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Identification of Methicillin-resistant *Staphylococcus aureus* Isolated from Dairy Cow's Milk in Tulungagung District, Indonesia

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ABSTRACT

Background: Mastitis is one of the factors contributing to the health-related decreased milk production and quality for dairy cows. Mastitis in ruminants is a serious bacterial disease caused by *Staphylococcus aureus.* Staphylococcal bacteria are becoming increasingly resistant to many classes of antibiotics, particularly β-lactam families like the MRSA strain. Laboratory tests are required to determine the level of bacterial resistance and to identify MRSA isolates sourced from dairy cows in Tulungagung District.

Methods: 110 milk samples were isolated on MSA media followed by Gram's staining and biochemical tests. Kirby-bauer diffusion test-based assessment of antibiotic sensitivity. The *S. aureus* isolates that underwent the MRSA identification test were *S. aureus* isolates that had developed a resistance to β-lactam antibiotics.

Result: A total 81 samples of the 110 isolated milk samples were determined to be positive for *S. aureus.* Out of total isolates, 25 isolates of *S. aureus* had the highest level of oxacillin resistance. As many as 4 isolates were confirmed to be MultiDrug Resistance (MDR) and 17 MRSA isolates were discovered from 100 samples of dairy cows. Early diagnosis of MRSA infection is crucial since it can be challenging to treat because this type of bacteria is known to be resistant to several drugs and spreads readily.

Key words: Milk, MRSA, Public health, *Staphylococcus aureus*.

INTRODUCTION

Milk is a nutrient dense (sources of nutrients mainly the protein). Even milk is regarded as a nutritious complement for the process of human growth and development. The reason for the significant requirement and desire for milk is its complete nutritious value (Smith *et al*., 2022). The production of nutritious and high-quality milk should be increased in order to boost revenue for everyone, especially dairy farmers and to promote public health by encouraging healthy milk consumption (Britt *et al*., 2018). However, Indonesia still has a high need and demand for milk that is inversely correlated with a low supply of milk, both in terms of quantity and quality (Khairullah *et al*., 2022). Mastitis is one of the factors contributing to the health-related decreased milk production and quality for dairy cows (Khairullah *et al*., 2020).

Dairy cows owned by businesses and small farmers alike frequently get mastitis, or udder inflammation, which results in significant losses (in milk production). In Indonesia, mastitis affects dairy cattle at a rate of 85% and because the majority of these cases are subclinical infections, prompt treatment or control is not always possible (Putra *et al*., 2023). This mastitis incidence may result in significant economic losses, particularly because of the decreased milk output, which may account for up to 25% of total production. Numerous pathogenic bacteria that enter the udder through the teat canal are one of the many causes that contribute to mastitis (Hughes and Watson, 2018). The incidence of mammary gland infection is caused by a number of predisposing factors, such as unsanitary milking, improper milking management, sores on the teats and the presence

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of pathogenic microorganisms in the cage environment (Widodo *et al*., 2022).

During manual milking, transmission of mastitis-causing pathogenic bacteria can take place between cows or from one udder's teat to another. This could happen because of unhygiene in milker's hands, the udder-washing water, the cloth used to dry the udder prior and after milking, or other tools used during milking (Goulart and Mellata, 2022).

Unhygienic handler adds *Staphylococcus aureus*, a causative agent of Mastitis in ruminants. Mastitis is a serious bacterial disease which is the primary cause of subclinical or clinical mastitis in dairy cows, leading to enormous losses for the dairy sector (Tarazona-Manrique *et al*., 2019). Mastitis brought on by *S. aureus* manifests as subacute or persistent inflammation. Despite the fact that these bacteria can grow and develop well in milk. *S. aureus* contamination might happen because of the presence of causative bacterium in fresh milk either during milking or processing. The teats of infected cows are the primary reservoir of *S. aureus* (Exel *et al*., 2023).

