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# Optimizing the Correlation between Results of Testing In Vitro and Therapeutic Outcome In Vivo for Fluconazole by Testing Critical Isolates in a Murine Model of Invasive Candidiasis

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**The trailing growth phenomenon seen when determining the susceptibilities of *Candida* isolates to the azole antifungal agents makes consistent endpoint determination difficult, and the M27-A method of the National Committee for Clinical Laboratory Standards addresses this problem by requiring an 80% reduction in growth after 48 h of incubation. For some isolates, however, minor variations of this endpoint criterion can produce up to 128-fold variations in the resulting MIC. To investigate the significance of this effect, isolates of *Candida* that exhibited various forms of trailing growth when tested against fluconazole were identified. The isolates were examined in a murine model of invasive candidiasis and were ranked by their relative response to fluconazole by using both improvement in survival and reduction in fungal burden in the kidney. The resulting rank order of in vivo response did not match the MICs obtained by using the M27-A criterion, and these MICs significantly overestimated the resistance of three of the six isolates tested. However, if the MIC was determined after 24 h of incubation and the endpoint required a less restrictive 50% reduction in growth, MICs which better matched the in vivo response pattern could be obtained. Minor variations in the M27-A endpoint criterion are thus required to optimize the in vitro-in vivo correlation for isolates that demonstrate significant trailing growth when tested against fluconazole.**

The Subcommittee for Antifungal Susceptibility Testing of the National Committee for Clinical Laboratory Standards (NCCLS) has collaborated with many other investigators to develop NCCLS document M27, entitled "Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts" (10). Now released in its approved level version (10) as document M27-A, this document describes reproducible macrodilution and microdilution methods for the susceptibility testing of *Candida* species and *Cryptococcus neoformans* against amphotericin B, flucytosine, ketoconazole, itraconazole, and fluconazole. The most recent step in the method's evolution has been the proposal of breakpoints for *Candida* when tested against fluconazole, itraconazole, and flucytosine (10, 16). By necessity, the primary goal of the M27 process was development of a method with good interlaboratory reproducibility. Only with this accomplished could the second, but actually more important, goal of proving that the method has a good correlation with in vivo treatment outcome fully proceed. In addition, the recent analysis of data sets correlating the MICs obtained by the M27 method with the outcomes for fluconazole and itraconazole (16) demonstrates that the M27-A method produces results that are comparable in predictive power to the results produced by antibacterial susceptibility testing (12).

However, these successes do not preclude the possibility of further improvements to the method. Like all susceptibility methods, the M27-A method contains a number of features that significantly influence the measured MIC. While the specific procedures used by the M27-A method and their rationale have been reviewed (18), two features are of particular interest with regard to this paper. First, the time of reading of the results for *Candida* spp. was fixed at 48 h on the basis of an initial study of the macrodilution variant of M27-A in which readings at 48 h improved interlaboratory reproducibility by ~20% over the reproducibility achieved by taking readings at 24 h for amphotericin B, flucytosine, and ketoconazole (9). Similar differences were also present but were somewhat less apparent in a parallel study that used both the macrodilution and the microdilution methods for the testing of amphotericin B, flucytosine, ketoconazole, and fluconazole (6). The second feature of the M27-A method that is of interest is the definition of the endpoint for the azole antifungal agents. On the basis of data from the two studies just mentioned (6, 9), the MICs of these agents were defined as the lowest drug concentrations that produced prominent reductions in growth. This endpoint, also known as MIC-2, was used to handle the well-known trailing growth phenomenon seen with the azole antifungal agents (18). The turbidity of this endpoint has been shown to be less than or equal to the turbidity of a fivefold dilution of the drug-free growth control from a macrodilution format assay (6, 8), and this convenient 80% reduction rule was rapidly incorporated into the M27-A method. This endpoint is obviously arbitrary, and it has been suggested that a less restrictive 50% reduction endpoint might be more relevant (1, 20). However, proof of this assertion has been lacking.

