

RAPID AND SIMPLE DETERMINATION OF MINIMUM BIOFILM ERADICATION CONCENTRATION BY A COLORIMETRIC MICROBIAL VIABILITY ASSAY BASED ON REDUCTION OF A WATER-TETRAZOLIUM SALT AND COMBINED EFFECT OF ANTIBIOTICS AGAINST MICROBIAL BIOFILM

Tadayuki Tsukatani^{*1}, Tomoaki Kawaguchi¹, Hikaru Suenaga¹, Masanobu Shiga² & Takashi Ikegami²

Address(es): Dr. Tadayuki Tsukatani,

¹Biotechnology and Food Research Institute, Fukuoka Industrial Technology Center, Kurume, Japan.

²Dojindo Laboratories, Kumamoto, Japan.

*Corresponding author: tukatani@fitc.pref.fukuoka.jp

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ABSTRACT

Rapid and simple method for the determination of minimum biofilm eradication concentration (MBEC) using a colorimetric microbial viability assay based on reduction of a tetrazolium salt WST-8 and the biofilm formation method on 96-pegs on the lid of a microtiter plate was developed. The biofilms formed on the 96 pegs were challenged by antibiotics, and the MBEC was then determined from the microbial viability of the biofilms formed on the 96 pegs, assessed by the WST-8 colorimetric assay. The MBECs obtained by the proposed and conventional methods favorably agreed. The most effective inhibitors of *S. aureus* and *P. aeruginosa* biofilms were vancomycin and ciprofloxacin, respectively. In addition, we clarified that *Staphylococcus aureus* biofilm was maximally suppressed by a combination of vancomycin, daptomycin, and teicoplanin. The proposed method yields similar performance to conventional methods, but is faster and more easily implemented. Therefore, the proposed method alleviates the tediousness and time-consuming nature of conventional biofilm susceptibility assay.

Keywords: Biofilm, minimum biofilm eradication concentration, susceptibility testing, antibiotics, tetrazolium salt

INTRODUCTION

Biofilms are formed when microorganisms aggregate with each other or adhere to a solid surface and then encase themselves in a self-produced matrix of extracellular polysaccharides and proteins. Once formed, biofilms are extremely difficult to eradicate and their cellular constituents develop increased antimicrobial resistance. Biofilm formation is thought to be directly attributable to various chronic or device-related infections (Bhinu 2005; Bridiera *et al.*, 2011; Burmølle *et al.*, 2010). Therefore, rapid antimicrobial susceptibility testing of microbial biofilm is increasingly important for appropriate patient management and clinical surveillance.

Biofilm-associated infections are treated with different concentrations of antibiotics. To evaluate biofilm resistance to various antibiotics, the biofilm minimum inhibitory concentrations (MICs), broth recovery-based biofilm minimum bactericidal concentrations (MBCs), and minimum biofilm eradication concentrations (MBECs) of the biofilms are assayed (Qu *et al.*, 2010). The biofilm MIC is the concentration that inhibits the immediate release of planktonic microbes from biofilms, and is administered during the acute early stage of biofilm-related infections (Ceri *et al.*, 2001). The biofilm MBC is the concentration that kills 99.9% of the bacterial cells in the biofilm or sterilizes most of them, and targets chronic biofilm infections (Aaron *et al.*, 2002; Zhang and Mah, 2008). The MBEC is the concentration that definitely eradicates all cells in the biofilm, and is administered for successful treatment of biofilm-related infections (Ceri *et al.*, 2001). However, a general procedure for the determination of these biofilm parameters is extremely complicated because it is difficult to wash the biofilms cultivated directly on the bottom of wells in microtiter plates and to collect the supernatant on the biofilms with pipetting (Nett *et al.*, 2011; Qu *et al.*, 2010). In addition, this method is prone to contamination.

Alternatively, biofilms can be formed on the 96 pegs on the lid of a microtiter plate (Harrison *et al.*, 2005, 2010). From an evaluation perspective, this approach offers several advantages over the conventional cultivation technique. First, the 96 peg lids coated with biofilms are easily shifted from one microtiter plate to another, enabling an easier wash process. Second, this method avoids accidental disruption of the biofilm by pipetting. However, to determine the MBEC, we need to count the number of bacteria in the established biofilms. For

this purpose, we must detach the bacteria from the biofilms established on the 96 pegs and incubate them on agar medium for 24 h.

