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Well diffusion for antifungal susceptibility testing

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Summary *Introduction:* The increasing clinical and microbiologic resistance of *Candida* spp. isolates to several antifungal agents is becoming a serious problem. It is now reasonable to propose the use of antifungal susceptibility testing in *Candida* spp. isolates from patients who have failed conventional therapy, before the selection of an empirical therapy.

Methods: One hundred and fifty eight isolates of *Candida* spp. were evaluated simultaneously by broth microdilution (NCCLS standard) and well diffusion testing (WD), a diffusion method similar to disc diffusion.

Results: According to the Wilcoxon Signed Ranks test performed, there was no significant difference ($p > 0.05$) between both methodologies for all antifungal agents tested (fluconazole, itraconazole, posaconazole, caspofungin and amphotericin B, with *C. tropicalis, C. krusei, C. dubliniensis, C. guillermondii, C. parapsilosis, C. albicans* and *C. glabrata*). A significant difference was observed when comparing well diffusion with NCCLS for fluconazole WD 80% ($p = 0.008$) in *C. glabrata*, as well as WD 80% ($p = 0.002$) and WD 50% ($p = 0.002$) in *C. albicans*.

Conclusions: The well diffusion test is simple, easy to reproduce, inexpensive, easy both to read and interpret, and has a good correlation to the reference NCCLS microdilution test and may represent an alternative method for antifungal drug susceptibility testing of *Candida* spp., mainly in laboratories with few resources.

Introduction

Serious fungal infections in immunocompromised patients are increasing in frequency and *Candida albicans* and non-*albicans* species remain the most common pathogens, with an increasing number of clinical and/or microbiological resistance of these species to several antifungal agents.[1—10]

It is now therefore reasonable to propose the use of antifungal susceptibility testing in order to analyze the causes of the failure of conventional therapy in patients with *Candida* isolates or even to predict 'in vivo' the response of mycoses to antifungal agents. As new antifungal agents are introduced for the treatment of infections caused by yeasts, it is important that reliable methods are available for the in vitro testing of both new and established agents.

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Intensive efforts are being made to develop standardized, reproducible and clinically relevant susceptibility testing methods for yeasts but susceptibility testing of fungi entails several methodological problems. In 1997 the National Committee for Clinical Laboratory Standards (NCCLS) published an approved reference macrodilution method (document M27-A) for in vitro testing.[11] This document describes a broth macrodilution method and its microdilution modifications. It is, however, laborious, time consuming, requires specialized personnel and has not eliminated the need for easier methods. Currently other simpler and more economic methods for routine clinical tests are being developed. Clearly, the disc diffusion method has the potential to provide a simple means of performing in vitro tests, but not all antifungal agents are available in discs. Furthermore, the discs are very expensive and their acquisition in developing countries is sometimes difficult. In an attempt to combat this, in 1997 Magaldi developed a modification of the disc diffusion method which she named the 'well diffusion' method (WD). The procedure is similar; the discs are supplemented with dilutions of the drug placed in wells which have been cut out in the agar. This allows the use and standardization of various concentrations of any drug for different fungal species. It has proven to be a cheap, simple and reliable method of antifungal drug susceptibility testing for *Candida* spp., and it produces results comparable with the disc diffusion test.[12–16]

The aim of this study was to compare the well diffusion method with the NCCLS broth microdilution method using several antifungal drugs including two new antimycotic drugs: posaconazole and caspofungin.

Materials and methods

Isolates

A total of 158 recent clinical isolates of *Candida* spp. were studied. The isolates were submitted to the Medical Mycology Section of the Instituto de Medicina Tropical, UCV, Caracas, Venezuela, for susceptibility testing. Thirteen control strains were included with a known susceptibility pattern: *C. glabrata* 90030, *C. parapsilosis* 22019, *C. krusei* 6258, *C. albicans* 90028 from the American Type Culture Collection (ATCC) and *C. tropicalis* S594, *C. tropicalis* S623B, *C. albicans* S621, *C. albicans* 623 and 5 *C. dubliniensis* (S-636, S2-14, S-645, S2-6, S2-5) from the Health Science Center, University of Texas. The identification of the isolated yeast colonies was established by the production of chlamydoconidia using modified Bilis agar, Feo,[17] and carbon source assimilation reactions, using a commercial kit, the API 32 C AUX identification system (BioMérieux). All isolates were subcultured onto Sabouraud Dextrose agar, 24 hours prior to identification.

