In Vitro Activity of Itraconazole against Fluconazole-Susceptible and -Resistant *Candida albicans* Isolates from Oral Cavities of Patients Infected with Human Immunodeficiency Virus

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A broth macrodilution technique, which was performed by following the recommendations provided by the National Committee for Clinical Laboratory Standards (document M27-P), was applied to study the in vitro activity of itraconazole against fluconazole-susceptible and -resistant *Candida albicans* isolates from the oral cavities of 100 patients infected with human immunodeficiency virus. The in vitro data demonstrated that itraconazole had good activity against the tested isolates; for 90% of all strains of *C. albicans*, MICs were 1 µg/ml, and only one isolate was highly resistant to this triazole (MIC, >16 µg/ml). However, the itraconazole MICs for the fluconazole-susceptible isolates were significantly lower than those for the fluconazole-resistant isolates; the MICs for 50 and 90% of the isolates tested were ≤0.03 and 0.25 µg/ml, respectively, for the fluconazole-susceptible isolates and 0.5 and 1 µg/ml, respectively, for the fluconazole-resistant isolates (P = 0.00001). Our findings could be of clinical relevance because human immunodeficiency virus-infected patients who fail fluconazole therapy for oral and/or esophageal candidiasis may require itraconazole at doses higher than those used in standard therapy.

Oropharyngeal candidiasis caused by *Candida albicans* is the most commonly diagnosed opportunistic infection in human immunodeficiency virus (HIV)-infected patients (17). Although this infection is not life-threatening, the sustained immunosuppression seen in these patients facilitates recurrences of infection (9). Consequently, several courses of acute- or long-term suppressive therapy are often required and may contribute to the development of resistant strains (3, 4, 21). Fluconazole and itraconazole are both used either in the treatment of acute oropharyngeal candidiasis or in the prophylaxis of oropharyngeal candidiasis in these patients (1). The lack of standardized methods for in vitro antifungal susceptibility testing presents difficulties in recognizing resistant strains. The Subcommittee on Antifungal Susceptibility Testing of the National Committee for Clinical Laboratory Standards (NCCLS) has recently proposed a broth macrodilution reference method for susceptibility testing of yeasts (10). In the study described here, we investigated the in vitro activity of itraconazole against fluconazole-susceptible and -resistant *C. albicans* isolates from the oral cavities of 100 HIV-infected patients by using the proposed NCCLS method.

MATERIALS AND METHODS

Isolates. A panel of 100 well-characterized clinical isolates of *C. albicans* submitted to the Fungus Testing Laboratory, Department of Pathology, University of Texas Health Science Center at San Antonio, was used in the study. All of the isolates were recovered from the oral cavities of HIV-infected

patients, and each strain represented a unique isolate from a patient. The isolates were chosen from among all *C. albicans* isolates sent to the laboratory for in vitro susceptibility testing with fluconazole by a previously published broth macrodilution method (15). In order to obtain isolates of *C. albicans* with different patterns of in vitro susceptibility to fluconazole, we included isolates for which fluconazole MICs ranged from ≤1.25 to >80 µg/ml. All of the isolates were retested for their susceptibilities to fluconazole and tested for their susceptibilities to itraconazole by a broth macrodilution method following the recommendations provided by the NCCLS subcommittee. In order to define isolates of *C. albicans* that were fluconazole susceptible and resistant by the NCCLS procedure, we arbitrarily chose a fluconazole breakpoint of 4 µg/ml, with isolates for which fluconazole MICs were ≤4 µg/ml considered susceptible (group A) and isolates for which fluconazole MICs were ≥8 µg/ml considered resistant (group B). Three reference strains, *C. albicans* ATCC 90028, *C. albicans* ATCC 90029, and *Torulopsis glabrata* ATCC 90030, were included in each run of the experiments.

Antifungal drugs. Both antifungal drugs were obtained from the manufacturers as standard powders. Fluconazole (Pfizer, Inc., New York, N.Y.) was dissolved in sterile distilled water to obtain a stock solution of 16,000 µg/ml. A stock solution of itraconazole (Janssen Pharmaceutica, Titusville, N.J.) of 5,000 µg/ml was prepared in polyethylene glycol 400 (PEG; Union Carbide, Danbury, Conn.) with the aid of heating at 75°C for 45 min.