In the context of these two features of the M27-A method, we observed that several *Candida* isolates had anomalous behavior with respect to the endpoint and time of reading when tested against fluconazole. The isolates shared a common behavior in that the MICs for the isolates were relatively low at

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TABLE 1. *Candida* isolates used in this study

| Isolate | Species | MIC behavior[a] | Time (h) | MIC frequency distribution[b] | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | ≤0.06 | 0.125 | 0.25 | 0.5 | 1 | 2 | 4 | 8 | 16 | 32 | ≥64 |
| 630-15.3 | *C. albicans* | Low/low | 24 | 1/− | 3/4 | −/2 | −/1 | | | | | | | |
| | | | 48 | | 2/2 | 2/2 | 1/1 | −/2 | | | | | | |
| 707-15 | *C. albicans* | Low/high | 24 | | | 5/5 | 1/− | 1/− | −/2 | | | | | −/3 |
| | | | 48 | | | −/4 | 2/− | 2/− | | | | | | 3/6 |
| UTR-14 | *C. albicans* | High/high | 24 | | | | | | | | −/3 | 2/2 | | |
| | | | 48 | | | | | | | | −/2 | 2/3 | | |
| 707-10 | *C. tropicalis* | Low/low | 24 | 2/2 | 2/5 | | | | | | | | | |
| | | | 48 | | 2/6 | 3/1 | | | | | | | | |
| 508-12.1 | *C. tropicalis* | Low/high | 24 | | | | 2/4 | 4/1 | | | | | | −/2 |
| | | | 48 | | | | | | 1/− | | | | | 6/7 |
| 623-20 | *C. tropicalis* | High/high | 24 | | | | 2/− | | | | | | 1/− | 2/7 |
| | | | 48 | | | | | | | | | | | 5/7 |

[a] On the basis of the modal MIC at each time point, the behavior of each isolate is summarized as low/low (24/48), low/high, or high/high MICs.

[b] The MIC for each isolate after 24 and 48 h of incubation was determined 7 to 17 times by the macro- and microdilution versions of the M27-A method, both with and without supplementing the medium with glucose at a concentration of 20 g/liter (see text). The resulting frequency distributions of the MICs indicate the number of macrodilution/number of microdilution MICs (−, none). The numbers of readings at 24 and 48 h are not identical for all isolates due to unequal numbers of replicates for some conditions.

24 h but were much higher after 48 h, with the difference in MICs sometimes spanning seven doubling dilutions. Strict application of the 80% growth reduction endpoint rule also strongly influenced the MIC. Such extreme variations in MICs would significantly affect the interpretation of the MIC and suggested that at least one measurement was likely in error. While approaches to selecting conditions which optimize in vitro-in vivo correlations have been reviewed (16), the strong effect of technical factors on the MICs for these isolates seemed to provide an additional avenue for validating or refuting some of the features of the M27-A method, and we now report on detailed studies with a selected set of such isolates.

(This work was presented in part at the 34th Annual Meeting of the Infectious Diseases Society of America, 1996 [15a].)

## MATERIALS AND METHODS

**Isolates.** Three isolates each of *Candida albicans* and *Candida tropicalis* were studied (see Table 1). Isolate UTR-14 was obtained from the oral cavity of a human immunodeficiency virus-infected adult with oropharyngeal candidiasis (7), while all of the other isolates were isolates from the bloodstream of patients enrolled in a trial of therapy for candidemia (14). The isolates were stored at −70°C and were subcultured at least twice on Sabouraud dextrose agar (Becton Dickinson Microbiology Systems, Cockeysville, Md.) at 35°C prior to testing. The isolates were identified to the species level by using the API 20C system (Analytab Products, Plainview, N.Y.).

**Susceptibility testing.** Susceptibility testing was performed by four different methods. First, the isolates were tested by using both the macrodilution and the microdilution versions of the NCCLS M27-A method in 0.165 M MOPS (morpholinepropanesulfonic acid)-buffered (pH 7) RPMI 1640 medium (10). In addition, the isolates were tested by using the macrodilution and microdilution methods of the M27-A method, but in 0.165 M MOPS-buffered (pH 7) RPMI 1640 medium supplemented with 20 g of D-glucose per liter, as suggested by Rodriguez-Tudela and Martinez-Suarez (19). The isolates were tested against fluconazole (supplied by Pfizer Pharmaceuticals) at serial twofold dilutions ranging from 0.06 to 64 μg/ml. For all methods, the MIC was the lowest concentration of fluconazole that produced an 80% reduction of turbidity when compared visually to the turbidity of the drug-free control.

