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Evaluation of Etest Method for Determining Voriconazole and Amphotericin B MICs for 162 Clinical Isolates of *Cryptococcus neoformans*

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The performance of the Etest for voriconazole and amphotericin B susceptibility testing of 162 isolates of *Cryptococcus neoformans* was assessed against the National Committee for Clinical Laboratory Standards (NCCLS) broth microdilution method. The NCCLS method employed RPMI 1640 broth medium, and MICs were read after incubation for 72 h at 35°C. MICs were determined by Etest for all 162 isolates with RPMI 1640 agar containing 2% glucose (RPG agar) and were read after incubation for 72 h at 35°C. The Etest results for both voriconazole and amphotericin B correlated well with reference MICs. Agreement was 94% for voriconazole and 99% for amphotericin B. When discrepancy was noted between the results obtained by Etest and broth microdilution for voriconazole, the Etest generally provided a higher MIC. The Etest method using RPG agar appears to be a useful method for determining the susceptibility of *C. neoformans* to voriconazole and amphotericin B.

Numerous studies have shown that when performed according to the manufacturer's instructions, the Etest stable agar gradient method (AB BIODISK, Solna, Sweden) provides excellent performance for testing *Candida* species against a variety of antifungal agents including polyenes, azoles, echinocandins, and flucytosine (4–6, 12, 13, 15–17, 21, 23). Some studies have also included isolates of *Cryptococcus neoformans* and have demonstrated the suitability of Etest using RPMI 1640 agar supplemented with 2% glucose (RPG agar) for determining the in vitro susceptibility of this pathogenic yeast to amphotericin B, fluconazole, itraconazole, and flucytosine (1, 4, 5, 8, 11, 13, 21, 23). These studies have been limited by the inclusion of low numbers of *C. neoformans* isolates, and none have included any of the new triazole antifungal agents.

Voriconazole is a new triazole with broad-spectrum activity against *Candida* spp., *Aspergillus* spp. and other filamentous fungi, and *C. neoformans* (7, 9, 14, 18–20, 22). Voriconazole has been tested in vitro against *Candida* spp. by both the broth microdilution (BMD) method and Etest (15), and voriconazole has been tested against *C. neoformans* by the BMD method (14). At this time, there are no studies evaluating the performance of Etest with voriconazole against *C. neoformans*.

In this study, we have evaluated the performance of the Etest for voriconazole using RPG by comparing the results with those obtained using the National Committee for Clinical Laboratory Standards (NCCLS) BMD method for testing 162 clinical isolates of *C. neoformans*. In addition, because a recent report by Aller et al. (1) found a very poor level of agreement between Etest and the BMD method for testing *C. neoformans* against amphotericin B, we have also included amphotericin B in the evaluation.

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MATERIALS AND METHODS

Test organisms. One hundred sixty-two clinical isolates of *C. neoformans* were selected for testing. These isolates were all recent clinical isolates from 50 geographically diverse medical centers worldwide. The *C. neoformans* organisms had all been isolated from either cerebrospinal fluid or blood samples from infected patients (14, 24). All isolates were stored as suspensions in sterile distilled water at room temperature until used in the study. Prior to testing, each isolate was subcultured at least twice on potato dextrose agar plates (Remel, Lenexa, Kans.) to ensure purity and optimal growth.

Antifungal agents. Etest strips containing voriconazole and amphotericin B were supplied by AB BIODISK. Voriconazole and fluconazole were obtained as reagent-grade powders from Pfizer, Inc. (Groton, Conn.), and amphotericin B was obtained from Sigma (St. Louis, Mo.). Stock solutions were prepared in dimethyl sulfoxide and further diluted in RPMI 1640 medium buffered to pH 7.0 with 0.165 M morpholinepropanesulfonic acid (MOPS) buffer (Sigma) and were dispensed into 96-well microdilution trays. Trays containing a 0.1-ml aliquot of the appropriate drug solution (two times the final concentration) in each well were subjected to quality control (QC) testing and then sealed and stored at −70°C until used in the study. The final concentrations of voriconazole and amphotericin B in the wells ranged from 0.007 to 8 μg/ml. Fluconazole concentrations in the wells ranged from 0.06 to 64 μg/ml.

Media. The agar formulation used for the Etest was RPMI 1640 (American Biorganics, Buffalo, N.Y.) supplemented with 1.5% agar and 2% glucose (RPG agar) and buffered with MOPS (8, 15). The RPMI 1640 broth medium used for microdilution testing was buffered with MOPS in accordance with the NCCLS M27-A method (10).

Antifungal susceptibility testing methods. BMD tests were performed according to NCCLS document M27-A (10). An inoculum suspension to match the turbidity of a 0.5 McFarland standard, diluted to a concentration of 1.0×10^3 to 5.0×10^3 cells per ml, was standardized spectrophotometrically, and an aliquot of 0.1 ml was added to each well of the microdilution tray (final inoculum, 0.5×10^3 to 2.5×10^3 cells/ml). In each case, the inoculum size was verified by colony counting. The microdilution trays were incubated at 35°C. The MIC endpoints were read visually following 72 h of incubation. The MICs of voriconazole and fluconazole were defined as the lowest concentration that produced a prominent decrease (~50%) in turbidity compared to the turbidity of the drug-free control well (10, 14, 15). The MIC of amphotericin B was defined as the lowest concentration that produced complete inhibition of growth (first clear well).