Previously, we developed a method for rapid susceptibility testing based on the reduction of a tetrazolium salt (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, or monosodium salt (WST-8)) as an indicator reagent (Tsukatani *et al.*, 2009, 2012). In this method, the microorganisms reduce the electron mediator 2-methyl-1,4-naphthoquinone (NQ) to naphthohydroquinone, which in turn reduces WST-8 to formazan. The final reduction product, formazan, maximally absorbs at 460 nm.

This study aims to develop a rapid and simple method for the determination of the MBEC. In our colorimetric microbial viability assay, biofilms are formed on 96 pegs on the lid of a microtiter plate, and the MBEC is evaluated from the reduction of WST-8 to formazan. The advantages of the proposed method are demonstrated by comparison with conventional methods. The proposed method provides a rapid and simple determination of the MBEC. In addition, we also applied our approach to the biofilm susceptibility of *Staphylococcus aureus* and *Pseudomonas aeruginosa* exposed to plural antibiotics.

MATERIAL AND METHODS

Biofilm growth

S. aureus and *P. aeruginosa* used in this study were obtained from the Biological Resource Center at the National Institute of Technology and Evaluation (NBRC, Chiba, Japan), and the American Type Culture Collection (ATCC, Rockville MD, USA). For *P. aeruginosa*, 180 µl of bacterial inoculum grown to approximately 10⁶ CFU ml⁻¹ in Mueller–Hinton broth (MHB) was added to each well of a 96-well microtiter plate. The plate was then covered with a 96 peg lid and incubated for 24 h, during which time biofilms formed on the 96 pegs. Polypropylene pegs (96 pin plate; Stem Co., Tokyo, Japan) was used for *P. aeruginosa* biofilm. For *S. aureus*, 180 µl of bacterial inoculum grown to approximately 10⁷ CFU ml⁻¹ in MHB was added to each well of a 96-well microtiter plate. The plate was then covered with a 96 peg lid and incubated for 72 h. In the experiments involving 72-h biofilms, the 96 peg lids were first incubated for 24 h, then transferred to 96-well microtiter plates containing fresh MHB, and incubated for another 48 h. Polystyrene pegs (Nunc-Immuno TSP; Thermo Fisher Scientific Inc., MA, USA) was used for *S. aureus* biofilm. The

amounts of established biofilm were measured by a crystal violet stain method (Antunes et al., 2010).

Detection reagents

The mixture of tetrazolium salt WST-8 (Dojindo Laboratories, Kumamoto, Japan) and 2-methyl-1,4-NQ (Sigma Chemicals, St. Louis, MO, USA) was used as the detection reagent. The WST-8 contains sulfonate groups giving them a net negative charge that reduces their ability to move across cell membranes. Thus, it is necessary to employ 2-methyl-1,4-NQ as an electron mediator to facilitate the cellular reduction of tetrazolium salts. WST-8 was dissolved in distilled water at a concentration of 11.1 mM and the solution was sterilized by passing it through a cellulose acetate membrane filter (pore size = 0.2 μm). 2-Methyl-1,4-NQ (Sigma Chemicals, St. Louis, MO, USA) was dissolved in dimethyl sulfoxide to a concentration of 1.0 mM, then mixed with WST-8 solution at a ratio of 1:9. The prepared detection reagent contained 10 mM WST-8 and 0.1 mM 2-methyl-1,4-NQ.

Determination of MBEC

The MBEC was determined as shown in Fig. 1. The 96 peg lids with established biofilms were placed onto 96-well microtiter plates containing sterile PBS and vortexed for 30 s to dislodge any unattached bacteria. After washing three times in sterile PBS, the 96 peg lids were placed onto 96-well microtiter plates containing 180 μl of 2-fold serial dilutions of antibiotics (range 1280 to 1 μg ml⁻¹) in Mueller–Hinton broth (MHB). After 20 h challenge at 35 °C, the 96 peg lids were removed and washed three times with sterile PBS, then placed onto new 96-well microtiter plates containing 180 μl recovery broth (MHB) and incubated for a further 24 h at 35 °C. After the recovery incubation, the 96 peg lids were washed three times with sterile PBS, then placed onto new 96-well microtiter plates containing 200 μl of reaction solution (190 μl MHB and 10 μl detection reagent). After incubation at 35°C, the generated formazan was measured at 460 nm with a microplate reader (VersaMax, Molecular Devices Co., Sunnyvale, CA, USA). The MBEC was read as the lowest concentration of antibiotics for which

the absorbance change (relative to the blank value without bacteria) was less than 0.1.