In selected experiments, growth reduction was estimated spectrophotometrically: plates from the microdilution format assay were agitated and the optical densities of the wells were determined at 530 nm (EIA Autoreader; model EL310; Bio-Tek Instruments, Burlington, Vt.). The background optical density of the drug- and organism-free sterility check well was subtracted from all the optical densities of all wells, and the resulting optical density values were divided by the optical density of the drug-free growth-control well to calculate the percentage of growth relative to the growth in the growth control.

**Animal model.** Four- to six-week-old healthy male CF1 mice (Harlan Sprague Dawley, Inc., Indianapolis, Ind.) were used. After the fashion of inoculum preparation for the MIC studies, fungi for inoculation in the animal model were prepared by growing the yeasts on Sabouraud dextrose agar at 35°C, suspending the organisms in saline, and adjusting the inoculum to the desired optical density. The inoculum was quantitated by plating 0.1 ml of serial 10-fold dilutions onto Sabouraud dextrose agar and counting the resulting colonies after 48 h of incubation. To produce infection, the mice were inoculated via the tail vein with a suitable inoculum in a volume of 0.2 to 0.25 ml. After infection, the mice were observed twice daily, and animals exhibiting profound inanition or an inability to reach food and water were sacrificed. All surviving mice were killed at 21 days after inoculation. In preliminary studies, groups of 10 mice each were infected with various inocula of each strain to determine an inoculum for each strain that produced approximately 80% mortality after 7 days. In the treatment experiments, groups of 15 to 16 mice were inoculated on day zero with the appropriate inoculum for each isolate. For the groups of treated mice, 1 or 5 mg of fluconazole per kg of body weight in 0.2 ml of saline was given intraperitoneally daily starting 1 h after intravenous infection, and treatment was continued for 5 days. On day 4, five mice from each group were sacrificed for determination of the numbers of CFU of *Candida* per gram from the kidney. This determination was made by removing and weighing both kidneys, homogenizing kidneys in 5 to 10 ml of saline with a Stomacher 80 (A. J. Seward, UAC House, London, England), and plating suitable dilutions on Sabouraud dextrose agar. The plates were incubated at 35°C, and the number of colonies was enumerated after 48 h of growth. All animal care procedures were supervised and approved by the University of Texas-Houston Animal Welfare Committee.

**Statistical methods.** Survival times were estimated by using the Kaplan-Meier method and were compared by the log-rank technique. Organ clearance data were compared by using analysis of variance and/or the *t* test, as appropriate. All calculations were performed by using SPSS for Windows, version 7.5.1 (SPSS, Inc., Chicago, Ill.).

## RESULTS

**In vitro susceptibility testing results.** The results of repeated testing of the six isolates by the four variations of the M27-A method are presented in Table 1. All isolates were tested at least once by each method. For isolates for which multiple MIC estimates differed widely (e.g., the 48-h readings for *C. albicans* 707-15), high MICs were somewhat more likely when testing was performed by the microdilution format. Glucose supplementation had no effect on the MIC distributions (data

