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Profile of Multidrug Resistance and Methicillin-Resistant *Staphylococcus aureus* (MRSA) on dairy cows and risk factors from farmer

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Abstract. *Khairullah AR, Sudjarwo SA, Effendi MH, Ramandininto SC, Gelolodo MA, Widodo A, Riwu KHP, Kurniawati DA, Rehman S. 2022. Profile of Multidrug Resistance and Methicillin-Resistant* Staphylococcus aureus *(MRSA) on dairy cows and risk factors from farmer. Biodiversitas 23: 2853-2858. Staphylococcus aureus* is a facultative anaerobic Gram-positive coccus that causes mastitis and is one of the most widespread pathogenic bacteria in dairy farming. These bacteria can quickly develop into antibiotic-resistant strains due to inappropriate antibiotic administration. *Staphylococcus aureus* bacteria that have Multidrug-Resistant (MDR) properties can be known as Methicillin-Resistant *S. aureus* (MRSA) if these bacteria are resistant to beta-lactam antibiotics. The high number of MDR and MRSA contamination in dairy farms as a result of excessive administration of antibiotics in the treatment of dairy cows and the spread of these bacteria cannot be separated from the sanitation management at the time of milking, it can be from milk that is milked from the udder and it can also be from the hands of farmers during the milking process. This study tests antibiotic resistance and detects Methicillin-Resistant *S. aureus* (MRSA) strains sourced from milk and hand swabs of farmers in Probolinggo, East Java, Indonesia. A total of 109 dairy cow's milk samples and 41 samples of farmers' hand swabs were taken from each farm. Samples were cultured and purified using Mannitol Salt Agar (MSA). *Staphylococcus aureus* resistance profile was performed by disk diffusion test using antibiotic disks such as Oxacillin, Cefoxitin, Tetracycline, Erythromycin, and Gentamicin. *Staphylococcus aureus* isolates that were resistant to Oxacillin and Cefoxitin antibiotics were then tested for Oxacillin Resistance Screening Agar Base (ORSAB) as a confirmation test for Methicillin-Resistant *S. aureus* (MRSA). The results of the isolation and identification found 80 (53.33%) *S. aureus* isolates, then the results of the resistance test found 8 (10%) *S. aureus* isolates that were Multidrug-Resistant (MDR), and in the Oxacillin Resistance Screening Agar Base (ORSAB) test there were 3 isolates of *S. aureus* MDR were ORSAB positive. It can be found that there are several *S. aureus* isolates that are Multidrug-Resistant (MDR) and Methicillin-Resistant *S. aureus* (MRSA) in dairy farms. The *S. aureus* that is MDR and MRSA can be spread to the community and will endanger public health. Thus, prevention and control measures are needed to suppress the spread of *S. aureus* infection on a dairy farm in Probolinggo, East Java, Indonesia.

Keywords: MDR, Milk, MRSA, public health, swab hand

INTRODUCTION

Staphylococcus aureus is one of the pathogenic bacteria that can cause public health problems because these bacteria often contaminate products of animal origin. As it causes contamination of milk, commonly known as Milk Borne Disease (MBD) (Ayele et al. 2017). *Staphylococcus aureus* is a facultative anaerobic Gram-positive coccus and is one of the most widespread pathogenic bacteria in dairy farming that causes mastitis (Dufour et al. 2012; Herlina et al. 2015; Mustopa et al. 2018). These bacteria can quickly develop into antibiotic-resistant strains due to inappropriate antibiotic administration (Manyi-Loh et al. 2018). *Staphylococcus aureus* has a defense system against antibiotics, namely by mutating the active and binding sites, forming trans membrane proteins known as efflux proteins and plasmids that code for antibiotic resistance genes (Fuda et al. 2005). *Staphylococcus aureus* is resistant to more than or equal to 3 antibiotics, hence said to be Multidrug-Resistant (MDR) (Hiramatsu et al. 2014; Khairullah et al. 2019).

