ISSN: 0975 -8542

Journal of Global Pharma Technology

Available Online at: www.jgpt.co.in

RESEARCH ARTICLE

Epidemiology of *Staphylococcus Aureus* Clinical Isolates Causing Skin and Soft Tissue Infections Among Patients in the Community Settings At Primary Healthcare Centres in Sidoarjo Region-East Java, Indonesia

Sugiharto[1, 2]*, Sanarto Santoso[3], Dewi Santosaningsih[3], Tita Hariyanti[4]

***Corresponding Author: Sugiharto**

1. *Doctoral Program of Medical Sciences, Faculty of Medicine, Brawijaya University, Malang, Indonesia.*
2. *Department of Public Health Science, Faculty of Medicine, Wijaya Kusuma University Surabaya, Indonesia.*
3. *Department of Clinical Microbiology, Faculty of Medicine, Brawijaya University / Dr. Saiful Anwar Hospital, Malang, Indonesia.*
4. *Department of Public Health Science, Faculty of Medicine, Brawijaya University, Malang, Indonesia.*

Abstract

Objectives To determine percentage of case finding of *Staphylococcus aureus*, methicillin resistant *Staphylococcus aureus* (MRSA) and panton–valentine leukocidin (PVL)-positive *Staphylococcus aureus* (*S. aureus*) isolated from skin and soft tissue infections in the community settings at Primary Healthcare Centres in Sidoarjo region, Indonesia and the relationship of risk factors of Staphylococcal skin and soft tissue infections. Methods *S. aureus* isolates from clinical cultures of patients in 2 Primary Healthcare Centres were included. Wound cultures were performed to identify the MRSA phenotypically using VITEK® 2 system. The *mecA* gene and genes encoding PVL were detected using Polymerase Chain Reaction (PCR). Clinical data including gender, age, hospitalization history in primary health care center and hospital, comorbidity (diabetes mellitus) history and antibiotic history, were collected from questionnaires and medical record. Statistical analysis was done by Chi-square-Fisher's exact test. Results In total, 45 patients involved in this study. The 20 patients consisted Staphylococcal strain, and 25 patients did not. Of the 20 Staphylococcal strains, 3 samples were *Staphylococcus* coagulase negative, 1 sample was *Staphylococcus gallinarum* and another sample was *Staphylococcus haemolyticus*. There were 15 patients (33.3%) were infected by *S. aureus*. Of these, 13 isolates were MRSA-positive (28,9%) and 1 isolate was PVL-positive (2,2%). Analysis showed no relationship between the risk factors and finding of *S. aureus*, neither MRSA and PVL in all patients ($p > 0.05$).Conclusions *S. aureus* is common in the community. The MRSA is clinical importance in Indonesia, not only in hospitals, but also in the community settings. High case of MRSA was found in this study. However, the case of PVL-positive *S. aureus* was low. It is very important to promote case finding by detecting MRSA in the community settings. A national surveillance system should be set-up for further monitoring this.

Keywords *Methicillin-resistant S. aureus, Panton–valentine leukocidin, Staphylococcus aureus, community setting, Prevalence.*

Introduction

Staphylococcus aureus is a Gram-positive bacterium [1].Two years after the introduction of penicillin, this *S. aureus* was resistant to it. In late 1950s, semisynthetic antibiotic methicillin was developed. In 1960, *S.aureus* began to resistant to it. This newly mutated bacterium introduced as MRSA [2].Staphylococci are variably susceptible to many antimicrobial drugs [1].

Staphylococcus aureus is recognized as an important pathogen, both in the hospital and community settings [3], a major cause of hospital acquired infections, causing high morbidity and mortality across the world [4].The rising colonization rates of *S. aureus* lead to the increasing of infection rates in the community and in hospitals [5].*Methicillin resistant Staphylococcus aureus* is the bacterium that causes a potentially fatal *Staphylococcus* infection that cannot be killed by commonly used antibiotics. *Staphylococcus* is normally present in the environment and

