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A modified microtiter-plate test for quantification of staphylococcal biofilm formation

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Abstract

The tube test and the microtiter-plate test are the most frequently used techniques for quantifying biofilm formation, an important indicator for the pathogenicity of staphylococci. The purpose of the present study was to develop a modified microtiter-plate technique for quantification of biofilm formation. This technique involves fixing the bacterial film with methanol, staining with crystal violet, releasing the bound dye with 33% glacial acetic acid, and measuring the optical density (OD) of the solution at 570 nm by using an enzyme immunosorbent assay reader. Biofilm formation of 30 *Staphylococcus* strains was estimated by the tube test, the standard microtiter-plate test and the modified microtiter-plate test. The modified microtiter-plate test, as a quantitative assay, is superior to the tube test in terms of objectivity and accuracy. It is also superior to the standard microtiter-plate test because it enables indirect measuring of bacteria attached both to the bottom and to the walls of the wells, while in the standard test only the dye bound to the bacteria adhered to the bottom of the wells is spectrophotometrically registered. Highly significant differences between OD values obtained by the standard microtiter-plate test and those obtained by the modified test suggest that large number of bacteria were attached to the walls of the wells. Therefore, the modification of the standard microtiter-plate test by introduction of an additional step of decolorization by acetic acid seems to be a useful improvement of the technique.

Keywords: Biofilm formation; Staphylococci; Microtiter-plate

1. Introduction

The rising incidence of hospital-acquired infections caused by staphylococci strongly correlates with the increasing use of implantable medical devices, primarily catheters and other plastic foreign bodies (Grosserode and Wenzel, 1991). The key step in the pathogenesis of staphylococcal foreign body-associated infections is the colonization and formation of stable biofilm on the surface of the foreign body. Colonized bacteria embedded in the amorphous extracellular material (slime) are referred to as biofilm (Heilmann et al., 1997). It has been speculated that testing for biofilm formation could be a useful marker for the pathogenicity of staphylococci, particularly coagulase-negative species (Christensen

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et al., 1985; Baddour et al., 1986; Davenport et al., 1986; Younger et al., 1987; Mulder and Degener, 1998). However, some authors found little or no correlation between biofilm formation in vitro and the clinical outcome of the infection (Kotilainen, 1990; Perdreau-Remington et al., 1998).

Biofilm formation may be determined in several different ways, but most frequently it is demonstrated by the tube test (Christensen et al., 1982, 1985), in which the bacterial film lining a culture tube is stained with a cationic dye and visually scaled, or by the microtiter-plate test (Christensen et al., 1985), in which the optical density (OD) of the stained bacterial film is determined spectrophotometrically. The main drawback of the former is its qualitative nature, while the latter suffers from incompleteness as it detects the biofilm formation on the bottom of the well only.

In this paper we describe the development of a simple and efficient method for quantification of biofilm formation based upon the standard microtiter-plate test (Christensen et al., 1985), and an immunological procedure used for the determination of the number of adherent cells (Takahashi et al., 1994).

2. Material and methods

2.1. Media

The media used in this study were tryptic soy broth (TSB) without glucose (Difco Laboratories, Detroit, MI, USA), tryptic soy agar (TSA) without glucose, TSB and TSA supplemented with 0.25%, 1% or 2.5% D(+)-glucose (Merck, Darmstadt, Germany). To obtain TSA, the original TSB was supplemented with 1.5% Bacto-agar (Difco Laboratories). All media were autoclaved 30 min at 116°C. Glucose was added before autoclaving.

2.2. Bacterial strains

Thirty strains of staphylococci were used in this study, as follows.

- The four reference strains: *S. aureus* ATCC 25923, *S. aureus* NCTC 8325, *S. epidermidis* ATCC 14990^T and *S. sciuri* ATCC 29062^T.
- Twenty-six clinical isolates: *S. aureus*-12 strains; *S. haemolyticus*-8 strains; *S. sciuri*-4 strains; *S. xylosus*-1, one strain; and *S. gallinarum*, one strain.

The reference strains were stored as lyophilized cultures, and the clinical strains (isolated at the Institute of Microbiology and Immunology, School of Medicine, University of Belgrade, Yugoslavia) were kept in the refrigerator on Columbia agar (CA) (Torlak, Belgrade, Yugoslavia) supplemented with 5% sheep blood for up to 4 months.

