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Correlation of In Vitro Fluconazole Resistance of *Candida* Isolates in Relation to Therapy and Symptoms of Individuals Seropositive for Human Immunodeficiency Virus Type 1

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Yeast strains isolated from the oropharynx of 87 consecutive patients infected with human immunodeficiency virus type 1 were examined for in vitro susceptibility to fluconazole. *Candida albicans* was isolated from 73 patients. Fifty-one patients had received antifungal therapy in the month preceding the yeast infection. Thirty-two patients had symptomatic oropharyngeal candidiasis. The MICs were correlated with azole use and with clinical symptoms and signs. Although there is overlap between groups, in vitro testing identified a large group of patients for whose yeast isolates the fluconazole MICs were high and who remained symptomatic while receiving azole therapy. This study supports the ability of in vitro testing to predict the clinical outcome of mucosal fungal infections. The study also demonstrates that azole resistance of oropharyngeal yeasts is a common problem in patients infected with human immunodeficiency virus type 1 and that this azole resistance has clinical relevance.

Candida species, particularly *Candida albicans*, are important opportunistic pathogens in patients infected with human immunodeficiency virus type 1 (HIV-1). Infections can range from relatively mild oropharyngeal or vulvovaginal candidiasis to severe debilitating mucocutaneous candidiasis including *Candida* esophagitis. In our institution, during the past 2 years *Candida* esophagitis has been the AIDS-defining illness in 7% of patients with HIV-1 infection. Fluconazole is used frequently for treatment and prophylaxis of oropharyngeal and esophageal candidiasis (13). Recently we noted several patients who developed severe oropharyngeal or esophageal candidiasis while receiving azole therapy. This finding prompted us to look for in vitro evidence of azole, specifically fluconazole, resistance. Our results show that clinically resistant candidiasis may be correlated with the detection of in vitro fluconazole resistance.

MATERIALS AND METHODS

Patient population. Initially, eight HIV-1-seropositive individuals with clinically resistant oropharyngeal or esophageal candidiasis were selected. Subsequently, a prospective study was initiated. HIV-1-seropositive patients seen in the adult HIV/Infectious Diseases Clinic, Duke University Medical Center, were randomly selected by three clinical physicians (M.L.C., J.A.B., and H.A.W.). In the prospective portion of the study, 79 patients were examined. A total of 87 HIV-1-positive individuals made up the study population. Patients were classified as asymptomatic or symptomatic. Patients with no symptoms or signs of oropharyngeal or esophageal candidiasis were classified as asymptomatic. Patients with oropharyngeal or esophageal candidiasis by inspection or those with symptoms (sore mouth or tongue, odynophagia, dysphagia) were classified as symptomatic. Antifungal therapy with azoles was defined as treatment, either prophylactic or active with clotrimazole troches, ketoconazole, or fluconazole, within the month preceding *Candida* isolation.

Fungi. Patient specimens consisted of oral rinses (15 to 30 ml of sterile normal saline); one specimen was an esophageal brushing. Samples (0.1 ml) were inoculated onto a Sabouraud agar plate containing chloramphenicol and incubated at 30°C. A single colony was chosen for subculture, susceptibility testing, and storage. Two strains of *C. albicans* served as control isolates; one (MCV 7.220) was resistant in vivo to fluconazole, and one (A39) was susceptible in vivo (19). Identification of isolates was based on germ tube production, carbohydrate fermentation, carbohydrate assimilation, and results of Dalmau morphologic studies as needed. Isolates were stored at room temperature in sterile deionized water.

Susceptibility testing. Fluconazole was obtained from Roerig-Pfizer, New York, N.Y., as a single lot of powder, 99.4% pure. A 1,250-μg/ml stock solution was prepared in distilled water, membrane filter sterilized, and stored in 1-ml aliquots at −20°C. MICs were determined by using filter-sterilized RPMI 1640 medium buffered to pH 7.0 with 0.165 M 3-*N*-morpholinopropanesulfonic acid (Sigma Chemical Co., St. Louis, Mo.).