TABLE 1. Antifungal activities of amphotericin B and voriconazole against 162 isolates of *C. neoformans* as determined by the BMD method and Etest

Antifungal agent	Method[a]	MIC (μg/ml)[b]			% Agreement[c]
		Range	50%	90%	
Amphotericin B	BMD	0.12–1	0.5	1	
	Etest	0.015–1	0.5	1	99
Voriconazole	BMD	0.015–2	0.06	0.12	
	Etest	0.015–>8	0.06	0.12	94

[a] The BMD method was performed as described in NCCLS document M27-A (10).

[b] 50 and 90%, MICs at which 50 and 90% of isolates tested are inhibited, respectively.

[c] Percentage of Etest MICs (read at 72 h) that are within 2 $\log_2$ dilutions of the reference microdilution MICs (RPMI 1640 broth, 72 h).

For the Etest, 90-mm-diameter plates containing agar to a depth of 4.0 mm were used. The agar surface was inoculated by using a nontoxic swab dipped in a cell suspension adjusted spectrophotometrically to the turbidity of a 1.0 McFarland standard. After excess moisture was absorbed into the agar and the surface was completely dry, the voriconazole and amphotericin B Etest strips were applied to each plate. The plates were incubated in air at 35°C and read at 72 h. The voriconazole MIC was read at the lowest concentration at which the border of the elliptical inhibition zone intercepted the scale on the strip. Any growth, such as microcolonies, throughout a discernible inhibition ellipse was ignored (15). The amphotericin B MIC was read at the point at which the zone of complete inhibition intersected the strip (8).

QC testing. QC testing was performed in accordance with NCCLS document M27-A using *Candida krusei* ATCC 6258 and *Candida parapsilosis* ATCC 22019 (10). QC determinations made on each day of testing were within the control limits for voriconazole, fluconazole, and amphotericin B established by Barry et al. (2).

Analysis of results. Etest MICs of voriconazole and amphotericin B read at 72 h were compared to reference MICs read at 72 h. Since the Etest scale has a continuous gradient of concentrations, the MICs between twofold dilutions were raised to the next twofold level of the reference method for comparison (12, 13, 15–17). Off-scale MICs at the upper limit were converted to the next higher concentration, and off-scale results at the lower limit were left unchanged. Discrepancies between MICs of no more than 2 dilutions were used to calculate the percent agreement.

RESULTS AND DISCUSSION

Table 1 summarizes the in vitro susceptibilities of 162 *C. neoformans* isolates to voriconazole and amphotericin B as determined by the reference BMD method and Etest. The voriconazole and amphotericin B MICs obtained were consistent with values reported previously for *C. neoformans* tested in RPMI 1640 medium (8, 14). Amphotericin B MICs did not exceed 1 μg/ml by either method. Voriconazole MICs of >1 μg/ml were observed for only one isolate (MIC, 2 μg/ml) as determined by the BMD method and for five isolates (MICs of 2 μg/ml [two isolates], 4 μg/ml [one isolate], and > 8 μg/ml [two isolates]) as determined by Etest. The distribution of fluconazole MICs was also representative of values reported previously (3, 14): MICs of ≤8 μg/ml for 92%, 16 to 32 μg/ml for 6.8%, and ≥64 μg/ml for 1.2% of the isolates (data not shown).

Table 1 also summarizes the percentage agreement of voriconazole and amphotericin B MICs at 72 h obtained by the Etest on RPG agar that were within 2 dilutions of the reference method. There was 99% agreement for amphotericin B and 94% agreement for voriconazole. The lone discrepancy with amphotericin B was due to an Etest result that was 3 dilutions lower than the companion BMD result. When a discrepancy was noted between the results obtained by Etest and BMD for voriconazole, the Etest generally provided a higher MIC.

The results of this study provide the first documentation of the applicability of the Etest method for determining the in vitro susceptibility of *C. neoformans* to the newly licensed triazole voriconazole. As noted by others (1, 8, 11), we found that RPMI 1640 agar with glucose (2% final concentration) supported optimal growth of isolates representing a wide range of geographic origins and provided excellent agreement with the MICs determined by the BMD method. Although the Etest for voriconazole gave higher MICs than the BMD method for some isolates, this was not as pronounced as has been noted by others for fluconazole tested by Etest against *C. neoformans* (4, 21, 23).

In contrast to the recent report of Aller et al. (1) who found an unacceptably low (13.5%) level of agreement with the BMD method when Etest was used to test 35 strains of *C. neoformans* against amphotericin B, we found essentially complete agreement between the Etest and BMD method when testing amphotericin B. This high level of agreement has also been reported by Chen et al. (4), Lozano-Chiu et al. (8), and Simor et al. (21). Furthermore, Lozano-Chiu et al. (8) found the Etest to be a useful method for detecting resistance to amphotericin B in *C. neoformans* isolates. These investigators reported that Etest identified strains of *C. neoformans* inhibited by 2 to 6 μg of amphotericin B per ml (8). We did not identify any strains with possible amphotericin B resistance (MIC, >1 μg/ml) among 162 representative clinical isolates.

In summary, we have provided the first evidence of the ability of the Etest to generate voriconazole MIC data for *C. neoformans* that are comparable to those obtained by the NCCLS microdilution method. Furthermore, we have confirmed the findings of others regarding the usefulness of the Etest for determining the in vitro susceptibility of *C. neoformans* to amphotericin B. RPG may be used to determine reference-quality MICs with voriconazole, amphotericin B, and flucytosine Etest reagents in tests with *C. neoformans* as well as *Candida* species (4, 5, 8, 11, 15). Etest reagents provide great flexibility in that they allow clinical laboratories to test one or more antifungal agents selectively against yeast isolates as the clinical situation dictates. Although it is not yet clear what role voriconazole may have in the treatment of cryptococcosis, the potent activity as demonstrated by both broth- and agar-based methods suggest that it may be a useful addition to the existing treatment regimens.

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