Preliminary identification of all clinical isolates was based upon Gram stain, morphological and cultural characteristics, positive catalase reaction, and resistance to bacitracin. *S. aureus* strains were identified by positive coagulase test with rabbit plasma and positive thermo-DNase test. Coagulase-negative strains were identified by Staphylococcus MIC/ID panel (Becton-Dickinson) or API Staph (bioMérieux, Marcy-l'Etoile, France).

Prior to inoculation, all strains were transferred from the stock cultures to CA and incubated aerobically at 37°C for 24 h. Then, all strains were subcultured one more time under the same conditions. Schleifer and Kroppenstedt (1990) found that the infecting bacteria are often surface-associated and that their cell surface can therefore be expected to be more similar to that of bacteria grown on a solid surface than to that found in organisms grown in liquid media. Thus, refreshed strains were transferred on TSA + 0%, TSA + 0.25%, TSA + 1% and TSA + 2.5% glucose and incubated aerobically at 37°C for 24 h.

2.3. Tube test

The qualitative assay for biofilm formation was performed according to the method described by Christensen et al. (1985), with certain modifications.

Glass tubes (13 × 100 mm) filled with 2.6 ml of TSB containing 0%, 0.25%, 1% or 2.5% glucose, were inoculated with a loopful of a pure culture of a strain from the TSA plates containing 0%, 0.25%, 1% or 2.5% glucose, respectively. A total of 0.6 ml

of inoculated broth was removed from each tube for the microtiter-plate test. Tubes containing only TSB were included in the test as negative controls.

After overnight incubation at 37°C in air, the content of each tube was carefully removed with a pipette, and 2 ml of a 0.25% safranin solution for Gram staining (bioMérieux) was immediately added. After 1 min the tubes were emptied with a pipette, and placed upside down without a wash step in between. Following overnight standing at room temperature the results of the test were read.

The test was considered positive when there was an adherent layer of stained material on the inner surface of the tube. The adherence was estimated as absent (0), weak (+), moderate (++), or strong (+++). The presence of stained material at the liquid–air interface was not considered to be indicative of biofilm formation. The test was repeated three times for each strain.

2.4. Microtiter-plate test

Three wells of a sterile 96-well flat-bottomed plastic tissue culture plate with a lid (Spektar, Čačak, Yugoslavia) were filled with 200 μl of bacterial suspension each. Negative control wells contained broth only. The plates were covered and incubated aerobically for 24 h at 37°C. Then, the content of each well was aspirated, and each well was washed three times with 250 μl of sterile physiological saline. The plates were vigorously shaken in order to remove all non-adherent bacteria. The remaining attached bacteria were fixed with 200 μl of 99% methanol (Destilacija, Teslić, Yugoslavia) per well, and after 15 min plates were emptied and left to dry. Then, plates were stained for 5 min with 0.2 ml of 2% Hucker crystal violet used for Gram staining (bioMérieux) per well. Excess stain was rinsed off by placing the plate under running tap water.

After the plates were air dried, the dye bound to the adherent cells was resolubilized with 160 μl of 33% (v/v) glacial acetic acid (Zorka Pharma, Šabac, Yugoslavia) per well. The OD of each well was measured at 570 nm by using an automated ICN Flow Titertek Multiscan Plus reader. The reading was performed two times: (i) before addition of glacial acetic acid, as in standard microtiter-plate test and (ii) after glacial acetic acid was added.

For the purposes of comparative analysis of test results, we introduced classification of adherence capabilities of tested strains into four categories. Commonly used Christensen et al. (1985) classification of the results obtained by the microtiter-plate test has only three categories. All strains were classified into the following categories: non-adherent (0), weakly (+), moderately (++), or strongly (+++) adherent, based upon the ODs of bacterial films. We defined the cut-off OD (OD_C) for the microtiter-plate test as three standard deviations above the mean OD of the negative control. Strains were classified as follows:

$OD \leq OD_C$	non-adherent
$OD_C < OD \leq 2 \times OD_C$	weakly adherent
$2 \times OD_C < OD \leq 4 \times OD_C$	moderately adherent
$4 \times OD_C < OD$	strongly adherent

All tests were carried out three times and the results were averaged.

2.5. Statistical methods

Statistical differences among the results obtained by the tube and the microtiter-plate tests with and without addition of acetic acid were examined by the nonparametric Friedman test, followed by the Wilcoxon paired test. Wilcoxon paired test was used to compare OD values obtained in the microtiter-plate tests performed with and without addition of acetic acid. P values of < 0.05 were considered significant.