Antifungal susceptibility testing

Antifungal agents

The antifungal agents amphotericin B (AMB), caspofungin (CAS), posaconazole (POS), itraconazole (ITC), and fluconazole (FLC) were used in their commercial presentation to prepare a stock solution adjusted to the concentration of 1.25 mg ml^{-1} (25 μg per well). Each agent was dissolved in its corresponding solvent (Table 1). No references are available to determine the minimum inhibitory concentration (MIC) of caspofungin. A test concentration of the drug necessary to visualize a clear inhibition zone was performed. A serial solution of caspofungin was prepared in distilled water and adjusted to a final concentration of 1.25 mg ml^{-1} (25 μg per well). In order to obtain appropriate measurements for carrying out the well diffusion and the NCCLS methods, MIC values were measured in Casitone and RPMI media by NCCLS criteria (document M27[11]). MICs were defined as the lowest concentration which inhibited 100%

Table 1 Antimycotic agents.

Licensed product generic name	Producer	Commercial name	Solvent
Posaconazole (POS)	Schering-Plough, Kenilworth, NJ		PEG-400*
Itraconazole (ITC)	Janssen Pharmaceutica, Titusville, NJ	Sporanox	DMSO**
Fluconazole (FLC)	Pfizer Inc., New York, NY	Diflucan	H_2O dest.
Amphotericin B (AMB)	ER Squibb & Sons, Princeton, NJ	Fungizone	PEG-400*
Caspofungin (CAS)	Merck & Co. Inc., Whitehouse, NJ	Cancidas	H_2O dest.

* PEG-400 polyethylene glycol (Union Carbide, Danbury, CT)
** DMSO 100% dimethylsulfoxide (Sigma Chemical Co, St.Louis, MO)

Table 2 Antifungal susceptibility breakpoints against *Candida* sp. for NCCLS microdilution and well diffusion methods.

Antifungal agent	NCCLS			Well diffusion		
	Susceptible ($\mu g\,ml^{-1}$)	Susceptible dose dependent ($\mu g\,ml^{-1}$)	Resistant ($\mu g\,ml^{-1}$)	Susceptible (mm)	Susceptible dose dependent (mm)	Resistant (mm)
Amphotericin B (AMB) Caspofungin (CAS)	≤1		≥1	≥15	14—10	≤9
Fluconazole (FLC)	≤8.0	16—32	≥64	≥19	18—13	≤12
Itraconazole (ITC) Posaconazole (POS)	≤0.125	0.25—0.5	≥1			

of visible growth after 24 hours. The breakpoint values for caspofungin obtained were comparable to those of amphotericin B (Table 2). We compared the baseline MIC with the microbiological outcome.

NCCLS microdilution method

This method was used as described in the National Committee for Clinical Laboratory Standards.[11] All the tests were performed twice. The MIC of amphotericin B (MIC_{90}) was defined as the lowest concentration resulting in a complete inhibition of growth. The same results were obtained with caspofungin. The MICs of all the other compounds (azoles) were defined as the lowest concentration which resulted in a prominent decrease in turbidity compared with that of growth-control wells, using the turbidity numerical score proposed by the NCCLS (MIC_{50} and MIC_{80}).

