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# Influence of Test Conditions on Antifungal Time-Kill Curve Results: Proposal for Standardized Methods

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**This study was designed to examine the effects of antifungal carryover, agitation, and starting inoculum on the results of time-kill tests conducted with various *Candida* species. Two isolates each of *Candida albicans*, *Candida tropicalis*, and *Candida glabrata* were utilized. Test antifungal agents included fluconazole, amphotericin B, and LY303366. Time-kill tests were conducted in RPMI 1640 medium buffered with morpholinepropanesulfonic acid (MOPS) to a pH of 7.0 and incubated at 35°C. Prior to testing, the existence of antifungal carryover was evaluated at antifungal concentrations ranging from 1× to 16× MIC by four plating methods: direct plating of 10, 30, and 100 μl of test suspension and filtration of 30 μl of test suspension through a 0.45-μm-pore-size filter. Time-kill curves were performed with each isolate at drug concentrations equal to 2× MIC, using a starting inoculum of approximately $10^5$ CFU/ml, and incubated with or without agitation. Last, inoculum experiments were conducted over three ranges of starting inocula: $5 \times 10^2$ to $1 \times 10^4$, $>1 \times 10^4$ to $1 \times 10^6$, and $>1 \times 10^6$ to $1 \times 10^8$ CFU/ml. Significant antifungal carryover (>25% reduction in CFU/milliliter from the control value) was observed with amphotericin B and fluconazole; however, carryover was eliminated with filtration. Agitation did not appreciably affect results. The starting inoculum did not significantly affect the activity of fluconazole or amphotericin B; however, the activity of LY303366 may be influenced by the starting inoculum. Before antifungal time-kill curve methods are routinely employed by investigators, methodology should be scrutinized and standardized procedures should be developed.**

Data collected from time-kill studies have provided critical information regarding the rate and extent of bactericidal activity, pharmacodynamic characteristics (i.e., relationship between concentration and effect and the postantibiotic effect), and potential antagonism or synergy between antibacterial agents administered concomitantly. These data have significantly enhanced our understanding regarding the dynamic relationships which exist between antimicrobial agents and their effects on bacteria. In fact, time-kill testing has become an indispensable tool for assessing the activity of antimicrobials against bacteria. Standardized methods providing instruction on the implementation of time-kill methods have been proposed by the National Committee for Clinical Laboratory Standards to ensure the reproducibility and accuracy of test results (11).

Despite widespread recognition of the value of data generated with time-kill studies against bacteria, similar data for fungi are virtually nonexistent. This, however, is not entirely surprising if one considers that it was only recently that guidelines for conducting and interpreting antifungal susceptibility test results were established and approved (13, 16). With the framework for in vitro testing of antifungal agents in place, it is only a matter of time before increased interest in antifungal time-kill testing is generated. Guidelines regarding procedures for antifungal time-kill testing have yet to be established. Significant interlaboratory variability, nonreproducible results, conflicting data, and unstandardized interpretation of results are only a few of the potential pitfalls that may be encountered if time-kill methods are not standardized. Similar problems were encountered and slowed the development and acceptance of in vitro antifungal susceptibility testing procedures.

There are currently relatively few published reports of antifungal time-kill studies. Our group has conducted several studies utilizing time-kill procedures for the study of antifungal activity (1–10, 17, 18). The methods used to conducted these studies were developed in our laboratory. During the course of these studies, we evaluated several variables for their effect on time-kill curve results. This report details our findings regarding the influence of various test conditions on antifungal time-kill results.

(A preliminary report of this work has been presented previously [7].)

## MATERIALS AND METHODS

**Antifungal agents.** Fluconazole (Pfizer Inc., New York, N.Y.), amphotericin B (Sigma Chemical Company, St. Louis, Mo.), and LY303366 (Eli Lilly and Co., Indianapolis, Ind.) were utilized for susceptibility determinations and time-kill studies. Stock solutions of each agent were prepared utilizing RPMI 1640 (Sigma) buffered to a pH of 7.0 with 0.165 M morpholinepropanesulfonic acid (MOPS) buffer (Sigma) as the solvent. Dimethyl sulfoxide (DMSO) was used to aid the solubilization of each of the drugs. The final concentration of DMSO in the time-kill test solutions was ≤1% (vol/vol) of the solution composition. To establish that exposure to DMSO did not affect the growth of the test isolates, fungi were grown in the presence of 1% (vol/vol) DMSO and compared with growth of fungi naive to DMSO. Stock solution were separated into unit-of-use aliquots and stored at −70°C until used.

