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Full Length Research Paper

A simple and rapid differentiation method for combating therapeutically challenging planktonic and biofilm-producing *Staphylococcus aureus*

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The aim of the current study is to establish a simple and fast method that can be easily applied in hospitals for the differentiation of biofilm and planktonic *Staphylococcus aureus* strains. To achieve the objective, two reference strains, methicillin resistant *S. aureus* (MRSA) and methicillin sensitive *S. aureus* (MSSA) were examined using three types of methods; adhesion capacity of bacteria on polystyrene surfaces was verified using the quantification method followed by scanning electron microscope (SEM) in order to confirm the production of biofilm. In addition, to make a meaningful comparison of biofilm cells in stationary phase with a planktonic phase, the minimum inhibitory concentration (MIC) method was used. The results from this study demonstrated that the cells of the biofilm and planktonic cells can be differentiated by the simple MIC method. This study led to the development and improvement of methods for differentiation and detection of biofilm production of *S. aureus.*

Key words: Biofilm, methicillin sensitive *Staphylococcus aureus* and methicillin resistant *S. aureus*, microtiter plate, scanning electron microscope, minimum inhibitory concentration, vancomycin.

INTRODUCTION

Historically, *Staphylococcus aureus* has been recognized as an important cause of disease around the world and it has become a major pathogen associated with both hospital- and community-acquired infections (Ozerdem et al., 2003; Beenken et al., 2004). Infections due to multiple drug resistant strains which include methicillin resistant *S. aureus* (MRSA) are increasing and becoming more critical due to their capacity to produce slime (Archibald and Gaynes, 1997). This slime is made up of bacteria in stationary phase, hydrophilic polysaccharides (glycocalyx) and minerals such as calcium which are essential to the structural integrity of the biofilm (Akiyama et al., 2002). It is advantageous for bacteria to form stable communities of protection rather than live as free planktonic cells (Yarwood et al., 2004). Most serious infections such as endocarditis, osteomyelitis and catheter-related infections are caused by biofilm-producing strains (Donlan, 2000; Gotz, 2002). Once these bacteria colonize patients, damaged tissue and the integument surrounding indwelling devices becomes an ideal target site for bacterial access, development of biofilm and ensuing infection (Donlan, 2001). For many patients who develop catheter-related bloodstream infections (CRBSI), the device must be removed, thus,

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causing loss of vascular access, additional invasive procedures and added health care cost (O'Grady et al., 2002). One of the biggest challenges in health care and specified goal of the Centers of Diseases and Control is the prevention of CRBSI (Dvorchik, 2004). Once staphylococcal biofilms form, they are difficult to eradicate and treated because they render the anti-microbial therapy and the immune response ineffective in controlling infections, compared to log phase planktonic cells which occur in the form of single free cells (Spoering and Lewis, 2001). Differentiating the planktonic from the biofilm-production may reduce drug use and its dosage which not only control the emergence of resistant strains, but also reduce side effects of drugs taken in high doses.

Evaluation in literature on locks solutions antibiotics is a major concern in biofilm studies since the currently available Kirby-Bauer method does not differentiate the biofilm from planktoniccells. Furthermore, some investigators did not make note of the biofilm producing capabilities of the clinical isolates tested. Therefore, the purpose of this study was to establish a simple and rapid method that can be easily used in hospitals for the differentiation of biofilm and planktonic strains of MRSA. To achieve the goal, the ability of bacterial adherence on polystyrene surfaces was examined using the method of quantification followed by scanning electron microscope (SEM) for the purpose of confirming biofilm production, and the effectiveness of vancomycin in the prevention of biofilm and planktonic free cell formation was evaluated.

MATERIALS AND METHODS

Collection of samples

The present study involved 2 reference strains ATCC35556 and ATCC700698 for MSSA and MRSA positive for biofilm production, respectively. These strains were obtained from the Department of Medical Microbiology and Parasitology, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, Malaysia.

