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Antifungal Susceptibility Survey of 2,000 Bloodstream *Candida* Isolates in the United States

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***Candida* bloodstream isolates (n = 2,000) from two multicenter clinical trials carried out by the National Institute of Allergy and Infectious Diseases Mycoses Study Group between 1995 and 1999 were tested against amphotericin B (AMB), flucytosine (5FC), fluconazole (FLU), itraconazole (ITR), voriconazole (VOR), posaconazole (POS), caspofungin (CFG), micafungin (MFG), and anidulafungin (AFG) using the NCCLS M27-A2 microdilution method. All drugs were tested in the NCCLS-specified RPMI 1640 medium except for AMB, which was tested in antibiotic medium 3. A sample of isolates was also tested in RPMI 1640 supplemented to 2% glucose and by using the diluent polyethylene glycol (PEG) in lieu of dimethyl sulfoxide for those drugs insoluble in water. Glucose supplementation tended to elevate the MIC, whereas using PEG tended to decrease the MIC. Trailing growth occurred frequently with azoles. Isolates were generally susceptible to AMB, 5FC, and FLU. Rates of resistance to ITR approached 20%. Although no established interpretative breakpoints are available for the candins (CFG, MFG, and AFG) and the new azoles (VOR and POS), they all exhibited excellent antifungal activity, even for those strains resistant to the other aforementioned agents.**

Candidemia is now the fourth-most-common bloodstream infection in the United States (11, 12, 23, 24). Antifungal susceptibility testing has become an important tool in the management of patients with invasive candidiasis, since both in vitro resistance and toxicity issues must be considered when selecting an antifungal agent (5, 10, 15, 30, 33). The NCCLS has developed the standardized and reproducible M27-A2 method for testing yeasts (18). This method is widely accepted and readily available in reference centers and specialized clinical laboratories. Although variations of this method have been proposed and intense investigation into the effects of different media and drug-solubilizing agents are ongoing, the basic method has proven to be a useful and reproducible standard (3, 4, 29, 36).

In this study, we examined the susceptibilities of 2,000 bloodstream *Candida* spp. isolates in the United States to currently licensed and newly available antifungal agents. Since small variations in the testing method have been shown to potentially increase the correlation of in vitro results with clinical response, three testing variations were studied: use of antibiotic medium 3 for testing amphotericin B (AMB), supplementation of the medium to 2% glucose (for all drugs), and use of polyethylene glycol (PEG) as a solvent (for drugs that are normally dissolved in dimethyl sulfoxide). (This work was presented in part as abstracts 642 and 643 at the 39th Annual Meeting of the Infectious Diseases Society of America, San Francisco, Calif., 2001.)

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MATERIALS AND METHODS

Isolates. The Mycoses Study Group (MSG) of the National Institutes of Health carried out two clinical trials for patients with candidemia between 1995 and 1999 in the United States. MSG 33 was a study of fluconazole (FLU) plus AMB versus FLU alone for the treatment of candidemia (32), and MSG 34 was an epidemiological study (R. J. Hamill, P. G. Pappas, J. H. Rex, J. Y. Lee, H. Horowitz, C. A. Kauffman, N. Hyslop, R. A. Larsen, D. K. Stein, E. A. Graviss, C. J. Thomas, and the Mycosis Study Group, Abstr. 38th Annu. Meet. Infect. Dis. Soc. Am., abstr. 36, 2000). The 39 participating centers shipped 2,947 *Candida* isolates from 1,911 patients to the Laboratory of Mycology Research at the University of Texas—Houston Medical School. Isolates were stored in sterile water at room temperature, with a backup in glycerol frozen at −70°C. Since some of the received isolates represented serial collection of isolates from a single patient, subsequent work focused on the 2,000 isolates that represented the first isolate of each species from each patient. Identification was carried out using the API 20C AUX method (bioMerieux Vitek, Inc., Hazelwood, Mo.), with supplemental standard morphological and biochemical testing for problem isolates using cultures in cornmeal agar, germ tube testing, and the Murex identification system (Murex Diagnostics, Norcross, Ga.). Identification of an isolate as *C. dubliniensis* was made based on (i) demonstration of absence of growth at 42°C, (ii) formation of abundant chlamydospores on cornmeal-Tween 80 agar, (iii) absence of assimilation of xylose or α-methyl-D-glucoside, (iv) DNA banding patterns characteristic of a type isolate following digestion of genomic DNA, and (v) amplification of a *C. dubliniensis*-specific 288-bp fragment (39).

