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Methicillin Resistant *Staphylococcus aureus* (MRSA) Isolation and *mec*A Gene Detection from Milk and Farmer Hand Swab in Tulungagung, Indonesia

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ABSTRACT

Dairy farms have a high incidence of MRSA infections due to the repeated use of the same medicines on dairy cows and the physical contact between farmers and cows during milking. This study evaluated the incidence of MRSA in dairy cow milk and farmer hand swabs in Tulungagung, Indonesia. Using oxacillin and cefoxitin diffusion disks, phenotypic detection approaches were evaluated, then transferred to the Oxacillin Resistance Screening Agar Base (ORSAB) test and genotypically verified using PCR to find the *mec*A gene encoding MRSA. One hundred ten dairy cow milk samples and 45 farmer's hand swabs were collected from Tulungagung, East Java, Indonesia. Mannitol salt agar (MSA) was used for cultivation and purification. The disk-diffusion test used oxacillin and cefoxitin to identify *S. aureus* resistance. Oxacillin and cefoxitin-resistant *S. aureus* isolates were tested for MRSA using ORSAB. In addition, MRSA isolates were PCR-tested for the *mec*A gene. *S. aureus* was found in 110 (70.97%) of 155 isolates. Of the total 110 isolates of *S. aureus*, 16 (14.54%) and 39 (35.45%) were known to be resistant to Cefoxitin and Oxacillin, respectively. When tested with ORSAB, 23 isolates from 55 resistant isolates showed positive results for MRSA. Dairy milk was the source of most MRSA, which is 15 isolates, while hand swabs only carried 8 isolates. However, PCR analysis only found *mec*A gene in two isolates. According to this study, many MRSA isolates were found in dairy farms in Tulungagung, Indonesia, but only a few have the *mec*A gene.

Keywords: milk, swab hand, mecA; MRSA; public health

INTRODUCTION

Cow's milk is an animal-derived product that can transmit a variety of harmful microorganisms which can impact public health, a condition called milk borne disease (MBD) (van den Brom *et al.*, 2020; Anshariet *et al.*, 2021). It is believed that *S. aureus* commonly contaminates milk and may potentially cause health issues (Khairullah *et al.*, 2020a). *S. aureus* can be isolated on the surface of the skin and mucous membranes of both animals and humans (Hanssen *et al.*, 2017). Colonization of *S. aureus* can

result in moderate to severe diseases, such as dermatitis, arthritis, endocarditis, septicemia, and foodborne illness (Reddy *et al.,* 2017). According to some previous studies, *S. aureus* infects people through tainted milk (Dittmann *et al.,* 2017; Ramandinianto *et al.,* 2020; Gebremedhin *et al.,* 2022). Typically, *S. aureus* is present on the surface of the udder skin of healthy dairy cows and those with mastitis; hence, they can serve as a source of *S. aureus* contamination in milk (Abril *et al.,* 2020). Due to the improper use of antibiotics, these bacteria can rapidly develop into strains resistant to such antibiotics (Keman & Soyer, 2019). Various types of beta-lactam antibiotics, including penicillin, monobactam, carbapenem, oxacillin, and cefoxitin, can be used to treat *S. aureus* (Chai *et al.,* 2020). However, specific antibiotics used in the detection of methicillin resistant *S. aureus* (MRSA) screening by disk diffusion are oxacillin and cefoxitin. MRSA is *S. aureus* that has developed β-lactam resistance (Bhawini *et al.,* 2019).

Previous research has revealed that MRSA can concern animal and human health (Anjum *et al.,* 2019). Most MRSA infections in dairy farms were linked to continuous antibiotics used on dairy cows that may not shift (Khairullah *et al.,* 2022a) and to the physical contact between dairy cows and milkers during milking (Gopal & Divya, 2017). Therefore, it is possible for this infection to be derived through milk from the hands of farmers when milking (Khairullah *et al.,* 2022b). Sendang District in Tulungagung, East Java Province, Indonesia, is home to one of Indonesia's main milk production hubs (Parmawati, 2019). In Tulungagung, antibiotics have been frequently used to treat infections on dairy farms, particularly in cases of mastitis (Widianingrum *et al.,* 2022). This frequent use of antibiotics has led to the potential of MRSA infection on Tulungagung dairy farms (Tibebu *et al.,* 2021).