**Test isolates.** Two clinical isolates of *Candida glabrata* (strains 582 and 350) and of *Candida tropicalis* (strains 2697 and 3829) were selected for testing. Additionally, one American Type Culture Collection strain (ATCC 90028) and one clinical isolate (OY31.5) of *Candida albicans* were utilized. Isolates were obtained from the Department of Pathology, The University of Iowa College of Medicine.

**Antifungal susceptibility testing.** The MIC of each antifungal was determined against test isolates by using broth microdilution techniques as described by the National Committee for Clinical Laboratory Standards (13). MICs were determined in RPMI 1640 buffered to a pH of 7.0 with MOPS. The starting inoculum was approximately $1 \times 10^3$ to $5 \times 10^3$ CFU/ml. Microtiter trays were incubated

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at 35°C in a moist, dark chamber, and MICs were recorded after 48 h of incubation. The susceptibility endpoints for fluconazole and LY303366 were defined as the lowest concentration of antifungal which resulted in an 80% reduction in visual growth compared with growth of the control (7, 13). In contrast, the MIC of amphotericin B was defined as the lowest concentration of drug which resulted in total inhibition of visual growth.

**Limit of quantitation.** The lower limit of accurately detectable CFU/milliliter or the limit of quantitation was determined for each of the six isolates. A fungal suspension was made in sterile water with each isolate and adjusted to a 0.5 McFarland turbidity standard (approximately $1 \times 10^6$ to $5 \times 10^6$ CFU/ml). A series of dilutions, using sterile water, were made with the standardized suspensions, resulting in three suspensions with fungal concentrations of approximately 100, 50, and 30 CFU/ml for each isolate. Thirty microliters was removed from each suspension and plated on potato dextrose agar (PDA) plates (Remel, Lexena, Kans.) for colony count determination. Plates were incubated at 35°C, and viable-colony counts were determined after 24 to 48 h. Experiments were conducted in quintuplicate.

**Antifungal carryover.** Antifungal carryover determinations were conducted as previously described (9). Briefly, fungi were obtained from stored samples and subcultured twice on PDA plates (Remel) prior to testing. Fungal suspensions were prepared in sterile water by touching one or two colonies from a 24- to 48-h-old culture plate and adjusting the resulting suspension to a 0.5 McFarland turbidity standard (approximately $1 \times 10^6$ to $5 \times 10^6$ CFU/ml) by spectrophotometric methods. The resulting suspension was then diluted via sequential dilutions of 1:100 and 1:2 with sterile water to yield a fungal suspension of approximately $5 \times 10^3$ CFU/ml. One hundred microliters of the diluted fungal suspension was then added to 900 μl of sterile water or sterile water containing fluconazole, amphotericin B, or LY303366, resulting in a starting inoculum of approximately $5 \times 10^2$ CFU/ml. Antifungal carryover was evaluated over a range of antifungal concentrations from 1× to 16× MIC. Immediately following the addition of the fungal suspension to the aqueous solutions, an aliquot was removed from each tube and streaked across PDA plates (Remel) or RPMI 1640 agar plates (135-mm diameter; Remel) (RPMI agar was used only for the plating of 100-μl samples) for colony count determinations. Because of problems in the acquisition of 135-mm-diameter PDA plates, RPMI agar was used. Three methods were initially evaluated for colony count determinations: direct plating of 10, 30, or 100 μl (amphotericin B and LY303366 only) of test solutions. If antifungal carryover was noted with any of these methods, the study was repeated adding a fourth plating method: dilution of 30 μl of the test sample in 10 ml of sterile water followed by vacuum filtration through a 0.45-μm-pore-size filter and subsequent placement of the filter onto a PDA plate. Colony counts were determined following incubation at 35°C for 24 to 48 h. Tests were conducted in quintuplicate. Reproducibility of results were evaluated by determining the coefficients of variation associated with results obtained with control samples by each of the sampling methods.

