ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, May 2002, p. 1455–1461 Vol. 46, No. 5
0066-4804/02/$04.00+0 DOI: 10.1128/AAC.46.5.1455–1461.2002

Potato Dextrose Agar Antifungal Susceptibility Testing for Yeasts and Molds: Evaluation of Phosphate Effect on Antifungal Activity of CMT-3

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Received 21 August 2001/Returned for modification 29 October 2001/Accepted 20 November 2001

The broth macrodilution method (BMM) for antifungal susceptibility testing, approved by the National Committee for Clinical Laboratory Standards (NCCLS), was found to have deficiencies in testing of the antifungal activity of a new type of antifungal agent, a nonantibacterial chemically modified tetracycline (CMT-3). The high content of phosphate in the medium was found to greatly increase the MICs of CMT-3. To avoid the interference of phosphate in the test, a new method using potato dextrose agar (PDA) as a culture medium was developed. Eight strains of fungi, including five American Type Culture Collection strains and three clinical isolates, were used to determine the MICs of amphotericin B and itraconazole with both the BMM and the PDA methods. The MICs of the two antifungal agents determined with the PDA method showed 99% agreement with those determined with the BMM method within 1 $\log_2$ dilution. Similarly, the overall reproducibility of the MICs with the PDA method was above 97%. Three other antifungal agents, fluconazole, ketoconazole, and CMT-3, were also tested in parallel against yeasts and molds with both the BMM and the PDA methods. The MICs of fluconazole and ketoconazole determined with the PDA method showed 100% agreement within 1 $\log_2$ dilution of those obtained with the BMM method. However, the MICs of CMT-3 determined with the BMM method were as high as 128 times those determined with the PDA method. The effect of phosphate on the antifungal activity of CMT-3 was evaluated by adding Na_2HPO_4 to PDA in the new method. It was found that the MIC of CMT-3 against a *Penicillium* sp. increased from 0.5 μg/ml (control) to 2.0 μg/ml when the added phosphate was used at a concentration of 0.8 mg/ml, indicating a strong interference of Na_2HPO_4 with the antifungal activity of CMT-3. Except for fluconazole, all the other antifungal agents demonstrated clear end points among the yeasts and molds tested. Nevertheless, with its high reproducibility, good agreement with NCCLS proposed MIC ranges, and lack of interference of phosphate, the PDA method shows promise as a useful assay for antifungal susceptibility testing and screening for new antifungal agents, especially for drugs that may be affected by high (supraphysiologic) phosphate concentrations.

Fungal infections have been reported to have dramatically increased in the past decade, and these often occur as systemic infections or as coinfections with other diseases, such as AIDS or cancer, or in patients who are immunocompromised (1, 2, 5, 18). In fact, candidiasis has become the most common infection in AIDS and cancer patients. Unfortunately, in addition to the limited number of antifungal drugs currently available, fungal infections tend to rapidly develop resistance to these drugs. For these reasons, fungal infections now show much higher mortality rates than bacterial infections (19).

The rapid increase in fungal infections and the growing number of new antifungal agents indicate an increasing need for rapid and accurate methods for antifungal screening and susceptibility testing. The National Committee for Clinical Laboratory Standards (NCCLS) recently approved a standardized method for antifungal susceptibility testing of yeasts, M27-A (7), and proposed a method for testing of filamentous fungi, M38-P (8), based on a broth macrodilution method (BMM). Subsequently, several modifications of these methods were proposed, including flow cytofluorometric detection (9, 20), colorimetric microdilution (4, 10), E-test (13, 16), modified agar dilution (21), semisolid agar test (14), and capacitance method (3). Most of these methods were developed for the determination of MICs of agents against yeasts.

