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Original Article

Biofilm formation and detection of A/D genes in MRSA (Methicillin-Resistant *Staphylococcus aureus*) and MSSA (Methicillin-Sensitive *Staphylococcus aureus*)

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Abstract

MRSA (Methicillin-Resistant *Staphylococcus aureus*) arises due to excessive use of methicillin antibiotics and complications from all medical practices that have been carried out lately. The prevalence of MRSA tends to increase and its ability to form biofilms so that it has the potential to significantly increase mortality and morbidity. Biofilms are considered one of the microbial virulence factors. Biofilm formation in S. *aureus* is regulated by the expression of *PIA* which mediates attachment between cells to cells and is a product of the ABDC *ica* gene. This study aims to determine the ability of biofilm formation as one of the virulence factors in MRSA compared to MSSA (Methicillin-Sensitive *Staphylococcus aureus*) . its relationship to the presence of A and D *ica* genes as biofilm coding genes. Biofilm formation *assay* on MRSA and MSSA using the Microtiter Plate (MtP) method and the A/D gene detection assay performed by PCR method. The results showed that the incubation end value of Optical Density (OD) for MSSA1 (0.75), MSSA2 (0.46), MRSA1 (0.53) isolates according to the formula were interpreted as moderate biofilm producer. The conclusions of this study were all of the test isolates (MSSA1, MSSA2 and MRSA1) were able to form a biofilm. Distribution of *ica* A and *ica* D genes in MSSA 1 and MSSA 2 was varied while MRSA1 had the *ica* A and *ica* D genes.

Keywords: Biofilm, *ica* A/D gene, MRSA, MSSA

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Introduction

Bacterial resistance to various antibiotics is a worldwide problem (pandemic) that is not less important than the problem of infection/virulence of the bacteria itself. One of the important bacterial resistances to be noticed is Methicillin-Resistant *Staphylococcus aureus* (MRSA) (Yuwono & Biomed, 2010). MRSA is a type of *S. aureus* or also called Methicillin Sensitive *Staphylococcus aureus* (MSSA) that is resistant to methicillin antibiotics and other class medications, such as penicillin, amoxicillin, and oxacillin.

A survey conducted in 2008 in ten cities in Indonesia shows that the prevalence of MRSA is quite high, at 27%, where Surabaya (40%) keeps the third-largest after Makassar (100%) and Jakarta (54%), followed by Semarang (36%), Yogyakarta (31%), Bandung (23%), Padang (21%), Malang (19%), Solo (17%) and Denpasar (7%) (The first multicenter study, 2008).

MRSA creates a large risk and has the potential to cause significant mortality and morbidity in the human population because it is able to form a biofilm. Biofilm has a considerable impact on health and are estimated to be associated with 65% of nosocomial infections (Kaur & Wankhede, 2014). Riemann and Oliver (2006), defines the biofilm as a collection of microorganisms and associated extracellular products on its surface and commonly attached to biological and non-biological substrates.

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The formation of biofilm in *S. aureus* is governed by PIA expression mediating cell-to-cell attachment and is the product of the genes of ABDC *ica* (Kaur & Wankhede, 2014). The product of the *ica* A gene is a homologous transmembrane protein with N-acetyl-glucosaminyltransferase, which requires the *ica* D gene product for its optimum activity. The N-acetyl-glucosamine Oligomer produced by *ica* AD reaches a maximum length of 20 residues and it occurs only when the *ica* AD is coexpressed with *ica* C, which allegedly encodes a membrane protein, whose oligomeric chain is longer. (O'Gara, 2007).

Infection accompanied by biofilm formation becomes a major problem, as it is difficult to be managed effectively by the host immune system and is resistant to treatment with antimicrobials. Such protection mechanisms are thought to preclude the absorption and penetration of antibiotics through the biofilm matrix (Cavaliere et al., 2014). Biofilm is known to be involved in various kinds of microbial infections in the body. It is estimated that nearly 80% of all infections (Ghafourian et al., 2013).

Eftekhar and Dadaei (2010) in their study showed that 55% of MRSA isolates were able to form biofilm and 75% of MRSA isolates contained *ica* operon. Previous research by Nuryastuti (2013) showed that the presence of *ica* A / D genes in chromosomes in MRSA and MSSA isolates is not always followed by the ability of MRSA bacteria to form biofilms. Operon *ica* in *S. epidermidis* and *S. aureus* is influenced by environmental factors but its expression is more closely controlled in *S. aureus*. The ability of the biofilm-forming in vitro greatly depends on the type of strain of *S. aureus* (Eftekha & Dadaei, 2010).

