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Evaluation of a Colorimetric Antifungal Susceptibility Test by 2,3-Diphenyl-5-Thienyl-(2)-Tetrazolium Chloride for Fluconazole in *Candida* Species Isolated from Clinical Specimens

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Background: The aims of this study were to evaluate the colorimetric antifungal susceptibility test to fluconazole using 2,3-diphenyl-5-thienyl-(2)-tetrazolium chloride (STC) for various *Candida* species isolated from clinical specimens and to compare the results with those of the CLSI M27-A2 standard method.

Methods: The fluconazole MICs for 204 clinical *Candida* isolates consisting of 100 *C. albicans*, 45 *C. glabrata*, 28 *C. tropicalis*, 22 *C. parapsilosis*, and 9 other *Candida* species were determined by the CLSI and STC colorimetric methods.

Results: All 204 *Candida* strains were grown on the growth control wells of CLSI standard plates, but 26 *Candida* strains (6 *C. albicans* and 20 *C. tropicalis*) were not grown on those containing STC. Therefore, those 26 *Candida* strains were excluded from the comparison of MICs in this report. Overall, the STC visual and spectrophotometric readings of fluconazole MICs showed 96.1% (N=171) and 89.9% (N=160) accordance with those obtained by the CLSI standard method within 2 dilutions, respectively. The STC visual reading of *C. albicans* showed 76.6, 92.6, and 95.8% accordance with the CLSI standard method within 1, 2, and 3 dilutions, respectively. The agreement between the two endpoint determinations of the STC colorimetric method (visual and spectrophotometric readings) was excellent, with 170 of the 178 MICs within 2 dilutions.

Conclusion: The STC colorimetric method to determine the MIC for *Candida* species except *C. tropicalis* showed high levels of agreement with CLSI method. And also, it is useful with objective and easy interpretation. **(Korean J Clin Microbiol 2007;10:90-95)**

Key Words: STC, Colorimetric method, Antifungal susceptibility, Fluconazole, *Candida*

INTRODUCTION

Reliable susceptibility testing of pathogenic and opportunistic fungi is of growing importance because of the development of new antifungal drugs, the emergence of drug resistance, and the high prevalence of serious fungal infections in critically ill and other immunocompromised patients[1-4]. In 2002, the National Committee for Clinical Laboratory Standards (NCCLS; now the Clinical and Laboratory Standards Institute)[5] published reference guidelines for susceptibility testing of *Candida* species and *Cryptococcus neoformans* as M27-A2. This method remains the standard for antifungal susceptibility testing in clinical microbiology laboratories.

Despite the considerable progress made using the CLSI standard method for broth dilution susceptibility testing of yeasts, problems still arise when determining the minimum inhibitory concentration (MIC), since the measurement of the MICs of such organisms is often subjective and variable[6-9]. Consequently, several modifications of the broth microdilution format, as well as alternative methodologies such as spectrophotometry[10,11], agar-based assay[12,13], and flow cytometry[14-16], have been developed for in vitro testing. There have been several attempts to use colorimetric determination of broth microdilution endpoints[17-21]. The colorimetric methods are attractive in that they generate clear-cut endpoints based on a visual color change.

2,3-diphenyl-5-thienyl-(2)-tetrazolium chloride (STC) (Kyokuto Seiyaku, Tokyo, Japan) is an oxidation–reduction indicator that, in the presence of growing organisms, changes from colorless to red[22,23]. Previously, we reported the evaluation of a colorimetric antifungal susceptibility test using STC for ketoconazole and itraconazole[24]. However, this study evaluated only *Candida albicans* and did not include fluconazole, a commonly used antifungal agent. The aims of the present study were to evaluate the colorimetric antifungal susceptibility test to fluconazole using STC for various *Candida* species isolated from clinical specimens

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and to compare the results with those of the CLSI M27-A2 standard method.

MATERIALS AND METHODS

1. Fungal isolates

A total of 204 nonduplicate isolates of *Candida* species were chosen from thsose isolated from various clinical specimens between 2003 and 2004 at a university hospital. The isolates were identified with conventional biochemical tests including the germ tube test and the API 20C system (bioMérieux Vitek, Hazelwood, MO, USA). The isolates were stored in skim milk at −70°C until used and were plated on Sabouraud dextrose agar (SDA) prior to testing. *Candida parapsilosis* ATCC 22019 and *Candida krusei* ATCC 6258 were used for quality control, as recommended by CLSI.