Microtiter plate method

Biofilm formation was assessed quantitatively in microtiter plates as previously described by Stepanovic et al. (2007) with some modifications. Briefly, MRSA and MSSA reference strains were grown in 6-well polystyrene tissue culture plates supplemented with Trypticase Soy Broth containing 1% glucose at 37°C for 48 h under aerobic conditions. After incubation, the plate was washed ten times by submerging in deionized water. The remaining attached bacteria were fixed with 2 ml of absolute methanol per well for 20 min before plates were emptied and left to dry overnight. The biofilm were stained with 1 ml of 0.1 Safranine for 15 min. Excess stain was rinsed off and the plates were washed three times with distilled water. After the plates were air-dried overnight, the dye bound to the adherent cells was dissolved with 95% ethanol per well and absorbance was measured at 490 nm (A490). The experiment was repeated three times. The absorbance (A490) of wells with sterile TSB was used as the negative control. A490 of 0.8 was taken as the cutoff point to differentiate between the biofilm formations. Biofilm formation for each strain and positive control were analyzed and categorized based on the absorbance of the safranine stained biofilm. Strains were considered as adherent (A490 >0.8) and non-adherent (A490 ≤0.8) biofilm formers on the basis of their absorbance property. Any OD value above the cut-off OD was considered as positive for biofilm formation.

SEM method

Biofilm formation was further confirmed by electron microscopy to distinguish planktonic free cells from biofilm. For this, bacteria were grown in six well tissue culture plates on glass coverslips in TSB containing 1% glucose at 37°C for 48 h. For free planktonic cells, the bacterial cells were grown in TSB containing 1% glucose at 37°C for 1h and then fixed onto the glass coverslip surfaces. Both samples were prepared for the scanning electron microscope using the conventional method described by Ganderton et al. (1992) in which the samples were fixed in 4% buffered glutaraldehyde for 5 h at 4°C and then rinsed three times with 0.1 M sodium cacodylate buffer and fixed with 0.1 M osmium tetroxide for 1 h at 4°C. The critical dehydration was performed with 35 to 100% ethanol in double distilled water and the samples were dried in a critical point dryer and coated with a gold layer. Three different points were examined by SEM at proper magnification. The biofilm producers were seen forming a close network layer among the cells, however no such structure was observed in the negative biofilm producers.

Minimum inhibitory concentration (MIC) method

In order to make a meaningful comparison of biofilm stationary phase cells with planktonic phase cells, one MRSA and one MSSA grown at stationary phase (biofilm producing phase) and planktonic phase were exposed to a serial dilution of vancomycin. Biofilm producers were essentially exposed to fresh serial dilution of vancomycin with concentrations starting from 0.125 to 64 µg/ml for 24 h, then incubated for another 24 h. Planktonic phase cells were treated with the same fresh serial dilution of vancomycin at zero time of incubation and then incubated for 24 h. Results for the microtiter plate assay were interpreted according to the manufacturer's instructions as earlier described.

RESULTS

In the highest biofilm producers (Figure 1), we show that the thick confluent layer of bacteria along the bottom of the microtiter plate well is related to biofilm accumulation of biomass (Figure 2). Use of scanning electron microscopy noted clearly visible glycoprotein network layer in both MRSA and MSSA (Figures 3 and 4) compared to the free planktonic cells in which non-released glycoprotein layer and biofilm matrix (Figure 5). For further investigation, the diluted concentrations of vancomycin from 0.125 to 64 µg/ml showed growth inhibition of the planktonic phase at 1 µg/ml (MIC), while bactericidal concentration was at 2 µg/ml (MBC) upon overnight incubation at 37ºC. In contrast to inhibitory and bactericidal activity on biofilm production that were observed at 16 µg/ml of minimum biofilm inhibition concentration (MBIC) and 32 µg/ml of minimum biofilm eradication concentration (MBEC) as shown in Figure 6.

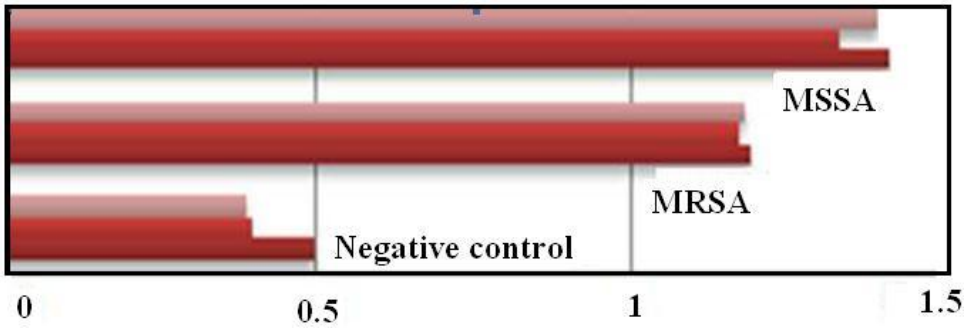

Figure 1. The average OD490 of three independent experiments on adherent-forming ability to the polystyrene surface of 6-well cell culture plates by MSSA and MRSA reference strains. 0.8 was taken as the cutoff point; any OD value above the cut-off OD was considered as positive for biofilm formation production.