usually remains harmless unless a drug-resistant form of it invades a wound or enters the body of a person with a compromised immune system [6].Asia is among the regions with the highest incidence of MRSA in the world, with an estimated proportion from 28% (in Hong Kong and Indonesia) to >70% (in Korea) among all clinical *S. aureus* isolates in the early 2010s [7]. MRSA carriage rate in three big cities in Indonesia (Malang, Semarang, Denpasar) among surgery patients screened at discharge was 8.0%, 5.9%, and 0.4%, respectively [8].*Staphylococcus aureus* acquires methicillin resistance by insertion into the chromosome of a mobile genetic element, designated SCC*mec*, carrying the *mec*A gene. This gene encodes an additional penicillin-binding protein, PBP2a, which is not inhibited by existing b-lactam antibiotics [9], rapidly develops for many new drugs [10].Progress in preventing MRSA infections in health care settings, assessment of the problem in both health care and community settings is needed [11].

Methicillin resistant Staphylococcus aureus has been recognized to be most often associated with various infections in patients exposed to nosocomial settings, which is known as healthcare-associated (HA-) MRSA. Community-associated (CA-) MRSA is often defined clinically through hospital stays and an absence of risk factors for HA-MRSA infections. It has also been distinguished from HA-MRSA through the possession of unique drug resistance patterns and molecular characteristics. Generally, HA-MRSA typically belongs to SCC*mec* I, II and III, while CA-MRSA carries SCC*mec* IV or V [1, 12].

The worldwide emergence of CA-MRSA strains has been linked to carriage of genes encoding PVL, a two-component leukolytic toxin [13].Another study by Lina, et al. mentioned that PVL gene is only exists in CA-MRSA [14].This toxin (PVL) is an important virulence factor in CA-MRSA infections [1].Vandenesh et al. (2003) concluded that HA-MRSA did not harbor PVL genes or the SCC*mec* IV element [15].This former research about the stability of PVL gene to determine CA-MRSA identity is important.

The PVL gene now can be used as a stable marker for identification of PVL gene due to its specificity to CA-MRSA isolates from different continents. This concept was used as reference in this study to prove that there was indeed CA-MRSA. In this study, we want to explain the epidemiology of CA-MRSA. In order to accomplish the purpose, we also genotypically identified the samples to prove the samples were MRSA, especially CA-MRSA instead of HA-MRSA.

Material and Methods

Sample

Sample was collected from Primary Healthcare Centres, Sidoarjo, Indonesia. The samples were collected with Amies Transport medium, which then transferred into Clinical Microbiology Laboratory of Faculty of Medicine, Brawijaya University, Malang, Indonesia. There were 45 samples of skin and soft tissue infections of patients. Then only 20 samples suspected of *S. aureus* were processed. The 20 samples were suspected CA-MRSA. Only the samples that phenotypically proved MRSA that would be continued to genotypic identification procedure.

Culturing Sample

The suspected CA-MRSA samples were transferred into thio-glycolate (TG) medium by streaking method. After 24 hours, the samples were transferred again from TG media to 400 ml brain heart infusion broth (BHIB) media and 9 ml nutrient broth (NB) media. Samples were incubated in 37°C for 48 hours before being harvested. The sample that cultured in BHIB media was used for protein isolation method while the one that cultured in NB media was used for genotypic identification.

Phenotypic-Genotypic MRSA Identification and DNA Isolation

Phenotypic identification was done by VITEK® 2 systems in Saiful Anwar Hospital, Malang. This system allows identification by antibiotic susceptibility testing. The antibiotic-antimicrobial that being used for this test were: cefoxitin, benzylpenicillin, ampicillin, cloxaxillin, oxacillin, cefadroxil, cefazolin, gentamicin, streptomycin, ciprofloxacin, levofloxacin, moxifloxacin, inducible clindamycin resistance, erythromycin, clindamycin, quinupristin/ dalvopristin, linezolid, vancomycin, tetracycline, tigocycline, nitrofurantoin, rifampicin and trimethoprim/ sulfamethoxazole. The result of this test was

used as first stage of identification before upheld to next step, genotypic identification. Genotypic identification was performed by PCR identification. All the samples were harvested after 48-hours incubation. Every sample in 10 ml tube was placed in microtube 1.5 ml (6 micro tubes for each code).All the tubes centrifugated at 12000 rpm for 1 minute 27°C. The supernatant contained media while the pellet was the bacteria. The media was discarded. The remaining pellet was recollected in one micro tube based on its codes. The next step referred to the instruction of Invitrogen Purelink® Genomic DNA Kit for Gram-Positive Bacteria without any major modification. The result, purified DNA was stored at -20°C for further application, PCR.

