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Detection of multidrug-resistant (MDR) *Staphylococcus aureus* isolated from raw milk in a dairy farm

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Abstract

Background: The presence of microorganisms in milk cannot be totally prevented, making it a crucial factor in determining its quality. *Staphylococcus aureus* is one of the germs that can be detected in milk and other dairy products. Many *S. aureus* related foodborne disease outbreaks have been reported to be caused by multidrug-resistant (MDR) strains.

Methods: In the Indonesian province of East Java, a total of 112 raw milk samples were taken from various dairy farms. 10 ml of milk was collected from each cow using a sterile bottle. The Milk samples were transported to the laboratory under a cold chain and analyzed using standard microbiological procedures. Evaluation of *S. aureus* isolates for antibiotic susceptibility using the Kirby-Bauer disc diffusion method.

Results: Findings from this study showed that 100 (89.2%) out of 112 samples of raw milk taken from dairy farms in 2 regions located in Pasuruan and Lumajang yielded positive for *S. aureus*. The antibiotic sensitivity profile showed that Cefoxitin was the most effective with 2% resistance. The highest resistance recorded was against Ampicillin (78%). Resistance recorded against other antibiotics include Tetracycline (36%), Erythromycin (11%), and Amoxicillin (12%). Multidrug resistance was recorded for *S. aureus* isolates from 10 samples of raw milk.

Conclusion: The study's conclusions highlight the risk of milk contamination and the potential for *S. aureus* to develop multidrug resistance, both of which are harmful to human health. It is strongly recommended that strict hygiene practices be maintained and improved in dairy farms.

Introduction

Milk of animal species contains many essential nutritional supplements such as carbohydrates, proteins, fats, vitamins and minerals that have beneficial effects on human life [1]. As consumers grow more drawn to microprocessed goods, the usage of raw milk is progressively rising. Nonetheless, zoonotic infections may spread through cow's milk [2]. It is established that a variety of foodborne bacteria can damage raw milk derived from animal sources [3–5].

Microbial contamination of milk is an important quality measurement, as it cannot be completely avoided. Several microorganisms are found in milk and other dairy products, one of which is *Staphylococcus aureus* [6]. There is a consistent correlation between the frequency of antibiotic resistance and foodborne *S. aureus* [7]. *S. aureus* is the most major bacterial pathogen of recent years and has caught the attention of international public health initiatives as the third most common cause of food poisoning worldwide [8]. *S. aureus* is a Gram-positive, facultative anaerobic, immobile, and ubiquitous bacterium in nature. These microorganisms naturally inhabit the skin and nasal cavities of warm-blooded animals and are considered to be the main cause of several health-related infections (including endocarditis, soft tissue abscesses and skin infections, osteomyelitis, and bacteraemia) [9].

Life-threatening infections in animals and humans can also be caused by *S. aureus.* In animals, these bacteria cause abscesses, mastitis, urinary tract infections, and dermatitis [10–12]. Dairy farmers suffer financial losses when their cows get mastitis due to the pathogenic bacteria *S. aureus* [13]. When dairy cows are milked, dairy products may become contaminated and enter the food chain. The mammary gland serves as the primary infection reservoir [14]. Serious health hazards for humans can occur when consuming contaminated milk [15]. The fact that it is connected to nosocomial infections in people serves as further evidence for this [16]. Mastitis is still treated with antibiotics, and the use of antibiotics can cause *S. aureus* to become resistant to antibiotics [17]. On the other hand, treatment failure comes with significant financial and health consequences, and antibiotic resistance is an increasing issue [18].

Multidrug-resistant (MDR) strains offer an efficient means of studying antimicrobial resistance in *S. aureus*, a significant global health issue [19]. MDR strains present significant hurdles, particularly in the twenty-first century [20,21]. Given the danger that MDR bacteria pose, it is imperative to control the threat and stop it from spreading [22]. Antimicrobial therapy is a crucial tactic for reducing human infection and mastitis [23]. Antibiotic-resistant *S. aureus* is becoming more common in animals and foods obtained from them as a result of the extensive use of antibiotics in the production of animal meals [24,25].

Methicillin-resistant *Staphylococcus aureus* (MRSA) is one of the MDR strains of *S. aureus* that have been linked to numerous outbreaks of foodborne illness [26–28]. The development of MDR strains as a result of human overuse of antibiotics presents challenges for the treatment of *S. aureus* infections, including MRSA in veterinary medicine [29–31]. Bacteria classified as MDR are isolates that exhibit resistance to at least one of three or more classes of antibiotics [32]. The majority of antimicrobials used in veterinary medicine are also used to treat infections in humans. For instance, *S. aureus* infections in humans and animals have been managed with the use of lincosamides, tetracyclines, beta-lactams, fluoroquinolones, streptomycins, rifamycins, sulfonamides, aminoglycosides, and macrolides [18]. The objective of this study is to identify raw milk from dairy farms in Pasuruan and Lumajang, East Java, Indonesia, that contains MDR *S. aureus*.

