

THE CORRELATION BETWEEN icaA AND icaD GENES WITH BIOFILM FORMATION *Staphylococcus epidermidis* IN VITRO

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ABSTRACT

This study was conducted to identify the presence of icaA and icaD genes in *S. epidermidis* and to analyze the relationship between the presence of icaA and icaD genes with the ability of in vitro biofilm formation in *S. epidermidis*. *S. epidermidis* isolates from patients and healthy people were collected and PCR was examined to detect icaA and icaD genes. which then continued to examine the ability of biofilm formation by the method of Congo Red Agar. The results of this genotypic and phenotypic examination were then tested for correlation with statistical tests using SPSS 23.0. A total of 40 *S. epidermidis* isolates were collected, consisting of 20 clinical isolates and 20 isolates of normal flora. The icaA gene was positive in 5 isolates (12.5%), and 8 isolates (20%) were positive for the icaD gene, 3 isolates with icaA and icaD were both positive. One hundred percent of isolates with icaA or icaD positively formed biofilms, but there were 15 isolates (42.9%) who did not have the icaA gene but showed the ability to form biofilms, while 12 isolates (37.5%) who did not have the icaD gene also formed biofilms. Fifty percent of *S. epidermidis* isolates showed the ability to form biofilms at CRA. The Fisher Exact test showed a significant relationship between the icaA gene and the ability of biofilm formation ($p=0.047$ ($p<0.05$)) as well as the icaD gene ($p=0.03$ ($p<0.05$)). The icaA and icaD genes have a significant relationship to biofilm formation in *S. epidermidis*. There was another mechanism in the formation of biofilms that are not dependent on the ica gene.

Keywords: ica gene; biofilm; *Staphylococcus epidermidis*

ABSTRAK

Penelitian ini dilakukan untuk mengidentifikasi keberadaan gen icaA dan icaD pada *S. epidermidis* serta menganalisis hubungan antara keberadaan gen icaA dan icaD dengan kemampuan pembentukan biofilm in vitro pada *S. epidermidis*. Isolat *S. epidermidis* yang berasal dari pasien dan orang sehat dikumpulkan dan dilakukan pemeriksaan PCR untuk mendeteksi gen icaA dan icaD. yang kemudian dilanjutkan pemeriksaan kemampuan pembentukan biofilm dengan metode Congo Red Agar. Hasil pemeriksaan genotif dan fenotif ini selanjutnya diuji hubungan dengan uji statistik menggunakan SPSS 23.0. Sejumlah 40 isolat *S. epidermidis* terkumpul, terdiri dari 20 isolat klinik dan 20 isolat flora normal. Gen icaA positif pada 5 isolat (12,5%), dan 8 isolat (20%) positif gen icaD, 3 isolat dengan icaA dan icaD yang keduanya positif. Seratus persen isolat dengan icaA atau icaD positif membentuk biofilm, namun terdapat 15 isolat (42,9%) yang tidak memiliki gen icaA tapi menunjukkan kemampuan membentuk biofilm, sementara 12 isolat (37,5%) yang tidak memiliki gen icaD juga membentuk biofilm. Lima puluh persen isolat *S. epidermidis* menunjukkan kemampuan membentuk biofilm pada CRA. Uji Fisher's Exact menunjukkan hubungan bermakna antara gen icaA dengan kemampuan pembentukan biofilm ($p=0,047$ ($p<0,05$)) demikian pula dengan gen icaD ($p=0,03$ ($p<0,05$)). Gen icaA dan icaD memiliki hubungan yang bermakna bagi pembentukan biofilm pada *S. epidermidis*. Terdapat mekanisme lain dalam pembentukan biofilm yang tidak tergantung gen ica.

Kata kunci: Gen ica; biofilm; *Staphylococcus epidermidis*

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INTRODUCTION

Biofilm are structured communities of bacteria, which are attached to the surface and embedded in a self-produced matrix of extracellular polymeric substances

(Abee et al 2011, Heilmann et al 1996). Nowadays biofilms become a seriously public health problems because biofilm-associated microorganisms exhibit dramatically decreased susceptibility to antimicrobial agents and their potency to cause medical devices

indwelling related infection and more resistant to host immune systems (Davey & O'toole 2000, Donlan 2001). In biomaterial associated infection, almost 80% of the cells involve *Staphylococcus epidermidis*. This fact can be explained by the ease access of them as the skin flora then occupied and attached to the wound and implant (von Eiff et al 2002).

