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Correlation Between Etest®, Disk Diffusion, and Microdilution Methods for Antifungal Susceptibility Testing of *Candida* Species From Infection and Colonization

Melyssa Negri,[1] Mariana Henriques,[1] Terezinha I.E. Svidzinski,[2] Claudete Rodrigues Paula,[3] and Rosário Oliveira[1]*

[1]*Centre of Biological Engineering, Institute for Biotechnology and Bioengineering, Universidade do Minho, Braga, Portugal*

[2]*Division of Medical Mycology, Teaching and Research in Clinical Analysis Laboratory, State University of Maringá, Maringá, Paraná, Brazil*

[3]*Department of Microbiology, Laboratory of Pathogenic Yeasts, Biomedical Science Institute, University of São Paulo, São Paulo, Brazil*

The correlation between the microdilution (MD), Etest® (ET), and disk diffusion (DD) methods was determined for amphotericin B, itraconazole and fluconazole. The minimal inhibitory concentration (MIC) of those antifungal agents was established for a total of 70 *Candida* spp. isolates from colonization and infection. The species distribution was: *Candida albicans* ($n = 27$), *C. tropicalis* ($n = 17$), *C. glabrata* ($n = 16$), *C. parapsilosis* ($n = 8$), and *C. lusitaniae* ($n = 2$). Non-*Candida albicans Candida* species showed higher MICs for the three antifungal agents when compared with *C. albicans* isolates. The overall concordance (based on the MIC value obtained within two dilutions) between the ET and the MD method was 83% for amphotericin B, 63% for itraconazole, and 64% for fluconazole. Considering the breakpoint, the agreement between the DD and MD methods was 71% for itraconazole and 67% for fluconazole. The DD zone diameters are highly reproducible and correlate well with the MD method, making agar-based methods a viable alternative to MD for susceptibility testing. However, data on agar-based tests for itraconazole and amphotericin B are yet scarce. Thus, further research must still be carried out to ensure the standardization to other antifungal agents. J. Clin. Lab. Anal. 23:324–330, 2009.

Key words: antifungal susceptibility; disk diffusion method; Etest® method; microdilution method; non-*Candida albicans Candida* species.

INTRODUCTION

Fungal hospital infections (FHI) incidence has increased significantly over the last decades. Yeasts from *Candida* genus are the most frequently isolated fungi, corresponding to 80% of FHI and being the fourth microbial agent responsible for blood stream infection (1–3).

Until some years ago, *Candida albicans* was the *Candida* species that held the most clinical attention. However, in parallel with the overall increase of fungal infections it has been observed that non-*Candida albicans Candida* (NCAC) species infections are emerging. *C. tropicalis*, *C. glabrata*, *C. parapsilosis*, *C. krusei*, *C. guilliermondii*, and *C. lusitaniae* (2,4,5) are the most prominent hospital isolated NCAC species. The reasons for this alteration in the pattern of *Candida* species distribution has not yet been completely understood but could be attributed to the resistance of those microorganisms to antifungal agents and their high use for relatively long periods during hospitalization (2,6,7).

For this reason, it is of major importance to monitor the susceptibilities of isolates recovered from compromised

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*Correspondence to: Rosário Oliveira, Department of Biological Engineering, Universidade do Minho, Campus de Gualtar 4710-057, Braga, Portugal. E-mail: roliveira@deb.uminho.pt

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patients in order to determine strains decreased susceptibilities. The development of standardized antifungal susceptibility testing methods has been the subject of numerous studies during the last decade. Thus, the Clinical and Laboratory Standards Institute (CLSI) developed several reference methods, including for yeasts M27-A2 method (8,9). However, in order to do this in a cost-effective way, simple and inexpensive testing procedures are needed, which must be accurate and precise (10,11).

Agar-based susceptibility testing methods have been a focus of interest for many researchers and include the classical disk diffusion (DD) method and the Etest® (ET) method (10,12–14). Those tests are very attractive owing to their simplicity, reproducibility, and lack of requirements for specialized equipment (15). Thus, this work aims to examine *Candida* susceptibility profile by applying three different techniques for studying antifungal resistance of *C. albicans* isolates and NCAC species in a hospital. Moreover it is also a goal to compare the susceptibility of *Candida* isolates obtained from colonization and infection.

MATERIALS AND METHODS

Hospital

This study was performed at the University Hospital (UH) of Maringá, Paraná, Brazil, which is a school hospital with 116 beds, including an Ambulatory, Emergency Room, Intermediate Centre, Intensive Care Unit (ICU), and Semi ICU. UH attends users of the public health system from Maringá and others municipalities of the 15th Regional Health Centre of Paraná.