Methods

Sample collection

This The Pasuruan and Lumajang Districts of East Java Province, Indonesia, were the locations of 112 raw milk samples that were taken during February and March of 2022. Fifty-two raw milk samples were taken from Pasuruan District and sixty raw milk samples were taken from Lumajang District. The number of cows in the two areas suspected of having mastitis determined the quantity of samples collected. Samples were collected during the morning milking time. Ten millilitres of samples were taken from each cow. Sampling was carried out by collecting the milk into sterile bottles. Samples were delivered to the laboratory using an ice box.

Bacteria isolation and identification

The isolation of *S. aureus* was carried out through enrichment in buffered peptone water; 1 ml of each sample was taken aseptically using a pipette and placed in a 10 ml test tube after which it was filled with 9 ml of Buffered Peptone Water. Test tubes containing the samples were subjected to incubation at 37°C for 24 hours [25]. Samples were streaked on Mannitol Salt Agar (MSA) (HiMediaPvt. Ltd., M118). Gram staining was performed for suspected yellow colonies [33] and examined microscopically. Gram-positive bacteria are cocci-shaped and clustered. Biochemical tests performed include catalase test and coagulase test. After incubating at 37°C for 24 hours, 200 µl of rabbit plasma was dispensed into a coagulase tube containing bacterial colonies to perform the coagulase test [34]. The reaction is positive if the clot does not move when

the tube is tilted (Figure 1). A catalase test was performed by adding 3% hydrogen peroxide (H_2O_2) to bacterial colonies on glass slides. The appearance of air bubbles indicated the presence of *S. aureus* (Figure 2).

Figure 1: The coagulase test on *S. aureus* shows a positive reaction and looks cloudy.

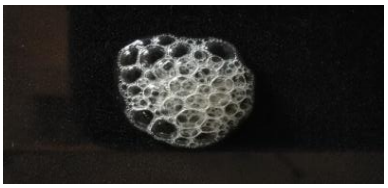

Figure 2: The appearance of air bubbles indicated the presence of *S. aureus*

Antibiotic sensitivity test

Mueller-Hinton agar was used to evaluate *S. aureus* isolates for antibiotic susceptibility using the Kirby-Bauer disc diffusion method. The test was conducted refer to the Clinical and Laboratory Standard Institute [35]. The isolate that was identified and purified on Mannitol Salt Agar (MSA) media (HiMedia Pvt. Ltd., M118) (Figure 3) and isolates was standardized by diluting to 0.5 McFarland's standard. Then wipe using a cotton swab sterile on the surface of Muller Hinton Agar (MHA) plates. The disc contains an antibiotic of known concentration (Oxoid, UK) antibiotics Cefoxitin (30 µg), Tetracycline (30 µg), Erythromycin (15 µg), Ampicillin (25 µg), Amoxicillin (20 µg) applied on Muller Hinton Agar (MHA) plates (HiMedia, M173) using a sterile tweezer and 24 hours incubation at 37°C. The measurement of the diameter of the inhibition zone was used as an interpretation result (Figure 4).

Results

Findings from this study show that 100 (89.2%) samples of the 112 samples of raw milk collected from dairy farms in 2 districts (Pasuruan and Lumajang) of East Java, Indonesia were positive for *S. aureus* out of 52 samples collected from Dairy farms in Pasuran, 48 (92.3%) were positive. In Dairy farms from Lumajang, 60 samples were collected and 52 (86.6%) were positive for *S. aureus.* collected and 52 (86.6%) were positive for *S. aureus.*

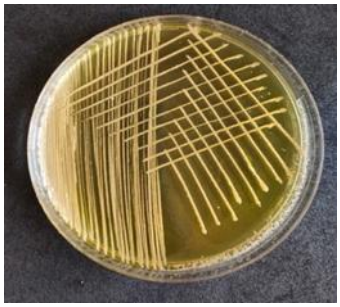

Figure 3: *S. aureus* isolates from raw milk on mannitol salt agar (MSA) shows golden-yellow pigment colonies of mannitol fermentation