S. epidermidis is prototypic bacteria forming biofilm that important cause infection (Mack et al 2013). Biofilm production of *Staphylococcus* depend on several regulator proteins, however the essential factors are presence and expression of icaADBC operon that encode PIA (Li et al 2005).

Molecular study have revealed that attachment stage which the microorganism were adhered each other ininitially and were elaborated formed biofilm, mediated PIA that synthesized product of icaADBC operon (Mack et al 1996, Chaieb et al 2005).Trough biofilm mediating ica operon, enabling this bacteria to colonize inert surfaces of medical devices (Zieburh et al 2006).

PIA positive strain can be distinguished from PIA negative strain by Congo Red Agar method (Heilman dan Gotz 1998). PIA positive strains formed black and dry crystal colonies, while PIA negative formed red colonies (Reid 1999, Gotz 2002). Several studies have demonstrated that CRA method are fast, sensitive, reproducible and colony growing in this medium can still be used for further analysis (Freeman et al 1989, Arciola et al 2002, Kaiser et al 2013).

MATERIALS AND METHODS

Bacterial strains

A number of 40 isolates of *S. Epidermidis* were obtained from blood specimen of sepsis patients and skin swab of health volunteers, then were identified in Clinical Microbiology Laboratory Dr. Soetomo Hospital Surabaya. DNA isolation and PCR were done in Institute of Tropical Disease Universitas Airlangga.

Rejuvenation of *S. epidermidis* isolates

Stock isolates were kept in TSB and glycerol 50% solutions, then were stored in-800C. *S. Epidermidis* bacteria were re-cultured on Blood Agar medium, incubated at 35±20°C for 18-24 hours. The pure colony growths on Blood agar plate were picked 3-5 colonies by disposable loop to perform DNA isolation, while biofilm formation were examined by Congo Red Agar method.

DNA extraction with boiling method

As many as 3-5 colonies were added into 200 µl TE solution pH 8.0 in 1.5 ml extraction tube and vortex thoroughly. Briefly, the bacteria suspension were boiled in thermostat at 99°C for 10 minutes, then the tube were chilled for 1-3 minutes at room temperature. The suspension were centrifuged at 10.000 g for 5 minutes. The supernatan obtained was used as DNA template for PCR process.

DNA amplification by PCR

PCR was performed by Cycler machine (Biorad) to amplify the icaA and icaD genes. The oligonucleotide primer for icaA were, forward 5'-TCT CTT GCA GGA GCA ATC AA-3' and reverse 5'-TCA GGC ACT AAC ATC CAG CA-3' and oligonucleotide primer for icaD were, forward 5'-ATG GTC AAG CCC AGA CAG AG-3' and reverse 5'-CGT GTT TTC AAC ATT TAA TGC AA-3' (Arciola et al 2001, El-Mahallawy et al 2009, Terki et al 2013; Zhou et al 2013). Mix PCR for uniplex PCR icaA and icaD consist of 12,5 µl Go Taq® Green Master Mix Promega, primers with each volume 1 µL, 5 µl template DNA and ddH2O until final volume 25 µl. The PCR cycle was as carried out follows: predenaturation at 94°C for 4 min; 40 cycles of denaturation at 94°C for 30 seconds, annealing at 57,6°C for 30 seconds, and extension at 72°C for 30 seconds; and a final extension step at 72°C for 10 min. The amplicon was electrophoresed into 3% agarose gel, stained with Ethidium bromid. *S. epidermidis* ATCC 35984 is used as positive quality control (slime-producing strain) and *S. epidermidis* ATCC 12228 as negative control (non-slime-producing strain) (Li et al 2009).

Biofilm formation examination

Biofilm formation were examined by Congo Red Agar (CRA) method. This medium was prepared by adding 0,8 g Congo Red (Sigma-Aldrich, Steinheim, Germany), 10 g Bacto agar (Becton, Dickinson and Co, Sparks, MD, USA), 37 g BHI (Oxoid, Basingstoke, UK) and 50 g Suchrose (Merck, Darmstadt, Germany) to 1.000 mL Aquades. *S. epidermidis* were cultured on Blood Agar Plate (BAP) and were incubated at 35°C during 18- 24 h. Bacteria suspension were prepared by 0,85% sterilized NaCl with 1,5x10⁸ CFU/mL (0,5 Mc Farland Standard). A 4-µL aliquot of a bacterial suspension was inoculated in a spot and incubated at 35°C under aerobic conditions for 24 h. Ten strains were inoculated per plate. The results were checked by 3 observers, and the experiments with CRA were performed at least twice. The colonies of bacteria on Congo Red Agar were evaluated by the criteria as follows: 1+: dark red

colonies, non biofilm producer, 2+: red colonies, non biofilm producer, 3+: brown colonies, biofilm producer, 4+ : black colonies, biofilm producer (Christensen et al 1982, Arciola et al 2005, Kaiser et al 2013).

