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Fluconazole Susceptibilities of Bloodstream *Candida* sp. Isolates as Determined by National Committee for Clinical Laboratory Standards Method M27-A and Two Other Methods

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The in vitro activity of fluconazole against 143 *Candida* spp. obtained from the bloodstreams of 143 hospitalized patients from 1995 to 1997 was studied. Susceptibility tests were carried out by two macrodilution methods, the M27-A and a modified M27-A method (0.165 M, pH 7/morpholinepropanesulfonic acid-buffered RPMI 1640 medium supplemented with 20 g of D-dextrose per liter), and by the agar diffusion method (with 15-μg fluconazole [Neo-Sensitab] tablets). With 2 μg of fluconazole per ml, 96.92% of 65 *C. albicans* isolates, 86.2% of 58 *C. parapsilosis* isolates 7 of 8 *C. tropicalis* isolates, and 1 of 6 *C. glabrata* isolates were inhibited. Only one strain of *C. albicans* and one strain of *C. tropicalis* were resistant. The agreement between the two macrodilution methods was greater than 90% within ±2 $\log_2$ dilutions for all strains except *C. glabrata* (83.3%) and *C. tropicalis* (87.5%). Generally, MICs were 1 $\log_2$ dilution lower in glucose-supplemented RPMI 1640 medium. No correlation between zone sizes and MICs was found. All strains susceptible by the diffusion test were susceptible by the dilution method, but the converse was not necessarily true. Interestingly, inhibition zones were smaller for *C. albicans*, for which the geometric mean MIC was 0.29 μg/ml and the mean inhibition zone diameter was 25.7 mm, while for *C. parapsilosis* the geometric mean MIC was 0.96 μg/ml and the mean inhibition zone diameter was 31.52 mm. In conclusion, the two macrodilution methods give similar results. The modified M27-A method with 2% dextrose has the advantage of shortening the incubation time and simplifying the endpoint determination.

Fungal infections represent a significant cause of morbidity and mortality in immunocompromised patients. The majority of these infections are reported to be caused by *Candida* spp., which, at this moment, are the fourth most common group of isolates recovered from blood cultures in the United States (10). Rates of candidemia are also increasing substantially in Europe.

Although *C. albicans* continues to be the most common cause of invasive mycoses, in recent years non-*C. albicans Candida* species (e.g., *C. parapsilosis*, *C. glabrata*, *C. tropicalis*, or *C. krusei*) have become increasingly important causes of fungemia in hospitalized patients and account for more than 60% of *Candida* species isolated from blood in several institutions (5, 11).

Although amphotericin B has been the most effective antifungal agent against systemic infections, the introduction of new azole compounds (fluconazole [FZ], ketoconazole, itraconazole) with increased potency and safety has made long-term and low-toxicity treatments possible. However, the availability and increased use of these compounds may lead, in some cases, to the development of drug resistance. This situation requires the use of in vitro antifungal susceptibility tests when a deep-stated mycotic infection is present.

Since publication in 1995 and 1997 of standard guidelines (M27-A) for testing of the susceptibilities of yeasts to antifungal agents by both macro- and microdilution methods by the National Committee for Clinical Laboratory Standards (NCCLS) (12, 13), it is possible to study and compare the results of susceptibility tests reported in the literature.

We studied the susceptibilities to FZ of the *Candida* spp. isolated from blood in our hospital during 1995, 1996, and 1997 by the M27-A broth macrodilution method, a modified M27-A broth macrodilution method, and the agar diffusion method.

MATERIALS AND METHODS

Antifungal agent. A solution of 2 mg of FZ per ml in saline (FZ for intravenous infusion; Pfizer, Madrid, Spain) was used. Further dilutions were made in the assay medium. Neo-Sensitab tablets of FZ (15 μg) were provided by Rosco Diagnostica (Taastrup, Denmark) and were stored at room temperature.

Assay media. Two media were used: (i) standard RPMI 1640 medium and (ii) a modified (2% dextrose) standard RPMI 1640 medium (RPMI-G medium; GIBCO BRL, Izasa, S.A., Barcelona) (18).