Well diffusion method

The well diffusion test[12—16] was performed using Casitone agar[18] (Bacto-casitone: 9 g; yeast extract: 5 g; tri-sodium citrate: 10 g; glucose: 20 g; bactoagar: 15 g; phosphate buffer: KH_2PO_4: 1 g; Na_2HPO_4: 1 g (Difco) (pH 6.6); H_2O 1000 ml). The inoculum used was prepared using the yeasts from a 24-hour culture on Sabouraud dextrose agar, a suspension was made in a sterile saline solution (0.85%). The turbidity of the suspension was adjusted with a spectrophotometer at 530 nm to obtain a final concentration to match that of a 0.5 McFarland standard ($0.5—2.5 \times 10^3$). 20 ml of Bacto-casitone were melted, cooled to 55 °C and than inoculated with 1 ml of the organism suspension. The inoculated agar was poured into the assay plate (9 cm in diameter), and allowed to cool down on a leveled surface. Once the medium had solidified, four wells, each 4 mm in diameter, were cut out of the agar, and 20 µl of the antifungal agent were placed into each well. A total of four antimycotics were placed into each plate and incubated at 35 °C for 24 hours. Because of disagreement over the criteria to determine the end point of the diameters of the clear zone of inhibition of growth for the azoles, it was measured both at 50% and 80%. However, NCCLS disc diffusion breakpoints were used for the interpretative breakpoints. The criteria used to determine MICs were different for caspofungin and amphotericin B because the diameters of the inhibition zones were clear. A dramatic reduction in growth was observed (>90%).

Overall, 158 clinical isolates were tested twice by each method. Results were interpreted according to NCCLS breakpoints for all the tests (Table 2),[3,4] except for caspofungin (see antifungal agents).

Statistical analysis

A statistical analysis was performed with the percentage analysis and the Wilcoxon Signed Ranks test ($p > 0.05$). There was significant difference between the two methodologies.

Results

One hundred and fifty eight isolates of *Candida* spp. were simultaneously evaluated by broth microdilution and well diffusion testing. A summary of the frequencies of susceptible, dose dependent and resistant isolates for both methods, is reported in Tables 3 and 4. According to the Wilcoxon Signed Ranks test performed, there was no difference between the methodologies for all antifungal agents

Table 3 Frequency of susceptible and resistant isolates of 158 *Candida* spp. with fluconazole, itraconazole and posaconazole.

	Drugs		
	FLC	ITC	POS
C. albicans (108 isolates)			
WD 80%			
S	73	76	82
DD	7	9	8
R	28	23	18
WD 50%			
S	99	98	102
DD	3	3	1
R	6	7	5
NCCLS			
S	82	81	87
DD	10	4	6
R	16	23	15
C. glabrata (30 isolates)			
WD 80%			
S	13	6	9
DD	6	9	6
R	11	15	15
WD 50%			
S	17	9	12
DD	5	9	6
R	8	12	9
NCCLS			
S	20	10	9
DD	4	9	7
R	6	11	16
C. tropicalis (7 isolates)			
WD 80%			
S	1	1	4
DD		1	2
R	6	5	1
WD 50%			
S	1	2	5
DD	1	1	2
R	5	4	
NCCLS			
S	1	1	4
DD			2
R	6	6	1
C. dubliniensis (5 isolates)			
WD 80%			
S	5	5	5
DD			
R			
WD 50%			
S	5	5	5
DD			
R			
NCCLS			
S	5	5	5
DD			
R			
C. krusei (4 isolates)			
WD 80%			
S	1	1	1
DD			1
R	3	3	2
WD 50%			
S	1	1	2
DD		1	1
R	3	2	1
NCCLS			
S			1
DD			1
R	4	4	2
C. parapsilosis (2 isolates)			
WD 80%			
S	2	2	2
DD			
R			
WD 50%			
S	2	2	2
DD			
R			
NCCLS			
S	2	2	2
DD			
R			
C. guillermondii (2 isolates)			
WD 80%			
S	1	1	2
DD			
R	1	1	
WD 50%			
S	1		2
DD		1	
R	1	1	
NCCLS			
S	1		2
DD			
R	1	2	

S = susceptible; DD = susceptible dose-dependent; R = resistant; WD = well diffusion.

tested against *C. tropicalis C. krusei, C. dubliniensis, C. guillermondii, C. parapsilosis, C. albicans* and *C. glabrata* ($p > 0.05$). There was, however, a significant difference observed when comparing well diffusion with NCCLS, for fluconazole WD 80% ($p = 0.008$) against *C. glabrata* and WD 80% ($p = 0.002$) and WD 50% ($p = 0.002$) against *C. albicans*.

Table 4 Frequency of susceptible and resistant isolates of 158 *Candida* spp. with amphotericin B and caspofungin.

