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Modified Agar Dilution Susceptibility Testing Method for Determining In Vitro Activities of Antifungal Agents, Including Azole Compounds

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In vitro activities of antifungal agents, including azole compounds, against yeasts were easily determined by using RPMI-1640 agar medium and by incubating the plates in the presence of 20% CO_2. The end point of inhibition was clear by this method, even in the case of azole compounds, because of the almost complete inhibition of yeast growth at high concentrations which permitted weak growth of some *Candida* strains by traditional methods. MICs obtained by the agar dilution method were similar to those obtained by the broth dilution method proposed by the National Committee for Clinical Laboratory Standards.

Various kinds of antifungal susceptibility testing methods have been reported, but it is still difficult to determine the MICs of azole compounds against yeasts such as *Candida albicans* and *Candida tropicalis*, which can grow at a reduced rate even in the presence of relatively high concentrations of azole compounds (1–3, 6–9, 11–13). The National Committee for Clinical Laboratory Standards (NCCLS), a nonprofit educational organization in the United States, proposed a broth dilution method (M-27P) for antifungal susceptibility testing in 1992, and this method is used widely throughout the world (2, 6, 9). Fungal growth inhibition by azole compounds in the NCCLS method is not perfect, however, and the MIC is defined as the lowest concentration giving 80% inhibition of fungal growth. We report here an agar dilution susceptibility testing method which gives a clear end point for determining MICs of azole compounds and other antifungal agents against yeast type fungi and which is useful for the determination of the susceptibilities of large numbers of fungal strains.

MATERIALS AND METHODS

Test Organisms. The yeast type fungi used are listed in the tables. *C. albicans* ATCC 90028 and ATCC 90029, *Candida parapsilosis* ATCC 90018, *Candida glabrata* ATCC 90030, and *Cryptococcus neoformans* ATCC 90112 and ATCC 90113 are quality control reference strains for determining the MICs of antifungal agents by the NCCLS method. Test organisms were grown on Sabouraud dextrose agar (Difco) at 35°C. *Candida* and *Saccharomyces cerevisiae* were grown for 20 h, and *C. neoformans* was grown for 48 h. Cells were suspended in saline, and the suspension was adjusted to 0.5 McFarland standard and then diluted 10-fold with saline to give a yeast suspension of 0.5×10^5 to 2.5×10^5 CFU per ml.

Antifungal agents. Fluconazole (Pfizer, Tokyo, Japan) was extracted from commercially available injection in our laboratory. The purity of fluconazole as determined by high-performance liquid chromatography was more than 99%. Amphotericin B and flucytosine were obtained from Squibb Japan and Sigma, respectively.

Antifungal susceptibility testing. MICs were determined by an agar dilution method using RPMI-1640 medium (Gibco BRL, Grand Island, N.Y.) which was recommended by NCCLS M27-P for broth dilution testing (2). A double concentration of RPMI-1640 was prepared with 0.3 M morpholinepropanesulfonic acid (MOPS; Dojindo, Tokyo, Japan) buffer (pH 7.0), was sterilized by filtration through a membrane filter (pore size, 0.45 μm), and was mixed with equal volume of 3.0% agar (Wako, Osaka, Japan) which had been autoclaved at 121°C for 15 min and kept at 55°C. The agar medium (9.9 ml) was then poured into petri dishes containing 0.1 ml of serial dilutions of antifungal agents dissolved in dimethyl sulfoxide (Wako) and was solidified. About 10^3 CFU of fungal cells suspended in saline was inoculated with a multiple inoculator (Sakuma, Tokyo, Japan) onto the agar plates prepared as described above. The plates were then incubated in an ordinary incubator, a CO_2 incubator, or a jar containing CampyPak (BBL Microbiology Systems, Cockeysville, Md.) at 35°C for 20 h. After the MICs for *Candida* species and *S. cerevisiae* were determined, the plates were incubated for an additional 48 h to determine the MIC for *C. neoformans*. The MIC was defined as the lowest concentration of antifungal agent giving no visible growth or causing almost complete inhibition of growth. MICs were also determined by the NCCLS broth dilution method (2).

