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# Antifungal Susceptibility Testing of Isolates from a Randomized, Multicenter Trial of Fluconazole versus Amphotericin B as Treatment of Nonneutropenic Patients with Candidemia

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**The antifungal susceptibilities of 232 pathogenic bloodstream *Candida* isolates collected during a recently completed trial comparing fluconazole (400 mg/day) with amphotericin B (0.5 mg/kg of body weight per day) as treatment for candidemia in the nonneutropenic patient were determined both by the National Committee for Clinical Laboratory Standards M27-P macrobroth methodology and by a less cumbersome broth microdilution methodology. For amphotericin B, M27-P yielded a very narrow range of MICs (0.125 to 1 μg/ml) and there were no susceptibility differences among species. For fluconazole, a broad range of MICs were seen (0.125 to >64 μg/ml), with characteristic MICs seen for each species in the rank order *Candida albicans* < *C. parapsilosis* ≅ *C. lusitaniae* < *C. glabrata* ≅ *C. krusei* ≅ *C. lipolytica*. The MIC distribution for *C. tropicalis* was bimodal and could not be ranked. Broth microdilution MICs were within one tube dilution of the M27-P MIC for ≥90% of isolates with amphotericin B and for ≥77% of isolates with fluconazole. For both methods, elevated MICs did not predict treatment failure. In the case of amphotericin B, the MIC range was too narrow to permit identification of resistant isolates. In the case of fluconazole, MICs for isolates associated with failure to clear the bloodstream consistently were equivalent to the median MIC for the given species. Successful courses of therapy were seen with four isolates from four patients despite MICs of ≥32 μg/ml. As MICs obtained by M27-P and similar methods correlate with responsiveness to fluconazole therapy in animal models and in AIDS patients with oropharyngeal candidiasis, the lack of correlation in this setting suggests that the MICs for these isolates are at or below the relevant fluconazole breakpoint for this dose of fluconazole and patient setting and that host factors such as failure to exchange intravenous catheters were more important than MIC in predicting outcome.**

Antifungal susceptibility testing remains in evolution. Recent work by the National Committee for Clinical Laboratory Standards has focused on the development of standard, reproducible methods for testing of yeast. The proposed method M27-P is a broth macrodilution method that has good inter- and intralaboratory reproducibility (5, 10). As MICs obtained by methods similar to M27-P have generally correlated well with outcome in various animal models of infection, it is anticipated that M27-P (or a method utilizing the principles of M27-P) will prove useful in prediction of the likelihood of response to a given antifungal agent (16). However, no breakpoints have yet been established for M27-P. During our recently completed trial of fluconazole versus amphotericin B for therapy of candidemia in nonneutropenic patients, we collected *Candida* bloodstream isolates from our patients (15). We now report the distribution of M27-P MICs seen in this collection of pathogenic *Candida* isolates, the correlation of these MICs with an easily performed broth microdilution variation of M27-P, and the correlation of all of these MICs with outcome.

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## MATERIALS AND METHODS

**Isolates.** *Candida* bloodstream isolates from patients enrolled in a trial comparing fluconazole with amphotericin B as treatment for candidemia in the nonneutropenic patient were collected during execution of the trial (15). In brief, patients in this trial were required to have a positive blood culture for *Candida* sp. and to be febrile or hypotensive or have a localized *Candida* infection. After enrollment, patients were randomized to receive either amphotericin B (0.5 mg/kg of body weight per day) or fluconazole (400 mg/day) for 14 days after the last positive blood culture. Fully evaluable patients (patients meeting all entry criteria—these patients are referred to as primary analysis patients in reference 15) were judged as having a successful outcome if blood cultures became negative and signs and symptoms of the bloodstream infection resolved. Outcomes were failures when patients had persistence of candidemia or developed unacceptable drug toxicity. Outcomes were relapses when patients during a 3-month follow-up period (i) developed recurrent fungemia, (ii) presented with a visceral *Candida* infection, or (iii) were given more than 4 days of antifungal therapy for an asymptomatic *Candida* urinary tract infection or any amount of antifungal therapy for any other cause.

While 224 candidemic patients were enrolled in the clinical trial, only 232 isolates from 146 patients were available for study. Of these, 119 isolates were from 73 aphotericin B-treated patients and 113 isolates were from 73 fluconazole-treated patients. Analyses of MIC distribution and comparison of MIC methodologies were performed using all 232 isolates. Correlation of MIC with outcome was, however, determined with only those isolates obtained from fully evaluable study patients and is thus limited to 112 isolates from 66 amphotericin B-treated patients and 104 isolates from 68 fluconazole-treated patients.