Candida Isolation and Identification

Yeasts were isolated from July 2006 to June 2007. The samples were classified in two groups: isolates from infections (INFEC) and from colonization (COL).

INFEC isolates were obtained from urine cultures (40) and from blood cultures (7), from all patients admitted to the ICU and presenting *Candida* infection symptoms. Isolates from urine were considered from infection following the Centre for Disease Control and Prevention definitions (16). Accordingly, Candiduria was considered to be established when the presence of *Candida* species in urine cultures was higher than 10^5 CFU/ml (in the absence of bacteria). Moreover, for patients with urinary catheters, the urine was collected after 24 hr of the device exchange.

Urine samples were spread by calibrated loop (10 μL) on CLED medium (Difco, Detroit, Michigan). Hemoculture samples were cultured suspending one volume of blood in ten volumes of trypticase soy broth (Difco) and then incubated in the automatic BACTEC (Becton Dickinson Microbiology Systems, Sparks, MD) system. Viable yeasts were subcultured on Sabouraud dextrose agar medium (SDA, Difco) at 37°C for 48–72 hr.

COL isolates were obtained from the hands of UH staff members (9), and from central venous catheters (CVCs) (14). The COL yeasts were isolated following the methods described by Bonassoli et al. (17) and Maki et al. (18).

After yeast growth, they were subcultured in CHROMagar *Candida*® (CHROMagar BioMerisc, Paris, France) to assess the purity of the culture and the color of the colonies. Yeasts grown in this selective and differential media were identified macroscopically, microscopically and physiologically according to Kurtzman and Fell (19). All samples were cultivated in SDA for 24 hr at 37°C before being assayed and were kept in the Mycology Section of the Microbiology Department of the Biomedical Sciences Institute of São Paulo in Brazil.

Antifungal Susceptibility Test

Microdilution method

Reference antifungal susceptibility testing of *Candida* spp. was performed using the microdilution (MD) method described in CLSI M27-A2 (8).

Reference powders of fluconazole (Pfizer Inc. New York, New York), itraconazole (Janssen Pharmaceutical) and amphotericin B (Squibb Pharmaceutical) were used. Stock solutions were prepared with a concentration 10 fold the final concentration and diluted with RPMI 1640 (Sigma, St. Louis, Missouri), with L-glutamine, without bicarbonate, supplemented with 2% dextrose and buffered to pH 7.0 with 0.165 *N*-morpholinopropanesulfonic acid (MOPS) to obtain twice the final concentration.

ET method

ET strips were provided by AB BIODISK (Solna, Sweden), with concentrations ranging from 0.002 to 256 μg/ml for fluconazole and from 0.002 to 32 μg/ml for itraconazole and amphotericin B.

Cells grown in SDA were suspended in saline solution (0.9%) and the turbidity was adjusted to 0.5 in a McFarland scale (1×10^6 to 5×10^6 cells/ml) before incubation at 37°C for 48 hr. Then a swab was immersed in the suspension spread on a RPMI 1640 (American Biorganics Inc. Buffalo, New York) supplemented with 1.5% agar and 2% glucose, and buffered with MOPS plate. The minimal inhibitory concentrations (MICs) of drugs were read asthe lowest concentration at which the border of the elliptical inhibition zone intercepted the scale of the strip. Any growth, such as microcolonies, throughout a discernible inhibition ellipse was ignored. MIC interpretative criterion was performed according to the CLSI M27-A2 (8). Briefly, fluconazole: MIC $\leq$ 8 μg/ml—susceptible

(S), $16 < MIC < 32\,\mu g/ml$—susceptible-dose dependent (S-DD), and $MIC \geq 64\,\mu g/ml$—resistant (R); Itraconazole: $MIC \leq 0.125\,\mu g/ml$—S, $0.25 < MIC < 0.5\,\mu g/ml$—S-DD, and $MIC \geq 1\,\mu g/ml$—R; Amphotericin B: $MIC > 1\,\mu g/ml$—R.

DD method

DD testing of fluconazole, itraconazole, and amphotericin B was performed as described by Barry et al. (14) and CLSI document M44-A (20). Fluconazole (25 μg), itraconazole (10 μg), and amphotericin B (100 μg) disks were obtained from CECON—*Centro de Controle e Produtos para Diagnóstico*, LTDA (São Paulo, Brazil). Plates containing Mueller–Hinton agar (Difco) supplemented with 2% glucose and methylene blue (0.5 μg/ml) were inoculated and incubated as described for the ET method. After cultivation, the inhibitory diameter zone (dz) was measured with a ruler. The interpretive criteria for the fluconazole disk test were those published by Barry et al. (14) and the CLSI M44 A (20): $dz \geq 19$ mm—S; $15 < dz < 18$ mm—S-DD; $dz \leq 14$ mm—R. The response to the other antifungal agents was interpreted according to the manufacturer's instructions, Itraconazole: $dz \geq 20$ mm—S, $12 < dz < 19$ mm—S-DD, $dz \leq 11$ mm—R; Amphotericin B: $dz \leq 10$ mm—R.

