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Detection of Resistance to Amphotericin B among *Cryptococcus neoformans* Clinical Isolates: Performances of Three Different Media Assessed by Using E-Test and National Committee for Clinical Laboratory Standards M27-A Methodologies

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Although reliable detection of resistance in vitro is critical to the overall performance of any susceptibility testing method, the recently released National Committee for Clinical Laboratory Standards M27-A methodology for susceptibility testing of yeasts discriminates poorly between resistant and susceptible isolates of *Candida* spp. We have previously shown that both substitution of antibiotic medium 3 for RPMI 1640 medium in the microdilution variant of the M27-A method and use of the E-test agar diffusion methodology permit detection of amphotericin B-resistant *Candida* isolates. To determine the relevance of these observations to *Cryptococcus neoformans*, we have evaluated the performances of both the M27-A and the E-test methodologies with this yeast using three different media (RPMI 1640 medium, antibiotic medium 3, and yeast nitrogen base). As with *Candida*, we found that only antibiotic medium 3 permitted consistent detection of resistant isolates when testing was performed in broth by the M27-A method. When testing was performed by the E-test agar diffusion method, both RPMI 1640 medium and antibiotic medium 3 agar permitted ready detection of the resistant isolates. Reading of the results after 48 h of incubation was required for testing in broth by the M27-A method, while the MIC could be determined after either 48 or 72 h when the agar diffusion method was used.

In vitro detection of resistance to antifungal agents is potentially useful when selecting the best therapy for a given patient. Unfortunately, it is not always easy to demonstrate a clear correlation between in vitro measures of susceptibility and in vivo response (32). In particular, detection of resistance to amphotericin B has proven to be technically difficult. For isolates of *Candida*, the M27-A methodology of the National Committee for Clinical Laboratory Standards (NCCLS) (24) is often unable to identify isolates that are resistant to amphotericin B (31) unless the NCCLS-specified RPMI 1640 medium is replaced with antibiotic medium 3 (also known as Penassay broth) (22, 31). Use of antibiotic medium 3 in combination with the E-test agar-based methodology has been shown to further enhance the ability to detect resistant isolates (38). Unfortunately, use of this medium is not without its difficulties. Because its components are not completely defined, the potential for significant lot-to-lot variability is present. Although such variation was found to be minimal, at least for current lots of antibiotic medium 3 (22), a recent study by Nguyen et al. (25) emphasizes the potential for interlaboratory variability when this medium is used. Unlike the results previously obtained in our laboratory with glucose-supplemented antibiotic medium 3 (22, 31), they found that glucose supplementation decreased the ability of the medium to accurately detect amphotericin B resistance among isolates of *Candida*. In addition, they found that the data obtained after 48 h of incubation predicted microbiologic failure more accurately than those obtained after 24 h. The reason for this difference is not clear.

Testing of the susceptibility of *Cryptococcus neoformans* to different drugs has been extensively studied with a wide variety of culture media (2, 4, 5, 9–12, 14, 15, 17, 27, 29, 30). However, none of the previous studies have specifically addressed the detection of amphotericin B-resistant isolates of *C. neoformans*. The M27-A document (24) does not address this issue either, although it does suggest that use of yeast nitrogen base (YNB) instead of RPMI 1640 medium may prove to be useful. In one pair of related studies, YNB was shown to enhance the growth of *C. neoformans* and improve the clinical relevance of fluconazole MICs (15, 39). Although infections caused by this organism are a cause of significant morbidity and mortality (1, 7, 26), the number of published reports of clinical resistance to amphotericin B by *C. neoformans* is surprisingly low (23, 30, 36). One of the possible reasons for this small number of reports may be the lack of a clinically relevant methodology for the detection of such isolates resistant to amphotericin B with a good correlation to in vivo results. In this work, using the same approach described above for *Candida* spp., we have evaluated the impact of test medium and format on the in vitro detection of amphotericin B-resistant *C. neoformans*.

(This work was presented in part at the 97th General Meeting of the American Society for Microbiology, Miami Beach, Fla., 4 to 8 May 1997 [21a], and at the 37th Interscience Conference on Antimicrobial Agents and Chemotherapy, Toronto, Ontario, Canada, 28 September to 1 October 1997 [21b].)