Isolates were stored at −70°C until testing and were passaged at least twice on Sabouraud dextrose agar at 35°C prior to testing. Isolates were identified to the species level by using the API 20C system (Analytab Products, Plainview, N.Y.).

**Susceptibility testing.** Broth macrodilution MICs were determined by the National Committee for Clinical Laboratory Standards M27-P methodology. In short, yeasts at a final concentration of $0.5 \times 10^3$ to $2.5 \times 10^3$ cells per ml were incubated in air at 35°C for 48 h with twofold dilutions of amphotericin B (0.0313 to 16 μg/ml) or fluconazole (0.125 to 64 μg/ml). The MIC was that concentration

TABLE 1. Amphotericin B MICs for *Candida* bloodstream isolates

| Species (no. of isolates tested) | Result by: | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | M27-P | | Microdilution method at: | | | | | |
| | | | 24 h | | | 48 h | | |
| | $MIC_{50}$ (μg/ml) | $MIC_{90}$ (μg/ml) | $MIC_{50}$ (μg/ml) | $MIC_{90}$ (μg/ml) | % Agreement[a] | $MIC_{50}$ (μg/ml) | $MIC_{90}$ (μg/ml) | % Agreement |
| *C. albicans* (129) | 0.5 | 1 | 0.5 | 0.5 | 93 | 0.5 | 1 | 96 |
| *C. glabrata* (31) | 1 | 1 | 0.5 | 1 | 90 | 1 | 1 | 90 |
| *C. tropicalis* (40) | 0.5 | 1 | 0.5 | 0.5 | 98 | 1 | 1 | 95 |
| *C. parapsilosis* (23) | 0.5 | 1 | 0.25 | 0.25 | 65 | 1 | 1 | 87 |
| *C. krusei* (5) | 1 | 1 | 1 | 4 | 80 | 2 | 4 | 60 |
| *C. lusitaniae* (3) | 0.5 | 1 | 0.25 | 0.25 | 66 | 0.5 | 0.5 | 100 |
| *C. lipolytica* (1) | 1 | 1 | 1 | 1 | 100 | 2 | 2 | 100 |

[a] Percentage of MICs that were equivalent to the M27-P MIC ± one tube dilution. Overall agreement was 90 and 94% at 24 and 48 h, respectively.

of drug that completely inhibited growth (amphotericin B) or produced an 80% reduction of turbidity by comparison to the drug-free control (fluconazole) (10).

Following the principles of the National Committee for Clinical Laboratory Standards M27-P methodology (10), broth microdilution testing was performed as described previously (11, 12) using spectrophotometric inoculum preparation, an inoculum of $0.5 \times 10^3$ to $2.5 \times 10^3$ cells per ml, and RPMI 1640 buffered to pH 7.0 with 0.165 M morpholinepropansulfonic acid (MOPS; American Biorganics, Inc., North Tonawanda, N.Y.). Microdilution trays containing serial twofold dilutions of the antifungal agents were prepared by Alamar Biosciences, Inc. (Sacramento, Calif.). The microdilution trays were dried, sealed in individual packages, and stored at ambient temperature. Microdilution wells were reconstituted by addition of the inoculum suspension to a final volume of 200 μl. Final concentrations of the antifungal agents were 0.0313 to 16 μg/ml for amphotericin B and 0.125 to 256 μg/ml for fluconazole. The trays were incubated in air in a moist chamber at 35°C. Broth microdilution endpoints were determined after 24 and 48 h of incubation with the aid of a reading mirror. The MIC of amphotericin B was the concentration of drug that completely inhibited growth (referred to as MIC-0 in references 11 and 12). The MIC of fluconazole was the lowest concentration at which comparison with the growth control (drug-free) well revealed prominent reduction in turbidity (referred to as MIC-2 in references 11 and 12).

**Statistical analysis.** The relationship between outcome and log(MIC) was tested with logistic regression: log(MIC) was entered into the equation, and the significance of its correlation was determined.