CMTs are derivatives of tetracycline in which the dimethylamino group at position 4 of the multiple rings has been removed. They show no effective antibacterial activity, unlike the parent drug, tetracycline, but do exhibit other therapeutic properties, such as the abilities to inhibit host-derived tissue destructive enzymes, the matrix metalloproteinases, and to reduce the levels of proinflammatory cytokines, such as interleukin 1β and tumor necrosis factor alpha (6, 15, 17). In the experiments described below, we unexpectedly found that the nonantibacterial CMTs exhibited antifungal activity in vitro and that this activity was severely compromised by the high (nonphysiologic) level of phosphate in RPMI 1640 medium, which significantly increased the MICs of this new group of antifungal agents. As a result, a new and rapid method using potato dextrose agar (PDA) as a culture medium was developed for antifungal susceptibility testing of CMTs. Therefore, the purpose of the current study was to evaluate the reproducibility and accuracy of this new method compared to those of the NCCLS macrodilution methods (M27-A and M38-P), to evaluate the effects of phosphate on the antifungal activity of

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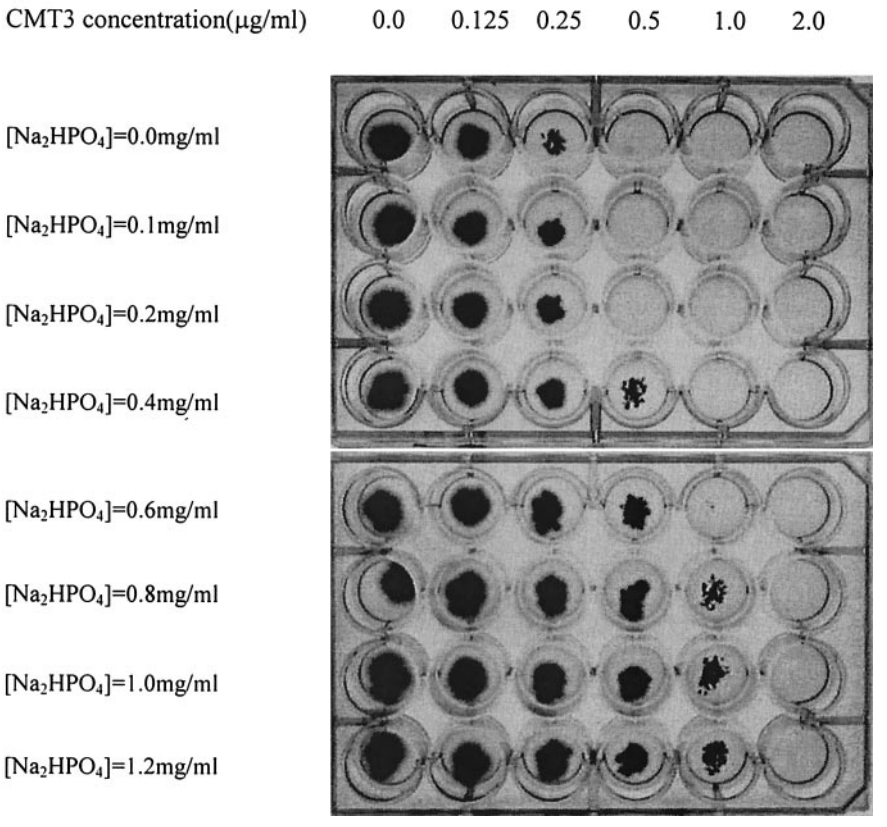

FIG. 1. Effects of phosphate on the antifungal activity of CMT-3 against a *Penicillium* sp. The 24-well plate was filled with PDA containing different concentrations of added phosphate and CMT-3, inoculated with a *Penicillium* sp., and incubated at 35°C for 48 h. Wells in the first lane (without CMT-3) were used as negative controls. The MICs were determined to be 0.5, 0.5, 1.0, and 2.0 μg/ml at phosphate concentrations of 0.0, 0.2, 0.4, and 0.8 mg/ml, respectively.

CMT-3, and to demonstrate the usefulness of the new method as an antifungal susceptibility test for both yeasts and molds.

MATERIALS AND METHODS

Study design. The in vitro studies described below assessed (i) the effect of different concentrations of phosphate in the culture medium on the antifungal activity of CMT-3; (ii) the reproducibility of the new method (PDA method) with amphotericin B (AMB) and itraconazole as antifungal agents against both yeasts and molds; (iii) the accuracy of the PDA method compared to that of the NCCLS model; and (iv) the feasibility of the PDA method for measuring the MICs of other antifungal agents, such as fluconazole, ketoconazole, and CMT-3.

