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Presence of Antibiotic-Resistant in *Staphylococcal* Subclinical Mastitis in Several Regencies of East Java, Indonesia

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ABSTRACT

Staphylococcal* mastitis has been reported as a serious dairy disease in various regions around the world. The occurrence of resistant strains in *Staphylococcus* species to antibiotics has triggered alternative treatment substituting antibiotic usage on the global scene. This study aimed to investigate the presence of antibiotic-resistant genes in *Staphylococcal* subclinical-mastitis cases present in several regencies of East Java Province, Indonesia. A total of 592 quarter milk samples were collected from 62 farms in the region with high dairy cattle populations in Lumajang, Banyuwangi, Malang, Sidoarjo, Jember, Pasuruan, Probolinggo, and Mojokerto. Subclinical-mastitis samples were screened using the California mastitis test (CMT). Positive CMT samples were grown on the selective *Staphylococcus* media and tested for their biochemical properties. The polymerase chain reaction was performed to detect the presence of antibiotic-resistant genes in all isolates (*Staphylococcus sp*) using a specific pair-primer for *mecA*, *blaZ*, *tetK,* and *tetM* genes. The result showed that about 67% of milk samples were subclinical mastitis in several regencies of East Java. About 17.12% of subclinical mastitis was caused by *Staphylococcus* species (*Staphylococcus aureus*, *Staphylococcus epidermidis*, and other non-*aureus Staphylococci* (NAS)). The most prevalent region of *Staphylococcal* subclinical-mastitis was recorded in Jember. However, only NAS species obtained from Mojokerto, Malang, Probolinggo, and Banyuwangi were detected to have a *blaZ* gene responsible for penicillin resistance. In conclusion, the appearance of the antibiotic-resistant gene in NAS species found in several regencies of East Java can be used as important information to evaluate *Staphylococcal* subclinical-mastitis treatment.

Keywords: Staphylococcal subclinical-mastitis; antibiotic-resistant genes; penicillin; dairy milk

INTRODUCTION

Mastitis is a potential zoonotic disease affecting the dairy industry and is divided into clinical and subclinical mastitis (Abebe *et al*., 2016). Clinical mastitis is defined as inflammation of the udder with swelling, redness, and fever symptoms, while subclinical mastitis is an asymptomatic inflammation (Oliviera *et al*., 2013; Koop *et al*., 2010). In addition, several infectious bacteria have been documented to be associated with clinical or subclinical mastitis, such as *Staphylococcus aureus*, non-*aureus Staphylococci* (NAS), *Streptococcus agalactiae, Streptococcus dysgalactiae, Streptococcus uberis*, *Escherichia coli*, *Corynebacterium bovis,* and *Pseudomonas aeruginosa* (Windria *et al*., 2016; Heikkilä *et al*., 2018; Cervinkova *et al*., 2013; Gonçalves *et al*., 2020; Yuan *et al*., 2017). According to its prevalence, subclinical mastitis becomes more challenging due to the diagnosis system, persistence, and impact on dairy production (Mbindyo *et al*., 2020).

Antibiotic is commonly used in several dairy farming to treat mastitis disease (Poizat *et al*., 2017). However, the use of antibiotics in livestock treatment adversely affects the benefits of further prevention and control of mastitis by increasing the antimicrobial-resistant (AMR) strain (Chandrasekaran *et al*., 2014), contaminating livestock products (Vishnuraj *et al*., 2016), and resulting in the economic losses (Aslam *et al*., 2018). Several mastitis-associated bacteria such as *S. aureus* isolated from human foodborne-disease cases and animal milk are resistant to several antibiotics. Moreover, the most methicillin-resistant *S. aureus* (MRSA) is also found in humans (Widianingrum *et al*., 2016). Notably, some mastitis-associated bacteria carry antibiotic-resistant genes such as the *mecA* gene responsible for methicillin-resistant, *blaZ* for penicillin, as well as *tetK* and *tetM* for tetracycline (Hoekstra *et al*., 2020). Consequently, the antimicrobial treatment is no longer effective in preventing and controlling mastitis in dairy farming (Chandrasekaran *et al*., 2014).

