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Comparison of Two Microdilution Methods for Testing Susceptibility of *Candida* spp. to Voriconazole

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The growing number of fungal infections, coupled with emerging resistance to classical antifungal agents, has led to the development of new agents, among them voriconazole. Susceptibility to voriconazole was tested by using two microdilution techniques: the reference method described in National Committee for Clinical Laboratory Standards document M27-A2 and a colorimetric method, Sensititre YeastOne. A total of 272 *Candida* isolates (132 of *Candida albicans*, 62 of *C. parapsilosis*, 33 of *Candida glabrata*, 21 of *C. krusei*, 15 of *C. tropicalis*, and 9 of *C. lusitaniae*) and two control strains (*C. parapsilosis* ATCC 22019 and *C. krusei* ATCC 6258) were tested. There was a high rate of agreement between the two methods used (97 to 100%).

The incidence of invasive mycosis in immunocompromised or surgically treated patients has risen in recent years (20). Growing primary and secondary resistance to classical antifungal agents has led to the need to develop new agents (7) such as voriconazole (3). However, the activity of these agents against a range of pathogens needs to be measured (11).

In 2002, in document M27-A2, the National Committee for Clinical Laboratory Standards (NCCLS) proposed a broth dilution reference method (RM) for antifungal susceptibility testing of yeasts and yeast-like organisms, especially *Candida* spp. and *Cryptococcus neoformans* (14). However, these standardized methods are complex and time-consuming; the growing demand for this type of testing has given rise to commercial testing methods that are both fast and easy to perform (6, 9, 16). One such method is the Sensititre YeastOne colorimetric antifungal panel.

The aim of the present study was to compare the susceptibility of *Candida* strains (from human clinical samples) to voriconazole as evidenced by two microdilution methods, the RM described in NCCLS document M27-A2 and Sensititre-Alamar YeastOne.

A total of 272 strains were isolated from 77 sputum samples, 64 pharyngeal exudate samples, 51 blood cultures, 38 abscesses, 22 vaginal exudate samples, and 20 urine samples. The following isolates were collected: 132 of *Candida albicans*, 62 of *C. parapsilosis*, 33 of *C. glabrata*, 21 of *C. krusei*, 15 of *C. tropicalis*, and 9 of *C. lusitaniae*.

Strains were identified by simple microbiological methods, including gross inspection for colony morphology and growth on chromogenic media such as CHROMagar, followed by microscopic examination (inspection, germ tube production, and chlamydospore formation). Identification was also confirmed by biochemical methods (10). Isolates were stored as suspensions in sterile distilled water at −70°C until the study was performed (11). Prior to testing, each isolate was subcultured on Candida CHROMagar to ensure purity and optimal growth.

The following antifungal agents were used. For the NCCLS method, standard voriconazole antifungal powder was supplied by the Pfizer Inc. Central Research Division (Groton, Conn.). Stock solutions were prepared in dimethyl sulfoxide and subsequently diluted with RPMI 1640 medium (Sigma Chemical Co., St. Louis, Mo.) buffered to pH 7 with 0.165 M morpholinepropanesulfonic acid (MOPS; Sigma), and the mixtures were dispensed into 96-well microdilution trays. Trays containing an aliquot of 0.1 ml in each well were sealed and frozen at −70°C until used in the study. The NCCLS recommendations were followed for voriconazole dilution. Final concentrations ranged from 0.03 to 16 μg/ml (14, 19).

The Sensititre YeastOne system is based on microdilution methodology with RPMI 1640 medium supplemented with a pH indicator (Alamar blue). Panels enable testing of in vitro susceptibility to a panel of antifungal agents, including voriconazole, with MICs being determined by color changes (7). It is a standardized investigational method and correlates well with the NCCLS method M27-A2 for a large number of experimental variables. Disposable trays were precoated with six antifungal agents: amphotericin B, fluconazole, itraconazole, ketoconazole, flucytosine, and voriconazole. The individual trays contained dried voriconazole concentrations (twofold serial dilutions) ranging from 0.008 to 16 μg/ml (13).