Susceptibility testing procedure. In the present study, RPMI 1640 (American Biorganics, Inc., Niagara Falls, N.Y.) medium with L-glutamine without sodium bicarbonate and buffered at pH 7 with morpholinepropanesulfonic acid (0.165 M; 46.5 g/liter) was used. Drug dilutions were prepared at 10 times the strength of the final drug concentration (640 to 1.25 µg/ml for

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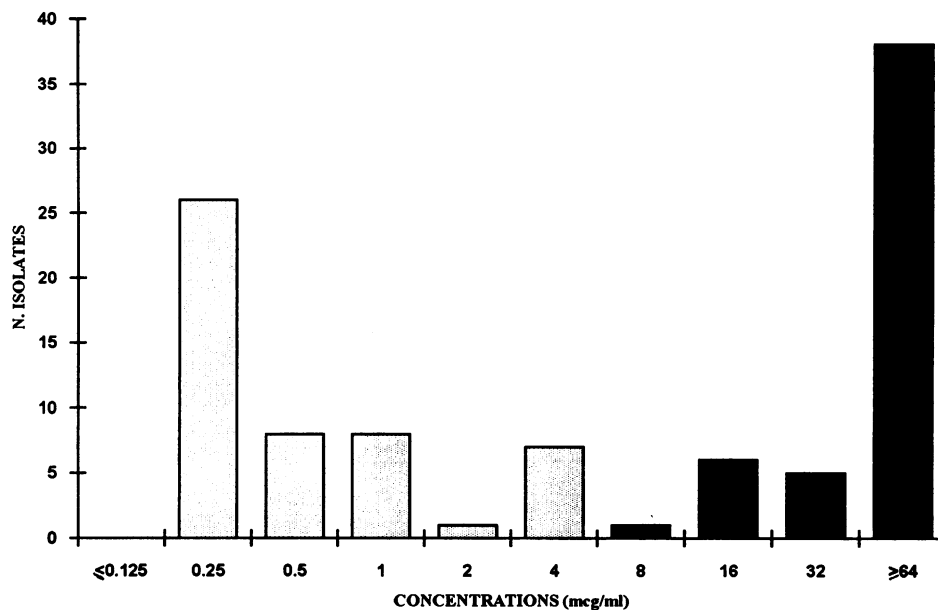
FIG. 1. Distribution of fluconazole MICs for 100 isolates of *C. albicans* tested by the NCCLS procedure (10). Gray bars indicate the isolates considered fluconazole susceptible (MICs, ≤4 μg/ml; group A); black bars indicate the isolates considered fluconazole resistant (MICs, ≥8 μg/ml; group B).

fluconazole and 160 to 0.3125 μg/ml for itraconazole) by an additive drug dilution schema for minimizing systematic pipetting errors. The drugs were stored at −70°C until they were used. Yeasts were grown on Sabouraud dextrose agar (BBL) for 24 h. The yeast inocula were prepared by a previously described method (11). Briefly, five colonies of at least 1 mm in diameter were suspended in 5 ml of sterile 0.85% saline. The turbidity of each suspension was measured at 530 nm and was adjusted to a final transmission of 85%. The working suspension was made by dilution to 1:100 in sterile distilled water; this was followed by dilution to 1:20 with the medium at a sufficient volume to directly inoculate each MIC tube with 0.9 ml. Confirmation of the inoculum sizes was determined with the final highest inoculum of all tested strains by the enumeration of the CFU per milliliter obtained by subcultures on Sabouraud dextrose agar. Yeast inocula (0.9 ml) were added to 10 times the strength of the final drug concentrations in polystyrene plastic tubes (12 by 75 mm; Falcon 2054; Becton Dickinson, Lincoln Park, N.J.), bringing the drug dilutions to the final test concentrations (0.125 to 64 μg/ml for fluconazole and 0.03 to 16 μg/ml for itraconazole). Drug-free and yeast-free control tubes were included for each isolate tested. All tubes were incubated without agitation at 35°C and were read at 48 h. Before reading, each tube was vortexed and its turbidity was compared with that of the growth control (drug-free) tube. For both azoles, MICs were defined as the lowest concentration which resulted in a visual turbidity of less than or equal to 80% inhibition when compared with that produced by the growth control (0.2 ml of growth control plus 0.8 ml of uninoculated RPMI 1640) (10).

