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Comparison of a 2,3-Bis(2-Methoxy-4-Nitro-5-Sulfophenyl)-5-[(Phenylamino)Carbonyl]-2*H*-Tetrazolium Hydroxide (XTT) Colorimetric Method with the Standardized National Committee for Clinical Laboratory Standards Method of Testing Clinical Yeast Isolates for Susceptibility to Antifungal Agents

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MICs for clinical *Candida* and *Cryptococcus* isolates were determined by a method incorporating the colorimetric indicator 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2*H*-tetrazolium hydroxide (XTT), and the results were compared with MICs obtained by the National Committee for Clinical Laboratory Standards approved standard method (M27-A). One hundred percent of all isolates demonstrated agreement within 2 dilutions between the MICs of amphotericin B, fluconazole, itraconazole, ketoconazole, and flucytosine obtained by the two methods. These data suggest that an XTT-based method could provide a useful means for the determination of antifungal susceptibility of yeasts.

The National Committee for Clinical Laboratory Standards (NCCLS) has recently developed a standardized broth-based method (M27-A) of testing pathogenic yeasts for susceptibility to antifungal agents (4). Although this method demonstrates good intra- and interlaboratory agreement, MICs of fungistatic agents such as fluconazole and itraconazole are often difficult to determine because of trailing endpoints caused by the partial inhibition of fungal growth. In attempts to overcome this shortcoming, investigators have developed a number of alternative testing methods, including modifications of the NCCLS method (8) or reading the MIC endpoints in a different manner, such as by spectrophotometry (7) and colorimetry (5, 6, 11). Colorimetric methods are attractive, since they have the potential to generate clear-cut endpoints, based on a visually detectable color change. A colorimetric method utilizing the oxidation-reduction colorimetric indicator Alamar blue, for example, has been shown to generate clear-cut endpoints for fluconazole and itraconazole for yeast clinical isolates and to yield MICs that correlate well with those determined by the NCCLS standard method (5, 6, 11). An alternative colorimetric method utilizes the tetrazolium salt 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2*H*-tetrazolium hydroxide (XTT). XTT is a yellow salt that is reduced by mitochondrial dehydrogenases of metabolically active yeast cells to a dark blue XTT formazan product (2, 10). This color change can be assessed visually, similar to the Alamar blue method. In order to evaluate the potential utility of an XTT colorimetric method, we determined the MICs of five antifungal agents for clinical yeast isolates and compared them to those obtained by the standard NCCLS microtiter method (M27-A).

Clinical isolates of *Candida* spp. and *Cryptococcus neoformans* were obtained from the Center for Medical Mycology, Cleveland, Ohio, and included *Candida albicans* ($n = 29$), *C. tropicalis* ($n = 17$), *C. lusitaniae* ($n = 11$), *C. guilliermondii* ($n = 6$), *C. glabrata* ($n = 12$), *C. parapsilosis* ($n = 18$), *C. krusei* ($n = 5$), and *C. neoformans* ($n = 20$). *C. parapsilosis* ATCC 22019 and *C. krusei* ATCC 6258 were tested concurrently as quality control (QC) isolates. Fungi were maintained on Sabouraud dextrose agar. Prior to susceptibility testing, the isolates were placed on fresh agar plates and grown for 24 h at 35°C.

The susceptibility of each isolate to amphotericin B (Sigma Chemical Co., Saint Louis, Mo.), fluconazole (Pfizer Pharmaceuticals Group, New York, N.Y.), itraconazole and ketoconazole (Janssen Pharmaceuticals, Beerse, Belgium), and flucytosine (Sigma) was assessed in two ways. (i) Susceptibility testing was performed with 96-well round-bottom tissue culture plates as described in the M27-A procedure for the susceptibility testing of yeasts. The MIC was read visually as described in the NCCLS M27-A document (4). (ii) The second assay of susceptibility testing was a modification of the previously described XTT colorimetric method (2). Briefly, the susceptibility plates were prepared as for the NCCLS method. Three hours prior to the endpoint reading, the plates were agitated, and 50 μl of an XTT-phenazine methosulfate (PMS) mix (final concentrations of 200 μg/ml for XTT and 19.13 μg/ml for PMS [both pur-

TABLE 1. Comparison of NCCLS- and XTT-based MIC data for antifungal susceptibility testing of QC strains of *Candida*

