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# Comparison of a Spectrophotometric Microdilution Method with RPMI–2% Glucose with the National Committee for Clinical Laboratory Standards Reference Macrodilution Method M27-P for In Vitro Susceptibility Testing of Amphotericin B, Flucytosine, and Fluconazole against *Candida albicans*

JUAN L. RODRÍGUEZ-TUDELA,[1]* JUAN BERENGUER,[2] JOAQUÍN V. MARTÍNEZ-SUÁREZ,[1] AND ROSARIO SANCHEZ[2]

*Unidad de Micología, Centro Nacional de Microbiología, Instituto de Salud Carlos III, 28220 Majadahonda,[1] and Servicio de Microbiología Clinica y Enfermedades Infecciosas, Hospital General Gregorio Marañon,[2] Madrid, Spain*

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**The National Committee for Clinical Laboratory Standards has proposed a reference broth macrodilution method for in vitro antifungal susceptibility testing of yeasts (the M27-P method). This method is cumbersome and time-consuming and includes MIC endpoint determination by visual and subjective inspection of growth inhibition after 48 h of incubation. An alternative microdilution procedure was compared with the M27-P method for determination of the amphotericin B, flucytosine, and fluconazole susceptibilities of 8 American Type Culture Collection strains (6 of them were quality control or reference strains) and 50 clinical isolates of *Candida albicans*. This microdilution method uses as culture medium RPMI 1640 supplemented with 18 g of glucose per liter (RPMI–2% glucose). Preparation of drugs, basal medium, and inocula was done by following the recommendations of the National Committee for Clinical Laboratory Standards. The MIC endpoint was calculated objectively from the turbidimetric data read at 24 h. Increased growth of *C. albicans* in RPMI–2% glucose and its spectrophotometric reading allowed for the rapid (24 h) and objective calculation of MIC endpoints compared with previous microdilution methods with standard RPMI 1640. Nevertheless, good agreement was shown between the M27-P method and this microdilution test. The MICs obtained for the quality control or reference strains by the microdilution method were in the ranges published for those strains. For clinical isolates, the percentages of agreement were 100% for amphotericin B and fluconazole and 98.1% for flucytosine. These data suggest that this microdilution method may serve as a less subjective and more rapid alternative to the M27-P method for antifungal susceptibility testing of yeasts.**

The frequency of serious fungal infections, especially yeast infections, is rising. This increasing incidence has been attributed to several factors, including the use of cytotoxic and immunosuppressive drugs, the prevalence of human immunodeficiency virus type 1 infection, and the widespread use of newer and more powerful antibacterial agents (2–4).

Although progress in standardizing antifungal susceptibility testing has been considerable as a result of the development and publication of a reference method by the National Committee for Clinical Laboratory Standards (NCCLS), Villanova, Pa. (the M27-P method) (11), several problems remain unsolved.

Most publications (5–7, 14, 15) have reported equivalence between the reference method (the M27-P method) (11) and the more user-friendly microdilution format to which it was adapted. However, this microdilution adaptation of the broth macrodilution method described in NCCLS document M27-P (11) also has drawbacks. The use of plain RPMI 1640 with a glucose concentration of 0.2% along with the small recommended inoculum ($0.5 \times 10^3$ to $2.5 \times 10^3$ CFU/ml) precludes the optimal growth of several species and/or strains of yeasts (12, 18, 19). Furthermore, the subjective visual assessment of endpoints according to a +1 to +4 scale is another handicap for standardization (7).

In a preliminary study (18) we demonstrated that, compared with the use of standard RPMI 1640 broth alone, the use of this medium supplemented with 18 g of glucose per liter (RPMI–2% glucose) facilitated reading of growth endpoints at 24 h without significantly affecting the MICs themselves. After comparing the RPMI–2% glucose broth microdilution method with the standard M27-P method (11), with good results (8), we proved that plain RPMI 1640 precluded the determination of the $MIC_{80}$ (lowest drug concentration giving rise to an inhibition of growth equal to or greater than 80% of that of the drug-free control) as the NCCLS recommended for endpoint estimation of azole drugs (11). The optical density of the so-called "trailing effect" produced by many *Candida albicans* strains was very close to the 80% inhibition optical density (OD) value, and therefore a less stringent criterion, e.g., a 50% transmission inhibitory concentration, as described by Galgiani and Stevens (9), should be used. However, the use of RPMI–2% glucose produced the same MIC, whatever method, $MIC_{80}$ or 50% transmission inhibitory concentration determination,