Fungal isolates. Four yeast strains, i.e., *Candida parapsilosis* ATCC 22019, *Candida tropicalis* ATCC 750, *Candida krusei* ATCC 6258, and *Candida albicans* ATCC 24433, and strains of four filamentous fungi, i.e., a *Penicillium* sp, *Aspergillus flavus*, *Aspergillus fumigatus* ATCC 1022, and a *Rhizopus* sp, were used to evaluate the reproducibility of the PDA method and its accuracy by comparing the MIC ranges obtained by this method and the NCCLS model. Other fungal isolates, such as *Pseudallescheria boydii*, *Paecilomyces variotii* ATCC 22319, *Yarrowia lipolytica*, and *Candida glabrata*, were also used to test the feasibility of the PDA method. Except for those strains described as being from the American Type Culture Collection, the fungi used in this experiment were clinical isolates from the Clinical Microbiology Laboratory, Department of Laboratories, University Hospital and Medical Center, State University of New York at Stony Brook.

All fungal strains were freshly cultured aerobically on PDA slants at 35°C for 48 to 72 h (or until they reached full growth). The yeast cells or fungal spores were collected with a sterile cotton-tipped applicator and suspended in sterile water, and the concentrations were adjusted to 5×10^3 to 2×10^4/ml before use in vitro in the susceptibility tests for the various antifungal agents.

Reagents and antifungal agents. PDA, MOPS [3(*N*-morpholino)propanesulfonic acid], RPMI 1640 medium, and AMB were purchased from Sigma Chemical Co. (St. Louis, Mo.). Itraconazole and ketoconazole were purchased from Research Diagnostics Inc. (Flanders, N.J.), fluconazole was obtained from ICN Biomedicals Inc. (Aurora, Ohio), and CMT-3 was provided by Collagenex Pharmaceutical, Inc. (Newtown, Pa.).

All antifungal agents were dissolved in dimethyl sulfoxide (DMSO) as 100× stock solutions just before use. The concentrations of the antifungal agents for use in the BMM were as follows: AMB, itraconazole, and ketoconazole at 0.03 to 16 μg/ml and fluconazole and CMT-3 at 0.06 to 64 μg/ml. For use in the PDA method, the concentrations of CMT-3 were 0.03 to 16 μg/ml, and all other agents were used at the same concentrations as in the BMM.

Determination of in vitro antifungal susceptibility. The experimental procedure for the BMM was carried out as described in the M27-A method for yeasts or the M38-P method for molds.

The procedure for the PDA method was as follows. PDA powder was dissolved in distilled water to a final concentration of 39g/liter and then sterilized at 121°C for 15 min. The sterilized PDA solution was placed in a water bath, and the temperature was cooled to and maintained at 55 to 60°C. The antifungal agent stock solutions (100×, dissolved in DMSO) were mixed with the PDA solution to produce a series of different final concentrations as described above. Drug-free agar containing only 1% DMSO was used as a control. The mixtures of antifungal agent and PDA solutions were poured directly into the wells (1.0 ml/well for 24-well plates or 0.5 ml/well for 48-well plates). After the plates were cooled to room temperature, 5 μl (for 48-well plates) or 10 μl (for 24-well plates) of freshly made fungal suspension (5×10^3 to 2×10^4/ml) was inoculated onto the agar of each well. The plates were incubated aerobically at 35°C until the fungi of the control cultures (containing no drug) reached a growth sufficient for identification, i.e., 48 h for yeasts and most filamentous fungi; a longer time was needed for slower-growing fungi (e.g., 72 h for *P. boydii*) until the visible colony

