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Flucytosine과 세 Azole의 Spectrophotometric Broth Microdilution법을 이용한 항진균제 감수성 검사를 위한 최적의 파장

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The Optimal Wavelength of Spectrophotometric Broth Microdilution Antifungal Susceptibility Test for Flucytosine and Three Azoles

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Background : There is no guideline for the appropriate wavelength at which to measure the optical density (OD) value in broth microdilution antifungal susceptibility testing, although a spectrophotometric reading method is commonly used. The present study aimed to analyze the difference in the OD values over the range of visible light and to ascertain the optimal wavelength for the spectrophotometric method of microdilution testing.

Methods : We measured the OD of background blank controls of broth medium, antifungal agents, and inocula of five type strains using a Synergy HT multi-detection microplate reader at 5-nm intervals from 380 nm to 760 nm. We also estimated the OD differences between the 50% of growth control and blank control.

Results : The OD of the blank control showed a parabola shape with two peaks and steadily decreased at longer wavelengths. The curves of the antifungal agent were similar to those of blank controls, and the influence of each antifungal agent on the OD was minimal. For the difference in OD between 50% of growth control and the blank control, the curve was the opposite of the blank control, and the OD increased steadily at the wavelengths above 600 nm.

Conclusions : The range between 600 nm and 700 nm was the optimal wavelength for broth microdilution antifungal susceptibility testing, although any wavelength within the visible light spectrum can be used. *(Korean J Lab Med 2009;29:324-30)*

Key Words : *Antifungal susceptibility test, Broth microdilution method, Spectrophotometer, Wavelength*

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INTRODUCTION

In vitro antifungal susceptibility tests provide a reliable measure of the relative activities of various drugs.

They predict the likely outcome of therapy, provide the means with which to monitor the development of drug resistance, and reveal the therapeutic potential of investigational agents [1-3]. The CLSI published reference guidelines for susceptibility testing of *Candida* species and *Cryptococcus neoformans* as M27-A2 in 2002 [4] and revised it as M27-A3 in 2008 [5]. However, this standard method for broth dilution susceptibility testing of yeasts still has some problems in the determination of the minimum inhibitory concentration (MIC). Especially for flucytosine and the azoles, endpoints are typically less well defined than for amphotericin B, which may be a significant source of variability in a broth microdilution test [5]. It is recommended that the MIC for azoles and flucytosine be defined as the lowest concentration at which a prominent decrease in turbidity is observed.

Recently, several alternative methods such as flow cytometry [6, 7], E test [8-10], colorimetric assay [11-13], and spectrophotometric reading of broth microdilution [14, 15] have been used in an attempt to obtain an objective and convenient interpretation of antifungal susceptibility results. Among these options, a modified broth microdilution format using spectrophotometry complemented by visual reading was accepted in CLSI M27-A2 for determining the MICs of such organisms, which is the first well that shows a 50% decrease in optical density (OD) relative to the drug-free growth control well [4]. This spectrophotometric reading provides an objective result concordant with traditional visual reading. However, we found that the wavelength for spectrophotometric reading was different in various articles, and the reason for the choice of a specific wavelength was not specified. Also, there are no guidelines for the appropriate wavelength to measure OD value anywhere in CLSI M27-A2. So, the present study aimed to analyze the difference of OD in relation to the measured wavelength at 5-nm intervals between 380 nm and 760 nm and to ascertain the optimal wavelength for determination of the MIC in spectrophotometric broth microdilution antifungal susceptibility testing of flucytosine and three azoles.

MATERIALS AND METHODS

We tested the ODs of the broth medium, RPMI 1640-morpholinopropanesulfonic acid (MOPS), at different wavelengths to determine the value of the background blank control. The medium was identical to those used for the broth microdilution antifungal susceptibility test; the absence of an antifungal agent and yeast inoculum were the only differences. We selected the range from 380 to 760 nm because it is within the visible spectrum and is used most commonly in the laboratory. The OD measurement was achieved by a Synergy HT multi-detection microplate reader (Bio-Tek, Winooski, VT, USA) at 5-nm intervals.

The influence of an antifungal agent on the OD is also of great importance. To confirm the influence of antifungal agents themselves, we included four agents, flucytosine, itraconazole, ketoconazole, and fluconazole. Stock solutions of itraconazole and ketoconazole were prepared by dissolving the powder in 100% dimethylsulfoxide), whereas fluconazole and flucytosine were dissolved in sterile distilled water. The solution was divided into 1-mL aliquots at a concentration of 1,280 μg/mL and frozen at -70℃ 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 3-N-MOPS. The broth microdilution testing was performed according-NMOPS to the guidelines of the CLSI using 96-well microplates [4]. The plates contained serial dilutions of the antifungal agents without a yeast inoculum. The final concentrations were 0.125-16 μg/mL for itraconazole and ketoconazole and 0.125-64 μg/mL for flucytosine and fluconazole. The blank control well contained 200 μL of RPMI 1640 medium. The OD for each concentration was likewise measured by the microplate reader between 380 and 760 nm.