* Corresponding author. Mailing address: Unidad de Micología, Centro Nacional de Microbiología, Instituto de Salud Carlos III, Ctra. Majadahonda-Pozuelo, km. 2, 28220 Majadahonda, Madrid, Spain. Phone: 34 1 5097961. Fax: 34 1 5097966. Electronic mail address: jlrodrgz@isciii.es.

was used (19). Two additional studies showed good intra- and interlaboratory reproducibilities for this method (16, 21). Furthermore, a good correlation with clinical outcome could be shown for ketoconazole and fluconazole (21). Other advantages are obvious, such as an earlier and objective MIC endpoint determination (at 24 h by the spectrophotometric method versus 48 h by the visual method), simplified by the large difference between the OD of the control growth and the OD of inhibited cultures, the fact that a dose-response curve could be obtained for each pair of strain-antifungal agent tested, and a good correlation with clinical outcome (21). Furthermore, a recent report by Odds et al. (12) indicated the clear advantages for antifungal susceptibility testing when RPMI–2% glucose medium and a larger inoculum were used.

We have demonstrated a good equivalence between our method and a microdilution technique with plain RPMI 1640 (16, 18, 19). Nevertheless, a mandatory step for validation of this spectrophotometric method with RPMI–2% glucose is its comparison with the reference method established by the NCCLS (11). In a previous study (8), we compared both methods, but only with fluconazole. The aim of the present study was to expand the previous experience with fluconazole and to test two additional antifungal drugs, amphotericin B and flucytosine, frequently used to treat human mycoses.

## MATERIALS AND METHODS

**Test organisms.** Six American Type Culture Collection (ATCC) strains, *C. krusei* ATCC 6258, *C. parapsilosis* ATCC 22019, *C. parapsilosis* ATCC 90018, *C. albicans* ATCC 90028, *C. albicans* ATCC 24433, and *C. tropicalis* ATCC 750, for which reference MIC ranges for amphotericin B, flucytosine, and fluconazole are well defined (13), were tested five times on different days by the spectrophotometric method with RPMI–2% glucose. Furthermore, two additional ATCC strains, *C. albicans* ATCC 64550 (*C. albicans* AD), a well-known azole-resistant strain (22), and *C. albicans* ATCC 64548 (1), a susceptible strain with a minimal trailing effect, were also evaluated 20 times on different days.

In addition, 50 clinical isolates of *C. albicans* from 50 different AIDS patients with oral candidiasis were also tested by both methods. Eighteen of these 50 isolates were isolated from prospectively studied patients who either failed or responded to fluconazole therapy. They are described elsewhere (21).

Each isolate was maintained in water solution at ambient temperature in the laboratory until testing was performed.

**Antifungal agents.** The following antifungal agents (from the indicated manufacturers) were used in the study: amphotericin B (Squibb Industria Farmaceutica S.A., Madrid, Spain), flucytosine (Productos Roche, S.A., Madrid, Spain), and fluconazole (Pfizer S.A., Madrid, Spain). The drugs were provided as standard powders of known potency. Amphotericin B and fluconazole were dissolved in 100% dimethyl sulfoxide (Sigma Aldrich Quimica, S.A., Madrid, Spain). Flucytosine was dissolved in sterile distilled water. All stock solutions were frozen at −70°C as 100× stocks until they were used.

**NCCLS macrodilution reference method (M27-P method).** The broth macrodilution test was performed by following the guidelines of the proposed reference method for yeasts, NCCLS document M27-P (11). The medium used to prepare the 10× drug dilutions and inoculum suspension was liquid RPMI 1640 with L-glutamine and 0.165 M morpholinopropanesulfonic acid (MOPS) without sodium bicarbonate (Bio-Whittaker, Walkersville, Md.). Inocula of the yeast suspensions were prepared by the spectrophotometric procedure and ranged from $0.5 \times 10^3$ to $2.5 \times 10^3$ CFU per ml. The yeast inoculum (0.9 ml) was added to each test tube that contained 0.1 ml of a 10× concentration of one of each of the antifungal agents used. The final concentrations of amphotericin B and flucytosine ranged from 16.0 to 0.03 μg/ml, and that of fluconazole ranged from 64.0 to 0.12 μg/ml. The growth control tube(s) contained a 0.9-ml volume(s) of an inoculum suspension and a 0.1-ml volume(s) of drug-free medium. In addition, 1 ml of uninoculated, drug-free medium was included as a sterility control.

