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- Content and Archives
- Aims and Scopes
- Editorial Board
- Guidance for Authors
- Ethical Guidelines
- Charges
- Membership
- Previously Reviewers
- Conference Events

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Biodiversitas - Vol 21 no 8

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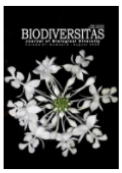

Biodiversitas Vol. 21, No. 8, August 2020

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TABLE OF CONTENT

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BIODIVERSITAS
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*Mec*A gene and methicillin-resistant *Staphylococcus aureus* (MRSA) isolated from dairy farms in East Java, Indonesia

SANCAKA CHASYER RAMANDINIANTO[1], ASWIN RAFIF KHAIRULLAH[2], MUSTOFA HELMI EFFENDI[3,4,♥]

[1]Program of Animal Diseases and Veterinary Public Health, Faculty of Veterinary Medicine, Universitas Airlangga. Jl. Mulyorejo, Surabaya 60115, East Java, Indonesia
[2]Doctoral Program in Veterinary Science, Faculty of Veterinary Medicine, Universitas Airlangga. Jl. Mulyorejo, Surabaya 60115, East Java, Indonesia
[3]Department of Veterinary Public Health, Faculty of Veterinary Medicine, Universitas Airlangga. Jl. Mulyorejo, Surabaya 60115, East Java, Indonesia
[4]Halal Research Center, Universitas Airlangga. Jl. Mulyorejo, Surabaya 60115, East Java, Indonesia. Tel./fax.: +62-31-5915551,
♥email: mheffendi@yahoo.com

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Abstract. *Ramandinianto SC, Khairullah AR, Effendi MH. 2020. MecA gene and methicillin-resistant* Staphylococcus aureus *(MRSA) isolated from dairy farms in East Java, Indonesia. Biodiversitas 21: 3562-3568.* Milk Borne Disease (MBD) can be caused by a variety of pathogenic bacteria, one of which is *Staphylococcus aureus* which has a large impact on aspects of public health. The therapy used to treat staphylococcal infection is Oxacillin preparations that can inhibit bacterial wall synthesis, but the adaptation of the mecA gene to staphylococcal cassette chromosome mec (SCCmec) causes the emergence of strains of methicillin-resistant *S. aureus* (MRSA). The purpose of this study was to detect the level of MRSA strain contamination in dairy cows in East Java by comparing the mecA gene, Oxacillin, and Cefoxitin Disc Diffusion Methods and Oxacillin Resistance Screen Agar (ORSA) detection methods. A total of 150 cow's milk samples were taken at 3 village dairy farms in East Java, samples were added to the enrichment media Buffer Pepton Water (BPW) and then isolates were planted and purified using Mannitol Salt Agar (MSA). The detection of MRSA was carried out by the Kirby Bauer disc diffusion preparation Cefoxitin 30 µg and Oxacillin 30 µg then confirmed by ORSA and the presence of mecA gene by the polymerase chain reaction (PCR) method. The results showed that from a total of 92 *S. aureus* isolates using Oxacillin disc test, 24 resistant isolates were obtained, using Cefoxitin disc test, 17 isolates were obtained, and using the ORSA test 18 MRSA isolates were obtained. MRSA isolates tested by PCR obtained evidence of 2 isolates of mecA gene. It can be concluded that the Oxacillin disc test was the highest sensitivity for detecting MRSA strain isolate, however, mecA gene was the golden standard to detect MRSA on the dairy farms.

Keywords: Cefoxitin disc, milk-borne disease, mecA gene, MRSA, Oxacillin disc, ORSA

INTRODUCTION

Milk borne diseases (MBD) are a very important problem in the public health sector which can be caused by a variety of pathogenic bacteria, one of which is *Staphylococcus aureus*. *S. aureus* opportunistic pathogens that are often found in humans and animals, these bacteria can cause a diverse spectrum of diseases from minor skin infections to systemic such as pneumonia and meningitis (Jangra and Singh 2010). Some researchers suggest that *S. aureus* can be transmitted to humans through contamination of milk, unprocessed milk, and milk products (Seifu et al. 2004; Swetha et al. 2017). *S. aureus* is commonly found in the skin and mucosa of ruminants, which have sub-clinical or clinical mastitis which is a source of contamination in dairy products (Sasidharan et al. 2011).