**Agitation.** Time-kill procedures were conducted as previously described (5, 9). Fungal suspensions, adjusted to a 0.5 McFarland turbidity standard, were prepared as described above. A 1:10 dilution of this suspension was made by adding 1 ml of fungal suspension to 9 ml of RPMI 1640 with or without (control) the desired amount of antifungal. This dilution yielded a starting inoculum of approximately $1 \times 10^5$ to $5 \times 10^5$ CFU/ml. Antifungals were tested at a concentration equal to 2× MIC for test isolates. Two identical sets of solutions were prepared for each isolate: control (drug-free) (tube 1), fluconazole (tube 2), amphotericin B (tube 3), and LY303366 (tube 4). All solutions were incubated at 35°C; however, one set of tubes was placed on an orbital shaker and incubated with agitation, whereas the other set of tubes was incubated without agitation. At predetermined time points (0, 1, 2, 3, 4, 6, 8, 12, and 24 h), a 100-μl sample was removed from each tube and serially diluted 10-fold with sterile water, and a 30-μl aliquot was plated onto a PDA plate for colony count determination. When colony counts were expected to be less than 1,000 CFU/ml, a 30-μl sample was taken directly from the test solution and plated onto a PDA plate without dilution. Plates were then incubated for 24 to 48 h at 35°C prior to examination. All experiments were conducted in duplicate.

**Inoculum.** Time-kill methods as detailed above were utilized with the following modifications. Three ranges of starting inocula for each isolate were studied against fluconazole, amphotericin B, and LY303366: $5 \times 10^2$ to $1 \times 10^4$, $>1 \times 10^4$ to $1 \times 10^6$, and $>1 \times 10^6$ to $1 \times 10^8$ CFU/ml. Antifungals were tested at concentrations equal to 2× MIC for each isolate. Test samples were incubated at 35°C with agitation. Aliquots were removed from each test solution for colony count determination at 0, 1, 2, 3, 4, 5, 6, 7, 8, 12, and 24 h following inoculation. The plating procedures described above were followed for high- and medium-inoculum samples; however, the limit of fungal quantitation was lowered to approximately 30 CFU/ml for each isolate in the low-inoculum group. This was accomplished by plating 100 μl of the test sample directly onto an RPMI 1640 agar plate without dilution. Plates were incubated at 35°C for 24 to 48 h prior to determination of colony counts. All experiments were conducted in duplicate.

**Analysis.** For the quantitation limit and antifungal carryover studies, intraspecies results were combined. Antifungal carryover colony count results for all three drugs, at each multiple of the MIC, were compared to the control value for each of the sampling methods. Significant antifungal carryover was defined as >25% reduction in CFU/milliliter compared to the control value (14).

Mean colony count data ($\log_{10}$ CFU/milliliter) data from agitation and inoculum studies were plotted as a function of time for each isolate and evaluated visually with respect to rate and extent of growth or growth reduction. Differences among experimental curves were determined and expressed as $\log_{10}$ values.

TABLE 1. Median MICs of fluconazole, amphotericin B, and LY303366 against test isolates

| Isolate | Median MIC (μg/ml) | | |
|---|---|---|---|
| | Fluconazole ($n$ = 6) | Amphotericin B ($n$ = 6) | LY303366 ($n$ = 6) |
| *C. albicans* ATCC 90028 | 0.25 | 1 | 0.015 |
| *C. albicans* OY31.5 | 0.25 | 1 | 0.015 |
| *C. glabrata* 350 | >128 | 1 | 0.06 |
| *C. glabrata* 582 | 4 | 1 | 0.045 |
| *C. tropicalis* 2697 | >128 | 1 | 0.03 |
| *C. tropicalis* 3829 | 0.5 | 1 | 0.03 |

## RESULTS

**Antifungal susceptibility.** Susceptibility data for each isolate are presented in Table 1. *C. glabrata* 350 and *C. tropicalis* 2697 both exhibit resistance to fluconazole, with MICs of >128 μg/ml (16).

**Limit of quantitation.** Using a 30-μl sampling volume, the lower limit of accurate and reproducible quantitation was 50 CFU/ml for each of the isolates. According to these sampling methods, the cumulative (all species) percent coefficient of variation (% CV) of colony counts resulting from control samples was 22.7. Cumulative % CVs calculated for the 10-μl, 100-μl, and filtered sampling methods were 37.2, 16.6, and 24.4, respectively. Sampling variability appeared to be slightly greater with *C. tropicalis* (% CV for 30-μl sampling = 34.2) compared with the other species (% CV = 17). The presence of antifungal in solution did not affect the reproducibility of sampling results.