Broth macrodilution MICs were determined after 48 h of incubation at 35°C. For amphotericin B, the MIC was read as the lowest drug concentration that prevented any discernible growth. For flucytosine and fluconazole, MICs were determined by visually comparing the turbidity of each MIC tube with an 80% inhibition standard (1:5 dilution of the corresponding growth control) (11). $MIC_{80}$ endpoints by the macrodilution method were the lowest drug concentrations with a turbidity less than or equal to that of the standard. *C. krusei* ATCC 6258, *C. albicans* ATCC 90028, *C. albicans* ATCC 64550, and *C. albicans* ATCC 64548 were used as controls in this susceptibility test.

**Microdilution spectrophotometric $MIC_{80}$ method with RPMI–2% glucose.** RPMI–2% glucose, an improved medium that facilitates the reading of growth inhibition by azole drugs (12, 18, 19), was used in all microdilution tests. Preparation of RPMI–2% glucose has previously been described in detail (10).

A starting inoculum of $10^6$ CFU/ml was prepared by the spectrophotometric method recommended by the NCCLS (11). The adjusted yeast suspensions were diluted 1:10 with RPMI–2% glucose medium, and microtiter plates were inoculated with this dilution by using an automatic pipettor programmed to dispense 10 μl into each well to obtain a final inoculum of approximately $10^4$ CFU/ml (6). The final concentrations of amphotericin B and flucytosine ranged from 16.0 to 0.03 μg/ml, and those of fluconazole ranged from 64.0 to 0.12 μg/ml. The inoculated plates were incubated at 35°C for 24 h in a humid atmosphere. After agitation of the plates with a microtiter plate shaker for 5 min (1), spectrophotometric readings were performed with a Mios Merck automatic plate reader (Merck Igoda, S.A., Madrid, Spain) set at 630 nm for amphotericin B and at 405 nm for flucytosine and fluconazole. For amphotericin B the MIC endpoint was considered the lowest drug concentration with no growth (OD < 0.1). For flucytosine and fluconazole, the MIC endpoint was calculated as the lowest drug concentration giving rise to an inhibition of growth equal to or greater than 80% of that of the drug-free control ($MIC_{80}$), similar to the visual endpoint criterion recommended by the NCCLS (11). All ATCC strains described above were evaluated by this spectrophotometric method.

**Analysis of results.** The microdilution MICs obtained by the RPMI–2% glucose microdilution method at 24 h were compared with the MICs obtained by the NCCLS reference macrodilution method at 48 h. Both on-scale and off-scale MIC results were included in the analysis. The high off-scale MICs (>16.0 or >64.0 μg/ml) were converted to the next highest concentration (32.0 or 128.0 μg/ml, respectively), and the low off-scale MICs (≤0.03 or ≤0.12 μg/ml) were left unchanged. Discrepancies among MIC endpoints of no more than 2 dilutions (two tubes or wells) were used to calculate the percent agreement.

## RESULTS

The MICs of the three antifungal agents obtained by the NCCLS macrodilution method for *C. krusei* ATCC 6258, *C. albicans* ATCC 90028, *C. albicans* ATCC 64550, and *C. albicans* ATCC 64548 were in the reference range (13) indicated in Table 1.

Table 1 summarizes the MICs determined by the spectrophotometric method with RPMI–2% glucose for eight ATCC strains (six of them were quality control or reference isolates) (13). The equivalence between both methods was excellent when the two recommended quality control strains (13) and *C. albicans* ATCC 24433 were compared.

As expected, some discrepancies were detected with reference strains, and they are indicated in Table 1. Three MIC determinations for amphotericin B, for *C. albicans* ATCC 90028 and *C. tropicalis* ATCC 750, fell outside the range (0.25 μg/ml). For *C. tropicalis* ATCC 750 and flucytosine, all MIC determinations fell outside the reference range. For fluconazole and *C. tropicalis* ATCC 750, two MIC determinations (both 0.25 μg/ml) were outside the range. In addition, a fluconazole MIC of 2.0 μg/ml for *C. parapsilosis* ATCC 90018 was also outside the reference range. The remaining MICs obtained for the reference strains were in the published range (13) and are presented in Table 1.