A quality control was performed in all tests in accordance with the CLSI document M27-A2 (8) by using *C. krusei* ATTC 6258 and *C. parapsilosis* ATCC 22019. These species were included in all runs, and all results were within published limits (14).

Analysis of results

The MIC values from ET and MD were considered in agreement when they were no more than two serial ten times dilutions apart. The interpretive breakpoints of ET and DD obtained were used to determine the categorical agreement in comparison to the results of the reference MD method for amphotericin B, fluconazole, and itraconazole. Major errors, were defined when, for the same isolate, it was obtained a classification of R in DD or ET methods and a classification of S in MD method. Very major errors were defined when a result of S in the DD or ET methods corresponded to R in the MD method. Minor errors were defined when a result of S or R was obtained in one of the tests and a result of S-DD in the other method (21). Amphotericin B does not have intermediate category established for CLSI M27-A2.

Statistical analysis

Comparison of MIC obtained with the three methods was performed using the Mann–Whitney *U*-test, at 95% confidence level.

RESULTS

A total of 70 *Candida* spp. isolates (Table 1) were used in this study, with 33% obtained from colonization and 67% from infection (candiduria or candidemia) in the UH of Maringá, Paraná, Brazil. NCAC species were predominant in relation to *C. albicans* corresponding to 61% of the isolated yeasts. Moreover, *C. tropicalis* and *C. glabrata* were identified in the same extent and were more prevalent than the other NCAC species.

Considering the ET method (Table 2), amphotericin B was the only antifungal agent that showed MIC values under the reference limit ($< 1\,\mu g/ml$) for all isolates except for one *C. albicans* (MIC = 32) and one *C. glabrata* (MIC = 6). All tested strains, when assessed by ET, presented higher MICs compared with MD for fluconazole, itraconazole, and amphotericin B, although this difference was not statistically significant.

Comparing isolates from colonization and infection it is possible to see (Table 2) that COL yeasts were more susceptible to the three drugs than INFC yeasts in the ET method, showing significant differences concerning amphotericin B ($P < 0.0001$) and itraconazole ($P = 0.001$). Although, NCAC species had higher MICs for fluconazole and itraconazole in the MD method compared with *C. albicans* MICs, the difference was not statistically significant. Moreover, NCAC species showed smaller inhibitory zone diameters (DD) for the three antifungal agents in relation to *C. albicans* ($P < 0.05$). In the three methods studied itraconazole was the antifungal agent with the highest number of resistant NCAC species.

Table 3 shows that the overall concordance (based on the MIC value obtained within two dilutions) between the ET and MD methods was 83% for amphotericin B, 63% for itraconazole, and 64% for fluconazole. Considering the breakpoint, ET and DD methods had high categorical agreement for the

TABLE 1. Distribution of Yeasts Isolates According to Their Origin and Species

	Origin				
	Colonization		Infection		
Yeast	Hand	Catheter	Urine	Blood	Total
C. albicans	2	6	16	3	27
C. tropicalis	2	3	11	1	17
C. glabrata	1	1	11	3	16
C. parapsilosis	3	3	2	0	8
C. lusitaniae	1	1	0	0	2
Total	9	14	40	7	70

TABLE 2. In Vitro Susceptibility of *Candida* spp. Isolates Obtained From Colonization (COL) or Infection (INFEC) to Fluconazole, Itraconazole, Amphotericin B Using Three Test Methods: Micro-Dilution (MD); Etest® (ET), and Disk-Diffusion (DD)

