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# Effects of Incubation Temperature, Inoculum Size, and Time of Reading on Broth Microdilution Susceptibility Test Results for Amphotericin B against *Fusarium*

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**In vitro antifungal susceptibility testing for filamentous fungi remains unstandardized and is unreliable for determining adequate therapy. A study was performed to evaluate the effect of inoculum size ($10^2$, $10^3$, $10^4$, and $10^5$ conidia/ml), incubation time (48 and 72 h), and temperature (25, 30, and 35°C) on MICs of amphotericin B for *Fusarium* spp. (20 strains). The inoculum size showed the clearest effect: when the inoculum was varied from $10^2$ to $10^5$ conidia/ml, the geometric mean MICs showed increases of between 10- and 19-fold in all the combined conditions of temperature and incubation time assayed. Time of incubation had less effect (increases of between two- and threefold in approximately half of the geometric mean MICs), and temperature especially had little effect (the increases were no higher than twofold). The effects of interaction between inoculum size and temperature on MICs were not statistically significant, while the combined effects of inoculum size and time of reading and of time of reading and temperature produced systematic variation in MICs.**

The incidence of serious infections caused by opportunistic fungi continues to increase (3). Among these fungi, *Fusarium* spp. have become important as pathogens of patients with compromised immune systems (7, 10). In the treatment of fusarial infections, amphotericin B (AMB) is practically the only antifungal drug that has shown some effect. However, not all clinical isolates are susceptible. An important number of the tested strains appear to be resistant to this drug (14–16), but comparisons among the data are difficult to make because the methods for performing these tests are not standardized. Variations in key technical factors such as inoculum size, incubation temperature, and time of reading have all been cited as reasons for variability in test results within a given laboratory and between laboratories (13). It has been suggested that analysis and standardization of each test condition might increase the reproducibility of the broth dilution method for fungal susceptibility testing. Great efforts are being made to standardize the in vitro parameters to obtain reproducibility of data on clinical yeasts at inter- and intralaboratory levels (8, 9). However, the in vitro activities of antifungal drugs against filamentous fungi have not yet been studied thoroughly (3), and in the case of *Fusarium* spp., the information available is sparse (11, 12, 14, 15). The present study was performed to evaluate the effects of inoculum size, incubation time, and temperature on variations of AMB MICs for *Fusarium* isolates in a broth microdilution method.

## MATERIALS AND METHODS

**Test organisms.** Twenty strains of *Fusarium* were used in the study (10 from clinical sources and 10 from environmental sources); these included *Fusarium oxysporum* ($n = 8$), *F. verticillioides* ($n = 6$), *F. solani* ($n = 2$), *F. dimerum* ($n = 1$), *F. proliferatum* ($n = 2$), and *F. equiseti* ($n = 1$). *Paecilomyces variotti* ATCC 36257 was included as the control.

**Medium.** RPMI 1640 medium powder with L-glutamine and without sodium bicarbonate (GIBCO BRL, Life Technologies, IZASA) was used. The medium was buffered to pH 7.0 morpholinepropanesulfonic acid (0.165 M; MOPS; Sigma Chemical Co.).

**Antifungal drug.** MICs of AMB were determined by a broth microdilution test. Fungizone (E. R. Squibb & Sons), the commercial preparation of AMB, was used, and stock solutions of 1,000 μg/ml were prepared with sterile distilled water. The drug dilutions were performed by using the recommendations of the National Committee for Clinical Laboratory Standards standard additive twofold drug dilution schema to reduce pipetting errors. The drug dilutions were prepared with sterile distilled water to be 10 times the strength of the final drug concentration and were further diluted 1:5 with RPMI medium to provide the 2× strength needed for the test. The broth microdilution test was performed in sterile, flat-bottomed 96-well microplates (Greiner Labortechnik). Aliquots (100 μl) of the 2× drug concentrations were dispensed into the wells of the microdilution plates with a multichannel pipette. Well 12 of each row served as the growth control. The microdilution plates were stored at −20°C until used.