Recently, the prevalence study of subclinical mastitis to evaluate regional treatment in East Java is limited to particular districts (Khairullah *et al*., 2019; Ramandinianto *et al*., 2020). Additionally, some reports have shown that antibiotic treatment in East Java is high and correlates with the incidence of antibiotic-resistant strains associated with mastitis (Khairullah *et al*., 2019). Meanwhile, clarification of the antibiotic-resistant genes in subclinical mastitis agents in East Java has not been widely reported. Therefore, this study aimed to investigate the presence of antibiotic-resistance genes in *Staphylococcal* subclinical-mastitis cases present in several regencies of East Java Province, Indonesia.

MATERIALS AND METHODS

The milk samples were obtained from lactating dairy cows, and each farmer carried out the milking according to their operational procedures. A laboratory study was conducted to discover the distribution of antibiotic-resistance genes on *Staphylococcal* subclinical-mastitis agents in several regencies of East Java.

Sampling of Subclinical Mastitis Milk

Subclinical-mastitis milk-samples were obtained by purposive sampling method with size 10% from a population (with criteria: all cow on lactating phase) in Regencies of Jember, Malang, Lumajang, Probolinggo, Mojokerto, Pasuruan, Banyuwangi, and Sidoarjo based on high dairy cattle populations in several regencies of East Java (Ministry of Agriculture, 2020). For each regency, the farms with the largest cattle population through stratified random sampling were chosen according to Windria *et al*. (2016). Samples of subclinical mastitis were selected by California mastitis test (CMT) analysis. A total of 10 mL milk per quarter of all individual lactating cows from 62 selected farms were collected on the tube and were identified by CMT reaction. The shape of the viscous mass on the CMT reaction was marked as a positive reaction with the reaction grade of -, +1, +2, +3 (Harjanti & Sambodo, 2020). The positive CMT samples were then processed for phenotypic identification of *S. epidermidis* and genotypic identification of *S. aureus* and NAS species.

Isolation and Identification of *Staphylococcal* Isolates

All subclinical mastitis samples were grown on Nutrient Agar plate (NA, CM0003B, Oxoid, England) following sub-culturing on *Staphylococcus* selective media such as Mannitol Salt Agar (MSA, CM0085W, Oxoid, England) and *Staphylococcus* Agar 110 (SA 110, M521, HiMedia, India). Each medium was incubated at 37 °C for 24 hours (Carter & Wise, 2004) for phenotypic determination. The colony representing *Staphylococcus* species was characterized by observing the colonies of microbial-based on the specifications in the Certificate of Analysis of Quality Control Laboratory Oxoid Limited, Basingstoke (2020) and HiMedia Laboratories Pvt, India. A colony with pink color on the MSA media was identified as *S. epidermidis*, whereas a colony with a color changed from pink to yellow was identified as *S. aureus*. In addition, the colony of *S. aureus* produced pigment on SA 110 media but not *S. epidermidis*.

Additionally, a molecular-based determination for particular *S. aureus* isolates was done using polymerase chain reaction (PCR) technique (Widianingrum *et al*., 2016). Briefly, the DNA of staphylococci isolates was isolated following Windria *et al*. (2016) and subjected to a 35 standard cycle PCR condition with a specific annealing temperature (Table 1). All isolates with no specific *23S rRNA* gene were then grouped into NAS species.

Determination of Antibiotic-Resistant Genes in *Staphylococcal* Isolates

The presence of antibiotic-resistant genes in bacterial isolates (*S. aureus, S. epidermidis,* and NAS species) was detected through standard PCR techniques using specific pair-primers (Table 1). The specific DNA sequence responsible for particular antibiotic-resistant genes in *Staphylococcus* species was amplified in standard PCR conditions. The condition was described previously by Widianingrum *et al.* (2016) with a temperature of pre-denaturation at 94 °C for 120 seconds, followed by 30 cycles of denaturation at 94 °C for 30 seconds, annealing (the temperature depends on pair-primers) for 30 seconds, and extension at 72 °C for 60 seconds before a post-extension at 72 °C for 200 seconds. The amplicon was analyzed based on the size of the am-

Table 1. Oligonucleotide primers coding *Staphylococcus aureus* and antibiotic-resistant genes

