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Evaluation of a Colorimetric Broth Microdilution Method for Antimicrobial Susceptibility Testing Using 2,3,5-Triphenyltetrazolium Chloride

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Background: The broth microdilution susceptibility testing method is considered a standard for determining minimum inhibitory concentrations, and the addition of the redox indicator 2,3,5-triphenyltetrazolium chloride (TTC) to the broth microdilution method simplifies and increases its objectivity. The current study evaluated the usefulness of a TTC-modified broth microdilution method for antimicrobial susceptibility test of frequently encountered clinical isolates.

Methods: The minimal inhibitory concentrations (MICs) of 10 antimicrobials for 111 clinical isolates of four bacterial species, *Staphylococcus aureus, Escherichia coli, Enterobacter cloacae,* and *Acinetobacter baumannii,* were investigated by a modification of the Clinical and Laboratory Standards Institute (CLSI)-recommended broth microdilution method with the addition of 2,3,5-triphenyltetrazolium chloride (TTC). The inhibitory effects of TTC against 192 strains of 22 bacterial species isolated from clinical specimens were also evaluated.

Results: The number of colonies of all 192 strains of 22 bacterial species grown on TTC-containing Mueller-Hinton agar did not differ from those grown on Mueller-Hinton agar only. The MICs with TTC were within 2 dilutions of those obtained by the CLSI method in 569 (97.6%) of 583 organism-antimicrobial agent combinations.

Conclusions: The colorimetric MIC method using TTC may be a useful surrogate of antimicrobial susceptibility testing for most of the frequently isolated bacteria. **(Korean J Clin Microbiol 2007;10:49-53)**

Key Words: Susceptibility tests, Colorimetric assay, 2,3,5-triphenyltetrazolium chloride, *Escherichia coli, Staphylococcus aureus, Enterobacter cloacae, Acinetobacter baumannii*

INTRODUCTION

Recently, infections caused by resistant microorganisms such as methicillin-resistant *Staphylococcus aureus* (MRSA) strains with reduced susceptibility to vancomycin[1,2], extended-spectrum β-lactamase (ESBL)-producing *Escherichia coli*, and imipenem-resistant *Acinetobacter baumannii* have been reported[3]. Reliable susceptibility testing of pathogenic microorganisms is of growing importance because of the development of new antibiotics and the emergence of drug resistance[4,5]. The broth microdilution method is considered as a standard for determining minimum inhibitory concentrations (MICs)[6], and it has been reported that the addition of the redox indicator, 2,3,5-triphenyltetrazolium chloride (TTC) (Sigma, St. Louis, MO, USA), to the broth microdilution method simplifies and increases its objectivity[7-9]. However, few bacterial species were included in these studies. In the present study, the inhibitory effects of TTC were tested against a battery of frequently encountered clinical isolates to determine a possible application of TTC to these organisms. In addition, we investigated the agreement between original and TTC-modified method for the determination of antibiotic susceptibility.

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MATERIALS AND METHODS

1. Microorganism strains

To investigate the inhibitory effect of TTC on microorganism growth, 192 isolates of 22 bacterial species isolated from clinical specimens at Pusan National University Hospital were used. For MIC determinations, 111 isolates composed of *Staphylococcus aureus* (n=30), *Escherichia coli* (n=28), *Enterobacter cloacae* (n=25), and *Acinetobacter baumannii* (n=28) were collected at Kosin University Gospel Hospital. These four species were chosen because they are commonly isolated from various clinical specimens. *S. aureus* ATCC 29213 and *E. coli* ATCC 25922 strains were used for quality control, as recommended by the Clinical and Laboratory Standards Institute (CLSI)[10,11]. All bacterial isolates were stored in skimmed milk at $-70^{\circ}C$ until use and plated on blood agar prior to testing.