RESULTS AND DISCUSSION

The antifungal activity of fluconazole against yeasts was determined by the agar dilution method in atmospheres with various oxygen concentrations (Table 1). The concentration of oxygen was decreased by increasing CO_2 concentration or by using CampyPak. *C. albicans* strains having different in vivo susceptibilities to fluconazole were included in the test.

The MICs of fluconazole for the many test strains were not greatly influenced by oxygen concentration, but 4 of 17 strains showed higher resistance in the normal atmosphere (in an ordinary incubator) than in a reduced-oxygen concentration. For example, *C. albicans* TA, which was highly susceptible to fluconazole in vivo, was resistant when the MIC was determined in the normal atmosphere. The MIC value for *C. albicans* ATCC 90028 obtained in the normal atmosphere was also much higher than that (≦0.13 to 0.5 μg/ml) expected from the NCCLS broth dilution method (2). The two strains, however, were susceptible to fluconazole in the presence of 10 or 20% CO_2. *C. albicans* TIMM1850 and *C. tropicalis* IFO 0587, which were resistant to fluconazole in the normal atmosphere, also became susceptible in the presence of CO_2. When determined in the presence of 20% CO_2, the MIC of fluconazole for *C. albicans* correlated well with its in vivo activity; the MICs of fluconazole for *C. albicans* TA, TIMM1756, TIMM1850, and CA383 were 0.13, 0.5, 0.5, and >64 μg/ml, respectively, and doses of orally administered fluconazole that kept 50% of mice with systemic infections alive were 0.3, 1.5, 4.0, and 51 mg/kg of body weight, respectively (data not shown). Although the two CO_2 concentrations gave fundamentally the same results, MIC determination in 20% CO_2 was easier than that in 10% CO_2 since the end points of growth inhibition were clearer at the higher CO_2 concentration. A representative result obtained in the presence of 20% CO_2 is shown in Fig. 1. The experiment with itraconazole gave a similar result (data not shown). The MICs of fluconazole for two of the above four strains were also

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TABLE 1. Effect of oxygen concentration on the MICs of fluconazole against yeasts

Organism	MIC[a] (μg/ml) determined in or with:			
	Atmosphere	10% CO_2	20% CO_2	CampyPak
C. albicans ATCC 90028	>64	1	0.5	>64
C. albicans ATCC 90029	2	1	1	1
C. albicans TA	>64	0.13	0.13	0.25
C. albicans TIMM1756	1	0.5	0.5	0.5
C. albicans TIMM1850	>64	>64	0.5	>64
C. albicans CA383	>64	>64	>64	64
C. parapsilosis ATCC 90018	1	1	1	1
C. glabrata ATCC 90030	16	32	32	16
C. glabrata IFO 0622	8	16	32	16
C. tropicalis IFO 0587	64	2	2	16
C. tropicalis IFO 10241	2	2	2	2
Candida krusei IFO 0584	32	64	64	32
C. krusei IFO 1162	>64	>64	64	>64
Candida utilis IFO 0619	4	4	4	4
S. cerevisiae IFO 0209	4	8	8	8
C. neoformans ATCC 90112	8	4	N.G.[b]	8
C. neoformans ATCC 90113	32	32	32	32

[a] MICs were determined by the agar dilution method using RPMI-1640 medium with inocula of 10^3 CFU per spot. The agar plates were incubated in an ordinary incubator (atmosphere), a CO_2 incubator (10% and 20% CO_2), or a jar with CampyPak for 20 h at 35°C. After the MICs for *Candida* and *S. cerevisiae* were determined, the plates were incubated for an additional 48 h to determine the MICs for *C. neoformans*.

[b] N.G., the organism did not grow enough for a MIC determination.

decreased by CampyPak, which produces a microaerobic condition (6 to 9% oxygen, 5.5 to 7% carbon dioxide, and 0.2% hydrogen after 24 h). These results suggest that the oxygen concentration in the environment is an important factor for testing the activities of azole compounds against yeasts.

