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Detection of mecA Genes from *Staphylococcus aureus* Bacteria inRaw Cow Milk in the Surabaya Regional Farm

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ABSTRACT

Milk is a very important food ingredient in meeting nutritional needs and a good growth medium for microorganisms. *Staphylococcus aureus* bacteria are pathogens that often contaminate milk. *Staphylococcus aureus* bacteria can cause a disease called mastitis. The acropolis is very susceptible to infections, treatment and prevention need to be done to avoid mastitis. Treatment of mastitis until now still uses antibiotics, improper use of antibiotics can cause *Staphylococcus aureus* resistance to antibiotics. The mecA gene is the gene responsible for beta-lactam antibiotic resistance. This study aims to determine the presence of the mecA gene in *Staphylococcus aureus* isolate from raw cow's milk. The samples from this study are 24 samples taken from dairy farms in the Surabaya Region. Isolation of *Staphylococcus aureus* bacteria by conventional microbiology based on culture properties, Gram staining and biochemical tests. The results of this study showed that there were 5 samples that were positive for Staphylococcus aureus. Molecular detection by PCR of the mecA gene in *Staphylococcus aureus* was not found in the five *Staphylococcus aureus* isolates. Based on this study has the result that the sample found *Staphylococcus aureus* but no mecA gene was found.

INTRODUCTION

Milk is one of the most important food ingredients in fulfilling the nutritional needs of the community. However, milk is also a good medium for the growth of microorganisms so that it can potentially be a potentialmeans for the spread of pathogenic bacteria (milkborne diseas) which are easily contaminated anytime and anywhere as long as the handling does not pay attention to hygiene (Kusumaningsih and Ariyanti, 2013).

Bacterial growth in milk can affect milk quality and food safety characterized by changes in taste, aroma,color, and appearance. Contamination of pathogenic bacteria also causes unwanted damage so that milk is not suitable for consumption. Pathogenic bacteria that can contaminate milk are *Staphylococcus aureus*. Bacterial contamination of milk starts from the milk expressed by the presence of bacteria growing around the udder or from the farmer himself, then through the air carrying particles from the environment. Therefore, milk is easilycontaminated with bacteria from the environment (Arjadi et al., 2018).

Staphylococcus aureus is one of the bacteria that cause mastitis which results in losses. *Staphylococcus aureus* is commonly present on the udder surfaces of healthy

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cows and cows suffering from mastitis and is therefore a potential source of contamination. *Staphylococcus aureus* resistance not only causes disease in the udder of cattle, but also has the potential to become a source of enterotoxins for humans who consume milk. The development of bacterial resistance is known to be associated with the inappropriate use of antibiotics. Recently, the resistance of *Staphylococcus aureus* to β-lactam antibiotics, especially penicillin, including methicillin, has grown rapidly, giving rise to a new strain called Methicillin resistant *Staphylococcus aureus* (MRSA) (Aziz et al., 2016).

Methicillin resistant *Staphylococcus aureus* (MRSA) is one of the strains of *Staphylococcus aureus* that is resistant to methicillin antibiotics. MRSA was first detected in cow's milk mastitis and is a pathogenic bacteria in humans. The mecA gene is the gene responsible for resistance to beta-lactam antibiotics such as methicillin, but mecA has also been reported in methicillin-sensitive *Staphylococcus aureus.*

Detection of mecA gene in *Staphylococcus aureus* isolated from raw milk samples was performed molecularly using PCR method which is known to be fast and accurate. The mecA gene in *Staphylococcus aureus* samples was amplified using gene-specific primers. In some studies, PCR detection of the mecA gene in raw milk was performed to determine the species of *Staphylococcus aureus*. Therefore, in this study, we investigated the presence of *Staphylococcus aureus* from raw milk produced by several traditional dairy farmers in the Surabaya area to detect signs of mastitis in cows and evaluate milking safety. The mecA gene was found to be an important virulence factor.

MATERIALS/METHOD

1. Materials

This research is ethical because it uses bacterial samples from raw cow's milk as biological samples.

