved the manuscript.

COMPETING INTERESTS

The authors declare that they have no competing interests.

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