Human affecting *S. aureus* can cause infection, particularly Methicillin-Resistant *Staphylococcus aureus* (MRSA) is known to be resistant to several medications (Yunita *et al*., 2020), it is challenging to treat this illness. There are several different types of Staphylococcal infections, including as pneumonia, bacteremia and postoperative wound contamination (Decline *et al*., 2020). *S. aureus* has a very high potential for producing a wide range of illnesses and food poisoning in both people and animals (Rahmaniar *et al*., 2020). It is known that *S. aureus* produces a variety of heat stable enterotoxins in milk. There have been reports of *S. aureus* enterotoxin food poisoning in milk and dairy products (Tyasningsih *et al*., 2022).

Treatment of *S. aureus* in cases of mastitis is known to be challenging, particularly due to the difficulty in selecting the appropriate type of antibiotic in the field and the ease with which resistance develops (Sharun *et al*., 2021). Staphylococcal bacteria are becoming increasingly resistant to many classes of antibiotics, particularly β-lactam families like the MRSA strain, its resistance to various types of antibiotics is developing rapidly (Alexander *et al*., 2023). The plasmid, which allows for quick transmission between Staphylococci, contains the gene for the penicillinase enzyme. Additionally, numerous hospitals have noted an increase in the prevalence of *S. aureus* and MRSA, which are often both types of these bacteria that are MDR (Waruwu *et al*., 2022).

Sendang (a sub district in Tulungagung district) is one of a major milk-producing regions of East Java (Khairullah *et al*., 2023) revealed that breeders poor care practices for dairy cows with subclinical mastitis eventually progresses to clinical mastitis. There have been numerous reports of subclinical mastitis cases in Tulungagung district, which is consistent with the high volume of milk production and the presence of dairy cattle (Ramandinianto *et al*., 2020). Mastitis instances are frequently caused by infections caused by the MRSA bacteria. Making the appropriate antibiotic choice can lower the number of mastitis cases (Wilm *et al*., 2021).

The incidence of MRSA infection can be a health problem for the community. Laboratory tests are required to determine the level of bacterial resistance and to identify MRSA isolates sourced from dairy cows in Tulungagung district, so that this research data can be useful for the prevention and treatment of mastitis incidents on cattle farms of dairy industry.

MATERIALS AND METHODS

Study area and sample collection

The study was carried out from March 2023 to May 2023. Dairy cow milk samples were collected from different dairy farms in the Sendang Subdistrict region of the Tulungagung district, while bacterial isolation and sensitivity testing were conducted at the Veterinary Microbiology Laboratory, Faculty of Veterinary Medicine, Airlangga University. A total of 110 milk samples were collected. A 60 ml sample bottle was used to hold each milk sample that was collected. The samples are then transported to the lab and were being kept under refrigerated conditions using a cooling box until analysis.

Isolation and identification of *S. aureus*

Milk samples obtained from enrichment media were purified and cultivated on Mannitol Salt Agar (MSA) medium before being incubated at 37°C for 24 hours (Dilnessa and Bitew, 2016). Identification was carried out by evaluation based on morphological cultural characteristics, followed by microscopic analysis using Gram's staining technique, which reveals clusters of Gram-positive bacteria in the shape of coccus (Sadiq *et al*., 2020). The catalase test and the coagulase test were used in biochemical assays to identify the *S. aureus* species. Hydrogen peroxide (H_2O_2) 3% was used to clean glass objects to perform catalase tests and the solution was then mixed with one colony's growth product (Lagos *et al*., 2016). For the coagulase test, *S. aureus*-related colonies were extracted from MSA media using ose (Check the ose), then these colonies were added to 3 ml of Nutrient Broth media and cultured at 37°C for 24 hours. A vortex was used to thoroughly mix 1 ml of rabbit plasma before incubation, which was followed by a 24-hour incubation period (Javid *et al*., 2018).