A research report reveals that the presence of multiple antibiotic resistance from *S. aureus* creates new problems for the world of health practitioners and researchers (Motamedi et al. 2010). There are studies on phenotypic and genotypic antibiotic resistance stating that since 1962 methicillin-resistant *Staphylococci* (MRS) have been found where the first case of methicillin-resistant *Staphylococcus aureus* (MRSA) has occurred in humans but has now been detected in animals (Kumar and Prasad 2010; Chajęcka-Wierzchowska et al. 2015). MRSA resistance to beta-lactam antibiotics is caused by various mechanisms, one of which is the production of unusual penicillin-binding protein (PBP), which forms PBP2 thereby weakening the affinity for the antibiotic β-lactam expressed by the *mec*A gene (Katayama et al. 2000). *Mec*A gene detection using the polymerase chain reaction (PCR) method is the gold standard for detecting MRSA, but cannot be done in all clinical laboratories due to various facilities, capabilities and costs (Fernandes et al. 2005). The difficulty of using PCR in an effort to detect the presence of MRSA can be reduced by using Cefoxitin disc diffusion, Oxacillin disc diffusion combination of Oxacillin Resistance Screen Agar (ORSA) (Tyansningsih et al. 2019; Decline et al. 2020).

The purpose of this study was to detect and evaluate the level of MRSA contamination in dairy cow milk in East Java and to compare the phenotypic detection method using screening using Cefoxitine disc diffusion, Oxacillin disc diffusion combination of Oxacillin Resistance Screen Agar (ORSA) and confirm genotypically using PCR to detect the MecA gene. The sensitivity and specificity of the test will show the effectiveness and ease of application of the

MRSA strain detection method. Also, this research information is very important to support strategic and technical decision making by relevant institutions for mitigation and prevention of impacts on aspects of public health in food safety.

MATERIALS AND METHODS

Ethical clearance

Raw milk was used in this study, hence ethical clearance was not necessary. Raw milk samples were collected from three dairy farms in East Java Province, Indonesia, namely Batu, Pasuruan, and Probolinggo districts.

Sampling

Total samples of 150 dairy cows were collected from 3 Village Unit Cooperatives in Probolinggo, Batu, and Pasuruan areas during October-November 2019. Dairy cows milk collected directly from milk can and as many as 15 mL were collected in centrifuge tubes of 50 mL (Biologix, BD-T0034). Samples were taken 1 mL aseptically to be put into 10 mL containing 4 mL of buffered water peptone buffer media (Oxoid, CM0509) using Syringe 3CC (AKD 20902900277) then incubated at 37oC for 24 hours with an incubator (Isuzu Model 2-2195, Jica) (Thaker et al. 2013).

Bacteria isolation and identification

Samples produced from enrichment media were cultured and purified on Mannitol Salt Agar media (HiMedia Pvt.Ltd, M118) and incubated at 37 ºC for 24 hours. Identification was done by examination based on morphological cultural characteristics, than microscopic examination using Gram's method of staining which shows Gram-positive bacteria in the form of coccus and clustering (Effendi et al. 2018). Biochemical tests were carried out to confirm the *S. aureus* species with the catalase test and coagulase test (Effendi et al. 2019). Catalase tests were carried out by dripping hydrogen peroxide (H_2O_2) 3% on clean glass objects and mixing with ose of the 1 colony (Tyasningsih et al. 2019). Coagulase test was carried out with Coagulase slide test was given 50 µl rabbit blood plasma dripped on a glass object, then mixed with 1 ose of a bacterial colony, and Coagulase tube test using 200 µl blood plasma was added with as much as 3-4 isolate colonies then incubated at 37ºC for 24 hours (Effendi et al. 2019).

