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Detection of Enterotoxin type B gene on Methicillin Resistant *Staphylococcus aureus* (MRSA) isolated from raw milk in East Java, Indonesia

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ABSTRACT

Background and Aim: Methicillin resistant *Staphylococcus aureus* (MRSA) has an exponential rate of spread throughout the world and its occurrence rates have been detected in animal / food samples of animal origin. The methicillin resistance (MR) strain is a significant factor in the potential virulence of *Staphylococcus aureus* such as the presence of Stapylococcal enterotoxin B (SEB) which induces a super antigenic effect. This study aims to detect the presence of SEB gene in MRSA isolates that isolated from cow milk in East Java province.

Materials and Methods: Raw cow's milk ingredients were inoculated in enrichment m[illegible] and mannitol salt agar (MSA) which were then tested using oxacillin and cefoxitin disc diffusion combined with oxacillin screen agar (ORSA) to detect MRSA strains. All MRSA strains were detected by the SEB gene by the polymerase chain reaction (PCR) method.

Results: In this study confirmed 18 MRSA from 150 samples of cow's milk and detected 4 MRSA isolates having SEB gene with product size 478 bp. In Conclusion: Detection of MRSA carrying the SEB gene in milk can have an impact on the public sector / animal health (milk borne disease) and the economy on livestock. It is very necessary to further analyze the relationship between various virulence factors in MRSA isolates that have the potential to be transmitted through cow's milk in East Java.

Keywords: Methicillin resistant *Staphylococcus aureus* (MRSA), Stapylococcal enterotoxin B (SEB), food safety, milk borne disease.

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INTRODUCTION

Cases of methicillin resistance Staphylo[illegible]ci (MRS) have been discovered since 1962 where the first case of methicillin resistance Staphylococcus aureus (MRSA) was detected in humans with exponential development [1]. Various studies have reported detecting MRSA strains in animals or food products of animal origin, one of which is cow's milk with different prevalence variations [2,3,4]. Basically methicillin resistant (MR) in S. aureus occurs due to changes in penicillin-binding protein (PBP2a) there[illegible] reducing the affinity of the β lactam antibiotic induced by the mecA gene in the staphylococcal cassette chromosome (SCCmec) [5].

Confirmation of methicillin resistance (MR) is a significant factor in the potential for S. aureus virulence, such as the relationship of MRSA with the presence of exotoxins-producing genes such as enterotoxin [6]. Staphylococcus aureus itself is a commensal bacterium that is commonly found in ruminant skin and mucosa, wh[illegible] has a connection with sub-clinical or clinical mastitis that can be transmitted to humans through contamination of milk, [illegible]rocessed milk and other dairy products [7], [8]. Ingestion of Staphylococcal enterotoxins (SE) produced[illegible] several strains of Staphylococcus through the consumption of raw milk and milk products can lead to the occurrence of milk borne disease (MBD), where such events have been reported in many studies [9,10]. SE has a good level of stability against heat and freezing / drying treatment, but also has resistance to proteolytic enzymes and low pH so that they can function fully in the digestive tract after consumption even at very low doses, which are 20 ng -1 µg / ml [11]. Staphylococcal enterotoxin B (SEB) is a dangerous type because it is able to induce a super [illegible]genic effect.

This study aims to detect the presence and evaluate the presence of MRSA strains carrying SEB gene that contaminate cow's milk in east Java by combining 2 methods to detect MRSA namely Cefoxitine disc diffusion and Oxacillin disc diffusion [illegible]n Oxacillin Resistance Screen Agar (ORSA), then the PCR method for detecting the presence of SEB gene in MRSA isolates. This research information is very important to support strategic and technical decision making by related institutions / institutions for mitigation and prevention of impacts on aspects of public health.