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This study aims to identify and analyze the ability of biofilm formation in MRSA and MSSA isolates, *ica* A/D genes distribution associated with biofilm formation, thus providing preliminary data on biofilm formation capacity ratio in these two strains of bacteria.

Methods

Bacterial Strain, Media and Growth Condition

The test bacterial isolates used in this study are MSSA1, MSSA2, and MRSA1. Bacterial MSSA1 isolates collection was obtained from microbiology laboratory, Faculty of Medicine, Wijaya Kusuma Surabaya University, bacterial MSSA 2 and MRSA 1 isolates collection were obtained from microbiology laboratory, Faculty of Medicine, Airlangga Surabaya University. All of the isolates were subcultures from stocks onto NA (Nutrient Agar) medium and incubated at 37 ºC overnight.

Study of Biofilm Formation on MRSA and MSSA by Microtiter Plate Method (MtP)

The microtiter plate method was conducted previously by Meritt et al. (2005) with modification. The overnight culture of MSSA1, MSSA2, and MRSA1 were inoculated in 5 mL of Luria Bertani broth supplemented with 2% sucrose and incubated overnight at 37 ºC. The overnight culture of the test isolates was diluted by fresh Luria Bertani broth supplemented with 2% sucrose medium to OD_{600}=1 (1%) then used to inoculated 96-well flat bottom polystyrene microtiter plates containing 150 µL fresh Luria Bertani broth supplemented with 2% sucrose. The plate was incubated in shaker (80 rpm, orbitak shaker) at room temperature for 4 h and then the plate were incubated at 37 ºC for ± 48 h. After incubation, the plates were carefully washed 4 times with sterile phosphate buffer saline (PBS) to remove planktonic bacteria and were air-dried in an inverted position before being stained. The plate was stained with 0.1% crystal violet solution of 100 µL for 30 min. The residual staining liquid is washed with sterile distilled water until no color in the rinse water, then allowed to air-dry. Finally100 µL DMSO 100% was added into each well before being read. Furthermore, the culture was analyzed by microtiter plate absorbance reader to analyze its absorbance at 590 nm. Each isolate performed twelve replications. The interpretation of results is described as follows: (If ODs ≤ ODc = nonbiofilm producer; ODc ≤ ODs ≤ 2 × ODc = weak biofilm producer; 2 × ODc ≤ ODs ≤ 4 × ODc = moderate biofilm producer; 4 × ODc <ODs = strong biofilm producer; (description: ODc = OD negative control, ODs = OD sample)). *Staphylococcus epidermidis* was used as the biofilm producer control strain, based on a preliminary test that showed no biofilm was formed (Gowrishhanker et al., 2016).

Detection of *ica* A/D Genes

Extraction of bacterial DNA was performed by the boiling process. After overnight culture Nutrient Agar medium 3-5 colonies were suspended in TAE buffer pH = 2, the suspension was boiled at 98 ºC for 10 min in the water bath then centrifuged at 10.000 rpm for 5 min. An aliquot of the supernatant was used as a DNA template for PCR or store at -20 ºC if not directly analyzed.

The selected primer is based on the base order available at the National Center for Biotechnology Information gene bank (locusAF086783) and Sequences of oligonucleotide primers for PCR amplification of biofilm-associated genes in MSSA1, MSSA2, and MRSA1 isolates are shown in the following Table 1.

The PCR assay for detection of *ica* A/D genes was performed using the primer (forward and reverse). Solution 1 (for 6 samples) with compositions: 75 µL PCR Mix, 6 µL forward primer, 6 µL reverse primer, and 3 µL H_2O. An aliquot of 5 µL of DNA template was added to 15µL solution 1 so that the total volume in microtube is 20 µL. Amplification was performed by using Thermal Sickle with thermal cycling profile as follows: initial denaturation at 94 °C for 2 minutes, (denaturation at 95 °C for 1 min, annealing at 56.9 °C for 1 min and extension at 72 °C during 1 min) with 30 cycles and a final extension at 72 °C for 5 min. (Eftekhar and Dadaei, 2011). Amplified PCR product was analyzed by 2% agarose gel, 1X TAE, 80 volts, 400 milliamperes for 20 min, stained with ethidium bromide 10 mg/mL and visualized under UV-illuminator. *Staphylococcus aureus* ATCC 25923 was used as a positive control strain or the model strain and sterile distilled water was used as a negative control (Yousefi et al., 2016).