2. Sample collection and phenotypic identification of *Staphylococcus aureus*

Raw cow milk samples collected from 3 traditional dairy farms in the Surabaya area of East Java province totaling 24 samples were used in this study. Samples were collected directly from each cow by the milkers using whole hand technology. Samples were collected in clean buckets and transferred to 100 ml labeled sterile milk bags. The samples were transferred into ice boxes and transported to the Microbiology Laboratory, Faculty of Health, Nadlatul Ulama University Surabaya (UNUSA). Isolation and phenotypic identification by dilution of samples followed by culture on Blood agar plate (BAP) media and incubation at 37°C for 24 hours. Colonies suspected of *Staphylococcus aureus* appear round, smooth, prominent, shiny, gray to dark golden yellow, forming gray to golden brown zones and hemolytic zones. Furthermore, bacterial colonies cultured on BAP media were Gram stained, the morphological characteristics of *Staphylococcus aureus* were Gram positive, the cocci were shaped like grapes, and purple in color. The colony is probably *Staphylococcus aureus*. Purified *Staphylococcus aureus* on nutrient agar (NA) is then subjected to biochemical tests with a catalase test, coagulase test, and mannitol test using mannitol salt agar (MSA) (Windria et al., 2023).

3. *Staphylococcus aureus* DNA Isolate

DNA molecules of *Staphylococcus aureus* bacteria obtained from raw milk samples were isolated using the ripening method. A total of 3 colonies were dissolved in 200 µL of Tris-EDTA buffer (pH 8), and the solution was homogenized by vortexing. The boiling process was carried out on a hot plate at 95-100°C for 15 minutes. At this temperature, heating occurs to promote lysis of the bacterial wall, so that DNA can react. The suspension was then centrifuged at speed. 13000 rpm for 1 minute until the supernatant and pellet are separated. The supernatant containing bacterial DNA is transferred to a new tube and can be used for quantitative DNA testing.

4. DNA Quantity Test

The quantity of DNA isolate was obtained by testing the purity and concentration of DNA using a *Nanodrop Spectrophotometer*. The purity and concentration of DNA at wavelengths of 260 and 280 nm are obtained automatically after testing the sample on the device. Sample testing with a nanodrop spectrophotometer was carried out by measuring 1 µl of blank solution (TE buffer) on the instrument. Then 1 µl of the isolated DNA sample was tested on the instrument. If the test results comply with the DNA purity standard, namely a ratio of 1.8 – 2.1 and the DNA concentration test results are > 100 ng/ µl, the DNA sample can be used for further testing (Koentjoro, 2018).

5. Amplification of mecA genes

Detection of the mecA gene by PCR using mecA specific gene primers, namely mecA-F primers (Forward: mecA 5'-AAA ATC GAT GGT AAA GGT TGG C-3') mecA-R primers (Reverse: 5'-AGT TCT GCA GTA

CCG GAT TTG C-3') with a PCR product length of 533 bp (Khairullah et al ., 2022). PCR amplification of DNA begins with making a mix solution. The reagent used is melted (thawed) first before use. The mix solution consists of 12.5 µl Go Taq mm 2X, 1 µl mecA gene primer (forward gene primer), 1 µl mecA gene primer (reverse gene primer), 8.5 µl Nuclease Free Water, 7 µl *Staphylococcus aureus* bacterial DNA isolate. The mixed solution is collected in a PCR tube, then centrifuged briefly (spin down) so that the solution on the tube wall drops down. The PCR tube is closed and then inserted into the Thermal Cycler for the PCR amplification of DNA. The temperature settings and PCR cycle to amplify the mecA gene are denaturation at 94 °C for 30 sec, annealing at 55°C for 30 sec, and extension at 72 °C for 1 min with a final extension at 72 °C for 5 min, for DNA amplification as many as 40 cycle (Khairullah et al ., 2022). After the PCR process is carried out, the DNA isolate resulting from PCR amplification with the mecA gene primer is stored in a freezer (Koentjoro, 2018).

