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# Paper 2

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## MOLECULAR GENOTYPING OF TSST-1 GENE *STAPHYLOCOCCUS AUREUS* FROM CLINICAL SPECIMEN

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### ABSTRACT

*Staphylococcus aureus* (*S. aureus*) is the most common pathogen in humans that causes various infections. Variations in infections due to *S. aureus* are related to the presence of virulence factors, one of which is Toxic Shock Syndrome Toxin-1 (TSST-1) which is the main cause of Toxic Shock Syndrome (TSS). This study aimed to explore the genetic pattern of the *tsst-1* genes among MSSA and MRSA from clinical isolates of patients at Dr. Soetomo General Academic Hospital, Surabaya-Indonesia. PCR examination was performed on 106 clinical samples of *S. aureus* isolates to determine the presence of the *tsst-1* genes; subsequently, phylogenetic analysis was performed. The positive *tsst-1* genes were found in 3 (5.7%) MSSA isolates and 2 (3.8%) MRSA isolates from 106 samples, of which were divided into 53 MSSA isolates and 53 MRSA isolates. No significant difference was noticed between the *tsst-1* genes on both the MSSA and MRSA bacteria ( $p = 0.648$ ). The positive *tsst-1* genes were found in 1 (4.5%) of pus specimens and 2 (16.7%) of blood specimens from MSSA isolates. However, the positive *tsst-1* genes were found in neither sputum nor urine specimens. The genes were found in 2 (16.7%) of blood specimens from MRSA isolates. Based on the phylogenetic tree, Indonesian *tsst-1* isolates belonged to the same clade as Japan, Iran, Iraq, India, Egypt and the United Kingdom isolates. The prevalence of *tsst-1* genes of both MSSA and MRSA from clinical isolates in Dr. Soetomo General Academic Hospital Surabaya are 5.7% and 3.8%, respectively. The *tsst-1* genes of Surabaya-Indonesia, Japan, Iran, Iraq, India, Egypt and the United Kingdom belonged to the same clade.

**Keywords:** MSSA; MRSA; toxic shock syndrome toxin-1; phylogenetic

### ABSTRAK

*Staphylococcus aureus* (*S. aureus*) merupakan patogen paling sering pada manusia yang menyebabkan berbagai macam infeksi. Variasi infeksi akibat *S. aureus* dihubungkan dengan adanya faktor virulensi salah satunya yaitu Toxic Shock Syndrome Toxin-1 (TSST-1) yang merupakan penyebab utama Toxic Shock Syndrome (TSS). Penelitian ini bertujuan untuk mengeksplorasi pola genetik gen *tsst-1* antara MSSA dan MRSA dari isolat klinis pasien di rumah sakit Dr. Soetomo, Surabaya-Indonesia. Dari 106 sampel isolat klinis *staphylococcus aureus* dilakukan pemeriksaan PCR untuk mengetahui adanya gen *tsst-1* dan selanjutnya dilakukan analisis phylogenetic. Dari 106 sampel terbagi menjadi 53 isolat MSSA dan 53 isolat MRSA didapatkan gen *tsst-1* positif sebanyak 3 (5,7%) isolat MSSA dan 2 (3,8%) isolat MRSA. Tidak didapatkan perbedaan yang bermakna antara gen *tsst-1* dengan bakteri MSSA dan MRSA. Distribusi gen *tsst-1* positif pada spesimen pus sebanyak 1 (4,5%) dari isolat MSSA dan spesimen darah 2 (10,5%) isolat akan tetapi tidak ditemukan pada spesimen sputum maupun urin sedangkan pada isolat MRSA hanya ditemukan pada spesimen darah sebanyak 2 (16,7%) isolat. Isolat *tsst-1* dari Indonesia dalam satu clade dengan Negara Jepang, Iran, Irak, India, Mesir dan Inggris mengacu pada hasil phylogenetic tree. Prevalensi gen *tsst-1* pada (MSSA) dari isolat klinis di RSUD Dr. Soetomo Surabaya sebesar 5,7% sedangkan MRSA sebesar 3,8%. Berdasarkan hasil phylogenetic tree didapatkan gen TSST-1 dari Surabaya dalam satu clade dengan negara Jepang, Iran, India, Irak, Inggris dan Mesir.