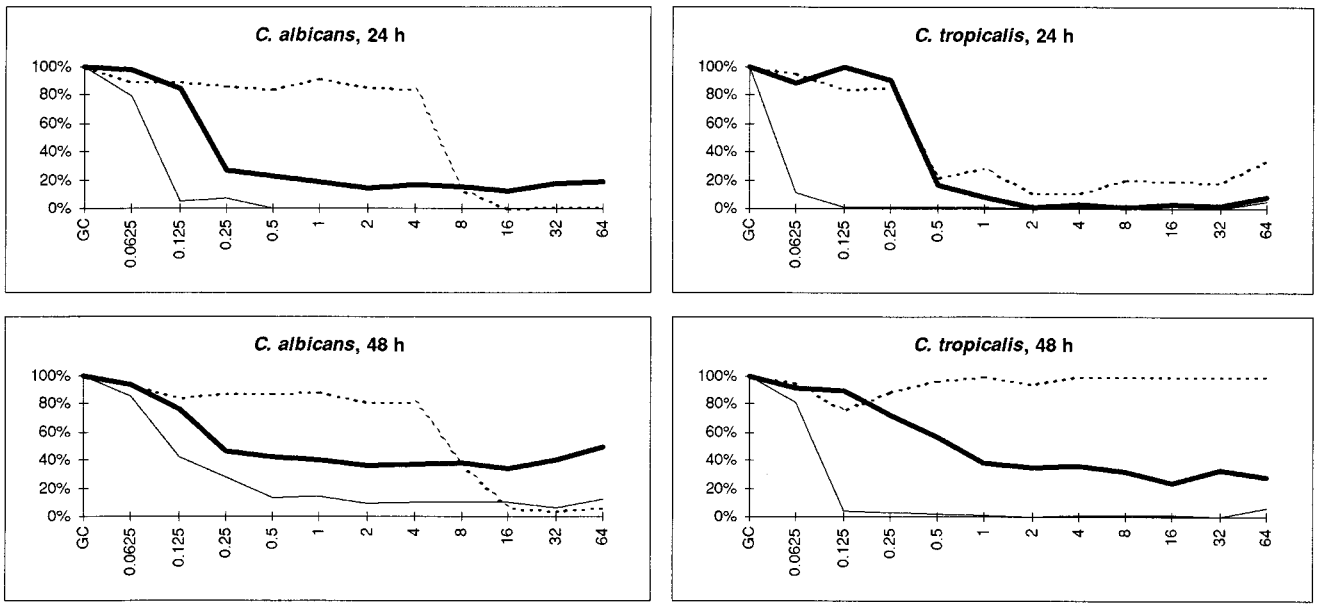


FIG. 1. Effect of fluconazole. Each isolate was tested by the microdilution version of the M27-A method, and the reduction in growth is expressed as a percentage of the growth in the drug-free growth control well (*y* axis) versus fluconazole concentration (in micrograms per milliliter, *x* axis) for the isolates for which the MICs were low/low (thin lines), the isolates for which the MICs were low/high (thick lines), and the isolates for which the MICs were high/high (dotted lines). Results for tests in 0.165 M MOPS-buffered (pH 7) RPMI 1640 medium are presented. GC, growth control. Similar results were obtained if testing was performed in medium supplemented with glucose at 20 g/liter (data not shown).

not shown). On the basis of these observations, the MICs for the isolates were classified as being relatively low at both 24 and 48 h (low/low), low at 24 h but higher at 48 h (low/high), or high at both time points (high/high).

The basis for the variability in the MIC estimates was clearly illustrated when the growth as a percentage of the growth of the growth control was determined by measuring the optical densities of the wells from the microdilution format assays (Fig. 1). As can be seen, both isolates for which the MICs were low/low had growth curves that broke sharply and fell to <10% of that for the growth control at 0.125 to 0.5 μg of fluconazole per ml. Both isolates for which the MICs were low/high had a break in their growth curves with fluconazole at 0.25 to 0.5 μg/ml, but the trailing growth was <20% of the growth for the growth control at 24 h and >20% of the growth for the growth control at 48 h. Finally, the two isolates for which the MICs were high/high were different in character. The *C. albicans* isolate for which MICs were high/high showed no response to fluconazole until the fluconazole concentration reached 8 to 16 μg/ml, but thereafter, its growth was sharply reduced to <5% of the growth for the growth control. On the other hand, the *C. tropicalis* isolate for which the MICs were high/high showed a break in its growth curve at 24 h (although not a value consistently <20% of the growth for the growth control) but demonstrated essentially no response to fluconazole at 48 h.

To aid in the interpretation of these disparate MIC estimates, the morphological effect of fluconazole on the fungi was assessed visually (Fig. 2). By comparison with the yeast cells of the growth control, all strains except the *C. albicans* strain for which the MICs were high/high demonstrated aberrant morphology (enlarged, swollen cells and/or clustering due to failure of cell separation) by 1 μg of fluconazole per ml. Such morphological effects are similar to those reported for azole antifungal agents (4, 5). In addition, a qualitative decrease in the number of fungi could be detected. By contrast, such effects were not clearly noted for the *C. albicans* isolate for which the MICs were high/high until the concentration of fluconazole reached 8 to 16 μg/ml.