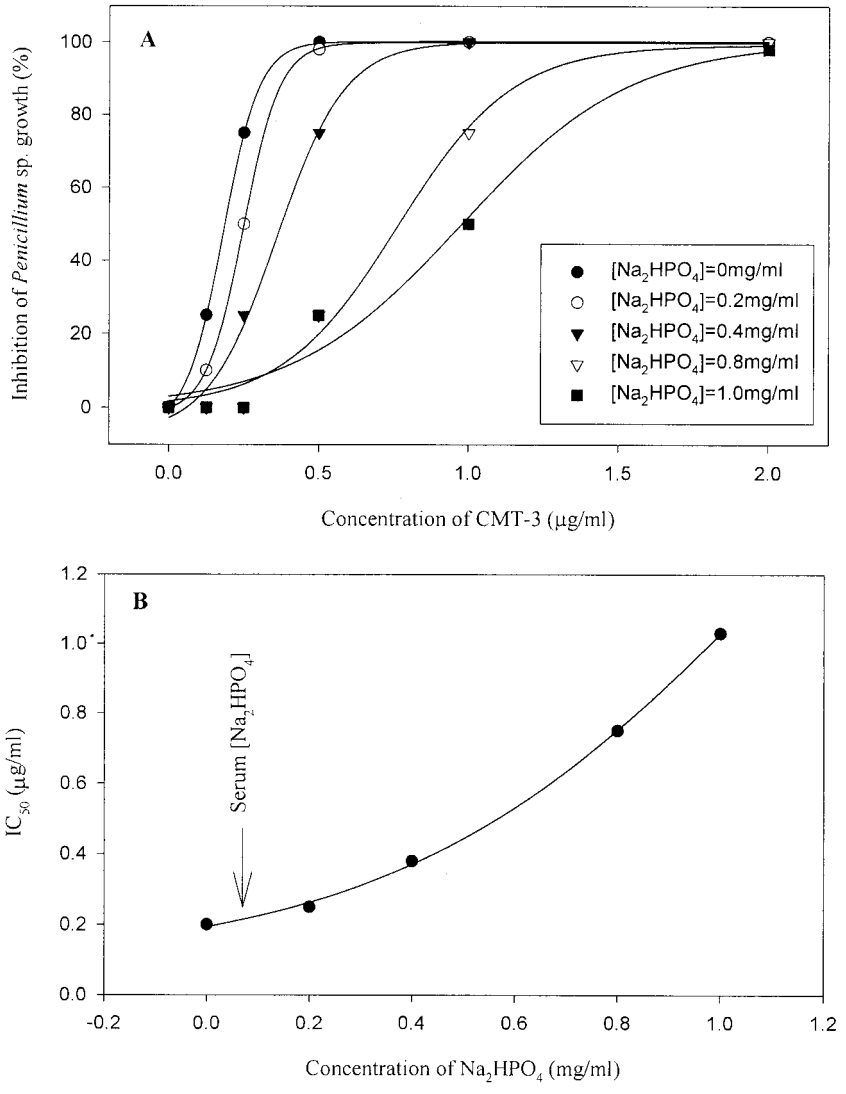

FIG. 2. Evaluation of the effects of phosphate on the antifungal activity of CMT-3 against a *Penicillium* sp. (A) Inhibition curves for CMT-3 against the fungus at different concentrations of added phosphate in PDA. (B) IC_{50}s of CMT-3 against the fungus. The IC_{50}s increased in a dose-dependent manner with the concentration of phosphate, showing a strong interference of phosphate with the antifungal activity of CMT-3.

size of the control cultures was evident. A four-grade scoring system was used (i.e., the control cultures were scored as +4; no detectable fungal growth was scored as 0; and +3, +2, and +1 represented 75, 50, and 25% the fungal growth of the control cultures, respectively). The MICs of CMT-3 and AMB were determined as the minimum concentration of the drug which completely inhibited fungal growth on the agar. The MICs of the azoles (fluconazole, itraconazole, and ketoconazole) were determined as the concentration which inhibited 75% or more of the fungal growth on the agar.

To determine the effect of phosphate on the antifungal activity of CMT-3, PDA solutions containing different concentrations of CMT-3 were mixed with a phosphate solution (Na_2HPO_4, pH 7.4, 100× stock solution in distilled water) to make the following series of final concentrations in the agar: 0.0, 0.1, 0.2, 0.4, 0.6, 0.8, 1.0, and 1.2 mg/ml. After the agar hardened, the fungal suspensions (*Penicillium* sp.) were inoculated onto the agar and incubated at 35°C, and the MICs were determined as described above.

For reproducibility and accuracy evaluation of the PDA method, all eight fungal strains were tested with AMB, and six strains were tested with itraconazole (except for *C. tropicalis* ATCC 750 and *C. albicans* ATCC 24433). Each test was repeated 15 times to obtain sufficient MIC data for statistical analysis. The same number of tests used for the BMM was carried out with the M27-A or M38-P method (7, 8) to obtain the standard MIC ranges for quality control (QC) use. The MICs of the other antifungal agents, i.e., fluconazole, ketoconazole, and CMT-3, were determined in one assay with both the PDA method and the BMM.

