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Comparison of E-Test and Broth Microdilution Methods for Antifungal Drug Susceptibility Testing of Molds

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We compared the E test with a broth microdilution method, performed according to National Committee for Clinical Laboratory Standards document M27-A guidelines, for determining the in vitro susceptibilities of 90 isolates of pathogenic molds (10 *Absidia corymbifera*, 10 *Aspergillus flavus*, 10 *Aspergillus fumigatus*, 10 *Aspergillus niger*, 10 *Aspergillus terreus*, 10 *Exophiala dermatitidis*, 10 *Fusarium solani*, 10 *Scedosporium apiospermum*, 5 *Scedosporium prolificans*, and 5 *Scopulariopsis brevicaulis*). Overall, there was 71% agreement between the results of the two methods for amphotericin B (E-test MICs within ±2 $\log_2$ dilutions of broth microdilution MICs) and 88% agreement with the results for itraconazole. The overall levels of agreement (within ±2 $\log_2$ dilutions) were ≥80% for 5 of the 10 species tested against amphotericin B and 8 of the 10 species tested against itraconazole. The best agreement between the results was seen with *A. fumigatus* and *A. terreus* (100% of results for both agents within ±2 $\log_2$ dilutions). The poorest agreement was seen with *S. apiospermum*, *S. prolificans*, and *S. brevicaulis* tested against amphotericin B (20% of results within ±2 $\log_2$ dilutions). In every instance, this low level of agreement was due to isolates for which the broth microdilution MICs were low but for which the E-test MICs were much higher. The E test appears to be a suitable alternative procedure for testing the susceptibility of *Aspergillus* spp. and some other molds to amphotericin B or itraconazole.

Antifungal drug susceptibility testing of molds has become more important because of the rising incidence of invasive mold infections in immunocompromised patients (1, 14, 22), and because of the emergence during treatment of mold strains resistant to antifungal agents (7). Although *Aspergillus* spp. are still the most common causes of mold infection, a growing number of other organisms have been reported to cause lethal infection in immunocompromised individuals. Among the more important of these emerging pathogens are *Fusarium* and *Scedosporium* species (1, 2, 11, 14, 22), many isolates of which are resistant in vitro to amphotericin B or itraconazole (17, 23).

The National Committee for Clinical Laboratory Standards (NCCLS) has developed a standardized broth macrodilution method of in vitro susceptibility testing for *Candida* spp. and *Cryptococcus neoformans* (15). In addition, interpretive breakpoints for *Candida* spp. have been proposed for itraconazole and fluconazole on the basis of a comparison of clinical outcome of treatment with the MICs of the agents for the organisms isolated (19). Standardization of antifungal susceptibility testing of molds is at a less advanced stage, but an initial multicenter study showed that consistent results could be obtained by either broth macrodilution or microdilution methods with buffered RPMI 1640 medium and a standardized inoculum concentration (10). A second and larger multicenter study showed a high level of agreement among the MICs of amphotericin B and itraconazole determined by a broth microdilution method (9). In addition, correlations between antifungal drug susceptibilities of some molds in vitro and treatment outcomes in patients as well as in animal models of infection have been reported (6, 12, 16). However, macrodilution and microdilution methods are time-consuming and labor-intensive and there is a need for simpler and more economical methods for susceptibility testing of molds.

The E test (AB Biodisk, Solna, Sweden) is a patented commercial method for the quantitative determination of antimicrobial drug MICs. It is set up in a manner similar to that of an agar disc diffusion test, but the disc is replaced with a calibrated plastic strip impregnated with a continuous concentration gradient of drug. Comparisons of the E-test method for *Candida* spp. and *C. neoformans* with the NCCLS broth macrodilution reference method and broth microdilution adaptations of it have demonstrated high levels of agreement between the results for most antifungal agents (3–5, 8, 20). In the present study, we compared the E test with a broth microdilution adaptation of the NCCLS reference method, using 90 mold isolates of 10 species.

MATERIALS AND METHODS

Test isolates. A total of 90 isolates were tested. These comprised 10 each of *Absidia corymbifera*, *Aspergillus flavus*, *Aspergillus fumigatus*, *Aspergillus niger*, *Aspergillus terreus*, *Exophiala dermatitidis*, *Fusarium solani*, and *Scedosporium apiospermum* and five each of *Scedosporium prolificans* and *Scopulariopsis brevicaulis*. The isolates tested came from the United Kingdom National Collection of Pathogenic Fungi (NCPF), held at the Mycology Reference Laboratory, Bristol, United Kingdom. Two *A. fumigatus* reference strains (NCPF 7097 and NCPF 7100) were included in each batch of tests to ensure quality control. NCPF 7097 had been isolated from a patient who had responded to amphotericin B treatment and was susceptible to amphotericin B and itraconazole; NCPF 7100 was isolated from a patient receiving itraconazole treatment and was found to be resistant to this agent (6).