**Antifungal carryover.** Antifungal carryover data are summarized in Table 2. No antifungal carryover was observed with LY303366 against test isolates with any of the three sampling volumes. For fluconazole, significant carryover was observed against *C. tropicalis* only. The effect began at concentrations of fluconazole equal to 16× and 8× MIC for the 10- and 30-μl sampling methods, respectively. Direct plating of 100 μl was not evaluated with fluconazole. In contrast, significant carryover was noted for amphotericin B against all of the species tested. At sampling volumes of 10 and 30 μl, carryover was observed only against *C. albicans* and only at concentrations equal to 16× and ≥8× MIC, respectively. Carryover was noted among all three species at a sampling volume of 100 μl, even

TABLE 2. Multiple of the MIC at which antifungal carryover was first noted

| Antifungal agent and sampling volume (μl) | Multiple of the MIC at which carryover was first noted in: | | |
|---|---|---|---|
| | *C. albicans* | *C. glabrata* | *C. tropicalis* |
| Fluconazole | | | |
| 10 | | | 16 |
| 30 | | | 8 |
| Amphotericin B | | | |
| 10 | 16 | | |
| 30 | 8 | | |
| 100 | 8 | 2 | 16 |

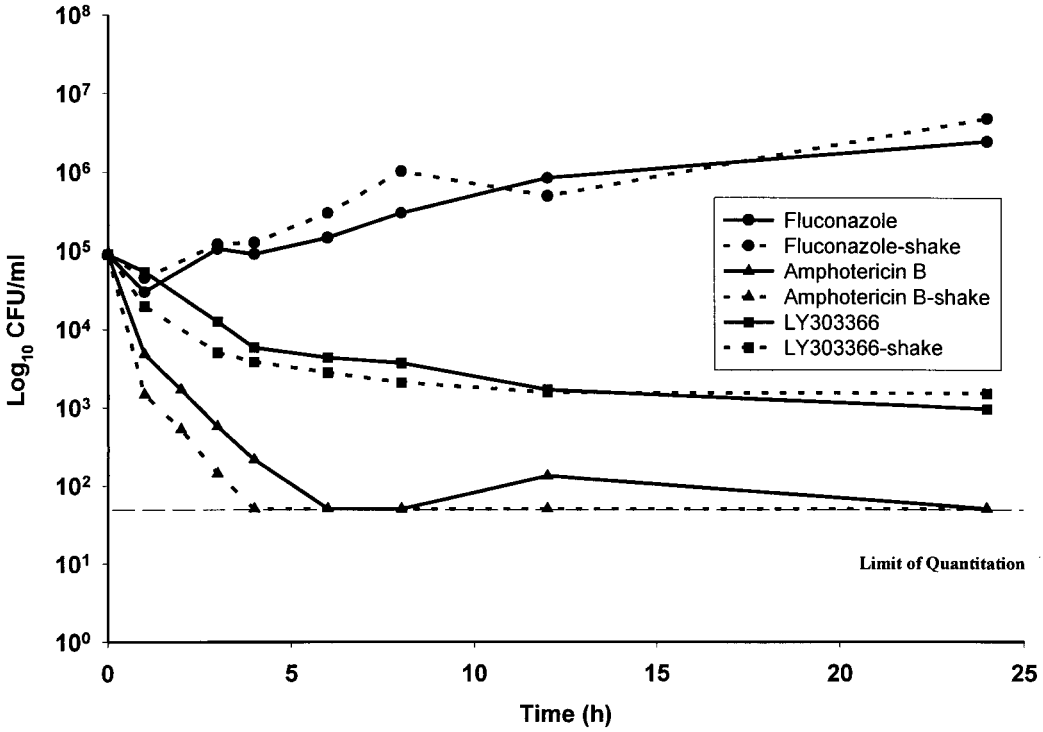


FIG. 1. Time-kill plot of the effect of agitation on *C. tropicalis* 3829.

with concentrations as low as 2× MIC (*C. glabrata*). Filtration was effective in eliminating the carryover noted with amphotericin B.

**Agitation.** The rate and extent of growth for each of the control samples appeared to be independent of agitation (Fig. 1). Likewise, agitation or the lack thereof did not affect the activity observed with any of the test agents. The difference between agitated and respective nonagitated samples did not exceed 0.7 $\log_{10}$ CFU/ml at any of the time points. Additionally, by the end of the 24-h study period, differences between samples were generally <0.3 $\log_{10}$ CFU/ml. Only for *C. albicans* OY31.5 was a difference of >0.3 $\log_{10}$ CFU/ml observed at 24 h. For this isolate, a difference of 0.7 $\log_{10}$ CFU/ml was detected between shaken and nonshaken samples of fluconazole. Against this isolate, slightly fewer CFU/milliliter were noted with the agitated sample than with the nonshaken sample.

