ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Apr. 1995, p. 906–909 Vol. 39, No. 4
0066-4804/95/$04.00+0

Detection of Amphotericin B-Resistant *Candida* Isolates in a Broth-Based System

JOHN H. REX,[1,2]* CHESTER R. COOPER, JR.,[3] WILLIAM G. MERZ,[4] JOHN N. GALGIANI,[5] AND ELIAS J. ANAISSIE[2]

Center for Infectious Diseases, Department of Internal Medicine, University of Texas Medical School,[1] and Department of Medical Specialties, M.D. Anderson Cancer Center,[2] Houston, and Medical Mycology Research Center, Department of Pathology, University of Texas Medical Branch, Galveston,[3] Texas; Department of Pathology, Johns Hopkins University, Baltimore, Maryland[4]; and Veterans Administration Medical Center, Tucson, Arizona[5]

Received 7 November 1994/Returned for modification 12 December 1994/Accepted 9 February 1995

Because of the limited ability of the National Committee for Clinical Laboratory Standards proposed M27P methodology to detect resistance to amphotericin B by *Candida* isolates, we sought to identify alternative media and pH conditions that could reliably identify resistant isolates. Antibiotic Medium 3 broth (also known as Penassay broth) buffered to pH 5 or pH 7 produced superior results and readily identified a series of resistant isolates.

Antifungal susceptibility testing is rapidly evolving. The M27P methodology currently under development by the National Committee for Clinical Laboratory Standards (NCCLS) gives reproducible results for testing of *Candida* and *Cryptococcus* isolates (3, 4, 6), and establishment of interpretive breakpoints is now beginning. However, it is becoming clear that the M27P methodology may be inadequate for certain organisms and antifungal agents. For example, *Cryptococcus neoformans* grows poorly in the RPMI 1640 medium suggested as part of the M27P methodology, and other media may therefore be preferable (5). In addition, concern has been raised that determination of susceptibility of *Candida* isolates to amphotericin B may be a problem (9). In this case, the M27P methodology yields a range of MICs that spans only three to four twofold serial dilutions. While MICs for putatively resistant isolates tend be at the high end of the range, the inherent variability of MIC determination precludes reliable discrimination between susceptible and resistant isolates. In this study, we have sought to resolve this problem by examining alternative media and pH conditions. We find that Antibiotic Medium 3 broth (also known as Penassay broth) buffered to pH 5 or pH 7 identifies amphotericin B-resistant isolates of *Candida* and is a candidate for standardization studies.

MATERIALS AND METHODS

Isolates. A series of putatively amphotericin B-resistant *Candida* isolates were obtained from a variety of sources (Table 1). In particular, a set of three *Candida* isolates previously characterized as amphotericin B resistant or susceptible on the basis of in vivo testing (1) was included. In addition, a pair of previously described bloodstream isolates (8) from a recent therapy trial comparing fluconazole with amphotericin B as therapy of candidemia (7) was also studied. Isolates were stored at −70°C and were passaged at least twice on Sabouraud dextrose agar at 35°C prior to being tested. Isolates were identified to the species level by using the API 20C system (Analytab Products, Plainview, N.Y.).

Susceptibility testing. Broth macrodilution MICs were determined by using the NCCLS M27P methodology. Briefly, yeasts at final concentrations of 0.5×10^3 to 2.5×10^3/ml were incubated in air at 35°C for 48 h with twofold dilutions of amphotericin B (0.0313 to 16 μg/ml). The MIC was defined as the lowest concentration of drug that completely inhibited growth (6).

While otherwise adhering to the procedures of the NCCLS M27P methodology, we carried out all other testing by using a broth microdilution format, reducing all volumes by 80% and performing the assay in a final volume of 200 μl in flat-bottomed 96-well microdilution trays (Falcon/Becton Dickinson, Lincoln Park, N.J.). Microdilution endpoints were determined after 24 and 48 h of incubation with twofold dilutions of amphotericin B (0.0625 to 64 μg/ml). The MIC of amphotericin B was the lowest concentration of drug that completely inhibited growth. Four different media were studied by this approach: Sabouraud broth (prepared as 10 g of neopeptone per liter of distilled water; Difco, Detroit, Mich.), Antibiotic Medium 3 broth (BBL/Becton Dickinson, Cockeysville, Md.), Casitone broth (prepared as 9 g of Casitone per liter of distilled water; Difco), and yeast nitrogen base (YNB; Difco). For each medium, glucose was added to