Antibiotic Sensitivity Test

Antibiotic sensitivity test results for 112 isolates of *S. aureus* showed that 2 samples (2%) were resistant to Cefoxitin (30µg) and 98 samples (98%) were sensitive. Tetracycline (30 µg) presented with 36% resistance and sensitivity of 60%. Erythromycin (15 µg) presented with 11% resistance and a sensitivity of 73%. Amoxicillin (25 µg) was resisted by 12 samples (12%) and was effective over 74 samples (74%). Antibiotic Ampicillin (20 µg) presented with 78% resistance and 22% susceptibility. Profile for multidrug resistance in *S. aureus* was summarized in (Table 1). The result showed that Staphylococcus isolates from 10 milk samples exhibited MDR traits, especially against more than 3 groups of antibiotics. The resistance profiles for Cefoxitin (20%), Tetracycline (90%), Erythromycin (40%), Amoxicillin (70%) and Ampicillin (100%) were noted (Table 1).

Location	Sample Code	Antibiotic disk inhibition zone diameter (mm)				
		Cefoxitin (FOX)	Tetracycline (TE)	Erythromycin (E)	Amoxicillin (AML)	Ampicillin (SAM)
Pasuruan	P16	21.64 (R)	19.52 (S)	12.32 (R)	14.52 (I)	19.92 (R)
	P18	29.14 (S)	8.12 (R)	23.14 (S)	12.72 (R)	19.22 (R)
	P38	31.94 (S)	9.44 (R)	21.24 (I)	11.22 (R)	15.44 (R)
Lumajang	L5	29.64 (S)	12.22 (R)	23.12 (S)	12.12 (R)	13.54 (R)
	L13	32.84 (S)	9.14 (R)	13.42 (R)	24.12 (S)	23.92 (R)
	L26	27.34 (S)	12.12 (R)	13.12 (R)	14.32 (I)	17.22 (R)
	L32	28.72 (S)	12.44 (R)	21.74 (I)	13.32 (R)	16.82 (R)
	L41	20.34 (R)	7.84 (R)	6.62 (R)	12.14 (R)	22.82 (R)
	L55	29.14 (S)	10.12 (R)	25.72 (S)	12.42 (R)	10.62 (R)
	L59	25.42 (S)	9.12 (R)	21.82 (I)	10.84 (R)	13.62 (R)

Table 1: Selected MDR profile of *S. aureus* isolated from milk with respect to location.

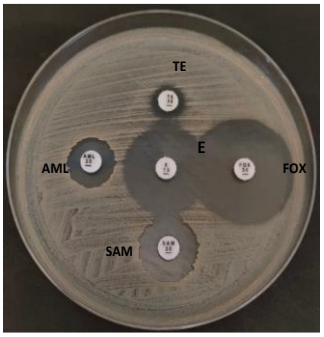

Figure 4: Antibiotic sensitivity test of *S. aureus* on Kirby-Bauer disc diffusion method was used for the measurement of the diameter of the inhibition zone.

Discussion

S. aureus is an important cause of animal and human diseases [36]. Colonization on dairy cows and contamination of raw milk with pathogenic *S. aureus* remains a significant problem for both dairy farmers and public health. The disease-causing potential of *S. aureus* stems from its ability to be very virulent after invasion [37]. The bovine mastitis problem results in a sharp decline in milk output, enormous financial losses for the dairy industry worldwide, as well as the loss of future calves, discarded milk, animal replacement, reduced milk production, and decreasing production on a quarter-by-quarter basis [38].

S. aureus milk contamination is usually associated with bovine mastitis or human carriers and can lead to contamination of the final products if proper food handling and animal husbandry practices are not used [39]. Strict precautions are required to keep away from infection of uncooked milk with *S. aureus*. On dairy farms, dairymen should be trained on how to reduce contamination (mastitis) from the raw milk environment and from the animals themselves. For example, milking can be done by a method that prevents contamination from udder and teat. In addition, it is very important to avoid temperature fluctuations via a well-controlled system in the milk supply chain on the cold chain. Competent authorities should impose strict control on surveillance [40].

In this study, out of 112 samples, 100 (89.2%) were contaminated with *S. aureus*. A higher percentage was shown in this study, compared to the research by Papadopoulos *et al*. [10] in which *S. aureus* was detected in 34 out of the 40 (85.0%) dairy farms. Another study by Javed *et al*. [41] showed that 37.14% (143/385) of *S. aureus* was isolated from bovine milk. Potential risk variables were taken into consideration in relation to the higher incidence of mastitis brought on by poor cleanliness during milking. Due to unclean udders and feet brought on by inadequate sewage systems in dairy farms, mastitis is highly likely to occur if basic hygiene precautions are not taken during milking. Unclean hands during the milking process have the ability to transmit pathogenic organisms that cause mastitis [42].