Tables 2, 3 and 4 provide the MICs of the three tested antifungal agents obtained by both methods. The comparison in each table is strain by strain. An excellent correlation was shown for amphotericin B and fluconazole because the percent agreement was 100%. For flucytosine, 98.1% agreement was obtained. For one strain (isolate 47) the flucytosine MIC was 1.0 μg/ml by the NCCLS method but 0.12 μg/ml by the RPMI–2% glucose method (Table 3). Furthermore, when for calculation of the percent agreement a discrepancy of no more than one twofold dilution was used, the results were as follows. For amphotericin B, the percent agreement was the lowest obtained, being 84%. For 47 (97%) isolates, the flucytosine and fluconazole MICs were the same or differed by one twofold dilution. In addition, strains for which fluconazole MICs were higher (Table 4, isolates 37 to 50) were detected by both methods. For all of the strains the MICs were the same or differed by one twofold dilution.

TABLE 1. Comparison of broth macrodilution MICs determined by the reference method (M27-P method) and an alternative microdilution test with RPMI–2% glucose against quality control, reference, and other ATCC strains

| Organism[f] | Antifungal agent | MIC (μg/ml) | |
|---|---|---|---|
| | | Reference range[a] | RPMI–2% glucose method |
| *C. krusei* ATCC 6258 (quality control strain) | Amphotericin B | 0.5–2.0 | 0.5–1.0[b] |
| | Flucytosine | 4.0–16.0 | 4.0–8.0[b] |
| | Fluconazole | 16.0–64.0 | 32.0–64.0[b] |
| *C. parapsilosis* ATCC 22019 (quality control strain) | Amphotericin B | 0.25–1.0 | 0.5–0.5[b] |
| | Flucytosine | 0.12–0.5 | 0.25–0.25[b] |
| | Fluconazole | 2.0–8.0 | 2.0–4.0[b] |
| *C. albicans* ATCC 24433 (reference strain) | Amphotericin B | 0.25–1.0 | 0.25–0.5[b] |
| | Flucytosine | 1.0–4.0 | 1.0–2.0[b] |
| | Fluconazole | 0.25–1.0 | 0.5–1.0[b] |
| *C. albicans* ATCC 90028 (reference strain) | Amphotericin B | 0.5–2.0 | 0.25 (3[c])–0.5[b] |
| | Flucytosine | 0.5–2.0 | 0.5–1.0[b] |
| | Fluconazole | 0.25–1.0 | 0.25–1.0[b] |
| *C. tropicalis* ATCC 750 (reference strain) | Amphotericin B | 0.5–2.0 | 0.25 (3)–1.0[b] |
| | Flucytosine | ≤0.12–0.25 | 0.5 (1)–1.0 (4)[b] |
| | Fluconazole | 1.0–4.0 | 0.25 (2)–4.0[b] |
| *C. parapsilosis* ATCC 90018 (reference strain) | Amphotericin B | 0.5–2.0 | 0.5–1.0[b] |
| | Flucytosine | ≤0.12–0.25 | 0.06–0.25[b] |
| | Fluconazole | 0.25–1.0 | 1.0–2.0 (1)[b] |
| *C. albicans* ATCC 64550 (22) | Amphotericin B | NA[d] | 0.25–1.0[e] |
| | Flucytosine | NA | 0.5–2.0[e] |
| | Fluconazole | NA | 16.0–128.0[e] |
| *C. albicans* ATCC 64548 (1) | Amphotericin B | NA | 0.12–1.0[e] |
| | Flucytosine | NA | 0.06–0.25[e] |
| | Fluconazole | NA | 0.25–0.5[e] |

[a] The reference range was established by the broth macrodilution method (the NCCLS M27-P method) (13).
[b] Five determinations for the three antifungal agents on different days.
[c] Values in parentheses are the number of MIC determinations outside the reference range.
[d] NA, not available.
[e] Twenty determinations for the three antifungal agents on different days.
[f] Numbers in parentheses are reference numbers.