The growth control well contained RPMI 1640-MOPS medium and dilutions of yeast inoculum without antifungal agent. This growth control is used to assess the viability of the organism in antifungal susceptibility testing and also serves as a turbidity control for reading endpo-

ints. We used five type strains of Candida species (*C. albicans* ATCC 64550, *C. albicans* ATCC 90028, *C. parapsilosis* ATCC 22019, *C. tropicalis* ATCC 20138, and *C. krusei* ATCC 6258). The isolates were stored in skim milk at −70℃ until used. They were grown on Sabouraud dextrose agar for 24 hr at 35℃ prior to testing, and the inoculum preparation followed the directions of document M27-A2 of the CLSI guidelines [4]. Five colonies with diameters of more than 1 mm on Sabouraud dextrose agar were suspended in sterile 0.85% saline and adjusted to a final concentration of 0.5-2.5×10^3/mL in RPMI 1640-MOPS. The microplates contained only the inocula of the five type strains with RPMI 1640. After incubation at 35℃ for 48 hr, we measured the OD of the growth control well between 380 and 760 nm. All procedures were repeated twice for reproducibility.

The MIC value of flucytosine and the three azoles corresponds to the first well that shows a 50% decrease relative to the OD in the drug-free growth control well. We verified the difference between the half OD of the growth control and the OD of the blank control to seek the optimal wavelength for spectrophotometric antifungal susceptibility testing.

RESULTS

The continuous OD curve of the blank control well is shown in Fig. 1. The curve was a parabola with two peaks.

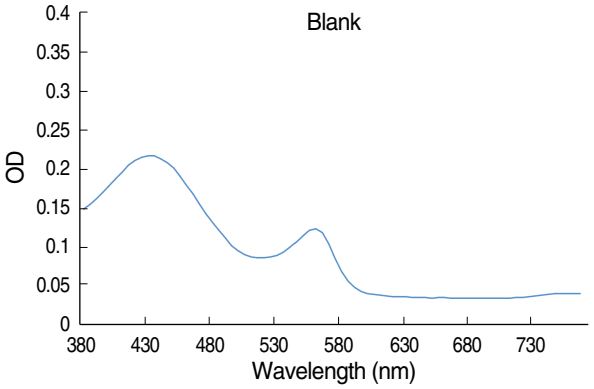

Fig. 1. Absorbance spectrum curve of blank control well.
Abbreviation: OD, optical density.