MATERIALS AND METHODS

Isolates. A collection of 12 isolates of *C. neoformans* identified as CN 1 to CN 12 was used. All organisms were obtained from AIDS patients with cryptococcal meningitis and were selected because the associated clinical history suggested relative susceptibility or resistance. Isolates CN 5 and CN 8 are thought to be amphotericin B susceptible and resistant, respectively, on clinical grounds (30), and were labeled in the original report as CN 1 and CN 3, respectively. Isolates CN 1 to CN 4, CN 6, CN 7, and CN 9 were found to be responsive to amphotericin B in a study of cryptococcal meningitis in patients with AIDS (21). Isolates

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TABLE 1. Susceptibility in RPMI 1640 broth[a]

Isolate	N	Putative status[b]	MIC (μg/ml)[c]					
			48 h			72 h		
			Range	Geo M	Mode	Range	Geo M	Mode
CN 1	31	S	0.0625–0.5	0.26	0.25	0.25–1	0.47	0.5
CN 2	32	S	0.0625–0.5	0.24	0.25	0.125–2	0.41	0.25
CN 3	29	S	0.0625–0.5	0.27	0.25	0.125–1	0.36	0.5
CN 4	30	S	0.125–0.25	0.18	0.25	0.125–2	0.4	0.25
CN 5	30	S	0.125–0.5	0.22	0.25	0.125–1	0.37	0.5
CN 6	34	S	0.125–1	0.24	0.25	0.125–8	0.45	0.5
CN 7	31	S	0.125–0.5	0.29	0.25	0.125–8	0.54	0.5
CN 9	37	S	0.0625–0.5	0.15	0.125	0.125–2	0.52	0.5
CN 12	20	S	0.125–0.25	0.24	0.25	0.25–0.5	0.31	0.25
CN 8	44	R	0.5–2	0.9	1	0.5–4	1.31	1
CN 10	20	R	1.0–2.0	1.27	1	2.0–4.0	2.07	2
CN 11	20	R	0.5–2	1.1	1	2	2	2
Clinical	72	S	0.125–0.5	0.36	0.5	0.125–1	0.46	0.5

[a] Eighty-four *C. neoformans* isolates were tested by the M27-A broth microdilution methodology in RPMI 1640 medium. Isolates CN 1 to CN 12 were tested the indicated number of times (*N*), while the 72 clinical isolates were tested once each. MICs were determined after 48 and 72 h of incubation.
[b] S, susceptible; R, resistant.
[c] Range, range of MICs; Geo M, geometric mean MIC; Mode, modal MIC.

CN 1 to CN 9 were recovered in the United States, whereas isolates CN 10 to CN 12 were Australian isolates kindly provided by Tanya Sorell (Center for Infectious Disease and Microbiology, Westmead Hospital, University of Sydney, Sydney, Australia). Isolates CN 10 and CN 11 were from an Australian human immunodeficiency virus-infected patient and developed resistance during treatment (23), and the last isolate, isolate CN 12, was a putatively susceptible organism. The amphotericin B resistance of isolate CN 8 has been shown to be due to reduced ergosterol content caused by defective sterol $\Delta^{8\rightarrow7}$ isomerase (18). Resistance to amphotericin B can be caused by other mechanisms (17), and the nature of the resistance of isolates CN 10 and CN 11 remains to be studied. In addition, 72 general clinical isolates of *C. neoformans* obtained from patients with cryptococcal meningitis were tested in order to determine the general population's behavior. As required by the NCCLS M27-A protocol, all organisms were kept at −70°C and were passed at least twice on Sabouraud dextrose agar at 35°C for 72 h prior to being tested. All isolates were identified as *C. neoformans* var. *neoformans* or *C. neoformans* var. *gattii* by use of canavanine-glycine-bromthymol blue agar (20). This agar permits growth of *C. neoformans* var. *gattii* (serotypes B and C) with a characteristic blue color but does not support the growth of *C. neoformans* var. *neoformans* (serotypes A, D, and AD). Quality control was ensured by testing the NCCLS-specified quality control isolates ATCC 90112 (*C. neoformans* [28]) and ATCC 22019 (*Candida parapsilosis* [24]), and results for these isolates were always within the defined quality control range (data not shown).