The Multidrug-Resistant (MDR) *S. aureus* can also be known as Methicillin-Resistant *S. aureus* (MRSA) when these are resistant to beta-lactam antibiotics (Guo et al. 2020). The Methicillin-Resistant *S. aureus* (MRSA) strain is associated with the acquisition of a mobile genetic

element called the Staphylococcal Cassette Chromosomal *mec* (SCC*mec*), which carries the *mec*A and *mec*C genes, which encode low-affinity penicillin-binding protein 2a (PBP2a) and confer resistance to beta-lactam antibiotics (Lee et al. 2018).

The high number of MDR and MRSA contamination in dairy farms is a result of excessive administration of antibiotics in the treatment of dairy cows. The spread of these bacteria cannot only be from the sanitation management at the time of milking, it can be from milk that is milked from the udder and it can also be from the hands of farmers during the milking process (Schnitt and Tenhagen 2020). Probolinggo Regency, precisely in Krucil District, is one of the largest dairy milk-producing centers in East Java (Bairosi et al. 2019). The use of antibiotics as a treatment in cases of infection in dairy cows has been widely practiced in Probolinggo, especially in cases of mastitis, so there may be contamination of *S. aureus* which is MDR and MRSA in dairy farms in Probolinggo (Soetji et al. 2012). Therefore, the aim of the present study was to detect Multidrug-Resistant (MDR) *S. aureus* and Methicillin-Resistant *S. aureus* (MRSA) sourced from dairy cow's milk and farmer's hand swabs in Probolinggo, East Java, Indonesia.

MATERIALS AND METHODS

Bacterial isolates

The samples of 109 dairy cow's milk samples and 41 farmer's hand swabs were taken at a dairy farm in the Probolinggo area, East Java, Indonesia between July2021to September 2021. Dairy cow's milk samples were taken from each cow in the third press as much as 30 ml which was then stored in a 60 ml sample bottle while the farmer's hand swab samples were taken from each farmer after the milking process using a sterile cotton swab which was then stored on Amies media. A total of 1 ml of each milk sample was put into a 20 ml test tube filled with 9 ml of Mannitol Salt Broth (MSB) media while, for Amies media containing hand swab samples. The Amies media was vortexed until it became liquid and then 1 ml of the Amies media was put into a 20 ml test tube filled with 9 ml of Mannitol Salt Broth (MSB) media. The test tube containing Mannitol Salt Broth (MSB) which had been mixed with the sample was incubated in an incubator (Isuzu Model 2-2195, Jica) at 37°C for 24 hours. The samples were cultured and purified using Mannitol Salt Agar (MSA) (Oxoid CM0085) and then incubated at 37°C for 24 hours.

Microscopic examinations of bacteria were carried out through Gram staining to obtain a picture of Gram-positive bacteria in the form of cocci and clusters. The biochemical examinations were carried out using a catalase test and a coagulase test. The catalase test was carried out by dripping 3% hydrogen peroxide (H_2O_2) on bacterial colonies that had been placed on the surface of the object glass (Mustafa 2014). The coagulase test was carried out by dripping 200 µl of rabbit plasma into a coagulase test tube containing bacterial colonies and then incubating at 37°C for 24 hours (Effendi et al. 2019).

Disc diffusion methods

The test was carried out concerning the Clinical and Laboratory Standards Institute (CLSI) 2020 guidelines in which *S. aureus* was tested for susceptibility to the antibiotics Oxacillin 30 µg, Cefoxitin 30 µg, Tetracycline 30 µg, Erythromycin 15 µg, and Gentamicin 10 µg (Oxoid) on Muller Hinton Agar (MHA) plates (Oxoid, CM0337). These antibiotics were selected based on various data on human veterinary therapy, the use of antibiotics in human medicine, and several epidemiological studies (Ramandinianto et al. 2020a). The isolates that have been isolated and identified will be purified on Mannitol Salt Agar (MSA) (HiMedia Pvt. Ltd., M118) and incubated at 37°C for 24 hours as a 0.5 Mc Farland suspension and then taken using a sterile cotton swab of size S (AKD 10903610549). Then wipe evenly on the surface of the Muller Hinton Agar (MHA) media (Oxoid, CM0337). Place the antibiotic disks Oxacillin 30 µg, Cefoxitin 30 µg, Tetracycline 30 µg, Erythromycin 15 µg, and Gentamicin 10 µg (Oxoid) side by side with a distance of 5 cm on Muller Hinton Agar (MHA) media that has been inoculated with isolates and then incubated at 37°C for 24 hours to measure the inhibition zone.