**Kata kunci:** MSSA; MRSA; toxic shock syndrome toxin-1; phylogenetic

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## INTRODUCTION

*Staphylococcus aureus* (*S. aureus*) is the most common pathogen found in humans that causes a wide variety of infections ranging from superficial skin and soft tissue infections to death-causing infections such as endocarditis, osteoarticular infections, pneumonia and bacteraemia. The therapeutic choice at the beginning of the development of *S. aureus* infection was the penicillin class of antibiotics. After some reported cases of penicillin resistance, new antibiotics such as methicillin was developed (Boswihi & Udo 2018). Methicillin is a semi-synthetic antibiotic of the  $\beta$ -lactam group, which kill bacteria by inhibiting bacterial cell wall synthesis. Short after usage of methicillin, it emerged methicillin resistant *Staphylococcus aureus* (MRSA), that resistance against all beta lactam antibiotics. MRSA resistance was identified encoded by *mecA* gene that expressing the PBP 2b that has no affinity against beta lactam antibiotic, and induce the beta lactam resistance (McGuinness et al 2017).

Variations in *S. aureus* infection are associated with virulence factors, such as adhesion and exo-protein factors which consist of exotoxins and enzymes, including nuclease, protease, lipase, hyaluronidase, and collagenase. Exotoxins produced by *S. aureus* are  $\alpha$ -haemolysin,  $\beta$ -haemolysin,  $\gamma$ -haemolysin, Pantone-Valentine leukocidin (PVL), Toxic Shock Syndrome Toxin-1 (TSST-1), Staphylococcal Enterotoxins (SE) and Exfoliative Toxins (EF) (Bien et al 2011, Motamedifar et al 2015, Oliveira et al 2018). Toxic Shock Syndrome Toxin (TSST-1) is an example of the SAgS exotoxin group that has the ability of pyrogenicity, super-antigenicity and crossing the surface mucosa. The *tst-1* encoding genes are present in the pathogenicity islands produced in the post-responsive phase. Toxic Shock Syndrome Toxin (TSST-1) is a small, non-glycosylated, polypeptide molecule weighing around 22 kD. The toxin is stable to chemicals, resistant to hot and dry conditions (Dinges et al 2000).

Toxic Shock Syndrome Toxin-1 (TSST-1) is one of the main virulence factors and the main cause of Toxic Shock Syndrome (TSS) (Teyhoo et al 2014). Toxic Shock Syndrome Toxin-1 (TSST-1) has the ability to activate T lymphocytes through a direct bond between the T Cell Receptor (TCR)  $V\beta$  with the Major Histocompatibility Complex class II (MHC II) molecules. The activation results on the production of pro-inflammatory cytokines, including Interleukin (IL-2), Interferon (IFN) and Tumor Necrosis Factor (TNF) causing symptoms of high fever, reddish rash, desquamation, hypotension, and acute, life-threatening multi organ failure of which are the symptoms of TSS

(Bien et al 2011, Motamedifar et al 2015, Oliveira et al 2018). The ability of bacteria to cause infection depends on the number of virulence factors from bacterial colonisation. The characteristics of virulence factors of each MSSA and MRSA may vary related to the length of treatment and the possibility of mortality (Motamedifar et al 2015). Research on MSSA and MRSA bacteria producing TSST-1 toxin from clinical isolates of patients in Indonesia is still rarely found.

This study aimed to explore the genetic pattern of the *tst-1* genes among MSSA and MRSA from clinical isolates of patients at Dr. Soetomo General Academic Hospital, Surabaya-Indonesia.

## MATERIALS AND METHODS

### Bacterial Isolates

All bacterial isolated, namely Methicillin sensitive *Staphylococcus aureus* (MSSA) and Methicillin resistant *Staphylococcus aureus* (MRSA) were collected from clinical isolates of hospitalized patients in Dr. Soetomo General Academic Hospital Surabaya. The bacterial were cultured and identification according to the routine procedure in this hospital. After sub-culture in primary medium, the growth colonies were to identify and AST by BD Phoenix™ Automated Microbiology System or Vitek 2 system, that had been carried out at the Clinical Microbiology Laboratory of Dr. Soetomo General Academic Hospital, Surabaya. All isolates were stored at deep freeze in -80 °C.