Materials and Methods

Sampling

Total milk samples of 150 dairy cows were collected from 3 Village Unit Cooperatives in Kediri, Probolinggo and Pasuruan areas during October - December 2019. Dairy dairy milk collected directly from milkcan and as many as 15 ml were collected in centrifuge tubes of 50 ml setril (Biologix, BD-T003434). 1 ml of milk is taken aseptically to be inserted into a glass tube steril 10 ml (onemed) containing 4 ml of media broth buffered peptone water by using Syringe 3cc (onemed, AKD 20902900277) [12], then

incubated at 37°C for 24 hours with incubator (Isuzu Model 2-2195, Jica).

Bacteria Isolation and Identification

The results of incubation of enrichment media were cultured on mannitol salt media, the colonies with yellow color that showed the indicator of S. aureus, incubated at 37°C for 24 hours. Identification was done by examination based on morphological cultural characteristics, then microscopic examination using Gram's method of staining which shows gram-positive bacteria in the form of coccus and clustering [13]. Biochemical examination was carried out to confirm the Staphylococcus aureus species with Catalase test and Coagulase test [14, 15], Catalase test was carried out by dripping 3% hydrogen peroxide (HO) on clean glass and mixing with 1 loop inoculum of bacterial colony [16]. Coagulase test is done by two methods namely Coagulase slide test / clumping factor and Coagulase tube test. Coagulase slide test / clumping factor gives 50 µl rabbit blood plasma dripped on a glass object, then mixed with 1 loop inoculum of bacterial colony, Coagulase tube test using 200 µl blood plasma is added with as many as 3-4 isolate colonies and then incubated 37°C for 24 hours.

Methicillin resistance Staphylococcus aureus (MRSA) phenotypic detection

MRSA detection is done by combining the disc diffusion test method using 2 preparations of cefoxitin and oxacillin and then the results are confirmed with the oxacillin screen agar test (ORSA) test. Testing with disc diffusion is performed on Muller Hinton agar (MHA) plates [17]. Isolates that have been isolated and identified will be purified on mannitol salt and incubated at 37°C for 24 hours and then made into 0.5 McFarland's suspension and then taken using Sterile Cotton Swab S (Onemed, AKD 10903610549). The applied evenly on the surface of the MHA media, cefoxitin 30 µg and oxacillin 30 µg was placed side by side with a distance of 4.5 cm and then incubated 37°C for 24 hours and the inhibition zone was measured. In cefoxitin and oxacillin disc diffusion test inhibition zone ≤21mm is an isolate of methicillin resistant (MR). All MR isolates by the disc diffusion test were tested by ORSA, where the ORSA test refers to previous studies [18, 19. 20]. Isolates are taken by several colonies to be used as 0.5 Mc Farland's suspension and subsequently will be taken using Sterile Cotton Swab S (Onemed, AKD 10903610549) and applied to Oxacillin Screen Agar Base added by Oxacillin Resistance Selective Supplement.

Detection of the SEB gene

All isolates confirmed as MRSA by the disc diffusion test and the ORSA test were tested using PCR to detect the presence of the SEB gene. The DNA extraction process is carried out in accordance with the boiling lysis method [21], taking several bacteria colony to be tested put into eppendorf safe-lock tubes containing 300 µl TE (10 mM Tris, pH 8, 10 mM EDTA) and then and put eppendorf thermoStat ™ at 98 ° C for 10 minutes. After that in the centrifuge with 10,000 rpm for 10 minutes. Reaction mixture contains Go tag green mastermix, SEB forward and reverse gene primers, DNase free water and DNA template. Amplification was carried out using a thermal cycler machine with a slight modification of the protocol [17], namely predenaturation at 95 ° C for 1 minute followed by 40 cycles of denaturation at 95 ° C for 1 minute, annealing at 55 ° C for 1 minute, and extension at 72 ° C for 1 minute. The amplification step ended with a final extension at 72 ° C for 2 minutes. Electrophoresis was carried out on the PCR product by taking 10 µl from each - each and put into the well and placing 100 bp markers as much as 6 µl at the edge of the well. Electrophoresis is run at 100 volts for 40 minutes. The positive control used was ATCC 25923 S. aureus subsp. aureus rosenbach.