Quality control isolates were used in every testing batch and included ATCC 750 (*C. tropicalis*), 5W31 (*C. lusitaniae*), ATCC 20019 (*C. parapsilosis*), ATCC 6258 (*C. krusei*), ATCC 90028 (*C. albicans*), and CL524 (*C. lusitaniae*). MICs for these isolates were compared with published control limits (20, 35) and used to guide quality control testing and validation per NCCLS guidelines (18).

Drugs. AMB, 5-flucocytosine (5FC), FLU, itraconazole (ITR), voriconazole (VOR), posaconazole (POS), caspofungin (CFG), micafungin (MFG), and anidulafungin (AFG) were obtained from their manufacturers as research powders and frozen (−70°C) or refrigerated (3°C) as required. Drug stocks (100×)

TABLE 1. MIC_{50} and MIC_{90} summary for the most common *Candida* spp. with nine antifungal agents[a]

Species (*n*)	MIC (μg/ml)																	
	AMB		5FC		FLU		ITR		POS		VOR		AFG		CFG		MFG	
	50%	90%	50%	90%	50%	90%	50%	90%	50%	90%	50%	90%	50%	90%	50%	90%	50%	90%
C. albicans (733)	0.06	0.25	0.13	1	0.25	2	0.06	0.5	0.03	0.13	0.03	0.06	0.03	0.03	0.5	0.5	0.03	0.03
C. glabrata (458)	0.13	0.5	0.13	0.13	8	32	1	4	1	2	0.25	1	0.03	0.13	0.5	1	0.03	0.06
C. parapsilosis (391)	0.13	0.5	0.13	0.13	1	2	0.13	0.25	0.03	0.13	0.03	0.06	2	2	2	2	1	2
C. tropicalis (307)	0.13	0.5	0.13	0.5	0.5	16	0.13	1	0.06	1	0.06	2	0.03	0.13	0.5	1	0.03	0.06
C. krusei (50)	0.25	0.5	4	32	32	>64	0.5	1	0.25	0.5	0.5	1	0.06	0.13	1	2	0.13	0.25
C. lusitaniae (20)	0.13	0.5	0.13	0.13	0.5	2	0.06	0.25	0.03	0.13	0.03	0.06	0.06	0.25	1	2	0.06	2
C. dubliniensis (18)	0.03	0.13	0.13	0.13	0.13	0.5	0.03	0.06	0.03	0.06	0.03	0.03	0.03	0.06	0.5	0.5	0.03	0.03

[a] Shown are the results for MIC-0 at 24 h for AMB and MIC-2 at 48 h for all other drugs. *n,* number of isolates.

were made following the NCCLS M27-A2 recommendations (18). AMB, ITR, VOR, POS, and AFG were diluted in dimethyl sulfoxide. 5FC, FLU, CFG, and MFG were diluted in deionized water. Additional testing was also carried out for a limited number of randomly selected strains (~15%) for ITR, VOR, and POS in PEG 400 (Sigma, St. Louis, Mo.). All drug stocks were frozen at −70°C until plate preparation. Testing ranges were 0.03 to 16 μg/ml for all drugs, except for 5FC and FLU, which were tested at 0.13 to 64 μg/ml.