Antibiotic sensitivity test

Kirby-bauer diffusion test-based assessment of antibiotic sensitivity. This method was carried out by taking *S. aureus* bacterial isolates from MSA media using a sterile cotton swab in a suspension containing physiological saline with standard Mc Farland turbidity of 0.5 and then wiping it evenly on the surface of the Mueller Hinton Agar (MHA) media. The MHA media surface was covered with antibiotic discs, which were subsequently incubated for 24 hours at 37°C (Mutmainnah *et al*., 2020). There were 4 types of antibiotics used for the sensitivity test in this study, namely the aminoglycoside group (Gentamicin 10 µg), the macrolide group (Erythromycin 15 µg), the tetracycline group (Tetracycline 30 µg) and the β-lactam group (Cefoxitin 30 µg and oxacillin 30 µg). Each antibiotic disc was attached to the MHA media's

surface at a distance of 25 to 30 mm. The Clinical and Laboratory Standard Institute (CLSI, 2020) provides the basis for the interpretive standard for determining the diameter of the inhibitory zone.

MRSA identification test

The *S. aureus* isolates that underwent the MRSA identification test were *S. aureus* isolates that had developed a resistance to β-lactam antibiotics (Cefoxitin and oxacillin). Oxacillin Resistance Screening Agar Base (ORSAB) media that had been combined with Oxacillin Resistance Selective Supplement was used to inoculate the bacterial colonies from MHA medium in order to confirm the presence of MRSA. Following that, ORSAB medium was incubated for 24 hours at 37°C. When the bacterial colonies on ORSAB media turn blue, it is a positive indicator of MRSA (Ibrahim *et al*., 2017).

RESULTS AND DISCUSSION

Bacterial isolates

Based on the results of the sample examination and the results of the morphological culture, Gram staining and biochemical testing, 81 samples (73.64%) of the 110 isolated milk samples were determined to be positive for *S. aureus* (Table 1). The emergence of golden yellow bacterial colonies on MSA media suggested successful morphological culture of *S. aureus* (Fig 1). The presence of purple colonies and globular, clustered forms during Gram staining indicates a positive Gram result (Fig 2). The formation of gas bubbles in the catalase test (Fig 3) and plasma clots in the coagulase test are signs of positive biochemical tests for *S. aureus* (Fig 4).

MSA media was utilized for the isolation in this study because *S. aureus* can ferment mannitol employing streak media with multilevel patterns for early culture (Pumipuntu *et al*., 2017). Gram-stained *S. aureus* bacteria displayed purple coloration under a 1000x magnification microscope and a clustered, spherical morphology that is typical of Staphylococcus germs (Kobayashi *et al*., 2020). A positive catalase test result means that the bacteria are either aerobic or facultatively anaerobic, meaning that they use oxygen to breathe (Linzner *et al*., 2022). A positive coagulase test result means *S. aureus* can produce coagulase enzymes that can cause the activation of nonproteolytic pro-thrombin and fibrinogen cleavage (Trivedi *et al*., 2018).

Antibiotic resistance of *S. aureus*

According to this investigation, 25 isolates of *S. aureus* had the highest level of oxacillin resistance. While there are 18 isolates of tetracycline, 11 isolates of erythromycin, 9 isolates of cefoxitin and 6 isolates of gentamicin-resistant *S. aureus* (Table 2).

Numerous factors contribute to the development of bacteria that are resistant to antibiotics, including inadequate use of antibiotics, a populace that is reluctant to use such medications, treatment antibiotics that are the same but have been used repeatedly, inadequate research into new antibiotics and inadequate government oversight of the manufacture and distribution of antibiotics (Cook and Wright

Fig 1: *S. aureus* colonies in MSA.

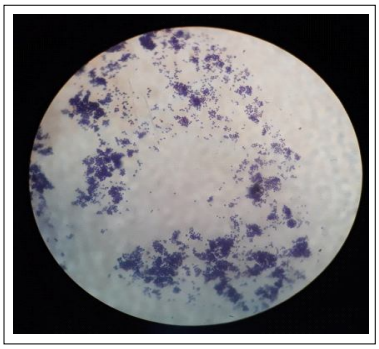

Fig 2: Gram-stained *S. aureus* colonies under a microscope with a magnification of 1000x.

Fig 3: Catalase test results indicate *S. aureus* positivity.