RESULTS

Effect of phosphate on the antifungal activity of CMT-3. At low concentrations of phosphate added to PDA, i.e., 0.1 and 0.2 mg/ml (0.07 and 0.15 mM, respectively), CMT-3 exhibited strong antifungal activity against a *Penicillium* sp. (Fig. 1); note that the physiologic concentration of phosphate, i.e., the concentration in human serum, is approximately 0.06 mg/ml. The MIC of CMT-3 at these phosphate concentrations was determined to be 0.5 μg/ml (1.37 μM), the same as that for the control, which contained no added phosphate. However, when the added phosphate concentration in PDA reached 0.4 mg/ml or above, significant interference with the antifungal activity of CMT-3 was observed. The MIC of CMT-3 against a *Penicil-*

TABLE 1. Reproducibility of MICs of AMB against eight fungal strains, as determined by the PDA method

Organism[a]	No. of occurrences at an MIC (μg/ml) of:							Tested range[b] (μg/ml)	% Reproducibility[c]
	0.12	0.25	0.5	1.0	2.0	4.0	8.0		
C. parapsilosis (ATCC 22019)	0	0	1	9	5	0	0	0.5–2.0	100
C. krusei (ATCC 6258)	0	0	0	3	11	0	1	1.0–4.0	93
C. tropicalis (ATCC 750)	0	0	1	11	2	1	0	0.5–2.0	93
C. albicans (ATCC 24433)	0	1	2	10	2	0	0	0.5–2.0	93
A. flavus (CI)	0	0	1	12	2	0	0	0.5–2.0	100
A. fumigatus (ATCC 1022)	0	0	1	8	5	0	0	0.5–2.0	100
Penicillium sp. (CI)	1	11	3	0	0	0	0	0.12–0.5	100
Rhizopus sp. (CI)	0	1	8	6	0	0	0	0.25–1.0	100

[a] CI, clinical isolate.
[b] The range was determined as the peak and 1 $\log_2$ dilution.
[c] Percentage of MICs within the tested range.

lium sp. increased from 0.5 to 2.0 μg/ml at 0.8 mg of added phosphate/ml in PDA, four times the MIC observed in the absence of added phosphate (Fig. 1). The inhibition curves and 50% inhibitory concentrations (IC_{50}s) for CMT-3 against a *Penicillium* sp. in the presence of different added phosphate concentrations are shown in Fig. 2. With an increase in the phosphate concentration in the agar, higher concentrations of CMT-3 were required to inhibit fungal growth (Fig. 2A) and, as a result, the IC_{50}s increased in a dose-dependent manner (Fig. 2B). The results demonstrated strong interference of phosphate with the antifungal activity of CMT-3 in vitro.

Reproducibility and accuracy of the PDA method. The MICs of AMB and itraconazole against six to eight fungal strains determined with the PDA method in 15 repeated tests fell mainly within 2 to 3 $\log_2$ dilutions, indicating sharp end points in the tests (Tables 1 and 2). For AMB, the range of MICs was 0.12 to 8.0 μg/ml, with a *Penicillium* sp. at the low end (0.12 μg/ml) and *C. krusei* at the high end (8.0 μg/ml) of the range. Ninety-three percent of the MICs were within the tested ranges for three of the eight fungal strains tested, and 100% were within the tested ranges for all the other strains (Table 1). In contrast, itraconazole showed a narrower range of MICs, from 0.06 to 1.0 μg/ml, with *C. parapsilosis* being the most sensitive (0.06 μg/ml) and the filamentous fungi being less sensitive. *C. krusei* and a *Rhizopus* sp. exhibited 93% reproducibility, while 100% of the MICs for the other four fungi were within the tested ranges (Table 2). Overall, the reproducibility of the MICs of both drugs in the PDA method was satisfactory (97%).

The level of agreement between MICs determined with the PDA method and the standard ranges obtained with the NCCLS proposed model or determined with the BMM was evaluated as shown in Table 3. For AMB, 50% (four of the eight strains) of the MIC ranges determined by the PDA method were the same as those determined by the BMM. For itraconazole, the MIC ranges determined with the PDA method were the same as those determined with the BMM for four of the six strains (67%). Except for the MIC of AMB against *C. krusei* and that of itraconazole against a *Rhizopus* sp. (both showed 93% agreement within 1 $\log_2$ dilution of the QC ranges), all other MICs of both drugs determined for the eight fungal strains by the PDA method showed 100% agreement within 1 $\log_2$ dilution of the QC ranges (determined by the BMM). Therefore, the MIC results obtained by the PDA method appear to be reliable, based on the NCCLS proposed model.