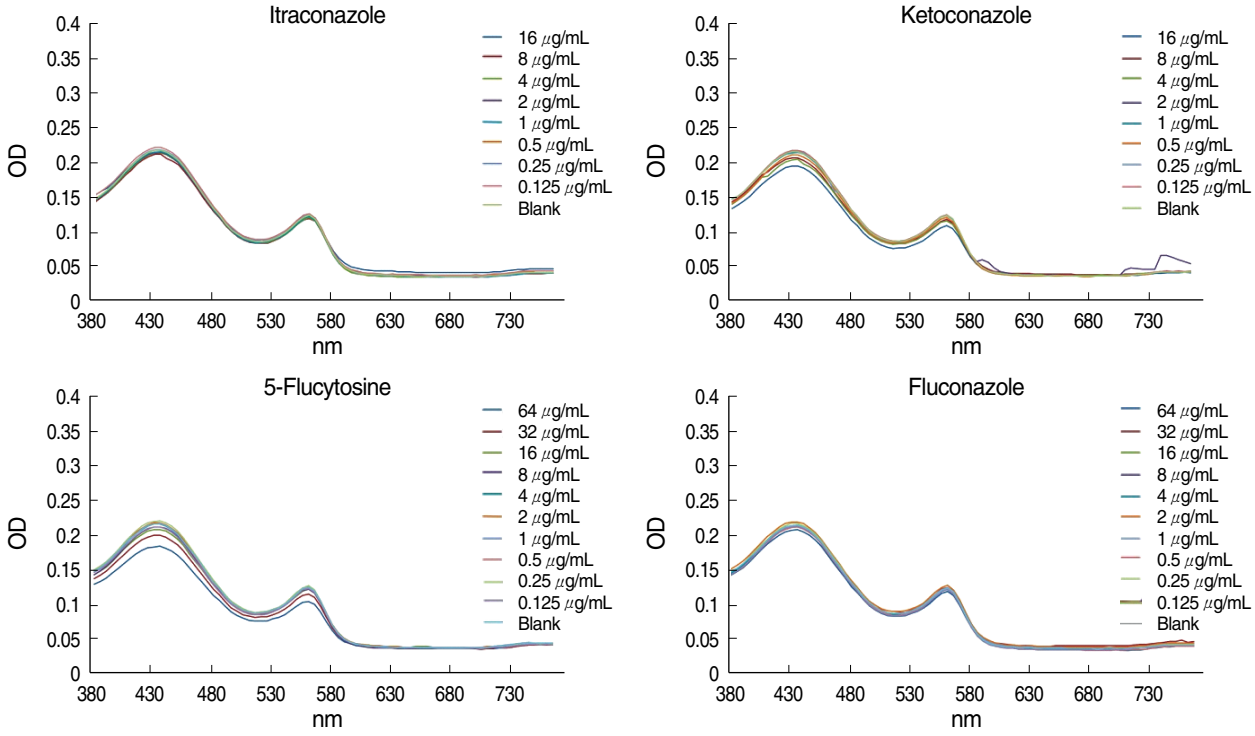

Fig. 2. Absorbance spectrum curves of antifungal agent-containing wells.
Abbreviation: See Fig. 1.

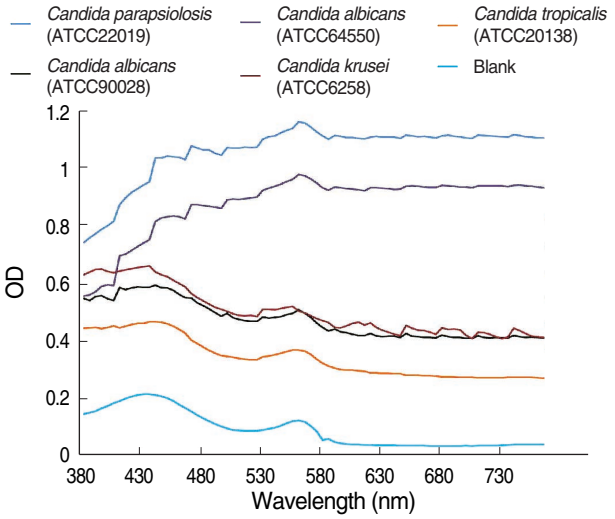

Fig. 3. Absorbance spectrum curves according to wavelength of five type strains after incubation at 35°C for 48 hr.
Abbreviation: See Fig. 1.

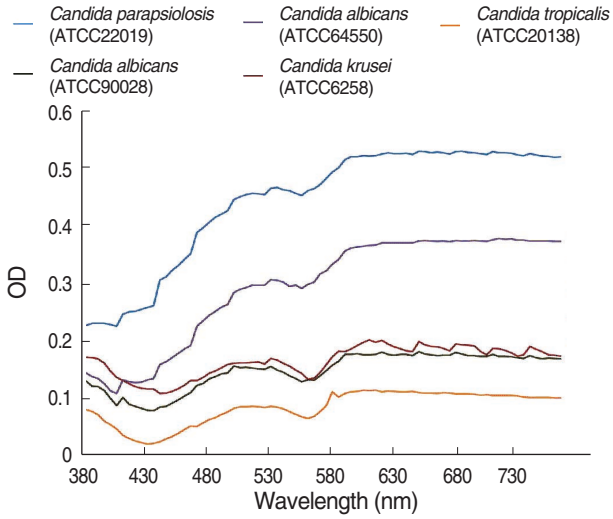

Fig. 4. Difference between half OD of growth control and the OD of the blank control.
Abbreviation: See Fig. 1.

The OD generally was higher at shorter wavelengths and lower at longer wavelengths. The OD at the starting wavelength, 380 nm, was relatively high. The values increased steadily to 435 nm and then decreased until the wavelength reached 520 nm. The values increased at 560 nm and decreased at about 600 nm. The OD values of second parabola and peak were lower than those of the first parabola and peak. The OD remained unchanged above 600 nm.

The ODs of four antifungal agent-containing wells with the previously mentioned concentrations are shown in Fig. 2. The shape and value of the curves for the wells containing itraconazole and fluconazole were nearly the same as those of the blank control well, whereas the values for flucytosine and ketoconazole were slightly higher than those of the blank control well in the two peak regions. However, the ODs had no relation to the drug concentrations of flucytosine and ketoconazole, and the difference was minor, so it could be ignored. The influence of these four antifungal agents on the OD was minute at any wavelength between 380 and 760 nm.