	MD				ET				DD			
	COL		INFEC		COL		INFEC		COL		INFEC	
Species (*n*) Antifungal agent	Range[a]	R[c]	Range[a]	R[c]	Range[a]	R[c]	Range[a]	R[c]	Range[b]	R[c]	Range[b]	R[c]
C. albicans (27)												
Fluconazole	0.125–0.5	0	0.25–32	0	0.032–≥256	1	0.094–≥256	8	15–40	0	12–42	1
Itraconazole	<0.030–1	1	0.03–4	1	0.004–≥32	1	0.008–≥32	7	20–32	0	11–29	1
Amphotericin B	0.03–0.125	0	0.06–0.5	0	0.016–0.5	0	0.004–≥32	1	19–35	0	17–28	0
C. tropicalis (17)												
Fluconazole	0.5–0.5	0	0.125–8	0	0.25–1.5	0	0.125–≥256	1	20–25	0	09–28	2
Itraconazole	<0.03–0.25	0	0.06–1	2	0.01–0.5	0	0.023–≥32	3	15–26	0	07–28	2
Amphotericin B	0.03–0.125	0	0.06–0.5	0	0.003–0.25	0	0.47–1	0	25–17	0	12–21	0
C. glabrata (16)												
Fluconazole	2–8	0	0.5–16	0	0.25–32	0	2–16	0	20–25	0	13–25	0
Itraconazole	0.06–0.125	0	0.06–4	6	0.012–0.5	1	0.094–32	11	12–20	0	01–20	6
Amphotericin B	0.03–0.03	0	0.06–0.25	0	0.004–1	0	0.064–6	1	16–23	0	15–36	0
C. parapsilosis (8)												
Fluconazole	0.25–2	0	2–4	0	0.047–32	0	32–32	0	09–32	0	15–19	1
Itraconazole	<0.03–0.125	0	0.5–1	1	0.004–0.25	0	8–24	2	22–35	0	15–10	1
Amphotericin B	0.03–0.125	0	0.5	0	0.002–0.038	0	0.19–0.38	0	14–20	0	20–15	0
C. lusitaniae (2)												
Fluconazole	1–4	0	–	–	6–8	0	–	–	09–25	1	–	–
Itraconazole	0.015–0.06	0	–	–	0.002–0.25	0	–	–	20–24	0	–	–
Amphotericin B	0.06–0.125	0	–	–	0.032–0.19	0	–	–	19–30	0	–	–

[a]Values expressed in micrograms per millilitre (MD and ET).
[b]Values expressed by inhibitory zone diameter in millimetres (DD).
[c]Number of resistant isolates.

TABLE 3. Percentages of Isolates Distributed by Susceptibility Category (% Isolates) and ET or DD Discrepancy Compared to MD (% discrepancy)

		% Isolates			% Discrepancy				
Antifungal	Method	S	SDD	R	Minor	Major	Very major	% Categorical agreement	% Agreement2 dilution steps
Fluconazole	MD	97	3	0					
	ET	70	16	14	16	13	0	71	64
	DD	73	20	7	24	7	0	67	–[b]
Itraconazole	MD	57	27	16					
	ET	30	33	36	31	10	0	57	63
	DD	61	26	13	21	3	4	71	–[b]
Amphotericin B	MD	100	–[a]	0	–[a]				
	ET	97	–[a]	3	–[a]	3	0	97	83
	DD	100	–[a]	0	–[a]	0	0	100	–[b]

Percentage of isolates classified in same ET and DD category as the MD reference method (% categorical agreement and agreement ≤2 dilution steps).
[a]Not applicable since no intermediate category is defined.
[b]Not applicable.

three antifungal agents. Although DD method showed the highest concordance with MD (71%) for itraconazole, this method presented a very major discrepancy rate (4%) for this agent (*C. albicans* two isolates and *C. glabrata* one isolate).

DISCUSSION

Several risk factors are associated with the occurrence of FHI, among which are the wide use of antimicrobial agents, yeast cross-colonization from the hands of health professionals and use of catheters (2).

Nosocomial dissemination of *Candida* species have similar routes to other pathogens, such as the transient carriage on the hands of the hospital staff followed by colonization or infection of patients and the involvement of the environment as a reservoir (22).

In a total of 70 *Candida* spp. isolated in the UH of Maringá (Table 1), 13% were obtained from colonization of the hands of health professionals; 20% from colonization of central venous catheters and 67% were found in patients with candiduria or candidemia. It is well known that yeast-colonization of health professional's hands can be a source of infection by translocation of those microorganisms to the patient, when handling or even by contamination of catheter surface (6,23). Thus, it is possible to understand why the major cause of *Candida* spp. infection is reported on critical patients admitted in ICU (2,22,24).

During the last decades the number of *Candida* spp. hospital infections has highly increased becoming the fourth most common infection pathogens in ICU; it has also been observed a significant increase in morbidity and mortality of patients who developed FHI (4,24). In this study it was observed (Table 1) that a high prevalence of *Candida* spp. recovered from candiduria, 85.11% of infected patients and only 14.89% from candidemia. Candiduria cases are increasing especially among ICU hospitalized patients, who undergo in prolonged use of urinary catheter or broad spectrum antibiotics (25). Furthermore, several studies show that this scenario can be significantly associated with candidemia, thus increasing even more the risk factor for developing complicated infections (26).