Antifungal agent	MIC (μg/ml) for strain by method			
	C. parapsilosis ATCC 22019		*C. krusei* ATCC 6258	
	NCCLS	XTT	NCCLS	XTT
Amphotericin B	0.25–0.5	0.25	1.0–2.0	2
Fluconazole	1.0–2.0	1.0–2.0	32–64	64
Itraconazole	0.25	0.25	0.25	0.25–0.5
Ketoconazole	0.06–0.125	0.06–0.13	0.5	0.5
Flucytosine	0.25–0.5	0.125–0.25	4.0–8.0	4.0–8.0

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TABLE 3. Agreement between XTT- and NCCLS-derived MICs

Species (no. of isolates) and antifungal agent	% Agreement within 1 well (no. with agreement/no. tested)[a]
C. albicans (29)	
Amphotericin B	97 (28/29)
Fluconazole	94 (27/29)
Itraconazole	94 (27/29)
Ketoconazole	97 (28/29)
Flucytosine	100 (29/29)
C. glabrata (12)	
Amphotericin B	100 (12/12)
Fluconazole	100 (12/12)
Itraconazole	100 (12/12)
Ketoconazole	100 (12/12)
Flucytosine	100 (12/12)
C. tropicalis (17)	
Amphotericin B	100 (17/17)
Fluconazole	100 (17/17)
Itraconazole	100 (17/17)
Ketoconazole	100 (17/17)
Flucytosine	100 (17/17)
C. guilliermondii (6)	
Amphotericin B	100 (6/6)
Fluconazole	100 (6/6)
Itraconazole	100 (6/6)
Ketoconazole	100 (6/6)
Flucytosine	100 (6/6)
C. parapsilosis (18)	
Amphotericin B	89 (16/18)
Fluconazole	89 (16/18)
Itraconazole	72 (13/18)
Ketoconazole	78 (14/18)
Flucytosine	83 (15/18)
C. lusitaniae (11)	
Amphotericin B	91 (10/11)
Fluconazole	91 (10/11)
Itraconazole	45 (5/11)
Ketoconazole	64 (7/11)
Flucytosine	91 (10/11)
C. krusei (5)	
Amphotericin B	80 (4/5)
Fluconazole	80 (4/5)
Itraconazole	100 (5/5)
Ketoconazole	100 (5/5)
Flucytosine	100 (5/5)
C. neoformans (20)	
Amphotericin B	100 (20/20)
Fluconazole	100 (20/20)
Itraconazole	100 (20/20)
Ketoconazole	100 (20/20)
Flucytosine	100 (20/20)

[a] Agreement is expressed as the percentage of isolates for which XTT MICs were within 1 dilution of the NCCLS MIC. Note that there was 100% agreement for all isolates and agents of XTT MICs within 2 dilutions of the NCCLS MIC.

chased from Sigma]) was added to all wells. The plates were incubated for 3 h at 35°C to allow for color development. The MIC in the XTT assay was determined visually as the lowest concentration of antifungal agent at which no color change occurred.

The XTT MICs of the NCCLS QC strains correlated strongly with those obtained by the M27-A method (Table 1) and were confirmed in duplicate and by retesting of these organisms up to five times on different days (Tables 1 and 2). Furthermore, the ranges of MICs obtained by the XTT and NCCLS methods were almost identical for all *Candida* spp. and *C. neoformans* (Table 2).

In order to more closely analyze the agreement between the

TABLE 2. Comparison of NCCLS- and XTT-based MIC data for antifungal susceptibility testing of clinical isolates of *Candida* and *C. neoformans*

