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NOTE

A Simple Colorimetric Method for Testing Antimicrobial Susceptibility of Biofilmed Bacteria

Shukho Kim, Mi Jin Kim, Hee Young Kang, Sung Yong Seol, Dong Taek Cho, and Jungmin Kim*

Department of Microbiology, Kyungpook National University, School of Medicine, Daegu 700-422, Republic of Korea

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This study introduces a simple colorimetric method which can measure the antimicrobial susceptibility of bacteria in biofilms using trimethyl tetrazolium chloride (TTC) as an indicator of viable bacteria. The new method was utilized for the evaluation of antibiotic susceptibility of *Escherichia coli*, *Klebsiella pneumoniae*, and *Staphylococcus aureus* biofilms.

***Keywords*:** biofilm, antimicrobial susceptibility, colorimetric

In medicine, biofilm associated infections have gradually increased with the expanded use of medical prosthetic devices (Habash and Reid, 1999; Donlan, 2001a; Trautner and Darouiche, 2004). Bacterial biofilms are known to be resistant to antimicrobial agents and the mechanisms of this resistance are generally explained by the following four hypotheses; poor penetration of the drugs, limitation of nutrient and slow growth, adaptive stress responses of bacteria, and formation of persister cells (Stewart, 2002). For some antimicrobial agents, the concentration required to kill biofilmed bacteria may be a thousand times greater than that required to kill planktonic cells of exactly the same strain (Donlan, 2001b; Donlan and Costerton, 2002). Therefore, the standard antimicrobial susceptibility test against planktonic bacteria has limited relevance in the determination of antimicrobial susceptibility against biofilmed bacteria. This study developed a new colorimetric antimicrobial susceptibility test which can measure the antimicrobial susceptibility of biofilmed bacteria very conveniently. This method uses trimethyl tetrazolium chloride (TTC) as an indicator of viable bacteria and is comprised of the following steps; preparation of a 1-day old biofilm, treatment with the antimicrobial agent, addition of 0.02% TTC and measurement of TTC absorbance at 540 nm. Since reduction of TTC by viable bacteria produces red formazan, bacterial growth inhibition can be measured quantitatively by colorimetric absorbance at 540 nm (Knezevic and Petrovic, 2008).

Preparations of 1-day old biofilm were done as follows. Bacteria were cultured overnight in 3 ml of Mueller-Hinton broth (MHB, Beckton Dickinson, USA) with shaking. Overnight cultures were adjusted to an OD_{600} of 1.0 and the adjusted bacterial suspension was diluted 100-fold using fresh MHB. One hundred microliter aliquots of the diluted bacterial suspension were inoculated into each well of a 96-well flat-bottom polystyrene plate (SPL Plastic Labware, Korea) and incubated in a humidified incubator for 24 h at 37°C. When the antimicrobial treatment was applied, the media was first removed and the plate was dried up-side-down on a sterile paper towel for 15 min at room temperature. Then, 200 μl of fresh MHB containing a serially diluted antimicrobial agent was added to each well and incubated in a humidified incubator for further 24 h. After incubation, 50 μl of 0.1% TTC (Sigma, USA) was added to a final concentration of 0.02%. After an 1 h incubation at 37°C, the OD_{540} was measured on a VERSA max microplate reader (Molecular Devices, USA). The mean OD_{540} value of 1-day biofilmed cells before treatment with the antimicrobial agent was set as the control and the mean OD_{540} values of each biofilm after treatment were expressed as % value with respect to the control. Then, the minimum biofilm inhibitory concentration (MBIC) was determined as the lowest concentration of drug that resulted in a mean % value less than 100%. The minimum biofilm eradication concentration (MBEC) was assessed with $MBEC_{50}$ and $MBEC_{90}$ which is the concentration of drugs that eradicates 50% and 90% of the bacteria in preformed biofilms, respectively. Therefore, $MBEC_{50}$ and $MBEC_{90}$ were determined as the lowest concentration of drug that resulted in a mean % value less than 50% and less than 10%, respectively.

Minimum inhibitory concentration (MIC) of planktonic bacteria was determined by broth microdilution method (CLSI, 2008) and minimum bactericidal concentration (MBC) of planktonic bacteria according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI, 1999).

Seven bacterial strains were tested for antimicrobial susceptibility by standard procedures (MIC and MBC) and newly developed procedures (MBIC, $MBEC_{50}$, and $MBEC_{90}$) as described above. *Escherichia coli* ATCC 25922 and two clinical isolates of *Klebsiella pneumoniae* (B170 and B172)

* For correspondence. E-mail: minkim@knu.ac.kr; Tel: +82-53-420-4845; Fax: +82-53-427-5664

were used as representative test strains of Gram-negative bacteria. *Staphylococcus aureus* ATCC 29213 and three clinical isolates of *S. aureus* (WS-3, WS-8, and WS-22) were used as representative test strains of Gram-positive bacteria. *E. coli* ATCC 25922 and *S. aureus* ATCC 29213 were from bacterial collection of the Department of Microbiology at Kyungpook National University and four clinical isolates were from patients admitted to Kyungpook National University Hospital in 2009.

Antimicrobial agents included were as follows. Ampicillin/sulbactam (Union Korea Pharm Co., Korea), cefotaxime (Duchefa, The Netherlands), amikacin (Sigma Chemical Co., USA), and ciprofloxacin (Fluka, Switzerland) were chosen for *E. coli* and *K. pneumonia*. Methicillin, vancomycin, teicoplanin (Sigma Chemical Co., USA), and azithromycin (Pfizer Inc., Korea) were chosen for *S. aureus*.