Gene	Primer sequence	Annealing (°C)	Target Size (bp)	Reference
23S rRNA	5′ AGCGAGTTACAAAGGAGGAC 3 ‘	64	1250	Straub *et al.*, 1999
	3′ AGCTCAGCCTTAACGAGTAC 5′			
MecA	5′ AAAATCGATGGTAAAGGTTGGC 3′	55	532	Strommenger *et al.*, 2003
	3′ AGTTCTGCAGTACCGGATTTGC 5′			
blaZ	5′ ACTTCAACACCTGCTGCTTTC 3′	61	173	Martineau *et al.*, 2000
	3′ TGACCACTTTTATCA GCAACC 5′			
tetK	5′ GTAGCGACAATAGGTAATAGT 3′	55	360	Strommenger *et al.*, 2003
	3′ GTAGTGACAATAAACCTCCTA 5′			
tetM	5′ AGTGGAGCGATTACAGAA 3′	55	158	Strommenger *et al.*, 2003
	3′ CATATGTCCTGGCGTGTCTA 5′			

plicon in 1% agarose visualized with UV transilluminator (Sakura). A 100 bp DNA ladder (Vivantis) was used as a molecular ruler (Widianingrum *et al.*, 2016).

Data Analysis

The percentage of incidence of subclinical mastitis and *Staphylococcal* subclinical mastitis were analyzed descriptively. The species were grouped according to the California Mastitis Test (CMT) then its relationship was analyzed through bivariate Pearson Correlation by a two-tailed significance test using SPSS version 21.0.

RESULTS

In this study, 397 out of 592 samples (67%) were identified as subclinical mastitis found in several regencies of East Java, Indonesia. This incidence of subclinical mastitis was in the range with the highest percentage of CMT +1 presented at 42%, while the lowest CMT +2 was at 21% (Table 2). In addition, the data showed that about 51% of subclinical mastitis incidents were caused by *Staphylococcus* species determined as NAS, *S. epidermidis*, and *S. aureus* at the portion of 47%, 14%, and 5%, respectively (Figure 1).

According to the data, *Staphylococcal* subclinical mastitis was distributed at eight regions in several regencies of East Java. The Region of Jember was the most prevalent area with all types of *Staphylococcus* species found in the area. The distribution of *S. aureus* was uncertainly present in mastitis, with a CMT score of +3 found in the Malang region. In addition, similar *S. aureus* was also found in Jember and Probolinggo regions but presented at lower CMT scores (Table 3). However, relationship analysis reveals a low correlation between CMT and the identification result of *Staphylococcus* species with a positive value of the correlation only between CMT and NAS species (Table 4). This result indicates that the *Staphylococcus* species did not always associate with the level of CMT result in several regions in several regencies of East Java (Figure 1, Table 3).

Interestingly, the data showed that only five samples positively carried an antibiotic-resistant *blaZ* gene found in the species isolated from farms in Mojokerto, Malang, Probolinggo, and Banyuwangi regions. All samples belonged to non-*aureus Staphylococci* (NAS) species. However, no *mecA, tetK,* and *tetM* antibiotic-resistant genes were found in all isolates (Table 5, Figure 2).

Table 2. Incidence of subclinical mastitis in several regencies of East Java, Indonesia

Total of samples	Subclinical mastitis	Number of samples on California Mastitis Test (CMT)		
		CMT +1	CMT +2	CMT +3
592	397 (67%)	169 (42%)	82 (21%)	146 (37%)

Note: CMT +1= low reaction, CMT +2= medium reaction, CMT +3= high reaction

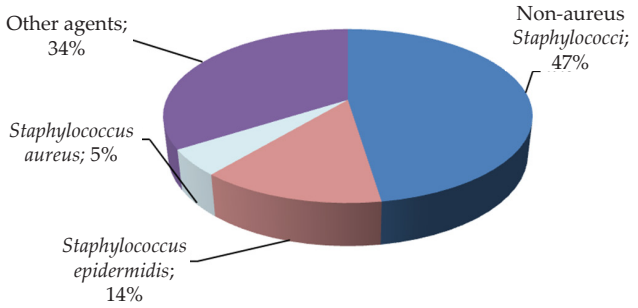

Figure 1. Presence of *Staphylococcal* subclinical mastitis in several regencies of East Java. (NAS= 49 isolates, *Staphylococcus aureus*= 5 isolates, *Staphylococcus epidermidis*= 14 isolates, other agents= 35 samples).