Inoculum preparation, reading, and interpretation were performed for each method as specified below. For the NCCLS method, the inoculum size was adjusted to yield concentrations of 0.5×10^3 to 2.5×10^3 CFU/ml by dilution in RPMI medium in accordance with the M27-A2 guidelines (14). An aliquot of 0.1 ml was added to each well of the microdilution tray. In each case, inoculum size was confirmed by colony counting. MIC endpoints were determined after incubation for 48 h at 35°C. As is customary for azole compounds such as voriconazole, the MIC was defined as the lowest concentration that produced a 50% reduction in growth compared with that of the drug-free control (4).

For the Sensititre YeastOne method, the inoculum was adjusted turbidimetrically and the inoculum size conformed to the M27-A2 guidelines. Results were read after 24 h of incu-

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TABLE 1. Susceptibility of the species studied

Organism and test[a]	MIC range (μg/ml)	MIC_{50} (μg/ml)	MIC_{90} (μg/ml)	% Agreement
C. albicans to FZ (n = 66)				
RM[b]	0.03–0.125	0.03	0.06	98
Sensititre	0.03–0.25	0.03	0.03	
C. albicans SDD FZ (n = 24)				
RM	0.03–0.5	0.06	0.5	97
Sensititre	0.03–0.125	0.03	0.06	
C. albicans resistant to FZ (n = 42)				
RM	0.03–16	0.03	1	98
Sensititre	0.03–16	0.06	1	
C. parapsilosis				
RM	0.03–0.06	0.03	0.06	98
Sensititre	0.03–0.125	0.03	0.125	
C. glabrata				
RM	0.03–1	0.125	0.25	100
Sensititre	0.03–1	0.125	0.25	
C. krusei				
RM	0.06–0.25	0.125	0.25	100
Sensititre	0.06–0.25	0.125	0.25	
C. tropicalis				
RM	0.03–0.25	0.03	0.25	98
Sensititre	0.03–0.25	0.03	0.125	
C. lusitaniae				
RM	0.03–0.06	0.03	0.03	98
Sensititre	0.03–0.06	0.03	0.06	

[a] FZ, fluconazole.
[b] Described in NCCLS document M27-A2.

bation at 35°C. Yeast growth was evident as a change in the Alamar blue growth indicator; the change from blue to pink facilitated clearer identification of breakpoints than the turbidimetric method, thus reducing the trailing effect characteristic of azole antifungal agents that hinders the interpretation of results when dilution techniques are used (6).

Two yeast-like fungal strains with known susceptibility to voriconazole were used as quality controls: *C. parapsilosis* ATCC 22019 and *C. krusei* ATCC 6258 (2). All clinical isolates grew on the drug-free growth controls when both test methods were used; MICs for quality control reference strains were within accepted limits for voriconazole (2).

Discrepancies between MIC pairs of no more than ±1 or ±2 dilutions were used for calculations of percent agreement.

Results are shown in Table 1, which includes the range of voriconazole MICs, the MIC for 50% of the strains tested (MIC_{50}), and the MIC_{90} in micrograms per milliliter recorded by using the two microdilution methods and the level of agreement. *C. albicans* isolates were classified into three groups based on susceptibility to fluconazole: of the 132 *C. albicans* isolates, 66 were classified as fluconazole susceptible, 24 were classified as fluconazole susceptible-dose dependent (SDD FZ), and 42 were classified as fluconazole resistant.

Of the 33 *C. glabrata* strains, 12 were resistant to fluconazole. All strains of *C. parapsilosis*, *C. tropicalis*, and *C. lusitaniae* were susceptible to fluconazole.

The highest rate of agreement between the two methods was found for *C. glabrata* and *C. krusei*. The MIC for *C. glabrata* ranged from 0.03 to 1 μg/ml by both methods, with a MIC_{50} and MIC_{90} of 0.125 and 0.25 μg/ml for the Sensititre method and the RM, respectively. Similarly, the MICs for *C. krusei* ranged from 0.06 to 0.25 μg/ml, with a MIC_{50} and MIC_{90} ranging from 0.125 to 0.25 μg/ml for the Sensititre method and the RM, respectively. Thus, there was 100% agreement between the two microdilution methods, which—significantly—indicated marked activity of voriconazole against *C. glabrata* and *C. krusei*.

The agreement between the methods for *C. tropicalis* and *C. lusitaniae* was 98%; the MIC and MIC_{50} ranges matched, but the MIC_{90} for both species differed by ±1 dilution. A difference of ±1 dilution between methods was also recorded for the MIC, MIC_{50}, and MIC_{90} ranges for fluconazole-susceptible *C. albicans* and *C. parapsilosis*, with 98% agreement.