A broth macrodilution technique with an end point of MIC 80% was used. MIC 80% is defined as the lowest drug concentration which showed a turbidity less than the turbidity of a 1:5 dilution of the growth control (6). Procedures for preparation of inoculum and drug dilutions have been described previously (6). Isolates were grown on Sabouraud agar plates at 30°C for 48 h. At least five colonies for each strain were vortexed in 5 ml of 0.85% sterile saline. By using a spectrophotometer set to 530 nm, turbidity was adjusted to match that of a McFarland 0.5 standard. Inocula were then diluted 1:100 in RPMI 1640 medium. A twofold dilution series of fluconazole was prepared at 10× strength by the method of NCCLS standard M7-A2 (17). A 100-μl portion of each drug dilution was dispensed to the corresponding tube

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TABLE 1. Number of patients receiving concurrent antifungal therapy

Primary drug	No. of patients receiving the secondary drug:			
	Nystatin[a]	Clotrimazole[a]	Ketoconazole	5-Flucytosine
Fluconazole	1	3	1	1
Ketoconazole	1			
Amphotericin B	1			

[a] One patient, shown in the clotrimazole column, was receiving triple antifungal therapy with nystatin, clotrimazole, and fluconazole.

(12 by 75 mm, polystyrene), and 0.9 ml of inoculum was then added to each tube. A drug-free control for each organism consisted of 0.9 ml of inoculum added to 0.1 ml RPMI 1640. Tests were incubated at 35°C for 48 h. A single fungal isolate would grow only at 30°C; therefore, for this isolate, drug susceptibility testing was carried out at 30°C. A sterility control consisted of 1 ml of RPMI 1640 medium incubated concurrently. The resistant and susceptible control strains with known MIC 80% of 31.2 and <0.5 μg/ml, respectively, were included in each trial.

For each isolate, the MIC 80% end point standard was prepared by thoroughly vortexing the drug-free control tube and diluting 0.2 ml of the control in 0.8 ml of RPMI 1640 medium. This dilution provides a standard that is equivalent to 80% inhibition of growth. The remaining tubes in the series were vortexed and visually compared with the MIC 80% standard. As a final control, a terminal subculture of the MIC 80% tube in each series was performed to assess purity of growth.

Statistical methods. The results for the different patient groups (with or without symptoms and signs, receiving or not receiving azoles) were compared by using a rank sum test (Mann-Whitney).

RESULTS

The patient profile for the individuals in the study showed that 21 of 87 patients had asymptomatic HIV-1 infection, 16 had AIDS-related complex (ARC), and 50 had AIDS. The mean age was 36.5 years. Twenty-two patients (25%) were female. Thirty-six patients (41%) were African American, two patients (2%) were Native American, and the rest were non-Hispanic Caucasian. These numbers approximate those in our HIV Clinic, except that a higher portion of people with AIDS were in the study than are seen in the clinic.

Yeast isolates consisted of *C. albicans* from 73 patients (84%), *Torulopsis glabrata* from 4 patients (5%), *C. krusei* from 4 patients (5%), *C. tropicalis* from 2 patients (3%), and *C. parapsilosis*, *Candida* species, *Hansenula anomala*, and *Saccharomyces cerevisiae* from 1 patient each. Serial isolates from nine patients were studied (two isolates each from eight patients and four isolates from one patient).

Antifungal therapy had been used in 51 patients (59%) in the month preceding collection of the fungal isolates. Eight patients were receiving multiple antifungal agents at the same time (Table 1). Of the patients receiving a single antifungal agent, 1 was treated with nystatin, 12 were treated with clotrimazole, 12 were treated with ketoconazole, and 18 were treated with fluconazole. Thirty-six patients were on no antifungal therapy during the month preceding collection of the fungal isolates. Of the total 87 patients, 31 had symptomatic oropharyngeal or esophageal candidiasis as defined above. One male patient had symptomatic genital candidal infection but was asymptomatic for oropharyngeal disease.