2. Antimicrobial agents and TTC

Ampicillin/sulbactam (A/S; Ilsung, Ansan, Korea), aztreonam (AZT; Donga, Ansan, Korea), cefepime (FEP; Choongwae, Seoul, Korea), cefoxitin (FOX; Merck Sharp & Dohme, Rahway, NJ, USA), cefotaxime (CTX; Handok, Seoul, Korea), ceftazidime (CAZ; Hanmi, Hwasung, Korea), cephalothin (CEP; Merck Sharp & Dohme), imipenem (IPM; Choongwae, Seoul, Korea), teicoplanin (TEI; Narion Merrell Dow, Seoul, Korea), and vancomycin (VA; Daewoong Lilly, Seoul, Korea) were used for MIC determinations. The antibiotic solutions were made by dissolving the drug powder in distilled water or phosphate buffer solution. Solutions were divided into 1-mL aliquots at a 100× final concentration of the antibiotics in the broth microdilution testing and frozen at −70°C until use. On the day the test was performed, the antibiotic stock solution was thawed and diluted in Mueller-Hinton broth (Becton Dickinson, Sparks, MD). The stock solution of TTC was made by dissolving the TTC powder in distilled water, filter sterilizing it, and dividing it into 500−μL aliquots at a concentration of 5 g/L. These were frozen at −70°C until needed. The stock solution was thawed and added to Mueller-Hinton broth on the day the test was performed. The final TTC concentration was 40 μg/mL.

3. Inhibitory effect of TTC

Countable amounts of all strains, representing between 10 and 1,000 colony-forming units (CFU), were inoculated onto Mueller-Hinton agar (Becton Dickinson) and Mueller-Hinton agar containing TTC 100 μg/mL, and colony numbers were counted after an overnight incubation. The concentration of TTC (100 μg/mL) was selected because it was high enough to determine bacterial inhibition, given that the final concentration of TTC is 40 μg/mL in the modified microdilution method used in this study. Mueller-Hinton agar was chosen instead of Mueller-Hinton broth because colonies were countable in this medium. The counts were compared statistically by the paired Student's t-tests.

4. Antimicrobial susceptibility test using the CLSI broth microdilution (CLSI-BD) and the modified CLSI broth microdilution using TTC (TTC-BD)

The colorimetric method using TTC was identical to the broth microdilution method described by the CLSI in terms of the reagents, medium preparation, inoculum, drug concentrations, and incubation time. The only exception was that TTC was added to the Mueller-Hinton broth at a final concentration of 40 μg/mL. The CLSI-BD and TTC-BD were performed according to the guidelines of the CLSI using 96-well microplates. The final drug concentrations were 0.06 to 256 μg/mL. Colonies of the 111 isolates with diameters of larger than 1 mm were suspended in sterile tryptic soy broth and adjusted to a final concentration of 2.5×10^5 to 5.0×10^5 cells/mL in Mueller-Hinton broth including TTC. This inoculum was added directly to wells containing antimicrobial agents using a multichannel pipette and incubated at 37°C. In TTC containing media, wells with microbial growth were colored pink to red. MICs were interpreted visually at 18 h or 24 h. All measurements were performed in duplicate for each isolate. Levels of agreement between the colorimetric broth microdilution methods with or without TTC were estimated.

RESULTS

1. Inhibitory effect of TTC

The number of colonies of all 192 isolates of bacteria grown in TTC-containing Mueller-Hinton agar did not differ from those grown on Mueller-Hinton agar without TTC (Table 1).

2. MICs in broth dilution method

The MICs of VA and TEI for 30 isolates of *S. aureus* were 0.5 to 2.0 μg/mL and 0.25 to 2.0 μg/mL, respectively. For 30 strains of *E. coli*, the MICs were as follows: A/S, 4 to >256 μg/mL; AZT, 0.12 to >256 μg/mL; CAZ, 0.25 to >256 μg/mL; CEP, 16 to >256 μg/mL; CTX, 4 to >256 μg/mL; FEP, 0.12 to >256 μg/mL; FOX, 2 to >256 μg/mL; and IPM, 1 to >2 μg/mL. Among 30 strains of *E. cloacae*, the MICs were as follows: CAZ, 16 to >256 μg/mL; CTX, 32 to 256 μg/mL; and FEP, 1 to 128

Table 1. Inhibitory effect of bacterial growth by TTC (100 μg/mL)