2. Antifungal drug and reagents

1) Fluconazole: The stock solution of fluconazole was made by dissolving the powder in water. The solution was divided into 1-mL aliquots at a concentration of 1,280 μg/mL and frozen at −70°C until use. On the day the test was performed, the stock solution was thawed and diluted in RPMI 1640 (with L-glutamine but without bicarbonate) buffered to pH 7.0 with 0.165 M MOPS. The fluconazole was provided by Pfizer Pharmaceuticals (Pfizer Inc., New York, N.Y., USA), and the chemicals were purchased from Sigma Chemical Company (St. Louis, MO, USA).

2) STC and solubilizing agent: The stock solution of STC was made by dissolving the powder in distilled water and dividing it into 200 μL aliquots at a concentration of 50 mg/mL that were frozen at −70°C. The stock solution was thawed and added to RPMI 1640 with MOPS on the day the test was performed. The solubilizing agent was 50% v/v dimethylformamide in water to which was added 20% v/v sodium dodecyl sulfate. It was stored unfiltered at room temperature.

3. Antifungal susceptibility testing by CLSI broth microdilution

The reference broth microdilution testing was performed according to the guidelines of the CLSI using 96 well microplates [5]. Briefly, fluconazole was diluted in RPMI 1640, and 100-μL aliquots were placed in the wells, which had clear, U-shaped bottoms. The final fluconazole concentrations were 0.125 to 64 g/mL. Five colonies of yeast strains with diameters of more than 1 mm on SDA were suspended in sterile 0.85% saline and adjusted to a final concentration of $0.5\sim2.5\times10^3$ cells/mL in RPMI 1640 with MOPS. This inoculum was added directly to the wells containing antifungal drug and incubated at 35°C. The endpoints were recorded visually after 48 h of incubation. The reference broth microdilution method was scored by comparing the growth in each test well with that in the growth control well. The MIC was defined as the drug concentration at which a prominent decrease of turbidity in visual readings.

4. Colorimetric broth microdilution using STC

The colorimetric method using STC was identical to the broth microdilution method described above in terms of the reagents, medium preparation, inoculum, drug concentrations, and incubation time, with two exceptions: that STC was added to RPMI 1640-MOPS medium with fluconazole at a concentration of 100 μg/mL (final STC concentration 50 μg/mL), and that the solubilizing agents were added at 48 h of incubation and then the plates were incubated for 2 h. The wells with fungal growth contained dark-lilac precipitates before addition of the solubilizing agent and

Table 1. Comparison of fluconazole MICs of 178 clinical *Candida* isolates determined by CLSI and STC colorimetric broth microdilution method with visual and spectrophotometric readings

Species	No. of isolates	Method	MIC of fluconazole (μg/mL)		
			Range	50%	90%
C. albicans	94	CLSI	≤0.125～≥128	0.25	≥128
		STC (Visual)	≤0.125～≥128	0.25	1
		STC (Spectrophotometric)	≤0.125～≥128	0.25	64
C. glabrata	45	CLSI	≤0.125～≥128	4	16
		STC (Visual)	≤0.125～≥128	8	16
		STC (Spectrophotometric)	≤0.125～≥128	16	32
C. tropicalis	8	CLSI	≤0.125～≥128	≥128	≥128
		STC (Visual)	≤0.125～≥128	≥128	≥128
		STC (Spectrophotometric)	≤0.125～≥128	≥128	≥128
C. parapsilosis	22	CLSI	≤0.125～2	1	2
		STC (Visual)	≤0.125～4	1	2
		STC (Spectrophotometric)	≤0.125～4	1	4
Other species	9	CLSI	≤0.25～≥128	32	≥128
		STC (Visual)	≤0.25～≥128	64	≥128
		STC (Spectrophotometric)	≤0.25～≥128	64	≥128

Table 2. Accordance of fluconazole MICs of 178 clinical *Candida* isolates determined by CLSI and STC colorimetric method with visual and spectrophotometric readings

Species	No. of agreement/No. of tested (%)					
	Within 1 dilution		Within 2 dilution		Within 3 dilution	
	STC (visual)	STC (spec)	STC (visual)	STC (spec)	STC (visual)	STC (spec)
C. albicans	72/94 (76.6)	70/94 (74.5)	87/94 (92.6)	83/94 (88.3)	90/94 (95.8)	88/94 (93.6)
C. glabrata	39/45 (86.7)	31/45 (68.9)	45/45 (100.0)	39/45 (86.7)	45/45 (100.0)	44/45 (97.8)
C. tropicalis	8/8 (100.0)	7/8 (87.5)	8/8 (100.0)	7/8 (87.5)	8/8 (100.0)	7/8 (87.5)
C. parapsilosis	22/22 (100.0)	20/22 (90.9)	22/22 (100.0)	22/22 (100.0)	22/22 (100.0)	22/22 (100.0)
Other species	9/9 (100.0)	9/9 (100.0)	9/9 (100.0)	9/9 (100.0)	9/9 (100.0)	9/9 (100.0)
Total	150/178 (84.3)	137/178 (77.0)	171/178 (96.1)	160/178 (89.9)	174/178 (97.8)	170/178 (95.5)