Results

This research was conducted at the Laboratory of Gastroentrenteritis and Salmonellosis, Institute of Tropical Disease (ITD), Airlangga University, to find out the ability of biofilm formation of MRSA (Methicillin-Resistant *Staphylococcus aureus*) and MSSA (Methicillin-Sensitive *Staphylococcus aureus*) by microtiter plate (MtP) method and detection of *ica* A/D genes as encoding of biofilm formation in MRSA and MSSA isolates.

The biofilm formation of MSSA1, MSSA2 and MRSA1 isolates by MtP method is shown in Figure 1. The bacterial cells were grown in 96-well flat-bottom polystyrene microtiter plates containing Luria Bertani Broth medium supplemented with 2% sucrose. The cells that adhered to the plate surface after washing with sterile PBS were visualized by crystals violet staining.

Determination of the ability of biofilm formation was conducted by quantitative analysis with MtP method that

analyzing the value of OD (Optical Density) at the beginning and end of incubation with the microtiter plate reader. Result of biofilm formation for MSSA1, MSSA2, and MRSA1 isolates is presented in Table 2.

According to Table 2, the biofilm formation ability for MSSA1, MSSA2, and MRSA1 isolates showed that all of the selected isolates were found moderate biofilm producer. The OD value of the end of incubation for MSSA1, MSSA2 and MRSA1 isolates is greater than or equal to 2 times of the OD value of the end of incubation on control (0.750) or greater than or equal to 1.501. It is also less than or equal to 4 times of the OD value of the end of incubation on control or less than or equal to 3.001 according to the formula used, it is interpreted as moderate biofilm producer.

Detection of *ica* A/D genes as encoding gene of biofilm formation for MSSA1, MSSA2 and MRSA1 isolates was conducted by using the PCR technique. The primer used was obtained from PT. Genetika Science Indonesia, Manufacturer: Integrated DNA Technologies. Sequences of oligonucleotide primers for PCR amplification of biofilm-associated genes in MSSA1, MSSA2, and MRSA1isolates are shown in Table 1. Result of PCR amplification for detection of *ica* A gene (A) and *ica* D gene (B) responsible for biofilm formation on (1) MSSA1, (2) MSSA2 and (3) MRSA1 isolates is shown in Figure 2.

Table 3 shows that the presence of *ica* A/D genes on *Staphylococcus* sp. bacteria shows varied results. MSSA1 isolate was found in *ica* A gene, but it was not found in *ica* D gene. While, the MSSA2 isolate was not found in *ica* A gene, but it was found in *ica* D gene, and MRSA1 isolate was found in both *ica* A and *ica* D genes.

Table 1. Sequences of oligonucleotide primers for PCR amplification of biofilm associated genes in MSSA 1, MSSA 2, and MRSA 1isolates.

Gene	Primer name	Primer Sequence (5'-3')	Product size (bp)	PCR Program	M-PCR Volume
ica	A	F : *GTTGTCGACGTTGGCTACTG* R : *CGACAAGAACTACTGCTGCG*	523 bp	1 cycle (94ºC----2mnt) (94ºC----1 mnt) (56,9ºC--1 mnt) (72ºC----1 mnt) (72ºC----5 mnt) (4ºC----∞)	20 µL
ica	D	F : *CCCAACGCTAAAATCATCGC* R : *TCGCGAAAATGCCCATAGTT*	212 bp	1 cycle (94ºC----2mnt) (94ºC----1 mnt) (56,9ºC--1 mnt) (72ºC----1 mnt) (72ºC----5 mnt) (4ºC----∞)	20 µL

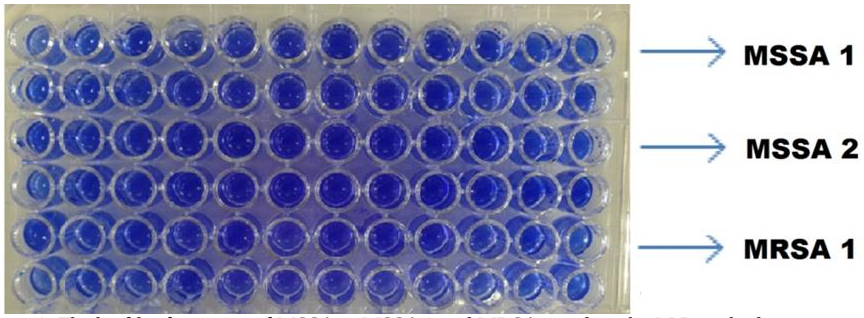

Figure 1. The biofilm formation of MSSA 1, MSSA 2 and MRSA 1 isolates by MtP method.