**Inoculum.** The maximum change in $\log_{10}$ CFU/milliliter observed over the study period for each isolate is presented in Table 3. The control growth curves for the low and medium starting inocula paralleled each other until the 12-h time point for each of the isolates (Fig. 2). By 24 h, the curves had converged to a common maximal fungal concentration. Control curves for the highest inocula exhibited minimal increases in CFU/milliliter, generally less than 1 $\log_{10}$ unit. Rather, colony counts remained relatively constant at levels near the concentration of maximal sustainable growth throughout the study period. The level of maximal sustainable growth varied among the test isolates and ranged from approximately $10^6$ CFU/ml for *C. albicans* 90028 to $10^8$ CFU/ml for *C. glabrata* 582.

Fungistatic activity (<99.9% reduction in CFU/milliliter compared to the control value) was observed with fluconazole against test isolates at each of the inocula examined (Fig. 2). A slight increase in CFU/milliliter over the starting inoculum was noted for each of the isolates. This elevation in CFU above the starting inoculum was greatest for *C. glabrata* 350 and the *C. tropicalis* isolates. At the highest inoculum, exposure to fluconazole resulted in the smallest increase in CFU/milliliter from the starting inoculum; however, at this inoculum, control and fluconazole curves exhibited the least amount of separation at

TABLE 3. Maximum change in colony count from the starting inoculum for test isolates

| Isolate | Maximum change in $\log_{10}$ CFU/ml from the following starting inoculum[a]: | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Low ($5 \times 10^2$–$1 \times 10^4$ CFU/ml) | | | Medium ($>1 \times 10^4$–$1 \times 10^6$ CFU/ml) | | | High ($>1 \times 10^6$–$1 \times 10^8$ CFU/ml) | | |
| | FLU | AMB | LY | FLU | AMB | LY | FLU | AMB | LY |
| *C. albicans* 90028 | 1.3 | −2.6 | −0.7 | 0.9 | −3.4 | −1.5 | 0.4 | −5.0 | −1.3 |
| *C. albicans* OY31.5 | 1.5 | −2.5 | −0.9 | 1.2 | −3.8 | −2.1 | 0.2 | −5.1 | −3.7 |
| *C. glabrata* 350 | 4.0 | −2.7 | −0.8 | 2.3 | −4.0 | −1.6 | 1.2 | −3.8 | −1.4 |
| *C. glabrata* 582 | 2.7 | −2.7 | −0.9 | 2.2 | −3.9 | −2.1 | 1.1 | −3.4 | −1.0 |
| *C. tropicalis* 2697 | 3.6 | −2.1 | −1.1 | 2.1 | −3.4 | −1.7 | 0.7 | −3.8 | −3.8 |
| *C. tropicalis* 38289 | 3.4 | −2.0 | −1.3 | 2.2 | −3.0 | −2.4 | 0.5 | −4.2 | −3.9 |

[a] FLU, fluconazole; AMB, amphotericin B; LY, LY303366.