### DNA Isolation and PCR Amplification of the *tst-1* Genes

DNA extraction was carried out using the TE boiling method. MSSA and MRSA samples were cultured on Manitol Salt Agar (MSA) media and incubated at 37°C for 24 hours. The colony was then inoculated with a sterile transfer loop in an Eppendorf Tube containing 100 $\mu$ L TE buffer. The suspension was then dissolved using a vortex and heated at thermostat (Eppendorf, North America) temperature of 95°C for 10 minutes, respectively. Following the heating, the tube was cooled to room temperature and was centrifuged at 10,000 rpm for 10 minutes. The extracted DNA was stored at -20°C before amplification (Kuntaman et al 2016). PCR amplification procedure was run in 25  $\mu$ L mixture, consisting of 3.5 $\mu$ L DNA template, 12.5 $\mu$ L PCR mix (DreamTaq Green, Thermo Scientific, USA), 0.5 $\mu$ L (50 pmol) for each of the primers for gene *tst-1* and 8  $\mu$ L of distilled water to make 25 $\mu$ L mixture of PCR reaction. Forward primers: 5'-ACC CCT GTT

CCC TTA TCA TC-3' and reverse primers: 5'-TTT TCA GTA TTT GTA ACG CC-3' were used to make an amplicon product of 326 bp (Mehrotra et al 2000). DNA was amplified using a thermal cycler PCR (Icycler, Biorad Thermal Cycler). An initial denaturation step was run at 18°C for 6 minutes and followed by a separate 32 cycles of denaturation at 95°C for 1 minute, annealing at 55°C for 35 seconds, and extension at 72°C for 30 minutes, and the cycles was ended by 72°C for 10 minutes respectively (Alni et al 2018). The reaction products were run in 1.5% agarose gel electrophoresis with ethidium bromide staining. DNA bands were then visualised and observed using a UV transilluminator (Spectroline, Spectronic, USA).

The amplicon of tsst-1 gene was sequenced using Applied Bio system ABI 3130 8 capillary sequencer as manufacture procedure. The result of sequence analyzed with CLC Sequence Viewer version 7.6 software, nucleotide and protein used Lasergene Megalign 7.0 software and build phylogenetic tree with MEGA X.

**Statistical Analysis**

The difference in the proportion of the tsst-1 genes between MSSA and MRSA bacteria was analysed using the SPSS Mann-Whitney test. The p value of <0.05 indicated a significant difference.

**RESULTS**

**Bacterial Isolates**

A total of 106 isolates were collected during the study since July until October. The bacterial isolates were consisting of 53 MSSA bacterial isolates and 53 MRSA bacterial isolates. Out of 53 MSSA bacterial isolates, 22 (41.5%), 19 (35.8%), 10 (18.9%) and 2 (3.8%) isolates were taken from pus, blood, sputum and urine specimens respectively. Meanwhile, out of 53 MRSA bacterial isolates, 24 (45.3%), 17 (32.1%), 12 (22.6%) isolates were taken from pus, blood and sputum specimens respectively. The 53 MSSA bacterial isolates were collected from 32 (60.4%) males and 21 (39.6%) females, whereas 53 MRSA came from 29 (54.7%) male and 24 (45.3%) females respectively. The patients ranged from baby below one year of age up to 80 years old, that mostly at 51-60 years old show in Table 1.

Table 1. The characteristics of specimens, genders and age groups of MSSA and MRSA isolates

	Bacterial Number	
	MSSA (%)	MRSA (%)
Specimens		
Pus	22 (41.5)	24 (45.3)
Blood	19 (35.8)	17 (32.1)
Sputum	10 (18.9)	12 (22.6)
Urine	2 (3.8)	0 (0.0)
Total	53 (100)	53 (100)
Genders		
Male	32 (60.4)	29 (54.7)
Female	21 (39.6)	24 (45.3)
Total	53 (100)	53 (100)
Age groups		
0-10 years	11 (20.8)	11 (20.8)
11-20 years	11 (20.8)	5 (9.4)
21-30 years	1 (1.9)	5 (9.4)
31-40 years	5 (9.4)	6 (11.3)
41-50 years	7 (13.2)	3 (5.7)
51-60 years	10 (18.9)	18 (33.9)
61-70 years	4 (7.5)	4 (7.5)
71-80 years	4 (7.5)	1 (1.9)
Total	53 (100)	53 (100)

MSSA: methicillin-sensitive *Staphylococcus aureus*; MRSA: methicillin-resistant *Staphylococcus aureus*

**The tsst-1 Genes in Clinical Isolates**

The tsst-1 genes were identified using the PCR method. The results of electrophoresis of tsst-1 genes are displayed in Figure 1. The positive tsst-1 genes were found in 5 (4.7%) isolates of 106 isolates, consisting of 3 (5.7%) MSSA isolates and 2 (3.8%) MRSA isolates. No significant difference was found between the tsst-1 genes on MSSA and MRSA bacteria (p = 0.648). Table 2 showed characteristic of the positive tsst-1 genes on MSSA and MRSA isolates based on specimens, genders and age groups. The positive tsst-1 genes on MSSA isolates, were found in 1 (4.5%) and 2 (10.5%) isolates, from pus and blood specimens, respectively. However, the positive tsst-1 genes were not found from both of sputum and urine specimens. The positive tsst-1 genes on MRSA isolates were only found in 2 (16.7%) isolates from blood specimens. The spread of tsst-1 distribution among specimens, sex and age show in Table 2.