## RESULTS

**Comparison of broth macrodilution (M27-P) and microdilution MICs.** The MICs at which 50% of the strains tested were inhibited ($MIC_{50}$s) and $MIC_{90}$s according to species and method are shown in Tables 1 and 2, along with the percentage of the broth microdilution MICs that were within one tube dilution of the M27-P MIC. Overall agreement was 85% at both 24 and 48 h. For amphotericin B, the agreement was ≥90% at both 24 and 48 h for all species except *Candida parapsilosis*. The microdilution MIC for this species tended to be one tube dilution lower than the M27-P MIC. The agreement between methods for fluconazole was not as close, with an overall agreement of 80% obtained with 24-h broth microdilution readings. In general, the discrepancies for fluconazole were due to small variations in endpoint determination rather than to gross MIC discrepancies: the percentage of microbroth MICs that were within two tube dilutions of the M27-P MIC was 89% at both 24 and 48 h.

**Correlation of MIC with outcome.** The correlation of M27-P MICs with outcome for each drug is shown in Fig. 1 and 2 for *Candida albicans*, *C. glabrata*, *C. tropicalis*, and *C. parapsilosis*. Failures are broken down into those associated with failure to clear the bloodstream as opposed to all other types of failure and relapse. Failures due to toxicity are problematic in this analysis, as they do not reflect drug efficacy. There were, however, only two cases of failure due to toxicity. The first involved a fluconazole-treated patient whose blood contained both *C. tropicalis* (fluconazole MIC, 0.5 μg/ml) and *C. parapsilosis* (fluconazole MIC, 1 μg/ml). The second involved an amphotericin B-treated patient whose blood contained *C. albicans* (amphotericin B MIC of 0.5 μg/ml). Reclassification of these patients as unevaluable does not alter the results. A small number of *Candida krusei*, *C. lusitaniae*, and *C. lipolytica* isolates were also available for testing (Tables 1 and 2). The five *C. krusei* isolates were from three patients and required amphotericin B MICs of 0.5 μg/ml (one isolate) and 1 μg/ml (four isolates); all required a fluconazole MIC of 32 μg/ml. Two of these patients were treated with amphotericin B and did well. The third patient was treated with fluconazole, cleared his bloodstream, and did well during primary therapy. However, *C. krusei* funguria was noted several weeks after the end of therapy, and although the patient was afebrile and did not have a leukocytosis, systemic

TABLE 2. Fluconazole MICs for *Candida* bloodstream isolates

| Species (no. of isolates tested) | Result by: | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | M27-P | | Microdilution method at: | | | | | |
| | | | 24 h | | | 48 h | | |
| | $MIC_{50}$ (μg/ml) | $MIC_{90}$ (μg/ml) | $MIC_{50}$ (μg/ml) | $MIC_{90}$ (μg/ml) | % Agreement[a] | $MIC_{50}$ (μg/ml) | $MIC_{90}$ (μg/ml) | % Agreement |
| *C. albicans* (129) | 0.25 | 1 | 0.25 | 1 | 86 | 0.5 | 1 | 72 |
| *C. glabrata* (31) | 16 | 32 | 8 | 16 | 90 | 16 | 32 | 94 |
| *C. tropicalis* (40) | 1 | >64 | 0.25 | 1 | 54 | 1 | >64 | 65 |
| *C. parapsilosis* (23) | 1 | 4 | 0.5 | 2 | 80 | 1 | 8 | 91 |
| *C. krusei* (5) | 32 | 32 | 32 | 32 | 100 | 64 | 64 | 100 |
| *C. lusitaniae* (3) | 2 | 4 | 2 | 2 | 100 | 2 | 2 | 100 |
| *C. lipolytica* (1) | 64 | 64 | 32 | 32 | 100 | 64 | 64 | 100 |

[a] Percentage of MICs that were equivalent to the M27-P MIC ± one tube dilution. Overall agreement was 80 and 77% at 24 and 48 h, respectively.