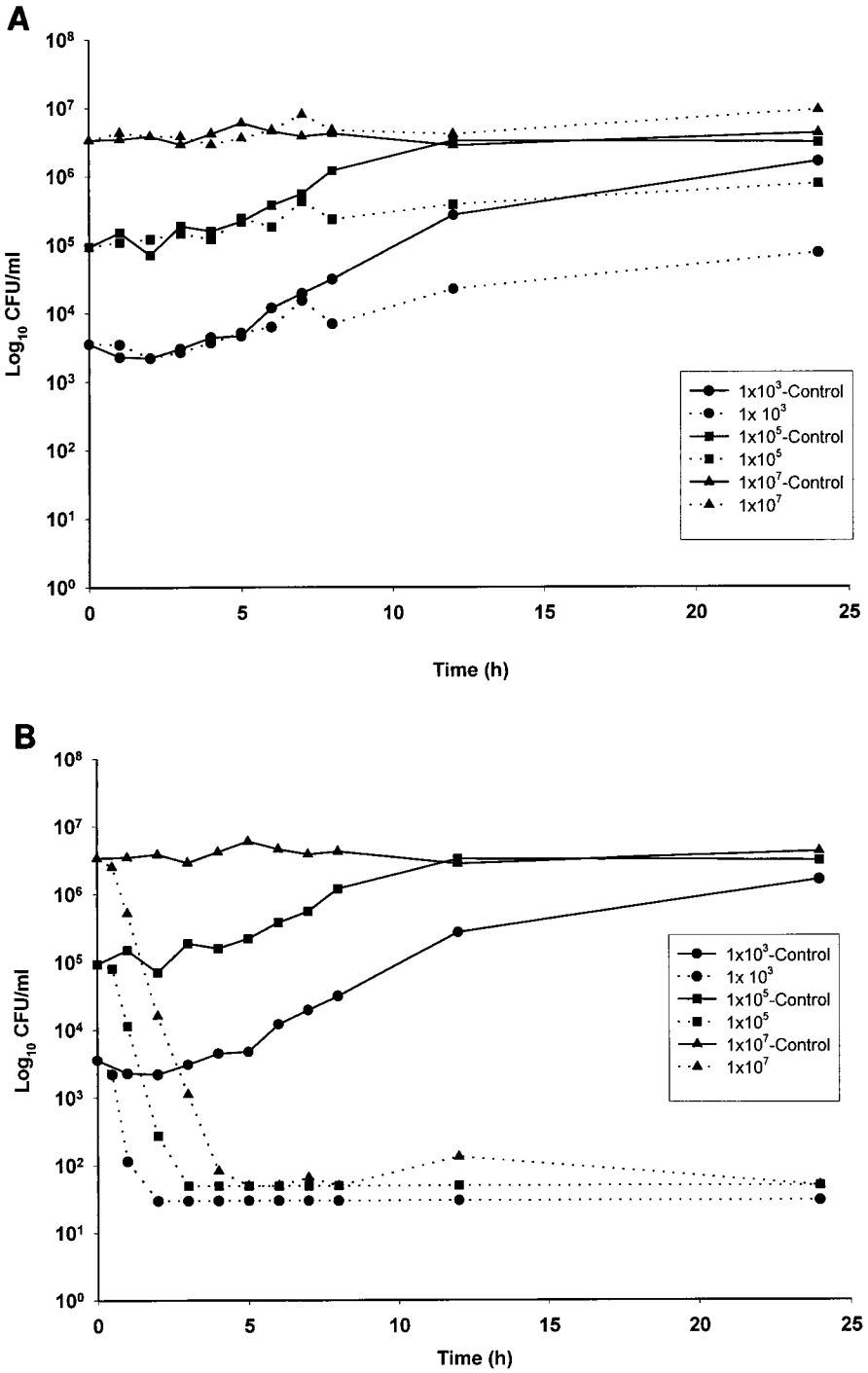


FIG. 2. Time-kill plots demonstrating the effects of starting inoculum on the activities of fluconazole (A), amphotericin B (B), and LY303366 (C) against *C. albicans* 90028.

the latter time points. In contrast, even though time-kill curves for isolates with the lower inocula exhibited both an upward trend and larger upward deviations from the starting inocula, these curves also demonstrated the greatest degree of separation between fluconazole and control curves. The observed rates of change in CFU/milliliter over the first 12 h of sampling were similar for isolates exposed to fluconazole at the two lower inocula.

Amphotericin B exhibited fungicidal activity, with a reduction in $\geq 3$ $\log_{10}$ CFU/ml compared to the starting inoculum, against each of the isolates at the two higher test inocula (Fig. 2). However, according to the sampling methods employed, we were unable to measure a 99.9% reduction in CFU/milliliter at the lowest starting inocula. For the two higher starting inocula, we were able to measure a 99.9% reduction in CFU/milliliter without concerns of approaching the lower limit of quantita-

