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Detection of multidrug-resistant *Staphylococcus aureus* and coagulase-negative staphylococci in cow milk and hands of farmers in East Java, Indonesia

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Abstract. *Khairullah AR, Kurniawan SC, Sudjarwo SA, Effendi MH, Afnani DA, Silaen OSM, Putra GDS, Riwu KHP, Widodo A, Ramandinianto SC. 2023. Detection of multidrug-resistant* Staphylococcus aureus *and coagulase-negative staphylococci in cow milk and hands of farmers in East Java, Indonesia. Biodiversitas 24: 658-664.* Cow milk is a nutrient-dense food containing various nutrients, i.e., carbohydrates, fat, and protein. However, it can potentially spread several pathogenic bacteria, such as staphylococci. Staphylococci cause public health problems. Staphylococcal strains were divided into two groups based on coagulase characteristics, namely coagulase-positive and coagulase-negative staphylococci (CoNS). Coagulase-positive was represented by *Staphylococcus aureus*. *S. aureus* and CoNS can become resistant to several antibiotics, known as multidrug resistance (MDR) bacteria. *S. aureus* and CoNS bacteria are known as causal agents in human infections. This study aims to identify the presence of multidrug resistance *S. aureus* and CoNS bacteria in several dairy farms in East Java, Indonesia. We collected 332 milk and 125 swab samples from the hand of farmers from several dairy farms in Probolinggo, Tulungagung, and Blitar of East Java, Indonesia. The collected *S. aureus* and CoNS were tested for antibiotic susceptibility using the disc diffusion method. The antibiotic used were cefoxitin, erythromycin, gentamicin, tetracycline, and oxacillin. The results of isolation and identification based on morphological and biochemical characteristics showed that out of 457 samples, 291 samples (63.68%) were positive for S. aureus, and 166 samples (36.32%) were positive for CoNs. There were 15 *S. aureus* isolates (5.15%) and 7 CoNS isolates (4.22%) confirmed as MDR; therefore, it is confirmed that *S. aureus* and CoNS resistance to various drugs was very high in the province of East Java. Intensive livestock systems, high livestock densities, and excessive use of antibiotics in modern dairy systems may predispose the developing growth of MDR *S. aureus* and CoNS infections. In conclusion, the results of this study indicate the presence of very high multidrug resistant *S. aureus* and CoNS in the East Java provinces. MDR bacteria can be prevented from spreading by improving biosecurity, hygiene in the milking process, and regular health care for animals and dairy workers.

Keywords: CoNS, hand swab, MDR, milk, public health, *Staphylococcus aureus*

INTRODUCTION

Foodborne disease (FBD) is caused by ingesting water and food contaminated with pathogens, poisons, or chemicals (Bintsis 2017). Bacteria (66%) are the primary cause of FBD, followed by chemicals (26%), viruses (4%), and parasites (4%) (Abebe et al. 2020). Cow milk is an animal-derived dietary ingredient that has the potential to spread several pathogenic microbes that could affect public health, often known as a milk-borne disease (MBD) (Ramandinianto et al. 2020a). According to several studies, numerous infections, including staphylococci strains, can be spread via cow's milk (Khairullah et al. 2022a; Asiimwe et al. 2017; Zigo et al. 2022). It is due to the chemical components of milk that are needed by the body, making it a nutrient-rich substrate favorable for the growth and spread of harmful germs (Górska-Warsewicz et al. 2019). Based on the coagulase characteristics, staphylococcal strains are divided into two main groups: coagulase positive and negative. The coagulase-positive staphylococci are represented by *Staphylococcus aureus. Staphylococcus aureus* is a pathogenic bacteria. It causes several human illnesses, including Staphylococcal, scalded skin syndrome, toxic shock syndrome, endocarditis, abscesses, and impetigo (Rahmaniar et al. 2020; Decline et al. 2020; Khairullah et al. 2022b), and various infectious diseases in immunocompetent hosts, from the skin to systemic infections (Rossi et al. 2020). The second group was numerous coagulase-negative staphylococci (CoNS) that are often present on the skin and mucous membranes of both animals and humans as a normal and natural microbiota (Michalik et al. 2020). Therefore, coNS is categorized as less or non-pathogenic, but its opportunistic pathogen contributes to infection (Heilmann et al. 2019).