**Response to therapy in vivo.** After establishing suitable inoculum sizes for each organism, three treatment trials were performed with the *C. tropicalis* isolate for which MICs were high/high and two trials were performed with the other isolates. For each isolate, the survival times of the untreated controls did not differ between treatment trials ($P > 0.1$), and thus, the data from the replicate treatment trials were pooled to provide a single estimate of survival for each isolate with each dose of fluconazole (Table 2). At 1 mg/kg/day, fluconazole prolonged survival significantly for all isolates except the *C. albicans* isolate for which the MICs were high/high. At 5 mg/kg/day, fluconazole prolonged the survival times significantly for all isolates, but the least prolongation was again noted for the *C. albicans* isolate for which the MICs were high/high.

The data on the reduction in the numbers of CFU per gram in the kidney by fluconazole therapy showed a pattern comparable to that obtained with the survival data (Table 3). Because statistically significant two- to fivefold differences in the numbers of CFU per gram of kidney tissue were noted for the untreated controls between replicate treatment trials, the mean numbers of CFU per gram of kidney tissue was computed for the untreated controls in each experiment, and all values from each experiment were then divided by this value and aggregated across experiments to produce a single estimate of the percent reduction in the numbers of CFU per gram of kidney tissue relative to the numbers of CFU per gram for the untreated controls for each dose of drug.

**Aggregate analysis of response from in vitro and in vivo data.** Considering the overall pattern of response based on prolongation of survival and the reduction in the numbers of CFU per gram of kidney tissue, the six isolates can be loosely classified as having good, fair, or poor responses to fluconazole therapy (Table 4). This classification is clearly arbitrary, but it

