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Comparative Evaluation of FUNGITEST and Broth Microdilution Methods for Antifungal Drug Susceptibility Testing of *Candida* Species and *Cryptococcus neoformans*

KATE G. DAVEY, ANN D. HOLMES, ELIZABETH M. JOHNSON, ADRIEN SZEKELY, AND DAVID W. WARNOCK*

Mycology Reference Laboratory, Public Health Laboratory Service, Bristol, United Kingdom

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The FUNGITEST method (Sanofi Diagnostics Pasteur, Paris, France) is a microplate-based procedure for the breakpoint testing of six antifungal agents (amphotericin B, flucytosine, fluconazole, itraconazole, ketoconazole, and miconazole). We compared the FUNGITEST method with a broth microdilution test, performed according to National Committee for Clinical Laboratory Standards document M27-A guidelines, for determining the in vitro susceptibilities of 180 isolates of *Candida* spp. (50 *C. albicans*, 50 *C. glabrata*, 10 *C. kefyr*, 20 *C. krusei*, 10 *C. lusitaniae*, 20 *C. parapsilosis*, and 20 *C. tropicalis* isolates) and 20 isolates of *Cryptococcus neoformans*. Overall, there was 100% agreement between the methods for amphotericin B, 95% agreement for flucytosine, 84% agreement for miconazole, 83% agreement for itraconazole, 77% agreement for ketoconazole, and 76% agreement for fluconazole. The overall agreement between the methods exceeded 80% for all species tested with the exception of *C. glabrata* (71% agreement). The poorest agreement between the results for individual agents was seen with *C. glabrata* (38% for fluconazole, 44% for ketoconazole, and 56% for itraconazole) and *C. tropicalis* (50% for miconazole). The FUNGITEST method misclassified as susceptible 2 of 12 (16.6%) fluconazole-resistant isolates, 2 of 10 (20%) itraconazole-resistant isolates, and 4 of 8 (50%) ketoconazole-resistant isolates of several *Candida* spp. Further development of the FUNGITEST procedure will be required before it can be recommended as an alternative method for the susceptibility testing of *Candida* spp. or *C. neoformans*.

The rising prevalence of serious fungal infections and antifungal drug resistance has created an increased demand for reliable methods of in vitro testing of antifungal agents that can assist in their clinical use. The National Committee for Clinical Laboratory Standards (NCCLS) has developed a standardized broth macrodilution method for the testing of *Candida* spp. and *Cryptococcus neoformans* (9). This reference method and microdilution adaptations of it have been reported to give almost identical results (1, 3, 4, 11, 18). However, these methods are time-consuming and labor-intensive and have not eliminated the need for simpler and more economical methods of routine testing.

The FUNGITEST method (Sanofi Diagnostics Pasteur, Paris, France) is a commercial procedure for the breakpoint testing of six antifungal drugs (amphotericin B, flucytosine, fluconazole, itraconazole, ketoconazole, and miconazole). Each 16-well microplate contains 2 negative control wells, 2 positive growth control wells, and 12 drug-containing wells. Each drug is provided at two concentrations in dehydrated form and is reconstituted by adding the inoculum suspension in RPMI 1640 medium. The FUNGITEST method is not available in the United States. In this study we compared the FUNGITEST method with a broth microdilution adaptation of the NCCLS reference method using 200 isolates of seven *Candida* spp. and *C. neoformans*.

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* Corresponding author. Mailing address: Mycology Reference Laboratory, Public Health Laboratory, Kingsdown, Bristol BS2 8EL, United Kingdom. Phone: (44) 117-928-5030. Fax: (44) 117-922-6611. E-mail: D.W.Warnock@PHLSBristol.btinternet.com.