Test media. Antibiotic medium 3 was obtained from BBL (lot JD4ZSG; Becton Dickinson Microbiology Systems, Cockeysville, Md.). This medium was supplemented with glucose to achieve a final glucose concentration of 2% (20 g/liter), the buffering capacity was increased by adding 1 g of dipotassium monophosphate per liter and 1 g of monopotassium monophosphate per liter, and the pH was adjusted to 7.0 with NaOH. RPMI 1640 medium (lot 85H46331; Sigma Chemical Co., St. Louis, Mo.) and YNB (lot 12183; Difco Laboratories, Detroit, Mich.) were prepared according to the manufacturer's instructions. After reconstitution, YNB was supplemented with glucose to obtain a final concentration of 0.5% (5 g/liter). Both RPMI 1640 medium and YNB were buffered to pH 7.0 with 3-(*N*-morpholino)propanesulfonic acid (MOPS; lot 75H5734; Sigma Chemical Co.), achieving a final concentration of 0.165 mol/liter. All three media were filter sterilized by passage through a 0.22-μm-pore-size filter system (Corning Inc., Corning, N.Y.).

For agar-based testing, all media were supplemented with glucose to a final concentration of 2% (20 g/liter). To prepare the agar plates, the double-strength, filter-sterilized medium was combined with an equal volume of heat-sterilized double-strength agar (Bacto Agar; Difco Laboratories) to yield the correct final concentration of medium in a 1.5% agar gel. E-test antimicrobial gradient strips were kindly provided by AB Biodisk (AB Biodisk, Solna, Sweden).

Susceptibility testing. Preparation of the yeast inoculum and drug was done by the NCCLS M27-A methodology, and MIC testing was performed by the microdilution variant of the method (24). We determined the MICs after both 48 and 72 h of incubation at 35°C by measuring the optical density at 530 nm with a plate reader (model EL-310; Bio-Tek, Burlington, Vt.). The amphotericin B concentration range used with antibiotic medium 3 and RPMI 1640 medium was 0.0625 to 64 μg/ml, while that used with YNB was 0.0312 to 16 μg/ml. The microdilution plates were mechanically agitated prior to reading (3), and the MIC was the lowest concentration of amphotericin B which completely inhibited fungal growth. The same fungal inoculum used for the broth-based assays was swabbed onto the surface of the agar plate and was allowed to dry for 15 min before the addition of the E-test strip. One E-test antimicrobial gradient strip containing amphotericin B (range, 0.002 to 32 μg/ml) was placed in each petri dish so that there was only one isolate and one drug strip per plate. The plates were incubated for 48 and 72 h, and the MIC was the point at which the zone of complete inhibition intersected the strip.

RESULTS

Susceptibility testing in broth: microdilution methodology with RPMI 1640. After 48 h of incubation, the modal MICs for three isolates, isolates CN 8, CN 10, and CN 11, were elevated (1.0 μg/ml) in comparison with those for all other isolates tested (Table 1). Despite this, the observed MIC ranges for the putatively susceptible and resistant isolates showed frequent overlaps. Qualitatively similar results were observed after 72 h of incubation.

Susceptibility testing in broth: microdilution methodology with antibiotic medium 3. After 48 h of incubation, the modal MICs for three isolates (isolates CN 8, CN 10, and CN 11) were consistently higher (1.0 μg/ml) than those for the other organisms tested (Table 2). Although the MIC range for one of the putatively resistant isolates, isolate CN 8, overlapped the observed MIC range for isolate CN 3, the range of observed MICs for the other putatively susceptible and resistant isolates did not overlap, thus permitting reliable discrimination between the groups. The overlap in MIC ranges for CN 3 and CN 8 was due to nine MIC readings of 0.5 μg/ml for CN 3. Data after 72 h of incubation produced similar results, and discrimination was also possible, but an increasing overlap in the range of MICs reduced the reliability of discrimination. The overlap at 72 h was due to a single elevated reading for each of isolates CN 6, CN 7, and CN 9, two elevated readings for isolate CN 2, and four elevated readings for isolate CN 3.

