

Short Communication: Investigation of *mecA*-positive and Methicillin-Resistant *Staphylococcus aureus* (MRSA) in dairy goat with subclinical mastitis from traditional farms in Banyuwangi, Indonesia

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Abstract. Praja RN, Edila R, Yudhana A, Saputro AL, Hamonangan JM, Praja SS. 2024. Short Communication: Investigation of *mecA*-positive and Methicillin-Resistant *Staphylococcus aureus* (MRSA) in dairy goat with subclinical mastitis from traditional farms in Banyuwangi, Indonesia. *Biodiversitas* 25: 1638-1643. Raw milk has the potential to transmit bacteria resistant to antibiotics and serve as a medium for the development of food-borne illnesses. *Staphylococcus aureus* is a harmful bacterium that can infect host cells and carry antibiotic-resistant genes. This study aims to investigate the occurrence of Methicillin-Resistant *Staphylococcus aureus* (MRSA) which contains *mecA* coding gene from raw goat milk in Banyuwangi, East Java, Indonesia. 150 raw milk samples were collected from five goat farms in Banyuwangi, East Java, Indonesia. *S. aureus* was isolated using selective media, and antibiotic susceptibility testing was conducted using the Mueller-Hinton agar diffusion method. MRSA was confirmed using the oxacillin and cefotaxime resistance screen agar test, with the presence of *mecA* genes identified through polymerase chain reaction. The results show that the prevalence of *S. aureus* was 30.6%. Of the 46 *S. aureus* isolates, 28 (60.87%) were categorized as MRSA. The *mecA* gene was observed in 9 (32.14%) MRSA isolates. The presence of MRSA in raw goat milk poses a significant public health threat, as bacteria carrying the *mecA* gene for antimicrobial resistance can spread through animal products like raw milk. Enhanced hygiene and sanitation protocols in dairy goat farms are essential prevention measures that must be strictly implemented.

Keywords: Antimicrobial resistance, infectious disease, methicillin-resistant *Staphylococcus aureus*, public health, raw milk

INTRODUCTION

Goat milk has surged in popularity due to its exceptional nutritional profile, marking a significant increase in public consumption. Renowned for its superior quality, it surpasses milk from other farm animals in various aspects. Rich in minerals, proteins, and vitamins, it boasts smaller fat globules, enhancing the digestive process. Furthermore, its lower lactose content renders it suitable for individuals with lactose intolerance or dairy allergies. Despite these advantages, challenges persist, notably mastitis, a predominant health concern in dairy goat farming. *Staphylococcus aureus* stands as the main cause, leading to decreased milk production and substantial economic losses on a global scale (Silva et al. 2023). As a pathogenic bacterium, *S. aureus* poses a dual threat, inducing both clinical and subclinical mastitis in farm animals while also instigating foodborne infections in humans. This risk arises from the potential contamination of goat or sheep milk, particularly in traditional caprine and ovine milk products that bypass

pasteurization processes. Such products, cherished for their authenticity, become vectors for transmitting *S. aureus* to consumers (Khairullah et al. 2020; Narayan et al. 2023). Methicillin-Resistant *Staphylococcus aureus* (MRSA) stands as a paramount concern in human healthcare, with its prevalence steadily escalating worldwide. Notably, MRSA infections are on the rise, posing formidable challenges due to their resistance not only to β -lactam antibiotics but also to a spectrum of other antimicrobial agents including aminoglycosides, macrolides, and quinolones. In recent times, MRSA has transcended the human domain, manifesting in various animals and their byproducts, such as milk and cheese (Ranjbar et al. 2018). Nevertheless, research into the presence of MRSA in dairy goats and milk products remains limited. Methicillin resistance in staphylococci is facilitated by the *mecA* gene, which encodes a penicillin-binding protein. This genetic mechanism results in reduced affinities to β -lactam antibiotics and is ubiquitous in all methicillin-resistant staphylococci strains. Despite the crucial role of this gene in conferring resistance,

comprehensive investigations into its prevalence and implications within dairy goat populations are still lacking. Expanding our understanding of MRSA dynamics in this context is imperative for devising targeted interventions and safeguarding both animal and human health (Sudhanthiramani et al. 2015).