Antifungal susceptibility testing. Antifungal susceptibility testing was carried out following the NCCLS M27-A2 microdilution method (18). Briefly, isolates were tested against all antifungal agents except AMB in RPMI 1640 (Sigma) buffered with 0.075 M 3-(*N*-morpholino)propanesulfonic acid, pH adjusted to 7.0. Supplemental testing was carried out on randomly selected (~15%) isolates in 3-(*N*-morpholino)propanesulfonic acid-buffered RPMI 1640 supplemented with glucose to 20 g/liter. AMB was tested in antibiotic medium 3 (Becton Dickinson, Cockeysville, Md.) buffered with NAH_2PO_4 H_20 plus $NAHPO_4$, pH adjusted to 7.0. Serial dilutions (2×) of the antifungals in the appropriate medium were performed, and 100 μl of the dilutions was dispensed on microdilution plates (Corning, Corning, N.Y.). Plates were frozen at −70°C until used. Validation of plate stability and potency was performed, running quality control organisms with each batch of tests. After growth on Sabouraud dextrose agar overnight, the fungal inocula were prepared as per M27-A2 to yield a 2× inoculum (1×10^3 to 5×10^3 CFU/ml), of which 100 μl was dispensed in each well of the microdilution plate for testing, resulting in the appropriate concentration of medium, drug, and microorganisms in each well. The plates were incubated at 35°C. MICs were assessed visually and by a spectrophotometer reading at 570 nm after agitation at 24 and 48 h. MICs are defined as follows: MIC-0 corresponds to the lowest drug concentration producing an optically clear well or 95% reduction in optical density compared with medium only and MIC-2, the lowest drug concentration producing prominent growth reduction or a 50% reduction in optical density. This report focuses on spectrophotometer readings, which were occasionally overridden by visual readings in cases of erroneous or technically deficient spectrophotometric readings. Unless otherwise noted, the MIC is the MIC-0 for AMB and MIC-2 for all other drugs. The choice of MIC-0 and antibiotic medium 3 for AMB is based on the fact that these testing conditions appear to discriminate more-resistant isolates (36).

Statistical analysis. Descriptive statistics were performed using Microsoft Excel and Access functions. r^2 for regression analysis between azole congeners was calculated using the $\log_{10}$ of the MICs with Epi Info 2002 software (Centers for Disease Control and Prevention, Atlanta, Ga.).

RESULTS

NCCLS-M27-A2-based data by drug. Table 1 shows the MICs at which 50% (MIC_{50}) and 90% (MIC_{90}) of the isolates tested were inhibited for each drug at 24 h (AMB) or 48 h (all other drugs) for the most commonly seen *Candida* spp. Table 2 shows MICs for the less frequent *Candida* spp. encountered in the survey. Table 3 shows the frequency of drug-resistant isolates identified in the survey for drugs that have established NCCLS interpretative breakpoints. While limited in numbers, the less commonly encountered *Candida* spp. showed uniform susceptibility to all of the drugs and are not specifically discussed in the paragraphs below.

AMB. Based on the medium and endpoints chosen, resistance to AMB appears to be rare. Using tentative breakpoints suggested in previous work (6, 34), 2 to 3% of *C. parapsilosis* and *C. krusei* isolates appeared to be resistant to this drug (Table 3). Higher MICs were not seen for *C. lusitaniae*, a species that is often, but not always, found to be resistant to AMB (16).

5FC. More than 95% of isolates of all species except *C. krusei* and *C. tropicalis* were susceptible to 5FC. Resistance to 5FC was noted for 12% of *C. krusei* isolates and 6% of *C. tropicalis* isolates.

FLU. Susceptibility to FLU was similar to that seen in other major surveillance surveys. *C. krusei* and *C. glabrata* showed the highest MICs. Overall resistance to FLU occurred in less than 10% of the tested strains. A separate analysis (data not shown) of FLU resistance by year failed to show an increase in resistance to FLU over the study years. Likewise, no regional variations in resistance were seen among the participating centers.

ITR. As with FLU, the susceptibility patterns of ITR were concordant with prior work. *C. glabrata* and *C. krusei* showed high MICs. Complete resistance was seen in 18% of isolates and thus was overall more common than for FLU.

POS. No interpretive breakpoints have been established for this compound. Most isolates had low MICs (0.03 to 0.13 μg/ml), with higher MICs noted for *C. glabrata* and *C. krusei*. The MIC_{90} for *C. tropicalis* was increased due to the trailing phenomenon (see below).

VOR. No interpretive breakpoints have been established. MICs were mostly in the range of 0.03 to 0.25 μg/ml. Higher MICs were noted for *C. krusei* and *C. glabrata*. The MIC_{90} for *C. tropicalis* was elevated due to trailing (see below).