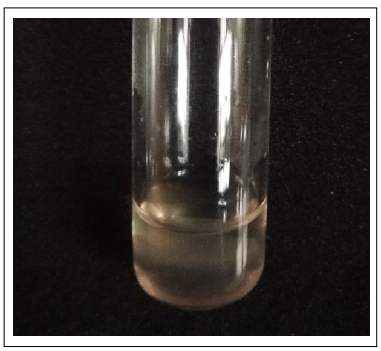

Fig 4: Coagulase test results indicate *S. aureus* positivity.

2022). An opportunistic pathogenic bacteria called *S.aureus* is frequently found in both people and animals (Howden *et al*., 2023). This bacterium may cause financial losses by reducing the quantity and quality of milk produced by dairy cows (Tesfaye *et al*., 2021).

The profile of antibiotic resistance derived from the results of the *S. aureus* resistance test to antibiotics revealed that 27 *S. aureus* isolates (33.33%) were found to be resistant to the 1 class of antibiotics tested out of a total of 81 *S. aureus* isolates. Whereas 9 isolates (11.11%) were resistant to 2 classes of antibiotics and 4 isolates (4.99%) were confirmed to be MDR because it was resistant to three or more classes of antibiotics (Table 2 and Fig 5).

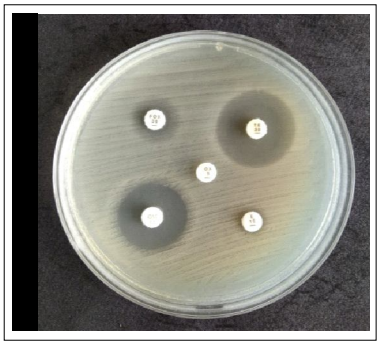

Fig 5: Analyze the susceptibility to antibiotics of a *S. aureus* isolate cultured on MHA.

As many as 4 out of every 81 *S. aureus* isolates were labeled as multidrug resistant because they exhibited resistance to at least 3 different antibiotic classes. A latent resistance gene's expression, a gene with a resistance determinant, or genetic mutation are the main ways that bacterial colonies survive in a threatened state (Palma *et al*., 2020). The majority of bacterial information is encoded by chromosomes and in MDR bacteria, this results in a multistep mutation that gradually increases resistance (Gogry *et al*., 2021). Extrachromosomal genes, which can be found in plasmids or bacteriophages, are present in some bacteria (Deutsch *et al*., 2018).

On transposons and integrons, where the factor R plasmids, also known as infectious plasmids, can be transferred, resistance factors can be transferred from chromosomes to plasmids or vice versa (Partridge *et al*., 2018). The Resistance Transfer Factor (RTF) segment and the r-determinant (r-unit) make up the R factor itself, with the RTF segment allowing the transfer of the R factor and the r-units each carrying characteristics associated with antibiotic resistance (Helinski 2022).

Dairy cows from Tulungagung district produced milk that included four isolate of *S. aureus* that was MDR (Table 3). This may account for the fact that Tulungagung district still had a low number of MDR cases of *S. aureus*, with 4 isolates from 110 milk samples analyzed.

Table 1: Isolation and identification of *S. aureus.*

Location	Sample size	Isolation test on MSA	Identification test			Positive *S. aureus* (%)
			Gram stain	Biochemical test		
				Catalase	Coagulase	
Tulungagung	110	98	98	87	81	81 (73.64%)

Note: % (Percentage of positive)

Table 2: Isolated *S. aureus* resistance profile by antibiotic group.

Group of antibiotics	Resistance profile	Number of isolates (n=81)	Total number of isolates (%)
		Resistant isolates (%)	
0	No one is resistant	41 (50.62%)	41 (50.62%)
1	GM	2 (2.47%)	27 (33.33%)
	E	5 (6.17%)	
	TE	7 (8.64%)	
	OX	12 (14.81%)	
	FOX - OX	1 (1.23%)	
2	GM - OX	1 (1.23%)	9 (11.11%)
	E - TE	1 (1.23%)	
	TE - OX	2 (2.47%)	
	FOX - OX - E	4 (4.99%)	
	FOX - OX - TE	1 (1.23%)	
≥3	E - TE - OX	1 (1.23%)	4 (4.99%)
	GM - E - FOX - OX	1 (1.23%)	
	GM - E - FOX – TE - OX	2 (2.47%)	

Note: GM = Gentamicin, E = Erythromycin, FOX = Cefoxitin, TE = Tetracycline, OX = Oxacillin.