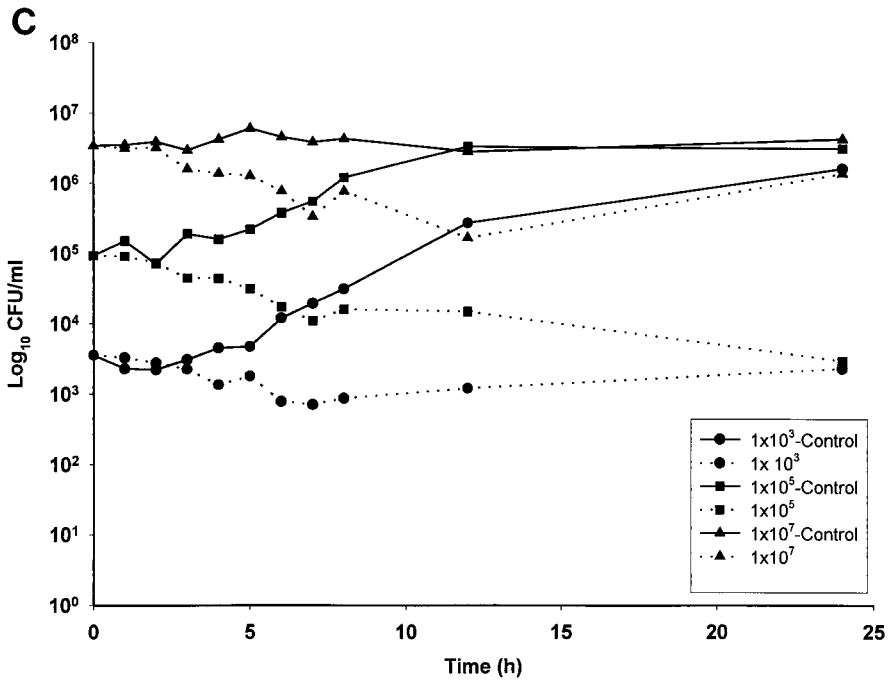


FIG. 2—*Continued.*

tion. The rates of fungicidal activity observed following exposure to amphotericin B were not dependent on the starting inoculum.

Testing with LY303366 yielded mixed fungistatic and fungicidal activity (Fig. 2). At the lower two starting inocula, fungistatic activity was exhibited against each of the isolates. Note, however, that curves resulting from the lowest inoculum demonstrated less drastic reduction in CFU/milliliter over the study period compared to curves resulting from the use of the medium inoculum. Additionally, colony counts resulting from low-inoculum samples consistently returned to or eventually exceeded starting inoculum levels by the 24-h time point. This failure to sustain antifungal activity was not present to the same degree with the two higher starting inocula. Although reductions in CFU/milliliter were observed at all inocula, fungicidal activity was exhibited by LY303366 only at the highest starting inocula and only against *C. albicans* OY31.5 and *C. glabrata* 350 and 582. For these same isolates, reductions in $\log_{10}$ CFU/milliliter observed at the lower two inocula were roughly 25 to 50% less than observed with highest inocula. For the remaining three isolates, *C. albicans* ATCC 90028 and *C. tropicalis* 2697 and 3829, LY303366 exhibited fungistatic activity at all starting inocula. Against these latter isolates, reductions in CFU/milliliter were similar for the high and medium inocula (Table 3).

## DISCUSSION

As clinical interest in fungi and antifungal therapies continue to grow, there is a pressing need to enhance our understanding of the fungicidal properties and pharmacodynamic characteristics of these agents. Despite the relative lack of data describing the use of time-kill methods for the study of fungi, we have found these techniques to be valuable in examination of antifungal dynamics. The methodology we employ for the study of antifungal agents is based upon adaptation of the procedures proposed for the time-kill evaluation of antibacterial agents (11). However, several methodological modifications to these procedures were required to facilitate the study of fungi and were based primarily upon the established guidelines for in vitro susceptibility testing of antifungals (13, 16). As a result, selection of test variables such as choice of growth medium and incubation temperature are common to both procedures. However, several test conditions specific to time-kill testing were identified as having the potential of significantly affecting test results. Therefore, the impact of variables, such as antifungal carryover, agitation, and starting inoculum, on test results are highlighted in this report.

For bacteria, the starting inoculum used for time-kill tests is similar to that recommended for in vitro susceptibility determinations, approximately $5 \times 10^5$ CFU/ml (12). Use of the same starting inoculum facilitates comparisons between MIC and time-kill data by precluding potential discrepancies resulting from an inoculum effect. However, a starting inoculum of $5 \times 10^2$ to $2.5 \times 10^3$ CFU/ml has been recommended for in vitro susceptibility testing of antifungals. Since the starting inoculum recommended for in vitro antifungal susceptibility testing is much lower than the inoculum recommendations for antibacterial time-kill testing, we felt that the following three questions needed to be answered. (i) What is the limit of quantitation for fungi? (ii) Does antifungal carryover occur with the test agents we have selected for evaluation and if so to what degree? (iii) Does an inoculum effect exist with fungi which would preclude comparisons between time-kill results and MICs if a higher starting inoculum were selected for time-kill studies?