MATERIALS AND METHODS

Isolates. A total of 200 isolates were tested. These comprised 50 *Candida albicans*, 50 *Candida glabrata*, 10 *Candida kefyr*, 20 *Candida krusei*, 10 *Candida lusitaniae*, 20 *Candida parapsilosis*, 20 *Candida tropicalis*, and 20 *C. neoformans* isolates. Of the 200 isolates, 180 were recent clinical isolates, submitted to the Mycology Reference Laboratory, Bristol, United Kingdom, for identification and susceptibility testing and 20 were obtained from the United Kingdom National Collection of Pathogenic Fungi, held at Mycology Reference Laboratory. Isolates were identified to the species level by the Auxacolor (Sanofi Diagnostics Pasteur) and API 20C (bioMerieux UK Limited, Basingstoke, England) yeast identification systems and by morphology on Oxoid cornmeal agar plates (Unipath Limited, Basingstoke, England). Two reference strains, *C. parapsilosis* ATCC 90018 and *C. krusei* ATCC 6258, were included in each batch of broth microdilution tests to ensure quality control.

Isolates were retrieved from storage in liquid nitrogen and were subcultured twice on plates of Oxoid Sabouraud dextrose agar supplemented with 0.5% (wt/vol) chloramphenicol to ensure optimal growth. Prior to testing, subcultures on Sabouraud dextrose agar were incubated at 35°C for 24 h (*Candida* spp.) or 48 h (*C. neoformans*).

Broth microdilution method. The broth microdilution method was performed according to the guidelines of NCCLS document M27-A (9). Analytical-grade powders of the six antifungal drugs were obtained from the respective manufacturers. Amphotericin B, ketoconazole, and miconazole were dissolved in dimethyl sulfoxide, and flucytosine and fluconazole were dissolved in sterile distilled water. A stock solution of itraconazole was prepared in polyethylene glycol 400 with the aid of heating to 75°C for 45 min (1). Stock solutions were diluted with RPMI 1640 medium (with L-glutamine but without bicarbonate) (Sigma Chemical Co., St. Louis, Mo.), supplemented with glucose (2%), and buffered to pH 7.0 with 0.165 M morpholinopropanesulfonic acid (MOPS; Sigma). The final concentration ranges were 0.03 to 16 μg/ml for amphotericin B and itraconazole and 0.125 to 64 μg/ml for flucytosine, fluconazole, ketoconazole, and miconazole.

Testing was performed in 96-well round-bottom microtiter plates. Cell suspensions were prepared in RPMI 1640 medium and were adjusted to give a final inoculum concentration of about 0.5×10^3 to 2.5×10^3 cells/ml. The plates were incubated at 35°C and were read after 48 h (*Candida* spp.) or 72 h (*C. neofor-*

mans). The MIC of amphotericin B was defined as the lowest concentration at which there was 100% inhibition of growth, and the MICs of flucytosine and the azoles were defined as the lowest concentrations at which there was 80% inhibition of growth compared with that in a drug-free control.

FUNGITEST method. The FUNGITEST microplates (Sanofi Diagnostics Pasteur) were set up according to the manufacturer's instructions. Cell suspensions were prepared in sterile distilled water and were adjusted to a turbidity corresponding to a 1.0 McFarland standard; 100 μl of this suspension was added to 1.9 ml of sterile distilled water, and this was further diluted by adding 20 μl to 3 ml of preprepared RPMI 1640 suspension medium. This gave a final inoculum concentration of 10^3 cells/ml. The microplates were inoculated by placing 100 μl of the appropriate cell suspension into each well. The plates were incubated at 37°C and were read after 48 h for *Candida* spp. or 30°C and 72 h for *C. neoformans.*

Each antifungal drug was tested at two concentrations, selected to distinguish resistant isolates from susceptible ones. The drug concentrations were as follows: amphotericin B, 2 and 8 μg/ml; flucytosine, 2 and 32 μg/ml; fluconazole, 8 and 64 μg/ml; itraconazole and ketoconazole, 0.5 and 4 μg/ml; and miconazole, 0.5 and 8 μg/ml.

Analysis of results. Tentative breakpoints have been established for fluconazole and itraconazole, allowing isolates of *Candida* spp. tested according to NCCLS guidelines to be classified as susceptible, susceptible dependent upon dose, or resistant (15). However, these breakpoints do not correspond to the breakpoint concentrations selected for the FUNGITEST method. Moreover, NCCLS breakpoints have not been established for the other four drugs included in FUNGITEST or for *C. neoformans*. For these reasons we classified isolates that were inhibited from growth at the lower of the two drug concentrations used in FUNGITEST as susceptible, those inhibited from growth at the higher of the two concentrations as intermediate, and those inhibited from growth at neither of the two concentrations as resistant. To permit comparisons with the broth microdilution reference method, the same breakpoints were applied to both tests. Major discrepancies were defined as results that classified the isolate as susceptible by one method and resistant by the other, and minor discrepancies were defined as variations from resistant to intermediate or intermediate to susceptible between the two methods.