In Banyuwangi, subclinical mastitis in dairy goats is seldom diagnosed by veterinarians due to the absence of clinical symptoms, and the California Mastitis Test (CMT), considered the gold standard for screening, is rarely conducted. Additionally, antibiotics are freely sold without health officer oversight, allowing dairy goat farmers easy access and usage. Administering antibiotics without proper dosage control can lead to antibiotic resistance, exacerbating the issue (Tamendjari et al. 2021). Antimicrobial resistance is a critical focus in both human and veterinary medicine, highlighting the necessity of monitoring resistance levels to gather insights and assess the effectiveness of interventions (Okoko et al. 2020). Emerging antimicrobial resistance across clinical, community, and veterinary domains is a global public health threat. MRSA, capable of interspecies transmission between animals and humans, aggravates this risk (Afnani et al. 2022). Treating MRSA infections is challenging due to the bacteria's ability to resist antibiotics, reducing treatment effectiveness. MRSA produces a Penicillin-Binding Protein (PBP2a), encoded by the *mecA* gene, which prevents beta-lactam antibiotics from binding to cell wall proteins, neutralizing their effects (Patel et al. 2021).

Several genes encoding the resistance properties of *S. aureus* have been identified and characterized such as *mecA* (methicillin/oxacillin), *blaZ* (penicillin G), *aacA-D* (aminoglycosides), *tetK* and *tetM* (tetracyclines), *ermA*, *ermB*, and *ermC* (macrolide-lincosamide-streptogramin B) (Aziz et al. 2016). Therefore, understanding the prevalence and antibiotic resistance patterns of *S. aureus* in goat milk is crucial for developing effective control and prevention strategies. Currently, there are no reports on the genotypic resistance of *S. aureus* isolates to methicillin, penicillin, and tetracyclines from dairy goats in Banyuwangi. Therefore, this study aimed to detect crucial antimicrobial-resistant gene encodings, such as the *mecA* gene, in Methicillin-Resistant *S. aureus* bacteria isolated from goats' milk with subclinical mastitis in Banyuwangi, East Java Province, Indonesia.

MATERIALS AND METHODS

Ethical approval

Ethical approval was reviewed and provided by the Animal Care and Use Committee (ACUC) of the Faculty of Veterinary Medicine, Universitas Airlangga, Indonesia with certificate number: 1.KEH.121.08.2023. The study was conducted from February to July 2023. Samples were collected from five dairy goat farms in the Siliragung Subdistrict, Banyuwangi District, East Java, Indonesia (latitude: -8.493277, longitude: 114.084479). Aseptic milk samples were obtained from each teat (individual udder) of visibly healthy nursing goats. In summary, the udders were cleansed using swabs soaked in a 70% ethanol solution, and a small amount of milk was discarded. Around 10-15 mL of milk was gathered in a sterile tube, appropriately

labelled, and promptly transported to the Laboratory of Microbiology at Faculty of Health, Medicine, and Life Sciences, Universitas Airlangga. The samples were maintained at a temperature of 4°C, promptly assessed for subclinical mastitis, and subsequently subjected to culturing within a day of collection.