The final MICs are the concentrations in the wells showing a 50% reduction relative to the OD in the drug-free growth control well. So we could presume that the wavelength showing a high OD for growth control and the greatest difference between the growth control and the blank wells would be the most suitable for the susceptibility test. The ODs of five type strains were sufficiently high compared with those of the blank control (Fig. 3). For two strains, *C. albicans* ATCC 64550 and *C. parapsilosis* ATCC 22019, the ODs increased steadily as the wavelength became longer, but the OD curve patterns from other three strains, namely, *C. albicans* ATCC 90028, *C. krusei* ATCC 6258, and *C. tropicalis* ATCC 20138, were similar to that of the blank control and could be attributable to the broth medium. The difference in the half OD of growth control and the OD of the blank control showed a characteristic feature resembling a blank control curve upside down (Fig. 4). For all type strains, it was decreased to the range of 380-440 nm, increased at wavelengths above 440 nm with a lower peak at 560 nm, and then increased steadily up to 560 nm. Although all ranges from 380 to 760 nm could be used for broth microdilution testing, we ascertained that the optimal wavelengths would be between 600 and 700 nm because the differences were exceedingly larger after 600 nm.

DISCUSSION

In vitro 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 [16, 17]. This has led to increasing standardization of the reference methods for *Candida* species in CLSI M27-A2 published in 2002, and for both *Candida* species and *C. neoformans* in the third revised CLSI M27-A3 published in 2008 [4, 5]. These methods have improved the reproducibility of in vitro susceptibility data and facilitated the establishment of interpretive breakpoints for antifungal agents.

Traditionally, the final MIC value is determined at the endpoint showing a prominent decrease in turbidity. However, this technique introduces a subjective decision that causes variable results from person to person and between laboratories [18]. To overcome this limitation, several modifications have been adopted as broth-based alternative approaches that may better serve practical clinical laboratory needs; such as spectrophotometric method [14, 15], colorimetric method [11-13], flow cytometry [6, 7], and E test [8-10]. Among these, the spectrophotometric reading of broth microdilution tests provides a more accurate and objective result and eliminates the subjective judgments that can confuse visual assessment of MIC endpoints [15]. Consequently, the broth microdilution method with spectrophotometric reading, which provided the least variable results among the different laboratories, is included in the CLSI as a reference method for the antifungal susceptibility testing of yeasts. The method has been in common use for several years.

It is important to select the optimal wavelength when spectrophotometry is used. However, in previous articles, the wavelength was diverse: 405 nm [15, 19], 450 nm [20], 492 nm [14], 530 nm [21, 22], and 570 nm [23]. There was no reason explained for the selection of a specific wavelength. Also, there has been no recommendation in the CLSI guideline for a standard wavelength. So, we measured the ODs of microplate wells at 5-nm intervals within the range of 380-760 nm, an area of visible light, by spectrophotometer to assess the optimal wavelength for MIC endpoint determination.

The blank control well contained only broth medium. The OD of this control is the background value, so the lower OD is more appropriate in microdilution susceptibility testing with spectrophotometric reading. In this study, the continuous OD curve of the blank control revealed a parabola shape with two peaks and decreased gradually with increasing wavelength. The continuous OD curve of the antifungal agent wells without yeast inocula closely resembled the pattern of the blank control. The ODs of flucytosine and ketoconazole were slightly greater than those of the blank controls. This could be caused by the color, the constituent, and the solvent of antifungal agents. We believed that this would not influence the determination of MIC because the increase is minute compared with those of the growth controls containing yeast. However, it could influence the final results if the OD difference between the well with a 50% decrease in growth and the blank control is small. We considered this point when selecting the optimal wavelength.

The growth control of five type strains revealed two characteristic patterns of the OD curves. We could not determine why these differences were observed, although we thought that it could be secondary to the difference of growth occasioned by various factors according to *Candida* species. However, for all type strains, the difference between the half OD of growth control and the OD of the blank control showed the same pattern. It decreased slightly to 435 nm, the lowest OD value. After that, the ODs increased gradually and were steady above 590 nm. If the OD of the growth control is sufficiently high, any wavelength between 380 nm and 760 nm could be used for susceptibility testing; however, if the OD of the growth control is low, the determination of the MIC could be difficult, and we should consider the influences of the blank control and the antifungal agents.

In conclusion, we suggest that the range between 600 nm and 700 nm is the optimal wavelength to determine the MIC, because the OD of the blank control well was steady and the lowest, and the difference between the OD of the growth control and that of the blank was the highest at this range of wavelength.

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