RESULTS

Table 1 summarizes the in vitro susceptibilities of the 200 isolates to the six antifungal drugs as measured by the reference broth microdilution method. The data are reported as MIC ranges and the MICs at which 50% and 90% of the isolates are inhibited (MIC_{50}s and MIC_{90}s, respectively). A broad range of MICs was observed for flucytosine and the four azole drugs, but the isolates tested were all susceptible to amphotericin B, with the MICs for the isolates ranging from 0.125 to 2.0 μg/ml. In each batch of tests, the MICs for the quality control strains were within the accepted limits for each of the antifungal drugs tested (data not shown).

Of the 200 isolates tested, for 12 isolates the broth microdilution MICs were >32 μg/ml for flucytosine and the isolates were classified as resistant to this agent. For 13 isolates the fluconazole MICs were >64 μg/ml, for 10 isolates the itraconazole MICs were >4 μg/ml, and for 8 isolates the ketoconazole MICs were >4 μg/ml. None of the isolates tested was classified as resistant to amphotericin B or miconazole on the basis of the broth microdilution MICs.

Table 2 presents a detailed analysis of the comparison between the FUNGITEST results and those of the broth microdilution method. Overall agreement between the results of the two methods ranged from 100% for amphotericin B to 95% for flucytosine, 84% for miconazole, 83% for itraconazole, 77% for ketoconazole, and 76% for fluconazole. The overall agreement between the methods ranged from 97% for *C. kefyr* to 95% for *C. albicans*, 94% for *C. parapsilosis*, 93% for *C. lusitaniae*, 89% for *C. neoformans*, 82% for *C. krusei* and *C. tropicalis*, and 71% for *C. glabrata.*

Major discrepancies between the results of the two methods were observed for 7 of the 48 different drug-organism combinations tested (Table 2). These discrepancies comprised eight individual results in which isolates of *C. albicans*, *C. glabrata*, or *C. tropicalis* that were classified as resistant to fluconazole, itraconazole, or ketoconazole by the broth microdilution MIC method were classified as susceptible by the FUNGITEST. Of the 12 isolates classified as resistant to fluconazole by the reference method, 2 (16.6%) were classified as susceptible by the FUNGITEST. For itraconazole and ketoconazole, two (20%) and four (50%) resistant isolates, respectively, were classified as susceptible by the FUNGITEST. The 12 isolates for which the flucytosine MICs fell within the resistant range by the broth microdilution test were also classified as resistant by the FUNGITEST.

Minor discrepancies were observed for 28 of the 48 different

TABLE 1. Antifungal drug susceptibilities of 200 isolates as determined by a broth microdilution method