Susceptibility testing in broth: microdilution methodology with yeast nitrogen base. Although higher MICs were recorded for putatively resistant isolates CN 8, CN 10, and CN 11, the MIC ranges for these isolates significantly overlapped those obtained for the putatively susceptible isolates (Table 3). Consequently, this medium was excluded from further consideration.

TABLE 2. Susceptibility in antibiotic medium 3 broth[a]

Isolate	*N*	Putative status[b]	MIC (μg/ml)[c]					
			48 h			72 h		
			Range	Geo M	Mode	Range	Geo M	Mode
CN 1	29	S	0.125–0.25	0.19	0.25	0.125–0.5	0.33	0.5
CN 2	27	S	0.0625–0.25	0.19	0.25	0.125–1	0.35	0.25
CN 3	27	S	0.125–0.5	0.26	0.25	0.125–2	0.42	0.5
CN 4	28	S	0.125–0.25	0.19	0.25	0.25–0.5	0.35	0.25
CN 5	29	S	0.0625–0.25	0.1	0.0625	0.0625–0.5	0.25	0.25
CN 6	28	S	0.0625–0.25	0.2	0.25	0.25–1	0.34	0.25
CN 7	29	S	0.0625–0.25	0.18	0.25	0.125–1	0.36	0.5
CN 9	38	S	0.0625–0.25	0.18	0.25	0.25–1	0.38	0.5
CN 12	20	S	0.125–0.25	0.14	0.125	0.125–0.25	0.18	0.25
CN 8	44	R	0.5–2	0.9	1	1.0–8.0	1.6	2
CN 10	20	R	1.0–2.0	1.1	1	1.0–2.0	1.18	1
CN 11	20	R	1.0–2.0	1	1	1.0–2.0	1.03	1
Clinical	72	S	0.0625–0.25	0.17	0.25	0.125–0.5	0.25	0.25

[a] Eighty-four *C. neoformans* isolates were tested by the microdilution methodology in broth in antibiotic medium 3. Isolates CN 1 to CN 12 were tested the indicated number of times (*N*), while the 72 clinical isolates were tested once each. MICs were determined after 48 and 72 h of incubation.
[b] S, susceptible; R, resistant.
[c] Range, range of MICs; Geo M, geometric mean MIC; Mode, modal MIC.

Susceptibility testing on RPMI 1640 agar. The results obtained by the E-test on RPMI 1640 agar for the putatively susceptible named isolates were similar to those obtained for antibiotic medium 3 by the microdilution method with broth, whereas the MICs for the putatively resistant isolates were noticeably increased (Table 4). The range of observed MICs for the putatively susceptible and resistant groups were widely separated: the highest MIC for a putatively susceptible isolate was 0.25 μg/ml, and the lowest MIC for a putatively resistant isolate was 1.5 μg/ml. The range of MICs for the 72 clinical isolates of *C. neoformans* was 0.006 to 0.38 μg/ml and the modal MIC was 0.094 μg/ml. Results after 72 h of incubation showed an overall increase of at least twofold over the values obtained after 48 h of incubation, but good separation of the MICs for the putatively resistant and susceptible isolates was still obtained.

Susceptibility testing on antibiotic medium 3 agar. The susceptibilities of the 12 named isolates of *C. neoformans* were determined after 48 h of incubation on antibiotic medium 3 supplemented with 2% glucose by using the E-test antimicrobial gradient strips. As with the microdilution method with broth and the E-test on RPMI 1640 agar, the three isolates for which MICs were higher (isolates CN 8, CN 10, and CN 11) were readily detected by this methodology (modal MICs, 3.0, 3.0, and 4.0 μg/ml, respectively, after both 48 and 72 h of incubation), while for the remaining isolates the modal MIC range was 0.064 to 0.25 μg/ml (Table 5). As with the results obtained on RPMI 1640 agar, the ranges of observed MICs obtained for the supposedly susceptible and resistant isolate groups on repeat testing were widely separated. As with RPMI 1640 agar testing, susceptibility values after 72 h of incubation were usually 1 twofold dilution higher than those obtained after 48 h, but they still permitted ready discrimination between the putatively resistant and susceptible isolates.