AFG. There are no established interpretive breakpoints for AFG. Most isolates exhibited MICs of 0.03 to 0.06 μg/ml, but *C. parapsilosis* strains showed MICs of 1 to 4 μg/ml.

CFG. There are no established interpretive breakpoints for caspofungin. Most isolates showed MICs of 0.5 to 2 μg/ml, with *C. parapsilosis* isolates tending to concentrate on the higher end. Paradoxical fungal growth at the highest drug concentrations, the so-called "Eagle" phenomenon, of unknown (but unlikely) in vivo significance (8, 40, 41) and slight trailing were occasionally observed when testing isolates with this drug.

TABLE 3. Resistance rates for antifungals with published interpretive breakpoints[a]

Species (*n*)	Frequency (%) of resistance to:			
	AMB	5FC	FLU	ITR
C. albicans (733)	0	3	5	8
C. glabrata (458)	0.8	0.2	8	51
C. parapsilosis (391)	2.5	2	2	4
C. tropicalis (307)	0.3	6	8	12
C. krusei (50)	2	12	34	20
C. lusitaniae (20)	0	0	0	0
C. dubliniensis (18)	0	0	0	0
Overall	0.8	3	6	18

[a] Resistance is defined as the following MICs (in micrograms per milliliter): AMB, >1; 5FC, ≥32; FLU, ≥64; and ITR, ≥1. *n,* no. of isolates.

MFG. As with the other candins, interpretive breakpoints are unknown, but most isolates exhibited MICs in the range of 0.03 to 0.06 μg/ml, except for *C. parapsilosis*, which had MICs of 0.5 to 4 μg/ml. *C. krusei* and *C. lusitaniae* also tended to have slightly higher MICs.

Cross-resistance. Cross-resistance among the azoles, particularly for pairs of congeners (FLU-VOR and ITR-POS), has been a concern (22, 43, 44). Table 4 shows summaries for the two combinations. As seen in Table 4, VOR MICs generally correlated with FLU MICs, although some dispersion was seen ($r^2 = 0.48$). This correlation was much better for ITR and POS ($r^2 = 0.65$) (Table 4). The ultimate significance of these relationships remains to be determined.

Variations by testing media and drug solubilizing agent. Table 5 shows MIC variations by testing medium and drug solubilizing agent when compared to standard RPMI and recommended solvents for 344 isolates. In general, adding more glucose to the medium tended to increase MICs of AFG and CFG and decrease the MICs of FLU and VOR while it increased those of ITR. Using PEG as a solvent decreased MICs of azoles by one to two dilutions for ~30% of isolates, whether glucose content was increased or not.

The trailing phenomenon. The trailing phenomenon is most often encountered with azoles and is characterized by incomplete inhibition of growth (14, 31, 42). Severe trailing can be identified by complete or partial growth inhibition at 24 h and a partial growth inhibition at 48 h, with an elevated MIC. The significance of this phenomenon and its impact on resistance have been studied and do not appear to correlate with clinical success or failure. Rather, trailing seems to be an artifact of the method (2, 14, 42). This particularly applies to *C. tropicalis*, which has a high frequency of trailing yet appears to be a consistently azole-susceptible species. In contrast, *C. glabrata* and *C. krusei* also show trailing, but they are truly known to be less susceptible or resistant to azoles, thus adding to the evidence that this phenomenon has no in vivo or clinical correlation. The true nature, ramifications, and mechanisms of this phenomenon are still under intense scrutiny. For this study, we arbitrarily defined trailing growth as an eightfold increase in MIC between 24- and 48-h results for any isolate. Table 6 shows the frequency of trailing for different drugs and *Candida* spp. Trailing was a particular issue for *C. krusei* with 5FC, for

TABLE 2. MICs for infrequent Candida spp.[a]

