

MOLECULAR DETECTION OF ENCODING ENTEROTOXIN C GENE AND PROFILE OF ANTIBIOTIC RESISTANT ON STAPHYLOCOCCUS AUREUS ISOLATED FROM SEVERAL DAIRY FARMS IN EAST JAVA, INDONESIA

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ABSTRACT : The purpose of this study were to identify the encoding gene for enterotoxin C and study the profile of antibiotic resistant on *Staphylococcus aureus* from raw milk in East Java, Indonesia. Raw milk samples of 148 samples obtained from four dairy farms. Bacterial identification was based on the growth in Mannitol Salt Agar (MSA) and Gram staining and catalase & coagulase tests. 24(16.2%) out of 148 milk samples were for positive *Staphylococcus aureus* isolation. Antibiotic sensitivity testing using Cefoxitin, Penicillin, Ampicillin, Erythromycin and Tetracyclin antibiotics showed 1(4%), 17(71%), 16(67%), 24(100%) and 8(33%) isolates were resistant to the antibiotics, respectively. MRSA isolate showed that 1 isolate was positive by using Cefoxitin disc diffusion test. The molecular identification on enterotoxin C gene by PCR showed that 2(8%) isolates were positive contain enterotoxin C gene. It was concluded that the raw milk can be a potential reservoir for *Staphylococcus aureus* strains to threat public health.

Key words : *Staphylococcus aureus*, antibiotic sensitivity test, MRSA, enterotoxin C gene.

INTRODUCTION

Staphylococcus aureus (*S. aureus*) is an important opportunistic pathogen in humans and in dairy cows (Haran *et al*, 2011). *S. aureus* is the main cause of intramammary infection, which is contagious in dairy cows (Prenafeta *et al*, 2014), which is the main reason for the use of antibiotics in dairy farms (Haran *et al*, 2011). Although, *S. aureus* has been successfully isolated from the body of cattle populations as well as from dairy farming environments, udder is the main source of *S. aureus* in mastitis (Hata *et al*, 2010). The presence of *S. aureus* in dairy cows and their environment is worth watching out for not only because it can cause mastitis, but also because of the potential of these bacteria to become contaminated in milk and the easy transfer of microorganisms through intermediary workers, water and production equipment (Prenafeta *et al*, 2014).

Efforts to control intramammary infection have been done a lot, but until now the obstacle that arises is the difficulty in eradicating intramammary infections caused by pathogens and it still causes substantial economic problems (Guler *et al*, 2005). The use of antimicrobial

agents in the dairy farming industry production systems and also in other livestock industries is a major focus on the phenomenon of the emergence of resistance to zoonotic pathogenic bacteria. Although, there are differences in the classes of antibiotics used in animals and humans, selection of resistance to one class of antibiotic has the potential for cross-resistance to another class of antibiotics (Haran *et al*, 2011).

Studies on the sensitivity profile of antibiotics in cow's milk have been carried out, but information on the profile of sensitivity to antibiotics in Indonesia especially in East Java is still very limited. This study aims to determine the sensitivity profile of the antibiotic *S. aureus* isolated from cow's milk in East Java from several dairy farms in several areas in East Java, namely Batu City, Pasuruan Regency, and Lumajang Regency. Information and data obtained from this study are expected to provide an overview of the antibiotic sensitivity profile of cow's milk and also to know the prevalence of encoding enterotoxin C gene in East Java, so that it can be useful as information material for control measures against *S. aureus* that are resistant to antibiotics in dairy cows.

MATERIALS AND METHODS

A total of 24 *S. aureus* isolates from raw milk obtained from several farms in three regions such as Pasuruan region for Nongkojajar farm (AP) and Grati farm (AG), Malang region for Batu farm (AB) and Lumajang region for Senduro farm (AS) in East Java, Indonesia, were used in this study that shown in Table 2. The isolation and identification were performed for counting bacteria using conventional phenotyping method involved mannitol salt phenol red agar growth (E. Merck, Darmstadt, Germany), Gram staining, microscopic observation, catalase test and tube *coa* test (Effendi *et al*, 2019).