**Preparation of inoculum suspension.** The isolates were maintained as a suspension in water at 4°C until testing was performed. For each experiment, strains were subcultured onto potato dextrose agar slants at 30°C for 15 days. The inoculum was prepared by removing the sporulated fungi from the agar slant with a loop and suspending them in 10 ml of sterile water. The fungal suspension was filtered once through sterile gauze to remove hyphae and was inspected microscopically. This resulting suspension contained >90% conidia. The conidial suspension was then concentrated by centrifugation and manually counted with a hemacytometer. The hemacytometer counts were verified by enumeration of colonies per milliliter of serial dilution on Sabouraud's dextrose agar plates that were incubated at 30°C for 48 h. The suspensions were then diluted in sterile distilled water to produce four different working suspensions ($10^6$, $10^5$, $10^4$, and $10^3$ conidia per ml). These working suspensions were further diluted 1:5 with RPMI medium to obtain 2× final suspensions.

**Broth microdilution test.** Twelve microdilution plates were necessary to evaluate the effects of four inoculum concentrations and three different temperatures on a panel of eight strains. Each well of the microdilution plates was inoculated on the day of the test with 100 μl of the corresponding inoculum suspension. This step brought the drug dilutions to the final test concentrations of 0.07 to 36.9 μg of AMB per ml and inoculum densities of $10^5$, $10^4$, $10^3$, and $10^2$ conidia per ml. Growth control wells (100 μl of inoculum suspension and 100 μl of drug-free medium) were included for each isolate tested. One well of the microdilution plates containing 100 μl of uninoculated drug-free medium was included as a sterility control. The control organism was handled in the same manner and was tested each time that a test was performed. The microdilution plates were incubated without agitation at 25, 30, and 35°C. Readings were made with the aid of a reading mirror at 48 and 72 h of incubation. The MIC was defined as the lowest drug concentration at which there was an absence of growth.

**Analysis.** Both on-scale and off-scale results were included in the analysis. The high off-scale MICs (>36.9 μg/ml) were converted to the next highest concentration (73.8 μg/ml), and the low off-scale MICs (≤0.07 μg/ml) were left unchanged (0.07 μg/ml). When skips (uneven patterns) were present, the MIC endpoint was the higher drug concentration. The data were analyzed by means

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TABLE 1. Geometric mean MICs of AMB at different inoculum concentrations, temperatures, and reading times

| Inoculum (conidia/ml) | MIC (μg/ml) at[a]: | | | | | |
|---|---|---|---|---|---|---|
| | 25°C | | 30°C | | 35°C | |
| | 48 h | 72 h | 48 h | 72 h | 48 h | 72 h |
| $10^2$ | 1.24 (1.01–1.52) | 1.75 (1.40–2.18) | 1.52 (1.29–1.79) | 2.47 (1.87–3.26) | 1.88 (1.52–2.32) | 2.66 (2.07–3.4) |
| $10^3$ | 1.94 (1.54–2.45) | 3.38 (2.33–4.9) | 2.23 (1.70–2.91) | 4.61 (3.16–6.74) | 2.31 (1.78–2.98) | 4.95 (3.56–6.9) |
| $10^4$ | 4.3 (2.76–6.72) | 10.62 (6.37–17.69) | 6.1 (4.35–9.21) | 17.22 (10.65–27.84) | 5.5 (3.71–8.14) | 15.52 (9.28–25.96) |
| $10^5$ | 16.07 (9.97–25.9) | 31.05 (19.66–49.03) | 19.14 (12.17–30.1) | 47.1 (31.64–70.1) | 19.14 (12.17–30.1) | 43.95 (30.14–64.09) |

[a] Figures in parentheses are 95% confidence intervals.

of multifactorial analysis of variance applied to a 4 by 3 by 2 (inoculum by temperature by time of reading) design with repeated measures (2). Previously, the data were transformed by taking logarithms. The means of the log data were back-transformed (antilog) to get the geometric mean. When sphericity conditions were not satisfied, a multivariate test based on the statistic Wilks's lambda was performed. The interactions were studied by means of simple effects analysis. Polynomial contrasts were used to analyze the MICs obtained with different inocula and temperatures.

## RESULTS

Table 1 summarizes the MICs against 20 strains of *Fusarium* obtained at different inoculum concentrations, temperatures, and reading times. The inoculum sizes, both at 48 h and at 72 h, affected the MICs markedly. When the starting fungal inoculum was varied from $10^2$ to $10^5$ conidia/ml, the geometric mean MICs increased in all cases between 10- and 19-fold. At 72 h in comparison with 48 h, all the geometric mean MICs were moderately increased, being between two- and threefold higher in approximately half of the cases. On the other hand, variations higher than twofold were not observed in any case when the temperature changed from 25 to 30°C or from 30 to 35°C. This happened when four different inocula and two incubation times were assayed.