Species (*n*)	Median (range) MIC (μg/ml)							
	AMB	5FC	FLU	ITR	POS	VOR	AFG	CFG	MFG
C. guilliermondii (9)	0.06 (0.03–0.25)	0.13 (0.13–64)	4 (0.25–8)	0.5 (0.03–2)	0.06 (0.03–0.25)	0.06 (0.03–0.13)	1 (0.06–2)	1 (0.5–2)	0.5 (0.06–0.5)
C. kefyr (4)	0.5 (0.13–0.5)	0.13	1 (0.13–1)	0.13 (0.03–0.5)	0.06 (0.06–0.25)	0.03	0.06 (0.03–0.5)	0.5 (0.5–1)	0.06 (0.06–0.5)
C. lipolytica (2)	1	4 (2–4)	16 (2–16)	2 (0.06–2)	0.25 (0.03–0.25)	0.06 (0.03–0.06)	1 (0.03–1)	2 (0.5–2)	0.5 (0.13–0.5)
C. rugosa (7)	0.13 (0.03–0.5)	0.25 (0.13–0.25)	4 (1–16)	0.06 (0.03–1)	0.03 (0.03–1)	0.03 (0.03–0.25)	0.03 (0.03–4)	2 (1–2)	0.06 (0.03–>64)
C. sphaerica (1)	0.13	0.13	4	0.13	0.13	0.03	0.13	2	0.25

[a] Shown are the results for MIC-0 at 24 h for AMB and MIC-2 at 48 h for all other drugs. *n,* no. of isolates.

TABLE 4. Cross-distribution of azole congener MICs[a]

Drug	MIC (μg/ml)	No. of isolates with MIC combination																				
		VOR MIC (μg/ml)											POS MIC (μg/ml)									
		0.03	0.06	0.13	0.25	0.5	1	2	4	8	16	>16	0.03	0.06	0.13	0.25	0.5	1	2	4	8	16	>16
FLU	0.13	191	5	1	1		1	1															
	0.25	368	15	3	2	2	2		1			5											
	0.5	293	53	9	3	3	2	1	1	1	1	3											
	1	182	46	26	5	3		2		3	1	4											
	2	60	27	20	5	3		1	1		2	3											
	4	14	30	43	25	4	4		1	1	1	2											
	8	6	15	55	66	35	5	4				1											
	16	2	3	8	25	48	35	2	1														
	32	3	2	4	21	15	27	5	1	1	2												
	64	5			4	13	3	9	2	1													
	>64	14	2	2	1	3	9	7	16	11	2	17											
ITR	0.03												374	17	1		1		1				1
	0.06												296	160	29	5	3	2	1	2	1		1
	0.13												116	109	27	10	6			1			
	0.25												24	76	52	31	17	6		1			
	0.5												8	23	17	59	109	42	8	3	1		2
	1												3	2	5	7	50	80	23	3			
	2												7	2	3	4	10	23	28	4	2		4
	4												1	2	1	1	2	20	17	2	1		3
	8															1		2	2	2		1	
	16															1		2	2	2		1	
	>16												4	1		2			5	6	4		12

[a] Distribution of VOR MICs by FLU MICs ($r^2 = 0.48$) and POS MICs by ITR MICs ($r^2 = 0.65$). All MICs are MIC-2 at 48 h.

all species except *C. parapsilosis* in the presence of azoles, and for *C. parapsilosis* when examining candins.

DISCUSSION

We present antifungal susceptibility data for a large survey of *Candida* spp. isolates causing bloodstream infections in the United States between 1995 and 1999. Our results confirm data from other surveys (21, 25, 27) and comprehensively present data on currently available and/or new antifungal agents.

While species-specific variations and occasional resistance were encountered, we can generally state that *Candida* spp. were susceptible to the traditional standards of treatment for primary infection: AMB and FLU. We found relatively low levels of FLU resistance and no geographic or temporal variations, as opposed to the findings previously described by Pfaller et al. (26). Resistance to ITR was found in nearly 20% of strains, which is a proportion compatible with previously published reports, and had species-specific trends. As shown in several earlier papers, resistance to older azoles is most commonly demonstrated for *C. krusei* and *C. glabrata* (2, 3, 22–24, 37). The new azoles (POS and VOR) have encouraging and potent antifungal activity against all *Candida* spp. including C. *glabrata* and *C. krusei*. Early in vivo and clinical experiences against infections caused by these organisms are encouraging as well (1, 9, 19, 27, 38).