Eighteen strains isolated from 18 AIDS patients with oropharyngeal candidiasis have been described elsewhere (21). Nine of them did not respond to fluconazole, being well-documented cases of clinical failure. For those strains the fluconazole MICs were ≥2.0 μg/ml by the NCCLS macrodilution method and ≥4.0 μg/ml by the spectrophotometric method with RPMI–2% glucose. For eight of the nine strains the fluconazole MICs were ≥8.0 μg/ml by both methods. On the other hand, nine AIDS patients with oropharyngeal candidiasis were well-documented cases of clinical success with fluconazole. For the strains from those patients, fluconazole MICs were ≤0.5 μg/ml by the NCCLS macrodilution method and ≤0.25 μg/ml by the spectrophotometric method with RPMI–2% glucose. No clinical data were available for the patients from whom the remaining isolates were obtained.

## DISCUSSION

Despite the introduction in 1992 by NCCLS of a proposed standard method for antifungal susceptibility testing (11), several unresolved drawbacks remained. The search for alternative methods has been constant, and several investigators (5–7, 14, 15) have published descriptions of microdilution methods comparable to the macrodilution technique recommended by NCCLS (11). The most popular of these microdilution methods, although in a more user-friendly format, is a reduced copy of the macrodilution format, but it had problems similar to those of the macrodilution format, such as visual endpoint determination, a small starting inoculum, and a long incubation period (48 h) (17).

The use of this method (7) in our laboratory indicated that the growth of many strains was suboptimal when the OD was

TABLE 2. Comparison of amphotericin B MICs obtained by NCCLS macrodilution method (M27-P method) with those obtained by microdilution with RPMI–2% glucose

| Isolate no. (no. of isolates) | MIC (μg/ml) | |
|---|---|---|
| | NCCLS (M27-P) | RPMI–2% glucose |
| 1 (1) | 0.06 | 0.12 |
| 2 (1) | 0.06 | 0.25 |
| 3, 4 (2) | 0.12 | 0.12 |
| 5 (1) | 0.12 | 0.25 |
| 6-8 (3) | 0.12 | 0.5 |
| 9, 10 (2) | 0.25 | 0.12 |
| 11 (1) | 0.25 | 0.25 |
| 12-18 (7) | 0.25 | 0.5 |
| 19, 20 (2) | 0.5 | 0.12 |
| 21-23 (3) | 0.5 | 0.25 |
| 24-33 (10) | 0.5 | 0.5 |
| 34, 35 (2) | 0.5 | 1.0 |
| 36, 37 (2) | 1.0 | 0.25 |
| 38-49 (12) | 1.0 | 0.5 |
| 50 (1) | 1.0 | 1.0 |

TABLE 3. Comparison of flucytosine MICs obtained by NCCLS macrodilution method (M27-P method) with those obtained by microdilution with RPMI–2% glucose

| Isolate no. (no. of isolates) | MIC (μg/ml) | |
|---|---|---|
| | NCCLS (M27-P) | RPMI–2% glucose |
| 1 (1) | 0.06 | 0.12 |
| 2-5 (4) | 0.12 | 0.06 |
| 6-22 (17) | 0.12 | 0.12 |
| 23 (1) | 0.12 | 0.25 |
| 24-42 (19) | 0.25 | 0.12 |
| 43, 44 (2) | 0.25 | 0.25 |
| 45 (1) | 0.25 | 1.0 |
| 46 (1) | 0.5 | 0.25 |
| 47 (1) | 1.0 | 0.12 |
| 48 (1) | 1.0 | 0.25 |
| 49 (1) | 1.0 | 0.5 |
| 50 (1) | 32.0 | 32.0 |

monitored by reading with a spectrophotometer (18, 19). Therefore, we developed a new microdilution method based on the standard broth macrodilution yeast susceptibility test method, but with several differences: (i) the same RPMI 1640 medium recommended by the NCCLS (11) was used, but it was supplemented with 18 g of glucose per liter (RPMI–2% glucose) (18), (ii) an inoculum of $10^4$ CFU/ml was used, and (iii) the microdilution plates were agitated before reading the results (1) and the MIC endpoint was estimated by spectrophotometric reading at 24 h.