Results and Discussion

Bacteria Isolation and Identification

The test results found 76 (50.7%) S.aureus isolates from 150 milk samples taken in 3 regions in East Java based on morphological cultural characteristics, gram's staining and biochemical tests. S. aureus has phenotypic characteristics of the colonies in the culture media results of mannitol salt agar (MSA), which changes the color of the media from red to yellow which indicates mannitol fermentation while the colonies have varying pigments including white, yellow, and orange [14].

Methicillin resistance Staphylococcus aureus (MRSA) phenotypic detection

Test of methicillin resistant using disc diffusion method (Figure - 1) on Muller Hinton Agar (MHA) media showed the total results of isolate resistance to cefoxitin preparations as many as 16 isolates (21%) while oxacillin resistance was 22 isolates (29%) of Staphylococcus aureus isolates (Table - 1). The results showed that there were no isolates that were only resistant to cefoxitin in the disc diffusion method, all isolates detected were resistant to cefoxitin also identified to be resistant to oxacillin but there were oxacillin resistant isolates and sensitive to cefoxitin (Table-2). MRSA confirmation is done by combining disc diffusion and ORSA test, where the expression of the blue culture indicator shows positive confirmation results while the white results show negative confirmation results. The ORSA test confirmed the presence of 18 MRSA isolates from the 22 isolates tested (Figure - 2), as shown on Table-2.

Detection of the SEB gene

Isolates that were confirmed as MRSA phenotypic by ORSA method were tested genotypically by PCR method to detect the presence of SEB genes in isolates. In total there were 20 MRSA isolates tested and detected 4 MRSA isolates had SEB gene with a distribution of 3 isolates from Kediri and 1 isolate from Probolinggo. Test results on isolates using positive control ATCC 25923 can be seen in figures 3-5 (Figures 3-5).

Discussion

Staphylococcus aureus (S.aureus) is a bacterium that is often found on the surface of the respiratory mucosa and in the urogenital tract of humans and animals. Staphylococcus aureus is a commensal bacterium that is opportunistic infectious in humans and animals [18]. Staphylococcus aureus is a pathogenic agent that can cause various infectious diseases from cutaneous to systemic infections in the immunocompetent host resulting in death [19]. The Pharma Innovation Journal research states that S. aureus can be transmitted through milk and cause milk borne diseases (MBD) [8]. In this study identified S. aureus contamination as many as 76 isolates (50.7%) of 150 samples of cow's milk, this percentage is quite high according to several studies reported in The Pharma Innovation Journal [8] which isolate 57% Staphylococcal strains and report the results a study in the Czech Journal of Food Sciences which isolated 47.5% Staphylococcal strains from milk samples

from dairy cows tested [20]. In addition, a research report by the Journal of Dairy Science revealed a level of positive Staphylococcus (S. aureus) coagulase contamination of 51% of milk samples tested [21], the Journal of Food Science reported 52.4% of S. aureus contamination in milk samples in milk trials [22] and the International Journal of Food Microbiology which reported 57.3% of S. aureus contamination in tested milk [23]. Variations in the percentage of S. aureus prevalence compared to other workers may be due to sample size, use of antibiotics in animal husbandry, and hygiene practices among dairy cows. The high incidence of S. aureus is an indication of poor hygienic during production, handling and distribution [24].