The *mec*A gene is a gene that mediates MRSA strains (Miragaia, 2018). According to Uehara (2022), this gene is located on the staphylococcal cassette chromosome *mec* (SCC*mec*), a cellular genetic component. This gene has a role in creating penicillin-binding protein 2a (PBP 2a) (Fishovitz *et al.,* 2014). PBP 2a has a lesser affinity for β-lactam antimicrobials than regular PBP, allowing MRSA to grow and construct cell walls even in the presence of high quantities of β-lactam (Fergestad *et al.,* 2020). Even though polymerase chain reaction (PCR) detection of the *mec*A gene encoding MRSA is widely used to confirm the presence of MRSA, this method cannot be carried out in all laboratories due to cost and resource limitations (Pournajaf *et al.,* 2014). Finding MRSA, it is possible to circumvent the limitations of PCR by employing the disk diffusion method with cefoxitin and oxacillin, followed by an inquiry using ORSAB (Khairullah *et al.,* 2022b).

This study aimed to investigate MRSA in farmer's hand swabs and dairy cow's milk in Tulungagung, East Java, Indonesia, and compared phenotypic detection methods using oxacillin and cefoxitin disc diffusion and genotypically utilizing PCR test to detect *mec*A gene encoding MRSA.

MATERIALS AND METHODS

Study Samples and Collection

The study has received approval from the Health Research Ethical Clearance Commission, Universitas Airlangga, with No. 353/HRECC.FODM/VI/2021 dated June 30, 2021. All methods in this study were performed following relevant guidelines and regulations. The sample collection was followed from September to October 2021. Samples were taken based on a completely randomized sampling design. A total of 110 dairy cow's milk samples and 45 samples of farmer's hand swabs were collected from 45 smallholder dairy farms in Sendang District, Tulungagung Regency, East Java Province, Indonesia. The farmers have informed consent and were on board with our research.

Isolation and Identification of *S. aureus* Isolates

Each cow had 30 ml of milk samples taken at the third milking, which were then placed in a 60 mL tube. Each farmer's hand was swabbed with a sterile cotton swab after milking, and the collection swabs were kept on Amies media. In a 20 mL reaction tube with 9 mL of Mannitol Salt Broth (MSB), 1 mL of each milk sample was added. For Amies media with a hand swab sample, the sample was vortexed once it became liquid, and then 1 mL of the liquid was added to a 20 mL reaction tube containing the same amount of MSB. The reaction tubes were then incubated at 37 °C for 24 h. The samples were recultured on Mannitol Salt Agar (MSA) (Oxoid) followed by overnight incubation at 37 °C.

Gram staining is used to obtain a picture of Gram-positive bacteria in the shape of clusters and cocci, like a bunch of grapes, during a microscopic analysis of bacteria (Effendi *et al.,* 2018). Catalase and coagulase tests were employed to conduct the biochemical analysis. The catalase test was conducted by dropping 3% hydrogen peroxide (H2O2) over bacterial colonies on top of an object glass (Effendi *et al.,* 2019). Leaking 200 µL of rabbit plasma into a coagulase reaction tube containing bacterial colonies and incubating it for 24 hours at 37 °C served as the coagulase test (Tyasningsih *et al.,* 2019).

Susceptibility Disc Diffusion Methods

Staphylococcus aureus susceptibility tests were examined in assent with the Clinical and Laboratory Standards Institute (CLSI) 2020 guidelines. Oxacillin and cefoxitin were standard antibiotics used to detect MRSA by disk diffusion because they have high sensitivity and specificity. On Muller Hinton Agar (MHA) (Oxoid) plates, the isolates' sensitivity to oxacillin 30 µg and cefoxitin 30 µg (Oxoid) was assessed. Isolates would be purified on MSA (HiMedia), incubated at 37 °C for 24 h at a 0.5 Mc Farland concentration, and then taken using a sterile cotton swab size S. The antibiotic discs oxacillin and cefoxitin were put 5 cm apart over MHA cultured with isolates and incubate at 37 °C for 24 h to determine the inhibition zone.

ORSAB Test

Staphylococcus aureus isolates resistant to oxacillin and cefoxitin were verified using Oxacillin Resistance Screening Agar Base-ORSAB test (HiMedia). On MHA, some *S. aureus* colonies were isolated and subsequently cultivated on ORSAB (HiMedia) with Oxacillin Resistance Selective Supplement (HiMedia) (Mustapha *et al.*, 2016).