Table 3: *S. aureus* isolates with a profile MDR.

Location	Sample code	Resistance profile	Antibiotic				
			GM	E	FOX	TE	OX
Tulungagung	T 17	GM - E - FOX - OX	✓	✓	✓	–	✓
	T 39	GM - E - FOX - TE - OX	✓	✓	✓	✓	✓
	T 70	E - TE - OX	–	✓	–	✓	✓
	T 77	GM - E - FOX - TE - OX	✓	✓	✓	✓	✓

Note: ✓= Resistant, - = Sensitive, T = Tulungagung, GM = Gentamicin, E = Erythromycin, FOX = Cefoxitin, TE = Tetracycline, OX = Oxacillin.

Table 4: Total number confirmed MRSA by ORSAB.

Location	A resistance group of antibiotics	Number of isolates tested ORSAB (n=25)	Positive ORSAB test	Number of MRSA
Tulungagung	1	13	11 (44%)	17 (68%)
	2	8	5 (20%)	
	≥3	4	1 (4%)	
Total number of MRSA			17 (68%)	17 (68%)

Note: *S. aureus* isolates screened for MRSA were only *S.aureus* isolates that were resistant to β-lactam antibiotics (cefoxitin and oxacillin).

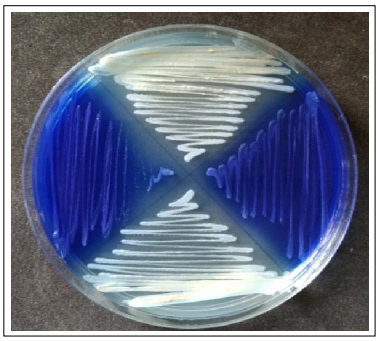

Fig 6: ORSAB test for MRSA identification.

Confirmation of MRSA

The findings of the MRSA identification of *S. aureus* isolates that were known to be resistant to cefoxitin and oxacillin revealed that 17 out of 25 isolates were positive on the ORSAB test (Table 4 and Fig 6). This demonstrates that 17 MRSA isolates (68%) were discovered from 110 samples of dairy cows that were analyzed, demonstrating that MRSA infection rates in dairy farms in Tulungagung district are quite high.

A significant DNA element called SCC*mec*, measuring 20-100 kb, is inserted into the *S. aureus* strain to transform it into an MRSA strain (Harkins *et al*., 2017). The Penicillin Binding Protein 2a (PBP 2a) genes, which are responsible for MRSA resistance, are encoded by the Staphylococcal Cassette Chromosome *mec* (SCC*mec*) proteins *mec*A and *mec*C (Miragaia 2018). Changes in the typical PBP, specifically PBP 2 to PBP 2a, cause MRSA resistance to all â-lactam class antibiotics (Da Costa *et al*., 2018). PBP 2a has a very low affinity for β-lactams, allowing the MRSA strain to survive and produce the bacterial cell wall even when it is cultivated in media containing high levels of β-lactams (Fergestad *et al*., 2020).

A dangerous bacterial strain called MRSA is frequently seen in people, but it can also colonize and infect other species, including livestock, wildlife, pets and poultry (Silva *et al*., 2023). MRSA in animals is significant not just from an economic and welfare point of view, but also because these strains have the potential to serve as a reservoir for zoonotic infections in humans (Correia *et al*., 2019). Early diagnosis of MRSA infection is crucial since it can be challenging to treat because this type of bacteria is known to be resistant to several drugs and spreads readily (Hassoun *et al*., 2017).

CONCLUSION

Milk from dairy cows in Tulungagung district, had up to 17 MRSA strains. A nearby veterinarian must assess the efficacy of using antibiotics to treat mastitis infection as a kind of therapy. Since no isolates of bacteria in this investigation were gentamicin-resistant, gentamicin antibiotics can still be utilized as an alternate treatment for mastitis infection.

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Conflict of interest

All authors declare that they have no conflicts of interest.

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