Organism (no. of isolates)	Antifungal agent	MIC (μg/ml)		
		Range	50%	90%
C. albicans (50)	Amphotericin B	0.12–0.5	0.25	0.5
	Flucytosine	≤0.12–>64	≤0.12	1
	Fluconazole	≤0.12–>64	0.25	8
	Itraconazole	≤0.03–>16	≤0.03	0.12
	Ketoconazole	≤0.12–8	≤0.12	1
	Miconazole	≤0.12–2	≤0.12	1
C. glabrata (50)	Amphotericin B	0.25–1	0.5	0.5
	Flucytosine	≤0.12–>64	≤0.12	≤0.12
	Fluconazole	4–>64	16	64
	Itraconazole	0.12–>16	1	8
	Ketoconazole	≤0.12–8	1	4
	Miconazole	≤0.12–2	0.5	1
C. kefyr (10)	Amphotericin B	1	1	1
	Flucytosine	≤0.12–1	≤0.12	≤0.12
	Fluconazole	0.25–1	0.25	0.25
	Itraconazole	≤0.03–0.25	0.06	0.12
	Ketoconazole	≤0.12–1	≤0.12	≤0.12
	Miconazole	≤0.12–1	≤0.12	≤0.12
C. krusei (20)	Amphotericin B	0.12–1	0.5	0.5
	Flucytosine	2–16	8	16
	Fluconazole	16–>64	32	>64
	Itraconazole	0.12–1	0.25	0.5
	Ketoconazole	≤0.12–2	1	2
	Miconazole	1–4	2	4
C. lusitaniae (10)	Amphotericin B	0.5–2	1	1
	Flucytosine	≤0.12–>64	≤0.12	>64
	Fluconazole	≤0.12–16	0.5	1
	Itraconazole	≤0.03–0.25	0.06	0.12
	Ketoconazole	≤0.12–0.25	≤0.12	≤0.12
	Miconazole	≤0.12–1	≤0.12	0.25
C. parapsilosis (20)	Amphotericin B	0.25–0.5	0.25	0.25
	Flucytosine	≤0.12–0.5	≤0.12	0.25
	Fluconazole	0.25–1	0.5	1
	Itraconazole	≤0.03–0.12	≤0.03	0.12
	Ketoconazole	≤0.12–0.25	≤0.12	≤0.12
	Miconazole	≤0.12–1	0.25	0.5
C. tropicalis (20)	Amphotericin B	0.5	0.5	0.5
	Flucytosine	≤0.12–>64	≤0.12	0.5
	Fluconazole	0.25–64	1	8
	Itraconazole	≤0.03–>16	0.06	0.5
	Ketoconazole	≤0.12–8	≤0.12	4
	Miconazole	≤0.12–4	1	2
C. neoformans (20)	Amphotericin B	0.12–0.5	0.25	0.25
	Flucytosine	1–16	8	8
	Fluconazole	2–8	4	8
	Itraconazole	0.06–0.5	0.25	0.5
	Ketoconazole	≤0.12–1	0.25	0.5
	Miconazole	≤0.12–1	0.25	0.5

TABLE 2. Percent agreement between FUNGITEST and reference broth microdilution method

Organism (no. of isolates)	Antifungal agent	% Agreement	% Minor discrepancies	% Major discrepancies[a]
C. albicans (50)	Amphotericin B	100	0	0
	Flucytosine	98	2	0
	Fluconazole	94	4	2
	Itraconazole	98	2	0
	Ketoconazole	88	8	4
	Miconazole	92	8	0
C. glabrata (50)	Amphotericin B	100	0	0
	Flucytosine	100	0	0
	Fluconazole	38	60	2
	Itraconazole	56	42	2
	Ketoconazole	44	54	2
	Miconazole	88	12	0
C. kefyr (10)	Amphotericin B	100	0	0
	Flucytosine	100	0	0
	Fluconazole	100	0	0
	Itraconazole	100	0	0
	Ketoconazole	90	10	0
	Miconazole	90	10	0
C. krusei (20)	Amphotericin B	100	0	0
	Flucytosine	80	20	0
	Fluconazole	75	25	0
	Itraconazole	70	30	0
	Ketoconazole	75	25	0
	Miconazole	90	10	0
C. lusitaniae (10)	Amphotericin B	100	0	0
	Flucytosine	90	10	0
	Fluconazole	80	20	0
	Itraconazole	100	0	0
	Ketoconazole	100	0	0
	Miconazole	90	10	0
C. parapsilosis (20)	Amphotericin B	100	0	0
	Flucytosine	100	0	0
	Fluconazole	100	0	0
	Itraconazole	100	0	0
	Ketoconazole	100	0	0
	Miconazole	65	35	0
C. tropicalis (20)	Amphotericin B	100	0	0
	Flucytosine	100	0	0
	Fluconazole	90	10	0
	Itraconazole	75	20	5
	Ketoconazole	75	20	5
	Miconazole	50	50	0
C. neoformans (20)	Amphotericin B	100	0	0
	Flucytosine	80	20	0
	Fluconazole	70	30	0
	Itraconazole	100	0	0
	Ketoconazole	95	5	0
	Miconazole	90	10	0
All isolates (200)	Amphotericin B	100	0	0
	Flucytosine	95	5	0
	Fluconazole	76	23	1
	Itraconazole	83	16	1
	Ketoconazole	77	21	2
	Miconazole	84	16	0