PCR Analysis

All *S. aureus* isolates that were resistant to cefoxitin and ORSAB-positive continued to a PCR assay to identify the appearance of the *mec*A gene (Mariyam & Gopinath, 2016). The DNA extraction procedure was conducted following the QIAamp DNA Mini Kit protocol (51306 and 51304), by which the isolates were previously purified on MSA (HiMedia) and inoculated on MHA (Oxoid). The primers utilized were 5'-AAA ATC GAT GGT AAA GGT TGG C-3' (*mec*A Forward) and 5'-AGT TCT GCA GTA CCG GAT TTG C-3' (*mec*A Reverse) (Nam *et al.*, 2019). The result PCR mixture was comprised of GoTaq® Green Master Mix (Promega), a ready-to-use solution including Taq DNA polymerase, dNTPs, MgCl2, and reaction buffer. Utilizing a Thermal Cycler T100 machine (Bio-Rad) for 40 cycles in 25 µL of the reaction mixture, DNA was amplified as follows: 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. Ten microliters of PCR product were evaluated by 2% agarose gel electrophoresis, and the gel was seen under ultraviolet light (Jiang *et al.*, 2021). A positive test indicates the presence of a PCR single band in the 533 base pair (bp).

RESULTS

Out of 155 samples taken from 45 dairy farms in the Tulungagung area of East Java, Indonesia, 110 (70.97%) of the isolation and identification tests discovered *S. aureus* isolates. Eighty-one isolates from dairy milk and 29 isolates from farmer's hand swab samples are shown in Table 1. On MSA media, *S. aureus* displayed colony phenotypic characteristics such as a change in medium color from red to yellow, which denoted the mannitol fermentation. As illustrated in Figure 1, the colonies possess a variety of colors, including white, orange, and yellow. The Gram staining test identifies cocci and clusters that are Gram-positive colonies; these colonies are then confirmed by the coagulase and catalase tests (Moraes *et al.*, 2021).

A total of 39 isolates (35.45%) were known as MRSA, which were resistant to oxacillin preparations based on the disc diffusion method using MHA media with details of 25 isolates from dairy cow milk samples, while 14 other isolates came from farmer's hand swab samples. Isolates resistant to cefoxitin preparations were obtained from as many as 16 isolates (14.54%), of which

Table 1. Quantity and total positive of *Staphylococcus aureus* of samples isolated from milk and swab hand

Sample type	Sample code	Quantity	Total of positive
Milk	TS	110	81 (73.64%)
Swab hand	TT	45	29 (64.44%)
Total		155	110 (70.97%)

Note: % (Percentage of positive *S. aureus*).

Figure 1. Mucoid white colonies on Mannitol Salt Agar were indicative of the presence of *Staphylococcus aureus*

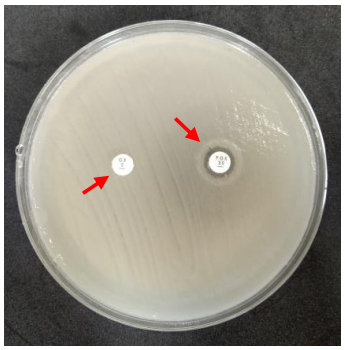

Figure 2. MHA disc diffusion test reveals resistance to oxacillin (OX) and cefoxitin (FOX) (Oxoid, CM0337). No zone of inhibition was found on the oxacillin antibiotic disk and only a small inhibition zone was found on the cefoxitin antibiotic disk.

Table 2. Diffusion disc test of *Staphylococcus aureus* isolated from milk and hand swabs against oxacillin and cefoxitin antibiotics

Sampel type	*Staphylococcus aureus* isolate (n=110)			
	Oxacillin		Cefoxitin	
	Resistant (%)	Sensitive (%)	Resistant (%)	Sensitive (%)
Milk	25 (22.73%)	56 (50.91%)	10 (9.09%)	71 (64.54%)
Swab hand	14 (12.73%)	15 (13.64%)	6 (5.45%)	23 (20.91%)
Total	39 (35.45%)	71 (64.55%)	16 (14.54%)	94 (85.45%)

Note: % Total percentage of *S. aureus* isolates with positive or resistant values; Oxacillin (30 µg) (Oxoid); Cefoxitin (30 µg) (Oxoid).

10 and 6 isolates came from samples of dairy cows and samples of farmer's hand swabs, respectively (Table 2, Figure 2).

Table 3 demonstrates that all *S. aureus* isolates that were resistant to cefoxitin were also found to be resistant to oxacillin. The results of the disk diffusion test revealed that no isolates of *S. aureus* were only cefoxitin resistant.