The analysis of variance applied to the study of the combined effect of the three factors on MICs showed that the effects of interaction between inoculum size and temperature on MIC were not statistically significant. On the other hand, first-order interactions between inoculum size and time of incubation ($\lambda_{(3,17)} = 0.323$, $P = 0.0005$) and between incubation time and temperature ($F_{(2,38)} = 8.88$, $P = 0.001$) were shown. Since the interactions between these factors were statistically significant, the effects of them on MICs do not have a simple interpretation, because the effect of each depends upon the level of the other factor.

The differences between the MICs obtained with the different inoculum sizes were always statistically significant at both times of reading (Fig. 1). The reading time had a differentiated effect at 48 and 72 h on MICs when the inoculum size increased. At 48 h, the increase in the geometric mean MICs was higher than 11-fold when the inoculum concentration was increased from $10^2$ to $10^5$ conidia/ml. At 72 h, under the same conditions, the increase was even higher: reading values which surpassed 17-fold. These changes in both cases showed a cubic trend represented by a curve which rose twice.

Time of incubation also conditioned the changes observed in MICs when the temperature was increased (Fig. 2). In this case, we observed the major increment when the temperature changed from 25 to 30°C at 72 h, although the means were statistically different at each time of reading. A more important effect was observed when the values obtained after 48 and 72 h of incubation were compared. In these conditions, approximately a twofold increase in the geometric mean MIC was observed both at 30°C and at 35°C.

## DISCUSSION

The goal of this study was to examine the influence of several variables on the broth microdilution MICs of AMB against 20 isolates of *Fusarium* spp. There is little published information regarding systematic differences in antifungal test results for filamentous fungi under different test conditions. A collaborative study was recently performed with different species of filamentous fungi, although *Fusarium* spp. were not included in it (4). That study was an important step on the way to the development of a reference method for antifungal susceptibility testing of the filamentous fungi and also to demonstrating that such development is possible. However, further studies with larger numbers of fungal isolates and with other species will be needed to evaluate the effect of the most important technical aspects on the MIC and to evaluate in vitro and in vivo correlations of drug efficacy. In the study by Espinel-Ingroff et al. (4), who used RPMI 1640, a temperature of 35°C, and a final test inoculum of $10^4$ CFU/ml, a good agreement between the broth macro- and microdilution methods was found. In a previous study with *Fusarium* spp., we reached a similar conclusion about the comparison of both methods

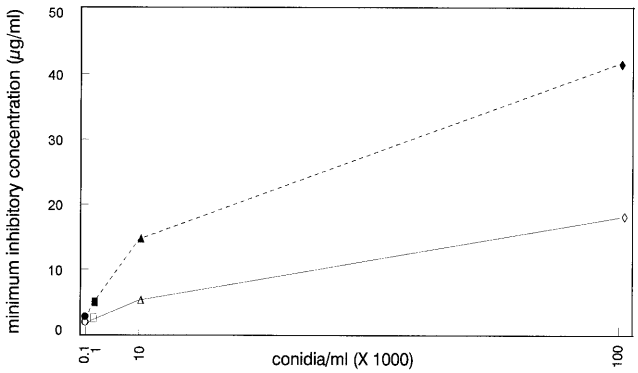


FIG. 1. Effects of inoculum on MICs of amphotericin B against 20 isolates of *Fusarium* at 48 h (——) and 72 h (– – –) of incubation. Each point represents the geometric mean of MICs at three temperatures. The concentrations (in micrograms per milliliter) at 48 h were 1.52 (○), 2.15 (□), 5.25 (△) and 18.03 (◇); those at 72 h were 2.25 (●), 4.26 (■), 14.16 (▲), and 40.09 (◆).