Oxacillin and Cefoxitin disc diffusion methods

The test was carried out referring to the Clinical and Laboratory Standards Institute guidelines where *S. aureus* was tested for susceptibility to the 30 µg Cefoxitin and Oxacillin 30 µg (Oxoid) antibiotic preparations on Muller Hinton agar plates (Oxoid, CM0337) (CLSI 2018). Isolates that have been isolated and identified will be purified on Mannitol Salt Agar (HiMedia Pvt. Ltd., M118) and incubated at 37 ºC for 24 hours as a 0.5 Mc Farland's suspension and subsequently taken using Sterile Cotton Swab S (AKD 10903610549). The swab was streaked evenly on the surface of the Muller Hinton agar medium (Oxoid, CM0337). Cefoxitin 30 µg and Oxacillin 30 µg were placed side by side with a distance of 4.5 cm on Muller Hinton agar medium which had been inoculated with isolate and then incubated 37 ºC for 24 hours and measured the inhibition zone. In the anefoxitin disc diffusion test inhibition zone ≤21mm is methicillin-resistant (MR) isolate, whereas in Oxacillin disc diffusion test isolates with an inhibition zone ≤10mm is MR.

Oxacillin resistance screen agar test

Oxacillin Resistance Screen Agar test (ORSA Test) is carried out referring to the Clinical and Laboratory Standards Institute guidelines aimed at confirming MRSA which is MR from Oxacillin and Cefoxitin Disc Diffusion Methods (CLSI 2018; Decline et al. 2020). Isolates were taken by several colonies to be used as a suspension of 0.5 Mc Farland and then by using Sterile Cotton Swab S (Onemed, AKD 10903610549) and making smears on the Oxacillin Screen Agar Base (HiMedia Pvt. Ltd., M1415) added with Oxacillin Resistant Selective Supplement (Supplements, HiMedia Pvt. Ltd., FD191).

Detection of the mecA gene

All confirmed *S. aureus* isolates were MRSA by the ORSA test were tested using PCR to detect the presence of the mecA gene (Rahmaniar et al. 2020). The DNA extraction process was carried out according to the QIAamp DNA Mini Kit protocol (51304 & 51306), where previously the isolate was purified first on Mannitol Salt Agar (HiMedia Pvt. Ltd, M118) and inoculated on Muller Hinton agar (Oxoid, CM0337). The primers used namely mecA F: 5 '-GAA ATG GAA CGT CCG ATA A-3' and mecA R: 5 '-CCA ATT CCA CAT TGT TTC CTA A-3' (Rajabiani et al. 2014; Rahmaniar et al. 2020). The master mixture uses GoTaq® Green Master Mix (Promega, 9PIM712) which is a premixed ready-to-use solution containing Taq DNA polymerase, dNTPs, $MgCl_2$, and buffers reaction. DNA was amplified using a Thermal Cycler T100 machine (Bio-Rad, 186-1096) with an initial denaturation step of 4 min at 94 °C than 35 cycles at 94°C for 1 min, annealing at 62 °C for 1 min, then the extension at 72°C for 45s. The final extension is carried out for 5 min at 72°C. Amplicons were processed with electrophoreses, where the gel will be visualized in ultraviolet illumination (Rahmaniar et al. 2020). Positive tests showed PCR products in the 310 bp band, with MRSA ATCC BAA 1026 as a positive control and *S. aureus* ATCC 25923 as a negative control.

RESULTS AND DISCUSSION

The test results found that there were 92 (61%) *S. aureus* isolates from 150 milk samples taken in 3 regions in East Java, Indonesia (Batu, Pasuruan, and Probolinggo districts) based on morphological characteristics and biochemical tests, as shown in Table 1. *S. aureus* has phenotypic characteristics of colonies on Mannitol Salt

Agar (MSA) media, which changes the color of the media from red to yellow which indicates mannitol fermentation while the colonies have varying pigments including white, yellow, and orange (Figure 1) (Effendi et al. 2019). Gram's method of staining test showed the form of the gram-positive colony and shaped like the genus Staphylococcus which was then confirmed by catalase tests and coagulase tests so that it was found that 61% of samples confirmed the presence of *S. aureus* contamination.