The PCR of *mec*A and PVL

The PCR identification was done to test the occurrence of PVL gene in the sample. The PVL gene identification process was done based on Lina, et al. (1999) [14]. The expected result was in the form of bands that can be compared with references, 433 bp. Primer was obtained from Humanizing Genomics Macrogen (www.macrogen.com). The sequences based on Murakami, et al. (1991) for *mec*A gene and Lina, et al. (1999) for the PVL gene test [14, 16]. The oligonucleotides that being used were: *mec*A (5' AAAATCGATGGTAAAGGTTGGC) and PVL (luk-PV-1: 5'ATCATTAGGTAAAATGTCTGGACATGAT CCA 3'; luk-PV-2: 5' GCATCAASTGTATTGGATAGCAAAAGC 3'). The PCR of PVL was started by mixing half the recipe of the instructions listed on Promega. The mixture for each well (DNA controls and samples) contained 6.25 µl of PCR mix Go Taq, DNA (0,5 µl for forward sample DNA (10 pmol), 0,5 µl for reverse sample DNA (10 pmol) and 1 µl for DNA control (10 pmol)) and 4.25 µl of Nuclease Free Water. DNA amplification was carried out for 30 cycles of reaction mixture as follows: denaturation of 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 1 min with a final extension at 72°C for 5 min. Ten microliters of PCR products was analyzed by 2% agarose gel electrophoresis. The result was analyzed using BioRad GelDoc system.

Results

Phenotypic and Genotypic Identification

Phenotypic identification result of 20 of total 45 patients that performed by VITEK® 2 test was described in Table 1. There were 15 samples that were proved *Staphylococcus aureus*. Three samples were *Staphylococcus* coagulase negative. Two other samples did not consist *Staphylococcus aureus,* but *Staphylococcus gallinarum* and *Staphylococcus haemolyticus* instead. The 15 samples of *Staphylococcus aureus* that proved MRSA were collected for further genotypic identification by PVL. We found 13 of 15 samples were MRSA-positive.

Table 1: VITEK® 2 & PVL Result

Sample code	VITEK® 2 result	Genotypic identification	PVL gene identification
01	*Staphylococcus gallinarum*	No	No
02	*Staphylococcus aureus*	Yes	-
04	*Staphylococcus haemolyticus*	No	No
07	*Staphylococcus aureus*	Yes	No
11	*Staphylococcus aureus*	Yes	-
13	*Staphylococcus aureus*	Yes	-
14	*Staphylococcus aureus*	Yes	-
15	*Staphylococcus coagulase negative*	No	No
17	*Staphylococcus aureus*	Yes	No
20	*Staphylococcus aureus*	Yes	-
21	*Staphylococcus coagulase negative*	No	No
23	*Staphylococcus aureus*	Yes	-
26	*Staphylococcus aureus*	Yes	-
27	*Staphylococcus aureus*	Yes	-
30	*Staphylococcus aureus*	Yes	-
32	*Staphylococcus coagulase negative*	No	No
35	*Staphylococcus aureus*	Yes	-
36	*Staphylococcus aureus*	Yes	-
37	*Staphylococcus aureus*	Yes	+
38	*Staphylococcus aureus*	Yes	-

The PVL gene identification of 13 samples of MRSA-positive showed that there was only one sample that produced a band in the gel, 433 bp. The sample code is 37. Although the band was merely visible, it still could be reassured that there was one (Figure 1a). Other lines showed no result at all. Referring to Vandenesh et al. (2003), PVL gene can be used as a stable marker for identification of CA-MRSA [15]. Therefore, 1 of 13 samples of MRSA-positive was proved CA-MRSA.