RESULTS

Forty isolates of *S. epidermidis* were checked the presence of icaA and icaD genes consist of 20 clinical isolates and 20 normal flora isolates. PCR revealed the presence of icaA and icaD genes in *S. epidermidis* (Figs. 1 and 2).

Electrophoresed results showed 5 (12.5%) icaA positive isolates, 8 (20%) icaD positive isolates, 3 (7.7%) isolates with both of icaA and icaD positive genes and 30 (75%) isolates with icaA and icaD were negative (Table 1). All of the ica positive genes were clinical isolates.

The biofilm producing phenotype of *S. epidermidis* were determined on CRA (Fig. 3). Fig. 5 shows the percentage of icaA gene related biofilm forming examination on CRA. All of the icaA positive isolates formed biofilm, while 42,9% of icaA negative isolates formed biofilm and 57,1% of them not formed biofilm.

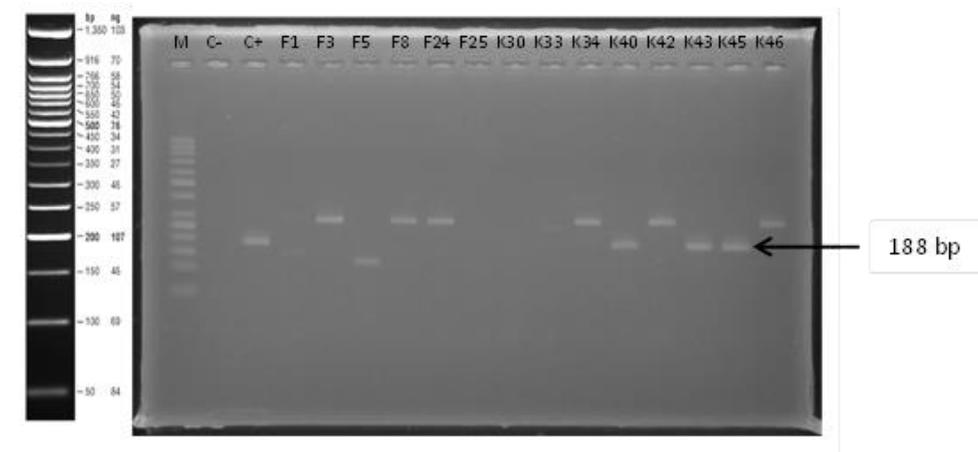


Fig. 1. PCR detection of icaA from *S. epidermidis* isolates were shown by amplicon of 188 bp; Lane 1 (M) = Marker (Marker gene ruler 50 bp DNA ladder (INTRON Sizer 50 plus DNA marker), Lane 2 (C -) = negative control, Lane 3 (C+) = icaA from control isolate (*S. epidermidis* ATCC 35984), Lane 4-14 = sample number.