The primary treatment of *S. aureus* and CoNS infections is antibiotic treatment or therapy; however, the overuse and misuse of antibiotics in veterinary medicine, animal husbandry, agriculture, and the treatment of human diseases increases the risk of bacterial antibiotic resistance (Economou and Gousia 2015). Due to their multidrug resistance (MDR) characteristics, which allow them to be resistant to several antibiotic classes, *S. aureus* and CoNS bacteria are frequently the cause of many recognized infections (Terreni et al. 2021). In addition, poor milk handling increases milk contamination with pathogenic microbes such as *S. aureus* and CoNS (Paradis et al. 2010). The widespread pathogenic microorganisms cause high-level contamination during milking (Msalya 2017).

Most of the cow milk production in Indonesia is in East Java (Ramandinianto et al. 2020b). The number of cattle breeders has increased from small to large scale in rural regions of East Java (Kalangi et al. 2014). Cases of infection in dairy cows are increasing in line with the increase in milk production and the dairy cattle population in East Java due to improper management of dairy farms (Khasanah et al. 2021). The overuse of antibiotics in managing dairy cows and the transmission of pathogenic bacteria during milking are the two primary causes of the high MDR bacterial contamination rate in East Java dairy farms. The MDR bacterial contamination might originate from the milk obtained from the cows' udders and the hands of farmers during the milking process (Ansharieta et al. 2021).

Laboratory tests are required because the prevalence of MDR *S. aureus* and CoNS infection can be a public health issue. Therefore, this study aimed to determine MDR *S. aureus* and CoNS bacteria isolated from milk and farmer hand swabs from several farms in East Java.

MATERIALS AND METHODS

Study design and sampling

This study was a surveillance study to detect multidrug resistance of *Staphylococcus aureus* and CoNS in East Java, Indonesia. A total of 332 milk samples and 125 hand swabs from farmers were collected from several dairy farms in East Java, including Probolinggo, Tulungagung, and Blitar districts. This study was carried out between July 2021 to February 2022. The milk samples were collected after the third milking pressure using a sterile cotton swab to collect samples from each hand of the farmer. Each milk sample was collected up to 30 mL, while the hand swab was subsequently preserved in Amies medium. No additional treatment, such as sterilization or disinfection of the udder, was carried out during sampling. All the samples were taken according to each farm's standard operational procedures for milking.

Bacterial strains isolation

One milliliter (1 mL) of each milk sample was added to a 20 mL test tube filled with 9 mL of the Mannitol Salt Broth (Oxoid, UK) medium. The Amies medium (Labware, China) containing the hand swab samples was vortexed until it became liquid before adding it to the 20 mL test tube. Then, the test tube was incubated for 24 hours at 37°C. Mannitol Salt Agar (MSA) (Oxoid, UK) was used to culture and purify the bacterial isolates from the samples and followed by a 24-hour incubation at 37°C.

Gram staining was performed to acquire an overview of Gram-positive bacteria in clusters and cocci under microscopic examination. The catalase test was performed by adding 3% hydrogen peroxide (H_2O_2) (Onemed, Indonesia) to bacteria colonies and placed on a glass slide. The coagulase test was performed by adding 200 µl of rabbit plasma to the coagulase test tube containing bacterial colonies that have been incubated for 24 h at 37°C.

Disc diffusion methods

The antibiotics were tested for their susceptibility against *S. aureus* and CoNS using Muller Hinton Agar (MHA) (Oxoid, UK). The antibiotics used were cefoxitin (30 µg), erythromycin (15 µg), gentamicin (10 µg), tetracycline (30 µg), and oxacillin (30 µg) (Oxoid, UK). The susceptibility test was performed by following the 2020 Clinical and Laboratory Standards Institute (CLSI). The isolates were cultured on Mannitol Salt Agar (MSA) and incubated at 37°C for 24 hours. MSA was a specific growth medium for *S. aureus.* Then, the bacterial suspension was taken using a size S sterile cotton bud, inoculated on the MHA medium, and spread evenly over the medium surface. After inoculation, the antibiotic discs were put next to one another at a distance of 5 cm apart.

