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In Vitro Activity of Voriconazole against *Prototheca wickerhamii*: Comparative Evaluation of Sensititre and NCCLS M27-A2 Methods of Detection

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A total of 104 *Prototheca wickerhamii* isolates and two control strains were tested for susceptibility to voriconazole using the Sensititre YeastOne colorimetric antifungal plate and NCCLS reference method. Voriconazole was highly active against all isolates, with an MIC at which 90% of isolates were inhibited of ≤0.5 μg/ml. Comparison of MICs obtained with the Sensititre product and the NCCLS method demonstrated agreement (100% ± 2 $\log_2$ dilutions) between the two methods. Voriconazole may offer an option for the treatment of *Prototheca* sp. infections, and its efficacy should be established through clinical experience.

Species of the genus *Prototheca* (family *Chlorellaceae*) are ubiquitous, unicellular, achlorophyllic algae closely related to the green algae *Chlorella* spp. (12). *Prototheca* spp. have been isolated from tree sap, potato peel, seawater, lakes, marshes, streams, and pond mud, as well as from rivers and wastewater (2). The first description of human infection attributed to *Prototheca* spp. was reported by Davies et al. in 1964 (6). Subsequently there have been numerous reports of localized cutaneous and subcutaneous protothecosis (5, 10, 18) and, in rare instances, systemic disease (9, 17). The involvement of *Prototheca* spp. in human disease (3), in both immunocompetent and immunocompromised patients, has been reported with increasing frequency (19).

Voriconazole is a monotriazole antifungal agent with activity against a broad spectrum of pathogenic fungi, among them *Candida* spp., including species displaying in vitro resistance to fluconazole, *Cryptococcus neoformans* and *Aspergillus* spp. (8). Our review of the literature did not reveal any studies that addressed the susceptibility of *Prototheca* spp. to voriconazole. This reports addresses the in vitro activity of voriconazole against strains of *Prototheca wickerhamii* and compares the results obtained using two microdilution methods: the Sensititre YeastOne method and the National Committee for Clinical Laboratory Standards (NCCLS) reference broth microdilution method (15).

One hundred four isolates of *Prototheca wickerhamii* were selected for testing. Strains were isolated from various sources, the majority coming from patients' fingernails, wastewater, and various culture collections. Cultures from collections were kindly provided by L. Ajello, Centers for Disease Control and Prevention, Atlanta, GA (2 strains); E. H. Ball, University of Glasgow, Scotland (2 strains); M. Feo, Instituto de Medicina Tropical, Universidad Central de Venezuela, Caracas, Venezuela (2 strains); P. Hocquet, Service des Maladies Parasitaires et Exotiques, Centre Hospitalier Regional, France (1 strain); H. Koeing, Institut de Parasitologie et Patologie Tropical, Université L. Pasteur, Strasbourg, France (1 strain); C. P. Kurtzman, Agricultural Research Service Culture Collection, Peoria, IL (1 strain); R. S. Pore, Department of Microbiology, West Virginia University, Morgantown, WV (1 strain); and our own laboratory (94 strains). Isolates were identified by standard methods (4, 12) and stored as suspensions in sterile distilled water at −70°C until use. Prior to testing, each isolate was subcultured at least twice on Sabouraud dextrose agar to ensure optimal growth.

For the NCCLS methods, 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 as recommended by NCCLS with RPMI 1640 medium (Sigma Chemical Co., St. Louis, Mo.) buffered to pH 7 with 0.165 M morpholinepropanesulfonic acid (MOPS; Sigma), with 2.000 g of glucose per liter of water, and an aliquot of 0.1 ml was dispensed into 96-well microdilution trays. Trays were sealed and stored frozen at −70°C until used in the study. Final concentrations ranged from 0.03 to 16 μg/ml (15).

The 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 (13). It is a standardized investigational method and correlates well with NCCLS method M27-A2 for a large number of experimental variables (13, 14). Disposable trays are precoated with six antifungal agents, yielding concentration across the range of clinical interest: 0.008 to 16 μg/ml for amphotericin B, 0.125 to 256 μg/ml for fluconazole, 0.008 to 16 μg/ml for itraconazole, 0.008 to 16 μg/ml for ketoconazole, 0.003 to 64 μg/ml for flucytosine and 0.003 to 16 μg/ml for voriconazole (13).

For the NCCLS method, the inoculum size was adjusted with a spectrophotometer to yield concentrations of 5×10^2 to 2.5×10^3 cells per ml by diluting in RPMI medium (15). An aliquot of 0.1 ml of each concentration was added to wells of the microdilution tray. In each case, inoculum size was confirmed by colony counts. MIC endpoints were determined vi-

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TABLE 1. In vitro activities of voriconazole against 104 *Prototheca wickerhamii* isolates as determined by the reference broth microdilution method[a] and Sensititre YeastOne system

Method	MIC (μg/ml)[b]		
	Range	50%	90%
NCCLS	≤0.008–0.5	0.12	0.5
Sensititre	≤0.03–0.5	0.12	0.5

[a] Performed according to NCCLS M27-A2 (15).
[b] MICs are 48-h MICs.

sually 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 (15).