The relative amounts of biofilms formed by the test strains were measured using gentian violet as a biofilm staining agent of the biofilm formation assay (Lee *et al*., 2008) for only the reference data.

As shown in Table 1, MICs and MBICs of *E. coli* ATCC 25922 and *K. pneumoniae* B170 strains for ampicillin/sulbactam, cefotaxime, amikacin, and ciprofloxacin were almost the same, whereas *K. pneumoniae* B172 strain showed

Table 1. The relative amounts of biofilms and antimicrobial susceptibilities of planktonic and biofilmed *E. coli*, *K. pnemoniae*, and *S. aureus*

Strains	GV stained biofilm (OD_{570})	Planktonic (mg/L)		Biofilm (mg/L)		
		MIC	MBC	MBIC	$MBEC_{50}$	$MBEC_{90}$
E. coli ATCC 25922	0.27					
Ampicillin/Sulbactam		4	4	4	16	>512
Cefotaxime		0.5	0.5	0.5	8	>512
Amikacin		0.5	0.5	1	8	>512
Ciprofloxacin		0.0078	0.5	0.0078	1	>8
K. pneumoniae B170	1.39					
Ampicillin/Sulbactam		2	8	2	64	256
Cefotaxime		0.5	4	0.5	16	128
Amikacin		0.5	2	0.5	2	>512
Ciprofloxacin		0.0625	0.0625	0.0156	1	>8
K. pneumoniae B172	1.21					
Ampicillin/Sulbactam		16	32	64	128	256
Cefotaxime		0.5	0.5	16	64	>512
Amikacin		0.5	0.5	8	>512	>512
Ciprofloxacin		0.125	2	0.25	>8	>8
S. aureus ATCC 29213	2.14					
Methicillin		4	16	0.5	8	>512
Vancomycin		1	2	4	8	>512
Teicoplanin		1	4	0.5	8	>256
Azithromycin		2	16	0.5	>512	>512
S. aureus WS-3	1.65					
Methicillin		64	>512	4	>512	>512
Vancomycin		2	8	4	8	>512
Teicoplanin		2	16	0.25	8	>256
Azithromycin		4	32	1	>512	>512
S. aureus WS-8	2.28					
Methicillin		8	>512	2	>512	>512
Vancomycin		1	8	4	16	>512
Teicoplanin		2	8	1	16	>256
Azithromycin		4	32	2	>512	>512
S. aureus WS-22	1.95					
Methicillin		8	>512	0.5	4	>512
Vancomycin		2	8	1	8	>512
Teicoplanin		1	4	0.5	8	>256
Azithromycin		4	32	0.5	32	>512

Abbreviations: GV, Gentian violet; OD_{570}, Optical density at 570 nm; MIC, Minimum inhibitory concentration; MBC, Minimum bactericidal concentration; MBIC, minimum biofilm inhibitory concentration; $MBEC_{50}$, minimum biofilm eradicatory concentration that kills 50% of bacteria in preformed biofilms and $MBEC_{90}$, minimum biofilm eradicatory concentration that kills 90% of bacteria in preformed biofilms.

higher values of MBIC than those of MIC for the drugs even though OD_{570} value of gentian violet stained *K. pneumoniae* B170 strain (1.39) was higher than that of *K. pneumoniae* B172 strain (1.21). Compared to the MBC values of *E. coli* ATCC 25922 for ampicillin/sulbactam, cefotaxime, amikacin, and ciprofloxacin, the $MBEC_{50}$ value of *E. coli* ATCC 25922 for each drug was 4 fold, 16 fold, 16 fold, and 2 fold higher, respectively. The $MBEC_{90}$ values for drugs were higher than the highest concentrations of the drugs tested.

MBIC values of *S. aureus* isolates for each drug were lower than MIC values. However, $MBEC_{50}$ values were higher than MBC values for most drugs and $MBEC_{90}$ values were higher than the highest concentrations of the drugs tested. To eradicate 50% of the biofilmed cells of *S. aureus*, vancomycin and teicoplanin were more efficient than azithromycin.

Staining with gentian violet in a biofilm formation assay does indicate not live bacteria, but attached dead and live cells with their biofilm matrix. TTC is known to be an adequate indicator for measuring microbial growth and used for bacterial and fungal susceptibility testing (Meletiadis *et al*., 2000; Rahman *et al*., 2004). In this study, TTC was used for measuring live bacteria in biofilms after treatment with an antimicrobial agent. Using microplates, inhibition of bacterial growth in biofilms was easily measured. In addition, estimation of the efficacy of the antimicrobial agents for the bacterial biofilms was possible by comparing the MBIC, $MBEC_{50}$, and $MBEC_{90}$ for each drug.

A new method, BioTimer assay, for counting *Staphylococcus* spp. in biofilms applied to the evaluation of antibiotic susceptibility of biofilm was recently developed (Pantanella *et al*., 2008). The authors used phenol red as an indicator of cell viability and measured the MBIC and MBEC of *Staphylococcus* isolates. The differences between the BioTimer assay and our TTC method were as follows: (1) Viable cell indicator, phenol red vs TTC. (2) Substance for biofilm formation, glass beads vs polystyrene microplates. (3) How the MBIC and MBEC were measured - recording the time of color change and extrapolation into a standard correlation line vs measuring the TTC absorbance using a spectrophotometer and a comparative analysis using a control value.

This TTC method could be useful for the convenient determination of antimicrobial susceptibility of Gram-negative and Gram-positive bacterial biofilms in hospital laboratories and for the screening of antimicrobial agents or biocides capable of eradicating bacterial biofilms for research purposes.

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