DISCUSSION

Farmers and researchers often ignore the incidence of mastitis with a CMT +1 score. This situation also oc-

Table 3. Distribution of *Staphylococcus* sp. causing subclinical mastitis in several regencies of East Java

Region	CMT	*Staphylococci* species		
		NAS	*Staphylococcus epidermidis*	*Staphylococcus aureus*
Jember	+++		√	
	++	√	√	√
	+	√	√	
Lumajang	+++			
	++		√	
Banyuwangi	+++	√		
	++	√		
Probolinggo	+++	√		
	++			√
	+			√
Malang	+++	√		√
Pasuruan	+++			
	++	√		
Sidoarjo	+++		√	
	++			
Mojokerto	+++	√		

Note: NAS= non-aureus Staphylococci; CMT= California Mastitis Test.

Table 4. Coefficient correlation of Bivariate Pearson (r)

Variable	*Staphylococci* species		
	NAS	*Staphylococcus epidermidis*	*Staphylococcus aureus*
CMT	0.129	-0.12	-0.301
Sig (2-tailed)	0.128	0.159	0

Note: NAS= non-aureus *Staphylococci*

Table 5. Detection of antibiotic-resistant genes in *Staphylococcus* sp. isolates

Antibiotic-resistant related genes	Number of *Staphylococci* species		
	NAS	*Staphylococcus epidermidis*	*Staphylococcus aureus*
mecA	0	0	0
blaZ	5	0	0
tetK	0	0	0
tetM	0	0	0

Note: NAS= non-aureus *Staphylococci*

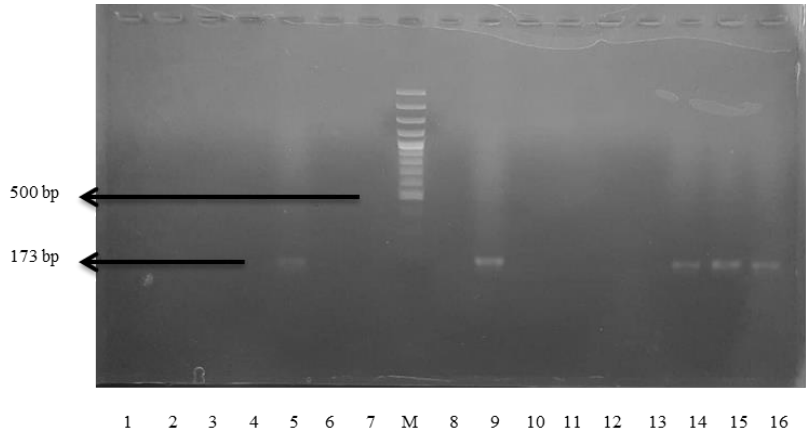

Figure 2. PCR electrophoresis results on antibiotic-resistant genes. (Line 1,2,3= *mecA* negative; 4,6= *blaZ* negative; 5,9= *blaZ* positive; 7,8= *tetK* negative; M= 100 bp molecular-size DNA ladder; 10,11,12,13= *tetM* negative; 14,15,16= *blaZ* positive).

curs throughout all regions of Indonesia and over the world. Cheng *et al.* (2021) only examined mastitis cases with scores ranging from CMT +2, Leitner *et al.* (2018) only from CMT +3. In our findings, it was known that at CMT +1 there was a higher bacterial variation in CMT +1 results. In other studies, Mekonnen *et al.* (2017) found 10 types with a total of 139 bacteria on CMT +1 compared to negative CMT results (5 types with a total of 86 bacteria), CMT +2 (8 types with a total of 116 bacteria), and CMT +3 (8 types with a total of 60 bacteria). Similar findings were also reported by Akter *et al.* (2020). This fact provides information that farmers should start giving medication to their livestock if the mastitis incident occurs at a CMT +1.