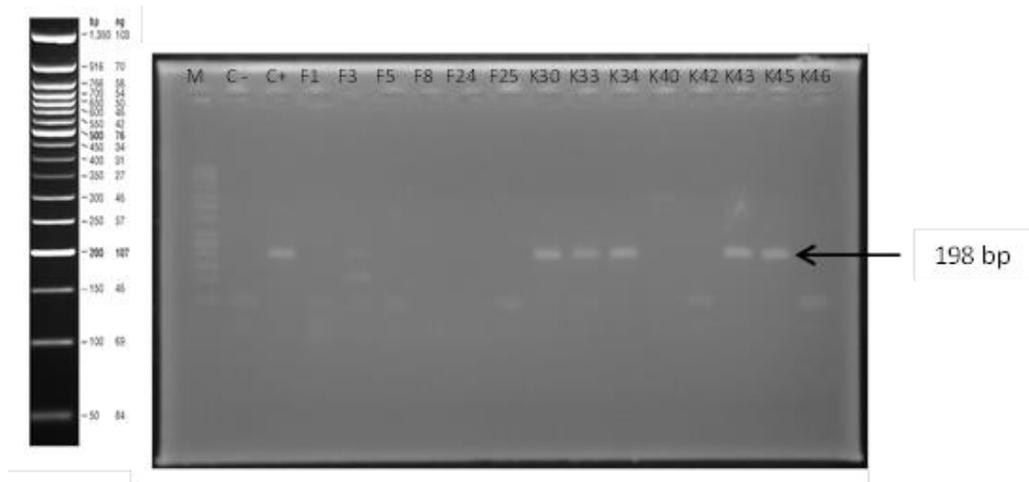


Fig. 2. PCR detection of icaD from *S. epidermidis* isolates were shown by amplicon of 198 bp; Lane 1 (M) = Marker (Marker gene ruler 50 bp DNA ladder (INTRON Sizer 50 plus DNA marker), Lane 2 (C-) = negative control, Lane 3 (C+) = icaA from control isolate (*S. epidermidis* ATCC 35984), Lane 4-14 = sample number.

Table 1. icaA and icaD genes detection results from *S. epidermidis* isolates

<i>icaD</i>	<i>icaA</i>		Positive		Negative		Total	
	Positive	Negative	Count	Percentage	Count	Percentage	Count	Percentage
Positive	3	5	8	20%	5	12.5%	13	32.5%
Negative	2	30	32	80%	30	75%	62	157.5%
Total	5	35	40	100%	35	87.5%	75	187.5%



Fig. 3. Biofilm forming examination results with CRA method. Black and brown colonies indicating biofilm producer strains, while red and dark red colonies indicating non biofilm producer strains.



Fig. 4. Colony colors on CRA with spot inoculation: A. Dark red; B. Red; C. Brown; D. Black.

One hundred percent of *icaD* positive isolates formed biofilm, while 37.5% of *icaD* negative isolates formed biofilm too and 62.5% of them were not formed biofilm. Biofilm forming examination results by Congo red agar method were shown 50% of *S. epidermidis* isolates were biofilm producer.

DISCUSSION

Genetic and molecular basics of biofilm formation *S. epidermidis* quite variable. Biofilm in *S. epidermidis* can be formed by means of several mechanism, but biofilm formation which were mediated by polysaccharide adhesin (PSA) capsule and polysaccharide intracellular adhesin (PIA) or polymeric N-acetyl-glucosamine (PNAG) production were one of the most important and now understood as one of the basic mechanisms of biofilm formation associated infection (Beenken et al 2003, Stanley & Lazizzera 2004, Yazdani et al 2006, O'Gara 2007). PIA synthesis involves complex processes and is influenced by various factors, such as the constitutional factors of

microbes, environmental factors and the host clinical situation (Agarwal & Jain 2011).

This study showed that 40 *S. epidermidis* isolates were examined, 5 (12.5%) were *icaA* positive gene and 8 (20%) were *icaD* positive gene, while 3 (7.7%) with both of *icaA* and *icaD* positive genes. This data are somewhat different with Pinheiro et al (2014) which showed the presence one of *ica* gene were detected in 89.7% isolates, the complete of *ica* genes were detected in 38,3% isolates, while 43.9% isolates were harbored *icaA* and *icaD* genes. In this study, the isolates were not all of them originated from clinical isolates that cause infection, then *ica* gene were not frequent.

Zhou et al. study (2013) revealed though *icaA* and *icaD* genes often overlap and cotranscription. The *icaD* has a higher positive detection than *icaA*, *icaB* and *icaC*. There were possible occurred because lose and mutated of these genes. This study demonstrated *icaD* positive gene more frequent (8 isolates) than *icaA* positive gene (5 isolates).

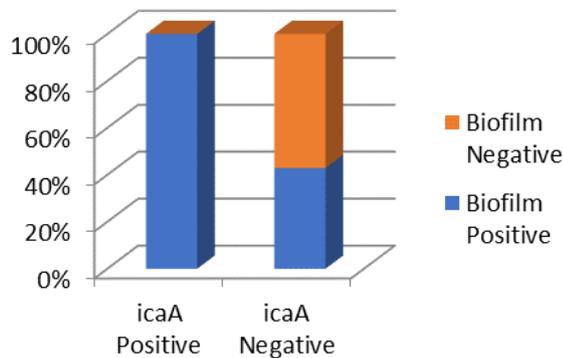


Fig. 5 The Percentage of icaA gene related biofilm forming capacity on CRA.