RESULTS

All of the tested organisms produced detectable growth after 48 h of incubation. The inoculum sizes of the working suspensions ranged from 0.5×10^3 to 2.3×10^3 CFU/ml for all pathogenic yeasts and the two reference strains of *C. albicans*. The inoculum sizes of *T. glabrata* ATCC 90030 ranged from 2.7

$\times 10^3$ to 6.7×10^3 CFU/ml, which was outside the limits of the NCCLS documents (0.5×10^3 to 2.5×10^3 CFU/ml) (10). This is explained by the facts that the inoculum is spectrophotometrically standardized and *T. glabrata*, being smaller in size than *C. albicans*, produces a higher CFU per milliliter at the same percent transmittance. Despite these higher numbers of CFU per milliliter for *T. glabrata*, each of the three reference control cultures resulted in the expected range for fluconazole in each run of the experiments: 0.25 to 0.5 μg/ml for the two strains of *C. albicans* and 8 to 16 μg/ml for *T. glabrata* ATCC 90030. Itraconazole MICs were ≤0.03 μg/ml for the two strains of *C. albicans*, and the itraconazole MIC was 0.5 to 1 μg/ml for *T. glabrata* ATCC 90030.

Fluconazole MICs. Figure 1 shows the distribution of fluconazole MICs for the 100 isolates of *C. albicans* tested by the NCCLS method. Fluconazole MICs ranged from 0.25 to >64 μg/ml. According to our definition, 50 isolates were fluconazole susceptible (group A) and 50 isolates were fluconazole resistant (group B). Fluconazole MICs for 50% (MIC$_{50}$s) and 90% (MIC$_{90}$s) of the group A isolates tested were 0.25 and 4 μg/ml, respectively. Fluconazole MIC$_{50}$s and MIC$_{90}$s for the group B isolates were 64 and >64 μg/ml, respectively.

Itraconazole MICs. Figure 2 shows the distribution of itraconazole MICs for the 100 isolates of *C. albicans* tested. Itraconazole MICs ranged from ≤0.03 to >16 μg/ml. There was a substantially different distribution of itraconazole MICs between the A and the B groups of *C. albicans* isolates. Itraconazole MIC$_{50}$s and MIC$_{90}$s were ≤0.03 and 0.25 μg/ml, respectively, for group A isolates and 0.5 and 1 μg/ml, respectively, for group B isolates. One strain in the latter group was highly resistant to itraconazole (MIC, >16 μg/ml). The same isolate, when tested for its susceptibility to ketoconazole by the NCCLS procedure, was resistant to this azole (MIC, 4 μg/ml) as well. When the Mann-Whitney U test was applied to determine the distribution of itraconazole MICs for the two groups of isolates, a statistically significance difference was found ($P = 0.00001$).
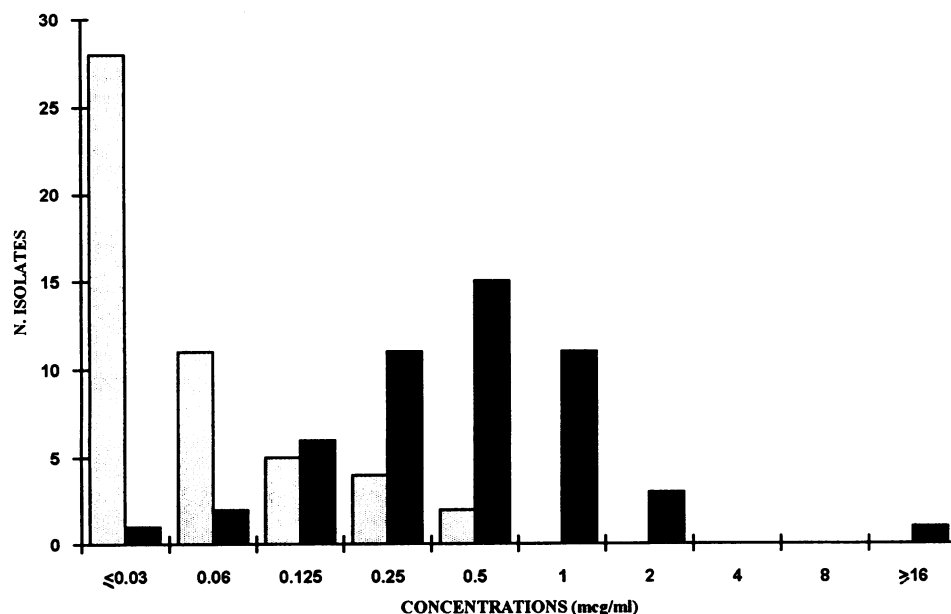
FIG. 2. Distribution of itraconazole MICs for 100 isolates of *C. albicans* tested by the NCCLS procedure. Gray and black bars indicate the distributions of the itraconazole MICs for group A and B isolates, respectively.

