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BioTimer Assay, a new method for counting *Staphylococcus* spp. in biofilm without sample manipulation applied to evaluate antibiotic susceptibility of biofilm

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ABSTRACT

The medical device-related infections are frequently a consequence of *Staphylococcus* biofilm, a lifestyle enhancing bacterial resistance to antibiotics. Antibiotic susceptibility tests are usually performed on planktonic forms of clinical isolates. Some methods have been developed to perform antibiotic susceptibility tests on biofilm. However, none of them counts bacterial inoculum. As antibiotic susceptibility is related to bacterial inoculum, the test results could be mistaken.

Here, a new method, BioTimer Assay (BTA), able to count bacteria in biofilm without any manipulation of samples, is presented. Moreover, the BTA method is applied to analyze antibiotic susceptibility of six *Staphylococcus* strains in biofilm and to determine the number of viable bacteria in the presence of sub-inhibitory doses of four different antibiotics. To validate BTA, the new method was compared to reference methods both for counting and antibiotic susceptibility tests. A high agreement between BTA and reference methods is found on planktonic forms. Therefore, BTA was employed to count bacteria in biofilm and to analyze biofilm antibiotic susceptibility. Results confirm the high resistance to antibiotics of *Staphylococcus* biofilm. Moreover, BTA counts the number of viable bacteria in the presence of sub-inhibitory doses of antibiotics. The results show that the number of viable bacteria depends on sub-inhibitory doses, age of biofilm and type of antibiotic. In particular, differently to gentamicin and ampicillin, sub-inhibitory doses of ofloxacin and azithromycin reduce the number of viable bacteria at lower extent in young than in old biofilm. In conclusion, BTA is a reliable, rapid, easy-to-perform, and versatile method, and it can be considered a useful tool to analyze antibiotic susceptibility of *Staphylococcus* spp. in biofilm.

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1. Introduction

Biofilm is a bacterial lifestyle widespread in microbial world and is a major concern in health care. Particularly relevant are the medical device-related infections caused by *Staphylococcus aureus* and *S. epidermidis* grown in biofilm (Bestul and Vandenbussche, 2005). In particular, biofilm catheter-related infections represent a great challenge for medicine as they are the most common device-related infections and represent the initial stage for catheter-related blood-stream infections that are life-threatening (Falagas et al., 2007).

Usually, antibiotic treatment of catheter-related and catheter-related bloodstream infections is based on antibiotic susceptibility tests performed on planktonic counterpart of the clinical isolates instead of on biofilm. It is well known that microorganisms organized in biofilm exhibit higher levels of antibiotic resistance than in planktonic form, so that a great part of therapeutic regimens based on susceptibility of planktonic forms fails to eradicate biofilm infections (Carratalà, 2002; Pascual et al., 1993). Therefore, it is imperative to set up a reliable method to detect antibiotic susceptibility of clinical isolated bacteria in biofilm, rather than in planktonic lifestyle.

Now, few methods are available to determine microbial antibiotic susceptibility of bacteria in biofilm (Clutterbuck et al., 2007; Moskowitz et al., 2004; Olson et al., 2002; Pettit et al., 2005). The Calgary Biofilm Device is the most popular method (Ceri et al., 1999) and determines the minimal biofilm eradication concentration (MBEC) as the concentration of antibiotic killing 100% of bacteria in biofilm. Unfortunately, none of these methods detects the actual number of bacteria in biofilm used as inoculum in MBEC tests. As inoculum size influences the results of susceptibility tests (Egervarn et al., 2007), MBEC values determined using the above mentioned methods, could be mistaken.

The enumeration of bacteria in biofilm is still a great challenge for microbiologist. As the standardized enumeration of bacterial population based on colony forming unit (CFU) method is not satisfactory to

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quantify bacteria in biofilm (Berlutti et al., 2003), several attempts have been carried out to enumerate bacteria in biofilm. Some methods suggest to detach bacteria by vortex or sonication and successively to count detached bacteria by CFU technique (Ceri et al., 1999; Sandoe et al., 2006). These procedures present disadvantages as they may not detach all bacteria or may alter bacterial viability leading to erroneous counts. Other methods count bacteria in biofilm through indirect assays based, for example, on ATP detection in bacteria (Chen and Godwin, 2006), or on the visible blue-to-red transformations of achromatic polymer induced by molecules released by multiplying bacteria (Silbert et al., 2006), or a colorimetric assay capable to count the number of adherent bacteria on abiotic surface. This last assay was employed to evaluate the adhesion ability of different bacteria to HEMA-based polymers without manipulation of samples (Berlutti et al., 2003).

Here, we describe a novel method named BioTimer Assay (BTA) that allows easily to count *Staphylococcus* in biofilm without any manipulation of samples. The new method, based on a previously described one (Berlutti et al., 2003), is applied for the evaluation of antibiotic susceptibility of *Staphylococcus* biofilm and for the contemporaneous enumeration of viable bacteria after exposure to sub-inhibitory doses of antibiotics.

2. Materials and methods

2.1. Strains

S. aureus ATCC6538, *S. aureus* 537 and *S. epidermidis* 1A were from bacterial collection of Department of Public Health Sciences, University Sapienza of Rome, and three clinical isolates: *S. aureus* (SAU1) from central venous catheter (CVC), *S. epidermidis* (SEP1) from abdominal catheter, and *S. epidermidis* (SEP2) from CVC. *Staphylococcus* clinical isolates were from patients with catheter-related infections admitted at Paediaric Hospital Bambino Gesù (Rome, Italy). Identification and antibiotic susceptibility of clinical isolates were performed using Vitek 2 (BioMerieux Italia s.p.a., Italy) apparatus. Strains were maintained in Trypticase soy broth (Difco Laboratories, MD) with glycerol (25%) at −80 °C and checked for purity on Columbia CNA agar (Difco Laboratories, MD, USA) with 5% red sheep cells before use.