As summarized in Table 1, NCAC species were predominant (61%) in relation to *C. albicans* (39%). According to Krcmery and Barnes (27), NCAC species are emerging as both colonizers and pathogens causing nosocomial fungal infections and are responsible for approximately 50% of fungal infections in ICU patients. In fact, Matsumoto et al. (5) isolated yeasts from blood and catheters of hospitalized children (0 to 7 years old) and observed that NCAC species were predominant.

The increase in NCAC species frequency is probably owing to the selection of species that are less susceptible to the most commonly used therapeutic antifungal agents. Species-specific differences clearly exist in the susceptibility to fluconazole and other antifungal agents, and may be significant for the emergence of NCAC species in some institutions (2,4,28,29).

Furthermore, NCAC species showed higher MICs than *C. albicans* for fluconazole and itraconazole (MD method). However, regarding amphotericin B, all isolates showed similar MICs, irrespective of the method employed, except two isolates when assessed by ET: one *C. albicans* (MIC = 32) and one *C. glabrata* (MIC = 6). Several studies (26,27) showed that usually NCAC species isolates present resistance to two or three antifungal agents, and some species, such as *C. tropicalis* and *C. lusitaniae* have inherent or secondary resistance to fluconazole. Moreover, other NCAC species behave as *C. albicans* and are more susceptible to azole antifungal agents (26,27).

Usually, *C. albicans* antifungal susceptibility can be predicted with accuracy but, in contrast, such prediction is not possible when NCAC species are concerned (27). Agar-based susceptibility tests such as the traditional DD and ET commercial methods have been the focus of interest of many researches (10,12–14). Those methods enable in vitro antifungal susceptibility determination and have shown excellent benefits such as technical simplicity, easy handling, quick results, and overcoming the use of costly and specialized equipment (13,15).

In this study the interpretative categorical agreement (Table 3) for ET method compared with MD reference method showed 71% for fluconazole, 57% for itraconazole, and 97% for amphotericin B. However the agreement, considering two fold dilutions, between the ET and MD were different: 64% for fluconazole, 63% for itraconazole, and 83% for amphotericin B. The differences achieved, considering categories (S, S-DD and R) or MIC in two different dilutions, can be owing to the differences in the range considered for ET and MD. For instance, for amphotericin B the MIC range in MD is 0.03–0.5 μg/L and in ET is 0.002–≥32 μg/L, leading to some disagreement of MICs within the "susceptible" group. This was also verified by other authors (10,14) who also compared agar-based methods with the MD reference method. Although the results obtained by both DD and ET methods were in acceptable concordance with those obtained by the MD method, the detection of resistance by agar-based methods correlates poorly with the detection of S-DD by the reference NCCLS M27-A2 method. Specifically, for fluconazole, some "false resistance" cases (major errors) were obtained with ET. Conversely, only 4% of the isolates that were classified as resistant by the MD method appeared to be susceptible when they were tested by DD method. This difference was principally owing to trailing growth associated with the MD method (8,9).

Recent studies have been showing that susceptibility tests, performed with both DD and ET methods, were just as reproducible as the results of the standard reference MD procedure (10,14,30). Furthermore, Pfaller et al. (21) studied fluconazole susceptibility of *C. glabrata* and considered a high agreement between agar-based and MD methods, with 64.7 and 52.3%, respectively for DD and ET. This can be explained by the level of proximity between susceptible and S-DD classification (14,21).

Based on these results and according to data provided by other studies (10,13,21,31), it is possible to highlight that ET and DD methods are useful for the determination of fluconazole resistance, but are not so reliable for differentiation between susceptible and S-DD yeast cells. Furthermore, it is known that the most important objective in the application of these methods is to detect antifungal resistance. Thus, both methods (ET and DD) can be extremely useful for such determination (9). However, concerning drugs as itraconazole and amphotericin B, data on agar-based tests are still scarce (10).

Currently, in vitro susceptibility tests can allow very important guidelines for candidiasis treatment, but the standard susceptibility test (CLSI M27-A2) is not always readily available in regular laboratories and is very time consuming, in opposition to the other more simple techniques such as ET and DD. The major feature of these agar-based tests is that they can allow a quick answer concerning *Candida* resistance to antifungal agents, preventing unnecessary patients drug abuse (13,21). In 2003, CLSI published a specific standard for DD testing of fluconazole (M44-A), which increases the possibility of implementing it in all routine laboratories. However, further research must still be carried out to ensure the standardization of this method for other antifungal agents.

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