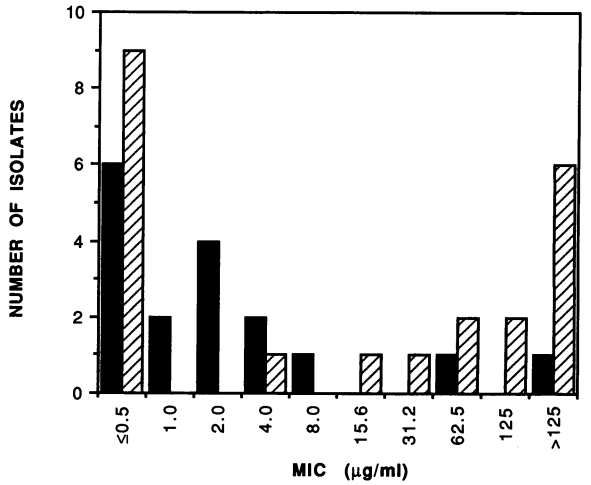

FIG. 1. MICs of fluconazole for *C. albicans* isolates from patients receiving azole therapy. Results from 17 asymptomatic (black bars) and 22 symptomatic (cross-hatched bars) patients are shown.

Figure 1 shows the distribution of fluconazole MICs among *C. albicans* isolates from patients receiving azoles. Results for both asymptomatic (n = 17) and symptomatic (n = 22) patients are shown. The MICs for patients with symptoms while receiving azoles were significantly different from those for patients without symptoms while receiving azoles (Mann-Whitney rank sum test $P < 0.0001$). Figure 2 shows the distribution of fluconazole MICs among *C. albicans* isolates from patients not receiving antifungal therapy. Results for both asymptomatic (n = 30) and symptomatic (n = 4) patients are shown. For *C. albicans* isolates, azole therapy and symptomatic candidiasis were significantly correlated with a distribution of higher MICs when compared with isolates from asymptomatic patients or symptomatic patients not taking therapy ($P < 0.0001$). If all four groups are compared, there is a significant difference in MIC distri-

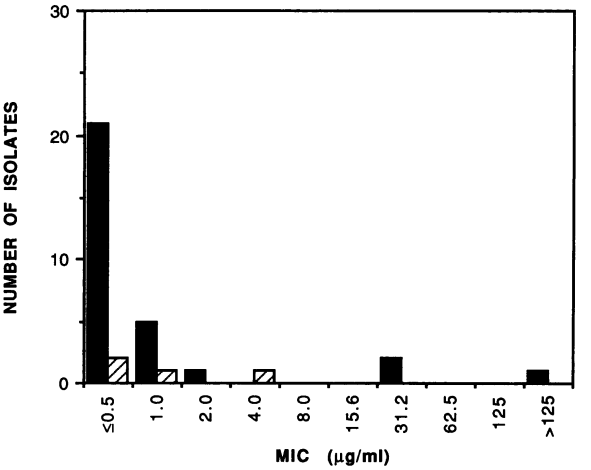

FIG. 2. MICs of fluconazole for *C. albicans* isolates from patients not receiving azole therapy. Results from 30 asymptomatic (black bars) and 4 symptomatic (cross-hatched bars) patients are shown.

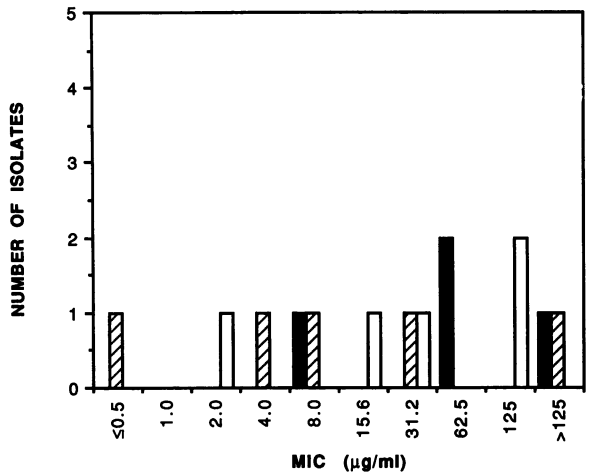

FIG. 3. MICs of fluconazole for non-*C. albicans* isolates. Results from five asymptomatic patients receiving azole therapy (cross-hatched bars), five symptomatic patients receiving azole therapy (white bars), and four asymptomatic patients not receiving azole therapy (black bars) are shown.