RESULTS AND DISCUSSION

Bacterial contamination from milk samples and hand swabs

Based on the number of collected samples (125 farmer hand swab samples and 332 milk samples), there were 208 milk samples (45.51%) and 83 hand swab samples (18.16%) were contaminated by *S. aureus.* In total, 291 samples (63.7%) were contaminated by *S. aureus*. At the same time, 42 samples (9.19%) of hand swab samples and 124 (27.13%) milk samples were positive for CoNS contamination. A total of 166 samples (36.32%) were positive for CoNS contamination based on morphological culture characteristics and biochemical tests (Table 1). Contamination of *S. aureus* and CoNS in milk samples and the hands of farmers could spread to humans. Contaminated milk can also originate from people who are milking cows (Yanuartono et al. 2020). There are many potential causes of *S. aureus* and CoNS infections, but farmers' unhygienic milking practices are the main ones (Kümmel et al. 2016; Taponen et al. 2017). The milk handling system, the udder's condition from the surface to the nipple, and the milk storage equipment are all potential sources of milk contamination (Calahorrano-Moreno et al. 2022).

Resistant bacteria to the available antibiotics

The results of the antibiotic resistance test revealed that 107 *S. aureus* isolates (36.77%) were resistant to 1 type of antibiotic used in this study, 59 isolates of *S. aureus* (20.27%) were resistant to two types of antibiotics, and 15 *S. aureus* isolates were resistant to 3 3 types of antibiotics. Therefore, 15 *S. aureus* isolates (5.15%) were verified as MDR. *S. aureus* which characterized by the OX-FOX-TE-E and OX-TE-E patterns of antibiotic resistance. Each antibiotic resistance pattern consists of 4 (3.75%) *S. aureus* isolates (Table 2). In addition, three *S. aureus* isolates (2.5%) have the OX-FOX-TE-E-CN antibiotic resistance pattern (Table 2).

Antibiotic resistance test also revealed that 67 CoNS isolates (40.36%) were resistant to one tested antibiotic. In comparison, 27 CoNS isolates (16.26%) were resistant to 2 classes, and due to their resistance to 3 classes of antibiotics, 7 CoNS isolates (4.22%) were determined as MDR isolates. In addition, four CoNS isolates (2.41%) had a pattern of OX-TE-E antibiotic resistance dominating the MDR of CoNS. In comparison, 2 isolates had a pattern of OX-FOX-TE-E antibiotic resistance (Table 3).

Genome-integrated genetic transfer from the primary source, namely CoNS, is the reason for the existence of MDR *S. aureus* (Osman et al. 2016). In contrast to CoNS, *S. aureus* has a higher pathogenicity and a tendency toward MDR characteristics (França et al. 2021). There were 15 *S. aureus* isolates (Table 4), and 7 CoNS isolates (Table 5) were MDR isolates taken from milk and farmers' hand swabs. It may be impacted by how dairy farms handle during milking process and milking management (Verdier-Metz et al. 2022). Inappropriate or excessive antibiotic use in treating infectious diseases in dairy cows may contribute to *S. aureus* and CoNS resistance to various antibiotic agents (Manyi-Loh et al. 2018). Cross-contamination by farmers during the milking process is another possible cause of antibiotic resistance. Breeders, veterinarians, and the community could all threaten the spread of *S. aureus* and CoNS with MDR characteristics (Jeżak and Kozajda 2022).

Table 1. The percentage of *Staphylococcus aureus* and CoNS contamination from milk and hand swab samples

Sample type	Number of samples	*S. aureus* contamination (%)	CoNS contamination (%)
Milk	332	208 (45.51%)	124 (27.13%)
Hand swab	125	83 (18.16%)	42 (9.19%)
Total	457	291 (63.68%)	166 (36.32%)

Table 2. Isolated *Staphylococcus aureus* that is resistant to the antibiotic group