S. aureus colonies were partly taken with sterile ose and then mixed with physiological NaCl solution in the test tube and divortex. The turbidity of the suspension is then compared with the standard turbidity of McFarland 0.5 until the same turbidity is achieved. This suspension is then rubbed evenly on the surface of the MHA media using a sterile cotton swab. The MHA media which had been rubbed with bacterial suspension was then allowed to dry for 15 minutes before the antibiotic discs were attached to ampicillin, penicillin, tetracycline, erythromycin, and cefoxitin with the appropriate distance. Media that have been given antibiotic discs are then incubated at 37°C for 24 hours. The reading of the results of the antibiotic sensitivity test using the disk diffusion method is done by measuring the diameter of the bright zone, or so-called inhibitory zone, which is formed around the antibiotic disk using a calipers. The diameter of the measurement results is then adjusted and classified into sensitive, intermediate and resistant groups based on the CLSI (2016) antibiotic sensitivity test standard as shown Figs. 1 and 2.

DNA extraction was carried out as described by Effendi *et al* (2018) with minor modifications: doubling the time of centrifugation, the amount of enzymes and addition of a final step for DNA precipitation by ethanol. Briefly, 1 ml of each sample was transferred to a microtube and centrifuged at 14,000 rpm for 4 min. The supernatant was discarded and the pellet was re-suspended and washed 2-3 times with Tris-EDTA buffer (Tris-HCL 10 mM, EDTA 1 ml, pH 8.8) until a clear solution was obtained. The pellet was washed with PCR buffer (Buffer 10X: Tris- HCl 100 mM, KCl 500 mM, pH 8.8) and finally resuspended in 100 μ l of PCR buffer. Thereafter, lysozyme (Merck, Germany) was added to each sample at a concentration of 2 mg/ml and the sample was incubated for 20 min at room temperature. After this, proteinase K (Fermentas, Germany) was added at a concentration of 400 μ g/ml and the sample was incubated

at 56°C for 1 h. The sample was then boiled for 15 min and centrifuged at 14,000 rpm for 45 sec. Approximately, 5 μ l of DNA extract was used as template for the PCR amplification of the *seC* gene fragment. In brief, 20 μ l of PCR reaction consisted of 12.5 μ l master mix, 0.5 μ l distilled water, 1 μ l of forward and reverse primers (Table 1) and template DNA. Thermocycling conditions were as follows: prewarming at 94°C for 45 sec, followed by 30 cycles at 94°C for 2 min, 55°C for 2 min and 72°C for 1 min and followed by final extension at 72°C for 2 min (Salasia *et al*, 2011). Electrophoresis was performed at 110V for 30min. PCR products were stained by ethidiumbromide and observed under ultraviolet light.

Table 1 : Primers for Encoding Enterotoxin C Gene.

Gene	Oligonucleatid sequence (5'-3')	Amplicon size (bp)
<i>SeC-1</i>	5'-GACATAAAAAGCTAGGAATTT-3'	257
<i>SeC-2</i>	5'-AAATCGGATTAACATTATCC-3'	

RESULTS AND DISCUSSION

The results of isolation and identification on 148 samples of raw milk from 4 dairy farms in East Java revealed at 24 (16.2%) positive samples of *Staphylococcus aureus* (Table 2). Twenty four positive samples for *Staphylococcus aureus* were subjected to antibiotic sensitivity test using five antibiotics and the results are presented in Table 3.

Table 2 : Number of raw milk samples and *Staphylococcus aureus* from several dairy farms.

Name of farm	Number of samples	Positive <i>Staphylococcus aureus</i>
Senduro (S)	34	6
Grati (G)	27	2
Batu (B)	45	5
Nongkojajar (P)	42	11
Total	148	24

Another goal of this study is to investigate and compare the antibiotic resistance profile of *S. aureus* isolated from milk samples that can enter the food chain and are transmitted to humans. Many recent studies shows an increasing tendency towards the occurrence of *S. aureus* that is resistant to several antibiotics throughout the world (Akindolire *et al*, 2015). Antibiotic sensitivity test results in this study showed that 100% of *S. aureus* isolates were susceptible or still sensitive to erythromycin. This is consistent with research conducted by Muslimin *et al* (2015), which states that *S. aureus* is a Gram-positive bacterium that is still sensitive to erythromycin. Erythromycin is a macrolide antibiotic that works by binding to the 50S ribosomal subunit, thus

Table 3 : Results of antibiotic sensitivity test.