MRSA infections spread throughout the world in a number that has continued to increase over the past 10 years. The prevalence of MRSA in the Asian region such as Japan and Singapore reaches more than 50% while in the Americas, Australia, some European countries range from 25-50% [25]. Prevalence in the Southeast Asian region, including Indonesia, is not widely known because research on MRSA is still small. The number of MRSA detected in this study was 20 isolates from 24 isolates tested or 13% of the total sample of cow's milk. These results are similar to several other studies reported which isolate the presence of 10.3% MRSA isolates [26] and the Journal of Food Protection report isolates 9.1% MRSA isolates in tested cow's milk [27]. However, the results differ from reports of advances in environmental biology which only detected 1.8% of MRSA in the samples tested [28]. The source of MRSA transmission is due to contact with humans transport animals, where cows infected with MRSA act as a reservoir and then transmit to other animals or humans [19]. MRSA colonization of cows can be a risk factor for people who have close contact with MRSA infected cows such as veterinarians, farmers, milkers and people who work in slaughterhouses [29]. The detection of MRSA in milk is a matter of concern and requires strict farm management practices, as well as proper sanitation procedures such as storage, handling and transportation.

Over the last few decades, the prevalence of MRSA has increased exponentially and is now considered a disease-causing bacterial in which the number of fatal infections caused by MRSA has been reported [17]. Increased pathogenicity of S. aureus may be due to the increased prevalence of strains that have the MCC SCC gene that causes transferable MRSA and the presence of toxins such as enterotoxins. The research report of the International Journal of Current Microbiology and Applied Sciences suggests that there is a relationship with the rate of detection of SEB genes in the MRSA strain [30]. In this study detected 4 isolates possessed SEB gene out of 20 MRSA isolates tested or about 20% of MRSA strains had SEB genes equal to the number reported in the Veterinary Institute Bulletin in Pulawy which detected 35% MRSA had SEB [31]. However, the amount is different from that obtained by the report of the International Journal of Current Microbiology and Applied Sciences where 75% of MRSA isolates have the SEB gene detected. SEB produced by MRSA is considered a major cause of staphylococcal toxic shock syndrome [32]. SEA and SEB enterotoxins are known to cause about 90% of staphylococcal food poisoning worldwide [33]. A research report in Uganda revealed that more than 90% of the isolates tested carried at least one enterotoxin-encoding gene that showed a high risk of MBD spread [34].

This research was carried out by PCR test to determine the presence of enterotoxin B (SEB) gene in MRSA isolates. Research conducted by Arifah, (2020) also found three positive isolates encoding the SEB gene [40], but from dog nose swab samples in Surabaya, while from raw milk the SEC gene was discovered [41]. Other researchers argue that SEC is the most common type of enterotoxin found in milk-derived samples [42]. In this study, some MRSA isolates did not encode the SEB gene, this is because isolates did not have enterotoxin genes or might have other types of enterotoxins [43].

Enterotoxins are known as superantigens because of their ability to activate polyclonal T cells. This activation causes proinflammatory cytokine production and excessive T cell proliferation, 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 intramamary infections [41]. 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 [44], so that the negative effects of milk can be avoided.

Conclusion

In this study it can be concluded that the presence of cow's milk contamination by MRSA is possible due to various factors, one of which is low milking hygine. Moreover, the detection of SEB genes in MRSA isolates is very dangerous for public health aspects, which will increase the potential spread of Staphylococcal food poisoning that is difficult to treat.

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Table 1. Results of isolation and identification and detection of MRSA.

Sampling Location	Sampel Size	*S.aureus* Isolate	MRSA Confirmed by ORSA
Kediri (Kr)	50	20	6
Probolinggo (P)	50	30	8
Pasuruan (G)	50	26	4
Total	**150**	**76**	**18**

Note : All FOX-resistant isolates (Cefoxitin 30 µg) had resistance to OX (Oxacillin 30 µg).