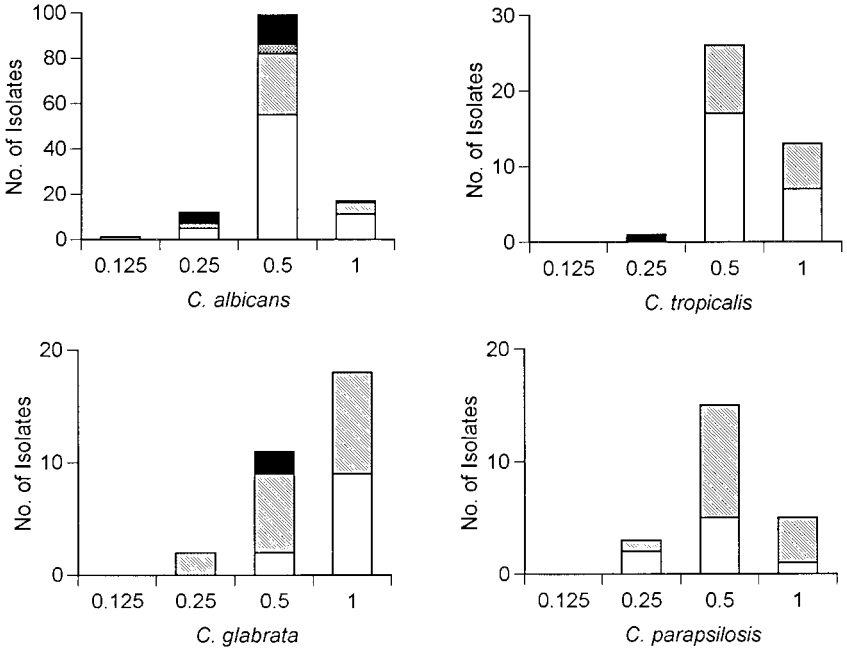


FIG. 1. Correlation of M27-P amphotericin B MICs with outcome. Outcomes are shown for isolates from the evaluable patients treated with the drug (■, failure to clear bloodstream; ▨, other failure; ▧, success). In addition, the MICs for isolates from the unevaluable patients (□) are shown so that the distribution of MICs may be appreciated.

antifungal agents were given for more than 4 days. The outcome for this patient is scored as a failure in the therapy trial, but this score obscures his initial response to therapy. The three *C. lusitaniae* isolates (amphotericin B MICs of 0.5, 0.5, and 1 μg/ml and fluconazole MICs of 2, 2, and 4 μg/ml) were from one patient who was treated with fluconazole and did well. The one *C. lipolytica* isolate (amphotericin B MIC of 1 μg/ml and fluconazole MIC of 64 μg/ml) was from one patient who was treated with fluconazole and did well.

As can be seen, high MICs did not predict failure. The

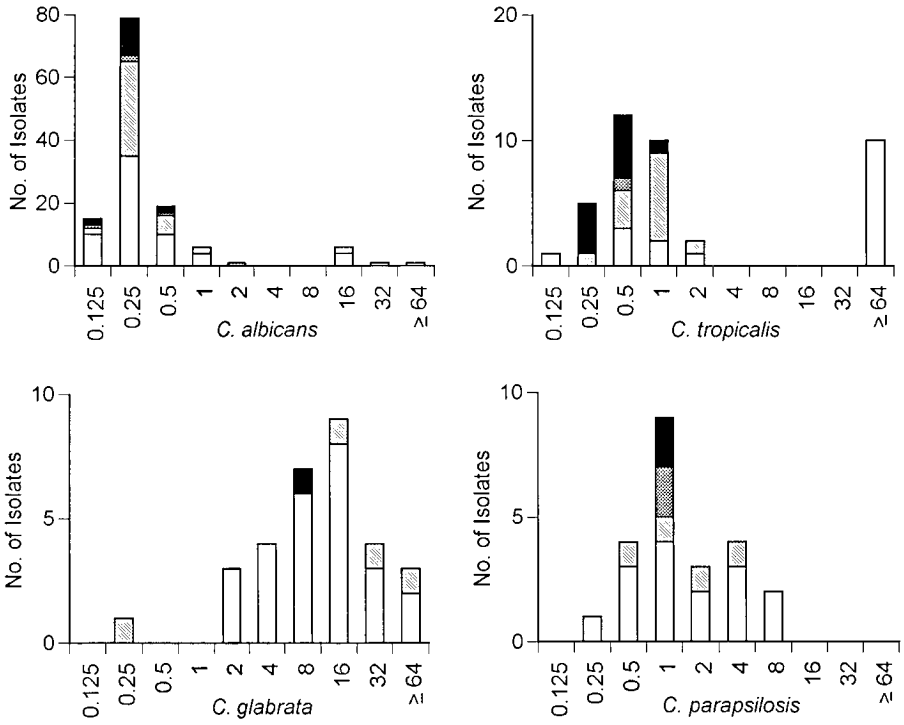


FIG. 2. Correlation of M27-P fluconazole MICs with outcome. Symbols are defined in the legend to Fig. 1.