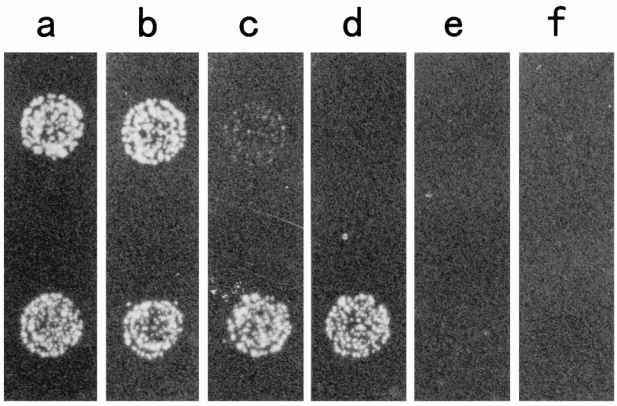

FIG. 1. *C. albicans* grown on an agar plate containing fluconazole in the presence of 20% CO_2. *C. albicans* TA (upper images) and TIMM1756 (lower images) were grown on agar plates containing 0 (a), 0.03 (b), 0.06 (c), 0.13 (d), 0.25 (e), and 0.5 (f) μg of fluconazole per ml in the presence of 20% CO_2. In this experiment, the MICs of *C. albicans* TA and TIMM1756 were determined to be 0.13 and 0.25 μg/ml, respectively.

C. neoformans grew slowly in 20% CO_2 since oxygen is a limiting nutrient for the growth of this microorganism (4, 10), and it was difficult to determine the MICs for some strains of *C. neoformans*, such as ATCC 90112, in 20% CO_2 (Table 1). We therefore cultured the organisms (by the method and at the inoculum concentration shown in Table 1) in an ordinary incubator after incubating them in the CO_2 incubator for 20 h. An additional 48 h of incubation in the ordinary incubator allowed the visible growth of *C. neoformans* ATCC 90112 and made the MIC determination possible (MIC, 8 μg/ml). In contrast, no growth was observed after an additional 48 h of incubation in the CO_2 incubator. The MIC for *C. neoformans*

TABLE 2. Comparison of MICs against yeasts determined by the agar dilution method with those determined by the NCCLS broth dilution method

Organism	MIC (μg/ml)[a] of:											
	Fluconazole				Amphotericin B				Flucytosine			
	Agar		Broth		Agar		Broth		Agar		Broth	
	Range	Geometric mean[b]	Range	Geometric mean	Range	Geometric mean	Range	Geometric mean	Range	Geometric mean	Range	Geometric mean
C. albicans ATCC 90028	0.5	0.50	0.13–0.5	0.20	1–2	1.1	0.5–1	0.89	0.25–2	1.0	0.5–1	0.71
C. albicans ATCC 90029	0.5–1	0.79	0.25–1	0.40	0.5–1	0.71	0.5–1	0.89	>64	>64	>64	>64
C. albicans TA	0.13–0.25	0.16	0.13–0.25	0.20	0.5–1	0.79	0.5–1	0.89	0.13–0.25	0.22	0.25–0.5	0.40
C. albicans TIMM1756	0.5	0.50	0.13–0.5	0.25	1–2	1.4	0.5–1	0.89	0.13–0.25	0.20	0.13–0.25	0.16
C. albicans TIMM1850	0.5–1	0.56	0.25–>64	32	0.5–2	1.0	0.5–2	1.0	>64	>64	>64	>64
C. albicans CA383	>64	>64	32–>64	64	0.5–1	0.79	0.5–1	0.79	0.06–0.25	0.18	0.25–0.5	0.28
C. parapsilosis ATCC 90018	1	1.0	0.5–1	0.63	2–4	2.2	1	1.0	0.13–0.5	0.20	0.13–0.5	0.18
C. glabrata ATCC 90030	32	32	8–>64	20	0.5–1	0.89	0.5–2	1.0	0.06–0.13	0.068	0.06–0.13	0.068
C. glabrata IFO 0622	16–32	29	8–16	10	0.5–2	0.89	0.5–1	0.71	≦0.03–0.06	0.053	0.06–0.25	0.11
C. tropicalis IFO 0587	1–2	1.8	0.5–2	0.89	2	2.0	0.5–1	0.89	0.13–0.25	0.16	0.25–0.5	0.32
C. tropicalis IFO 10241	1–2	1.8	0.25–2	1.0	1–2	1.8	0.5–1	0.89	0.13	0.13	0.13–0.5	0.28
C. krusei IFO 0584	64–>64	>64	32–>64	51	2–4	2.2	1–2	1.4	8–16	14	8–16	14
C. krusei IFO 1162	64–>64	>64	64–>64	>64	2	2.0	1–2	1.3	8	8.0	8–16	14
C. utilis IFO 0619	4–8	4.5	2–4	2.5	0.5–1	0.63	0.25–0.5	0.45	0.5–4	1.6	0.5–2	1.0
S. cerevisiae IFO 0209	8	8.0	4–16	8.0	1–2	1.4	0.5–1	0.89	≦0.03–0.13	0.079	0.06–0.25	0.098
C. neoformans ATCC 90112	4–8	6.4	1–4	2.5	2	2.0	0.5–1	0.71	2–8	4.0	2–8	3.6
C. neoformans ATCC 90113	32–64	40	4–8	5.0	2–4	2.5	0.5–1	0.56	>64	>64	>64	>64