TABLE 1. *Candida* isolates used in this study

Isolate	*Candida* species	Putative status[a]	Comment (reference)
5W31	*C. lusitaniae*	Resistant	Proven in animal model (1)
CL2819	*C. lusitaniae*	Resistant	Proven in animal model (1)
Y533	*C. lusitaniae*	Resistant	Relatively high MIC noted
Y534	*C. lusitaniae*	Resistant	Relatively high MIC noted
CL524	*C. parapsilosis*[b]	Susceptible	Proven in animal model (1)
Y537	*C. albicans*	Resistant	Relatively high MIC noted
625-3	*C. albicans*	Susceptible	Bloodstream isolate responsive to amphotericin B (7)
MY1012	*C. tropicalis*	Resistant	Relatively high MIC noted
90B-4	*C. tropicalis*	Susceptible	Bloodstream isolate responsive to amphotericin B (7)

[a] Putative susceptibility status assigned on the basis of isolate's source and any known studies of that isolate.
[b] Previously described as *C. lusitaniae* but since reidentified as *C. parapsilosis.*

* Corresponding author. Mailing address: 6431 Fannin, 1728 JFB, Houston, TX 77030. Phone: (713) 792-4929. Fax: (713) 792-4937. Electronic mail address: jrex@heart.med.uth.tmc.edu (Internet).

TABLE 2. Failure of M27P methodology to detect resistance to amphotericin B

Time of reading and MIC (μg/ml)	Frequency of indicated MIC for:			
	Bloodstream isolates[a]	5W31 (R)[b]	CL2819 (R)[b]	CL524 (S)[b]
24 h				
0.125	5			3
0.25	41	2	2	1
0.5	44	1	4	1
1		3		
48 h[c]				
0.125	1			
0.25	18			4
0.5	154			3
1	59	3	2	1
2		5	4	

[a] Two hundred thirty-two bloodstream isolates were each tested once by the M27P methodology, and the distribution of MICs is shown. The MIC was also determined at 24 h for 90 of these isolates.
[b] The MICs for three isolates known to be either susceptible (S) or resistant (R) in an animal model were determined five to eight times, and the resulting frequency distribution of MICs is shown.
[c] Standard reading time for the M27P methodology.

achieve a final concentration of 2% (2 g/100 ml). All media were buffered to the pH indicated in the text. Buffering to pH 7 was achieved with 10 mM phosphate, while buffering to pH 5 was performed by using 10 mM acetate.

RESULTS

Failure of M27P to detect resistance to amphotericin B. In initial studies, the MICs for the three isolates tested in an animal model were compared with the MIC distribution for a large collection of previously described bloodstream isolates (Table 2). While the MIC for the two resistant isolates was at times 2 μg/ml, a value higher than any for the bloodstream isolate collection, a value of 1 μg/ml was obtained about one-third of the time, thus precluding consistent identification of the resistant isolates. When the M27P method was modified by reading at 24 h, resistant isolates could not be identified even upon repeated testing. Also of interest, the values for isolate MY1012, a putatively resistant isolate for which the amphotericin B MIC is >64 μg/ml under other conditions (see below), were only 1 to 2 μg/ml at 24 and 48 h by the M27P method. In other preliminary studies with RPMI 1640, we found (as have others [10]) that supplementation with 2% glucose enhanced the rate of fungal growth but did not alter MICs. While it was possible to slightly alter the absolute MICs, we were also unable to significantly alter the range of MICs by growing the inoculum on YNB with 2% glucose, altering the medium pH to values between 3.0 and 7.4, or incubating at 30°C (data not shown).

Survey of alternative media. Several possible alternative media and two possible pH values were examined in a microbroth format with the collection of putatively susceptible and resistant isolates (Tables 3 and 4). Failure of several isolates to grow in Casitone broth at pH 7 or in Sabouraud broth at either pH makes these conditions unsuitable. YNB at pH 5 provided moderate discrimination at both 24 and 48 h, but this medium tended to elevate the MICs for all isolates. The best discrimination was obtained with Casitone broth at pH 5 and Antibiotic Medium 3 broth at both pH values. In particular, Antibiotic Medium 3 broth at both pH 5 and pH 7 provided a separation of at least three twofold dilutions between resistant and susceptible isolates, even at 24 h. Also of interest is the strikingly high MIC recorded for isolate MY1012, and Antibiotic Medium 3 broth consistently produced the highest MIC for this isolate.

TABLE 3. Microbroth MICs at 24 h

Isolate[a]	MIC (μg/ml) with medium:						
	Antibiotic Medium 3		Casitone		Sabouraud		YNB (pH 5)
	pH 7	pH 5	pH 7	pH 5	pH 7	pH 5	
5W31 (R)	2	2	NG[b]	2	1	1	4
CL2819 (R)	1	1	1	2	1	0.0625	4
Y533 (R)	2	1	0.5	2	NG	NG	2
Y534 (R)	1	0.5	0.25	1	NG	NG	2
Y537 (R)	4	2	0.0625	1	0.5	1	2
MY1012 (R)	>64	64	2	8	NG	NG	4
CL524 (S)	0.125	0.0625	0.0625	0.25	0.125	0.25	1
625-3 (S)	0.125	0.125	0.0625	0.25	0.0625	0.25	1
90B-4 (S)	0.0625	0.25	0.0625	0.25	0.25	0.5	1

[a] R, putatively amphotericin B resistant; S, susceptible.
[b] NG, no growth.