Figure 1. Electrophoresis of the tsst-1 genes from MSSA and MRSA bacteria.

Note: M: Marker 100bp DNA ladder, K-: negative control, K+: positive control of *Staphylococcus aureus* N315, 101-118: sample codes. 326bp: positive band of DNA of tsst-1 genes

Table 2. Characteristic of the positive tsst-1 genes on MSSA and MRSA isolates based on specimens, genders and age groups

	MSSA (%) Positive tsst-1 genes	MRSA (%) Positive tsst-1 genes
<b>Specimens</b>		
Wound	1 (4.5%)	0
Blood	2 (10.5%)	0
Sputum	0	2 (16.7%)
Urine	0	0
<b>Genders</b>		
Male	1 (3.1%)	1 (3.4%)
Female	2 (9.5%)	1 (3.4%)
<b>Age groups</b>		
0-10 years	1 (9.1%)	0

11-20 years	1 (9.1%)	1 (20%)
21-30 years	0	0
31-40 years	0	0
41-50 years	0	0
51-60 years	0	1 (5.6%)
61-70 years	1 (9.1%)	0
71-80 years	0	0

MSSA: methicillin-sensitive *Staphylococcus aureus*; MRSA: methicillin-resistant *Staphylococcus aureus*; tsst-1: toxic shock syndrome toxin-1

**DNA Sequencing and Phylogenetic Analysis of the tsst-1 Genes**

The sequence of tsst-1 genes, one of the examples in Figure 4 analysis of the nucleotide sequences and amino acid sequences of the tsst-1 genes was run using the Tamura-Nei neighbour-joining method.

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101-F_G11_20_Sby_Ina AAGGGGAAAA AGTTGACTTA AACACAAAAA GAACTAAAAA AAGCCAACAT ACTAGCGAAG 239
111-F_H11_23_Sby_Ina AAGGGGAAAA AGTTGACTTA AACACAAAAA GAACTAAAAA AAGCCAACAT ACTAGCGAAG 240
K+F_F11_17_Sby_Ina AAGGGGAAAA AGTTGACTTA AACACAAAAA GAACTAAAAA AAGCCAACAT ACTAGCGAAG 239
239-F_B12_06_Sby_Ina AAGGGGAAAA AGTTGACTTA AACACAAAAA GAACTAAAAA AAGCCAACAT ACTAGCGAAG 240
250-F_C12_09_Sby_Ina AAGGGGAAAA AGTTGACTTA AACACAAAAA GAACTAAAAA AAGCCAACAT ACTAGCGAAG 240
134-F_A12_03_Sby_Ina AAGGGGAAAA AGTTGACTTA AACACAAAAA GAACTAAAAA AAGCCAACAT ACTAGCGAAG 239
    