This investigation found that 10 (10%) of the several *S. aureus* isolates were MDR. The frequency of MDR *S. aureus* isolates in this study is in line with earlier research that demonstrated a significant percentage of MDR *S. aureus* isolates from animals. Raw milk and milk products have been found to contain MDR isolates. The study expressed the opinion that the existence of *S. aureus* isolates resistant to antibiotics in milk and dairy products, as well as the spread of the bacteria through contaminated food, could potentially pose a threat to public health [43].

Antibiotics are commonly used to treat livestock infections in farm animals caused by *S. aureus*; nevertheless, their usage is linked to the transmission of resistant germs to people through the ingestion of animal proteins [44]. Furthermore, there are substantial selection pressures due to the widespread misuse of antibiotics in all contexts, which has allowed resistant bacteria to survive and persist [45]. This presents a problem for dairy producers, veterinarians, and medical experts since it has a negative impact on how well microbial illnesses respond to therapy [46].

MDR bacterial infections are on the rise and represent a significant public health concern [47]. Isolates that are resistant to at least one medication from three or more antimicrobial classes are referred to as MDR bacteria [48]. The most significant pathogen within the Staphylococcus genus is *S. aureus*. The rise in public health concerns has been attributed to the recent discovery of MDR *S. aureus*, a bacteria that can infect both humans and animals [49]. Studies have shown that one of the most frequent causes of food poisoning globally is *S. aureus* [50]. *S. aureus* can cause infections in humans and animals as well as food poisoning in settings where food is processed [51].

Antibiotic usage has significantly raised the prevalence of antibiotic-resistant *S. aureus* strains and complicated the treatment process [52]. The emergence of antibiotic resistance in microorganisms poses a significant threat to public health since these germs can infect humans when handled incorrectly or when contaminated milk or meat products are consumed [53]. Penicillin was first believed to be effective against a variety of staphylococcal infections, but by the middle of the 1940s, certain *S. aureus* strains started to become resistant to the antibiotic [54]. As penicillin resistance rises, methicillin, a semi-synthetic beta-lactam antibiotic resistant to beta-lactamase enzymes, emerges and is thought to be effective against penicillin-resistant Sulfurous acid [41].

The study observed widespread use of antibiotics. This correlated with the high resistance of *S. aureus* from milk to penicillins and tetracyclines. The overuse of tetracyclines and penicillins in Kenya and other countries to treat and prevent mastitis and other livestock illnesses is the cause of the high level of resistance of *S. aureus* to these antibiotics [55]. It is possible for *S. aureus* to quickly become resistant to antibiotics. Since *S. aureus* resistance is developed by chromosomal changes and external horizontal gene transfer, the choice of antibiotics is crucial in treating the infection [56]. In the research area, dairy farms frequently utilize ampicillin. The assessment of *S. aureus*'s susceptibility to other beta-lactam antibiotics is based on its resistance to penicillin [57].

The high percentage of multidrug resistance suggests that *S. aureus* MDR is common in the dairy farms under investigation. This growth can have been influenced by numerous reasons. The majority of dairy producers in the area are ignorant of the dangers that *S. aureus* infestation poses to milk production and human health. Since no hygiene precautions were taken before or after milking, *S. aureus* on the udder surface may have made it easier for the bacteria to enter the mammary glands and other body parts [58]. The existence of subclinically infected cows and the disregard for sanitary conditions, such as inadequate milking methods, handling procedures, and storage, contribute to the reported prevalence of *S. aureus* in milk [59–61].

Findings from this study have established prospects for microbial contamination of milk by *S. aureus* and also established the possibility of MDR in dairy farms from East Java. Hence, the hygienic quality of raw milk and dairy products must be continuously monitored and improved. Therefore, the application of Good Manufacturing Practices is essential to ensure the safety of raw milk.

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Author Contributions

Dian Ayu Permatasari and Mustofa Helmi Effendi: Conceived, designed, and coordinated the study. Ajeng Rizki Ariati: Designed data collections tools, supervised the field sample and data collection, and laboratory work as well as data entry. Jola Rahmahani and Agumah Nnabuife Bernard: Contributed reagents, materials, and analysis tools. Wiwiek Tyasningsih and Aswin Rafif Khairullah: Carried out the statistical analysis and interpretation and participated in the preparation of the manuscript. All authors have read, reviewed, and approved the final manuscript.

Conflict of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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