Evaluation of a Colorimetric Antifungal Susceptibility Test by Using 2,3-Diphenyl-5-Thienyl-(2)-Tetrazolium Chloride

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A colorimetric antifungal susceptibility test was performed using 2,3-diphenyl-5-thienyl-(2)-tetrazolium chloride. Among 24 strains of Candida species, no trailing growth was found. In 22 and 20 strains, the MICs obtained in the colorimetric assay were within two dilutions of those obtained by the National Committee for Clinical Laboratory Standards method for ketoconazole and itraconazole, respectively.

The reference guidelines for susceptibility testing of Candida species and Cryptococcus neoformans (5), published by the National Committee for Clinical Laboratory Standards (NCCLS) in 2002, mark a great advance in the standardization of antifungal susceptibility testing. However, there are several problems in the determination of the MICs for yeasts (3). Therefore, several modifications of the NCCLS standards have been evaluated and adopted as alternative approaches that may better serve practical clinical laboratory needs. Among these modified methods, the colorimetric and spectrophotometric MIC endpoint determinations are particularly worthy of notice. 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. This indicator has been used to test the susceptibilities of Mycobacterium tuberculosis to antimycobacterial drugs (7, 8). The present study aimed to develop a colorimetric method of antifungal susceptibility testing using STC.

Five type strains (Candida parapsilosis ATCC 22019, Candida albicans ATCC 90028, C. albicans ATCC 64550, C. krusei ATCC 6258, and C. tropicalis ATCC 201380) and 19 strains of C. albicans isolated from the blood of patients admitted to Pusan National University Hospital were used. Among the type strains, C. parapsilosis ATCC 22019 and C. krusei ATCC 6258 were used for quality control, as recommended by NCCLS. To investigate the inhibitory effect of STC on the growth of Candida species, countable amounts of all 24 strains, representing between 10 and 200 CFU, were inoculated onto Sabouraud dextrose agar (SDA) alone and SDA containing 50 μg of STC/ml, and the number of colonies was examined after 2 days of incubation. The values were compared statistically by paired Student’s t tests. The reference broth microdilution was...
performed according to the guidelines of the NCCLS, using 96-well microplates for ketoconazole and itraconazole (5). All measurements were performed in duplicate for each yeast strain. The colorimetric method using STC was identical to the broth microdilution method with two exceptions: STC was added to RPMI 1640-morpholinepropanesulfonic acid medium with antifungal agents at a concentration of 100 g/ml, and the solubilizing agents were added at 48 h of incubation and plates were incubated for 2 h. The MICs were determined by three methods: visual reading before the addition of solubilizing agents (method 1), visual reading after the addition of solubilizing agents (method 2), and spectrophotometer determination after solubilization at 540 nm (method 3). The levels of agreement between the NCCLS and colorimetric methods were calculated for each MIC endpoint.

Inhibitory effect of STC against yeasts. The numbers of colonies of all 24 strains in STC-containing SDA was not different from those in SDA only (44.3 ± 14.3 versus 45.4 ± 14.2; P = 0.7). Comparisons of the growth of some strains on the two media are shown in Fig. 1.

MICs of broth microdilution method by NCCLS. The MICs of ketoconazole and itraconazole for the quality control strains were within the permissible ranges. The MIC ranges of ketoconazole and itraconazole for the quality control strains were 0.03 to 1.0 and 0.03 to 16 g/ml, respectively. Among the 24 strains, 18 and 9, respectively, demonstrated the trailing phenomenon with ketoconazole and itraconazole.

Agreement between NCCLS and colorimetric methods. Examples of microplates representing the colorimetric MIC assay with STC are shown in Fig. 2. Trailing growth was not seen. The MICs of ketoconazole and itraconazole obtained with STC correlated well with those obtained by the NCCLS standard method (Table 1). Overall, for 22 (92%) and 20 (83%) of the 24 strains, the ketoconazole and itraconazole MICs, respectively, were within two dilutions of those obtained by the NCCLS method. Also, the MICs obtained with the three different methods correlated very well. All of the MICs were within two dilutions. Two and four strains showing MIC differences of more than two dilutions from the NCCLS method for ketoconazole and itraconazole, respectively, did not overlap, and those MICs were lower in the colorimetric method.

The broth microdilution method with spectrophotometric reading has been widely studied and is included in the NCCLS as a reference method for antifungal susceptibility testing of yeasts (5). 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 tetratolium salts, such as 3-(4,5-dimethyl-2-thiazly)-2,5-diphenyl-2H-tetrazolium bromide (MTT) and 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2H-tetrazolium hydroxide (XTT) (1, 4, 6). Because of their convenience, the MTT and XTT methods have been employed as assays of yeast viability (1, 4, 6). However, the use of these tetratoliums has several drawbacks. In the MTT assay, a large number of cells is necessary, especially if they have low metabolic activity (2). XTT is too expensive for routine use in clinical laboratories. In both methods, the substrate should be added after growth has begun, and the time of addition is not well defined (3). Also, in our laboratory's experience, the developed color disappears within a day. The agitation step may cause a reading error as well. Agitation of plates may simulate reading of the endpoints, but the estimated absorbance is variable according to the degree and method of agitation. The trailing phenomenon also interferes with determination of the MIC endpoints. Therefore, in the search for a sensitive and precise method that assists the reading of MICs, we decided to develop the STC assay as a colorimetric visual or spectrophotometric method. In the STC method, the substrate can be added to media before autoclaving, the agitation step is not necessary, and no trailing was observed. In six strains showing discrepancy between the NCCLS and colorimetric assays, all MICs were lower in the colorimetric assay. However, there is no standard assessing susceptibility or resistance to a specific MIC, even in the NCCLS guidelines (5). Therefore, we could not estimate the differences as a major or minor error.

In conclusion, the colorimetric method using STC was objective and easy to interpret and showed high levels of agree-
ment with the NCCLS method for assessing sensitivity to ketoconazole and itraconazole.

REFERENCES