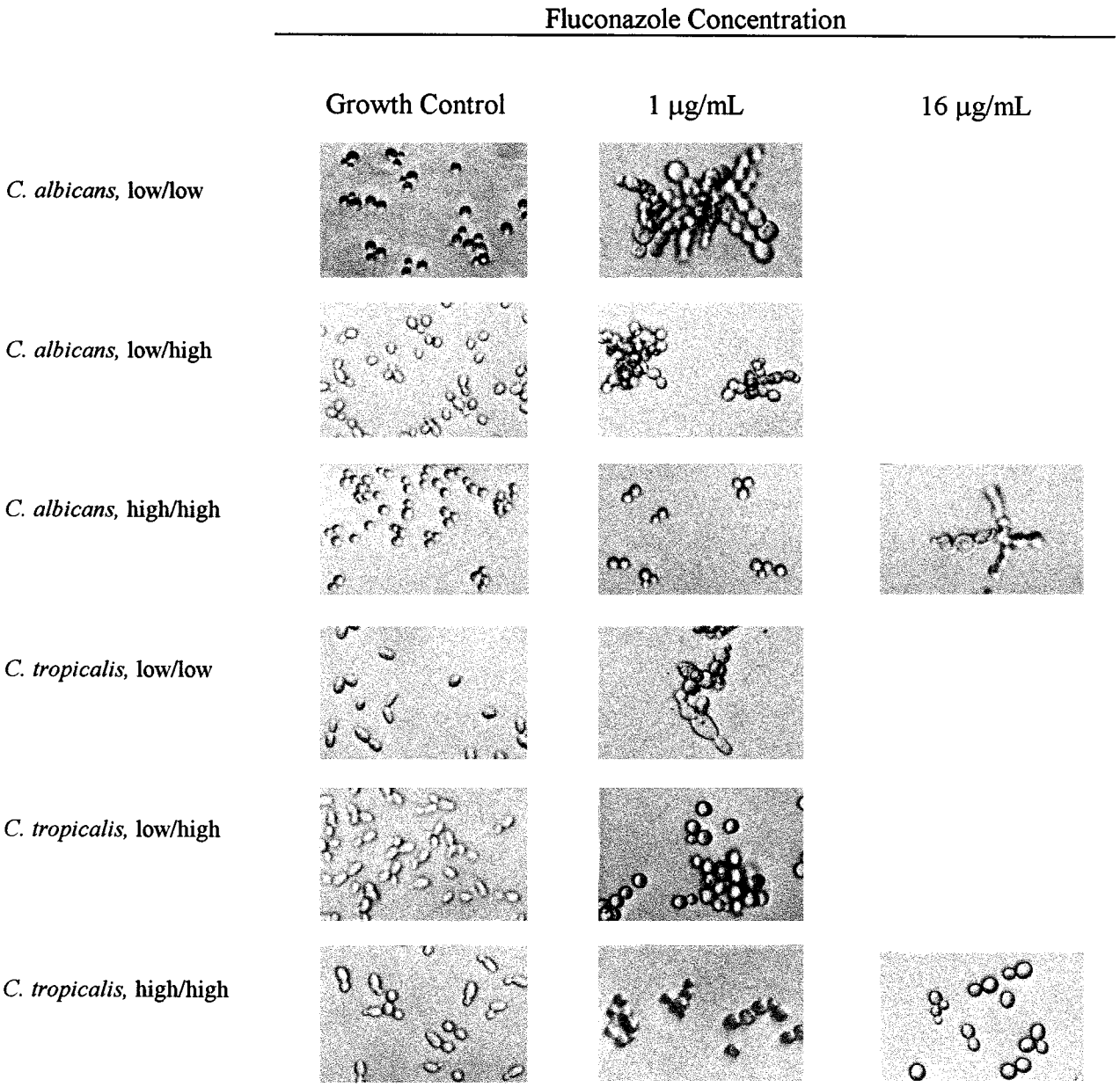


FIG. 2. Morphological effect of fluconazole on the fungi. After 24 h of incubation, samples of the material in the susceptibility assays were removed and examined microscopically. Note the aberrant morphology (clustering and/or ballooning of cells) that was readily seen by 0.5 to 1 μg/ml for all isolates except the *C. albicans* isolate for which the MICs were high/high. Such changes were not evident until a concentration of 8 to 16 μg/ml was reached for the *C. albicans* isolate for which the MICs were high/high. Magnification, ×500.

is helpful in generating a summary of the results and comparing them with the MIC data. Neither of the mouse model-based response rank orders were well captured by the MICs obtained at 48 h by the M27 method with an endpoint of 80% reduction of growth. Two problems are apparent. First the MICs obtained at 48 h by the M27-A method with an endpoint of 80% reduction of growth would make both isolates for which the MICs were low/high appear to be more resistant than the *C. albicans* isolate for which the MICs were high/high. This clearly contradicts the animal model data, and for these isolates the MICs obtained at 24 h by the M27-A method with an endpoint of an 80% reduction of growth best matched the response in vivo. Although this effect was seen for the MICs obtained by both the macrodilution and the microdilution methods, it was somewhat more prominent for readings determined in the microdilution format (Table 1). Second, the *C. tropicalis* isolate for which the MICs were high/high presents an additional problem. The MIC at neither 24 nor 48 h consistently correctly matched the response in vivo if an 80% reduction in growth was the endpoint criterion. Indeed, when the MIC was determined at 48 h, this isolate was the most resistant of the six isolates when any possible endpoint criterion was used. However, the growth of the isolate was clearly reduced at a fluconazole concentration of 0.5 μg/ml after 24 h, but capturing this result required use of a less restrictive endpoint of 50% reduction of growth relative to the control.

TABLE 2. Effect of fluconazole therapy on survival

| Isolate and MICs | Inoculum[a] | Mean ± SE survival time (days at the following dose of fluconazole (mg/kg/day): | | | Survival Δ (days)[b] | | $P$[c] | | |
|---|---|---|---|---|---|---|---|---|---|
| | | 0 | 1 | 5 | 0 vs 1 mg/kg/day | 0 vs 5 mg/kg/day | 0 vs 1 mg/kg/day | 0 vs 5 mg/kg/day | 1 vs 5 mg/kg/day |
| *C. albicans* | | | | | | | | | |
| Low/low | $2.5 \times 10^5$ | 5.5 ± 0.3 | 8.6 ± 1.0 | 12.2 ± 1.6 | 3.1 | 6.7 | 0.006 | 0.004 | 0.028 |
| Low/high | $2.9 \times 10^5$ | 5.4 ± 0.3 | 10.5 ± 0.6 | 11.1 ± 0.7 | 5.1 | 5.7 | <0.001 | <0.001 | 0.667 |
| High/high | $1.7 \times 10^6$ | 5.7 ± 0.3 | 6.2 ± 0.6 | 8.9 ± 0.8 | 0.5 | 3.2 | 0.345 | 0.005 | 0.015 |
| *C. tropicalis* | | | | | | | | | |
| Low/low | $5.2 \times 10^6$ | 3.9 ± 0.4 | 10.9 ± 1.1 | 10.9 ± 1.0 | 7.0 | 7.0 | 0.002 | 0.002 | 0.775 |
| Low/high | $3.3 \times 10^6$ | 3.1 ± 0.2 | 7.0 ± 0.9 | 9.9 ± 0.9 | 3.9 | 6.8 | 0.068 | <0.001 | 0.019 |
| High/high | $1.2 \times 10^7$ | 5.6 ± 0.8 | 11.8 ± 0.9 | 14.2 ± 0.9 | 6.2 | 8.6 | <0.001 | <0.001 | 0.080 |