Study design

For this study, sample collection utilized Purposive Sampling, wherein the largest farm in the district was selected, and all productive farms out of the 5 farms were included, resulting in a total of 150 samples. Subclinical mastitis was initially assessed using the California Mastitis Test (CMT) (Kruuse, UK). CMT scores were categorized into 0, +1, +2, and +3 grades based on the reaction intensity observed. Milk samples with scores of 0 and +1 were classified as negative, while those with scores of +2 or +3 were considered positive. The CMT procedure involved mixing 3-4 mL of untreated goat milk with an equal volume of CMT reagent and gently swirling to observe gel formation and color change, indicating the reaction intensity. Samples scoring +2 and +3 underwent further isolation and identification processes for *S. aureus* (Schmidt and Póvoa 2023). The collected samples were streaked on Mannitol Salt Agar (Merck KGaA, Germany) and incubated for 24-48 hours at 37°C. Colonies showing both typical and atypical characteristics of *Staphylococcus* were microscopically examined using Gram staining. Confirmed *Staphylococcus* genus isolates underwent further identification using a biochemical panel, including catalase, oxidase (Millipore 1.00181.0002, Canada), Voges-Proskauer, coagulase, urease, and mannitol fermentation tests, to determine the species. Based on cultural, Gram staining, and biochemical examination, all examination focused on the identification of *S. aureus* (Praja et al. 2023).

Antimicrobial susceptibility test

The antimicrobial susceptibility test was conducted using the Kirby-Bauer disc diffusion method with interpretation in Mueller-Hinton agar (Oxoid CMO337, UK). Discs containing oxacillin (30mcg) and cefoxitin (30mcg) were used. The culture turbidity was adjusted to the 0.5 McFarland standard, and a sterile cotton swab was dipped in the suspension and evenly applied to the entire surface of the Mueller-Hinton agar. Antibiotic discs were then placed on the inoculated plate surface and incubated at 37°C for 16-18 hours. The diameter of the inhibition zone was measured in millimeters and interpreted as sensitive or resistant according to the Clinical and Laboratory Standards Institute (CLSI 2018) guidelines. Additionally, *S. aureus* ATCC 25923 was used as a standard control and compared with pure cultures of *S. aureus* local isolates from raw goat milk samples (Harijani et al. 2020).

Polymerase chain reaction

DNA extraction adhered to the QIAamp DNA Mini Kit protocol (51304 and 51306), preceded by initial purification on Mannitol Salt Agar. The specific primers, *mecA* F: 5'-GAA ATG GAA CGT CCG ATA A-3' and *mecA* R: 5'-CCA ATT CCA CAT TGT TTC CTA A-3', were utilized

as described by Rajabiani et al. (2014) and Rahmaniari et al. (2020). The PCR reaction utilized GoTaq® Green Master Mix (Promega, 9PIM712) as a ready-to-use solution. Amplification was conducted on a Thermal Cycler T100 machine (Bio-Rad, 186-1096) under specified conditions. Following amplification, gel electrophoresis was performed, and the results were visualized under ultraviolet illumination. Positive tests showed PCR products with a size of 310 base pairs. MRSA ATCC BAA 1026 was used as a positive control, while *S. aureus* ATCC 25923 served as a negative control (Tyasningsih et al. 2022).

RESULTS AND DISCUSSION

The examination of 150 milk samples collected from five dairy goat farms in the Siliragung Subdistrict, Banyuwangi District, East Java, Indonesia, revealed that 46 samples (30.6%) were contaminated by *S. aureus* bacteria as described in Table 1. Confirmation of *S. aureus* was achieved through phenotypic traits observed in biochemical test results and Gram staining, following a standard identification protocol (Praja et al. 2023). Therefore, it was confirmed that 30.6% of the samples were contaminated by *S. aureus*.

Based on the present findings, the prevalence rate of *S. aureus* contamination in the Siliragung Subdistrict was higher compared to a previous study conducted in different traditional farms within the same Subdistrict (Praja et al. 2023). Additionally, it exceeded the reported prevalence rates from Yogyakarta, Central Java, Indonesia (Suwito et al. 2022) as well as from other countries such as Kenya (Okoko et al. 2020) and Nigeria (Danmallam and Pimenov 2019). However, the results align with a study conducted in Pekanbaru, Indonesia, where the prevalence of *S. aureus* was recorded at 30% (Widianingrum et al. 2021). The high prevalence of *S. aureus* in the Siliragung Subdistrict is attributed to the pathogen's effective transmission through various routes. In regions where traditional hand milking is prevalent, such as the Siliragung Subdistrict, there is a heightened risk of *S. aureus* spread during milking procedures. This is because hand milking may involve direct contact between the milker's hands and the cow's teats, facilitating bacterial transmission (El-Khabaz et al. 2015).