Species (No. of strains)	No. of colonies (mean±SD)	
	MHA only	TTC-containing MHA
Acinetobacter baumannii (30)	87.7±16.4	87.6±18.7
Aeromonas hydrophila (2)	30.0±4.2	33.0±2.8
Chryseobacterium indologenes (2)	25.0±1.4	24.8±1.0
Citrobacter freundii (3)	79.7±4.5	75.7±3.1
Enterobacter aerogenes (2)	67.0±1.4	65.0±2.8
Enterobacter cloacae (30)	32.5±5.6	30.5±7.5
Enterococcus faecalis (3)	770.0±42.4	720.0±56.6
Enterococcus faecium (3)	148.5±2.1	136.5±16.3
Escherichia coli (30)	178.9±12.5	175.6±8.9
Flavobacterium odoratum (2)	19.5±2.1	21.0±1.4
Klebsiella oxytoca (2)	126.5±0.7	125.5±0.7
Klebsiella ozaenae (2)	49.0±2.8	45.0±2.8
Klebsiella pneumoniae (30)	105.5±8.8	105.8±8.0
Morganella morganii (2)	120.0±7.1	125.0±15.6
Proteus mirabilis (2)	120.0±2.8	115.0±2.8
Providencia stuartii (4)	23.0±1.8	22.0±1.8
Pseudomonas aeruginosa (4)	128.3±3.0	127.5±3.5
Pseudomonas mendocina (2)	27.5±0.7	26.0±2.8
Serratia marcescens (2)	151.0±1.4	147.5±3.5
Staphylococcus aureus (30)	44.3±2.2	45.0±2.7
Staphylococcus, coagulase-negative (3)	25.0±2.0	25.2±3.8
Stenotrophomonas maltophilia (2)	120.0±4.2	125.0±5.7

Abbreviations: SD, standard deviation; MHA, Mueller-Hinton agar.

Table 2. Agreement within two-fold dilutions of colorimetric broth microdilution method with CLSI standard broth dilution method

		No. (%) of agreement				Total	
		S. aureus (n=30)	*E. coli* (n=28)	*E. cloacae* (n=25)	*A. baumannii* (n=28)	No. of tested	No. (%) of agreement
Teicoplanin		30 (100)				30	30 (100)
Vancomycin		30 (100)				30	30 (100)
Ampicillin/sulbactam			27 (96.4)		28 (100)	56	55 (98.2)
Aztreonam			27 (96.4)		28 (100)	56	55 (98.2)
Ceftazidime			24 (85.7)	25 (100)	28 (100)	81	77 (95.1)
Cephalothin			27 (96.4)		28 (100)	56	55 (98.2)
Cefotaxime			28 (100)	25 (100)	28 (100)	81	81 (100)
Cefepime			27 (96.4)	25 (100)	28 (100)	81	80 (98.8)
Cefoxitin			25 (89.3)		28 (100)	56	53 (94.6)
Imipenem			25 (89.3)		28 (100)	56	53 (94.6)
Total	No. of tested	60	224	75	224	583	
	No. (%) of agreement	60 (100)	214 (93.8)	75 (100)	224 (100)		569 (97.6)

μg/mL. Among 30 strains of *A. baumannii*, the MICs were as follows: A/S, 16 to >256 μg/mL; AZT, 32 to >256 μg/mL; CAZ, 64 to >256 μg/mL; CEP, >256 μg/mL; CTX, 256 to >256 μg/mL; FEP, 32 to 64 μg/mL; FOX, 128 to >256 μg/mL; IPM, 16 to >64 μg/mL.

3. Agreement between CLSI broth dilution and modified broth dilution with TTC

The MICs of antimicrobial agents obtained using TTC correlated well with those obtained by the CLSI broth dilution method. A total 583 organism-antimicrobial agent combinations were analyzed. Overall, in 569 (97.6%) of the total 583 combinations, the MICs were within 2 dilutions of those obtained by the CLSI method (Table 2). For each of the four species, the overall agreement at ± 2 $\log_2$ dilution ranged from 93.8% to 100% with no significant difference between any two species.

DISCUSSION

The redox indicator TTC has been used to enhance microbial viability and MIC determination for *Bilophila wadsworthia, Helicobacter pylori*, gram-negative fermentative organisms, and MRSA [7-9,12,13]. When TTC is added to the medium, bacterial growth results in a red color, making it easy to interpret the results. Because each method was applied to only one or a few strains in the earlier studies, however, the methods were not well established for commonly isolated bacteria. In this study we investigated 22 bacterial species isolated from clinical specimens for the evaluation of inhibition effect of TTC, and found that TTC itself was not inhibitory against the commonly isolated bacteria. So, it was demonstrated that TTC may be applied for the colorimetric methods in most of frequently cultured bacterial isolates.