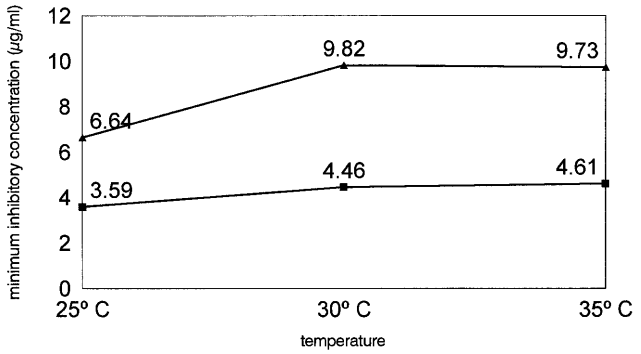


FIG. 2. Effects of temperature and time of incubation on geometric mean MICs of amphotericin B against 20 isolates of *Fusarium*. Each point represents the geometric mean of all inocula. The incubation times were 48 h (■) and 72 h (▲).

(11). We used the microdilution test because it is cheaper and simpler.

The inoculum size has been considered to be a critical variable in the development of susceptibility methods for bacteria (17), yeasts (13), and filamentous fungi (5), but some reports also demonstrate a lack of effect of inoculum concentrations in yeasts (1, 6) or even in filamentous fungi such as in *Fusarium* spp. (12). In our study, we have demonstrated a marked influence of this factor on MICs. With the final inoculum suggested by Espinel-Ingroff et al. (4) ($10^4$ CFU/ml), 35°C, and 48 h of incubation, we obtained a mean MIC of 5.5 μg/ml, which means an increase of approximately threefold in comparison to that obtained, under the same conditions, with an inoculum of $10^2$ conidia/ml (1.88 μg/ml).

In this work we have demonstrated that the combined effect of inoculum size and temperature on MICs is not significant. Therefore, we consider that when testing filamentous fungi, and especially *Fusarium* spp., the temperature of incubation should preferably be 25 or 30°C, because those fungi grow better at such temperatures than at higher ones, so the reading of results is easier.

Several studies on antifungal susceptibilities of *Fusarium* spp. have tested a representative number of strains (12, 14, 15), but they used different methods, media, temperatures of incubation, incubation times, medium pH, scoring system, etc., and therefore the results are difficult to compare. In a similar and recently performed study, Gehrt et al. (5) evaluated the effect of increasing inoculum sizes on filamentous fungi, among which they included three strains of *F. solani*. The mean MICs of AMB obtained against these strains in this study exhibited only a slight increase across the range of inocula ($10^2$ to $10^5$ conidia/ml), ranging from 1.17 μg/ml for the $10^2$ conidia/ml inoculum to 2.33 μg/ml for $10^5$ conidia/ml, while in our case, at the same temperature (35°C) and incubation time (48 h), the increase was much more marked (from 1.88 to 19.14 μg/ml). This significant difference between the two studies, despite the almost identical experimental conditions, could be explained by the very variable response of the different *Fusarium* strains to AMB. For example, in the present study, the range of MICs at 35°C with an inoculum size of $10^5$ conidia/ml was 4.62 to >36.9 μg/ml. With the other fungi tested, Gehrt et al. (5) obtained a more marked increase in MICs with increasing inoculum. In the same study, MICs against *F. solani* at 24 h of incubation were also provided; however, in our study, visible growth was not observed after 24 h of incubation. In the other studies in which *Fusarium* spp. were tested, the first reading was performed at 48 h of incubation.

In the present study, the MICs were also significantly affected by the duration of incubation. The incubation time also affected MICs in the study by Gehrt et al. (5), although *F. solani* was minimally affected.

In our study, we have emphasized the critical influence of the variables inoculum and incubation time, and under some conditions the temperature, on microdilution susceptibility testing of AMB against *Fusarium* spp. Perhaps these, together with the method of endpoint determination, are the most important variables to resolve before antifungal testing can be standardized. In susceptibility testing of yeasts, important progress has been made in recent years, and the methodology proposed (8, 9), with minor adaptations, can be generalized for several species. In the case of filamentous fungi, the differences in the type of reproductive structures between genera and even between species of a same genus and the different growth conditions required probably make it necessary to develop different methods for each particular fungal group. The tendency of the first steps made in the development of a reproducible test for filamentous fungi is toward the use of conidia, probably because they are much easier to work with. However, considering that hyphae are the predominant and practically the exclusive form found in the tissues of an infected individual, the relevance of the data obtained by testing conidial growth compared to that obtained by testing hyphal growth is arguable.

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