Yeast isolates. A total of 143 yeast isolates were tested. They comprised 65 *C. albicans*, 58 *C. parapsilosis*, 6 *C. krusei*, 6 *C. glabrata*, and 8 *C. tropicalis* isolates. All isolates were recovered from cultures of blood from 143 patients (95% adults) with severe underlying illnesses or clinical conditions (leukemia, diabetes, human immunodeficiency virus infection, trauma, solid-organ transplantation, or surgery) hospitalized in the intensive care, hematology, burn, cardiac surgery, trauma, and oncology units in the Hospital La Fe, Valencia, Spain, from 1995 to 1997. Strains subsequently isolated from the same patient were excluded. Culture, identification, and preservation of strains were carried out by standard methods (1). *C. albicans* ATCC 64548, *C. albicans* ATCC 90028 (FZ-susceptible strains), and *C. krusei* ATCC 6258 (FZ-resistant strains) were included as controls with each batch of organisms tested.

Susceptibility testing. (i) Broth macrodilution method. The broth macrodilution method was performed as described in NCCLS document M27-A (13) with RPMI 1640 and RPMI-G media by the spectronic method for inoculum preparation. The final inoculum varied between 1×10^3 and 5×10^3 CFU/ml and was confirmed by plating 10 and 100 μl from the drug-free control tube onto Sabouraud dextrose agar (Difco). The final volume was 1 ml. The FZ concentrations tested ranged from 64 to 0.03 μg/ml. Drug-free and yeast-free controls were included. Readings were made visually after 48 h of incubation at 35°C in RPMI 1640 medium and after 24 h in RPMI-G medium, according to the criteria determined for the NCCLS proposed standard (1:5 dilution of the corresponding growth control) (12). The MIC was defined as that concentration of drug that

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TABLE 1. Distribution of fluconazole MICs tested by broth macrodilution methods

Organism (no. of isolates)	Medium	No. of isolates with indicated MIC (μg/ml):											
		0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64
C. albicans (65)	RPMI 1640	1	3	17	23	14	3	2			1		1
	RPMI-G	1	4	25	24	6	3	1	1				
C. parapsilosis (58)	RPMI 1640		1	1	2	17	26	3	4	3	1		
	RPMI-G			1	7	21	18	6	1	3	1		
C. glabrata (6)	RPMI 1640					1				1	1	2	1
	RPMI-G					1				3	2		
C. krusei (6)	RPMI 1640											1	5
	RPMI-G										2	3	1
C. tropicalis (8)	RPMI 1640					5		2					1
	RPMI-G			1	2	4							1

produced an 80% reduction in turbidity compared with that of the drug-free control after 48 h of incubation in RPMI 1640 medium and after 24 h of incubation in RPMI-G medium. Discrepancies among MIC endpoints of no more than 2 dilutions were used to calculate the percent agreement. The MICs for all yeast strains that were inhibited by FZ concentrations of ≥1 μg/ml were redetermined. Isolates were classified as susceptible if the MIC was ≤8 μg/ml, susceptible, dose dependently, if the MIC was 16 to 32 μg/ml, and resistant if the MIC was ≥64 μg/ml (13).

(ii) Agar diffusion method. Disk diffusion tests were performed on modified Shadomy agar by following the manufacturer's recommendations (6). An FZ tablet (15 μg) was placed on the agar surface; subsequently, the diameters of the inhibition zones were measured with a metric vernier caliper after 24 h of incubation by following the criteria of the manufacturer both for the measurement of inhibition zones and for the interpretation of the diameters of the inhibition zones. For *Candida* spp. isolated from blood and sterile body fluids, strains with inhibition zones of ≥30 mm were considered susceptible, those with inhibition zones of <22 mm were considered resistant, and those with inhibition zones of 29 to 23 mm were considered intermediate.

RESULTS

The mode MICs of FZ for the reference strains were as follows: for *C. albicans* ATCC 90028 in RPMI 1640 and RPMI-G media, 0.25 and 0.12 μg/ml, respectively (ranges 0.12 to 0.5 μg/ml in both media); for *C. albicans* ATCC 64548, 0.25 and 0.25 μg/ml, respectively (range, 0.25 to 0.5 and 0.12 to 0.25 μg/ml, respectively); and for *C. krusei* ATCC 6258, 64 and 32 μg/ml, respectively (ranges, 64 and 16 to 32 μg/ml, respectively).