Group of antibiotics	Resistant to	Number of isolates (n=291)	Total number of isolates (%)
		Resistant isolates (%)	
0	Not resistant	110 (37.80%)	110 (37.80%)
1	OX-FOX	15 (5.15%)	107 (36.77%)
	OX	32 (10%)	
	TE	46 (15,81%)	
	E	10 (3.44%)	
	CN	4 (1.37%)	
2	OX-FOX-TE	5 (1.72%)	59 (20.27%)
	OX-FOX-E	11 (3.78%)	
	OX-TE	23 (7.90%)	
	OX-E	8 (2.75%)	
	OX-CN	3 (1.03%)	
	TE-E	8 (2.75%)	
	TE-CN	1 (0.34%)	
≥3	OX-FOX-TE-E	4 (1.37%)	15 (5.15%)
	OX-FOX-E-CN	1 (0.34%)	
	OX-TE-E	4 (1.37%)	
	OX-TE-CN	2 (0.69%)	
	OX-E-CN	1 (0.34%)	
	OX-FOX-TE-E-CN	3 (1.03%)	

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

Table 3. Isolated CoNS that is resistant to the antibiotic group

Group of antibiotics	Resistant to	Number of isolates (n=166)	Total number of isolates (%)
		Resistant isolates (%)	
0	Not resistant	65 (39.16%)	65 (39.16%)
1	OX	37 (22.29%)	67 (40.36%)
	TE	27 (16.26%)	
	E	1 (0.60%)	
	CN	2 (1.20%)	
2	OX-FOX-CN	1 (0.60%)	27 (16.26%)
	OX-TE	15 (9,04%)	
	OX-E	3 (1.81%)	
	OX-CN	1 (0.60%)	
	TE-E	6 (3.61%)	
	TE-CN	1 (0.60%)	
≥3	OX-FOX-TE-E	2 (1.20%)	7 (4.22%)
	OX-TE-E	4 (2.41%)	
	OX-FOX-TE-E-CN	1 (0.60%)	

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

Table 4. *Staphylococcus aureus* isolates with MDR characteristics to antibiotics

Location	Sample type	Sample code	Resistant to				
			OX	FOX	TE	E	CN
Probolinggo	Milk	AS 3	✓	–	✓	–	✓
		AS 16	✓	–	✓	–	✓
		AS 45	✓	–	✓	✓	–
		AS 64	✓	–	✓	✓	–
		AS 80	✓	✓	✓	✓	–
	Swab hand	AT 27	✓	–	–	✓	✓
		AT 28	✓	✓	✓	✓	–
		AT 40	✓	–	✓	✓	–
Tulungagung	Milk	TS 17	✓	✓	–	✓	✓
		TS 39	✓	✓	✓	✓	✓
		TS 70	✓	–	✓	✓	–
		TS 77	✓	✓	✓	✓	✓
	Swab hand	TT 37	✓	✓	✓	✓	✓
Blitar	Milk	SS 9	✓	✓	✓	✓	–
	Swab hand	ST 29	✓	✓	✓	✓	–

Note: ✓= Resistant, FOX: Cefoxitin, E: Erythromycin, CN: Gentamicin, TE: Tetracycline, OX: Oxacillin

Table 5. CoNS isolates with MDR characteristics to antibiotics

Location	Sample type	Sample code	Resistant to				
			OX	FOX	TE	E	CN
Probolinggo	Milk	AS 59	✓	–	✓	✓	–
	Swab hand	AT 6	✓	–	✓	✓	–
		AT 39	✓	–	✓	✓	–
Tulungagung	Milk	None	–	–	–	–	–
	Swab hand	None	–	–	–	–	–
Blitar	Milk	SS 1	✓	–	✓	✓	–
		SS 25	✓	✓	✓	✓	✓
		SS 26	✓	✓	✓	✓	–
		SS 29	✓	✓	✓	✓	–
	Swab hand	None	–	–	–	–	–

Note: ✓: Resistant, FOX: Cefoxitin, E: Erythromycin, CN: Gentamicin, TE: Tetracycline, OX: Oxacillin

Discussion

Milk-borne disease (MBD) affects not only human health and dairy cows but also the quality of dairy milk, so MBD has become a public health concern (Cheng and Han 2020). Previous studies showed the increased prevalence of *S. aureus* contamination in milk in developed and developing nations and antibiotic resistance (Akindolire et al. 2015; Khairullah et al. 2019; Ramandinianto et al. 2021). Staphylococci are pathogenic bacteria due to their ability to produce enterotoxins (Zeaki et al. 2019). Ingesting foods or milk containing enterotoxins produced by staphylococci is called staphylococcal poisoning (Johler et al. 2015). The enterotoxins need one to eight hours to develop toxic symptoms (Grispoldi et al. 2019). Symptoms of enterotoxin poisoning are stomach pain, vomiting, and diarrhea (Argudin et al. 2010).