No.	Sample Code	Location of sample	Antibiotics Disk				
			Ampicillin (10 µg)	Erythromycin (15 µg)	Tetracycline (30 µg)	Penicillin (10 U)	Cefoxitin (10 µg)
1.	AS7	Lumajang	31 (S)	27.1 (S)	26 (S)	30.38 (S)	25.6 (S)
2.	AS8		34 (S)	27.6 (S)	8 (R)	33.7 (S)	27 (S)
3.	AS11		18.5 (R)	29.32 (S)	24.42 (S)	18 (R)	22.38 (S)
4.	AS17		30.2 (S)	28.34 (S)	9 (S)	31 (S)	28.7 (S)
5.	AS18		27.20 (R)	27.30 (S)	23.34 (S)	25.6 (R)	27.64 (S)
6.	AS23		20.5 (R)	27.68 (S)	25.68 (S)	19.4 (R)	17.4 (R)
7.	AG17	Pasuruan	43 (S)	27.5 (S)	25 (S)	44.5 (S)	30.56 (S)
8.	AG20		38.74 (S)	27 (S)	24.52 (S)	44.4 (S)	29 (S)
9.	AB7	Batu	18.10 (R)	26 (S)	23.8 (S)	20 (R)	30.2 (S)
10.	AB8		23 (R)	30 (S)	30 (S)	22.46 (R)	24.52 (S)
11.	AB20		15.8 (R)	25.6 (S)	23 (S)	15.12 (R)	26.88 (S)
12.	AB21		31 (S)	30.56 (S)	19 (S)	14 (R)	23 (S)
13.	AB36		43.52 (S)	28.7 (S)	26.88 (S)	43 (S)	27.6 (S)
14.	AP5		Pasuruan	15.38 (R)	26.2 (S)	24.34 (S)	16.10 (R)
15.	AP7	18.44 (R)		27 (S)	24.4 (S)	16.2 (R)	25.56 (S)
16.	AP8	17.78 (R)		28.7 (S)	10 (R)	16.58 (R)	28.54 (S)
17.	AP9	15.2 (R)		25.56 (S)	11.28 (R)	14.32 (R)	29 (S)
18.	AP13	16.26 (R)		28.44 (S)	22.38 (S)	15.2 (R)	23 (S)
19.	AP31	15.6 (R)		26.38 (S)	19.42 (S)	19.5 (R)	24.52 (S)
20.	AP33	16.34 (R)		27.64 (S)	11.3 (R)	16 (R)	26 (S)
21.	AP35	16.9 (R)		30 (S)	13.12 (R)	15.2 (R)	28.54 (S)
22.	AP37	16.38 (R)		28.54 (S)	12.6 (R)	14.62 (R)	24.52 (S)
23.	AP39	16.4 (R)		29 (S)	11 (R)	15.2 (R)	25.6 (S)
24.	AP40	41 (S)	33 (S)	30.8 (S)	47.26 (S)	31 (S)	

Note: S is susceptible and R is resistant. Inhibition zone diameters in the table above are in millimeters (mm) according to CLSI (2016).

inhibiting bacterial protein synthesis, by blocking the translocation of amino acyl translocations in the ribosomal, binding of bacterial ribosomes whose strength depends on the antibiotic structure and RNA of the bacterial ribosome. Functional groups in erythromycin such as 112-hydroxy and 9-ketone in the lactone ring, 2-hydroxy and 3—dimethylamino in desosamine and 3"-chlorinated cladinose will bind to the 50S bacterial ribosome, so that it will interfere with protein synthesis. Macrolide class antibiotics are effectively used against Gram positive bacterial infections both aerobic and anaerobic (Gaynor and Mankin, 2003). The level of sensitivity of *S. aureus* tetracycline is 67%. Tetracycline works by inhibiting protein synthesis by preventing the attachment of aminoracilli-tRNA to the ribosome acceptor, causing the process of bacterial synthesis to fail so that it is unable to develop (Candrasedkaran *et al*, 2014).

Based on the results of sensitivity tests for penicillin and ampicillin, *S. aureus* isolates resistant to penicillin reached 71% and *S. aureus* isolates resistant to ampicillin reached 67%. This is similar to the study of Shiferaw and Ahmad (2016) *S. aureus* isolates resistant to penicillin reached 94%. Chandrasekaran *et al* (2014) also reported that penicillin resistance reached 63%. The β -lactam ring is important in the mechanism of action of this antibiotic because it allows inactivation of transpeptidase which catalyzes at the end of the cross-linking reaction of peptidoglycan synthesis in bacteria. The effectiveness of this antibiotic lies in its ability to reach penicillin-binding-protein (PBP) and its ability to bind PBP. Resistance to antibiotics is usually due to the hydrolysis of antibiotics by the β -lactamase enzyme, due to PBP modification, as well as due to changes in cellular permeability (Candrasedkaran *et al*, 2014).