We quickly realized that use of a starting inoculum, similar to that used for antifungal susceptibility determinations, was not viable for time-kill testing. Because of limitations imposed by the limit of quantitation, the use of a low starting inoculum affected our ability to detect a 99.9% reduction in CFU/milliliter with fungicidal agents. Additionally, with amphotericin B, if methods to lower the limit of quantitation are employed, antifungal carryover would be a concern unless samples were filtered.

Since we deemed the use of a low starting inoculum to be

unacceptable, we evaluated the possibility of using two higher starting inocula. Using a starting inoculum between $10^4$ and $10^6$ CFU/ml, we were better able to characterize the kill curves of fungicidal agents. At the highest inoculum, however, we noted that the level of maximal sustainable growth, the plateau of the growth curve, was approximately 1 $\log_{10}$ CFU/ml higher than the starting inoculum. As a result, detection of fungistatic activity with fluconazole was hindered because of the minimal separation observed between control and fluconazole curves. As a result of the limitations encountered with the highest and lowest inocula regarding the characterization of fungistatic and fungicidal activities, respectively, we recommend that a starting inoculum of approximately $10^5$ CFU/ml be employed for antifungal time-kill studies. Additionally, since the activity observed did not differ between the low and medium test inocula, MIC and time-kill results should be reflective of each other. In previous studies, we have been able to correlate MICs with observed time-kill activities of several agents including fluconazole, amphotericin B, and LY303366 (4, 7–9, 15).

In this study, we also attempted to examine the impact of sample agitation on time-kill results. Although we did not observe an appreciable difference among test samples, this may have been an artifact created by our sampling methods. Prior to the removal of each sample for colony count determination, all time-kill tubes were vortexed. In this study, colony count samples were obtained at 2, 4, 6, 8, 12, and 24 h. As a result, vortexing may have had a greater impact on results than did sample agitation. Three isolates were subsequently selected for reevaluation of the effect of agitation on results using a much less aggressive sampling schedule (data not shown). These data again failed to demonstrate an appreciable effect of agitation on results of any of the antifungal agents tested. Against some strains, however, agitation may have resulted in an increased rate of growth of control samples. Agitation did not affect the level of maximally sustainable fungal growth. Therefore, even though we did not detect a difference in results between agitated and nonagitated samples, we do recommend that time-kill samples be incubated with agitation.

Time-kill studies conducted so far have been conducted with *Candida* species and nonmucoid strains of *Cryptococcus neoformans*. We have selected these species for evaluation because of their clinical importance and because in vitro susceptibility tests were standardized primarily with these species. Prior to use in time-kill studies, we evaluated all isolates to confirm favorable growth characteristics. Strains were selected only if control cultures exhibited rapid and sustained growth and produced relatively large well-defined colonies on PDA plates. Therefore, discretion should be exercised in extrapolating time-kill methodology to mucoid strains of *C. neoformans*, filamentous fungi, and molds until methods are carefully evaluated with these organisms.

We have evaluated several time-kill variables and assessed their impact on test results. Factors such as starting inocula and sampling method can significantly influence time-kill results and/or the interpretation of results. Therefore, in an effort to minimize interstudy variation, we propose that the following procedures be adhered to when conducting antifungal time-kill studies. (i) A starting inoculum of $10^4$ to $10^6$ CFU/ml should be used. (ii) RPMI 1640 buffered to a pH of 7.0 with MOPS (or the medium used for susceptibility testing) should serve as the growth medium. (iii) Time-kill samples should be incubated at 35°C with agitation. (iv) Sampling methods should be evaluated for effect on antifungal carryover prior to implementation. (v) Sampling should continue for at least 24 h. Additionally, criteria used to describe bacterial time-kill data such as cidal ($\geq$99.9% reduction in CFU/milliliter from the starting inoculum) and static (<99.9% reduction in CFU/milliliter from the starting inoculum) activity or synergy (reduction of $\geq 2$ $\log_{10}$ CFU/ml by the combination over the most active agent alone), should employed when describing antifungal time-kill results. In our laboratory, we have found that results generated following these procedures are reproducible; however, multicenter validation of these methods should be conducted.

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