Based on the results of the Kirby-Bauer disc diffusion method using Mueller-Hinton agar, the assessment of Methicillin-Resistant *S. aureus* (MRSA) resistance to oxacillin and cefoxitin yields some key points for discussion. Firstly, the overall resistance rate to oxacillin was high at 60.87% (28/46), indicating a significant challenge in treating MRSA infections with this antibiotic. The cefoxitin resistance rate was lower at 19.56% (9/46), suggesting that cefoxitin may still be effective against some MRSA strains, as indicated in Table 2 and Figure 2. Interestingly, all isolates that exhibited cefoxitin resistance also demonstrated resistance to oxacillin. This suggests a link in resistance mechanisms for these two antibiotics, as they are both beta-lactam antibiotics. However, the subset of isolates showing resistance to oxacillin but remaining sensitive to cefoxitin is particularly notable. This discrepancy may indicate different resistance mechanisms at play within MRSA strains, which could have implications for treatment strategies.

The findings highlight the importance of employing multiple antibiotics in testing to accurately assess MRSA resistance profiles. The high resistance rate to oxacillin underlines the need for careful monitoring and strategic treatment approaches to manage and mitigate the spread of oxacillin-resistant MRSA infections. Further investigation is required to understand the genetic and molecular basis of the resistance variations observed between oxacillin and cefoxitin.

The isolates confirmed to be resistant to both oxacillin and cefoxitin underwent further genotypic testing using PCR to identify the presence of the *mecA* gene. Out of the 28 isolates confirmed to be resistant, nine isolates (32.14% of the tested isolates) tested positive for the *mecA* gene, as shown in Table 2. The findings suggest that the *mecA* gene is a key factor in MRSA resistance to beta-lactam antibiotics, including oxacillin and cefoxitin. The presence of this gene in approximately one-third of the tested isolates indicates its significant role in the resistance mechanism. However, the fact that not all isolates resistant to oxacillin and cefoxitin tested positive for the *mecA* gene suggests the possibility of other resistance mechanisms being involved in the remaining isolates. These results underscore the complexity of MRSA resistance and the need for comprehensive testing to fully understand the genetic basis of resistance. The identification of the *mecA* gene in MRSA isolates can provide valuable information for guiding targeted treatment strategies and for monitoring the spread of resistance within healthcare settings. Further research may be required to uncover additional genetic factors contributing to resistance in isolates that do not possess the *mecA* gene.

Table 1. Isolation and identification of *Staphylococcus aureus* from raw goat milk samples in Siliragung Subdistrict, Banyuwangi District, Indonesia

Farm	Samples (n)	<i>Staphylococcus aureus</i> positive (n)	Percentage (%)
1	20	6	30
2	38	11	28.95
3	26	9	34.61
4	34	13	38.23
5	32	7	21.87
Total	150	46	30.6%