DISCUSSION

The development of methods for routine susceptibility testing of yeasts is important to facilitate the selection of appropriate current and proposed antifungal agents for testing. However, susceptibility testing with these drugs, and especially with the azoles, is highly influenced by medium composition, length of incubation, temperature, and inoculum size (13, 14). Although the in vitro method for susceptibility testing of yeasts proposed by NCCLS was developed only for flucytosine, amphotericin B, ketoconazole, and fluconazole, we applied the same procedure for testing the susceptibilities of 100 strains of *C. albicans* isolated from the oral cavities of HIV-infected patients to itraconazole. Our in vitro data demonstrated that itraconazole has good activity against the tested isolates; for all 100 strains of *C. albicans*, MIC_{90}s were of 1 μg/ml, and only one isolate (1%) exhibited resistance to this triazole (MIC, >16 μg/ml). Resistance to the azoles was first reported by Holt and Azmi (5) in a strain of *C. albicans* from a patient with urinary candidiasis who had been undergoing prolonged oral treatment with miconazole. The isolate was resistant not only to miconazole but also to econazole and clotrimazole. Such ketoconazole-resistant strains of *C. albicans* were isolated from patients with chronic mucocutaneous candidiasis (6, 16, 20). More recently, ketoconazole- and fluconazole-resistant strains of *C. albicans* have been isolated from AIDS patients with oral and esophageal candidiases (2, 4, 12, 18, 19, 21). In these patients, it is often difficult to establish whether the failure of antifungal therapy is due to host factors associated with the immunodeficiency state or to true fungal resistance to the drugs. Interestingly, in our study, we found that for isolates for which fluconazole MICs were high, itraconazole MICs were proportionally higher, which may represent cross-resistance. This phenomena was also observed with ketoconazole-resistant strains of *C. albicans* to ticonazole and miconazole (8). Hughes et al. (7) showed that fluconazole was fungistatic in vivo against ketoconazole-resistant strains of *C. albicans*, but only when high doses were administered. Our findings could be of clinical relevance since the HIV-infected patients who failed

fluconazole therapy for oral and/or esophageal candidiasis may require itraconazole at doses higher than those used in standard therapy. It must be highlighted, however, that the clinical role of these in vitro tests is not well documented, and more studies are needed to evaluate the in vitro and in vivo correlations of drug efficacy. We arbitrarily chose a fluconazole breakpoint MIC of 4 μg/ml to define isolates that were fluconazole susceptible and resistant and to compare itraconazole MICs. This does not mean that patients infected with isolates for which fluconazole MICs were higher necessarily fail fluconazole therapy, or vice versa. In conclusion, the results of the present in vitro study suggest that itraconazole has good activity against *C. albicans* by using the procedures recommended by NCCLS (10). However, further assessment of the correlation of these MIC endpoints and the efficacy of this compound in vivo should be accomplished, especially with fluconazole-resistant *C. albicans* isolates from HIV-infected patients.

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