We compared the fluconazole MICs for *C. albicans* by both methods, with very good results (8). In the present study we tested eight ATCC strains five times on different days. Six of them were the quality control or reference strains recommended by the NCCLS (13). The remaining two strains were *C. albicans* ATCC 64548, a susceptible strain (1) with a minimal trailing effect, and *C. albicans* 64550 (*C. albicans* AD), a well-known azole-resistant strain (22). When the MICs of the three antifungal agents tested for *C. krusei* ATCC 6258, *C. parapsilosis* ATCC 22019, and *C. albicans* ATCC 24433 obtained by both methods were compared, excellent results were obtained because all MICs fell in the reference range (Table 1) (13). However, several discrepancies were detected with *C. albicans* ATCC 90028, *C. tropicalis* ATCC 750, and *C. parapsilosis* ATCC 90018.

Three amphotericin B MIC determinations for *C. albicans* ATCC 90028 were 0.25 μg/ml instead of between 0.5 and 2.0 μg/ml, which is the reference range (Table 1) (13). Pfaller et al. (15), using a broth microdilution method, obtained similar results; their modal MIC of amphotericin B for *C. albicans* ATCC 90028 estimated at 24 h was 0.25 μg/ml. In that study (15), the modal MIC visually determined at 48 h was 0.25 μg/ml, being 0.5 μg/ml when the endpoint estimation was by the spectrophotometric method. Furthermore, in the study that followed determination of the reference range, Pfaller et al. (13) obtained an amphotericin B MIC of 0.25 μg/ml 16 times.

A fluconazole MIC of 2.0 μg/ml for *C. parapsilosis* ATCC 90018 was outside the reference range (Table 1) (13). The inoculum of $10^4$ CFU/ml used in the spectrophotometric method with RPMI–2% glucose compared with that used in the NCCLS broth macrodilution method ($0.5 \times 10^3$ to $2.5 \times 10^3$ CFU/ml) could be an explanation for this MIC. In addition, Pfaller et al. (13) obtained a fluconazole MIC of 2.0 μg/ml four times in the study establishing the reference range. This result was obtained only 1.8% of the time. However, it was the only fluconazole MIC outside the reference range for this ATCC strain (13).

For *C. tropicalis* ATCC 750, three amphotericin B MICs of 0.25 μg/ml were outside the reference range. Although, the reference range was established to be between 0.5 and 2.0 μg/ml, an amphotericin B MIC of 0.25 μg/ml was obtained 13 times in that study (13).

All flucytosine MICs for *C. tropicalis* ATCC 750 were outside the reference range. The inoculum used in this spectrophotometric method ($10^4$ CFU/ml) could be the explanation for the MICs that were obtained. For fluconazole and *C. tropicalis* ATCC 750, MICs of 0.25 μg/ml were obtained twice, an MIC of 1.0 μg/ml was obtained once, and an MIC of 4.0 μg/ml was detected once. By a microdilution method, Pfaller et al. (15) obtained fluconazole MICs of 0.5 μg/ml when the reading method was visual and 0.25 μg/ml when the spectrophotometer was used to read the results. At 48 h, a fluconazole MIC of 1.0 μg/ml was estimated by both methods. So, it seems that the MIC endpoint for fluconazole, detected by the broth microdilution method at 24 h, for *C. tropicalis* ATCC 750 could be lower than those obtained by the NCCLS broth macrodilution reference method.

As a whole, with *C. tropicalis* and flucytosine as an exception, the MIC endpoints for the quality control and reference strains for amphotericin B, flucytosine, and fluconazole presented here have also been obtained by other investigators in different studies (13, 15). Therefore, our results are not unusual and could be accepted as adequate.

On the other hand, 50 isolates of *C. albicans* from patients with oropharyngeal or esophageal candidiasis were tested by both methods. The percent agreement between both methods was excellent, being 100% for amphotericin B and fluconazole. Agreement of 98.1% was reached for flucytosine. Furthermore, when a stricter percent agreement was used (e.g., one twofold dilution), good results were also obtained. For flucy-

