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The journal publishes original full length research papers, short communications and review articles in all areas of agricultural, biological and medical sciences. Emphasis is given to manuscripts which present novel finding pertinent to the biochemical basis of cellular structure and function as well as mechanism of cellular organelles. Investigations directed towards electron microscopy and localization of biomolecules in cells / tissues are particularly encouraged. This Journal is abstracted/ indexed by Clarivate Analytics (Biological Abstract, Zoological Records, BIOSIS Previews, Entomology Abstract, etc), ISI Master list, Scopus (Elsevier), CAB Intl (CAB Health Abstract, Nematology Abstract, Entomology Abstract, Forest Product Abstract, etc), Indian Science Abstract, All Russian Academy of Sciences, Medicinal and Aromatic Plant Abstract, etc. NAAS rating of year-2017 for this journal is 4.46 on a scale of 1 to 10. Also included in UGC list of Journals 2017.

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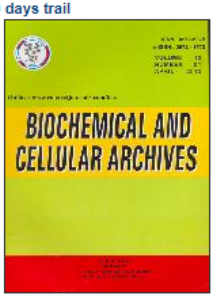

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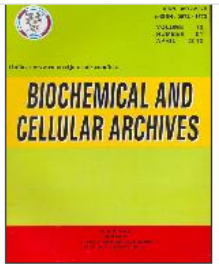

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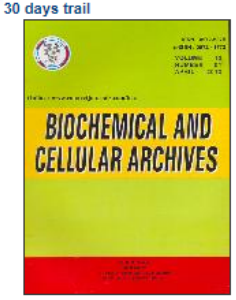

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MOLECULAR DETECTION OF ENCODING ENTEROTOXIN C GENE AND PROFILE OF ANTIBIOTIC RESISTANT ON *STAPHYLOCOCCUS AUREUS* ISOLATED FROM SEVERAL DAIRY FARMS IN EAST JAVA, INDONESIA

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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 resistanton *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, Amphicillin, 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 opurtunis pathogen in humans and in dairy cows (Haran *et al*, 2011). *S. aureus* is the main cause of intramammae 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 intramammae infection have been done a lot, but until now the obstacle that arises is the difficulty in eradicating intramammae 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 outas 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 ìl 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 ìg/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 ìl of DNA extract wasused as template for the PCR amplification of the *seC* gene fragment. In brief, 20 µl of PCR reaction consisted of 12.5 µl master mix, 0.5 µl distilled water, 1 µl of forward andreverse 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 minand 72°C for 1 min and followed by final extension at 72ºC 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)
SeC-1	5'-GACATAAAAGCTAGGAATTT-3'	257
SeC-2	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 *Staphylococcus aureus*
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)	28.7 (S)
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 to 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 (Candrasekaran *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 (Candrasekaran *et al*, 2014).

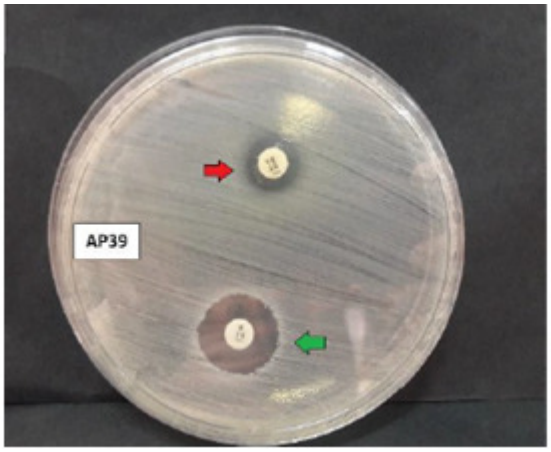

Fig. 1 : Antibiotic sensitivity test results against *S. aureus*. P is penicillin (resistant) (green arrow), TE is tetrasilin (resistant) (red arrow).

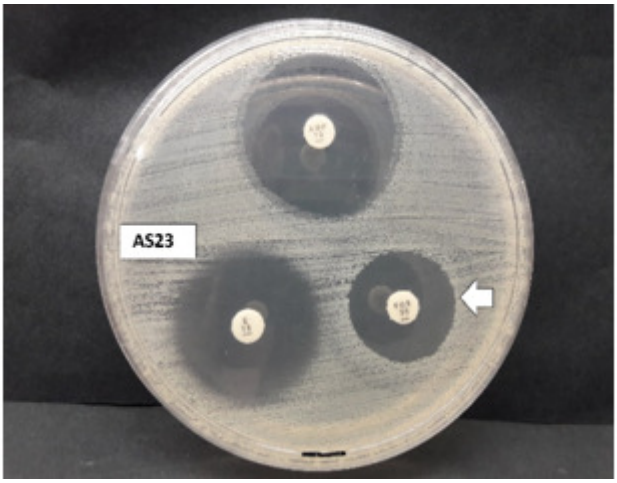

Fig. 2 : Results of a positive MRSA detection test performed by the Kirby-Bauer disk diffusion method using cefoxitin 30 µg with a 21 mm inhibition zone on the MHA. Description: FOX is Cefoxitin (resistant) (white arrow).

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

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; Rahmaniar *et al*, 2020). The accurate determination of MRSA in this study used the Cefoxitin disk diffusion test (CLSI, 2016). Tests with

also suppress livestock immunity and contribute to chronic intramamary 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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