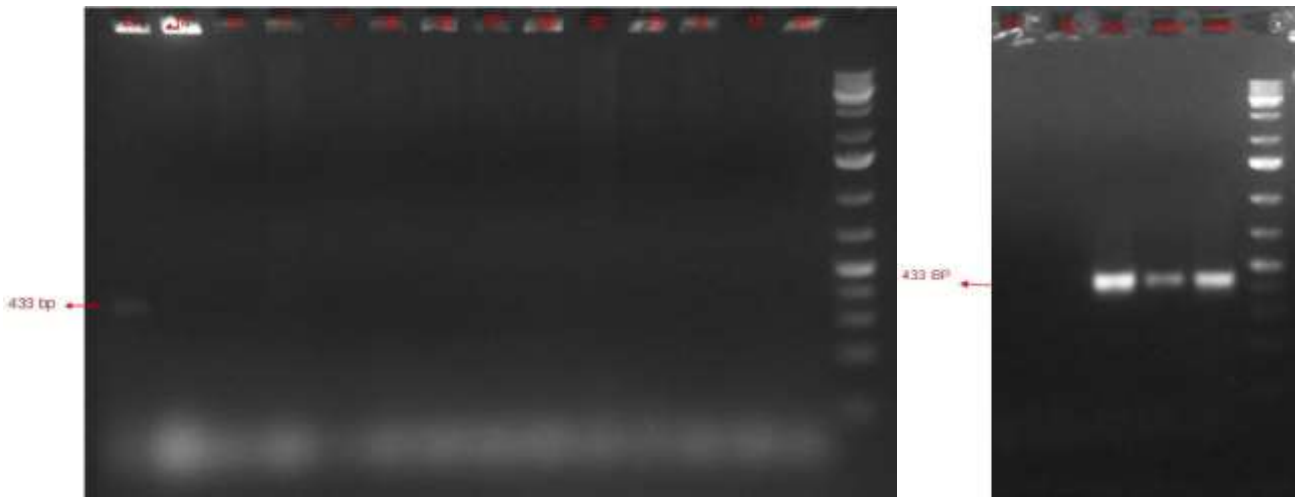

Figure 1: PCR result of MRSA samples. Each line contains different codes. The codes respectively for figure 1a (left to right) are 37, 14, 27, 17, 21, 26, 36, 30, 38, 32, 13, 11, 15, and 20. The code for figure 1b (from left to right) are 23, 35, DK (DNA control) 1, DK (DNA control) 2 and DK (DNA control) 3

Risk Factors of *S. aureus*, MRSA and PVL

We observed the risk factors of *S. aureus*, MRSA and PVL of the 45 patients as described in Table 2. There were 6 factors: gender, age, hospitalization history in primary health care center and hospital, comorbidity (diabetes mellitus) history and antibiotic history.

Table 2: Risk factors of *Staphylococcus aureus* skin and soft tissue infections in primary healthcare centers in Sidoarjo, Indonesia

Factors	Number of subject (%)		*p-value*	Number of subject (%)		*p-value*	Number of subject (%)		*p-value*
	***S. aureus* infection (n=15)**	**Not *S. aureus* infection (n=30)**		**MRSA (+) (n=13)**	**MRSA (-) (n=2)**		**PVL (+) *S. aureus* (n=1)**	**PVL (-) *S. aureus* (n=12)**	
Gender:									
Male	7(46.7)	16(53.3)	0.673*	5(38.5)	2(100.0)	0.200**	0(0.0)	5(41.6)	1.000**
Female	8(53.3)	14(46.7)		8(61.5)	0(0.0)		1(100.0)	7(58.3)	
Age (yo):									
<66	12(80.0)	22(73.3)	0.726**	10(76.9)	2(100.0)	1.000**	0(100.0)	10(83.3)	0.231**
≥66	3(20.0)	8(26.7)		3(2.3)	0(0.0)		1(0.0)	2(16.7)	
History of hospitalization: in primary health care center									
Yes	8(53.3)	10(33.3)	0.197*	8(61.5)	0(0.0)	0.200**	1(100.0)	7(58.3)	1.000**
No	7(46.7)	20(66.7)		5(38.5)	2(100.0)		0(0.0)	5(41.7)	
in hospital									
Yes	3(20.0)	11(36.7)	0.321**	2(15.4)	1(50.0)	0.371**	0(0.0)	2(16.7)	1.000**
No	12(80.0)	19(63.3)		11(84.6)	1(50.0)		1(100.0)	10(83.3)	
Comorbidity (diabetes mellitus):									
Yes	9(60.0)	11((36.7)	0.138*	9(69.2)	0(0.0)	0.143**	1(100.0)	8(66.7)	1.000**
No	-	-		-	-		-	-	
Unknown	6(40.0)	19(63.3)		4(30.8)	2(100.0)		0(0.0)	4(33.3)	
History of antibiotics use:									
Yes	7(46.7)	13(43.3)	0.832*	7(53.8)	0(0.0)	0.467**	1(100.0)	6(50)	1.000**
No				-	-				