TABLE 4. Comparison of fluconazole MICs obtained by NCCLS macrodilution method (M27-P method) with those obtained by microdilution with RPMI–2% glucose

| Isolate no. (no. of isolates) | MIC (μg/ml) | |
|---|---|---|
| | NCCLS (M27-P) | RPMI–2% glucose |
| 1-6 (6) | ≤0.12 | ≤0.12 |
| 7-12 (6) | ≤0.12 | 0.25 |
| 13 (1) | ≤0.12 | 0.5 |
| 14-22 (9) | 0.25 | ≤0.12 |
| 23-26 (4) | 0.25 | 0.25 |
| 27 (1) | 0.5 | ≤0.12 |
| 28, 29 (2) | 0.5 | 0.25 |
| 30 (1) | 0.5 | 0.5 |
| 31 (1) | 1.0 | 0.25 |
| 32 (1) | 1.0 | 1.0 |
| 33, 34 (2) | 2.0 | 4.0 |
| 35 (1) | 4.0 | 4.0 |
| 36 (1) | 4.0 | 8.0 |
| 37, 38 (2) | 8.0 | 8.0 |
| 39 (1) | 8.0 | 16.0 |
| 40, 41 (2) | 16.0 | 8.0 |
| 42 (1) | 16.0 | 32.0 |
| 43 (1) | 32.0 | 32.0 |
| 44, 45 (2) | 32.0 | 64.0 |
| 46 (1) | 64.0 | 32.0 |
| 47, 48 (2) | 64.0 | 64.0 |
| 49, 50 (2) | 128.0 | 128.0 |

tosine and fluconazole agreement of 97% was reached. This meant that for only three isolates was there a two twofold dilution difference between both tests. However, the percent agreement was lower for amphotericin B. For only 84% of the strains was there a one twofold dilution difference. Nevertheless, the MIC range for amphotericin B and *C. albicans* was very narrow, being between 0.06 and 1.0 μg/ml for all strains.

As was shown in another report (21), the MIC results obtained by this spectrophotometric microdilution technique with RPMI–2% glucose correlated with the clinical outcome for AIDS patients with oropharyngeal or esophageal candidiasis treated with ketoconazole or fluconazole. Eighteen strains included in that investigation (21) were also included in the present study. The MICs obtained varied no more than one twofold dilution compared with those obtained in the previous study (21). Also, the MICs were very similar to those obtained by the NCCLS method. At least for fluconazole, *C. albicans*, and oropharyngeal candidiasis, both tests identified strains for which the MICs were low and high. The interpretation of these MICs in this clinical setting indicates that strains for which fluconazole MICs (≤0.5 μg/ml) were lower responded to fluconazole treatment, and the opposite was also true, in that isolates for which fluconazole MICs (≥ 8.0 μg/ml) were higher did not respond. Although the number of patients is small, an in vivo correlation was achieved by both tests because similar MICs were obtained.

Our previous studies (8, 10, 16, 18, 19, 21) were based mainly on the antifungal susceptibility testing of *C. albicans*. However, in this report and in a previous one (8), we tested quality control and reference strains of other species (*C. krusei* ATCC 6258, *C. parapsilosis* ATCC 22019 and ATCC 90018, and *C. tropicalis* ATCC 750). The growth of these representative strains from these species is optimal in RPMI–2% glucose (20). The growth of *C. glabrata* and *C. lusitaniae*, two medically important species, is also optimal at 24 h in RPMI–2% glucose (20). However, the growth of *Cryptococcus neoformans* in either RPMI or RPMI–2% glucose is suboptimal. Therefore, the endpoint estimation should be performed at 48 to 72 h (20). In our opinion an alternative susceptibility procedure should be found for *C. neoformans* and other slowly growing species. In conclusion, the microdilution method with RPMI–2% glucose described here is easier to perform and more objective than the macrodilution reference method and the results are obtained in 24 h. Therefore, it can be recommended as a good alternative to the macrodilution reference method, the M27-P method (11).

Finally, although great advances have been achieved since the introduction in 1992 of the reference standard method for antifungal susceptibility testing (11), it is still too early to assume that all the problems of standardization of tests for yeasts have been solved. Because endpoint selection remained one main problem for standardization, the use of an objective method of determination (e.g., spectrophotometric) is mandatory. This method would allow independent and objective comparisons between laboratories. Furthermore, it is necessary to perform antifungal susceptibility testing with a synthetic medium that supports good growth of yeasts (e.g., RPMI–2% glucose) and that shows large differences between the OD of the control growth and the OD of inhibited cultures, helping in differentiating between susceptible and resistant strains and therefore the selection of an objective MIC endpoint. In addition, the in vitro data obtained by this method have a good correlation with clinical outcome (21). Although more studies are necessary to reaffirm this point, our present experience indicates that this assumption is feasible.

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