Identification of bacteria using MSA selective media is commonly used to determine among *Staphylococci* species (Ramandinianto *et al*., 2020; Arjyal *et al*., 2020). In MSA media, the *S. epidermidis* grow as a pink-color colony. In contrast, *S. aureus* grows as a yellow-color colony surrounded by a yellow halo on the pink-colored MSA medium (Subramanian *et al*., 2017). In addition, a *Staphylococcus* Agar 110 media was used to select particular species that belonged to *S. aureus* because of pigment formation during the bacteria growth. The detection for *23S rRNA* sequence using specific primers (Table 1) in all isolates reveals that not all *Staphylococci* isolates belong to *S. aureus* (Figure 1). This result suggested that a combination approach to determine, identify, and distinguish *Staphylococcus* species can be done using *Staphylococci* selective media and detection of *23S rRNA* sequence simultaneously. This approach has been used to detect the high sensitivity and unambiguity contaminants in food and distinguish *S. aureus* from other related bacteria (Straub *et al*., 1999). In addition to the approach, the *Staphylococci* species with no specific *23S rRNA* sequence are grouped into NAS species instead of *S. epidemidis*. Windria *et al.* (2016) reported that the species with similar phenotypic features growth in *Staphylococci* selective media are *S. aureus*, *S. pasteuri, S. haemoliticus*, and *S. xylosus*.

The correlation between CMT and species of *Staphylococci* can be used as early warning support of subclinical mastitis incidents. The low correlation in this study (Table 4) indicates the dangerous pathogen *S. aureus* associated with mastitis is present and should start to be considered, although in the lower score of CMT (CMT +1). *S. aureus* is an agent that needs to be considered because of its pathogenicity and is highly contagious (Song *et al*., 2020). More than 40 virulent *S. aureus* were reported (Yang *et al.,* 2015). In addition, *S. aureus* has pathogenicity factors incorporated into its ability in producing enterotoxins (Mama *et al*., 2021; Cretenet *et al*., 2011; Aziz *et al*., 2020), forming a biofilm (Raza *et al*., 2013), and encoding two conserved proteins glyceraldehyde-3-phosphate dehydrogenase-B (GapB) and -C (GapC) (Kerro-Dego *et al*., 2012).

CMT was used for subclinical mastitis detection because it is effective, cheap, and easy to apply in the field. The basic CMT reaction has been described by Bachaya *et al.* (2011) that CMT causes leakage of somatic cells on milk resulting in the release of DNA. The released DNA (acid) is then reacted with alkyl-arylsulfonate (alkaline) to form a gel, where the gel consistency depends on the total somatic cell count (SCC). The results of the CMT reaction are of greater value (from negative, +1, +2, +3) with increasing SCC in milk (Harjanti & Sambodo, 2020). However, the amount of SCC produced does not depend on the number of bacteria instead of bacterial species that induce SCC. For example, *Bacillus spp* induces the highest SCC at the population of 713.67 × 10^3 cells/mL compared to *S. aureus* at the population of 373.82 × 10^3 cells/mL and coagulase-negative *Staphylococcus* (CNS) at the population of 182.67 × 10^3 cells/mL (Sumon *et al*., 2017).

S. aureus was found in only 5% in this study. The low percentage of this species cannot be ignored because *S. aureus* was reported to cause chronic infections (Mohandes *et al.,* 2021) and is potentially resistant to various antibiotics (Widianingrum *et al*., 2016).

This study found several NAS species carry the *blaZ* gene (Table 5), suggesting that penicillin-resistant NAS species exist in mastitis in several regencies of East Java. The discovery of the *blaZ* gene in NAS species in East Java can be an illustration of the situation that may

occur in Indonesia, as we know the lack of research data on NAS. NAS species are often underestimated as the cause of mastitis. Researchers in almost all of Indonesia focus more on the type of *S. aureus* (Widianingrum *et al.,* 2016; Salasia *et al.,* 2011; Lucia *et al.,* 2017).