Reference MBECs are determined according to the conventional method reported by Harrison et al. (Harrison et al., 2005, 2010). The conventional method computes the MBECs from the number of bacteria in the established biofilms. After the recovery incubation described above, the 96 peg lids are placed in another 96-well microtiter plates containing growth recovery broth, and then sonicated in an ultrasonic sonicating bath. Viable cells in the biofilm can be detected by plating onto appropriate medium, or by measuring increased turbidity over time, using a microplate reader. However, some of viable cells in the biofilm established on the 96 peg lids might be left behind for lack of sufficient sonication. Thus, to implement the conventional method, we collected the biofilm matrix by carefully swabbing each peg with a sterile cotton swab. The tip of the cotton swab was then inserted into a tube containing sterile PBS. The tube was vortexed and sonicated to disintegrate the bacterial clumps and to dislodge the bacteria from the tip of the cotton swab. The bacterial suspension was plated onto agar medium and the number of viable bacteria were counted after 24 h incubation. The MBEC was taken as the minimum concentration of antibiotics for which no visible colonies appeared.

RESULTS AND DISCUSSION

Biofilm formation on the 96-pegs

To establish the biofilm on the 96 pegs, we first noted the optimal growth conditions of *S. aureus* and *P. aeruginosa*, such as peg materials, growth medium, and incubation time. *S. aureus* more readily formed biofilms on polystyrene pegs than on polypropylene (data not shown). On the other hand, *P. aeruginosa* established biofilms more effectively on polypropylene pegs. The biofilm formation medium was MHB because this medium is most commonly used in susceptibility testing. *P. aeruginosa* formed biofilms on the 96 pegs within 24 h, *S. aureus* required 72 h. The biofilms established on the 96 pegs under the optimum conditions were tested for the determination of the MBECs as shown in Fig. 1.