Isolates were retrieved from storage in liquid nitrogen or water, subcultured on plates of Oxoid Sabouraud dextrose agar (Unipath Ltd., Basingstoke, United Kingdom), supplemented with 0.5% (wt/vol) chloramphenicol, and incubated at 30°C until adequate growth was obtained. To induce spore formation, the isolates were subcultured on slopes of Oxoid potato dextrose agar and incubated at 35°C for 5 to 7 days.

E-test method. The E test was performed in accordance with the manufacturer's instructions. Spore suspensions were prepared in sterile saline and adjusted to a concentration of 10^6 spores/ml, corresponding to 78 to 82% transmission (*Aspergillus* spp.) or 68 to 70% transmission when a spectrophotometer set at 530 nm was used. The medium used was RPMI 1640 agar (1.5%), supplemented with glucose (2%) and buffered to pH 7.0 with MOPS (morpholinepropanesulfonic acid). The molten medium was dispensed in 20-ml amounts into 90-mm-diameter petri dishes, giving an agar depth of 4 ± 0.5 mm (mean ± standard deviation).

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TABLE 1. In vitro susceptibilities of 90 isolates to two antifungal agents as determined by E-test and broth microdilution methods

Organism (no. tested)	Antifungal agent	MIC (μg/ml)					
		E test			Microdilution method		
		Range	50%	90%	Range	50%	90%
A. corymbifera (10)	Amphotericin B	0.003–0.5	0.25	0.5	0.06–0.5	0.25	0.25
	Itraconazole	1–4	1.5	2	0.25–1	0.25	0.5
A. flavus (10)	Amphotericin B	0.5–8	4	8	0.5–2	1	1
	Itraconazole	0.19–0.75	0.5	0.75	0.125–0.25	0.25	0.25
A. fumigatus (10)	Amphotericin B	0.38–2	0.75	2	0.5–1	0.5	1
	Itraconazole	0.5–>32	0.75	>32	0.25–>16	0.25	>16
A. niger (10)	Amphotericin B	0.25–1	0.5	1	0.25	0.25	0.25
	Itraconazole	0.38–6	1.5	2	0.25–0.5	0.5	0.5
A. terreus (10)	Amphotericin B	0.75–4	1	3	1–2	1	2
	Itraconazole	0.19–0.5	0.25	0.38	0.25	0.25	0.25
E. dermatitidis (10)	Amphotericin B	0.004–0.25	0.125	0.19	0.125–1	0.5	1
	Itraconazole	0.25–0.5	0.25	0.5	0.125–0.5	0.25	0.5
F. solani (10)	Amphotericin B	0.38–8	2	8	1–2	1	2
	Itraconazole	>32	>32	>32	>16	>16	>16
S. apiospermum (10)	Amphotericin B	2–>32	>32	>32	1–>16	2	8
	Itraconazole	1–>32	1.5	>32	0.25–4	1	2
S. prolificans (5)	Amphotericin B	>32			2–16		
	Itraconazole	>32			>16		
S. brevicaulis (5)	Amphotericin B	>32			2–16		
	Itraconazole	>32			>16		

The plates were inoculated by dipping a sterile swab into the appropriate cell suspension and streaking it across the entire surface of the agar in three directions. The plates were dried at room temperature for 15 min before the E-test strips were applied. The plates were incubated at 35°C and read at 24 (*A. corymbifera*) or 48 (other species) h. The E-test MIC was read as the drug concentration at which the border of the elliptical inhibition zone intersected the scale on the antifungal test strip.

Broth microdilution method. The broth microdilution method was performed according to NCCLS guidelines (15). Amphotericin B was obtained from Sigma Chemical Co. (St. Louis, Mo.), and itraconazole was from Janssen Research Foundation (Beerse, Belgium). Amphotericin B was dissolved in dimethyl sulfoxide. A stock solution of itraconazole was prepared in polyethylene glycol 400 with the aid of heating to 70°C. Further dilutions of both drugs were made with RPMI 1640 medium (with L-glutamine; without bicarbonate) (Sigma), buffered to pH 7.0 with 0.165 M MOPS (Sigma). The antifungal agents were tested over a final concentration range of 0.03 to 16 μg/ml.

Broth microdilution MICs were determined in 96-well round-bottom microtiter plates. Spore suspensions were prepared in RPMI 1640 medium and adjusted to a final inoculum concentration of 0.4×10^4 to 5×10^4 spores/ml. The plates were incubated at 35°C and read after 24 h (*A. corymbifera*) or 48 h (other species). The MIC of amphotericin B was defined as the lowest concentration at which there was complete inhibition of growth; the MIC of itraconazole was defined as the lowest concentration at which there was prominent or complete inhibition of growth compared with that of the drug-free controls.