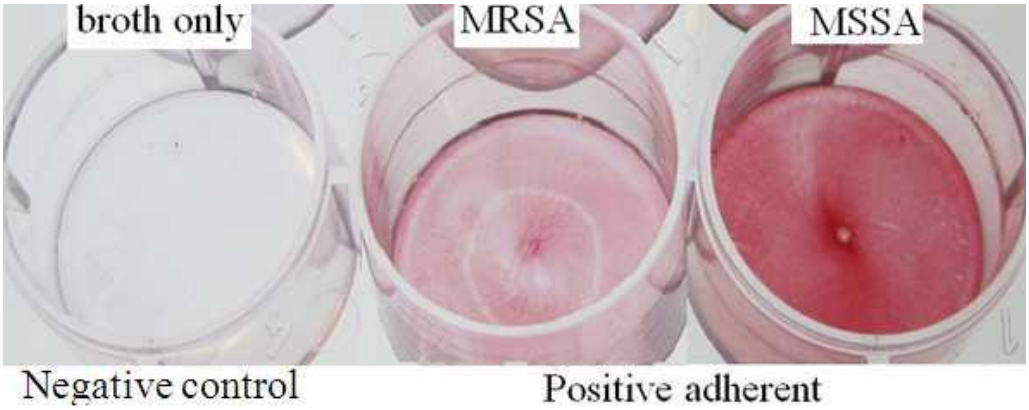

Figure 2. The quantification of MSSA and MRSA biofilm producing reference strains corresponding to 48 h in static 6-well tissue culture plate assay after staining with 0.1% safranin.

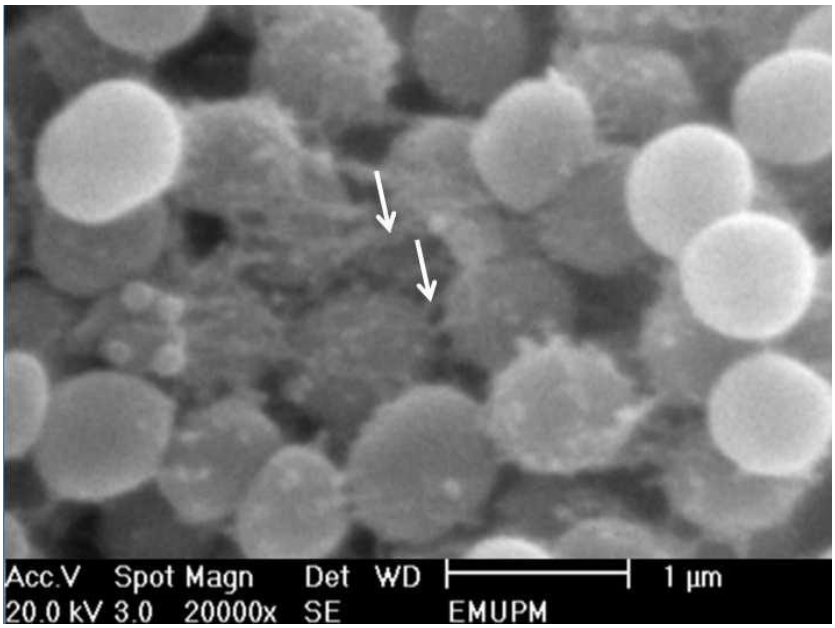

Figure 3. Scanning electron micrographs reveal biofilm producers by MSSA forming a close network layer among the cells.