narrow range of amphotericin B MICs makes identification of resistant isolates difficult: all 112 isolates from the 66 fully evaluable amphotericin B-treated patients required an amphotericin B MIC of ≤1 μg/ml, and of these failure was associated with 26 isolates from 14 patients. On the other hand, the broad range of fluconazole MICs is intriguing. However, of the 100 isolates from 64 fully evaluable fluconazole-treated patients for which fluconazole MICs were ≤16 μg/ml, 36 (from 19 patients) were associated with failure. Conversely, four isolates for which fluconazole MICs were ≥32 μg/ml were obtained from four patients who responded to initial fluconazole therapy (*C. glabrata*, two isolates; *C. krusei*, one isolate; and *C. lipolytica*, one isolate). Similar results were obtained when the 24- and 48-h microbroth MICs were correlated with outcome (data not shown).

When investigated by logistic regression, an inverse correlation was found between log(MIC) and outcome: lower MICs correlated with failure rather than success ($P = 0.001$ [amphotericin B] and $P = 0.05$ [fluconazole]). This inverse correlation is due to the clustering of treatment failures among the more numerous isolates for which MICs were low and the paucity of failures among the few isolates for which MICs were high.

**MICs for sequential isolates.** Sequential isolates obtained over periods of 6 to 26 days were available from six fluconazole-treated patients who failed to clear their bloodstreams. The fluconazole MICs for the sequential isolates remained within one tube dilution of each other and showed no tendency to rise during therapy (data not shown).

## DISCUSSION

The distribution of M27-P MICs for this collection of bloodstream *Candida* isolates was similar to that seen in previous studies (5). The narrow range of amphotericin B MICs is characteristic of results obtained with M27-P (15), and there is no obvious interspecies MIC rank order. For fluconazole, a broad range of MICs is obtained, with characteristic MICs for each species. The approximate rank order of fluconazole MICs is *C. albicans* < *C. parapsilosis* ≅ *C. lusitaniae* < *C. glabrata* ≅ *C. krusei* ≅ *C. lipolytica*. The MIC distribution for *C. tropicalis* appears to be bimodal, with one peak at 0.5 μg/ml and at ≥64 μg/ml, and is thus difficult to rank.

The broth microdilution method produced an acceptable level of agreement with M27-P. Overall, the agreement between the two methods was 85% at both 24 and 48 h. For amphotericin B, the agreement was ≥90% at both 24 and 48 h, whereas for fluconazole the agreement was 80 and 77% at 24 and 48 h, respectively. Although somewhat low, this level of agreement with fluconazole is consistent with that in previous interlaboratory studies (4, 12). In evaluating these results, it is important to keep the state of the art of antimicrobial susceptibility testing in perspective. In one of the earliest comparisons of broth microdilution and macrodilution testing methods for bacteria, Gerlach (6) reported agreements ranging from 72 to 100%, depending on the antibiotic tested. More recently, Baker et al. (1) demonstrated agreements among various antibacterial testing methods ranging from 69 to 98% for selected antimicrobial agents. Thus, the level of agreement between broth microdilution and macrodilution methods observed in our study is not too different from that observed for contemporary antibacterial testing methods.

The lesser agreement noted with fluconazole appears to be a fundamental property of susceptibility testing for this class of agents. Partial inhibition of fungal growth in vitro often takes place over a range of fluconazole concentrations and makes MIC endpoint determination both difficult and subjective (13). Less definite, or trailing, endpoints are also observed with antibacterial agents such as sulfonamides and trimethoprim. The recommended method of determining MIC endpoints when testing these antibacterial and antifungal agents is to estimate the concentration of drug that reduces growth by ≥80% relative to the growth control (10, 18). Such trailing endpoints certainly contribute to lower levels of agreement among susceptibility testing methods observed with antifungal and antibacterial agents alike (6, 13). Thus, it is important to recognize that in vitro testing of some organism-drug combinations may be quite problematic and that susceptibility or resistance may not be recognized by any in vitro susceptibility test method with the same level of reproducibility or accuracy that can be expected with most other organism-drug combinations (8).