The ORSAB test was used to validate the phenotypic test's finding of oxacillin and cefoxitin resistance. Positive confirmation findings were represented by the blue color, whereas the white color indicated negative confirmation results. According to the results of the ORSAB test, out of 39 *S. aureus* isolates that were oxacillin-resistant, 23 isolates (58.97%) from the disc diffusion method were positively identified as MRSA, as can be shown in Table 4.

A molecular test using the PCR was then performed on *S. aureus* isolates suspected of being MRSA (phenotypically positive for ORSAB). A total of 13

Table 3. Confirmation test of *Staphylococcus aureus* is MRSA isolated from milk and hand swabs based on the disk diffusion test, ORSAB test, and *mec*A gene detection using PCR

Sample type	Sample code	Resistance on disc diffusion test		ORSAB Test	*mec*A detection utilizing PCR	Number positive of MRSA isolates by *mec*A detection (%)
		OX	FOX			
Milk	TS 4	+	+	+	+	
	TS 7	+	-	+	Not tested	
	TS 9	+	+	-	Not tested	
	TS 11	+	-	+	Not tested	
	TS 17	+	+	+	-	
	TS 19	+	-	-	Not tested	
	TS 20	+	+	+	-	
	TS 33	+	-	-	Not tested	
	TS 34	+	-	-	Not tested	
	TS 39	+	+	+	-	
	TS 42	+	+	-	Not tested	
	TS 50	+	+	+	-	
	TS 51	+	-	-	Not tested	1 (7.69%)
	TS 55	+	+	+	+	
	TS 57	+	-	-	Not tested	
	TS 61	+	-	+	Not tested	
	TS 68	+	+	+	-	
	TS 70	+	-	-	Not tested	
	TS 77	+	+	-	Not tested	
	TS 82	+	-	+	Not tested	
	TS 84	+	-	+	Not tested	
	TS 85	+	-	-	Not tested	
	TS 86	+	-	+	Not tested	
	TS 88	+	-	+	Not tested	
	TS 95	+	-	+	Not tested	
Swab hand	TT 1	+	-	+	Not tested	
	TT 2	+	-	-	Not tested	
	TT 4	+	+	+	+	
	TT 9	+	-	-	Not tested	
	TT 13	+	+	+	-	
	TT 14	+	+	+	-	
	TT 16	+	+	+	-	1 (7.69%)
	TT 20	+	-	-	Not tested	
	TT 24	+	-	-	Not tested	
	TT 26	+	+	+	-	
	TT 37	+	+	+	-	
	TT 39	+	-	+	Not tested	
	TT 40	+	-	-	Not tested	
	TT 45	+	-	-	Not tested	
Total		39	16	23		2 (15.38%)

Note: MRSA= methicillin resistant *Staphylococcus aureus*; ORSAB= oxacillin resistance screening agar base; OX= Oxacillin 30 μg; FOX= Cefoxitin 30 μg (Oxoid); % (percentage): Total positive percentage of MRSA from *S. aureus* isolates by PCR at the sampling location; + = Resistant.

isolates suspected of being MRSA were checked using the PCR. Two isolates (15.38% of all the isolates that underwent PCR testing) were positive for the *mec*A gene, as indicated in Figure 3.

DISCUSSION

The contamination with *S. aureus* bacteria affects human health and dairy cows. This contamination's loss in milk output and grade is completely apparent (Wang *et al.*, 2018). Previous research has documented antibiotic-resistant *S. aureus* infections in dairy farms around the world that contaminate milk (Hassani *et al.*, 2022). One of the primary causes of *S. aureus* contamination in milk is a lack of hygiene during milk processing (Regasa *et al.*, 2019). Farmer's hands that are not clean when milking can be a risk factor for the transmission of *S. aureus* bacteria in milk (Tigabu *et al.*, 2015).

S. aureus is a pathogenic bacterium that might induce several infectious disorders ranging from mild to severe (Guo *et al.*, 2020). In this study, 110 samples (70.97%) were indicated as *S. aureus* out of 155 samples tested. This proportion was more significant than that found in the study by Lemma *et al.* (2021), which isolated 175 milk samples, 43 of which (24.57%) were *S. aureus*, and another study researched by Kou *et al.* (2021), which dissociated 37 milk samples, of which 60 samples (61.67%) were *S. aureus*. In addition, MRSA isolates were detected in milk and hand swabs on the same dairy farm. This proves that horizontal transfer of MRSA transmission can occur from the hands of farmers to milk or vice versa. However, not all MRSA isolates carried the gene encoding *mec*A. Therefore, purposeful sampling was performed in this study to identify the prevalence of *S. aureus* strains in dairy farms with poor milking hygiene, which can potentially increase bacterial contamination in milk (Tegegne & Tesfaye, 2017).