Unknown	- 8(53.3)	- 17(56.7)		6(46.2)	2(100.0)		- 0(0.0)	- 6(50)	

*Pearson Chi-Square test

Fisher's Exact Test

There were 15 of 45 (33.3%) samples from skin and soft tissue infections consisted *S. aureus*. Of these, 13 samples were MRSA-positive (28.9%) and 2 samples MRSA-negative. There was only 1 sample (2.2%) of PVL-positive and 14 samples were PVL-negative. The PVL-positive showed as CA-MRSA. The percentage of case finding of *S. aureus* was 33.3%, with characteristic as female (53.3%), <66 years old (80%), had hospitalization history in primary health care center (53.3%), no history of hospitalization in hospital (80%), comorbidity (diabetes mellitus) history (60%), and unknown antibiotic history (53.3%).

The percentage of case finding of MRSA was 28.9%, with characteristic as female (61.5%), <66 years old (76.9%), had hospitalization history in primary health care center (61.5%), no history of hospitalization in hospital (84.6%), had comorbidity (diabetes mellitus) history (69.2%) and antibiotic history (53.8%). There was not a significant relationship between *S. aureus*, neither MRSA and PVL in all patients and all the risk factors in this study according to Chi-Square-Fisher's Exact test (p value > 0.05).

Discussion

In this community settings study, the MRSA carriage rate was 28.9%, higher than the study in three hospitals in Indonesia by Santosaningsih et al. (2014) [8]. It may show that exposures to MRSA in community trends to raise up nowadays. We must concern and prepare for further research about it. Among 20 from 45 samples, there was only one sample that proved CA-MRSA. The CA-MRSA is genetically distinct from HA-MRSA, being resistant to fewer no-lactam antibiotics, carrying a smaller version of SCC*mec*, and often producing a cytotoxin, PVL [2]. Four of the samples were Staphylococcal strain, but not the *Staphylococcus aureus*. Only 13 samples of the remaining 15 samples were MRSA-positive. Based on PVL gene result, 12 samples were proved as HA-MRSA. Observing back to the questionnaire result, from 12 patients that the samples were taken, 58.3% have ever been hospitalized in primary health care center. Meanwhile for hospital data, only 2 of them that had ever been hospitalized in hospital and the remaining were not. It is very interesting for further discussion that 83.3% of the 12 samples of HA-MRSA had no history of hospitalization in hospital.

The only CA-MRSA sample coded 37 was a female, age more than 66 years old, had hospitalization history in primary health care center, no history of hospitalization in hospital, had comorbidity (diabetes mellitus) history and antibiotic history. We think that the MRSA of this patient is pure from community, because he had no history of hospitalization in hospital, but in primary health care center only. We need further discussion for this fact as CA-MRSA, because of the current definition of HA-MRSA is healthcare - not hospital anymore-acquired MRSA. We hope a large sample will be collected for further study of CA-MRSA and HA-MRSA.

Acknowledgements

We thank to Naqiyah Afifah Mulachelah, Department of Biology, Faculty of Mathematics and Natural Sciences, Brawijaya University, Malang, Indonesia and Suci Megasari, Laboratory of Biomedic, Brawijaya University, Malang, Indonesia for assisting the project. Also, to RR Ettie Rukmigarsari, Islam University, Malang for data analysis that is very helpful for this study.

References

1. Brooks GF, Carroll CK, Butel JS, Morse SA, Mietzner TA (2013) Jawetz, Melnick, & Adelberg's Medical Microbiology. 26th edition. Lange-The McGraw-Hill Companies, Inc., USA.