```

Figure 4. Sequence of tsst-1 genes sample

The phylogenetic tree of the isolates obtained in the current study and isolated from other countries, using the Tamura-Nei method showed in Figure 5. Two clades were found. The first clade consisted of animals from Egypt and Switzerland, and consisted of food from Egypt. The second clade consisted of clinical isolates of MSSA and MRSA bacteria from Surabaya-Indonesia, Japan, United Kingdom, Iran, Egypt, Iraq and India; animals from Thailand and India; as well as animal products from Egypt. Although MSSA isolate samples with code 111-F belonged to the same clade as samples from Japan, they still had a genetic variation of 0.35%. Other MSSA isolate samples with code 101-F had similar genetic variation to MSSA isolate samples with code 134-F. The MSSA isolate samples with codes 134-F and 239-F have a genetic variation of 0.35%. Two MRSA isolate samples with codes 239-F and 250-F had the same genetic variation as the 2 latter samples.



Figure 5. Phylogenetic tree of positive tsst-1 samples.  
Notes: 101-F, 111-F, 134-F, 239-F and 250-F: bacterial isolates of MSSA INA-SBY

## DISCUSSION

*Staphylococcus aureus* is one of the important pathogenic bacteria that causes infection in humans, in both of the hospital and the community environment (Teyhoo et al 2014). Infections due to *S. aureus* caused a long hospital stay, antibiotic use, high cost and mortality (Alni et al 2018). The ability of the bacteria in causing infection was supported by the presence of virulence factors, one of which was Toxic Shock Syndrome Toxin (TSST-1) which can cause Toxic Shock Syndrome (TSS) (Teyhoo et al 2014).

The prevalence of tsst-1 gene in MSSA and MRSA were low, 3 (5.7%) in MSSA and 2 (3.8%) in MRSA respectively, and there was no significant difference between MSSA and MRSA. The results were similar to a study conducted in Malaysia in 2013; of which found that the proportion of tsst-1 genes in MSSA strains was 13.3% greater than the proportion of the genes in MRSA strains. However, the Malaysia study found no MRSA bacterial strains that had tsst-1 genes. In 2011, 6.8% positive tsst-1 genes of 518 MSSA strains were reported (Sapri et al 2013). As many as 7.5%, 5% and 23% positive tsst-1 genes were also reported from Lybia (El-Ghodban et al 2016), Lebanon (Tokajian et al 2011) and Iran (Sabouni et al 2014) respectively. In 2015, positive tsst-1 genes were discovered in 14.3% MRSA and 19.3% MRSA isolates in an Iran study with the total sample of 345 *Staphylococcus Aureus* (Motamedifar et al 2015).

In a study performed in Thailand, was not identified of tsst-1 genes neither in 36 isolates MSSA nor 56 isolates MRSA bacterial strains (Indrawattana et al 2013). Recent report in 2014 from two Iran hospital, found positive tsst-1 genes in 2 MSSA isolates and 18 MRSA isolates from 100 isolates *Staphylococcus Aureus* and another hospital found 12 MRSA isolates positive tsst-1 genes in from 84 isolates *Staphylococcus Aureus* (Teyhoo et al 2014). In 2015, another Iranian study discovered positive tsst-1 genes in 56% MSSA isolates and 69.8% MRSA isolates (Koosha et al 2015). Besides, in 2018, the positive tsst-1 genes were mostly noticed in the MRSA bacterial strain except for one isolate derived from the MSSA bacterial strain (Alni et al 2018).

A study carried out in Japan displayed a larger proportion of the tsst-1 genes that was 75% of 152 samples of MRSA bacterial strain isolates (Nagao et al

2009). The difference between virulence factors in MSSA and MRSA bacteria was still debatable of which depended not only on genotype, but also on geographical distribution.

Wound is a type of specimen that is the main source of the production of the tsst-1 genes from *Staphylococcus Aureus* (Teyhoo et al 2014). The current study found 3 specimens with positive tsst-1 genes, including isolates from the specimens of wound, blood and sputum. Similar results were also obtained in Iran and Austria. Both studies in two latter countries discovered the positive tsst-1 genes were mostly derived from blood and wound (Eftekhar et al 2017, Roetzer et al 2016). The different results were obtained in Nigeria and Malaysia that the tsst-1 genes were not found in wound samples, but were found in samples of both vaginal fluid and respiratory (Ezeamagu et al 2017, Lim et al 2012). The tsst-1 genes could be found from diverse specimens of different substances of the patient's body. The present study described that the presence of positive tsst-1 genes was not comparable with the incidence of TSS; hence, the tsst-1 gene expression was influenced by various regulatory factors.

The tsst-1 isolates from Indonesia were categorised in the same clade as tsst-1 isolates from Japan, Iran, Iraq, India, Egypt and United Kingdom. The Indonesian specimens were also found to be similar to animals and animal product specimens from India, Thailand and Egypt. Nevertheless, isolates from Indonesia differed in clade from specimens found in animals and food originated from Egypt and Switzerland. The results of the present study displayed a diversity that was characterized by changes in the composition of proteins that affected differences in the expression of the tsst-1 genes. The findings still required further research on mutagenesis.

## CONCLUSION

The prevalence of tsst-1 genes of both MSSA and MRSA from clinical isolates in Dr. Soetomo General Academic Hospital Surabaya were 5.7% and 3.8% respectively. Based on tsst-1 gene sequencing showed that the MSSA isolates with code 111-F need further mutagenesis analysis, because there were changes of amino acid. The phylogenetic tree, tsst-1 genes from Surabaya-Indonesia was categorised as the same clade with tsst-1 genes from Japan, Iran, India, Iraq, Egypt and United Kingdom.

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