Methicillin-resistant (MR) *S. aureus* based on disc diffusion method on Muller Hinton Agar (MHA) media showed that the total results of isolate resistance to Oxacillin preparations were 26% with the highest percentage detected in Probolinggo, as well as the results of isolate resistance to Cefoxitin preparations which was found to be 18.5% and Probolinggo was 26%. the highest detected percentage, as shown in Table 2 and Figure 2. The results obtained showed that there were no isolates that were only resistant to Cefoxitin in the disk diffusion method, all isolates that were detected were resistant to Cefoxitin were also identified to be resistant to Oxacillin but there were isolates that were Oxacillin resistant and still sensitive to Cefoxitin, as shown in Table 3. The phenotypic MR test confirmation was continued using the Oxacillin Resistance Screen Agar (ORSA) test with a blue culture indicator showing positive confirmation results while the white color results were negative confirmation results (Rahmaniar et al. 2020). ORSA test showed that of 24 *S. aureus* isolates resistant to Oxacillin, the disc diffusion method was found that was positively confirmed MRSA were 18 isolates, as shown in Table 3.

Isolates confirmed as MRSA phenotypically by the ORSA method were further tested genotypically by the PCR method to detect the presence of the *mec*A gene in the isolate. A total of 18 MRSA isolates confirmed by ORSA were tested using the PCR method and 2 isolates (11.1% of the tested isolates) were detected positive *mec*A gene, so the 2 isolates were genotypically confirmed as MRSA strains, as shown on Figure 3 and Figure 4. Results from PCR tests showed that the isolates detected have the *mec*A gene which has resistance to Cefoxitin and Oxacillin antibiotics, as shown in Table 3.

Table 1. Positive *Staphylococcus aureus* isolates from milk samples

Location	Location code	Total	Positive *S. aureus* (%)
Probolinggo	A	50	30 (60%)
Batu	H	50	38 (76%)
Pasuruan	G	50	24 (48%)
Total		Total	92 (61%)

Note: %: Number of percentages of *S. aureus* in milk samples

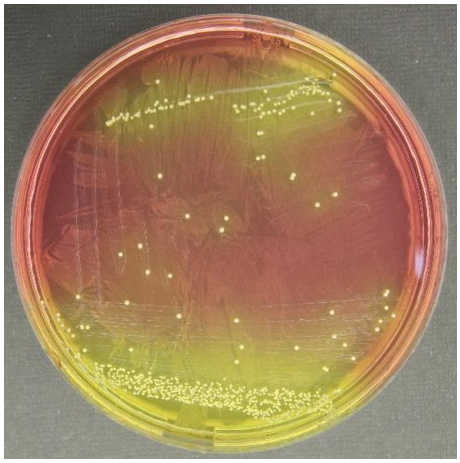

Figure 1. The results of positive yellow Mannitol Fermentation and *S. aureus* colonies appear mucoid white on Mannitol Salt Agar medium (HiMedia Pvt. Ltd, M118) (Cannon 600D DSLR). 72 x 72 dpi

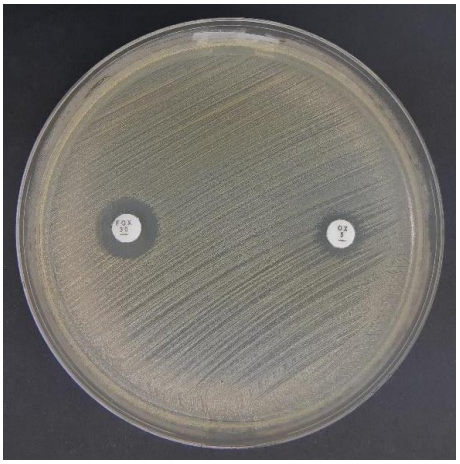

Figure 2. Cefoxitin (FOX) and Oxacillin (OX) Disc Diffusion Test in Mueller Hinton Agar (Oxoid, CM0337). FOX = Cefoxitin (Break Point 21-22 *), OX = Oxacillin (Break Point 21-22 *). * Clinical and Laboratory Standards Institute (Canon 600D DSLR). 72 x 72 dpi