The candins, a new class of antifungal agent, seem to have excellent in vitro activity against these organisms. It might be important to note here that the interpretation of MICs for these drugs is still a matter of some debate. Of note are the

TABLE 5. MIC variations by differences in test medium and solvent when compared to RPMI and standard solvent for 344 *Candida* spp. isolates[a]

Drug	% of isolates for which result of MIC comparison applies in:								
	RPMI–2% GLU			RPMI–2% GLU + PEG solvent			RPMI + PEG solvent		
	Identical	Up to 2 dilutions higher	Up to 2 dilutions lower	Identical	Up to 2 dilutions higher	Up to 2 dilutions lower	Identical	Up to 2 dilutions higher	Up to 2 dilutions lower
AFG	48	50	2						
CFG	51	30	18						
FLU	54	1	42						
5FC	83	1	16						
ITR	28	57	14	52	0	43	60	6	34
POS	68	14	14	66	1	26	69	2	27
VOR	81	3	14	69	1	27	73	1	25

[a] Data are shown for MIC-2 at 48 h. GLU, glucose.

TABLE 6. Frequency of the trailing phenomenon by drug and *Candida* spp.[a]

Species	Frequency (%) of trailing with:							
	5FC	FLU	ITR	POS	VOR	AFG	CFG	MFG
C. albicans	9	12	13	6	6	1	3	2
C. glabrata	1	14	29	30	32	2	2	2
C. krusei	18	14	25	20	14	2	6	4
C. parapsilosis	3	7	11	4	2	16	12	11
C. tropicalis	4	22	19	17	22	1	3	2

[a] Trailing growth was defined as an eightfold increase of the MIC-2 between 24 and 48 h.

relatively high MICs that were seen for *C. parapsilosis.* While these MICs are comparatively higher than those for the other species, there are no in vitro or in vivo data to suggest that this represents resistance, and the achievable blood concentrations of candins at the currently recommended doses generally equal or slightly exceed these MICs (13). In fact, a recent study of treatment of candidiasis with CFG versus AMB failed to show significant variation in response rates by species (17). The present survey also presents susceptibility data for less common *Candida* spp. While the numbers are limited, generally good activity for most of the drugs was shown.

This survey also provides information on the performance and reliability of the NCCLS M27-A2 method and its variations. Adding glucose to the medium tended to increase candin and ITR MICs and decrease FLU MICs. Adding PEG as a solvent tended to decrease MICs of ITR, POS, and VOR, perhaps due to better solubility and delivery of the drug. Nevertheless, the vast majority of MICs consistently remained within two dilutions of the MIC obtained by the standard NCCLS M27-A2 method.

The frequencies of the trailing phenomenon are consistent with what has been previously reported (2). Our definition was strict and very sensitive. It is also important to consider that trailing isolates were not excluded from the resistance analysis; thus, the true frequency of resistance may be slightly overestimated. The nature of the trailing phenomenon is unknown, as is its contribution to the perception of resistance in vitro and the ultimate possibility of in vivo resistance translation (14, 31, 33, 36, 42).

Cross-resistance between the old and newer azoles deserves further exploration. This phenomenon as been previously considered (28), and this study showed a proportional increase of FLU-VOR and ITR-POS MICs, with r^2 values of 0.48 and 0.65, respectively. While the MICs of the newer azole agents are lower than achievable concentrations, the clinical significance of these observations remains to be determined. Early experience shows good in vivo and clinical activity of these two new compounds against azole-resistant strains, classically azole-resistant species like *C. krusei*, and species with dose-dependent susceptibility like *C. glabrata* (7; L. Ostrosky-Zeichner, A. M. L. Oude Lashof, B. J. Kullber, and J. H. Rex, Abstr. 40th Ann. Meet. Infect. Dis. Soc. Am., Abstr. 352, 2002).

While correlation with clinical outcomes is still needed to validate the method for new drugs and establish interpretive breakpoints, this study provides evidence of the reproducibility and reliability of the NCCLS M27-A2 method and MIC trends and patterns for these drugs.

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