The susceptibilities of the yeast isolates to FZ obtained by the broth macrodilution method with the two media are presented in Table 1. As expected, all six isolates of *C. krusei* were resistant and susceptible, dose dependently, to FZ, with MICs of ≥32 μg/ml. The range of MICs for the *Candida* spp. isolated was wide for *C. albicans* and *C. parapsilosis*; *C. albicans* was the most susceptible organism tested. By the reference method, 1 μg of FZ per ml inhibited 93.85% of *C. albicans* isolates, 81.03% of *C. parapsilosis* isolates, 62.5% of *C. tropicalis* isolates, and 16.6% of *C. glabrata* isolates. For two strains (3.07%) of *C. albicans* and one strain (1.72%) of *C. parapsilosis*, FZ MICs were ≥16 μg/ml. The percent agreement between the MICs obtained in RPMI-G medium and those obtained by the reference broth macrodilution method ranged from 62.5 to 84.5% within ±1 $\log_2$ dilution and 83.3 to 100% within ±2 $\log_2$ dilutions. Except for *C. krusei*, for which agreement was 100% within ±2 $\log_2$ dilution, *C. parapsilosis* was the species for which the best agreement was achieved for all criteria used (the same result for ±1 and ±2 $\log_2$ dilutions). The major discrepancies were found for two strains of *C. albicans*: one was susceptible, dose dependently, in RPMI 1640 medium and the other was resistant in RPMI 1640 medium (MICs, 16 and >64 μg/ml, respectively), and both were susceptible in RPMI-G medium (MICs, 0.12 and 0.25 μg/ml, respectively). By the diffusion test, the inhibition zones for these strains were 23.2 and 22.3 mm, respectively. The MIC for the resistant strain was determined three times, and the same result was always obtained. However, when a less restrictive endpoint criterion (50% reduction of growth) was applied, the MICs were 4, 0.5, and 0.5 μg/ml in RPMI 1640 medium, but if the reading was taken at 24 h, the MIC was 0.12 μg/ml. In RPMI-G medium the MIC was 0.25 μg/ml all the three times. For the susceptible, dose dependently, strain, the MIC diminished to 0.06 μg/ml when the 50% reduction of growth criterion was applied.

The results of the agar diffusion test are presented in Table 2. A distribution of two well-defined populations can be observed: one dominant population with zone diameters of 23 to 29 mm for *C. albicans* and >30 mm for *C. parapsilosis* and a smaller population with zone diameters of <22 and <30 mm for *C. albicans* and between 23 and 29 mm for *C. parapsilosis*.

No correlation was found between MICs and inhibition zone

TABLE 2. Comparison of FZ MICs obtained by reference method with agar diffusion inhibition zones

FZ MIC (μg/ml)	No. of strains with the indicated inhibition zone diam (mm):														
	C. albicans			*C. parapsilosis*			*C. glabrata*			*C. krusei*			*C. tropicalis*		
	<22	23–29	≥30	<22	23–29	≥30	<22	23–29	≥30	<22	23–29	≥30	<22	23–29	≥30
0.03		1													
0.06		3				1									
0.12	1	13	1		1										
0.25	4	20	2		1	1									
0.5	4	6	3	1	2	14	1						2	2	1
1	1	1	1	1	8	17									
2		1	1	1		2							1	1	
4					2	2									
8				1	2		1								
16		1			1		2	1							
32							1			1					
≥64	1									5			1		
Total	11	46	8	4	17	37	5	1	0	6	0	0	4	3	1

[a] FZ MICs were determined by the NCCLS reference method.

diameters. In a comparison of the rank order of FZ susceptibilities according to MIC determined by the M27-A method with that according to inhibition zone diameters determined by the disk diffusion method, a major agreement for *C. parapsilosis* was found, in that for 37 strains that were susceptible according to their zone diameters, MICs were ≤4 μg/ml and for those that were resistant according to their zone diameters, MICs were between 0.5 and 8 μg/ml. Only 8 *C. albicans* strains were susceptible and 11 were resistant by the disk diffusion method. The only strain resistant by the agar dilution method was also resistant by the disk diffusion method.