Identification of *C. neoformans* var. *neoformans* and *C. neoformans* var. *gattii*. Two isolates of the general clinical isolates were found to be of *C. neoformans* var. *gattii*. The MICs for these two isolates were equal to or less than the modal MICs

TABLE 3. Susceptibility in yeast nitrogen base broth[a]

Isolate	*N*	Putative status[b]	MIC (μg/ml)[c]					
			48 h			72 h		
			Range	Geo M	Mode	Range	Geo M	Mode
CN 1	10	S	0.25–0.5	0.43	0.5	0.5–1	0.63	0.5
CN 2	10	S	0.25–1	0.5	0.5	0.5–1	0.66	0.5
CN 3	8	S	0.5–1	0.6	0.5	0.5–1	0.66	0.5
CN 4	8	S	0.5	0.5	0.5	0.5–1	0.82	1
CN 5	7	S	0.25–1	0.41	0.25	0.5–1	0.64	0.5
CN 6	8	S	0.25–0.5	0.35	0.25	0.5–1	0.64	0.5
CN 7	8	S	0.25–0.5	0.42	0.5	0.5	0.5	0.5
CN 9	11	S	0.5–2	0.68	0.5	0.5–1	0.75	1
CN 12	12	S	0.25–0.5	0.35	0.25	0.25–0.5	0.35	0.25
CN 8	9	R	1.0–2.0	1.36	1	1.0–2.0	1.86	2
CN 10	12	R	2	2	2	2	2	2
CN 11	12	R	1.0–2.0	1.85	2	1.0–2.0	1.74	2

[a] Twelve *C. neoformans* isolates were tested by the microdilution methodology in broth in YNB. The isolates were tested the indicated number of times (*N*). MICs were determined after 48 and 72 h of incubation.
[b] S, susceptible; R, resistant.
[c] Range, range of MICs; Geo M, geometric mean MIC; Mode, modal MIC.

TABLE 4. Susceptibility on RPMI 1640 agar[a]

Isolate	N	Putative status[b]	MIC (μg/ml)[c]					
			48 h			72 h		
			Range	Geo M	Mode	Range	Geo M	Mode
CN 1	3	S	0.19–0.25	0.23	0.25	0.38	0.38	0.38
CN 2	3	S	0.125	0.125	0.125	0.25	0.25	0.25
CN 3	3	S	0.19–0.125	0.14	0.125	0.19–0.38	0.26	0.19
CN 4	3	S	0.094–0.25	0.14	0.094	0.25	0.25	0.25
CN 5	3	S	0.064–0.094	0.07	0.064	0.19	0.19	0.19
CN 6	3	S	0.125–0.19	0.16	0.19	0.19–0.25	0.23	0.25
CN 7	3	S	0.094–0.125	0.11	0.125	0.19	0.19	0.19
CN 9	3	S	0.125	0.125	0.125	0.25	0.25	0.25
CN 12	3	S	0.125	0.125	0.125	0.19	0.19	0.19
CN 8	3	R	2.0–4.0	2.8	2	4.0–6.0	5.2	6
CN 10	3	R	1.5–2	1.65	1.5	2.0–4.0	2.5	2
CN 11	3	R	2	2	2	2	2	2
Clinical	72	S	0.006–0.38	0.086	0.094	0.064–0.5	0.162	0.19

[a] Each named *Cryptococcus* isolate was tested the indicated number of times by the E-test methodology in 2% glucose-supplemented RPMI 1640 agar. Isolates CN 1 to CN 12 were tested the indicated number of times (*N*), while the 72 clinical isolates were tested once each. MICs were determined after 48 and 72 h of incubation.
[b] S, susceptible; R, resistant.
[c] MIC range, range of MICs; Geo M, geometric mean MIC; Mode, modal MIC.

obtained for the other general clinical isolates tested in both RPMI 1640 medium and antibiotic medium 3.