bution if ranked according to use or nonuse of azole therapy. Also, there is a significant difference in MIC distribution based on the presence or absence of symptoms ($P < 0.0001$).

Figure 3 demonstrates the distribution of fluconazole MICs for non-*C. albicans* isolates collected from asymptomatic patients receiving azole therapy ($n = 5$), symptomatic patients receiving azole therapy ($n = 5$), and four asymptomatic patients not on therapy. There were no symptomatic patients, not on therapy, who had non-*C. albicans* isolates. For non-*C. albicans* strains there was no significant correlation of symptoms and azole therapy with fluconazole MIC. However, for all isolates together (*C. albicans* and non-*C. albicans*), there was a significant difference between distribution of MICs on the basis of use or nonuse of azole therapy ($P < 0.0001$). In addition, there was a significant difference in distribution of MICs on the basis of the presence or absence of symptoms ($P < 0.0001$).

Table 2 shows the fluconazole MICs for serial *C. albicans* isolates from nine patients. For most isolates the MIC did not change from low to high or vice versa. The patient from whom fungi were isolated four different times over 8 months developed concurrent *C. albicans* and *T. glabrata* infection after 8 months of fluconazole therapy.

TABLE 2. Fluconazole MICs for serial *C. albicans* isolates

Patient	Symptoms[a]	Isolate	Azole[b]	MIC (μg/ml)
1	+	1	–[b]	4.0
	+	2	–[b]	4.0
2	+	1	C	62.5
	+	2	K	62.5
3	+	1	C	62.5
	+	2	C+F[b]	62.5
4	–	1	K	8.0
	–	2	–	ND[c]
5	–	1	K	8.0
	+	2	K	8.0
6	+	1	F[b]	125
	+	2	F	>125
7	–	1	–	≤0.5
	–	2	–	≤0.5
8	+	1	C	≤0.5
	–	2	–	≤0.5
9	+	1	F	4.0
	+	2	F	4.0
	+	3	F	8.0
	+	4	F	ND

[a] The symbol + means that the symptom(s) or drug was present; the symbol – means that it was absent.

[b] Patient 1 was using nystatin along with intravenous and oral amphotericin B after failing to respond to fluconazole (F) and ketoconazole (K). Patient 3 received clotrimazole and fluconazole (C+F) at the same time. Patient 6 was receiving fluconazole and 5-flucytosine at the time of collection of the first isolate. Patient 9 developed concurrent *C. albicans* and *T. glabrata* (isolate 4) oral candidiasis after 8 months of fluconazole therapy.

[c] ND, not done.

DISCUSSION

This study was undertaken to examine the predictive value of antifungal susceptibility testing in relation to clinical symptoms and use of antifungal drugs. Several factors made this study necessary. First, in vitro-in vivo correlation must be approached on a clinical basis to justify in vitro testing. Although animal models have shown for both flucytosine (28) and azole compounds (19, 25) that in vitro testing can correlate with outcome of infection (12), relatively few reports have shown clinical relapse and development of in vitro resistance of yeasts to polyenes and azoles (5, 8, 10, 15, 27). Second, it has become clear that during this second decade of the AIDS epidemic, oral and gastrointestinal (luminal) infections with *Candida* species are becoming increasingly refractory to therapy. Third, although these mucosal infections do not generally progress to disseminated candidiasis in AIDS patients, they occur in a large number of patients in whom it may be easier to judge specific outcomes than for disseminated candidiasis, in which other factors from the underlying disease may cause confusion in determining efficacy. Fourth, these infections are troublesome clinically, and in vitro testing results potentially could help in clinical management and, if validated with outcome, could also be helpful in understanding the effect of antifungal prophylactic regimens on colonizing flora. It is interesting that we did not find significantly more non-*C. albicans* yeasts in patients receiving azoles, as have been found in other immunocompromised patients receiving fluconazole therapy (1, 7, 11, 20, 31). This finding may be explained by the small numbers and/or the inability to completely determine azole use in the months preceding the last month. Although the classifications of our patients into these specific groups may be less precise than for completely controlled groups, they do reflect common clinical practice, and the trends seen within these groups are likely to be reliable.