[a] The mice were inoculated with the indicated numbers of CFU of the given *Candida* species, and the mean ± SE survival time of each group was estimated by the Kaplan-Meier technique.
[b] The increase in survival (survival Δ) due to fluconazole therapy was computed as the difference between the survival estimate for the group receiving no drug and the group receiving the indicated treatment.
[c] The survival estimates for the indicated treatment groups were compared by the log-rack technique, and the resulting $P$ value is shown.

## DISCUSSION

The striking behavior of the test isolates in the assays described here provides yet another demonstration of the methodology dependence of all susceptibility test approaches. While these results validate the fundamental principles of the M27-A method and demonstrate that the method can produce results that correlate with clinical outcome, the results also indicate that minor variations in the M27-A method are required to optimize the in vitro-in vivo correlation for isolates that demonstrate significant trailing growth.

The data suggest two possible refinements of the M27-A method. First, the time of reading could be shortened from 48 to 24 h. Such reading times have previously been suggested on a practical basis (1, 2, 20), but these results demonstrate that the MIC at 24 h is superior to the MIC at 48 h, at least for some isolates. Although these data apply to fluconazole, it has also been shown that the meaning of amphotericin B MICs in a broth-based system is improved by reading the MIC at 24 h rather than at 48 h (15). It thus seems possible that these changes would be helpful for other drugs as well. Such reading time is also consistent with work now being reported for agar-based testing of fluconazole (3) and amphotericin B (21). The second improvement suggested by these data is that the endpoint criterion, at least for fluconazole, could be loosened so that the MIC is read as the lowest drug concentration that produces a significant effect on growth. The precise definition of "significant effect" may require additional work, but a 50% reduction would appear to be a reasonable starting point and has been conveniently used in prior work (1, 20). Adoption of these suggestions would also require that reference ranges for the quality control strains defined by the M27-A method (11, 17) be determined.

These data also illustrate the inherent limitation of using a single number (an MIC) to try to predict the global response to an infection. None of the MIC rank orders completely matches the responses obtained in vivo (Table 4). In particular, the *C. tropicalis* isolates tended to be more difficult to clear from the kidney and also to have less clear-cut morphologic responses to fluconazole (Fig. 2). This is most apparent with the *C. tropicalis* isolates for which the MICs were low/high and high/high, and this raises the possibility that the differences in the growth inhibition patterns seen in Fig. 1 are indicative of small, but real differences in fluconazole susceptibility. As can be seen, capturing these differences in a single measure of in vitro susceptibility is difficult.

This study has two principal limitations. First, animal models are a valuable model for investigating drug efficacy, but they do not necessarily mimic every aspect of the infection in humans (16). Second, it is difficult to prove that the results obtained with the various isolates are completely comparable. These isolates were from widely scattered geographic locations, and five of the six isolates have been shown to be unrelated by a DNA typing method (13) (the *C. albicans* isolate for which the MICs were high/high, isolate UTR-14, was not included in this survey). Thus, there is always the potential that the isolates might differ in more ways than just their MIC. In addition, production of exactly the same mortality in the untreated control mice infected with each isolate was not possible, but the

TABLE 3. Effect of fluconazole on colony count in kidney tissue

| Isolate and MICs | Mean ± SE % of control CFU/g kidney tissue at the following fluconazole dose (mg/kg/day): | | | $P$[b] | |
|---|---|---|---|---|---|
| | 0 | 1 | 5 | 0 vs 1 mg/kg/day | 0 vs 5 mg/kg/day |
| *C. albicans* | | | | | |
| Low/low | 100 ± 28 | 2 ± 1 | 1 ± 1 | 0.002 | 0.002 |
| Low/high | 100 ± 35 | 5 ± 1 | 1 ± 1 | 0.014 | 0.011 |
| High/high | 100 ± 28 | 530 ± 260 | 56 ± 29 | 0.118 | 0.287 |
| *C. tropicalis* | | | | | |
| Low/low | 100 ± 16 | 14 ± 3 | 4 ± 1 | <0.001 | <0.001 |
| Low/high | 100 ± 19 | 14 ± 5 | 9 ± 3 | <0.001 | 0.001 |
| High/high | 100 ± 18 | 24 ± 5 | 6 ± 1 | <0.001 | <0.001 |