6. Electrophoresis and Visualization of DNA bands

The results of DNA amplification were analyzed by agarose gel electrophoresis with a concentration of 2% (w/v). The agarose gel in the stem that has been filled with TAE 1X buffer is added with marker solution (DNA Ladder), negative control solution, and DNA sample solution. The first well was filled with a positive control solution consisting of 4 µl DNA Ladder size 100 - 1500 bp and 2 µl loading dye. The second well was filled with a negative control solution consisting of 4 µl distilled water and 2 µl loading dye. The third and subsequent wells are filled with 4 µl of sample DNA and 2 µl of loading dye.

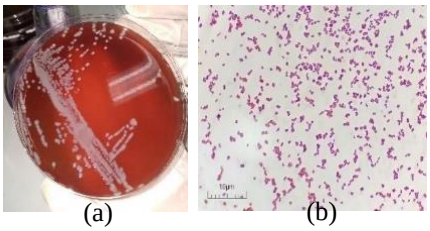
Electrophoresis was run with an electric voltage of 100 volts with an interval of 30 minutes. After the running is complete, the DNA bands are then visualized using a UV Transilluminator, then documented and the sample DNA bands compared with the DNA Ladder (Koentjoro, 2018).

RESULTS AND DISCUSSION

This study aims to determine whether there is contamination of *Staphylococcus aureus* bacteria carrying the mecA virulence factor gene in raw cow's milk. The virulence factor of *Staphylococcus aureus* is important in the pathogenicity of bacteria in posing a public health threat. The samples used in this study were raw cow milk from 3 traditional dairy farms in the Surabaya area. Raw cow milk samples were taken from groups of sick and healthy cows from each farm. The purpose of using purposive sampling method was to determine the prevalence of *Staphylococcus aureus* that has mecA gene in both groups of samples.

Before the milking process, it is necessary to pay attention to its sterilization, considering the large number of microbes that contaminate milk due to the high protein content, therefore all milking equipment and cows are clean to reduce the factor of bacterial contamination of milk being milked. Cows are milked directly by the milkers using the whole hand technique, which is manual milking using the whole hand and is the best technique because it does not cause elongated teats (Susilaningrum et al., 2022). A total of 24 milking samples from each cow were then transported to the laboratory using an ice box. Raw cow milk samples should be analyzed immediately a maximum of 2 hours after sampling to avoid excessive bacterial growth because every 30 minutes bacterial growth will double at room temperature (Susilaningrum et al., 2022).

The first stage for the isolation of *Staphylococcus aureus* bacteria from the sample is to dilute the sample gradually to prevent excessive bacterial growth when cultured on solid media. The diluted samples were cultured on Blood Agar Plate (BAP) media to determine the ability of bacteria to hemolyzes red blood cells which is characterized by the formation of a clear zone around the colony (Kurniasari et al., 2022). Colony growth of S. aureus isolates on BAP media growing colonies appear large, medium, small, white to yellowish in color, convex, shiny surface and a clear hemolysis zone is formed around the colony (Krihariyani et al., 2016). According to Nurhidayanti & Sari (2022), the results of microscopic observations of *Staphylococcus aureus* isolates are classified as Gram positive, cocci-shaped, purple in color, and have an irregular arrangement in groups like grapes. This is in accordance with the results of macroscopic and microscopic observations on 10 bacterial isolates with characteristics that refer to *Staphylococcus aureus* as in Figures 1(a) and 1(b). Based on the results of research on isolates in BAP culture, cell colonies appear round, convex, with flat, shiny edges, and have a variety of sizes and types of hemolysis (Figure 1(a)). The results of Gram staining of *Staphylococcus aureus* isolates from BAP culture showed Gram-positive bacteria, round in shape, clustered like grapes (Figure 1(b)).



(a) (b)

Figure 1 Observation results of bacteria culture in BAP (Blood Agar Plate) media (a). Characteristics of colonies on BAP (Blood Agar Plate) media (b). Colony Gram staining results from BAP.