MRSA confirmation by ORSAB

Staphylococcus aureus isolates resistant to Oxacillin 30 µg and Cefoxitin 30 µg (Oxoid) were confirmed by Oxacillin Resistance Screening Agar Base (ORSAB) (HiMedia M1415) using *S. aureus* isolates from Muller Hinton Agar (MHA) media, several colonies are taken and then cultured on Oxacillin Resistance Screening Agar Base (ORSAB) (HiMedia Pvt Ltd., M1415) plus Oxacillin Resistance Selective Supplement (Supplement, HiMedia Pvt. Ltd., FD191) (Decline et al. 2020).

RESULTS AND DISCUSSION

Results

The results of the sample examination showed that from 109 milk samples and 41 farmer hand swab samples taken at each farm, there were 54 samples (49.54%) found positive for *S. aureus* from milk samples and 26 samples (63.41%) for *S. aureus* from hand swab samples (out of a total of 150 samples of milk and hand swabs, total 80 samples (53.33%) positive for *S. aureus*) (Figure 1) based on morphological culture characteristics and biochemical tests (Table 1). Basically, *S. aureus* can be transmitted to humans through milk contamination, but milk contamination can be sourced from humans during milking (Dhanashekar et al. 2012). The positive number of *S. aureus* which is more than 40% can be caused by several factors, mainly due to the unhygienic factor of milking carried out by farmers. Contamination in milk can be sourced from the milk handling system, udder health from the surface of the udder to the teat, and milk storage equipment (Reta et al. 2016).

The antibiotic resistance profile from the results of the antibiotic resistance test showed that 23 *S. aureus* isolates (28.75%) were detected resistant to 1 class of antibiotics

tested, while, 30 *S. aureus* isolates (37.5%) were resistant to 2 classes of antibiotics, and 8 *S. aureus* isolates (10%) were confirmed to be Multidrug-Resistant (MDR) because they were resistant to 3 classes of antibiotics (Figure 2) which were dominated by the pattern of antibiotic resistance OX–TE–E (Oxacillin, Tetracycline, Erythromycin) with a total of 3 (3.75%) *S. aureus* isolates followed by antibiotic resistance pattern OX–TE–CN (Oxacillin, Tetracycline, Gentamicin) and OX–FOX–TE–E (Oxacillin, Cefoxitin, Tetracycline, Erythromycin) with 2 *S. aureus* isolates (2.5%) each (Table 2).

The existence of Multidrug-Resistant (MDR) *S. aureus* isolates was caused by the occurrence of an integrated genetic transfer of the genome from the main source, namely Coagulase-Negative *Staphylococci* (CoNS). *S. aureus* has a higher pathogenic level than Coagulase-Negative Staphylococci (CoNS) but in addition, *S. aureus* has a higher tendency to have Multidrug-Resistant (MDR) properties (Table 3). There was the same number of *S. aureus* isolates with Multidrug-Resistant (MDR) characteristics (Figure 2) in the isolates taken from milk and hand swabs, namely 4 isolates (5%) each. This can be influenced by the post-mastitis management system and post-milking treatment in dairy farms. The percentage pattern of *S. aureus* resistance to several antibiotic agents can be caused by inappropriate antibiotic administration or excessive administration of antibiotics in the treatment of infectious diseases in dairy cattle (Chowdhury et al. 2021) and this can also be caused by cross-contamination from farmers when carrying out a milking process in which the risk factors for transmission of *S. aureus* with Multidrug-Resistant (MDR) characteristics may be sourced from breeders, veterinarians, and the surrounding community (Tong et al. 2015).