NAS becomes a concern because they have 40 to 50% virulence genes of *S. aureus* (Åvall-Jääskeläinen, 2018). A study by Mahmmod *et al.* (2018) shows that 76% of NAS can cause infection in the mammary glands (*S. epidermidis, S. haemolyticus, S. chromogenes*) and 73% of NAS species infecting teat skin (*S. equorum, S. haemolyticus, S. cohnii*). Despite the penicillin-resistant NAS species exist in few numbers, the NAS species carrying the *blaZ* gene should be considered to address the other alternative treatments for *Staphylococcal* subclinical mastitis. Moreover, according to the interview during sampling, the information reveals that most farmers did not know the type of antibiotic used, but some farmers mentioned that they used Penicillin to treat sick cattle. The emergence of resistant strains makes bacterial infections more difficult to treat (Zaman *et al.*, 2017).

Previously, the use of other types of antibiotics has been recommended to treat *Staphylococcal* subclinical mastitis (Tahmasedi *et al.*, 2017). However, this recommendation is no longer suitable because 87.5% of MRSA strains developed by carrying the *blaZ* gene. These facts suggest that alternative treatments are needed to combat *Staphylococcal* subclinical mastitis in dairy farms (Widianingrum *et al.*, 2016; Widianingrum *et al.*, 2019). Resistance mechanisms were summarized by Handayani *et al.* (2017), including inappropriate use, wrong knowledge of the user, the use of monotherapy, easy to get and the massive sales by pharmaceutical companies, also antibiotics use for animals and livestock.

The implication from our finding is milk produced will transmit antibiotic-resistant microbes to humans who consume the milk so that the impact can be on the prevalence of antibiotic-resistant microbes in humans. Widianingrum *et al.* (2016) observed the resistance that occurred in samples of livestock (goat and cow's milk) and humans (vomit and skin infections) due to *S. aureus*, it was found that 80% of isolates in humans, 76.92% in bovine, and 41.67% in goat have been resistant to ampicillin (ampicillin is one of the penicillin antibiotics group). Resistance to ampicillin and penicillin in clinical and subclinical mastitis cases was investigated by Saini *et al.* (2012). Their study reported that 35.4% of *S. aureus* in herds were resistant to penicillin and half to ampicillin.

In the study of dairy products, Spanu *et al.* (2012) investigated the pattern of resistance genes from *S. aureus* isolated from cheese. It was known that from 20 kinds of cheese from 10 sheep cheese dairy companies in Sardinia (Italy), 19 isolates have the *blaZ* gene, 5 isolates have the *TetL* gene, 21 isolates have the *TetM* gene, 1 isolate has the *TetS* and *TetW* genes. Biovar analysis (probable origin of contamination) was also investigated in their study. It was known that 81% came from "animal" biovars and 16% from "human" biovar.

The pattern of virulence genes and the data of genetic polymorphism can be used to determine the relationship origin of the isolates. All the statements that have been described illustrate a resistance relationship between animals, the environment, and bacteria in processed products and in humans. So, we summarize several strategies for prevention and treatment measures, including 1. Early diagnostics to determine strategies earlier (Griffioen *et al.,* 2021) 2. Antibiotic residues in milk selectively support bacterial antibiotic resistance (Brown *et al.,* 2020), so the use of antibiotics in treating mastitis cases must be selective, careful, and wise. 3. The use of natural ingredients containing immunomodulators and antibacterial that do not cause antimicrobial resistance, such as virgin coconut oil (Widianingrum *et al.,* 2019), binahong (Widodo *et al.,* 2020), or probiotics (Barker *et al.,* 2020). 4. Milking management improvements such as using milking machines and supplementation with vitamin E and selenium (Ruegg, 2017). 5. Using modern technology such as genetic engineering and biotechnology (Cardoso *et al.,* 2019), genomic selection (Kaniyamattam *et al.,* 2020), and nanoemulsion formula (Machado *et al.,* 2020), etc.

CONCLUSION

This study concludes that a *blaZ* gene (encoding to penicillin resistance) in NAS bacteria causes subclinical mastitis in Mojokerto, Malang, Probolinggo, and Banyuwangi. This finding indicates that this species needs to be considered besides *S. aureus* and *S. epidermidis*. NAS becomes concerned because it can cause infection of the mammary glands and teat skin, increasing the incidence of mastitis. Alternative mastitis treatments are needed to avoid future risks.

CONFLICT OF INTEREST

We certify that there is no conflict of interest with any financial, personal, or other relationships with other people or organizations related to the material discussed in the manuscript.

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