The desired pure culture is obtained without any contaminants from other microbes (Rahayu, et al., 2019). According to Krihariyani, et al., (2016) *Staphylococcus aureus* colony growth on NA culture plates appears golden yellow, round, convex, with shiny flat edges, and has a soft texture. This in accordance with some of the macroscopic observations of *Staphylococcus aureus* colonies in this study shown in Figure 2. Varitions in colony properties in bacteria are caused by differences in starin expression of *Staphylococcus aureus* bacterial species (Jawetz, 2015). Microscopic observation of the colonies was carried out again to ensure that the bacterial isolates were uniform (pure). Pure bacterial isolates were then growth on NA slant media for biochemical testing.

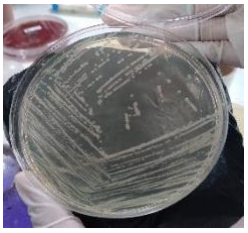


Figure 2 The results of purifying the bacterial colony weresuspected to be *S.aureus* on NA cup media.

Biochemical tests are carried out to determine the physiological properties of isolated bacterial colonies. Bacterial identification cannot be done by knowing its morphological properties alone, but must know the physiological properties of bacteria as well (Liempepas, 2019). Identification of the type *Staphylococcus aureus* bacteria from raw cow's milk samples was carried out with a series of biochemical tests including catalase test, coagulase test, and mannitol fermentation test.

According to Karimela, et al. (2017) the identification of *Staphylococcus aureus* bacteria is characterized by positive catalase, positive coagulase, and positive mannitol fermentation results. In this study, all bacterial isolates referring to *Staphylococcus aureus* were identified as producing catalase and coagulase enzymes. Meanwhile, the results of the mannitol test showed that 5 isolates could ferment mannitol from a total of 10 bacterial isolates referring to *Staphylococcus aureus*. The biochemical test results of *Staphylococcus aureus* isolates using the catalase test (Figure 3(a)), coagulase test (Figure 3(b)), and mannitol test showed positive results (Figure 3(c)). The results of phenotypic identification of *Staphylococcus aureus* from the samples can be seen in Table 1.

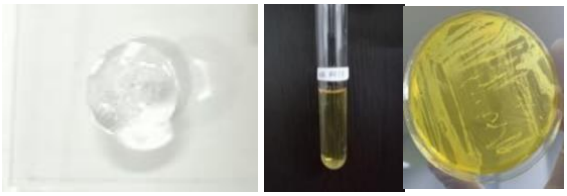


Figure 3 The results of the biochemical test of the isolate are suspected to be *Staphylococcus aureus* (a). Catalase positive (b). Coagulase positive (c).Mannitol positive.

Table 1. results of biochemical tests on bacterial isolates suspected to be *Staphylococcusaureus*

Sampel's code	Coagulase Test	Catalase Test	Mannitol Test
A4 (1)	+	+	+
B1(1)	+	+	-
B3 (1)	+	+	+
A3 (2)	+	+	+
A4 (2)	+	+	+
B1(2)	+	+	-
A4(3)	+	+	-
B2(3)	+	+	-
B3(3)	+	+	-
B4 (3)	+	+	+

Based on the results of observing culture characteristics in BAP, Gram staining, and biochemical test results, 5 sample isolates had similarities ranging from 83-100% to the characteristics of *Staphylococcus aureus*based on the Jawetz literature (2015) as in table 2. A total of 4 samples were identified as *Staphylococcus aureus* with a similarity of 83% and 1 bacterial isolate of the sample was identified as S. aureus with a similarityof 100%.