DISCUSSION

RPMI 1640 medium, the medium recommended by NCCLS for use in the susceptibility testing of yeasts, has been extensively studied and used over the years. However, Ghannoum et al. (15) have reported that YNB buffered with MOPS to pH 7.0 was a better choice than RPMI 1640 medium for standard susceptibility testing of *C. neoformans* against fluconazole, amphotericin B, and flucytosine. Witt et al. (39) subsequently used YNB and a microtiter method to accurately predict treatment failure in fluconazole-treated patients who suffered from AIDS-associated cryptococcal meningitis. Substitution of YNB for RPMI 1640 medium is now mentioned as a possible alternative method in the NCCLS M27-A document (24).

Nonetheless, some investigators have pointed out that use of YNB is problematic. Independent of the buffer used, one group reported that YNB prolonged the doubling times of some isolates at physiologic pH (8), and a second group reported that the growth of some strains was poor in this medium (16). In our study and in agreement with other workers (15), we found that YNB supported enough growth of *C. neoformans* to permit determination of antifungal susceptibility with broth. However, YNB broth was found not to be a suitable culture medium for the detection of amphotericin B-resistant isolates of *C. neoformans* due to a significant overlap in the MIC ranges obtained for putatively amphotericin B-susceptible and -resistant isolates. YNB broth was capable of distinguishing resistant and susceptible isolates by examination of the modal MICs obtained upon repeat testing, but the great degree of overlap of the observed MIC ranges prevented reliable discrimination with single MIC determinations. Taken with the data of Witt et al. (39), these data suggest that YNB, while useful for the testing of fluconazole, is not a good choice for the testing of amphotericin B.

Use of antibiotic medium 3 has been shown to be useful

TABLE 5. Susceptibility on antibiotic medium 3 agar[a]

Isolate	N	Putative status[b]	MIC (μg/ml)[c]					
			48 h			72 h		
			Range	Geo M	Mode	Range	Geo M	Mode
CN 1	3	S	0.25	0.25	0.25	0.38	0.38	0.38
CN 2	3	S	0.25	0.25	0.25	0.38	0.38	0.38
CN 3	3	S	0.25	0.25	0.25	0.38–0.5	0.15	0.5
CN 4	3	S	0.094	0.094	0.094	0.19	0.19	0.19
CN 5	3	S	0.094	0.094	0.094	0.19	0.19	0.19
CN 6	3	S	0.064	0.064	0.094	0.125–0.25	0.18	0.125
CN 7	4	S	0.094–0.125	0.1	0.094	0.19	0.19	0.19
CN 9	3	S	0.064	0.064	0.064	0.19–0.25	0.13	0.19
CN 12	3	S	0.19	0.25	0.19	0.38	0.38	0.38
CN 8	3	R	2.0–3.0	2.6	3	3.0–4.0	3.3	3
CN 10	3	R	3.0–4.0	3.3	3	3.0–4.0	3.6	4
CN 11	3	R	2	4	4	4	4	4

[a] Each named *Cryptococcus* isolate was tested the indicated number of times by the E-test methodology in 2% glucose-supplemented antibiotic medium 3 agar after 48 and 72 h of incubation.
[b] S, susceptible; R, resistant.
[c] MIC range, range of MICs; Geo M, geometric mean MIC; Mode, modal MIC.

when trying to identify amphotericin B-resistant isolates of *Candida* (25, 31, 38), and our data indicate that this pattern was again evident for *C. neoformans*. When used in the microdilution format, good separation between the putatively susceptible and resistant isolates was obtained. Only one putatively susceptible isolate, isolate CN 3, showed an overlap with a single putatively resistant isolate, isolate CN 8. The correct interpretation of these results is uncertain. CN 3 may have reduced susceptibility to amphotericin B, given the elevated geometric mean MIC for the isolate in antibiotic medium 3 broth. However, testing on agar with the E-test system provided even better discrimination between these groups of isolates, suggesting that use of an agar-based methodology is a better predictor of amphotericin B resistance. In fact, on agar, a greater MIC range than that in broth was obtained when testing the 72 clinical isolates in either RPMI 1640 medium or antibiotic medium 3. The reason for such a wider range, which permits better discrimination between susceptible and resistant isolates, is unknown, but a similar effect has been observed for *Candida* (38). This happened independently of the time of incubation (48 and 72 h). The modal MICs obtained after 48 and 72 h by both the broth and the agar methodologies showed excellent consistency in either medium.