Methicillin-Resistant *S. aureus* (MRSA) testing was performed on *S. aureus* isolates that were resistant to Oxacillin and Cefoxitin antibiotics. The results of this study found 42 isolates suspected to be Methicillin-Resistant *S. aureus* (MRSA) because they were resistant to the antibiotics Oxacillin and Cefoxitin, then from the 42 isolates after testing Oxacillin Resistance Screening Agar Base (ORSAB) (Table 4). A total of 20 isolates (47.62%) were found positive for Oxacillin Resistance Screening Agar Base (ORSAB) (Figure 3). Not all strains of Methicillin-Resistant *S. aureus* (MRSA) in this study were Multidrug-Resistant (MDR) to the antibiotics tested, of which 3 MRSA isolates were MDR while 17 other MRSA isolates were not.

Discussion

Milk Borne Disease (MBD) is a quite common public health problem because MBD not only has an impact on human health but also has an impact on the health of dairy cows, especially in the milk production and quality sector (Berhe et al. 2020). Several previous studies have reported the incidence of *S. aureus* in milk in both developed and developing countries and the resistance to antibiotics (Abdi et al. 2018). Improper and unhygienic handling of milk, especially during the milking process has an important role in the occurrence of milk contamination (Amenu et al. 2019). The use of the unhygienic hands of farmers during the milking process can also potentially transmit pathogenic bacteria in milk including *S. aureus* (Nyokabi et al. 2021). Dairy cow's milk contaminated with *S. aureus* can be potentially harmful to the health of consumers including human beings (McMillan et al. 2016).

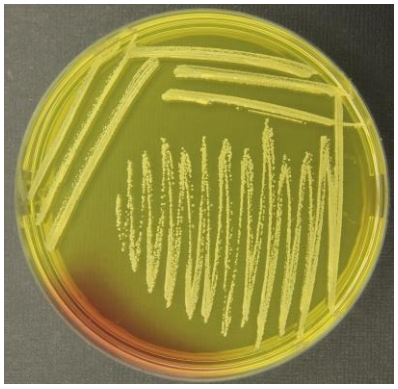

Figure 1. *Staphylococcus aureus* isolates in Mannitol Salt Agar

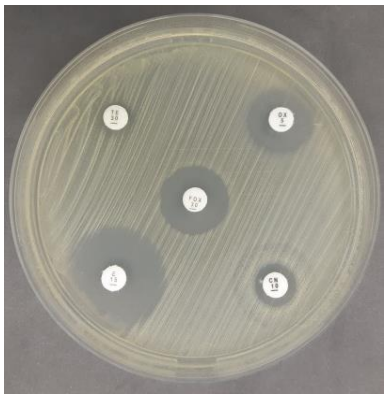

Figure 2. Antibiotic susceptibility test of *Staphylococcus aureus* isolate of Multidrug-Resistant (MDR) in Mueller Hinton Agar (MHA)

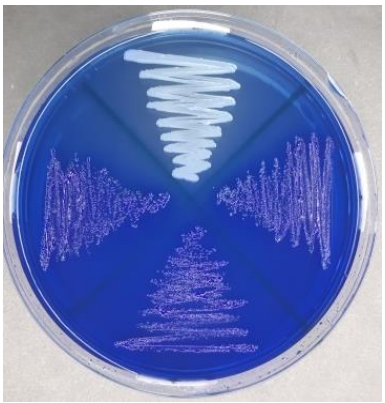

Figure 3. Confirmation for Methicillin-Resistant *Staphylococcus aureus* (MRSA) by Oxacillin Resistance Screening Agar Base (ORSAB). The blue color shows positive confirmation results, white results show negative confirmation results

Table 1. Isolation of *Staphylococcus aureus* by type of sample

Sample type	Sample code	Sample size	Positive *Staphylococcus aureus* (%)
Milk	AS	109	54 (49.54%)
Swab hand	AT	41	26 (63.41%)
Total		150	80 (53.33%)

Note: % (Percentage of positive *Staphylococcus aureus*)

Table 2. Resistance profile of isolated *Staphylococcus aureus* by group of antibiotic