Antifungal agent	MIC (μg/ml) for isolate (no. tested) by method																					
	C. albicans (29)		*C. glabrata* (12)		*C. tropicalis* (17)		*C. guilliermondii* (6)		*C. parapsilosis* (18)		*C. lusitaniae* (11)		*C. krusei* (5)		*C. neoformans* (20)	
	NCCLS	XTT	NCCLS	XTT	NCCLS	XTT	NCCLS	XTT	NCCLS	XTT	NCCLS	XTT	NCCLS	XTT	NCCLS	XTT
Amphotericin B	0.25–4.0	0.25–4.0	0.5–2	0.5–2	0.5–2	0.5–2	0.25–1	0.5–1	0.125–2	0.25–1	0.125–0.5	0.06–0.5	1.0–2.0	0.5–2.0	0.5–2	0.25–2.0
Fluconazole	0.125–>256	0.25–>256	8–256	8–128	0.25–>256	0.25–>256	2.0–16.0	2.0–32	1.0–64	0.5–32	2.0–8.0	2.0–8.0	32–128	32–64	2.0–>256	1.0–>256
Itraconazole	0.125–>16	0.125–>16	0.5–>16	0.5–>16	0.06–>16	0.06–>16	0.25–4	0.125–4	0.125–1	0.06–0.5	0.06–1	0.06–1	0.125–16	0.125–16	0.125–>16	0.06–>16
Ketoconazole	0.03–>16	0.015–16	0.5–4.0	0.5–8	0.015–16	0.015–16	0.125–2	0.125–4	0.125–0.5	0.03–0.125	0.03–0.5	0.06–0.5	0.25–8.0	0.5–8.0	0.25–>16	0.125–>16
Flucytosine	0.125–>64	0.125–>64	0.03–1	0.03–0.5	0.03–64	0.03–64	0.03–0.25	0.03–0.25	0.125–0.5	<0.03–0.125	0.03–>64	0.06–>64	2.0–8.0	1.0–8.0	0.25–0.5	0.125–1.0

XTT and NCCLS readings, agreements were expressed as percentage agreement in terms of (i) having the same MIC or a MIC within 1 dilution or (ii) having a MIC within 2 dilutions. Overall, there was an excellent agreement between the two MIC readings. For example, for *C. albicans* isolates, the levels of agreement were 97% for amphotericin B, 94% for itraconazole, 100% for flucytosine, 94% for fluconazole, and 97% for ketoconazole, as determined for those isolates for which the XTT-based MIC was either the same as or within 1 dilution of the NCCLS MIC (Table 3). Furthermore, the remainder of the XTT MICs fell within 1 dilution of the NCCLS MICs (Table 3).

Analysis of the agreement between the XTT and NCCLS MICs for the non-*C. albicans* species indicated that 100% of the *C. glabrata*, *C. tropicalis*, and *C. guilliermondii* isolates exhibited XTT MICs that were equal to or within 1 dilution of the NCCLS MIC (Table 3). Agreement was also excellent for *C. krusei* (80 to 100%). However, agreement was more variable for *C. parapsilosis* and *C. lusitaniae* isolates, 72 to 89% and 45 to 91% of which exhibited agreement within 1 dilution, with the remainder of the isolates exhibiting agreement within 2 dilutions (Table 3). With regard to *C. neoformans*, all of the XTT MICs fell within 1 dilution, suggesting 100% agreement between the two methods.

Tetrazolium salts have previously been used to determine the metabolic rates of higher eukaryotic cells and to assess the effects of cytotoxic agents (3), to provide a nondestructive and continuous spectrophotometric measurement of cell respiration (9), and to describe differences between the susceptibilities of adherent and nonadherent *Candida* cells (2). The M27-A MIC (4) is a useful means for the determination of MICs, although some difficulty may still be encountered in determining the MICs of certain agents, for example, azoles. Therefore, in the search for a sensitive method that assists the reading of MICs, we decided to develop the XTT assay as a colorimetric visual method. Our data show that the advantage of the XTT MIC is that one reads a colored endpoint, making reading of MICs easier. In contrast, the disadvantage of the XTT-based method is that it requires the additional step in order to add the XTT-PMS mix to the microtiter plate wells.

In this study, we have shown that agreement between the XTT and NCCLS MICs was excellent for the majority of the *Candida* isolates tested. For example, all of the XTT MICs fell within 2 dilutions compared to the NCCLS MICs, thus representing 100% agreement between the two methods. In addition, comparison of the MIC ranges further demonstrated the strong correlation between the two assay procedures. The only case of modest variation between these MIC determinations was observed for certain strains of *C. parapsilosis* and *C. lusitaniae*. The small variation among these isolates was not unexpected, given that previous studies with the Alamar blue reagent also showed some variation in comparison to the NCCLS reading (11). The XTT MIC was also useful for determining the susceptibility of *C. neoformans*. Excellent agreement between the values obtained by the two methods was observed.

In conclusion, the XTT-based method could prove to be a useful alternative to the NCCLS method. Taken together, alternative means of reading the MICs for yeast isolates such as the XTT assay, Alamar blue (5, 6), and the E-test (1) would provide a choice to the clinical microbiology laboratory when performing antifungal susceptibility testing of yeasts. Work comparing the XTT and NCCLS methods to determine the suitability of using an XTT-based method for the evaluation of the antifungal susceptibilities of filamentous fungi is currently under way.

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