Feasibility of the PDA method in antifungal susceptibility testing. Three antifungal agents, i.e., fluconazole, ketoconazole, and CMT-3, were tested by using both the BMM and the PDA method in order to compare the MICs determined by the methods (Table 4). Eighty-seven percent of the MICs (13 of 15) of fluconazole and ketoconazole determined by the PDA method were the same as or 1 $\log_2$ dilution higher than those obtained by the BMM. The MICs of ketoconazole against *Y. lipolytica* and a *Rhizopus* sp. obtained by the PDA method showed a 2 $\log_2$ dilution difference from those determined by the BMM. It does appear that MICs determined by the PDA method were slightly higher than those determined by the BMM. For these two azole drugs, the BMM/PDA method MIC ratios were between 0.25 and 1 (no more than a 2 $\log_2$

TABLE 2. Reproducibility of MICs of itraconazole against six fungal strains, as determined by the PDA method

Organism[a]	No. of occurrences at an MIC (μg/ml) of:							Tested range[b] (μg/ml)	% Reproducibility[c]
	0.06	0.12	0.25	0.5	1.0	2.0	4.0		
C. parapsilosis (ATCC 22019)	1	11	3	0	0	0	0	0.06–0.25	100
C. krusei (ATCC 6258)	1	6	8	0	0	0	0	0.12–0.5	93
A. flavus (CI)	0	1	12	2	0	0	0	0.12–0.5	100
A. fumigatus (ATCC 1022)	0	0	11	4	0	0	0	0.12–0.5	100
Penicillium sp. (CI)	0	0	4	10	1	0	0	0.25–1.0	100
Rhizopus sp. (CI)	0	1	5	8	1	0	0	0.25–1.0	93

[a] CI, clinical isolate.
[b] See Table 1, footnote *b*.
[c] See Table 1, footnote c.

TABLE 3. Comparison of the MICs of AMB and itraconazole determined by the PDA method and the BMM

Organism[a] and antifungal agent	MIC (μg/ml) determined by:		% Agreement[d]
	BMM[b]	PDA method[c]	
C. parapsilosis (ATCC 22019)			
AMB	0.25–1.0	0.5–2.0	100
Itraconazole	0.06–0.25	0.06–0.25	100
C. krusei (ATCC 6258)			
AMB	0.5–2.0	1.0–4.0	93
Itraconazole	0.12–0.5	0.12–0.5	100
C. tropicalis (ATCC 750), AMB	0.5–2.0	0.5–2.0	100
C. albicans (ATCC 24433), AMB	0.25–1.0	0.5–2.0	100
A. flavus (CI)			
AMB	0.5–4.0	0.5–2.0	100
Itraconazole	0.12–0.5	0.12–0.5	100
A. fumigatus (ATCC 1022)			
AMB	0.5–2.0	0.5–2.0	100
Itraconazole	0.12–1.0	0.12–0.5	100
Penicillium sp. (CI)			
AMB	0.12–0.5	0.12–0.5	100
Itraconazole	0.25–1.0	0.25–1.0	100
Rhizopus sp. (CI)			
AMB	0.25–1.0	0.25–1.0	100
Itraconazole	0.5–2.0	0.25–1.0	100

[a] CI, clinical isolate.
[b] For ATCC strains, the MIC ranges were obtained from NCCLS M27-A and M38-P; otherwise, the MIC ranges for filamentous fungi were obtained in our laboratory with the BMM.
[c] MIC ranges were determined from 15 repeated assays for each strain with the PDA method.
[d] Percentage of MICs obtained with the PDA method that were within 1 $\log_2$ dilution of the BMM MIC ranges.