In this study, we have shown two important trends which were identified by in vitro testing. First, there was a significant upward distribution of higher MICs in patients who were receiving azole therapy. These results suggest that exposure to azole compound will probably produce resistant yeasts in these patients. The magnitude, mechanisms, and prevention of this resistance await further studies. The second trend is that in vitro susceptibility testing was significantly correlated with signs and symptoms of infection. Patients with persistent symptoms and signs who were receiving azole therapy were more likely to have higher azole MICs for their yeasts. This finding strengthens the clinical utility of in vitro testing to identify a reason for treatment failure and shows that these azole-resistant organisms remain pathogenic in this immunosuppressed population.

The design of this study incorporated several features that may be useful for confirmation studies. First, the method used was that of a previously published protocol for testing of yeasts against amphotericin B, flucytosine, and azoles (21), which has now been modified by the National Committee on Clinical Laboratory Standards (18). This work has helped validate the reproducibility of results within and between laboratories and has a simplicity which makes it feasible for general laboratory use. Second, in all assays, simultaneous internal quality control strains were used, azole-resistant *C. albicans* MCV 7.220 and azole-sensitive *C. albicans* A39. MICs for these strains were not significantly different in each assay (range, 31.2 to 62.5 μg/ml versus ≤0.5 μg/ml, respectively). Third, one azole, fluconazole, was examined, since its use in AIDS patients has significantly increased. Further studies are needed to determine whether cross-resistance with other azole compounds is noted with these strains, as has been found in animal models (19). Alternatively, there could be differences in the mechanisms of drug resistance of these yeasts, allowing selective azole resistance, which may make in vitro testing even more important. Fourth, the study attempted to include consecutive patients within a clinic population with no attempt to bias selection but only to place them in four groups in relationship to symptoms and prior azole use. Although we attempted not to bias the selection of patients, it is possible that there was an emphasis on choosing those who were symptomatic. With the use of this strategy, we tried to understand the magnitude of clinically significant resistance within the clinical context of uncontrolled azole use and to determine whether in vitro testing could predict the outcome when antifungal drug use was not controlled. Indeed, a variety of antifungal agent combinations were used in this study.

In summary, in vitro susceptibility to fluconazole appears to correlate with clinical resistance. Although there is overlap between the groups in the study, the trend is apparent, and it is likely that in vitro testing will become a clinical tool in the management of these yeast infections. Further studies, with more patients from other institutions, larger studies of serial isolates from individual patients with clinical correlation, and studies of isolates from other sites such as the vulvovaginal area, are needed to confirm or reject our findings. There are related questions to be answered. First, does azole and/or polyene therapy select for changes in the mucosal mycoflora (1, 9, 16, 23, 26), such as for the inherently resistant organisms *C. krusei* and *T. glabrata* (1, 3, 8, 11, 20, 22, 29–31)? Second, does azole therapy induce the development of azole and/or polyene resistance in the mycoflora present during its use (2, 4, 10, 14, 23, 24, 27), or is azole therapy even necessary for resistant *C. albicans* to be present (5, 10)? Finally, what is the effect of drug dosage and length of exposure to azoles on the development of clinical resistance? It is essential to answer these questions correctly to establish clinically useful in vitro susceptibility testing for fungi and to help design appropriate strategies for antifungal therapy and prophylaxis.

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