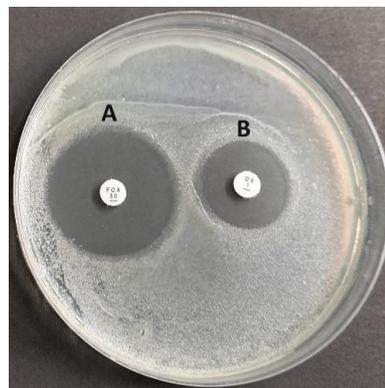
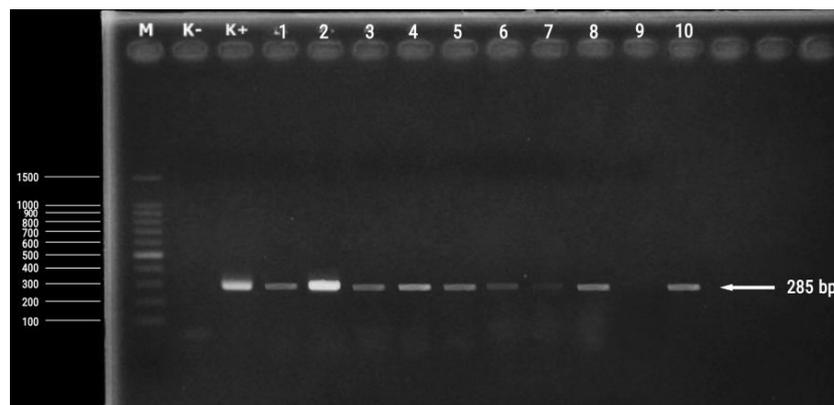


Figure 1. The result of the antibiotic diffusion test. A. Cefoxitin indicated sensitive (31 mm), and B. Oxacillin indicated resistance (20 mm)

Table 2. Oxacillin (OX) and Cefoxitin (FOX) disc diffusion test, and the occurrence of *MecA* positive *Staphylococcus aureus* based on Polymerase Chain Reaction (PCR) method

Farm	<i>Staphylococcus aureus</i> (n = 46)				<i>MecA</i>
	OX disc diffusion		FOX disc diffusion		
	Sensitive (%)	Resistant (%)	Sensitive (%)	Resistant (%)	
1	2 (4.35%)	4 (8.7%)	5 (10.87%)	1 (2.17%)	1
2	6 (13.04%)	5 (10.87%)	9 (19.56%)	2 (4.35%)	2
3	3 (6.52%)	6 (13.04%)	7 (15.22%)	2 (4.35%)	2
4	5 (10.87%)	8 (17.39%)	10 (21.73%)	3 (6.52%)	3
5	2 (4.35%)	5 (10.87%)	6 (13.04%)	1 (2.17%)	1
Total	18 (39.13%)	28 (60.87%)	37 (80.43%)	9 (19.56%)	9

**Figure 2.** *MecA* PCR results with positive bands at 285 bp. Line K-: *Staphylococcus aureus* ATCC 25923 (Negative Control), Line K+: MRSA ATCC BAA 1026 (Positive Control), Line 1-8: Positive isolate for *mecA* gene and 10: Negative isolate for *mecA* gene

The primary cause of oxacillin resistance in MRSA is the presence of the *mecA* or *mecC* genes. These genes encode Penicillin-Binding Protein 2a (PBP2a), which exhibits reduced binding affinity to beta-lactam antibiotics like oxacillin. As a result, these genes alter the antibiotic's target site, rendering it ineffective against the bacteria (Hughes et al. 2022). After reviewing the PCR test results, it's important to note that out of the 28 oxacillin-resistant *S. aureus* strains, 9 were found to carry the *mecA* gene. However, it's crucial to acknowledge that the presence of *mecA* or *mecC* genes doesn't always lead to oxacillin resistance. In some cases, *S. aureus* strains with the *mecA* gene may still exhibit oxacillin Minimum Inhibitory Concentration (MIC) levels considered susceptible. These strains are referred to as Oxacillin-Susceptible *mecA*-positive *S. aureus* (OS-MRSA) (Ramandinianto et al. 2010). Furthermore, there are oxacillin-resistant phenotypes of *S. aureus* that do not involve *mecA*, known as Borderline Oxacillin-Resistant *S. aureus* (BORSA). These strains are capable of conferring resistance to oxacillin through mechanisms other than the *mecA* gene (Messias et al. 2023). Moreover, genomic adaptations to oxacillin can occur through the acquisition of mutations associated with low-level resistance in various genes. These genes include those involved in c-di-AMP signal transduction pathways and the clpXP chaperone-protease complex (Santos et al. 2021).