Group of antibiotics	Resistance profile	Number of isolates (n=80) Resistant isolates (%)	Total number of isolates (%)
0	No one is resistant	19 (23.75%)	19 (23.75%)
1	OX	6 (7.5%)	23 (28.75%)
	TE	13 (16.25%)	
	E	4 (5%)	
2	OX – FOX	2 (2.5%)	30 (37.5%)
	OX – FOX – E	4 (5%)	
	OX – TE	11 (13.75%)	
	OX – E	8 (10%)	
	OX – CN	3 (3.75%)	
	TE – E	1 (1.25%)	
	TE – CN	1 (1.25%)	
≥3	OX – TE – E	3 (3.75%)	8 (10%)
	OX – TE – CN	2 (2.5%)	
	OX – E – CN	1 (1.25%)	
	OX – FOX – TE – E	2 (2.5%)	

Note: OX: Oxacillin 30 µg, FOX: Cefoxitin 30 µg, TE: Tetracycline 30 µg, E: Erythromycin 15 µg, CN: Gentamicin 10 µg, %: percentage. Info: Total 8 isolate Resistance with ≥3 Group of Antibiotic are Multidrug-Resistant (MDR)

Table 3. Profile Multidrug Resistance of *S. aureus* isolates

Sample type	Sample code	Resistance profile				
		OX 30 µg	FOX 30 µg	TE 30 µg	E 15 µg	CN 10 µg
Milk	AS 3	✓	-	✓	-	✓
	AS 45	✓	-	✓	✓	-
	AS 64	✓	-	✓	✓	-
	AS 80	✓	✓	✓	✓	-
Swab hand	AT 21	✓	-	✓	-	✓
	AT 27	✓	-	-	✓	✓
	AT 40	✓	-	✓	✓	-
	AT 41	✓	✓	✓	✓	-

Note: ✓: Resistant, OX: Oxacillin 30 µg, FOX: Cefoxitin 30 µg, TE: Tetracycline 30 µg, E: Erythromycin 15 µg, CN: Gentamicin 10 µg

Table 4. Total Number Confirmed MRSA by ORSAB

Sample type	A resistance group of antibiotics	No. of isolates tested ORSAB (n=42)	Positive ORSAB test	No. of MRSA
Milk	1	5	4 (9.52%)	15 (35.71%)
	2	19	11 (26.19%)	
	≥3	4	0 (0%)	
Swab hand	1	1	0 (0%)	5 (11.9%)
	2	9	2 (4.76%)	
	≥3	4	3 (7.14%)	
Total no. of MRSA		42	20 (47.62%)	20 (47.62%)

Note: %: Percentage of positive ORSAB

Staphylococcus aureus is a pathogenic bacterium that can cause various infectious diseases ranging from skin to systemic infections (Cheung et al. 2021). In this study, out of 150 samples (milk and swab hand), 80 samples (53.33%) were found to be contaminated with *S. aureus*. This percentage was higher than the research conducted by Wang et al. (2018) in which they analyzed 195 milk samples, of which 90 samples (46.15%) were found positive with *S. aureus.* Another study conducted by Jahan et al. (2014) evaluated 47 milk samples, of which 12 samples (25.53%) showed contamination of *S. aureus. S. aureus* has properties that are sensitive to antibiotics, but *S. aureus* has the potential to quickly develop to become resistant to several kinds of antibiotics (Chambers and DeLeo 2009). The incidence of bacterial resistance to antibiotics is a natural mechanism for the survival of bacteria so their presence can have a negative impact on society (Fair and Tor 2014). In this study, several isolates of *S. aureus* were found that were Multidrug-Resistant (MDR) against more than equal to 3 groups of antibiotics, namely 8 isolates (10%) in the disk diffusion method, this percentage is still lower than the research conducted by Kou et al. (2021) isolated 62 milk samples, of which 29 isolates (46.77%) were *S. aureus* that was resistant to more than equal to 3 groups of antibiotics or Multidrug-Resistant (MDR).