Table 2. Oxacillin and Cefoxitine Disc Diffusion test of *Staphylococcus aureus* by location

Location	*Staphylococcus aureus* (n=92)			
	OX Disc Diffusion		FOX Disc Diffusion	
	Resistant (%)	Sensitive (%)	Resistant (%)	Sensitive (%)
Probolinggo	10 (10.9%)	20 (21.7%)	8 (8.7%)	22 (23.9%)
Batu	8 (8.7%)	30 (32.6%)	6 (6.5%)	32 (34.8%)
Pasuruan	6 (6.5%)	18 (19.5%)	3 (3.3%)	21 (22.8%)
Total	24 (26%)	68 (74%)	17 (18.5%)	75 (81.5%)

Note: % (Percentage): Total percentage of positive or resistant resistant values in *S. aureus* isolates. FOX = *Cefoxitin* 30 µg, OX = *Oxacillin* 30 µg (Oxoid)

Table 3. Positive MRSA confirmed by OX and FOX Disc Diffusion, ORSA and *MecA* gene detection

Location	Sample code	Resistance on disc diffusion test		ORSA test	MecA detection using PCR	Number positive of MRSA isolates by MecA detection (%)
		OX	FOX			
Probolinggo	A04	+	+	+	-	-
	A07	+	+	+	-	
	A17	+	+	+	-	
	A21	+	+	+	-	
	A31	+	+	+	-	
	A32	+	-	+	-	
	A35	+	+	-	Not tested	
	A40	+	+	-	Not tested	
	A45	+	+	+	-	
	A49	+	+	+	-	
Batu	H02	+	+	-	Not tested	2 (2.2)
	H27	+	+	+	+	
	H28	+	-	-	Not tested	
	H37	+	+	+	-	
	H41	+	-	+	-	
	H48	+	+	+	+	
	H49	+	+	+	-	
	H50	+	+	+	-	
Pasuruan	G06	+	+	+	-	-
	G16	+	+	-	Not tested	
	G18	+	-	-	Not tested	
	G24	+	-	+	-	
	G33	+	+	+	-	
	G37	+	-	+	-	
Total number positive of MRSA Isolate by *MecA* detection						2 (2.2)

Note: FOX: *Cefoxitin* 30 µg, OX: *Oxacillin* 30 µg (Oxoid). % (percentage): Total positive percentage of MRSA from *S. aureus* isolates by PCR at the sampling location. +: Resistant, No: No tested

Discussion

Milk-borne diseases (MBD) are problems that must be controlled in the public health sector but not only affect human health but also have an impact on the economic sector. Research on antimicrobial drug resistance of *S. aureus* reports that related dairy product contamination is not only limited to developing countries but also occurs in developed countries. Some researchers report that bacterial outbreaks in milk and dairy products in countries going around 2-6% (De Buyser et al. 2001; Sasidharan et al. 2011). Improper food handling and unhygienic practices during the production process, packaging to distribution have a significant role in the occurrence of food poisoning (De Buyser et al. 2001). Another researcher stated that cow's milk can transmit various pathogens including *Staphylococci* strains (Angelillo et al. 2000).

Staphylococcus aureus is a pathogenic agent that can cause various infectious diseases from cutaneous to systemic infections in immunocompetent hosts, resulting in death (Vyas et al. 2015). In this study of 150 milk samples found as many as 61% detected *S. aureus* contamination, this percentage was higher compared to Swetha et al. (2017) which isolates 57% of *Staphylococci* strains, of which 73.6% were *S. aureus*; and reports by VyletěloVá et al. (2011) that isolates *Staphylococci* strains as much as 47.5% of dairy cow's milk, while the dominant strain is *S. aureus* with 46.4% of the total *Staphylococci* strains isolates. This study has a purposive design conducted specifically to detect the presence of *Staphylococci* strains in dairy cows farms that have low hygiene milking thereby increasing the potential for contaminants in cows milk. In line with this, research in North India states that differences in the number of isolates found can be influenced by differences in study design such as population and geographic distribution of samples, types of antibiotics used, and infection control practices (Oberoi et al. 2012).