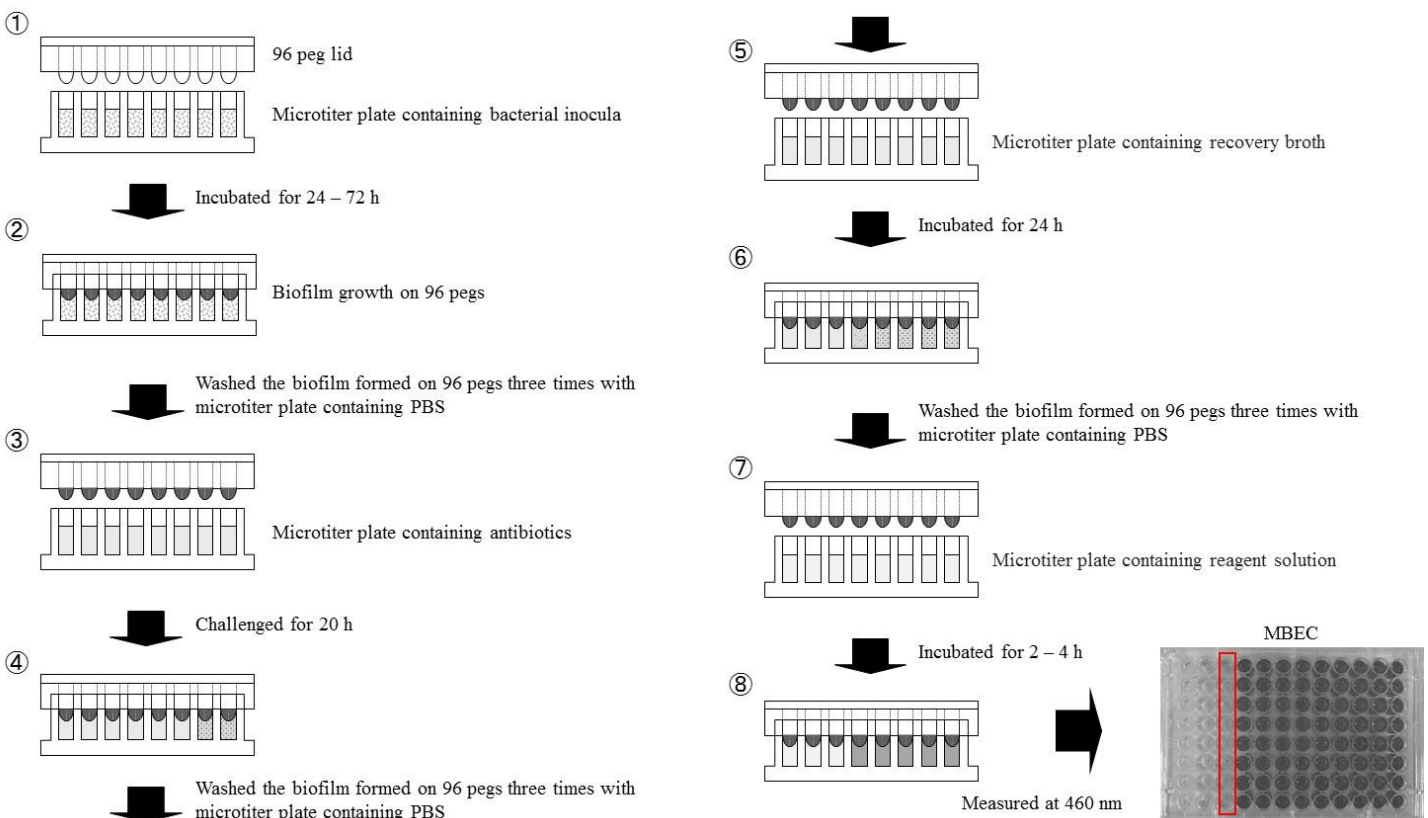


Figure 1 Procedures of biofilm susceptibility tests

Susceptibility of biofilms exposed to a single antibiotic

To evaluate the applicability of the WST-8 colorimetric method to rapid determination of the MBEC, we determined the biofilm susceptibility of *S. aureus* and *P. aeruginosa* in the presence of various single antibiotics by the proposed method.

Figure 2 shows the effects of the WST-8 reduction time on the susceptibility curves of *S. aureus* NBRC13276 and *P. aeruginosa* NBRC13275. For *S. aureus* exposed to vancomycin, the MBEC was estimated as 512 μg/ml at a WST-8 reduction time of 2 h (Fig. 2 (A)). The MBECs obtained at 2 h and 4 h were almost equal. The MBEC for *P. aeruginosa* exposed to ciprofloxacin was estimated as 256 μg/ml at 4 h, but the absorbance at 2 h was relatively low (Fig. 2

(B)). From these results, the WST-8 reduction time was decided as 2 h for *S. aureus* and 4 h for *P. aeruginosa*.

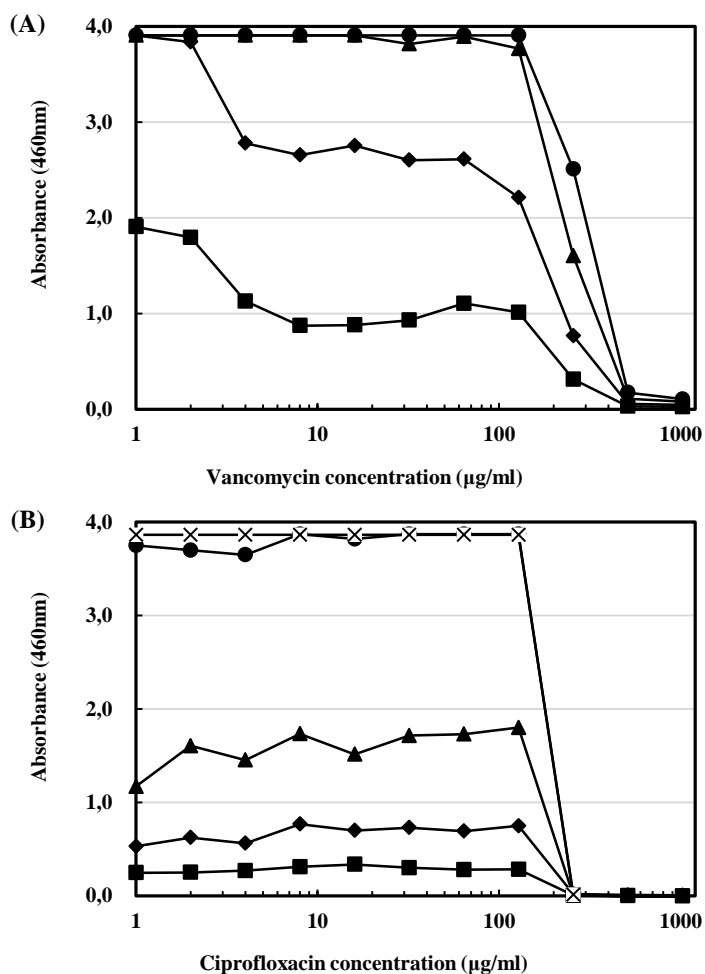


Figure 2 Absorbance (used to determine the MBEC) versus antibiotic concentration for different reduction times of WST-8. Biofilms of *S. aureus* NBRC13276 (A) and *P. aeruginosa* NBRC13275 (B).