Table 2 Percentage similarity of *Staphylococcus aureus* isolate according to Jawetz et al. (2015)

Sampelcode	Types of hemolysis	Types of Gram bacteria	Shape	Catalase	Coagulase	Mannitol	% Similarities
A4(1)	Gamma	Positif	Bulat, bergerombol	Positif	Positif	Positif	83%
B3(1)	Gamma	Positif	Bulat, bergerombol	Positif	Positif	Positif	83%

Sample code	Types of hemolysis	Types of Gram bacteria	Shape	Catalase	Coagulase	Mannitol	% Similarities
A3(2)	Gamma	Positif	Bulat, bergerombol	Positif	Positif	Positif	83%
A4(2)	Gamma	Positif	Bulat, bergerombol	Positif	Positif	Positif	83%
B4(3)	Beta	Positif	Bulat, bergerombol	Positif	Positif	Positif	100%

Bacterial isolates identified as *Staphylococcus aureus* then proceeded to the molecular analysis stage to detect the presence of the mecA gene. Detection of the mecA gene in *Staphylococcus aureus* bacterial isolates begins with isolating *Staphylococcus aureus* bacterial DNA using the conventional Boiling method. Quantitative DNA testing using NanoDrop spectrophotometer to determine the quality and quantity of DNA isolates. The purity test results of DNA isolates in 5 consecutive samples were 1.79; 1.39; 1.32; 1.90; and 2.20. The purity value of DNA can be influenced by protein and RNA contamination factors during the DNA isolation process (Koentjoro, 2021). The DNA concentration test results were obtained sequentially, namely 15.8 ng/µl; 10.4 ng/µl; 62.0 ng/µl; 22.9 ng/µl; and 196.4 ng/µl. The concentration of DNA isolates in this study is good enough to be used for DNA PCR amplification. According to Setyawati & Zubaidah (2021) the concentration of template DNA in the range of 0.01 - 0.1 ng/µl is good enough to be used in the DNA PCR amplification process. The use of sample DNA isolates in this study can proceed to the PCR stage. PCR amplification is performed using gene primers that are specific for each detected gene. PCR temperature and cycle settings for mecA gene detection are in accordance with the research of Khairullah et al. (2022). Furthermore, the PCR samples were electrophoresed, then the electrophoresis results were visualized with a UV Transilluminator and documented with GelDoc.

The presence of the mecA gene was not found in all samples which indicated the absence of DNA bands that appeared in the bp range which meant a negative result was obtained, and at the bottom of the 100 bp marker a dimer primer band was also seen. This band is believed to be a dimer because dimers are also found in other wells. This is in accordance with research conducted by Saraswati (2019), which states that dimers are structures formed between primer pairs, where they stick to each other and not to template DNA, because they have complementary bases. If this dimer bond is too strong, it will interfere with the DNA extension process and will result in a low product. The selection of a good primer must also pay attention to one of them is self complementary. Preferably, Self complementary has a low value because it will cause the primer pair to not bind to itself and also a hairpin structure can be formed. One way to minimize dimer formation is by optimizing PCR conditions involving annealing temperature and primer concentration optimization.

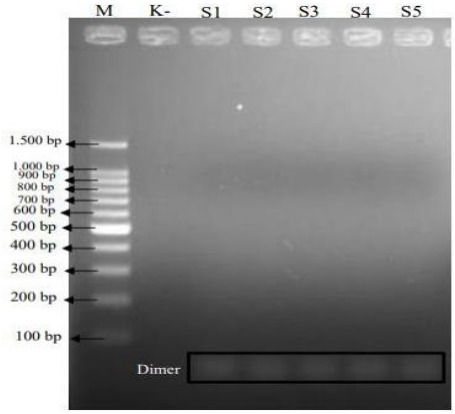


Figure 4 mecA gene visualization results from 2% agarose electrophoresis PCR results; M: Marker (100-1500 bp), K(-): Negative Control (sterile distilled water), S1: DNA sample of isolate A4(1), S2: DNA sample of isolate B3(1), S3: DNA sample of isolate A3(2), S4: DNA sample of isolate A4(2), and S5: DNA sample of isolate B4(3).

CONCLUSIONS

Based on the detection of the mecA gene of the *Staphylococcus aureus* bacteria obtained from raw cow milk in farmsin the Surabaya Region, 5 samples were identified as *Staphylococcus aureus* and all (100%) samples were not detected to have the mecA gene.

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