2. Lakhundi S, Zhang K (2018) Methicillin resistant Staphylococcus aureus: molecular characterization, evolution, and epidemiology. Clinical Microbiology Rev., 31:e00020-18.

3. Santosaningsih D, Santoso S, Budayanti NS, Suata K, Lestari ES, Wahjono H (2016) Characterisation of clinical Staphylococcus aureus isolates harbouring mecA or panton-valentine leukocidin genes from four tertiary care hospitals in Indonesia. Tropical Medicine and International Health, 21(5): May, 610–8.

4. Deepigaa M, Gopinath P (2018) Detection of vancomycin resistance among clinical isolates of Staphylococcus aureus. Research J. Pharm. and Tech., 11(2): February.

5. Niyas FM, Gopinath P (2016) Comparative study on detection of MRSA using oxacillin agar screening method, cefoxitin disc diffusion method and mecA gene by PCR among clinical isolates of Staphylococcus aureus. Research J. Pharm. and Tech. 9(9): September.

6. Williams ME (2012) Perspective on disease and disorder MRSA. Farmington Hills: Greenhaven Press.

7. Chen CJ, Huang YC (2014) New epidemiology of Staphylococcus aureus infection in Asia. European Society of Clinical Microbiology and Infectious Diseases. Clin Microbiol Infect, 20: 605-23.

8. Santosaningsih D, Santoso S, Budayanti NS, Kuntaman K, Lestari E.S, Farida H, et al (2014) Epidemiology of Staphylococcus aureus harboring the mecA or panton-valentine leukocidin genes in hospitals in Java and Bali, Indonesia. In: Am. J. Trop. Med. Hyg., 90(4): 728-34.

9. Cuny C, Witte W (2005) PCR for the identification of methicillin resistant Staphylococcus aureus (MRSA) strains using a single primer pair specific for SCCmec elements and the neighbouring chromosome-borne or fX. Clinical Microbiology Infection. 11: 834-7.

10. Pastagia M, Kleinman LC, Lacerda de la Cruz EG, et al (2012) Predicting risk for death from MRSA bacteremia. Emerging Infectious Diseases, 18(7): 1072-80. doi:10.3201/eid1807.101371.

11. Athena PK, Hatfield K, Baggs J, Mu Y, See I, Epson E, et al (2019) *Vital signs:* epidemiology and recent trends in methicillin-resistant and in methicillin-susceptible *Staphylococcus aureus* bloodstream infections-United States. Morbidity and Mortality Weekly Report. Centers for Disease Control and Prevention. *Weekly*/ March 8/ 68(9): 214-9.

12. Peng H, Liu D, Ma Y, Gao W (2018) Comparison of community- and healthcare-associated methicillin-resistant Staphylococcus aureus isolates at a Chinese tertiary hospital, 2012-2017. Scientific reports 8: 17916 doi: 10.1038/s41598-018-36206-5. www.nature.com/scientificreports.

13. Diep BA, Palazzolo-Ballance AM, Tattevin P, Basuino L, Braughton KR, et al (2008) Contribution of panton-valentine leukocidin in community associated methicillin-resistant Staphylococcus aureus pathogenesis. PLoS ONE 3(9): e3198. doi:10.1371/journal.pone.0003198.

14. Lina G, Piemont Y, Godail-Gamot F, Bes M, Peter M-O, Gauduchon V, et al (1999) Involvement of panton-valentine leukocidin-producing Staphylococcus aureus in primary skin infections and pneumonia. Infectious Diseases Society of America, 29: 1128-32.

15. Vandenesch F, Naimi T, Enright MC, Lina G, Nimmo GR, Heffernan H, et al (2003) Community-acquired methicillin-resistant Staphylococcus aureus carrying panton-valentine leukocidin genes: worldwide emergence. Emerging Infectious Disease, 9(8): 978-84.

16. Murakami K, Minamide WW, Koji N, Etuo T, Watanabe H, Watanabe S (1991) Identification of methicillin-resistant strains of staphylococci by polymerase chain reaction. Journal of Clinical Microbiology, 29(10): 2240-4.