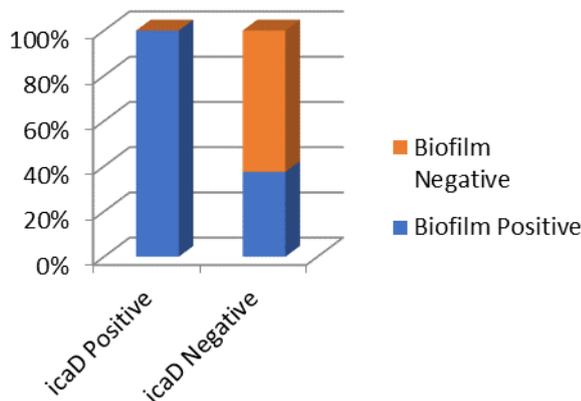


Fig. 6. The Percentage of icaD gene related biofilm forming capacity on CRA.

Biofilm which were detected through CRA method in this study were 20 isolates (50%). This results are similar with Satorres dan Alcaraz study (2007) that showed 41.3% of staphylococci were biofilm producer. Nasr et al study (2012) demonstrated biofilm forming examination with CRA method, and showed that 46% of staphylococcus isolates were detected as biofilm producer. Foka et al (2006) and Arslan et al (2007) in their study revealed in which the percentage of slime-producing strains of *S. epidermidis* ranged from 31% to 89%.

All of the isolates which icaA gene conceived have biofilm forming capacity on the CRA (1 isolate formed brown colony and 4 isolates formed black colonies), as well as the icaD gene positive isolates, all over also have biofilm forming capacity (2 isolates formed brown colonies and 6 colonies formed black colonies), while ica negative strains have variable colonies, there were red, dark red, brown and black.

The results of this study are similar with Los et al study (2010) revealed that most of ica-positive *S. epidermidis* showed biofilm forming capacity with CRA method. Similar data were demonstrated by several previous studies (Arciola et al 2002, El-Mahallawy et al 2009, Mariana et al 2009, Zhou et al 2013, Kaiser et al 2013). The results of these studies have shown the presence of ica gene was strong related with biofilm phenotype.

In this study, 15 of 35 (42.9%) isolates without icaA and 12 of 32 (37.5%) isolates without icaD can be formed biofilm on the CRA. Similar with Duggirala et al. study (2007) which that strain showed biofilm formation by CRA, some of them did not have ica genes. Nasr et al (2012) also demonstrated that 36% icaAD negative strain, capable to produce biofilm on the CRA, while Arciola et al (2006) found this phenomenon in 16% *S. epidermidis* isolate.

Oliviera and Cunha (2010) in their study revealed there are biofilm production in ica negative strain. This results indicated biofilm formation were complex network of several factors and ica genes not just the one of regulator that biofilm formation. Meaning even though does not have ica gene, this bacteria still form biofilm through other mechanism. Beside ica genes independent mechanism, there are others mechanism which are possible that owned by *S. epidermidis* to form of biofilm on the CRA.

Mechanism that involved in regulation of locus ica transcription only part of it has been understood and still other different regulated mechanism which is known (Arciola et al 2012). Staphylococcus produce exopolysaccharide Polysaccharide Intercellular Adhesin (PIA) or Polymeric N-Acetyl-Glucosamine (PNAG) is synthesized and excreted by protein are encoded icaADBC genes, but in vitro there are ica independent biofilm production (O'gara et al 2007).

In a study have proved PNAG/PIA not always absolutely necessary for *S. epidermidis* biofilm formation, and its may occur in biofilms formations with lack of ica and ica-negative genes are isolated from biofilm related infections. In many strain, biofilm formation can be mediated by specific surface proteins, called by Bap/Bhp (Biofilm associated protein) and Aap (Accumulation associated protein) and a number of Extracellular Teichoic Acids (ECTA), all of which are involved in the ica-independent biofilm formation mechanism, which is also under the control of the sarA regulator gene (Ziebuhr 2006, Otto 2008).

Other mechanism of ica-independent biofilm formation were demonstrated by O'Gara (2007), that atl gene have

an important role in initially adherence stage of biofilm developing. The alternative mechanism of biofilm synthesis relies on the ability of *S. epidermidis* to express a variety of adhesin proteins that allow cells to attach and colonize a number of different surfaces. These bacteria can also form biofilm using proteins (eDNAs) even though they do not produce polysaccharides.