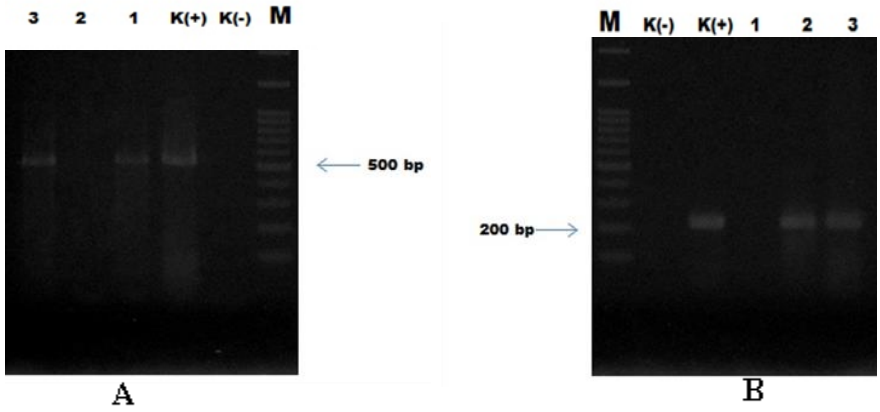

Figure 2. Result of PCR amplification for detection of genes ica A, 523 bp, (A) and ica D, 212 bp, (B) responsible for biofilm formation for (1) MSSA1, (2) MSSA2 and (3) MRSA1 isolates.

Table 2. Result of biofilm formation (Optical Density) for MSSA1, MSSA2, and MRSA1

Replication	Strain ID			
	Negative control	MSSA1	MSSA2	MRSA1
1	0.902	1.015	2.615	2.223
2	1.079	1.753	2.204	1.634
3	1.416	2.960	2.324	1.628
4	0.847	3.460	2.579	2.156
5	0.918	1.367	3.369	2.591
6	0.908	2.688	2.704	1.956
7	0.244	3.174	3.149	2.653
8	0.577	1.957	2.732	2.960
9	0.464	2.575	2.427	2.533
10	0.313	2.130	3.205	1.516
11	0.388	1.825	1.693	3.100
12	1.198	1.841	2.259	2.013
Average	0.771	2.229	2.605	2.247
Standard Deviation	0.356	0.746	0.476	0.526
Category	-	Moderate biofilm producer	Moderate biofilm producer	Moderate biofilm producer

Table 3. Presence of *ica* A/D genes on MSSA 1, MSSA 2, and MRSA 1 isolates

Bacterial Isolate	Result	
	***ica*A**	***ica*D**
MSSA 1	(+)	(-)
MSSA 2	(-)	(+)
MRSA 1	(+)	(+)

Discussion

Using excessive methicillin antibiotic causes MRSA formation with its adhesive properties. The occurrence of bacterial resistance is a complication of all the usual medical practices in recent times, so that the CA-MRSA (*Community-Associated methicillin-resistant S. aureus*) and HA-MRSA (*Hospital-acquired methicillin-resistant S. aureus*) appear (Cihaliva et al., 2015). The prevalence of MRSA in various hospitals in the world ranges from 2-70% with an average rate of 20% (Yuwono & Biomed, 2010) and causes major risk and also potentially causes significant mortality and morbidity rates in human populations because of the ability to form a biofilm. Biofilm have an enormous impact on healthcare and are estimated to be associated with 65% of nosocomial infections (Kaur & Wankhede, 2014).

Biofilm is structured communities of bacterial cells enclosed in a self-produced polymeric matrix and adherent to an inert or living surface (Phil, 2011). Infection followed by biofilm formation becomes a major problem, as it is difficult to be handled effectively by the host's immune system and resistant to treatment with antimicrobials (Cavaliere et al., 2014). Several infections caused by gram-positive bacteria, including those are caused by *S.epidermidis*, *S. aureus* and *Enterococci* are proved very difficult to treat with partial antibiotic therapy due to their very high natural resistance levels and partly because they form a biofilm (O'Toole et al., 2000). Biofilm formation of *S.aureus* is managed by PIA expression which mediates the adherents between cell to cell and it is a product from a gene of *ica* ABCD (Kaur & Wankhede, 2014).