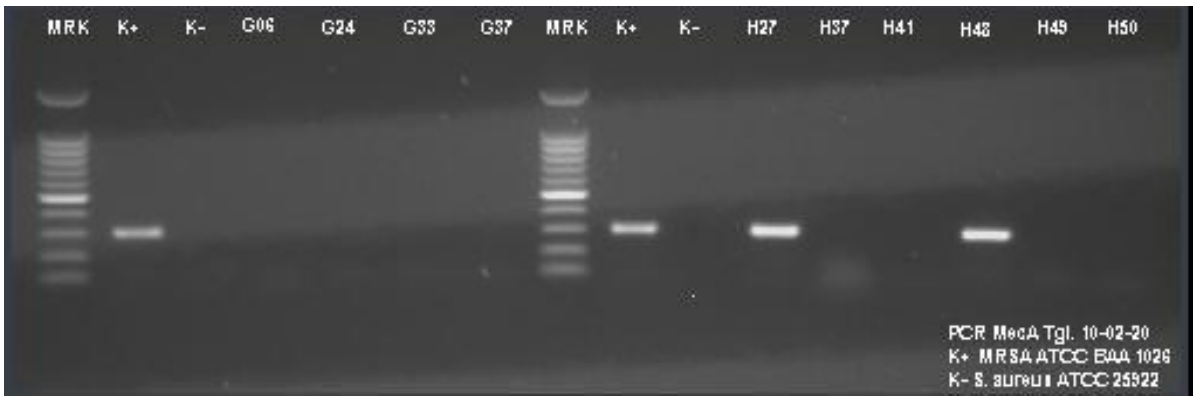

Figure 3. MecA PCR results with positive bands at 310 bp. MRK Line: 100-bp molecular-weight markers, Line K +: MRSA ATCC BAA 1026 (Positive Control), Line K-: *Staphylococcus aureus* ATCC 25923 (Negative Control), Line H27 and H48: Positive isolate for mecA gene from Batu, Line G06, G24, G33, G37, H37, H41, H49, and H50: Negative isolate for mecA gene.

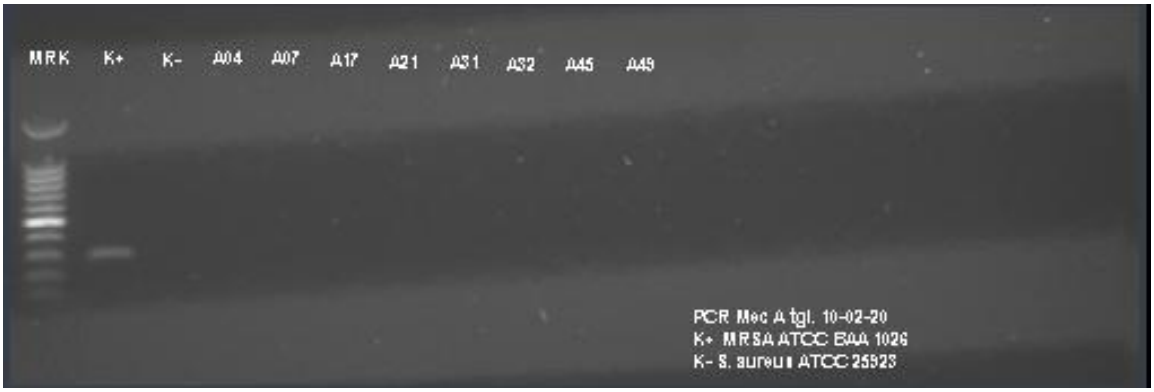

Figure 4. Meca PCR results with positive bands at 310 bp. MRK Line: 100-bp molecular-weight markers, Line K +: MRSA ATCC BAA 1026 (Positive Control), Line K-: *Staphylococcus aureus* ATCC 25923 (Negative Control), Line A04-A49: Negative isolate for mecA gene