Statistical analysis in this study indicate that there is a relationship between the presence of icaA and icaD genes with the biofilm forming capacity. The icaD gene (coefficient association of $\phi = 0.5$) has a stronger relationship when compared with the icaA gene (coefficient association of $\phi = 0.37$) to the biofilm formation on CRA. Based on statistical analysis, when icaA and icaD coincide in one bacteria, the strength of its relationship with biofilm forming capacity is increasing (coefficient association of $\phi = 0.577$), this corresponds to a study by McCann et al (2008) and Otto (2008) who stated that the simultaneous expression of icaA and icaD genes were shown to increase N-acetylglucosamine transferase. It is likely that the icaA protein requires icaD to translate an active conformation (Mack et al 1999, Otto, 2008).

PIA is synthesized from UDP-N-acetylglucosamine by N-acetylglucosaminyltransferase, which is encoded by ica loci, especially icaA. The single expression by this gene induces a low enzymatic activity resulting in the production of polysaccharide as well. The simultaneous expression of icaA and icaD would significantly increase N-acetylglucosaminyltransferase, and consequently an increase in the number of polysaccharide, forming a 10-20 β -1.6-Nacetylglucosamine oligomeric residue (Dobinski et al 2002, Gotz 2002).

Research of Zhou et al (2013) showed similar results with this study, that there is a stronger relationship between the presence of both the icaA and icaD genes and biofilms forming capacity compared to single expression icaA or icaD alone. Cafiso (2004) suggests that the overall existence of genes is not necessarily related to biofilm production, but there is an indication that there appears to be a correlation that can occur when at least two genes (icaAD) undergo co-transcription.

In contrast to the ica negative bacteria but its able to form biofilms that were found in some isolates in this study, there is sometimes a phenomenon in which ica is present, but the biofilm phenotype is negative, as described by Cafiso (2004) in a study that found that although the icaA or icaD genes was found *S. epidermidis*, nor can biofilms form. The reason for not producing biofilms in some isolates is that although

there are all ica genes, the icaC gene being considered necessary for PIA elongation is lack of transcription, although this is unclear.

The product of the icaR gene, together with changes in environmental conditions, may affect the expression of the ica genes (Cafiso 2004). Although staphylococci indicates the presence of ica genes, the expression of ica and biofilm production are strictly dependent on environmental conditions associated with gene expression involved adhesion process (Rachid et al 2000, Toledo Arana et al 2005, O'Gara 2007). Some environmental signals such as CO₂, O₂, iron limitations, antibiotic exposure, anaerobic conditions, ethanol, glucose levels, and osmotic stress (high osmolarity) may alter the regulation of ica expression and biofilm formation (Rachid et al 2000, Yazdani et al 2006, Otto 2008). Other factors affecting biofilm formation vary widely, such as humidity, temperature, pH of the environment, weather conditions, and chemical composition of the substrate nutrition (Kaali et al 2011).

The presence of the icaA and icaD genes are not always correlated with biofilm formation in vitro. The forming capacity in some isolates are also shared by strains lacking the icaA and icaD genes, so further investigation of genetic and biofilm-forming mechanisms independent of ica is still needed (Nasr et al 2012).

S. epidermidis has the potential ability to form biofilms with either ica-dependent and/or ica-independent mechanisms (Los 2010). Early detection and handling of biofilm formation of staphylococci are important step for the prevention and treatment of device-associated nosocomial infections, thus requiring simple phenotypic evaluation for the detection of biofilm producers (Jain & Agarwal 2009).

Moretro et al (2003) suggests that it is more appropriate to use biofilm formation methods and whether present or without locus ica is one of the criteria for determining potentially virulent strains, since biofilm formation on inert surfaces is highly sensitive to environmental and nutritional conditions, such as the presence of ethanol, iron, various concentrations of glucose and NaCl.

The combination of phenotypic and genotypic methods allow screening of strains that are potentially able to express the genes of biofilm formation of the Staphylococcus. Further study of regulatory mechanisms of biofilm formation by Staphylococcus in environmental processes, especially the genetic variation between strains is still needed (Ferreira et al 2014).

CONCLUSION

icaA and icaD genes have a statistically significant correlation with regards to biofilm formation in *S. epidermidis*. There are others biofilm formation mechanism that ica independent

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