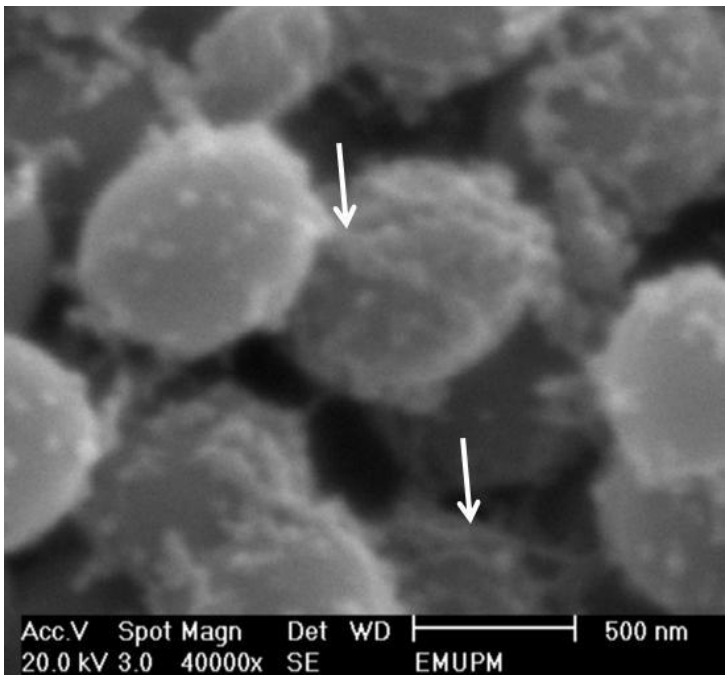

Figure 4. Scanning electron micrographs reveal biofilm producers by MRSA forming a close network layer among the cells.

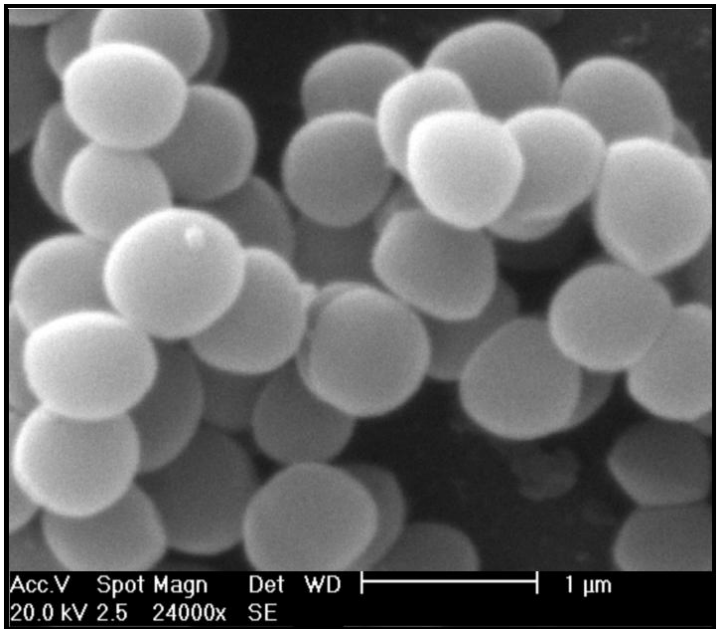

Figure 5. Scanning electron microscope showing no presence of the network layer by free planktonic cells of MRSA and MSSA.

DISCUSSION

Anti-staphylococcal agents such as linezolid has been evaluated in many *in vitro* biofilm models (Curtin et al., 2003; El-Azizi et al., 2005; Wiederhold et al., 2005). This agent, like others do not demonstrate significant activity in established biofilms due to either a lack of penetration, drug inactivation or due to the state of bacterial cell division within the biofilm (Donlan, 2000; Ceri et al., 1999; Oie et al., 1996). Promising agents must penetrate into the biofilm extracellular layer and have bactericidal activity irrespective of the bacteria's physiological state.