	Drugs	
	AMB	CAS
C. albicans (108 isolates)		
WD 90%		
S	106	108
DD		
R	2	
NCCLS		
S	106	108
DD		
R	2	
C. glabrata (30 isolates)		
WD 90%		
S	29	30
DD	1	
R		
NCCLS		
S	29	29
DD	1	1
R		
C. tropicalis (7 isolates)		
WD 90%		
S	7	7
DD		
R		
NCCLS		
S	7	7
DD		
R		
C. dubliniensis (5 isolates)		
WD 90%		
S	5	5
DD		
R		
NCCLS		
S	5	5
DD		
R		
C. krusei (4 isotates)		
WD 90%		
S	4	4
DD		
R		
NCCLS		
S	4	4
DD		
R		
C. parapsilosis (2 isolates)		
WD 90%		
S	2	2
DD		
R		
NCCLS		
S	2	2
DD		
R		
C. guillermondii (2 isolates)		
WD 90%		
S	1	1
DD	1	1
R		
NCCLS		
S	2	1
DD		1
R		

S = susceptible; DD = susceptible dose-dependent; R = resistant; WD = well diffusion.

Discussion

Methods for determining minimum inhibitory concentration (MIC) and interpretative breakpoint guidelines for fungi were only recently standardized.[3,11–19,20–23] Due to their relatively recent introduction and troublesome development, antifungal susceptibility tests and clinical interpretation remain somewhat controversial. As a result, it can be difficult to use antifungal susceptibility test results to define resistance. Although there is no consensus regarding the interpretation of breakpoint values for all antifungal agents, the standard NCCLS methods for antifungal susceptibility testing are considered a reference method.[11] Our geometric mean MICs for caspofungin against the tested isolates were similar to those found by Pfaller.[20] According to the Wilcoxon Signed Ranks test performed, there was no significant difference between both methodologies for all antifungal agents tested with *Candida* spp. ($p > 0.05$), therefore this simple well diffusion test is highly reproducible for pathogenic yeasts and it strongly agrees with the NCCLS method.

The discrepancy observed for fluconazole WD 80% ($p = 0.008$) against *C. glabrata* as well as WD 50% ($p = 0.002$) and WD 80% ($p = 0.002$) against *C. albicans*, when comparing the well diffusion test with NCCLS may be due to inadequate end point determination for fluconazole, as can be observed in the different results obtained by measuring 80% or 50% inhibition of growth. Fluconazole shows a diffuse zone of inhibition which is difficult to read and measure. It may be advisable to read well diffusion for fluconazole at 50% inhibition of growth

as this gives a clearer zone of inhibition compared to the NCCLS method.

In the group of *Candida* spp. studied with caspofungin, even though no definite cut-off points for caspofungin are yet established, we found that the average measurement of the inhibition zone diameter fits better with the endpoint established for amphotericin B by the disc diffusion method. The inhibition (>90% reduction) zone diameters of the well diffusion method were clear with a good definition. They were easy to measure after 24 hours because of the homogeneous distribution and growth of the strain obtained including the inoculum into the culture medium (Casitone) before pouring onto the plate.

The use of both pure and commercial formulations of the fluconazole disc has been compared to determine susceptibility of the *Candida* strains. Magaldi et al.[13] showed similar results for both presentations. Additionally, the use of the commercial formulation also allows the testing of any drug and the standardizing of various concentrations for different fungal species even when the discs for new drugs are not yet commercially available or are too expensive.

In previous studies the well diffusion method, as compared to the reference method (NCCLS[11]), showed a specificity of 100% for the susceptibility of the strains to fluconazole, with a positive predictive value of 100%. Even though the sensitivity was only 84.85%, the high specificity allows the identification of all resistant strains. This may be helpful to the physician when choosing a successful treatment.[16]

With well diffusion being a qualitative method, it is very difficult to establish the difference between resistant strains and dose-dependent strains. The NCCLS micro and macro broth dilution method is a quantitative technique that allows the discrimination of these.

The comparison of the two methods showed that the well diffusion test may represent an alternative method for antifungal drug susceptibility testing of *Candida* spp. using both new and established agents, mainly in laboratories with few resources. Nevertheless a clinical correlation needs to be established.

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