Incubation time: 1 h, ■; 2 h, ◆; 3 h, ▲; 4 h, ●; 5 h, ×.

The data represent the means for 4 identical wells of a microtiter plate from each experiment.

Tables 1 and 2 show the MBECs of *S. aureus* NBRC13276 and *P. aeruginosa* NBRC13275, respectively, exposed to various antibiotics and evaluated by the proposed and conventional methods. The most effective inhibitors of *S. aureus* and *P. aeruginosa* biofilms were vancomycin and ciprofloxacin, respectively. In addition, reference MBECs were also determined by the conventional method. There was good agreement between the MBECs obtained at 2–4 h using the WST-8 colorimetric method and those obtained after 24 h using the conventional method. In both *S. aureus* and *P. aeruginosa* biofilms, the percentage of the MBEC values located at $\pm 1 \log_2$ difference was 100%. To better assess the degree of agreement between the MBEC results obtained from the present method and the conventional method, the Wilcoxon signed-ranked test was performed. P values derived from the Wilcoxon signed-rank test demonstrated no significant differences ($P = 0.317$) between the proposed method and the conventional method.

The conventional method is complicated and the MBEC assays are time consuming because the microbial cells must be first extracted from the biofilm matrix and then incubated for another 24 h (Harrison et al., 2005, 2010). On the other hand, the proposed method is simple to implement and yields the MBEC results within a few hours. In addition, the proposed method provides a fast assay of the MBEC. These findings indicate that the proposed method could potentially circumvent the tedious and time-consuming conventional method.

Table 1 MBECs of *Staphylococcus aureus* NBRC13276 biofilms exposed to single antibiotics

Antibiotics	Proposed method	Conventional method
Azithromycin	1024<	1024<
Cefazolin	1024<	1024<
Chloramphenicol	1024<	1024<
Ciprofloxacin	1024<	1024<
Clarithromycin	1024<	1024<
Clindamycin	1024<	1024<
Daptomycin	1024<	1024<
Doxycycline	1024	1024
Erythromycin	1024<	1024<
Gentamicin	1024<	1024<
Imipenem	1024<	1024<
Kanamycin	1024<	1024<
Linezolid	1024<	1024<
Meropenem	1024<	1024<
Minocycline	1024	1024
Oxacillin	1024<	1024<
Phosphomycin	1024<	1024<
Rifampicin	1024<	1024<
Streptomycin	1024<	1024<
Teicoplanin	1024<	1024<
Tigecycline	1024	1024<
Tylosin	1024<	1024<
Vancomycin	512	512

(µg/ml)

Table 2 MBECs of *Pseudomonas aeruginosa* NBRC13275 biofilms exposed to single antibiotics

Antibiotics	Proposed method	Conventional method
Amikacin	1024<	1024<
Azithromycin	1024<	1024<
Aztreonam	1024<	1024<
Ceftazidime	1024<	1024<
Chloramphenicol	1024<	1024<
Ciprofloxacin	256	256
Clarithromycin	1024<	1024<
Colistin	1024<	1024<
Doxycycline	1024<	1024<
Enrofloxacin	1024	1024
Gentamicin	1024<	1024<
Imipenem	1024<	1024<
Kanamycin	1024<	1024<
Levofloxacin	1024	1024
Meropenem	1024<	1024<
Ofloxacin	1024<	1024<
Polymyxin B	1024<	1024<
Streptomycin	1024<	1024<
Tobramycin	1024<	1024<

(µg/ml)

Susceptibility testing of biofilms exposed to plural antibiotics

To investigate the combined effect of antibiotics against microbial biofilm, we applied the proposed method to the biofilm susceptibility of *S. aureus* exposed to combinations of plural antibiotics. Tables 3 and 4 present the MBECs of *S. aureus* in the presence of two and three antibiotics, respectively.

In the two-antibiotic combinations, each antibiotic was compared with vancomycin, the most effective inhibitor of *S. aureus* biofilm in the single-antibiotic study. Vancomycin combined with ciprofloxacin, daptomycin, and teicoplanin effectively reduced the MBEC (Table 3). Therefore, in the three-antibiotic combination, several antibiotics were combined with vancomycin and daptomycin. As shown in Table 4, vancomycin, daptomycin, and teicoplanin most effectively reduced the MBEC of *S. aureus* biofilm. The effectiveness of the vancomycin–daptomycin–teicoplanin combination was also investigated for other *S. aureus* strains. Clearly, biofilms established by other *S. aureus*, including methicillin-resistant *S. aureus* (MRSA), were largely inhibited by this drug combination. In the absence of teicoplanin, the combination of vancomycin and

daptomycin hardly inhibited other *S. aureus* with the exception of *S. aureus* NBRC13276 (data not shown). Therefore, it is thought that the addition effect of teicoplanin is very important for *S. aureus* biofilm eradication.