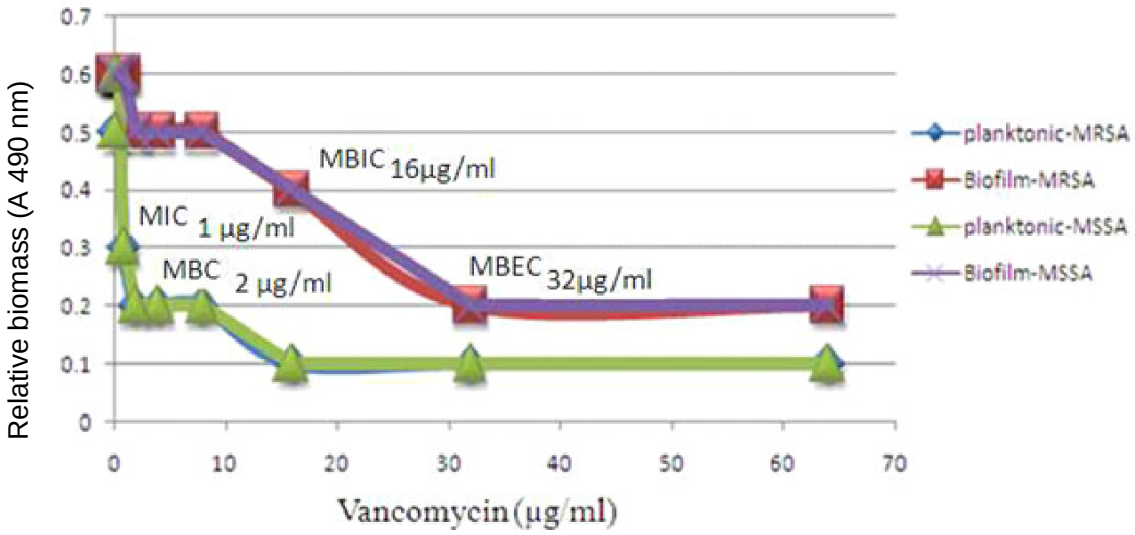

Figure 6. Graph shows effect of vancomycin serial dilution on the prevention of planktonic and biofilm formation by MRSA and MSSA grown in 96-well polystyrene plates exposed to increasing concentrations of vancomycin.

They must also prevent further biofilm formation. The problem involving the issue of biofilm identification has been found since the availability of the phenotypic approach of growth on highly selective or differential medium. Congo red agar (CRA) developed by Freeman et al. (1989) has long been known as a highly selective media for evaluation of slime production but several researchers noticed that use of the published CRA by Freeman did not seem reasonable as a means of screening clinical *S. aureus* isolates for biofilm production because contradictory results were obtained between phenotypic and genotypic approaches. Thus, the reason why Mariana et al. (2009) modified Freeman-CRA, whereby the modification of agar constituents allowed stability in complementing between the phenotypic and genotypic characterization, but not between the phenotypic and quantification method. The microtiter plate method remains among the most frequently used assays for investigating quantity of biofilm produced spectrophotometrically, but it is not known whether the bacteria in aggregated conditions coincide with the biofilm formation. With such shortcomings, our study was conducted to establish and improve methods for the identification and detection of biofilm producing *S. aureus* to combat therapeutically challenging planktonic and biofilm *S. aureus.* In the highest biofilm producers, we showed that formation of adherent cells occur first before converging to form a thick confluent layer of bacteria along the bottom of the microtiter plate. This is important for biofilm biomass accumulation, thus, increased OD490 values were associated with an incremental ability to form bacterial aggregates following initial attachment to the plastic surface (Figures 1 and 2).

Examination of biofilm layer images revealed that early biofilm formation as determined by OD490 stating intensity coincided with the formation of bacterial aggregates suggesting that the formation of bacterial aggregates is a critical event during early biofilm formation. As noted in *S. aureus* ATCC 35556 and ATCC 700986, bacterial aggregates were clearly visible within the glycoprotein network layer as part of the biofilm matrix by using SEM (Figures 3 and 4) compared to free planktonic cells where no layer was produced as shown in Figure 5. Further investigation showed the ability of vancomycin to inhibit growth of the planktonic phase cell at 1 µg/ml for both the methicillin sensitive and methicillin resistant *S. aureus* strains. Growth of the planktonic phase was eradicated at 2 µg/ml in contrast to inhibition and eradication of biofilm production growth in both reference strains at 16 µg/ml MBIC and 32 µg/ml MBEC, respectively as shown in Figure 6. During this study of biofilm producers, both the strains were found to be sensitive to vancomycin according to the Kirby Bauer susceptibility testing. It was also observed that the level of minimum inhibition concentration was inversely proportional to the level of biofilm production in high concentration of antibiotic. Our study was in agreement with Spoering and Lewis (2001) who observed that when the same bacterial cells were checked for antibiotic sensitivity (after liberating them from biofilm), the cells showed the same sensitivity as of planktonic bacteria indicating that biofilm producers themselves are not antibiotic resistant but their resistance against antibiotics can be due to several factors such as slow growth, presence of penetration barriers or maybe presence of some enzymes. In conclusion, this study has led to the development and improvement of methods for the differentiation and detection of biofilm producing *S.*

aureus for combating therapeutically challenging planktonic and biofilm-producing *S. aureus*.

The establishment of methods highly associated to biofilm formation can lead to further studies in developing such methods towards obtaining a predictive value potential to predict aggressiveness of biofilm producers. The ability to predict will allow for improved patient management through accurate and rapid determination of clinical biofilm isolates.

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