2.2. BioTimer Assay

BioTimer Assay (BTA) is an improvement of a previously described method (Berlutti et al., 2003). BTA employs BioTimer medium with phenol red (BT-PR medium) prepared as follows: Mueller Hinton broth (MH) (OXOID Ltd., UK) 21 g, glucose 10 g, phenol red (Sigma Aldrich, Italy) 25 mg, and distilled water to 1000 ml. After sterilization at 121 °C for 15 min, pH was checked and adjusted at 7.2±0.1. The final medium appeared clear and red. BTA measures microbial metabolism: the time required for colour switch of phenol red indicator in BT-PR medium (red-to-yellow) (Fig. 1), due to *Staphylococcus* metabolism, is correlated to initial bacterial concentration. Therefore, the time required for colour switch determines the number of bacteria present in a sample at Time 0 through a correlation line. To draw the correlation line specific for *Staphylococcus* spp., 0.2 ml of MH-overnight broth cultures were mixed with 1.8 ml of BT-PR medium. Serial two-fold dilutions in 1 ml of BT-PR medium were performed in 24-well plates (BD, Italy) and simultaneously counted using colony forming unit (CFU) method. Incubation was performed at 37 °C without shaking. The colour of the inoculated BT-PR medium was checked at regular time intervals. For each two-fold dilution, the time required for colour switch of BT-PR medium was recorded and plotted versus the $\log_{10}$ of CFUs.

2.3. Count of bacteria in biofilm expressed as planktonic-equivalent colony forming units (PE-CFUs)

To grow *Staphylococcus* in biofilm, the strains were incubated at 37 °C in Hearth Infusion (HI) (Oxoid) broth without agitation in the presence of sterile glass beads (diameter 5 mm). After incubation, colonized glass beads were washed three times in sterile saline solution and used to inoculate 1 ml of BT-PR medium. The inoculated BT-PR media were incubated at 37 °C and checked for colour switch. The time required for colour switch was used to determine the number of bacteria in biofilm on the glass beads using the correlation line. As the correlation line links the time for colour switch of BT-PR medium and the CFUs of planktonic bacteria, the number of *Staphylococcus* in biofilm is defined as planktonic-equivalent CFUs (PE-CFUs).

2.4. Biofilm detection

To evaluate biofilm development, *Staphylococcus* strains were cultured in the presence of glass beads as above described. After incubation, colonized glass beads were washed three times in sterile saline and biofilm was quantified (Berlutti et al., 2005; Peeters et al., 2008). Briefly, colonized glass beads were soaked in aqueous solution of crystal violet (1% w/v) and after 20 min of incubation at room temperature the glass beads were thoroughly washed to remove the colour excess and then air-dried. Biofilm was quantified by eluting crystal violet with a mixture of ethanol and acetone (80:20 v/v) and by determining the absorbance of the eluted dye at 570 nm using a DU70 spectrophotometer (Beckman, Milan, Italy).

2.5. Antibiotic susceptibility tests

Pure gentamicin, ofloxacin, azithromycin, and ampicillin (Sigma Aldrich, Italy) were used for antibiotic susceptibility tests. Final drug concentrations ranged from 1000 to 0.03 μg/ml. Antibiotic susceptibility tests were performed by broth dilution method in 24-well plates. Each well contained 1 ml of appropriate antibiotic dilution.

2.6. Planktonic form

Antibiotic susceptibility tests on planktonic forms were performed as broth dilution method both in MH broth following Clinical and Laboratory Standards Institute (CLSI) guidelines for susceptibility testing (CLSI, 2006) (reference method), and in BT-PR medium broth following BTA method. A total of $5\pm0.5*10^5$ CFUs/ml from HI-

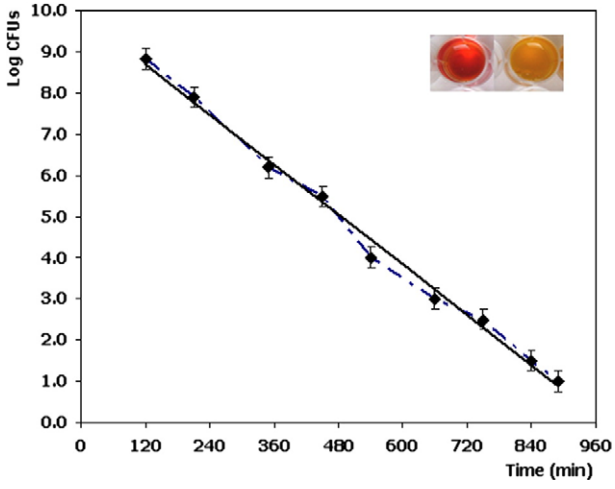

Fig. 1. Correlation line linking the time for colour switch of BioTimer Medium (BT-PR medium) and the number of planktonic *Staphylococcus*. Serial two-fold dilutions of overnight broth culture of *Staphylococcus aureus* ATCC6538 were prepared in 1-ml volumes of BT-PR medium and simultaneously counted using colony forming unit (CFU) method. For each dilution in BT-PR medium, the time required for colour switch (red-to-yellow) of the medium was plotted versus the $\log_{10}$ of CFUs. Linear regression analysis was used to calculate the equation describing the correlation line that relates time for colour switch/number of bacteria (for details see Materials and methods). In the insert, the red-to-yellow colour switch of BT-PR medium is depicted.