[a] As discussed in the text, the numbers of CFU per gram of kidney tissue from mice treated with 0, 1, or 5 mg of fluconazole per kg/day were expressed as a percentage of numbers of CFU per gram in the group not treated with drug. In an analysis of variance for all three doses, the overall $P$ value for the effect of therapy was 0.069 for *C. albicans* for which the MICs were high/high and <0.002 for all other isolates. In addition, the post-hoc Student-Newman-Keuls procedure found that the reduction in growth for the 1- and 5-mg/kg/day treatment groups did not differ from each other for any isolate but did differ from the reduction in growth for the group receiving no treatment ($P < 0.05$) for all isolates except the *C. albicans* isolate for which the MICs were high/high.
[b] For reference purpose, a simple $t$ test comparing the no-treatment group with the indicated treatment group was performed, and the resulting $P$ value is presented.

TABLE 4. Summary of responses in vivo and in vitro in fluconazole

| Isolate, MICs | In vivo response ranking[a] | | Modal MIC (μg/ml) at the following time and endpoint[b]: | | |
|---|---|---|---|---|---|
| | By survival | By nos. of CFU/g in kidney | 48 h, 80% | 24 h, 80% | 24 h, 50% |
| *C. albicans*, low/low | Fair | Good | 0.25 | 0.125 | 0.06 |
| *C. tropicalis*, low/low | Good | Fair | 0.125 | 0.125 | 0.06 |
| *C. albicans*, low/high | Good | Good | ≥64 | 0.25 | 0.25 |
| *C. tropicalis*, low/high | Fair | Fair | ≥64 | 0.5 | 0.5 |
| *C. tropicalis*, high/high | Good | Fair | ≥64 | ≥64 | 0.5 |
| *C. albicans*, high/high | Poor | Poor | 16 | 16 | 8 |

[a] On the basis of the response to 1 mg of fluconazole per kg/day, the isolates were sorted by best to worst response and were then grouped by inspection into descriptive categories of good, fair, or poor response to therapy. For example, good response in the survival ranking corresponds to a ≥5-day increase in survival, fair corresponds to a 3- to 4-day increase, and poor corresponds to a <1-day increase.

[b] Modal MICs read at 48 and 24 h and determined by the four variations of the M27-A method that use an 80% growth reduction endpoint (data in Table 1) along with the MIC that would be obtained after 24 h if only a 50% reduction in growth compared with the growth control were required (data in Fig. 1).

mean survival times were similar. When a difference exists (the survival times of the untreated mice infected with the *C. tropicalis* isolates for which the MICs were low/low and low/high are shorter than those of mice infected with the other isolates), this bias actually places the treatment at an additional disadvantage with respect to that agent and thus serves to strengthen the results. In addition, the results obtained in the animal studies are also supported by microscopic inspection of the fluconazole-exposed yeast. Thus, despite the limitations of studies of this type, the aggregate data suggest that it is reasonable to conclude that the rank orders for response derived from the animal studies (Table 4) are a true estimate of the relative susceptibilities of the isolates to fluconazole in vivo.

The implications of these results for users of the M27-A method are clear. For compliance with the M27-A method, results should be reported on the basis of the MICs at 48 h and the corresponding published breakpoints. While initial data from our laboratory suggest that isolates with discordant MICs at 24 and 48 h are only a minority of tested isolates, the MIC should ideally be determined at both time points, and special care in reporting of results should be taken with any isolate that produces strongly discrepant results. Likewise, interpretation of the results for isolates with significant trailing is difficult, and the results obtained for such isolates should be carefully reviewed. Such caution is especially warranted when testing is done by the more convenient microdilution version of the M27-A method. It might, for example, be appropriate to provide a supplemental report indicating that the MIC for the isolate shows substantial variation with the technique used to determine the MIC and that the predictive significance of the reported MIC is less certain than usual.

## ACKNOWLEDGMENT

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