Table 4. ORSAB test of *Staphylococcus aureus* isolated from milk and hand swabs

Sample type	Sample code	Number of isolates tested ORSAB (n=39)	Positive ORSA test
Milk	TS	25 (64.10%)	15 (38.46%)
Swab hand	TT	14 (35.90%)	8 (20.51%)
Total		39 (100%)	23 (58.97%)

Note: MRSA= methicillin resistant *Staphylococcus aureus*; ORSAB= oxacillin resistance screening agar base; % = Percentage of positive ORSAB.

In accordance with this study, Schechner *et al.* (2013) observed that variances in the amounts of isolates detected might be impacted by variables in research design, like as the sample's demographic, geographic distribution, techniques for infection control, and the type of antibiotic used. Figure 3 shows *mec*A PCR findings with a single positive band at 533 bp. Routes for 100-bp molecular weight indicators, TT4 and TS4: Positive *mec*A gene isolates, TT13, TT14, TT16, TT26, TT37, TS68, TS17, TS20, TS39, TS50, and TS55 isolates: Negative *mec*A gene isolates.

The availability of MRSA, which is resistant to all β-lactam antibiotics, has contributed to the worsening of the *S. aureus* infection problem, such as cephalosporins and monobactams, commonly used to treat Gram-positive bacterial infections (Foster, 2017). The spread of MRSA induces medical issues and spreads rapidly; thus, an early diagnosis was required to precisely identify MRSA (Green *et al.*, 2012). The disk diffusion technique used in this investigation revealed that 39 *S. aureus* samples were resistant to disk oxacillin (35.45%) and disk cefoxitin (14.54%). According to Panda *et al.* (2016), phenotypic detection of MRSA using a diffusion disk remains not reliable, and *mec*A genotyping by PCR is still

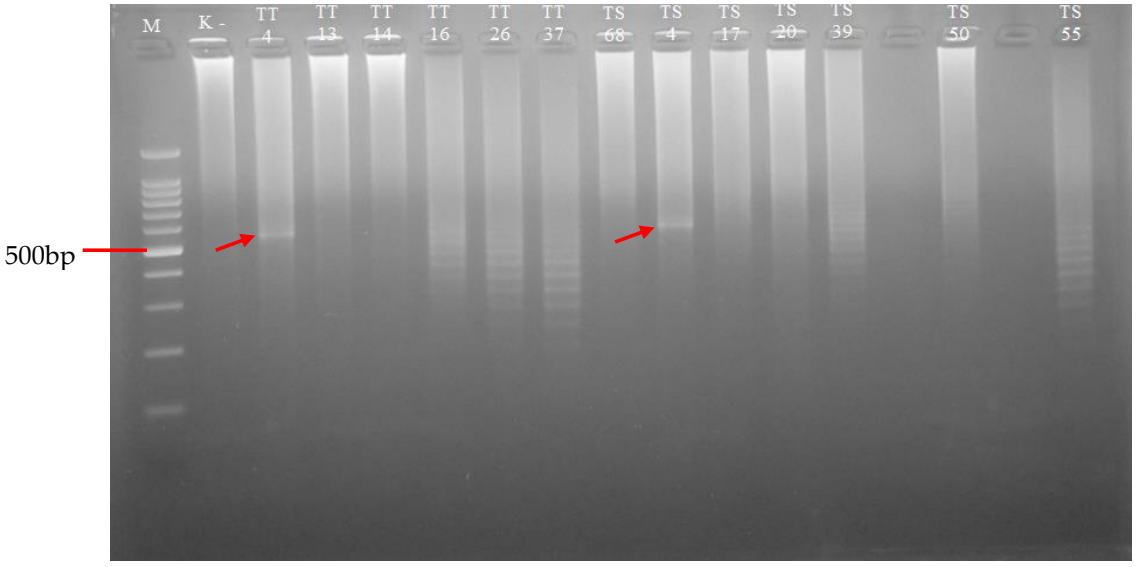

Figure 3. Results of *mec*A gene electrophoresis showed a positive single band at 533 bp (appointed with red arrow). Marker line= Markers with a 100-bp molecular weight; Line K-= *Staphylococcus aureus* ATCC 25923 (Negative Control); Line TT4 and TS4= Positive isolate for *mec*A gene; Line TT13, TT14, TT16, TT26, TT37, TS68, TS17, TS20, TS39, TS50, and TS55= Negative isolate for *mec*A gene.