3. Results and discussion

The experiments performed in our study enabled us to measure the rate of adherence and subsequent biofilm formation of tested bacteria. Summarized results of the tube and microtiter-plate tests are presented in Table 1. The tube test showed significantly different results from the results obtained by the standard microtiter-plate test. On the contrary, statistical analysis showed no significant difference between the results obtained by the tube test and results obtained by the modified microtiter-plate test with acetic acid. However, the modified microtiter-

Table 1
Overall results of the tube and microtiter-plate tests[a]

Test	Number of strains showing			
	No adherence (0)	Weak adherence (+)	Moderate adherence (++)	Strong adherence (+++)
Tube test				
TSB w/o G	0	17	9	4
TSB + 0.25% G	0	4	21	5
TSB + 1% G	0	4	17	9
TSB + 2.5% G	1	4	17	8
Standard microtiter-plate test				
TSB w/o G	24*	6*	0**	0
TSB + 0.25% G	11*	19*	0*	0***
TSB + 1% G	8*	21*	1*	0*
TSB + 2.5% G	5	25*	0*	0*
Modified microtiter-plate test				
TSB w/o G	1	17	6	6
TSB + 0.25% G	0	5	16	9
TSB + 1% G	0	2	17	11
TSB + 2.5% G	0	2	17	11

[a] G, glucose. *Statistically significant difference compared to both the tube test and the modified microtiter-plate test; **statistically significant difference compared to the tube test; ***statistically significant difference compared to the modified microtiter-plate test.

plate test, as a quantitative assay, is superior to the tube test in terms of objectivity and accuracy. The results of all three tests used in the study show that the presence of glucose significantly enhances the adherence of a majority of the strains, which is in agreement with results of other studies (Christensen et al., 1985; Deighton and Balkau, 1990; Mulder and Degener, 1998). However, this effect is largely independent of the concentration of glucose used.

According to the results of the tube test, almost all tested strains produced biofilm. The tube adherence assay is simple and easy to perform, but reading of the results may be difficult, especially if the observer is not familiar with this technique. Moreover, observers frequently disagree in the interpretation of the tube test, particularly in interpreting weak reactions (Christensen et al., 1985). However, we noted that certain modifications of the procedure might improve accuracy of interpretation of the results obtained by the tube test, e.g., after the dying process tubes should be emptied with a pipette and not by pouring. Reading of results after completely drying tubes is superior to examination immediately after staining.

According to the literature, the quantitative microtiter-plate assay eliminates subjectivity in reading of obtained results and predicts clinical relevance more reliably than the tube test (Deighton and Balkau, 1990). In the present study, however, significant disagreement between results of the tube test and results of the standard microtiter-plate test was observed. Significantly more strains were classified as nonadherent by the quantitative microtiter-plate test. Factors that may influence the adherence capability of staphylococci include different adherence mechanisms for glass and plastic surfaces, minor imperfections of glass test tubes and presence of grease (Deighton and Balkau, 1990).

In our modification of the microtiter-plate method, addition of acetic acid enables indirect measuring of bacteria attached both to the bottom and walls of the wells. Acetic acid resolubilizes dye bound to the adherent cells at the bottom of the well as well as dye bound to the cells attached to the walls of the wells. Only 160 μl of 33% (v/v) glacial acetic acid was added per well, to avoid interference with stained material at the liquid–air interface, which was not considered to be indicative of biofilm production in the tube test (Christensen et al., 1985).

Adherence determined by the microtiter-plate test

Table 2
Adherence of various *Staphylococcus* species in the modified microtiter-plate test in the presence of 0.25% glucose

Species	No. of strains tested	No. of strains showing			
		No adherence (0)	Weak adherence (+)	Moderate adherence (++)	Strong adherence (+++)
S. aureus	14	–	2	8	4
S. epidermidis	1	–	–	–	1
S. haemolyticus	8	–	2	4	2
S. sciuri	5	–	1	3	1
S. xylosus	1	–	–	1	–
S. gallinarum	1	–	–	–	1

performed with addition of acetic acid (Table 2) significantly differs from that obtained by the standard microtiter-plate test. Although the analysis of adherence capabilities of different *Staphylococcus* species is beyond the scope of this study, it is interesting to note moderate to high degree of adherence of *S. sciuri*, and one tested strain of *S. gallinarum*, a species rarely isolated from human specimens. Obtained results indicate that the modification of the standard microtiter-plate test by introduction of an additional step of decolorization by acetic acid seems to be a useful improvement of the technique and provides better correlation with the tube test.

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