Staphylococcus aureus problem develops with the presence of methicillin resistance (MR) so that the methicillin resistance group *Staphylococcus aureus* (MRSA) has resistant properties to all β-lactam antibiotics including cephalosporins and monobactams which are important antibiotic groups for the treatment of staphylococcal infections (Graveland et al. 2011; Vyas et al. 2015). MRSA infection not only causes therapeutic problems but also makes it easy to spread, so a rapid and early diagnosis and accurate identification of MRSA are needed (Yamazumi et al. 2001). In this study, several *S. aureus* isolates were detected that were resistant to the preparation of Oxacillin by 26% and Cefoxitin 18.5% in the disc diffusion method. Brown and Walpole (2001) state that basically MRSA detection with phenotypic methods still has not shown optimal results and *mec*A genotypic tests are still the main recommendation even though it cannot be applied to routine tests. So that accurate, rapid, and cost-effective identification of MRSA can use phenotypic methods (Krishnan et al. 2002). Cefoxitin disc diffusion and Oxacillin disc diffusion have the same sensitivity level of 100%, specificity where Cefoxitin disc diffusion is 92.59% while Oxacillin disc diffusion is 74.07% (Vyas et al. 2015). This value is in line with various studies that have been reported by Oberoi et al. (2012), Velasco et al. (2005), Tiwari et al. (2009), and Mathews et al. (2010). However, several studies suggest that the Cefoxitin disc method has a better level of sensitivity compared to the Oxacillin disc method in detecting MRSA, so the Oxacillin disc method still has a level of false positives (Skov et al. 2003; Boubaker et al. 2004; Velasco et al. 2005). Vyas et al. (2015) state that the rate of false positives can be influenced by the hyperproduction of β-lactamase, giving rise to Oxacillin resistance phenotypic expression but does not have a genetic resistant mechanism.

In this study, all isolates that were detected to be resistant to Cefoxitin also had resistance to Oxacillin, but some isolates were detected to be resistant to Oxacillin and sensitive to Cefoxitin. All isolates detected were resistant to Oxacillin confirmed by the ORSA test, in line with Datta et al. (2011) which stated that the ORSA test had 100% specificity and from this study found 18 (75%) isolates out of 24 isolates expressed positive results as MRSA. The sensitivity level will validate the homogeneity of the resistant strains tested while specificity will be related to the borderline of the minimum inhibitory concentration (MIC) of a strain (Swenson et al. 2001). ORSA positive isolates were all genotypically tested using PCR to detect the presence of the *mec*A gene, which became the golden standard for detecting MRSA. Two isolates from Batu were detected having the *mec*A gene, where the isolates also had positive results in all phenotypic methods (Cefoxitin and Oxacillin disc diffusion and ORSA), these results were similar to the results of research conducted by researchers from Southern Province of India(Venugopal et al. 2019). Cefoxitin is a good inducer for expressing the presence of the *mec*A gene because it can increase the expression of the protein penicillin-binding protein 2a (PBP2a) encoded by the *mec*A gene, the statement was also in accordance with Cauwelier et al. (2004) and Velasco et al. (2005).

In this study, it can be concluded that the presence of milk contamination by MRSA can be caused by various factors, one of which is low hygiene milking. Besides, MRSA contamination is very dangerous for public health aspects, which will increase the potential spread of Staphylococcal infection which is difficult to treat. So the urgency of clinical microbiology laboratories identify is very important to be able to do accurate, rapid and cost-effective identification of MRSA contamination in transmission media such as food from animal origin. Genotypic detection using PCR to detect the presence of the *mec*A gene is the golden standard of MRSA detection, but in a laboratory that unable to do molecular testing can use Cefoxitin disk diffusion as a marker for MR detection. This is based on the ability of the Cefoxitin disc diffusion test in detecting *mec*A gene expression so that it can be a solution as an MRSA screening instrument more effectively and efficiently in cost, technical applications, and media preparation.

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