Careless and unhygienic handling of milk, especially during pumping, could result in contaminated milk (Khairullah et al. 2020a). Unclean hands while milking can potentially spread pathogenic bacteria, such as staphylococci, to the milk (Gebremedhin et al. 2022) and cow udders, cow bodies, airborne dust, unsanitary milking equipment (Robles et al. 2020). Due to frequent contact between farmers and dairy cows while milking, unhygienic farmers' hands could be a source of *S. aureus* transmission (Regasa et al. 2019). Staphylococci-tainted dairy cow milk can potentially endanger customers' health, including humans (Kou et al. 2021).

Staphylococcus aureus and CoNS bacteria-contaminated milk are frequently linked to subclinical mastitis in dairy cows (Suwito et al. 2021; Khairullah et al. 2020b). *Staphylococcus aureus* was present in 291 samples (63.68%), and 166 samples (36.32%) were contaminated with CoNS out of the 457 samples (milk and hand swabs) in this study. A study by El-Jakee et al. (2013) revealed that out of 632 milk samples, there were 313 samples (49.52%) were contaminated with *S. aureus*, and 111 samples (17.56%) were contaminated with CoNS. Therefore, the presence of *S. aureus* and CoNS in milk samples is in line with the results of this study. Raspanti et al. (2016) showed that out of 441 milk samples, 222 samples (50.34%) contained *S. aureus,* and 219 samples (49.66%) contained CoNS.

Although *S. aureus* and CoNS are susceptible to some antibiotics, they can quickly develop resistance to many drugs (Pantosti et al. 2007; John and Harvin 2007). A natural survival strategy for bacteria is the development of antibiotic resistance, which can have a detrimental effect on society (Larsson and Flach 2022). This study showed that several CoNS and *S. aureus* isolates were categorized as MDR due to their resistance to three or more classes of antibiotics, namely 15 isolates of *S. aureus* (5.15%) and 7 isolates of CoNS (4.22%). A previous study by Petróczki et al. (2021) revealed that out of 62 bacteria isolated from milk samples, 17 isolates (27.42%) were MDR *S. aureus*. Another study by Phophi et al. (2019) showed that 142 bacteria were isolated from milk, and 73 isolates (51.41%) were found to be MDR CoNS.

Expressions of latent resistance genes, genes with resistance determinants, or genetic mutations are the main ways bacterial colonies survive in a threatened state (Munita and Arias et al. 2006). A multistep mutation gradually increases resistance in MDR bacteria (Vogwill and MacLean 2015). Resistance genes can be passed from chromosomes to plasmids or vice versa on transposons and integrons, where factor R plasmids, also known as infectious plasmids, can be transferred (Partridge et al. 2018). In addition, extrachromosomal genes in plasmids or bacteriophages are present in some bacteria (Deutsch et al. 2018).

The future development of MDR *S. aureus* and CoNS infections may be influenced by intensive husbandry, high livestock density, and overuse of antibiotics in modern dairy farming systems (Manyi-Loh et al. 2018). Setting the distance between cows, placing pens far from the kitchen, isolating cows in separate pens, brooming manure, and regularly washing dairy cows might prevent the spread of MDR bacteria in dairy farms (Pandit et al. 2021). However, groups of dairy cows close to one another can result in MDR bacterial infections (Widodo et al. 2022). Control of bacterial diseases requires surveillance and laboratory tests (Langley et al. 2015). Therefore, the MDR of *S. aureus* and CoNS must be prevented through improved biosecurity and hygiene in the milking process and environments in dairy sheds and following the standard animal and human health care procedures (Garcia et al. 2019).

In conclusion, results of this study indicate the presence of very high multidrug resistant *S. aureus* and CoNS in dairy farms in the East Java provinces. 15 MDR *S. aureus* isolates and 7 MDR CoNS isolates were detected in milk and farmer hand swabs. The spreading of multidrug resistance bacteria can be controlled by increasing biosecurity, hygiene in the milking process, and regular health care measures for animals and dairy workers.

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