Detailed studies using Antibiotic Medium 3 broth. As Antibiotic Medium 3 broth supported good growth of all yeast isolates and appeared to have potential for discrimination between susceptible and resistant isolates, the general distribution of MICs for *Candida* isolates was determined by testing isolates from the bloodstream collection at both pH 5 (218 isolates tested) and pH 7 (221 isolates tested). These results are shown in Table 5, along with the frequency distribution of the MICs obtained when the putatively resistant isolates were tested repeatedly. The MICs for isolates 625-3 and 90B-4 are included in the MICs for the bloodstream isolate collection and are not separately reported. By the same methodology as that used in the previous study of these isolates (8), correlation of outcome for the bloodstream isolates with MIC by all four methods demonstrated that all failures were due to the more numerous low-MIC isolates: with the 24-h reading, MICs for all failures were <0.25 μg/ml, while with the 48-h reading, MICs for all failures were <0.5 μg/ml. These data suggest that this group of isolates can be regarded as susceptible to amphotericin B. Antibiotic Medium 3 broth generally provided discrimination between resistant isolates and these susceptible ones, and the best discrimination of the resistant isolates

TABLE 4. Microbroth MICs at 48 h

Isolate[a]	MIC (μg/ml) with medium:						
	Antibiotic Medium 3		Casitone		Sabouraud		YNB (pH 5)
	pH 7	pH 5	pH 7	pH 5	pH 7	pH 5	
5W31 (R)	2	2	NG[b]	2	1	1	4
CL2819 (R)	2	2	2	4	2	2	8
Y533 (R)	2	1	1	2	1	NG	4
Y534 (R)	2	2	0.5	2	0.5	0.5	4
Y537 (R)	16	4	0.25	2	1	2	2
MY1012 (R)	>64	>64	8	>64	NG	NG	8
CL524 (S)	0.25	0.125	0.25	1	0.25	1	2
625-3 (S)	0.5	0.25	0.125	0.25	0.25	0.25	1
90B-4 (S)	0.125	0.5	0.25	0.5	0.5	0.5	2

[a] R, putatively amphotericin B resistant; S, susceptible.
[b] NG, no growth.

TABLE 5. Extended studies of Antibiotic Medium 3 broth[a]

Condition and MIC (μg/ml)	Frequency of indicated MIC for:							
	Bloodstream isolates	5W31 (R)	CL2819 (R)	Y533 (R)	Y534 (R)	Y537 (R)	MY1012 (R)	CL524 (S)
pH 7, 24 h								
0.0625	44							1
0.125	89		2					6
0.25	83							
0.5	5			3	5	1		
1		2	3	3	1			
2		5	3			1		
4						4		
8								
16								
32								
≥64							9	
pH 7, 48 h								
0.0625								
0.125	8							1
0.25	66		1					3
0.5	132							3
1	14	2	2	5	2	1		
2	1	2	1	1	4			
4		3	4					
8						1		
16						2		
32						2		
≥64							9	
pH 5, 24 h								
0.0625	60							2
0.125	89		1					
0.25	61							
0.5	7				2			
1			1	2				
2	1	2	1			2		
4								
8								
16								
32							1	
≥64							3	
pH 5, 48 h								
0.0625	5							
0.125	26							2
0.25	93							
0.5	88		1					
1	5			2	1			
2	1	2	2		1			
4						2		
8								
16								
32								
≥64							4	

[a] Isolates from the bloodstream isolate collection were each tested once, and the resulting distribution of MICs is shown. Putatively resistant (R) and susceptible (S) isolates were tested multiple times, and the resulting frequency distribution of MICs for the given isolate is shown.

among the bloodstream isolates was noted after incubation for 24 h.

DISCUSSION

In this report, we have confirmed that the NCCLS M27P reference methodology has a limited ability to identify amphotericin B-resistant *Candida* isolates. Although an adaptation of the M27P methodology to a microdilution format was not studied, comparable results have been reported for the two methods (2, 8). Other possible modifications (addition of glucose, growing the inoculum on a different medium, incubating at 30°C, or reading at 24 h) also did not appear to represent viable approaches. We thus sought alternative media that

might provide improved discrimination. It was also desirable that the selected medium be readily available, be usable at a neutral pH, and yield adequate results after 24 h of incubation.