were pink to red after addition of the agent. The MICs were determined as the lowest drug concentration that prominently decreased the color change in visual readings and the lowest drug concentration showing a more than 50% reduction of absorbance at 540 nm. The agreement between the CLSI and STC colorimetric methods was calculated for MIC endpoint of each strains. In order to analyze the agreement of MICs between CLSI and STC readings, the accordance was expressed as percentages in terms of having the same MIC, an MIC within 1, 2, or 3 dilutions.

RESULTS

204 clinical *Candida* isolates, consisting of 100 *C. albicans*, 45 *C. glabrata*, 28 *C. tropicalis*, 22 *C. parapsilosis*, and 9 other *Candida* species (5 *C. krusei*, 2 *C. guilliermondii*, 1 *C. lusitaniae*, 1 *C. pelliculosa*), were tested to evaluate MICs for fluconazole by the CLSI and STC methods. The control strains of *C. parapsilosis* ATCC 22019 and *C. krusei* ATCC 6258 were included in all runs, and all results were consistent with published data.

All 204 *Candida* strains were grown on the growth control wells of the CLSI standard plates, but 26 *Candida* strains (6 *C. albicans* and 20 *C. tropicalis*) were not grown on those containing STC. So, those 26 *Candida* strains were excluded from the comparison of MICs in this report. Table 1 summarizes the in vitro susceptibilities of the 178 isolates of *Candida* species to fluconazole as determined by the CLSI broth microdilution method and the STC colorimetric broth microdilution method with visual and spectrophotometric endpoint determination after 48 h. The data are reported as MIC ranges and the MICs at which 50% and 90% of the isolates are inhibited. The MIC ranges of *Candida* strains for fluconazole by CLSI standard method were $\leq$0.125 to $\geq$128 μg/mL except *C. parapsilosis*.

Table 2 details the agreement between CLSI standard method and the STC colorimetric method with visual and spectrophotometric. The fluconazole MICs determined by the STC colorimetric method correlated well with those obtained by the CLSI standard method. Overall, for 171 (96.1%) and 160 (89.9%) of the 178 strains, MICs of fluconazole by the visual and spectrophotometric readings, respectively, were within 2 dilutions of those obtained by the standard method. The proportions of isolates for which the MICs of STC with visual reading were within 2 dilutions of those of CLSI standard method were 92.6% for *C. albicans* and 100% for *C. glabrata*, *C. tropicalis*, *C. parapsilosis*, and the other species. The proportions of isolates for which the MICs of STC with spectrophotometric reading were within 2 dilutions of those of the standard method were 88.3% for *C. albicans*, 86.7% for *C. glabrata*, 87.5% for *C. tropicalis*, and 100% for *C. parapsilosis* and the other species. Overall, the visual reading of STC showed slightly higher accordance with the CLSI method than did the spectrophotometric reading. The STC visual reading of *C. albicans* showed 76.6, 92.6, and 95.8% accordance with the CLSI method within 1, 2, and 3 wells, respectively. The agreement between the two endpoint determinations of the STC colorimetric method (visual and spectrophotometric) was excellent: 170 of 178 (95.5%) MICs were within 2 dilutions (data not shown). The spectrophotometric reading of the STC colorimetric method tended to give slightly higher fluconazole MICs than the visual reading.

DISCUSSION

Despite great advances in the standardization by the CLSI, there are several problems in the determination of the MICs of antifungal agents, such as the subjectivity of visual reading, trailing effects caused by partial inhibition of fungal growth, and interlaboratory variability[25]. In addition, antifungal susceptibility testing is influenced by a number of technical variables, including inoculum size and preparation, medium formulation and pH, duration and temperature of incubation, and the criterion used for MIC endpoint determination[26-28]. Therefore, several modifications of the CLSI standards have been evaluated and adopted as alternative approaches that may better serve practical clinical laboratory needs.

Previously, we demonstrated the clinical usefullness of the STC colorimetric method for antifungal susceptibility testing to itraco-

nazole and ketoconazole with *C. albicans*[24]. The colorimetric method using STC does not differ from the CLSI standard, except that STC is added to the microdilution tray in a preincubation step at a final concentration of 50 μg/mL. The use of STC for assessing the inhibitory effect against *C. albicans* has also been demonstrated in the previous study. However, the *Candida* species was restricted to *C. albicans*, the number of isolates was small, and the evaluation included only itraconazole and ketoconazole[24].