RPMI 1640 medium is the standard medium used with the NCCLS M27-A method, and some workers have reported the suitability of RPMI 1640 medium for susceptibility testing of *C. neoformans*. Anaissie et al. (2) found that RPMI 1640 medium gave more consistent results than Eagle's minimum essential medium or YNB when testing clinical isolates of *C. neoformans* in a microdilution format in broth, and a recent eight-center collaborative study (3) showed that RPMI 1640 medium provided satisfactory growth for both *C. albicans* and *C. neoformans*. Likewise, Franzot and Hamdan (11) reported the ability of RPMI 1640 medium to support the growth of 53 cryptococcal isolates, and Gadea et al. (13) found that supplementation of RPMI 1640 medium with 2% glucose provided enough growth of *C. neoformans* for the determination of MICs after 48 h of incubation. In agreement with those investigators, we found that RPMI 1640 medium supported the growth of all the *C. neoformans* isolates tested in the study, permitting the performance of susceptibility testing. In addition, the putatively amphotericin B-resistant isolates were identified by both the broth and the agar formats. However, as with YNB, the RPMI 1640 broth data showed frequent overlaps between the MIC ranges for putatively amphotericin B-susceptible and -resistant isolates, rendering the separation unreliable under this testing format. Conversely, testing on RPMI 1640 agar produced excellent discrimination between the two groups of strains, and an overlap in the observed MIC ranges was not noted after either 48 or 72 h of incubation. The results obtained with RPMI 1640 agar were thus qualitatively comparable to those obtained with antibiotic medium 3 broth, and RPMI 1640 agar thus appears to be acceptable for use in the testing of *C. neoformans*.

Interestingly, the MICs obtained with RPMI 1640 medium in the microdilution format were generally 1 twofold dilution higher than those obtained with antibiotic medium 3, while the situation was reversed when the agar format was used. The reason for this is unknown, but supplementation of RPMI 1640 agar with 2% glucose (20 g/liter) may play a role. Glucose supplementation of RPMI 1640 medium has been shown to be more useful than RPMI 1640 medium without glucose supplementation for the enhancement of growth, simplification of endpoint determinations, and detection of strains with decreased susceptibility to fluconazole (24, 34).

The ubiquitous variety *C. neoformans* var. *neoformans*, as opposed to *C. neoformans* var. *gattii*, which has a limited geographical distribution, has been the most frequent cause of the cryptococcal infections found in AIDS patients and immunocompromised patients (6, 33, 35, 37). This observation has been true even in those locations in which *C. neoformans* var. *gattii* is endemic (19). Infection with *C. neoformans* var. *gattii* is associated with longer hospital stays and a need for more prolonged courses of antifungal therapy, while infection with *C. neoformans* var. *neoformans* has been associated with an increased mortality rate (37). In our survey, only two isolates in the collection were identified as *C. neoformans* var. *gattii*. The susceptibilities of these two strains to amphotericin B were found to be similar to those of the other isolates tested. Although the number of *C. neoformans* var. *gattii* strains tested in this study is low, this suggests that the same treatment should be equally effective for both varieties.

On the basis of the aggregate results, we believe that isolates CN 8, CN 10, and CN 12 are resistant to amphotericin B, suggesting that isolates for which E-test MICs are $\geq$1 μg/ml on antibiotic medium 3 and on RPMI 1640 agar may be considered resistant. E-test MICs of $\leq$0.25 μg/ml on antibiotic medium 3 and of $\leq$0.5 μg/ml on RPMI 1640 agar suggest susceptibility to amphotericin B. The implications of MICs between these values are unclear and will require further work. In antibiotic medium 3 broth, amphotericin B MICs of $\geq$0.5 μg/ml may suggest resistance.

In conclusion, we have found that the identification of amphotericin B-resistant *C. neoformans* is possible using antibiotic medium 3 by the microdilution format with broth, the E-test on glucose-supplemented RPMI 1640 agar, or the E-test on glucose-supplemented antibiotic medium 3 agar. YNB is not a suitable medium. The time of reading of the results depends on the testing format used: reading of results after 48 h of incubation is required for the broth-based test, while reading of the results of tests done on agar can be performed after either 48 and 72 h. The reproducibility of these results would require validation in a multicenter study.

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