Table 2. Results from the detection of SEB gene in MRSA isolates

Location	Sampel Number	Resistance on Disc Diffusion Test		ORSA Test	SEB Detection	
		OX	FOX			
Kediri	Kr03	✓	✓	Positive	**Positive**	
	Kr04	✓	✓	Positive	**Positive**	
	Kr05	✓	✓	Positive	**Positive**	
	Kr07	✓	Sensitive	Positive	Negative	
	Kr13	✓	Sensitive	Positive	Negative	
	Kr37	✓	✓	Positive	Negative	
Probolinggo	P04	✓	✓	Positive	Negative	
	P07	✓	✓	Positive	Negative	
	P17	✓	✓	Positive	Negative	
	P21	✓	✓	Positive	Negative	
	P31	✓	✓	Positive	Negative	
	P32	✓	Sensitive	Positive	Negative	
	P35	✓	✓	**Negative**	Not tested	
	P40	✓	✓	**Negative**	Not tested	
	P45	✓	✓	Positive	Negative	
	P49	✓	✓	Positive	**Positive**	
Pasuruan	G06	✓	✓	Positive	Negative	
	G16	✓	✓	**Negative**	Not tested	
	G18	✓	Sensitive	**Negative**	Not tested	
	G24	✓	Sensitive	Positive	Negative	
	G33	✓	✓	Positive	Negative	
	G37	✓	Sensitive	Positive	Negative	

Note : ✓= resistant; FOX = *Cefoxitin* 30 µg, ; OX = *Oxacillin* 30 µg (Oxoid).

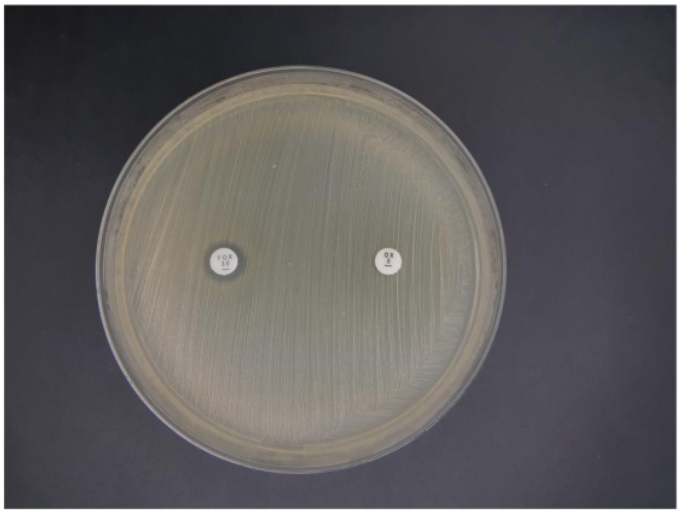

Figure-1. Detection of MRSA with oxacillin and cefoxitin disc diffusion method.

Figure-2. Confirmation of MRSA with ORSA test, the blue indicator is a positive result.

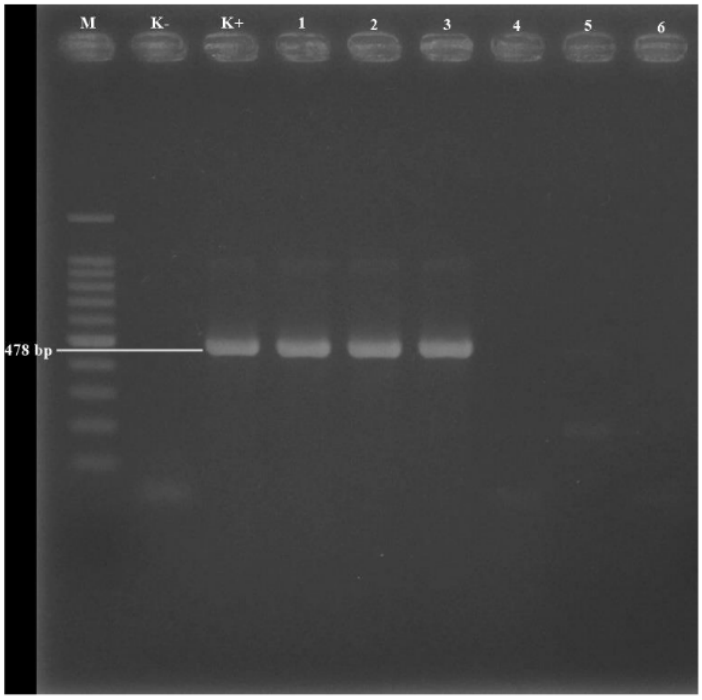

Figure-3. Results of PCR detection of SEB gene from MRSA isolates on the Kediri location. There were three SEB gene detected