In the present study, we evaluated the STC colorimetric method to fluconazole for 204 *Candida* strains isolated from various clinical specimens and compared it with the CLSI M27-A2 standard method. Overall, the MIC values using the STC colorimetric method showed excellent correlations with those obtained by the standard method except some strains of Candida. Therefore, MIC values obtained using STC may be treated as equivalent to those obtained by the CLSI method.

Some *Candida* strains showing high MICs were included in this study. We could assume that the difference was due to high MICs of *Candida* strains isolated from other than blood specimens, including respiratory specimens. Also, these differences could be attributable to trailing growth associated with the CLSI standard method. Arthington-Skaggs et al. reported that approximately 16% to 18% of *C. albicans* isolates display trailing growth when tested against itraconazole and fluconazole[10]. The trailing phenomenon is largely responsible for the difficulties in endpoint determination, especially with *Candida* species[7-9]. Among 11 *C. albicans* with a MIC of more than 64 μg/mL in the CLSI standard method, three strains had values of 0.5 or 1 g/mL in the STC colorimetric method by both visual and spectrophotometric readings (data not shown). The STC colorimetric method thus may have eliminated the trailing phenomenon of antifungal susceptibility testing for fluconazole. For the verification of trailing phenomenon, comparison with the 24 h incubation results is instructive; however, MIC values were determined only after 48 h of incubation for all the *Candida* species in this study, and we did not compare the 24 h incubation results.

Colorimetric assays of viability are important tools in the study of eukaryotic cell activity. The STC colorimetric method has another merit when compared with other colorimetric methods in that STC is added in the media-preparation step, so it is convenient. It produces dark-lilac precipitates, so the growth of yeasts is observable before the extraction step. Absorbance does not change for several days after solubilization, so the time of reading is not strictly limited. However, we found an important pitfall in the use of STC colorimetric method. In the previous report, STC did not interfere the growth of *C. albicans* in any way, but many (20/28) *C. tropicalis* strains were not grown on the MIC plates containing STC in this study. The mechanism responsible for this observation was not ascertained, even though it was clearly demonstrated with *C. tropicalis* strains.

In summary, the colorimetric method using STC was objective and easy to interpret and showed high levels of agreement with the CLSI method, making it a reliable alternative to the standard method for *Candida* species. However many *C. tropicalis* strains were not grown on the MIC plates containing STC, so it is considered to be necessary to evaluate the test further, and it should be restricted in use until more data become available.

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=국문초록=

임상검체에서 분리된 칸디다 균종에서 2,3-Diphenyl-5-Thienyl-(2)-Tetrazolium Chloride 비색법을 이용한 Fluconazole 항진균제 감수성 검사의 평가

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배경: 임상검체에서 검출되는 다양한 칸디다 균종에서 2,3-diphenyl-5-thienyl-(2)-tetrazolium chloride (STC) 비색법을 이용한 fluconazole에 대한 항진균제 감수성 검사를 평가하고 CLSI M27-A2의 표준방법의 결과와 비교하고자 하였다.
방법: 임상검체에서 검출된 204균주의 칸디다를 연구대상으로 하였다. *C. albicans* 100균주, *C. glabrata* 45균주, *C. tropicalis* 28균주, *C. parapsilosis* 22균주 및 기타 *Candida* species 9균주로 시행하였다. Fluconazole 항진균제 감수성 검사는 CLSI법과 STC 비색법으로 시행하였다.
결과: CLSI 표준방법에서는 *Candida* 204균주 모두 양성 대조군에서 증식하였으나 STC가 포함된 양성 대조군에서는 *Candida* 26균주 (*C. albicans* 6균주와 *C. tropicalis* 20균주)가 증식하지 않았다. STC를 이용한 비색법의 fluconazole 최소억제농도를 육안 및 흡광도로 판정하였을 때 양성대조군에서 증식하지 않은 26균주를 제외한 178 균주 중 171주 (96.1%)와 160주 (89.9%)에서 CLSI 표준방법과 두 단계 희석범위 내의 일치율을 보였다. *C. albicans*에 대한 STC 육안판정은 표준방법과 비교하였을 때 1, 2, 3단계 희석범위에서 각각 76.6, 92.6, 및 95.8%의 일치율을 보였다. STC 비색법의 두 가지 판정법(육안 및 흡광도)은 178균주 중 170주 (95.5%)에서 두 단계 희석범위 내의 값을 나타내어 높은 일치율을 보였다.
결론: STC를 이용한 비색법은 *C. tropicalis*를 제외한 칸디다 균종에서 CLSI 표준방법과 높은 일치율을 보였으며 결과판정이 객관적이고 용이하였다. **[대한임상미생물학회지 2007;10:90-95]**

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