[a] MICs were determined six times by the agar dilution method (agar) and the NCCLS broth macrodilution method (broth). In the agar dilution method, the agar plates were incubated for 20 h in the presence of 20% CO_2 to determine the MICs for *Candida* and *S. cerevisiae*, and then incubated for 48 h in the normal atmosphere to determine the MICs for *C. neoformans.*

[b] MIC values of >64 and ≦0.03 μg/ml were regarded as 128 and 0.03 μg/ml, respectively, to calculate the geometric means, and the calculated means exceeding 64 μg/ml were expressed as >64 μg/ml.

ATCC 90113 was found to be 32 μg/ml under both incubation conditions. The plates should be incubated in 20% CO_2 for 20 h and then in the normal atmosphere for 48 h when *C. neoformans* and *Candida* species are tested at the same time, though it is unnecessary to include incubation in 20% CO_2 when only *C. neoformans* is tested.

MICs of fluconazole, amphotericin B, and flucytosine for 17 strains of yeast type fungi were determined by both the agar dilution method and the NCCLS broth dilution method. In the agar dilution method, the plates inoculated with the test microorganisms were incubated in the presence of 20% CO_2 for 20 h and then incubated in the ordinary incubator for 48 h. MICs were determined six times, and the ranges and geometric means of the MICs are shown in Table 2.

Both the agar dilution method and the broth dilution method gave reproducible results in determining the MICs of fluconazole, amphotericin B, and flucytosine for most strains. The ranges of fluctuation in the MICs determined six times for each strain were within a factor of four with some exceptions. The MICs for *Candida* species obtained by the agar dilution method were very similar to those obtained by the NCCLS method. However, the agar dilution MICs of fluconazole for *C. neoformans*, infection with which is more refractory to fluconazole treatment than that with *C. albicans*, were somewhat higher than those determined by the NCCLS method.

The agar dilution susceptibility testing method described here has two advantages over the NCCLS method. The first is a clear end point of inhibition. The second is that susceptibility testing of large numbers of organisms is easier; multiple strains can be tested with one set of agar plates in the agar dilution method, whereas each strain needs one set of test tubes in the NCCLS method. The most important point for the agar dilution method is to incubate organisms in the reduced oxygen concentration produced by 20% CO_2. Reduction of oxygen concentration, which reduces the growth rate of yeasts, is considered to be the optimum condition for in vitro susceptibility tests. The oxygen pressures in tissues and blood vessels where deep-seated mycoses often occur are lower than that in air (5). The antifungal susceptibility testing method described here may be a reasonable method for predicting in vivo activities and clinical efficacies of antifungal agents against infections with yeast type fungi, though it should be evaluated more prospectively in studies designed to ascertain in vitro-in vivo correlations.

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