DISCUSSION

FZ is a bistriazole antifungal agent that distributes widely in body tissues (9) and which has been shown to have few side effects coupled with good therapeutic activity. The increased use of this drug has given rise to the development of resistance among *Candida* spp.; as a result, it is important to monitor susceptibility. Since the NCCLS has a reference method and tentative breakpoints for three antifungal agents, results in the literature can be compared and any increase in the range of MICs can be detected. Performance of susceptibility tests by the NCCLS method on a large scale is difficult, but the agar diffusion test is easier for small laboratories. We have evaluated the ability of the agar diffusion method to separate strains that are susceptible and resistant to FZ with disks containing 15 μg of FZ. Previously, other investigators have evaluated a FZ diffusion test, but they used disks containing 25 μg (2, 15). Furthermore, the method described by Rodriguez-Tudela (18) was also evaluated but they used a microdilution method. That method, which is very similar to that of NCCLS, has the advantage of a shorter incubation time and simplified endpoint determination.

The MIC at which 90% of strains are inhibited (MIC_{90}) for *C. albicans* is 1 μg/ml, the MIC_{90} for *C. parapsilosis* is 4 μg/ml, and the MIC_{90} is for both *C. glabrata* and *C. tropicalis* is 64 μg/ml. According to the breakpoints stated by NCCLS, only one strain of *C. albicans* is resistant and another is susceptible, dose dependently; for *C. parapsilosis* only one strain is susceptible, dose dependently. These results are similar to those reported by other investigators who used the same method (3, 7, 20, 21).

The two dilution methods tested gave very similar results. Generally, MICs are 1 $\log_2$ dilution lower, endpoints are easier to determine, and the trailing effect is minor in RPMI-G medium. *C. albicans* is the species that shows the greatest trailing effect. The overall agreement within 2 $\log_2$ dilutions is 93.7%, which is lower than that reported by Espinel-Ingroff et al. (8), 97.7%, but they compared the M27-P macrodilution method with a microdilution method, using for the latter method RPMI-G medium as the culture medium, an inoculum size ranging from 1×10^4 to 5×10^4 CFU/ml, and *C. albicans* as the target yeast. The major discrepancy that we have found is for two strains of *C. albicans*: one susceptible, dose dependently, and the other resistant in RPMI 1640 medium (MICs, 16 and >64 μg/ml, respectively) and both susceptible in RPMI-G medium (MICs, 0.12 and 0.25 μg/ml, respectively). When a less restrictive endpoint criterion (50% reduction of growth) is applied for RPMI 1640 medium, the MICs decrease to 0.06 and 0.5 μg/ml, respectively, whereas for RPMI-G medium no changes are detected. This is a case in which the strain is susceptible at 24 h and resistant at 48 h, and Rex et al. (17) denominated a low/high strain.

The ability of the diffusion test to separate the strains susceptible and resistant to FZ is less. There is no correlation between zone size and MIC. Interestingly, inhibition zones are smaller for *C. albicans*, for which the geometric mean MIC was 0.29 μg/ml the mean inhibition zone was 25.7 mm, while for *C. parapsilosis* the geometric mean was 0.96 μg/ml and the mean inhibition zone was 31.52 mm. On the basis of the manufacturer's recommendations to interpret the inhibition zones and comparison of the MICs obtained by the reference method, only 13.8% of *C. albicans* strains and 65.5% of *C. parapsilosis* strains are well classified. Sandren et al. (19), using tablets of 15 μg, concluded that the diffusion test is an appropriate method for detection of FZ-resistant *C. albicans* strains. In our case, all strains susceptible by the diffusion test were also susceptible by the dilution method. The converse was not necessarily true. On the basis of our results we recommend, as Bille suggests (4), the determination of MICs of FZ for those strains resistant by the diffusion method.

When our results and those reported recently by other investigators (3, 5, 21, 22) are compared with those obtained by Price et al. (16) for *Candida* strains isolated over 5 years (1987 to 1992), it is interesting that the MIC range and MIC_{90}s have increased; for example, the MIC_{90} for *C. albicans* has gone from 0.25 to 1 μg/ml.

The increasing use of FZ as antifungal therapy is resulting in a reduction in susceptibility and the appearance of resistant strains. For this reason, it would be convenient to carry out antifungal susceptibility studies in order to establish the in vitro activities of antifungal agents against local isolates and also to detect shifts toward resistance as early as possible.

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