The emergence of cefoxitin resistance in *S. aureus* arises from mutations occurring in the genes responsible for encoding Penicillin-Binding Proteins (PBPs) and their associated proteins. This includes alterations in the promoter region, contributing to resistance development (Messias et al. 2023). All the isolates resistant to cefoxitin also exhibit resistance to oxacillin, but not all oxacillin-resistant isolates display resistance to cefoxitin. Different mechanisms of resistance can result in certain MRSA strains being resistant to oxacillin while remaining susceptible to cefoxitin. Studies conducted over the years have revealed that MRSA isolates positive for the *mecA* gene, yet susceptible to oxacillin and cefoxitin, display mutations in *mecA* that cause premature stop codons and frameshift mutations. These alterations make them susceptible to cefoxitin but resistant to oxacillin. These findings highlight the concept that various genetic mutations and variations in penicillin-binding proteins contribute to the diverse patterns of resistance observed in MRSA strains (Schmidt and Póvoa 2023).

Resistance to beta-lactam sensitive antibiotics and related antibiotics among *S. aureus* is commonly observed and may be facilitated by defensive mechanisms inherent within *S. aureus* colonies. Since Staphylococci can express Penicillin-Binding Protein 2a (PBP2a), it can be speculated that the *S. aureus* isolates produce this defensive enzyme to protect themselves from beta-lactam related antibiotics

(Chai et al. 2020). Confirmed *S. aureus* isolates through PCR are considered more reliable compared to those confirmed by phenotypic screening methods. This is not surprising, as phenotypic screening methods often yield ambiguous results due to inconsistent test performances, a large number of field isolates, and varying biochemical characteristics among bacterial isolates that deviate from the known genus and species patterns. Additionally, it's crucial to employ the most effective screening method using molecular identification to confirm MRSA strains that may contaminate the environment and be transmitted to animal products, including raw milk (El-Deeb et al. 2018; Qian et al. 2019; Harijani et al. 2020).

The variation in antibiotic resistance among different countries could be influenced by various factors, including farm management practices, particularly the intensity of antibiotic use. Careless and excessive use of antibiotics by dairy goat farmers can pose a potential risk factor leading to resistance. Penicillin, being the most common antibiotic, is capable of destroying the cell wall of Gram-positive bacteria (Suwito et al. 2022; Praja et al. 2023). The chemical structure of penicillin includes a beta-lactam ring. Resistance to oxacillin and cefoxitin, which are also derivatives of penicillin, in dairy goats with subclinical mastitis in Banyuwangi may be caused by the production of beta-lactamase enzymes, which can destroy the beta-lactam ring. Consequently, it is necessary to substitute antibiotics other than penicillin or its derivatives. The rise in antibiotic resistance among bacterial populations is often attributed to the widespread use of antibiotics in veterinary medicine (Njoroge et al. 2018), medicine and agriculture (Danmallam and Pimenov 2019). Monitoring antibiotic susceptibility profiles is essential for carefully selecting antibiotics, thereby improving treatment efficacy and minimizing the rise of antibiotic resistance.

In conclusion, this study reveals a 30.6% prevalence of *S. aureus* bacteria in raw milk from dairy goats with subclinical mastitis. The detection of the *mecA* gene in 28 isolates confirms the presence of Methicillin-resistant *S. aureus* (MRSA). The existence of MRSA in raw goat milk poses a significant threat, as these strains are pathogens not only in animals but also in humans. Consequently, there is a need for antibiotic substitution to prevent resistance mechanisms among dairy goats with subclinical mastitis. Strict policies regarding antibiotic use and veterinarian supervision are crucial for implementing disease control, treatment, and managing antibiotic usage, especially in farm animals.

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