Analysis of results. Because the E-test strips contain a continuous gradient of each drug tested instead of the $\log_2$ drug dilution scheme of the broth microdilution method, the E-test MICs were elevated to the next drug concentration which matched the microdilution scheme to facilitate comparison of the results. The percentage of agreement between the E test and the reference broth microdilution method was defined as the proportion of E-test results which fell within ± 1 or 2 $\log_2$ dilutions of the standard MIC results. In addition, the E-test results were examined to determine whether this method tended to produce higher or lower MICs than the broth microdilution method.

RESULTS

Table 1 summarizes the in vitro susceptibilities of the 90 isolates to amphotericin B and itraconazole as measured by the E-test and broth microdilution methods. The data are presented as MIC ranges and, where appropriate, as the drug concentrations required to inhibit 50 and 90% of the isolates of each species (MIC_{50}s and MIC_{90}s, respectively). In each batch of broth microdilution tests, the MICs of the antifungal agents for the two quality control strains were within the accepted limits (MICs of amphotericin B for NCPF 7097, 0.5 to 1 μg/ml, and for NCPF 7100, 1 to 2 μg/ml; MICs of itraconazole for NCPF 7097, 0.12 to 0.5 μg/ml, and for NCPF 7100, 4 to 16 μg/ml).

The reproducibility of the E-test procedure was investigated by testing six mold strains on five occasions (Table 2). In 9 of the 12 drug-organism combinations tested, the MIC endpoints fell within a three-step concentration range. In one of the three remaining combinations, the endpoints fell within four steps of each other (16 to >32 μg/ml); in the others, the endpoints were within a five-step range (0.25 to 1 μg/ml).

Table 3 presents a detailed analysis of the comparison be-

TABLE 2. Reproducibility of E-test method with six mold strains

Strain	MIC endpoint (μg/ml)	
	Amphotericin B	Itraconazole
A. fumigatus NCPF 7097	0.5–1	0.25–1
A. fumigatus NCPF 7100	2–4	32–>32
A. niger NCPF 2023	0.5–1	1–1.5
A. terreus NCPF 7445	3–6	0.19–0.38
S. apiospermum NCPF 2565	>32	0.25–1
S. prolificans NCPF 2871	16–>32	>32

TABLE 3. Agreement between E-test and broth microdilution methods for 90 isolates

Organism (no. tested)	Antifungal agent	% Agreement[a]	
		±1 $\log_2$ dilution	±2 $\log_2$ dilutions
A. corymbifera (10)	Amphotericin B	90	90
	Itraconazole	20	50
A. flavus (10)	Amphotericin B	50	60
	Itraconazole	50	100
A. fumigatus (10)	Amphotericin B	100	100
	Itraconazole	70	100
A. niger (10)	Amphotericin B	70	100
	Itraconazole	40	90
A. terreus (10)	Amphotericin B	90	100
	Itraconazole	100	100
E. dermatitidis (10)	Amphotericin B	10	70
	Itraconazole	90	90
F. solani (10)	Amphotericin B	50	80
	Itraconazole	100	100
S. apiospermum (10)	Amphotericin B	20	20
	Itraconazole	50	60
S. prolificans (5)	Amphotericin B	20	20
	Itraconazole	100	100
S. brevicaulis (5)	Amphotericin B	0	20
	Itraconazole	100	100
All organisms (100)	Amphotericin B	54	71
	Itraconazole	69	88

[a] Percentage of agreement between the results is defined as proportion of E-test MIC results that were within ±1 or 2 $\log_2$ dilutions of the broth microdilution MIC results.

tween the results of the E-test and broth microdilution methods. The overall levels of agreement between the results of the two methods (E-test MICs within ±2 $\log_2$ dilutions of broth microdilution MICs) were 71% for amphotericin B and 88% for itraconazole. The overall levels of agreement (within ±2 $\log_2$ dilutions) were ≥80% for five of the 10 species tested against amphotericin B and eight species tested against itraconazole. The lowest individual levels of agreement were seen with the two *Scedosporium* spp. and with *S. brevicaulis* tested against amphotericin B (20% of results within ±2 $\log_2$ dilutions). In general, the discrepancies between E-test and broth microdilution results for this agent were due to higher E-test MICs (data not shown).