the major recommendation, even if it is not performed regularly. Nonetheless, MRSA detection using disk diffusion is still extensively employed since it is rapid and cheaper (Bonjean *et al.,* 2016). Diffusion disks containing cefoxitin and oxacillin have the same sensitivity level of 100%. However, the oxacillin diffusion disk has a specificity of 74.07%, and the cefoxitin diffusion disk has a specificity of 92.59% (Sahai *et al.,* 2014). However, according to some past studies, the cefoxitin disk diffusion approach was more sensitive than the oxacillin disk diffusion method when looking at MRSA, due to the high rate of false positives in the oxacillin disk diffusion method (Sultana *et al.,* 2019). According to Bhutia *et al.* (2012), Oxacillin resistance can result from false positives being affected by β-lactamase hyperproduction; however, there is no genotypic resistance mechanism.

The results of the current study demonstrated that all isolates resistant to cefoxitin discs were also resistant to disc oxacillin. The ORSAB assay was used to confirm that each isolate was resistant to oxacillin and cefoxitin, in line with Pourmand *et al.* (2014) claim that the ORSAB test has a 100% specificity. In this study, 23 out of 39 isolates (58.97%) were positive for MRSA. The resistance strain being tested will be confirmed by the sensitivity level, and the specificity will be proportionate to the minimal inhibitory concentration (MIC) (Catalán *et al.,* 2022). Using PCR, the genotype of *S. aureus* isolates resistant to cefoxitin and positive for ORSAB was investigated to evaluate the presence of the *mec*A gene encoding. These isolates were also positive in all phenotypic assays (positive in the ORSAB test and resistance to disk cefoxitin in the disk diffusion method). Since it promotes the expression of the penicillin-binding protein 2a (PBP 2a) that the *mec*A gene codes for, the antibiotic cefoxitin is a potent inducer of the *mec*A gene's expression (Müller *et al.,* 2015).

According to the study's findings, MRSA infection in milk might be induced by various factors, including milking equipment, farmer hand hygiene, and a history of administering antibiotics to dairy cows (Schnitt & Tenhagen, 2020). Unhygienic hands of farmers when milking is one of the biggest risk factors for MRSA contamination in milk (Khairullah *et al.,* 2020b). In addition, MRSA contamination is very risky for human health, especially for humans who live and work on dairy farms (da Silva *et al.,* 2020). Therefore, laboratory microbiological examination is very important for the fast, accurate, and cost-effective identification and isolation of MRSA isolates from milk samples and farmer hand swabs (Girmay *et al.,* 2020). MRSA genotype identification utilizing PCR to detect the appearance of genes encoding *mec*A is the most reliable molecular MRSA test. However, if molecular testing is not accessible in a laboratory, the cefoxitin plate diffusion approach can be performed as an alternative (Koupahi *et al.,* 2016). This is predicated on the cefoxitin disc diffusion test›s capacity to identify *mec*A gene expression, which might facilitate and reduce the cost of MRSA screening (Bonjean *et al.,* 2016). Based on the results of this study, from 13 MRSA isolates examined using PCR, only 2 isolates encode *mec*A gene. This illustrates that the condition of dairy farms in Tulungagung, Indonesia was still good because of the lack of MRSA transmission gene. The two isolates encode *mec*A gene and were not from the same farm.

CONCLUSION

This study showed that MRSA isolates were more commonly found in milk, as many as 15 (38.46%) isolates, while in farmer hand swabs, only 8 (20.51 %) isolates were found. Based on 13 MRSA isolates examined using PCR, only 2 isolates encoded the *mec*A gene. MRSA contamination is very risky for human health, especially for humans who live and work on dairy farms. The primary source of MRSA infection in milk might come from farmer's unhygienic hands during milking. Laboratory microbiological examination is very important for the fast, accurate, and cost-effective identification and isolation of MRSA in dairy farms.

CONFLICT OF INTEREST

The authors confirm that there is no conflict of interest regarding this manuscript.

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