The glycopeptide antibiotics, such as vancomycin and teicoplanin have similar mechanisms of action on bacterial cell wall synthesis. Vancomycin and teicoplanin are both widely used in the treatment of infections caused by Gram-positive bacteria. Teicoplanin has a longer half-life than vancomycin (Murphy and Pinney, 1995; Wood, 1996). Thus, vancomycin requires multiple dosing to maintain adequate serum levels. As shown in Table 1, vancomycin alone showed higher biofilm eradication activity than teicoplanin. In the combination of vancomycin and teicoplanin, it is thought that vancomycin acts *S. aureus* biofilms potentially at an early stage, and then teicoplanin mainly affects the biofilms after the effectiveness of vancomycin is attenuated.

Saginur et al. (2006) reported that most of the effective antibiotic combinations against *S. aureus* (including MRSA) include rifampicin. Rifampicin is especially effective when combined with vancomycin and/or fusidic acid (Saginur et al., 2006). Vancomycin is often combined with a secondary antibiotic, usually rifampicin or gentamicin, for the treatment of serious MRSA infections (Deresinski, 2009). We demonstrated that a new combination of vancomycin, daptomycin and teicoplanin was most effective against *S. aureus* biofilms in vitro.

In the last decade, the role of biofilms in various chronic bacterial infections has been recognized (Burmölle et al., 2010). The findings of the present study suggest the utility of the proposed method as a rapid and simple assay of microbial biofilm. In the future, we hope that our method will be adopted as an alternative to the conventional method for rapid and simple susceptibility testing of microbial biofilm.

Table 3 MBECs of *Staphylococcus aureus* NBRC13276 biofilms exposed to combinations of two antibiotics, determined by the proposed method

Antibiotics	Vancomycin			
	16	32	64	128
Cefazolin	128<	128<	128<	128<
Ciprofloxacin	128	128	128	64
Clarithromycin	128<	128<	128<	128<
Clindamycin	128<	128<	128<	128<
Daptomycin	128<	128<	64	4
Gentamicin	128<	128<	128<	128
Linezolid	128<	128<	128<	128<
Meropenem	128<	128<	128<	128<
Minocycline	128<	128<	128<	128<
Rifampicin	128<	128<	128<	128<
Teicoplanin	128<	128<	2	1
Tigecycline	128<	128<	128<	128<

(µg/ml)

Table 4 MBECs of biofilms of various *Staphylococcus aureus* strains exposed to combinations of three antibiotics, determined by the proposed method

Antibiotics	Vancomycin / Daptomycin			
	8/4	16/4	32/4	64/4
<i>S. aureus</i> NBRC13276				
Ciprofloxacin	32<	32<	32<	8
Gentamicin	32<	32<	32	2
Minocycline	32<	32<	32<	32<
Rifampicin	32<	32<	32<	32
Tigecycline	32<	32<	32<	32<
Teicoplanin	8	4	4	1
<i>S. aureus</i> ATCC29213				
Teicoplanin	32<	32<	32<	32
<i>S. aureus</i> NBRC14462				
Teicoplanin	32<	32<	32<	32
<i>S. aureus</i> ATCC43300 (MRSA)				
Teicoplanin	32<	32<	32	16

(µg/ml)

CONCLUSION

We have develop the rapid and simple method for the determination of MBEC using a colorimetric microbial viability assay based on reduction of a tetrazolium salt WST-8 and the biofilm formation method on 96 pegs on the lid of a microtiter plate, and demonstrated the advantages of the proposed method as compared to the conventional methods. There was good agreement between the MBECs obtained at 2–4 h using the WST-8 colorimetric method and those

obtained after 24 h using the conventional method. In addition, by the proposed method, we clarified that a combination of vancomycin, daptomycin and teicoplanin was most effective for *Staphylococcus aureus* biofilm. The proposed method yields similar performance to conventional methods, but is faster and more easily implemented. Therefore, the proposed method alleviates the tediousness and time-consuming nature of conventional biofilm susceptibility assay.

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