For the YeastOne method, the inoculum conformed to the M27-A2 guidelines as noted above. Quality control was performed in accordance with NCCLS document M27-A2 using *Candida krusei* ATCC 6258 and *Candida parapsilosis* ATCC 22019 (15). MICs for quality control reference strains were within accepted limits for voriconazole (8). Results were read visually after 48 h of incubation 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, thus reducing the trailing effect characteristic of azole antifungal agents that hinders the interpretation of results when dilution techniques are used (7, 8, 13, 14).

Table 1 summarizes the in vitro susceptibilities of 104 *Prototheca wickerhamii* isolates to voriconazole as determined by the YeastOne and reference broth microdilution methods (15). Overall, voriconazole was highly active against all isolates, with an MIC at which 90% of isolates are inhibited (MIC_{90}) of ≤0.5 μg/ml. The activities of amphotericin B, fluconazole, itraconazole, ketoconazole and flucytosine only were assessed by the YeastOne system (Table 2). Voriconazole was more active than amphotericin B (MIC_{90} = 1 μg/ml) and itraconazole (MIC_{90} = 1 μg/ml) against all strains. All isolates exhibited very high MICs to both fluconazole (MIC_{90} = 256 μg/ml) and flucytosine (MIC_{90} = 64 μg/ml).

Comparison of MICs obtained using the YeastOne technique and the NCCLS method disclosed general agreement between the two methods for voriconazole (100% ± 2 $\log_2$ dilution steps). MICs within 2 dilutions of each other were considered to be in agreement (13, 14). The in vitro activities of voriconazole against a large number of yeasts and yeast-like species are well documented, with reported MICs of <2 μg/ml (8). A paper (11) dealt with the in vitro activities of five antifungal agents (amphotericin B, ketoconazole, flucytosine, itraconazole, and fluconazole) against *Prototheca* spp. using the Etest. *Prototheca* sp. strains presented resistance to the antifungal agents tested, with the exception of amphotericin B, to which all strains but *Prototheca stagnora* were susceptible.

TABLE 2. In vitro activities of antifungal agents against *Prototheca wickerhamii* (104 strains) using the Sensititre YeastOne system

Antifungal agent	MIC[a] (μg/ml)		
	Range	50%	90%
Amphotericin B	0.03–4	0.25	1
Fluconazole	64–256	64	256
Itraconazole	0.5–16	1	1
Ketoconazole	8–32	8	32
Flucytosine	32–64	32	64

[a] MICs are 48-h MICs.

Treatment of protothecal infections remains controversial. There is no standard treatment regimen (9, 10, 19). In immunocompromised patients, cutaneous protothecosis is particularly difficult to eradicate; lesions tend to recur and often spread (17). Clinical isolates are generally susceptible to amphotericin B (9) but resistant to flucytosine and fluconazole (10). The activities of ketoconazole and itraconazole against *Prototheca wickerhamii* are unclear; some authors report this species to be sensitive (9, 10), while others consider it resistant (17). Systemic itraconazole has been successfully used to treat cutaneous protothecosis in a number of patients (5, 10). Fluconazole has been used in cutaneous protothecosis (10) and also in a patient with systemic disease (19).

We found the MICs for voriconazole to be similar for all isolates tested. This is probably due not only to the extended potency of this new antifungal agent but also to its limited use outside clinical trials. However, although voriconazole remained highly effective against fluconazole-resistant strains of yeast (8, 13), there was a significant correlation between voriconazole and fluconazole MICs. Whether this drug will remain effective against fluconazole-resistant strains after widespread use remains to be determined. Its primary action is the inhibition of ergosterol biosynthesis by suppression of cytochrome P-450 activity, which is necessary for the demethylation of 14-α methylsterol to ergosterol. Ergosterol constitutes 4% of *Prototheca* cell membranes, and this probably relates to the drug's efficacy (1).

Voriconazole would seem at present to offer a new option for the treatment of *Prototheca* infections. It is probably the broadest-spectrum antifungal agent currently available; its spectrum is even broader than that of amphotericin B, adverse effects are fewer, and patient compliance higher; moreover, it is suitable for both oral and parenteral administration (16). The high rate of agreement found between the NCCLS reference method and YeastOne for in vitro determination of MICs echoes that reported for yeast-like (13) and filamentous (14) fungi. Given the results obtained here, and the excellent agreement with the NCCLS method, the YeastOne technique may be a reliable tool for the in vitro determination of voriconazole MICs and deserves further evaluation. In view of the potent in vitro activity demonstrated here against *Prototheca*, as well as promising early in vivo information, voriconazole also warrants further investigation for the treatment of protothecosis.

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