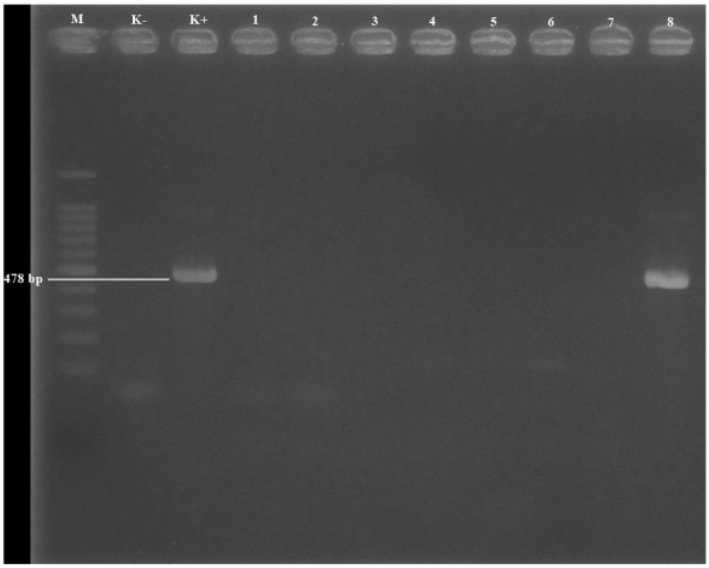

Figure-4. Results of PCR detection of SEB gene from MRSA isolates on Probolinggo location. There was one SEB gene detected

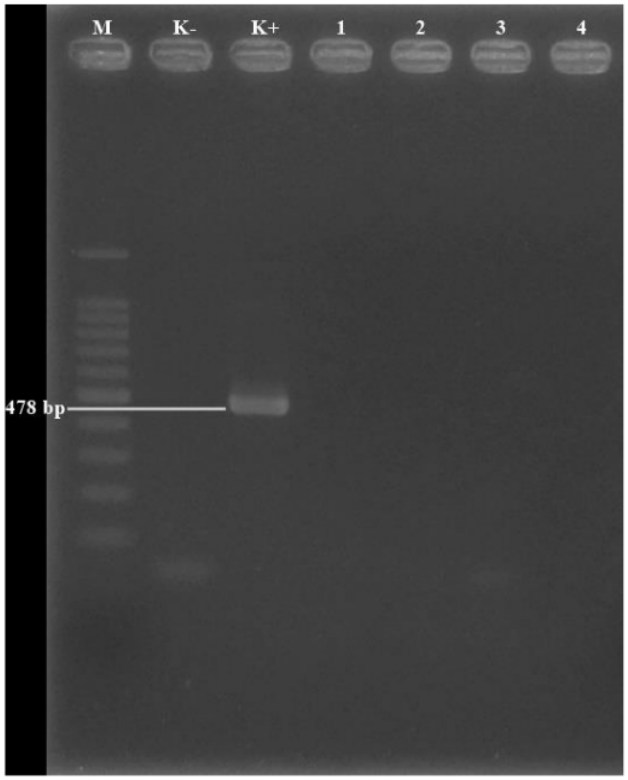

Figure-5. Results of PCR detection of SEB gene from MRSA isolates on Pasuruan location. There was no SEB gene detected.

Detection of Enterotoxin type B gene on Methicillin Resistant Staphylococcus aureus (MRSA) isolated from raw milk in East Java, Indonesia

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