Our data do not demonstrate a correlation between high MIC and outcome. Indeed, the concentration of failures among the numerous isolates for which MICs were low and the paucity of failures among the few isolates for which MICs were high produces an inverse statistical correlation of log(MIC) with outcome. This lack of a correlation of failure with high MIC has several related causes. The narrow range of amphotericin B MICs makes detection of resistant isolates difficult and as previously noted is a recognized problem with M27-P (16). On the other hand, the broad range of fluconazole MICs suggests that correlation with outcome might be possible. It is increasingly clear that M27-P and methods like it do detect intrinsic differences in susceptibility of *Candida* isolates to fluconazole in both animal models (16) and AIDS patients with oropharyngeal candidiasis (3, 17, 21). While a correlation might be demonstrated with a different test methodology, the lack of correlation with outcome observed in our study suggests that factors other than intrinsic antifungal susceptibility were operative in our patients. First, while no data from M27-P are yet available, preliminary data generated by methods which would likely produce similar results suggest that oropharyngeal candidiasis in AIDS patients treated with ~100 mg of fluconazole per day will not clear if the MIC for the infecting organism is ≥12 to 16 μg/ml (3, 17). Studies by Cameron et al. (2) and Redding et al. (14) using the M27-P methodology confirm the lack of clinical response when patients having isolates requiring higher fluconazole MICs are treated with such doses of fluconazole. Extrapolating from these data to our nonneutropenic patients treated with 400 mg/day, it is possible that our observed MICs of ≤64 μg/ml were at or below the relevant breakpoint for this dose of fluconazole in this patient setting. The second major consideration is host factors such as failure to adequately exchange catheters. Analysis of data from the clinical trial demonstrated that complete catheter exchanges had a powerful effect on the duration of candidemia, and many of the cases of failure to clear the bloodstream by both drugs appeared due to failure to exchange catheters (15).

These results are not surprising given previous experience with antibacterial susceptibility testing (7, 19, 20). As noted above, the discrepancies in the present study manifested both as clinical failures among isolates requiring low MICs and as several instances of patients responding to fluconazole despite being infected with an organism requiring a high (≥32-μg/ml) fluconazole MIC. Numerous clinical studies of bacterial infections have demonstrated that the use of an antibiotic judged to be effective in vitro (low MIC) does not ensure recovery. Conversely, the use of an agent that appears relatively ineffective in vitro (high MIC) does not preclude recovery (7, 19, 20). A major reason for the potential discrepancy between in vitro and in vivo results is that in vitro susceptibility tests are designed, for the most part, to determine the activity of antimi-

crobial agents in a static test situation that is convenient in the laboratory (20). These artificial conditions are so remote from those that exist in vivo that the tests can be expected to offer only a crude estimate of the likely outcome of treatment (7). Clearly the intrinsic antimicrobial properties of various agents may be profoundly influenced by pharmacological considerations, by the underlying condition of the patient, and by the patient's immunologic status (7, 19, 20). Generally speaking, in vitro susceptibility tests have been reasonably good predictors of therapeutic success.

However, because laboratory tests may also underestimate intrinsic antimicrobial activity and fail to take into account the patient's native ability to cope with the infection, even "inappropriate" agents (those with high MICs) may produce a favorable outcome (7). The situation is thus complex, and all tests of antimicrobial susceptibility, whether they involve fungi or bacteria, merely provide data that must be interpreted in terms of the pharmacologic properties of the drug and the condition of the patient (7, 20).

There is currently considerable concern about fluconazole resistance. Such resistance may occur via selection of species that are intrinsically less susceptible to fluconazole (e.g., *C. glabrata*) or by mutation of previously susceptible isolates to resistance. Our failure to see changes in MICs for sequential isolates is consistent with data suggesting that prolonged therapy (>90 days) is required before intrinsic resistance develops in AIDS patients with oropharyngeal candidiasis (9) and suggests that changes in susceptibility are unlikely to be problematic with the shorter courses of therapy used in this setting.

In summary, our data describe the range of M27-P MICs seen in *Candida* bloodstream isolates and demonstrate that a less cumbersome broth microdilution methodology can give comparable results. The lack of correlation of elevated MICs with failure prevents us from assigning an interpretive MIC breakpoint for fluconazole or amphotericin B. Our data suggest that the relevant MIC breakpoint for nonneutropenic candidemic patients treated with 400 mg of fluconazole per day is at or above 64 μg/ml and that patient management factors such as failure to exchange intravascular catheters can overshadow intrinsic antifungal susceptibility.

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