First, a series of putatively resistant and susceptible isolates was assembled and several different media were considered. During this screening process, special emphasis was given to the results produced for three isolates that were known from animal studies (CL524, 5W31, and CL2819) to be relatively amphotericin B resistant or susceptible (1). Next, when Antibiotic Medium 3 broth was found to be potentially suitable, an extended study of a collection of bloodstream isolates was undertaken.

These data demonstrate that Antibiotic Medium 3 broth buffered to pH 5 or pH 7 provides discrimination between resistant and susceptible isolates. Further, when the general distribution of MICs for *Candida* isolates was determined for a collection of bloodstream isolates, it was found that the resistant isolates were clearly separated from those for which MICs were within the range typical for *Candida* isolates, particularly after 24 h of incubation. It is, however, noteworthy that the distribution of results obtained with Antibiotic Medium 3 for the putatively susceptible isolates was similar to that obtained by the M27P method, and this suggests that testing with Antibiotic Medium 3 does not produce an overall shift of MICs but rather serves to highlight resistant isolates.

As Antibiotic Medium 3 broth is not a defined medium, issues of standardization and reproducibility are especially important and will require careful testing to identify appropriate quality control isolates and to demonstrate interlaboratory reproducibility. These studies are currently under way.

ACKNOWLEDGMENTS

We thank K. A. Bartizal for isolates Y533, Y534, Y537, and MY1012 and Page W. Nelson for technical assistance.

REFERENCES

1. **Anaissie, E. J., N. C. Karyotakis, R. Hachem, M. C. Dignani, J. H. Rex, and V. Paetznick.** 1994. Correlation between in vitro and in vivo activity of antifungal agents against *Candida* species. J. Infect. Dis. **170:**384–389.
2. **Espinel-Ingroff, A., T. M. Kerkering, P. R. Goldson, and S. Shadomy.** 1991. Comparison study of broth macrodilution and microdilution antifungal susceptibility tests. J. Clin. Microbiol. **29:**1089–1094.
3. **Espinel-Ingroff, A., C. W. Kish, T. M. Kerkering, R. A. Fromtling, K. Bartizal, J. N. Galgiani, K. Villareal, M. A. Pfaller, T. Gerarden, M. G. Rinaldi, and A. Fothergill.** 1992. Collaborative comparison of broth macrodilution and microdilution antifungal susceptibility tests. J. Clin. Microbiol. **30:**3138–3145.
4. **Fromtling, R. A., J. N. Galgiani, M. A. Pfaller, A. Espinel-Ingroff, K. F. Bartizal, M. S. Bartlett, B. A. Body, C. Frey, G. Hall, G. D. Roberts, F. B. Nolte, F. C. Odds, M. G. Rinaldi, A. M. Sugar, and K. Villareal.** 1993. Multicenter evaluation of a macrobroth antifungal susceptibility test for yeasts. Antimicrob. Agents Chemother. **37:**39–45.
5. **Ghannoum, M. A., A. S. Ibrahim, Y. Fu, M. C. Shafiq, J. E. Edwards, and R. S. Criddle.** 1992. Susceptibility testing of *Cryptococcus neoformans*: a microdilution technique. J. Clin. Microbiol. **30:**2881–2886.
6. **National Committee for Clinical Laboratory Standards.** 1992. Reference method for broth dilution antifungal susceptibility testing of yeasts; proposed standard. M27-P. National Committee for Clinical Laboratory Standards, Villanova, Pa.
7. **Rex, J. H., J. E. Bennett, A. M. Sugar, P. G. Pappas, C. M. Van der Horst, J. E. Edwards, R. G. Washburn, W. M. Scheld, A. W. Karchmer, A. P. Dine, M. J. Levenstein, C. D. Webb, the Candidemia Study Group, and the NIAID Mycoses Study Group.** 1994. A randomized trial comparing fluconazole with amphotericin B for the treatment of candidemia in patients without neutropenia. N. Engl. J. Med. **331:**1325–1330.
8. **Rex, J. H., M. A. Pfaller, A. L. Barry, P. W. Nelson, and C. D. Webb for the NIAID Mycoses Study Group and the Candidemia Study Group.** 1995. Antifungal susceptibility testing of isolates from a randomized, multicenter trial of fluconazole versus amphotericin B as treatment of nonneutropenic patients with candidemia. Antimicrob. Agents Chemother. **39:**40–44.
9. **Rex, J. H., M. A. Pfaller, M. G. Rinaldi, A. Polak, and J. N. Galgiani.** 1993. Antifungal susceptibility testing. Clin. Microbiol. Rev. **6:**367–381.
10. **Rodriguez-Tudela, J. L., and J. V. Martinez-Suarez.** 1994. Improved medium for fluconazole susceptibility testing of *Candida albicans*. Antimicrob. Agents Chemother. **38:**45–48.