[a] In all instances major discrepancies involved isolates that were classified as resistant by the reference broth microdilution method but that were classified as susceptible by the FUNGITEST.

drug-organism combinations (Table 2). These discrepancies exceeded 10% of the results for 15 drug-organism combinations, 20% for 9 combinations, 30% for 5 combinations, 40% for 4 combinations, and 50% for 2 combinations. The highest proportion of minor discrepancies occurred with *C. glabrata* and fluconazole (60%) or ketoconazole (54%) and with *C. tropicalis* and miconazole (50%).

Minor discrepancies between the results of the two methods comprised 164 individual results, 109 (66%) of which involved isolates that were classified as susceptible or intermediate by the FUNGITEST but for which the MICs were found to fall within the intermediate or resistant range by the broth microdilution MIC method. Among the eight different organisms tested, the largest numbers of minor discrepancies ($n = 84$) were recorded with tests involving *C. glabrata* (Table 3). Among the six antifungal agents tested, the largest numbers of minor discrepancies were noted in tests with fluconazole or ketoconazole (Table 4).

DISCUSSION

In the past, in vitro testing of antifungal agents has been regarded as problematic, but standardized methods have now been developed for *Candida* spp. and *C. neoformans* (9). For these methods to be useful, the results should provide a reliable prediction of the response to treatment for humans with infections. In particular, a high MIC should often correlate with therapeutic failure (15). Numerous reports have demonstrated that the ability to predict clinical outcome differs from agent to agent and depends on the patient population studied (5). For instance, high MICs of fluconazole are often predictive of therapeutic failure in human immunodeficiency virus-positive patients with oral candidiasis (12, 16, 17) but do not correlate with the clinical outcome in patients with candidemia (14). The situation with other antifungal agents is even less clear, but a number of investigations have reported that for the amphotericin B MICs for isolates of *Candida* spp. recovered during prolonged treatment with this agent, high or rising MICs appeared to correlate with therapeutic failure (2, 8, 10). It is evident that in vitro testing is useful in some clinical situations but not in others.

Although MIC determination is often perceived as the ideal method of antimicrobial drug susceptibility testing, much routine information on antibacterial agents is derived from much simpler methods, such as the agar disc diffusion test or breakpoint testing. The FUNGITEST method is designed to provide a simple and rapid method for the breakpoint testing of isolates of *Candida* spp. and *C. neoformans* with six antifungal agents. Our results suggest that although performance of the FUNGITEST method is straightforward, the level of agreement with a broth microdilution adaptation of the NCCLS reference method is much greater for some drug-organism combinations than it is for others.

Major discrepancies between the results of the FUNGITEST method and the broth microdilution test were uncommon, but they occurred for 7 of the 48 drug-organism combinations studied in this work. These major discrepancies involved isolates of *C. albicans*, *C. glabrata*, or *C. tropicalis* that were classified as susceptible to fluconazole, itraconazole, or ketoconazole by the FUNGITEST method but for which the MICs were found to fall within the resistant range by the broth microdilution method. The FUNGITEST method misclassified as susceptible 2 of 12 (16.6%) fluconazole-resistant isolates, 2 of 10 (20%) itraconazole-resistant isolates, and 4 of 8 (50%) ketoconazole-resistant isolates.

Minor discrepancies between the results of the two methods occurred for 28 of the 48 drug-organism combinations studied. These discrepancies affected 164 individual results, 84 (51%) of which involved tests with *C. glabrata* isolates. Of the 164 discrepant results, 109 (66%) involved isolates that were clas-

TABLE 3. Minor discrepancies (by organism) between FUNGITEST and reference broth microdilution method

Organism	No. of discrepant test results[a]				
	F susceptible, M intermediate	F intermediate, M resistant	M susceptible, F intermediate	M intermediate, F resistant	Total
C. albicans	7	1	3	1	12
C. glabrata	67	7	9	1	84
C. kefyr	2	0	0	0	2
C. krusei	11	2	7	7	22
C. lusitaniae	1	1	2	0	4
C. parapsilosis	0	0	7	0	7
C. tropicalis	6	0	12	2	20
C. neoformans	4	0	9	0	13
Total	98	11	49	6	164

[a] F, FUNGITEST; M, broth microdilution test.

sified as susceptible or intermediate by the FUNGITEST method, but for which the MICs were found to fall within the intermediate or resistant range by the broth microdilution test. An increase in the inoculum concentration or incubation time for the FUNGITEST method might help to reduce the number of discrepant results.