For *C. albicans* SDD FZ, there was 97% agreement between methods, with a difference of ±2 dilutions for MICs. For fluconazole-resistant *C. albicans*, both methods showed strong agreement for the MIC_{50} and MIC_{90} and both indicated a MIC range of 0.03 to 16 μg/ml.

The tests performed for all species, except for RM testing of fluconazole-resistant *C. albicans*, yielded MIC, MIC_{50}, and MIC_{90} ranges of <1 μg/ml for voriconazole, thus indicating excellent activity (17). For fluconazole-resistant *C. albicans*, the MIC_{50} and MIC_{90} were ≤1 μg/ml, also indicative of good activity, although the MIC range was wider.

In the present study, voriconazole was active (MIC, ≤1 μg/ml) against all of the species studied (*C. glabrata*, *C. krusei*, *C. tropicalis*, *C. lusitaniae*, *C. parapsilosis*, and *C. albicans*), especially strains that were fluconazole susceptible and dose dependently fluconazole susceptible, although not against a small proportion of fluconazole-resistant strains (MIC, >1 μg/ml) (17); this could be due to cross-resistance to azoles through sharing of resistance mechanisms.

One advantage of the Sensititre YeastOne method is its ease of use as a diagnostic tool in clinical microbiological laboratories; the contrasting technical complexity and the technical training necessary for the reading and interpretation of results when using the RM should be borne in mind when evaluating this commercial method (15). The results of susceptibility studies may be affected by many experimental variables, which can be reduced by eliminating certain handling processes, including preparation of dilutions and growth media, and thus increasing automation (1).

With the RM, the MIC was defined as the lowest concentration producing 50% inhibition of growth (4). The so-called trailing effect, or residual fungal growth, observed with azole antifungal agents, which hinders the interpretation of results with the RM, is partly overcome by the use of commercial techniques that facilitate reading via the change in color of a pH indicator in the growth medium (12). In the Sensititre system, readings are interpreted by color change, since wells with growth turn pink while growth-negative wells remain blue. With azoles, however, because of the trailing effect, the change in color is less evident and there is occasionally a change from pink (growth) to purple (partial inhibition of growth) (7); in this case, the MIC is the drug concentration in the first purple well. Trailing isolates are difficult to read by this method as well. When it comes down to it, both are microdilution methods and have very comparable levels of difficulty. The only difference in terms of performance is that one may purchase the YeastOne system but must prepare the RM panels. A key feature of the Sensititre YeastOne system is the possibility of incorporating new antifungal agents as they are introduced into clinical practice; for that reason, it is the only currently available commercial technique that enables in vitro determination of susceptibility to voriconazole.

The rate of agreement between Sensititre YeastOne and the microdilution RM has not yet been established for voriconazole. However, studies show considerable agreement between the two methods for other antifungal agents. Messer and Pfaller (12) reported 83% agreement for itraconazole and 93% agreement for flucytosine. Arikan et al. (1) obtained similar results for fluconazole and amphotericin B, as did Posteraro et al. (18). The agreement between the two methods is influenced by a number of experimental variables, including incubation time, which affects different yeast genera and species differently. Espinel-Ingroff et al. (6), for example, reported that agreement between the methods for *C. albicans* was greater at 24 h and ranged from 97% for amphotericin B to 87% for fluconazole, while with other *Candida* species, agreements rose to 97 to 100% for other antifungal agents.

The MIC_{90} for *C. glabrata* obtained here with the RM (0.25 μg/ml) does not agree with the results reported by other authors. Espinel-Ingroff (5) recorded a MIC_{90} of 4 μg/ml with the RM, while Hoban et al. (8) reported a value of 2 μg/ml for the same species and method. However, this may be because the populations of this isolate were different.

Voriconazole represents an interesting alternative antifungal agent for use against fluconazole-resistant species such as *C. krusei* and some strains of *C. glabrata* and is a particularly valuable option for the treatment of fluconazole-resistant candidiasis in AIDS patients (17).

The two methods studied show an agreement rate of 97 to 100%; since the Sensititre method requires less processing time and is easy both to perform and to read, it may be a valuable option for testing the activity of voriconazole against the yeast-like organisms commonly isolated in clinical mycology laboratories.

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