DISCUSSION

Until 1990, amphotericin B was the agent of choice for the treatment of most invasive mold infections. Although ketoconazole had been used with success in the treatment of some forms of histoplasmosis and coccidioidomycosis, and miconazole was sometimes effective in treating *S. apiospermum* infections, neither agent was regarded as first-line treatment for invasive infections in immunocompromised patients. Because few other antifungal agents were available, and because amphotericin B had a broad spectrum of action, there was limited interest in developing in vitro methods of testing that could predict the clinical outcome of amphotericin B treatment. Furthermore, acquired drug resistance did not appear to be a significant factor in treatment failure (21).

The introduction of new antifungal agents, such as itraconazole and voriconazole, and the recent detection of itraconazole-resistant strains of *A. fumigatus* emerging during treatment (7) have highlighted the need for reliable methods of in vitro testing for molds. The NCCLS reference method for *Candida* spp. and *C. neoformans* has acted as a useful starting point for the development of a standardized method for testing of antifungal agents against molds. However, this broth macrodilution method and microdilution adaptations of it are time-consuming and labor-intensive and have not eliminated the need for more convenient methods of routine testing.

The present evaluation is one of the first to investigate whether or not the E test is a suitable method for antifungal drug susceptibility testing of molds. Our results (Table 2) indicate that the E-test procedure is reproducible. Furthermore, our results (Table 3) demonstrate 100% agreement (results within ±2 $\log_2$ dilutions) between the E test and a standardized broth microdilution method for 10 isolates each of *A. fumigatus* and *A. terreus* tested against amphotericin B and itraconazole. The results of the two methods for six of the eight other molds tested against itraconazole showed at least 90% agreement. The overall level of agreement between the results of the two methods for amphotericin B was lower (71% of results within ±2 $\log_2$ dilutions), with some drug-organism combinations showing much lower levels of agreement than others. For instance, in tests with the two *Scedosporium* spp. and with *S. brevicaulis*, only 20% of E-test results were within ±2 $\log_2$ dilutions of the broth microdilution MICs. In every instance, this low level of agreement was due to isolates for which the broth microdilution MICs were low but for which the E-test MICs were much higher.

It is well 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 detect drug-resistant isolates of *Candida* spp. (13, 18). Wanger et al. (24) have demonstrated that the E test is superior to the broth macrodilution reference method as a means of distinguishing amphotericin B-resistant and -susceptible isolates of *Candida* spp. Many *S. apiospermum* and *S. prolificans* isolates are resistant to amphotericin B in vitro (2, 23). In this evaluation, the E-test MICs of amphotericin B for 7 of 10 *S. apiospermum* isolates and the five *S. prolificans* isolates were >32 μg/ml. In contrast, the broth microdilution MICs of amphotericin B for five of the *S. apiospermum* isolates were ≤2 μg/ml, while the MICs for four of the five *S. prolificans* isolates were ≤8 μg/ml. These results suggest that the E test permits much better discrimination in vitro of amphotericin B-resistant molds, such as *Scedosporium* spp., than does the broth microdilution method with RPMI 1640 medium.

As part of this evaluation, we tested three itraconazole-resistant *A. fumigatus* isolates. Two of these isolates (NCPF 7099 and NCPF 7100) were obtained from patients who failed itraconazole treatment and were shown to be resistant to this agent in vitro and in an animal model of infection (6, 7). The E-test and broth microdilution MICs of itraconazole for all three resistant *A. fumigatus* isolates were similarly high (>32 and >16 μg/ml). *F. solani* and *S. prolificans* isolates are often resistant to this azole antifungal agent in vitro (2, 17). The E-test and broth microdilution MICs of itraconazole for all 15 isolates of *F. solani* and *S. prolificans* were >32 and >16 μg/ml, respectively. In addition, the E-test MICs of itraconazole for 2 of the 10 *S. apiospermum* isolates tested were >32 μg/ml: the broth microdilution MIC for one of these isolates was 2 μg/ml,

and that for the other was 4 μg/ml. Our results suggest that the E test is able to detect itraconazole resistance in vitro in a wide range of molds.

It remains to be seen to what extent the MICs generated in this and other in vitro investigations will be predictive of clinical outcome in patients with invasive mold infections. Although standardization of mold susceptibility testing is less well developed than that of *Candida* spp. or *C. neoformans*, correlations between antifungal drug susceptibilities of some molds in vitro and treatment outcomes in patients, as well as in animal models of infection, have been reported (6, 16). However, further studies will be needed before firm conclusions can be drawn.

In conclusion, this investigation has demonstrated that the E-test method is a reproducible method of antifungal drug susceptibility testing with molds. It is less labor-intensive and much simpler to set up than the broth dilution test, and the results for few isolates are difficult to read. The results of this investigation showed a good level of overall agreement between the E-test method and a broth microdilution test performed according to NCCLS guidelines. Our results suggest that the E test is suitable for routine use in susceptibility testing of *Aspergillus* spp. and some other molds against amphotericin B or itraconazole.

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