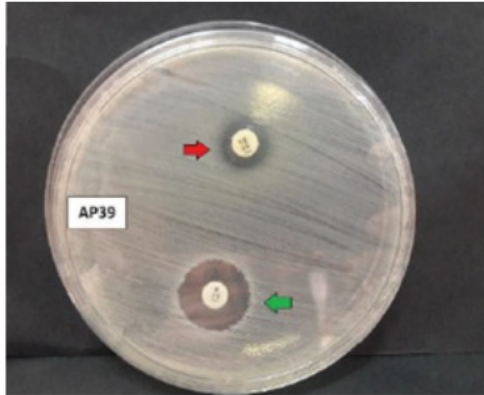


Fig. 1 : Antibiotic sensitivity test results against *S. aureus*. P is penicillin (resistant) (green arrow), TE is tetracycline (resistant) (red arrow).

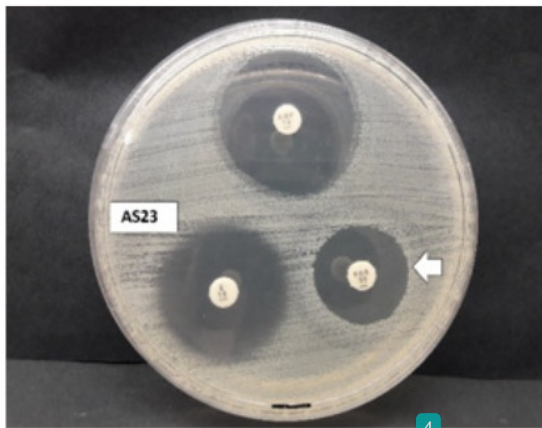


Fig. 2 : Results of a positive MRSA detection test performed by the Kirby-Bauer disk diffusion method using cefoxitin 30 µg with a 21 mm inhibition zone on the MHA. Description: FOX is Cefoxitin (resistant) (white arrow).



Fig. 3 : PCR test results for enterotoxin C encoding genes in MRSA and MSSA isolates were shown in the presence of band bands at 257 bp. Note: 1. MRK: Marker, 2. K (-): Negative control.

The presence of MRSA in animals is considered to be a big problem, especially from food products derived from animals because it is a health hazard for humans as consumers (Haran *et al*, 2011; Rahmiani *et al*, 2020). The accurate determination of MRSA in this study used the Cefoxitin disk diffusion test (CLSI, 2016). Tests with

cefoxitin disks have a sensitivity of 98.5% and specificity of 100%. This high sensitivity to cefoxitin can occur due to increased expression of *mecA*, which encodes the PBP2a protein, cefoxitin is able to induce the *mecA* gene. Therefore, it can be used as a screening test to control the spread of MRSA and alternative tests other than PCR as the gold standard (Datta *et al*, 2011, Tyasningsih *et al*, 2019). The results of this study only contained one positive MRSA isolate or about 4% of the 24 *S. aureus* isolates tested. This showed that the prevalence of MRSA in cattle on several farms in East Java is still low.

This research was conducted by PCR test to determine the presence of enterotoxin C (SEC) genes in MRSA and MSSA isolates. PCR test results showed that from 24 isolates there were two MSSA isolates encoding the SEC gene. Research conducted by El-Jakee *et al* (2012) also found one positive isolate encoding the SEC gene only from raw milk samples while from pasteurized milk samples, yogurt, cheese, meat, and chicken did not encode the SEC gene. This is evidence that the SEC is the most common type of enterotoxin found in milk-derived samples (El-Fatah *et al*, 2013). In this study, some MSSA isolates and MRSA isolates did not encode the SEC gene, this is because the isolates did not have enterotoxin genes or possibly have other types of enterotoxins (Khudor *et al*, 2012).

Enterotoxins are known as superantigens because of their ability to activate polyclonal T cells. This activation causes proinflammatory cytokine production and excessive proliferation of T cells, causing systemic release of proinflammatory cytokines, which can cause clinical signs such as fever, hypotension and shock. Superantigens

also suppress livestock immunity and contribute to chronic intramammary infections. Milk and milk products are considered as the main sources of transmission to humans so it is necessary to emphasize the existence of hygiene practices during processing, distribution and consumption (Oliveira *et al*, 2011; Bendahou *et al*, 2009.).

CONCLUSION

Twenty four *Staphylococcus aureus* isolates could be isolated from raw milk of dairy cows in several farms from East Java, Indonesia. One *S. aureus* was classified as MRSA bacteria. Through PCR testing, Enterotoxin C encoding gene of *S. aureus* could be identified in two isolates. The presence of Enterotoxin C encoding gene in *S. aureus* has the potential to spread its virulence factor to impact on the human health.

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