The CLSI agar dilution method is a standard test for MIC testing of microorganisms but it is laborious[6,10] due to the difficulties and the large amount of manual handling, and media needed to run it. On the other hand, the broth microdilution is convenient and widely used for rapid susceptibility testing to several antibiotics on a large number of bacterial isolates in a short time[5,6,8]. However, the MIC of the broth microdilution is determined by visual turbidity reading, making the test subjective and variable. To avoid this drawback, the redox indicator TTC was used in the broth microdilution method. The results could be read visually or by spectrophotometer, and the indicator makes reading easier because of the obvious color change. This overcomes the problems frequently encountered in the conventional broth microdilution test, such as inoculum sedimentation or very scant or transparent growth, which occurs with some species of bacteria[8].

When we compared the MICs of 10 antimicrobial agents determined by the TTC broth microdilution method with the MICs determined by the reference agar dilution method for 111 strains of four species in duplicate, the overall agreement of MICs were 97.6%, indicating that TTC broth microdilution could be a reliable method.

There are a few colorimetric redox indicators that can serve as alternatives to the standard method of visually grading turbidity. A mainstay of such techniques is assays involving the use of tetrazolium salts and alamar blue[5,8,14-16]. Some examples of tetrazolium salts are 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2H-tetrazolium hydroxide (XTT), 3-(4,5- dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), and 2-(4-iodophenyl)-3-(-4 nitrophenyl)-5-phenyl tetrazolium chloride (INT). Because of their convenience, the MTT and XTT methods have been employed in assays of yeasts viability[15,16]. However, the use of these tetrazoliums has several drawbacks. In the MTT assay, a large number of cells are necessary, especially

if they have low metabolic activity[16]. Both MTT and INT are more toxic than TTC or XTT to all the reference strains[8,16], and XTT is far more expensive than TTC. Furthermore, XTT exhibits a background color, which makes it less suitable for analysis. In addition, TTC has another advantage: whereas other dyes are added after incubation, TTC can be added before incubation, making it possible to observe the growth of microorganisms continuously and to detect the reading point easily. Moreover, reduced tetrazolium is water-soluble, so no solubility agent is necessary for visual or spectrophotometric reading.

In conclusion, TTC is not inhibitory for most commonly isolated bacteria. For susceptibility tests, the broth microdilution method using TTC for *S. aureus, E. coli, E. cloacae*, and *A. baumannii* is reliable, easy to perform, and economical. The colorimetric MIC method using TTC may be a useful surrogate of antimicrobial susceptibility testing for most of the frequently isolated bacteria.

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=국문초록=

2,3,5-Triphenyltetrazolium Chloride 첨가 미량액체배지희석법에 의한 항균제 감수성 검사 방법의 평가

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배경: 최소억제농도의 표준 측정법인 미량액체배지희석법에 환원지시제 2,3,5-triphenyltetrazolium chloride (TTC)의 첨가는 간단하고 객관적이다. 본 연구에서는 임상에서 자주 분리되는 균종에 대하여 감수성 검사를 할 때 TTC 첨가의 유용성을 확인하고자 하였다.

방법: *Staphylococcus aureus, Escherichia coli, Enterobacter cloacae, Acinetobacter baumannii* 등 4종 111개의 임상분리균주에 대해서 비색법을 이용한 감수성 검사를 실시하여 미량액체배지희석법의 결과와 일치정도를 확인하였다. 임상분리균주 22종에 대하여 TTC가 검사대상이 되는 균주의 억제효과를 평가하였다.

결과: 일반세균 22종 192균주에 대하여 TTC가 검사대상이 되는 균주를 억제하지 않는 것을 확인하였다. 감수성 검사 결과는 4종 111균주에 비색법을 첨가한 미량액체배지희석법의 항균제 감수성 시험 결과 CLSI법과 2희석단계 내에서 97.6% 일치하였다.

결론: TTC를 이용한 미량액체배지희석법은 흔한 임상분리균주의 MIC 측정에 이용할 수 있을 것으로 생각한다. **[대한임상미생물학회지 2007;10:49-53]**

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