Methicillin-Resistant *S. aureus* (MRSA) testing can be done using Oxacillin and Cefoxitin antibiotic disks followed by Oxacillin Resistance Screening Agar Base (ORSAB) test (Mustikawati et al. 2015), in line with Pourmand et al. (2014) which stated that the ORSAB test has a specificity of 100%. In this study, 20 isolates (47.62%) of *S. aureus* were found positive for ORSAB; so, 20 isolates could be said to be MRSA isolates. Oxacillin and Cefoxitin are beta-lactam antibiotics often used in the treatment of mastitis in dairy cattle caused by *S. aureus* (Khairullah et al. 2020; Pu et al. 2014). Detection of MRSA using disk diffusion is still widely used because it can be done quickly at a lower cost (Bennett and Sharp 2008). Diffusion disk testing using Oxacillin and Cefoxitin has the same sensitivity level of 100%, specificity where Oxacillin diffusion disk is 74.07% while Cefoxitin disk diffusion is 92.59% (Vyas et al. 2015). In a study conducted by Ramandinianto et al. (2020a) reported that isolates that were resistant to Oxacillin and Cefoxitin

antibiotics could be continued with Oxacillin Resistance Screening Agar Base (ORSAB) testing to confirm the presence of MRSA isolates. The mechanism of action of *S. aureus* resistance to beta-lactam antibiotics is a modification of the Penicillin-Binding-Protein (PBP) target, inactivation of antibiotics with beta-lactamases, and impaired drug penetration to achieve PBP goals (Hamilton et al. 2017). PBP2a is expressed by the gene encoding *mec*A and *mec*C which has a very low affinity for beta-lactams so even if these bacteria are cultured in a medium containing high concentrations of beta-lactams, MRSA can survive (Rolo et al. 2017).

In addition to this, 3 isolates were found to be Methicillin-Resistant *S. aureus* (MRSA) as well as Multidrug-Resistant (MDR) because the three isolates were positive on the Oxacillin Resistance Screening Agar Base (ORSAB) test and resistant to more than equal to 3 classes of antibiotics. These results are in line with the study conducted by Neyra et al. (2014) which stated *S. aureus* isolates are Methicillin-Resistant *S. aureus* (MRSA) is not necessarily Multidrug-Resistant (MDR) depending on the type of antibiotic group used in the study. This also applies to the contrary that *S. aureus* isolates that are Methicillin-Resistant *S. aureus* (MRSA) are not necessarily Multidrug-Resistant (MDR) because there are *S. aureus* isolates that are only resistant to beta-lactam antibiotics but are still sensitive to other classes of antibiotics (Kot et al. 2020).

The dairy farming industry can act as a potential reservoir for the emergence of MRSA strains that are easily spread and are adaptive when infecting hosts both animals and humans (Khairullah et al. 2020). The presence of MRSA based on the ORSAB test from milk and hand swabs of farmers is a source of bacterial zoonotic infections that can appear and attack humans (Ramandinianto et al. 2020b; Rahmaniar et al. 2020). Bacterial zoonotic diseases can be transferred from animals to humans in various ways, including through animal products such as milk (Tyasningsih et al. 2019) or zoonotic bacteria originating from animals can reach humans via the direct fecal-oral route, contaminated food products, improper food handling, and inadequate cooking (Harijani et al. 2020). Thus in the One Health concept, humans who are close to animals will be able to contract zoonotic pathogenic bacteria that can spread to other humans in the community (Decline et al. 2020). These studies showed that the ORSAB test is more sensitive but less specific than the Cefoxitin test. This finding is very important to detect the presence of MRSA so that it can be the basis for taking steps to control the spread of MRSA in dairy farms.

In conclusion this study, it can be found that there are several *S. aureus* isolates that are Multidrug-Resistant (MDR) and Methicillin-Resistant *S. aureus* (MRSA) in dairy farms that can be spread to the community and endanger public health. Risk factors for MDR and MRSA infection in livestock, especially in dairy cattle, need to be investigated and these data are very important for the preparation of specific guidelines for MDR and MRSA control in veterinary practice. In addition, prevention and control measures are needed to suppress the spread of Multidrug-Resistant (MDR) and Methicillin-Resistant *S. aureus* (MRSA) infection on a dairy farm in Probolinggo, East Java, Indonesia.

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