Like other methods for breakpoint testing of antimicrobial agents, the FUNGITEST method is based on the principle that growth of isolates is measured in cultures each containing one of just two drug concentrations that are capable of distinguishing resistant isolates from intermediate or susceptible ones. However, with the exception of the NCCLS reference method for fluconazole and itraconazole, breakpoint concentrations have not been validated for antifungal agents (15). Isolates of *Candida* spp. for which the fluconazole MICs are ≤8 μg/ml are classified as susceptible, while those for which the fluconazole MICs are ≥64 μg/ml are classified as resistant. Isolates for which MICs were 16 to 32 μg/ml have been termed susceptible dependent upon dose. Tentative breakpoints for itraconazole, but only for mucosal infections, have also been proposed (15): susceptible, ≤0.125 μg/ml; susceptible dependent upon dose, 0.25 to 0.5 μg/ml; and resistant, ≥1 μg/ml.

The breakpoint concentrations selected for the FUNGITEST method were based on the MIC distribution patterns for 193 representative isolates (including some resistant isolates) in tests with the six antifungal agents conducted in two laboratories in France (7). With the exception of the lower of the two concentrations of fluconazole, the breakpoints selected for testing this agent and itraconazole differ from the recommendations of the NCCLS subcommittee on antifungal drug susceptibility testing (15). To bring the FUNGITEST breakpoints into line with NCCLS recommendations, the itraconazole concentrations of 0.5 and 4 μg/ml would need to be reduced to 0.125 and 1 μg/ml, respectively, and the upper fluconazole concentration of 64 μg/ml would need to be changed to 32 μg/ml.

Our results suggest that the breakpoint concentrations selected for the other antifungal agents included in the FUNGITEST method may also need to be adjusted. In particular, the concentrations of 2 and 8 μg/ml selected for testing with amphotericin B appear to be too high. The 200 isolates tested in this study were all classified as susceptible by the FUNGITEST method, even though differences in MIC ranges, MIC_{50}s, and MIC_{90}s were apparent by the broth microdilution method. For instance, the MICs for 50 *C. albicans* isolates ranged from 0.125 to 0.5 μg/ml, while those for 10 *C. lusitaniae* isolates ranged from 0.5 to 2.0 μg/ml. It is recognized that the NCCLS method with RPMI 1640 medium does not perform as well for amphotericin B as it does for other antifungal agents, and there is concern that it does not distinguish drug-resistant isolates (6, 13). Nevertheless, reducing the concentrations of amphotericin B used in the FUNGITEST method might better differentiate susceptible and resistant isolates.

Although the FUNGITEST method is a simple procedure for antifungal drug susceptibility testing, it cannot be recommended for routine use in its present form. The results of the method showed good overall agreement with those of a broth microdilution test performed according to NCCLS guidelines (>70% for all species tested; >75% for all agents tested), but it misclassified as susceptible a significant proportion of azole-resistant isolates of several *Candida* spp. In addition, the FUNGITEST method misclassified a large number of inter-

TABLE 4. Minor discrepancies (by agent) between FUNGITEST and reference broth microdilution method

Antifungal agent	No. of discrepant test results[a]				
	F susceptible, M intermediate	F intermediate, M resistant	M susceptible, F intermediate	M intermediate, F resistant	Total
Amphotericin B	0	0	0	0	0
Flucytosine	5	1	3	1	10
Fluconazole	34	3	8	8	47
Itraconazole	10	4	17	1	32
Ketoconazole	38	3	1	0	42
Miconazole	11	0	20	2	33
Total	98	11	49	6	164

[a] F, FUNGITEST; M, broth microdilution test.

mediate isolates as susceptible and a smaller number of resistant isolates as intermediate.

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