The test of biofilm formation by MtP method which was obtained from the result of all tested *Staphylococcus sp.* (MSSA1, MSSA2, and MRSA1 isolates), and it is included in the category of moderate biofilm producer. The microtiter plate (MtP) method is used for measuring and quantifying the ability of bacterial isolate adherent. Bacterial cells adhere to the substrate or well wall on a microtiter plate and form a multicellular community which is a core stage in the occurrence of infection and these characteristics become as one of the virulence factors in microorganisms, especially bacteria (Moghada et al., 2014). This research result is a little bit different from the result that was obtained by Moghadam, Pourmand, and Aminharati (2014) where their research shows that there was 62,5% of MRSA isolate that was able to form a moderate biofilm, while 40% of MSSA did not form biofilm. This observation shows that the biofilm formation on bacteria very depended on the condition of growth and indicated that the use of adding various sugars was essential for biofilm formation (Terki, 2013). Gowrishhanker et al. (2016) in his research shows that

there were 44 (69,8%) positive isolates of a total of 63 MRSA isolates that formed biofilm by biofilm formation test with MtP method.

In this research, *ica* operon expression and biofilm formation on *Staphylococcus* sp. bacteria show varied results. It is suspected that biofilm expression was affected by environment signal and could induce an external stress response. Biofilm expression could be also affected by the presence of iron, where its maximum expression occurs at low iron concentration (Terki, 2013). The product of *ica* A gene is a homologous transmembrane protein with N-acetyl-glucosamin yltransferase which requires *ica* D gene product for its optimum activity. N-acetyl-glucosamine oligomer that is produced by *ica* AD reaches a maximum length of 20 residues and it occurs only when *ica* AD is coexpressed with *ica* C, which allegedly encodes a membrane protein whose oligomeric chain is longer (O'Gara, 2007).

Detection of *ica* A and D genes in this research shows that it is positive for both genes on MRSA1 isolate. This is in accordance with the results obtained from Moghadam, Pourmand and Aminharati (2014) which of the 40 MRSA isolates tested show that all those isolates are positive to *ica* A and *ica* D genes. Several studies have also shown the importance of the role of *ica* A and *ica* D genes in biofilm formation. *Ica* A and *ica* D genes are needed to form PIA, while PIA is needed to adhere and produce biofilm. MRSA isolates is a resistant isolate to some antibiotics which is too much to form biofilm structure, so that it could be suspected that most pathogens that MDR (Multi-Drug Resistant) often becomes biofilm producer (Moghadam et al., 2014). The cells that form biofilm have increased resistance to antibiotics compared to the resistance shown by planktonic cells that protection mechanism is considered as a barrier of absorbing and penetrating antibiotics through the biofilm matrix (Cavaliere et al., 2014).

On MSSA1 and MSSA2 isolates show *ica* A and *ica* D genes distribution which are different from MRSA, although both of them (MSSA 1 and MSSA 2) are known as forming moderate biofilm in biofilm test with MtP method. On MSSA 1 isolate, there is *ica* A gene, but there is no *ica* D gene. While on MSSA 2 isolate, there is no *ica* A gene, but there is *ica* D gene. In the research of Chokr et al., 2006 has also found this phenomenon, and it is suspected that this strain has variability in the sequence of locus *ica* gene which results in the production of polysaccharides reacting with serum anti-PIA. This is different from the results shown in MRSA isolates that are positive for the ica A and D genes. It is suspected that there is involvement of another ica gene other than ica A / D to biofilm formation, for example, ica B and ica C. PIA production is stimulated by action sensor proteins that are bound to membranes on bacterial cell walls. The PIA synthesis is catalyzed by a protein encoded by the ica operon, a gene group consisting of ADBC ica. The ica C gene may also be involved in the translocation of the extension of the polysaccharide on the cell surface. The ica B protein attached to the surface is then responsible for the deacetylation of the poly-N-acetylglucosamine molecule (O'Gara, 2007). Many of reports has demonstrated the significance of surface components in the biofilm formation of *S. aureus* such as the product of *ica ADBC* operon, which encodes proteins for the synthesis of polysaccharide, poly-N-acetyl β-1-6-glucosamine (PNAG). Also, few extracellular proteins, as well as cellboundadhesins (also called MSCRAMMs) are considered essential for the pathogenicity of *S. aureus* (Gowrishanker et al., 2016).

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