TABLE 4. Comparison of the MICs of fluconazole, ketoconazole, and CMT-3 determined by the BMM and the PDA method

Organism[a] and antifungal agent	MIC (μg/ml) determined by:		Difference[b] ($\log_2$ dilutions)
	BMM	PDA method	
C. parapsilosis (ATCC 22019)			
Fluconazole	2.0	4.0	+1
Ketoconazole	0.12	0.12	0
C. krusei (ATCC 6258)			
Fluconazole	32	32	0
Ketoconazole	0.5	1.0	+1
P. boydii (CI)			
Fluconazole	8	16	+1
Ketoconazole	1.0	2.0	+1
CMT-3	32	0.25	−7
C. glabrata (CI)			
Fluconazole	8	16	+1
Ketoconazole	4.0	4.0	0
CMT-3	32	4	−3
Y. lipolytica (CI)			
Fluconazole	2.0	4.0	+1
Ketoconazole	0.25	1.0	+2
CMT-3	32	2.0	−4
C. albicans (ATCC 24433)			
Fluconazole	4.0	4.0	0
CMT-3	16	0.5	−5
P. variotii (ATCC 22319)			
Ketoconazole	0.12	0.25	+1
CMT-3	16	1.0	−4
A. fumigatus (ATCC 1022)			
Ketoconazole	4.0	4.0	0
CMT-3	>64	2.0	<−5
Penicillium sp. (CI)			
Ketoconazole	1.0	2.0	+1
CMT-3	16	0.5	−5
Rhizopus sp. (CI)			
Ketoconazole	1.0	4.0	+2
CMT-3	32	1.0	−5
A. flavus (CI), CMT-3	64	2.0	−5

[a] CI, clinical isolate.
[b] Difference between PDA method result and BMM result.

dilution difference), values which represent an allowable shift in laboratory practice.

Although the results for both fluconazole and ketoconazole showed good agreement between the PDA method and the BMM, the MICs of CMT-3 determined by the BMM were much higher than those determined by the PDA method. For CMT-3, the MIC differences between the two methods were generally more than 3 $\log_2$ dilutions. The BMM/PDA method MIC ratios were 8 to 128; i.e., the MIC of CMT-3 determined by the BMM could be as much as 128 times higher than that determined by the PDA method (7 $\log_2$ dilution difference). These results reflect the fact that the antifungal activity of CMT-3 was severely inhibited by the high phosphate level in the medium used in the BMM (Fig. 1 and 2). In other words, the results obtained for CMT-3 antifungal susceptibility testing with the PDA method appear to be more reasonable and reliable than those obtained with the NCCLS proposed method.

In this study, both 24- and 48-well plates were used in the PDA method for all five antifungal agents tested against the yeasts and molds. Both plates showed clear MIC end points for either yeasts or molds (Fig. 1 and 3).

DISCUSSION

The results of this study demonstrated that the relatively high phosphate levels in the media used in current standardized methods strongly interfere with the determination of the MIC of CMT-3 as an antifungal agent. The possible inhibitory effect of phosphate on antifungal agents was predicted in M27-A (7) and might pose a problem in assessing other newly developed antifungal agents in the future. Therefore, one of the important objectives of the current study was to design a method to prevent the confounding effects of phosphate in culture media on the assessment of the in vitro potencies of antifungal agents. PDA was chosen for this new method not only because it is a commonly used medium for the growth of

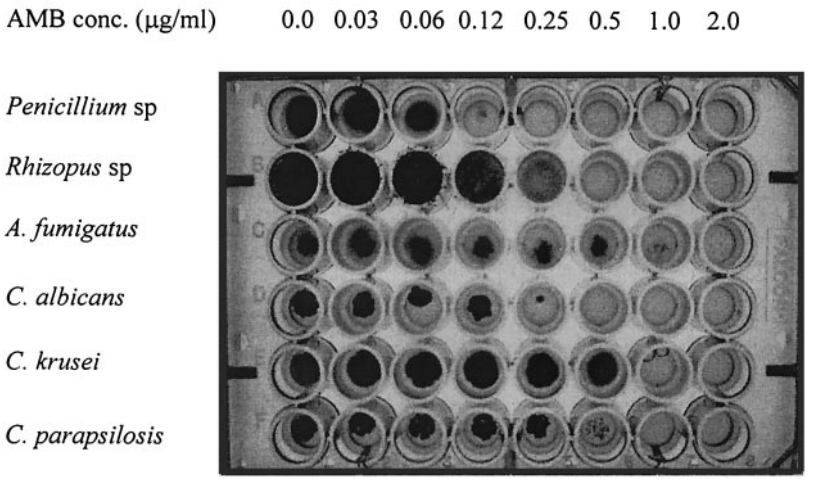

FIG. 3. MICs of AMB against six fungal strains determined on a 48-well plate. The wells were filled with PDA and different concentrations of AMB, separately inoculated with each of the fungi, and incubated at 35°C for 48 h. Wells in the first lane (drug free) were used as negative controls to score the growth in other wells. The end points were determined as no growth of the fungus. The MICs of AMB against a *Penicillium* sp., a *Rhizopus* sp., *A. fumigatus* (ATCC 1022), *C. albicans* (ATCC 24433), *C. krusei* (ATCC 6258), and *C. parapsilosis* (ATCC 22019) were determined to be 0.25, 0.5, 2.0, 0.5, 1.0, and 1.0 μg/ml, respectively.

fungi in the laboratory but also because it does not have a high level of phosphate. SABHI agar and Sabouraud's dextrose agar were also tested with CMT-3. However, to different extents, both culture media also showed interference with the antifungal activity of CMT-3 (data not shown).

It is interesting that when the phosphate concentration in PDA was increased to 0.8 mg/ml (the same as in RPMI 1640 medium), the MIC of CMT-3 against a *Penicillium* sp. increased from 0.5 to 2.0 μg/ml (Fig. 1), 2 $\log_2$ dilutions higher than that in the control cultures, which did not contain added phosphate. In contrast, the MIC of CMT-3 against the same fungus assessed by the BMM was found to be 16 μg/ml, 5 $\log_2$ dilutions higher than that observed with the PDA method. It seemed that in addition to phosphate, other components in RPMI 1640 medium might have altered the CMT-3 MIC determination. Possible components in BMM that might affect a CMT-3 MIC assay include MOPS, phenol red, glutathione (reduced), calcium nitrate, vitamins B_{12}, B_1, B_2, and B_6, biotin, *p*-aminobenzoic acid, niacinamide, and folic acid. However, when these compounds were tested in the PDA method with several fungal strains (i.e., C. *albicans*, a *Penicillium* sp., and *A. fumigatus*) as described for phosphate testing in Fig. 1, none of these compounds had any effect on the MICs of CMT-3 (data not shown). Whether other compounds in RPMI 1640 medium might have effects on CMT-3 antifungal activity is still under study.

The physiological phosphate level (as Na_2HPO_4) in human serum is 0.05 mg/ml, or 2 meq. Obviously, the phosphate concentration in RPMI 1640 medium is far above the physiological level. The negative effect of the phosphate from RPMI 1640 medium on CMT-3 antifungal activity should not occur in vivo. Therefore, for any antifungal agents that may interact with phosphate, such as CMT-3, the PDA method should be considered as an alternative to the current BMM.

Beyond the current NCCLS methods M27-A (for yeasts) and M38-P (for filamentous fungi), recent publications have proposed several modifications for antifungal susceptibility testing. Examples include the colorimetric broth microdilution method (10, 12) and the more recent capacitance method (3); both provide a fast and direct MIC end-point reading by using either a colorimeter or a Bactometer. Like M27-A, these two methods use the broth dilution technique and were designed to measure the MICs of antifungal agents against yeasts. The recently developed modified agar dilution susceptibility test (21), the commercially available technique E-test (11, 13, 16), and the most recently proposed semisolid agar antifungal susceptibility test (14) using agar-containing culture media all provide clearly visible MIC end-point readings. The former two methods are used mainly for testing MICs of antifungal agents against yeasts, whereas the last one can be used for both yeasts and molds. Although all of these methods have shown good reproducibility, accuracy, and excellent feasibility for clinical use, they do not test both yeasts and molds at the same time (except for the semisolid agar antifungal susceptibility test), and they do not prevent the interference of phosphate with antifungal agents such as CMT-3. To our knowledge, CMTs are the first discovered antifungal agents that are severely affected by the phosphate in the BMM.

The PDA method has been demonstrated to be an economical, practical, accurate, and reliable technique for antifungal susceptibility testing. Like BMM, it can be easily used for large-batch sample screening for antifungal agents or screening for preidentified drug-resistant fungal isolates. Additional characteristics of the PDA method include (i) its use to determine the MICs of antifungal agents for both yeasts and molds at the same time; (ii) its prevention of the inhibitory effect of phosphate on antifungal agents such as CMTs; and (iii) its ease of operation, which may provide a fast and effective screening method for the discovery of new types of antifungal agents in the future.

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