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# Evaluation of Etest for Direct Antifungal Susceptibility Testing of Yeasts in Positive Blood Cultures

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**The performance of the Etest (AB BIODISK, Solna, Sweden) for direct antifungal susceptibility testing of yeasts in positive blood cultures was compared with that of the macrodilution method for determining the MICs of five antifungal agents. Culture broths with blood from bottles positive for yeasts were inoculated directly onto plates for susceptibility testing with the Etest, and the MICs were read after 24 and 48 h of incubation. A total of 141 positive blood cultures (72 cultures of *Candida albicans*, 31 of *Candida tropicalis*, 14 of *Candida glabrata*, 11 of *Candida parapsilosis*, 3 of *Candida krusei*, and 3 of *Cryptococcus neoformans*, 4 miscellaneous yeast species, and 3 mixed cultures) were tested, and the rates of MIC agreement (±1 $\log_2$ dilution) between the direct Etest (at 24 and 48 h, respectively) and macrodilution methods were as follows: amphotericin B, 81.8 and 93.5%; flucytosine, 84.8 and 87.7%; fluconazole, 89.4 and 85.5%; itraconazole, 69.7 and 63.8%; ketoconazole, 87.9 and 79.0%. By a large-sample *t* test, the difference in $\log_2$ dilution between the direct Etest and the macrodilution method was found to be small ($P < 0.05$). The lone exceptions were ketoconazole at 48 h of incubation and itraconazole at both 24 and 48 h of incubation ($P > 0.05$). By Tukey's multiple comparisons, the difference between the direct Etest (48 h) and reference methods among different species was found to be less than 1 $\log_2$ dilution. When the MICs were translated into interpretive susceptibility, the minor errors caused by the direct Etest (at 24 and 48 h, respectively) were as follows: flucytosine, 2.3 and 1.4%; fluconazole, 3.0 and 3.6%; itraconazole, 21.2 and 21.3%. Itraconazole also produced an additional 3.0 and 3.6% major errors as determined by the direct Etest at 24 and 48 h, respectively. It was concluded that, except for itraconazole, the Etest method was feasible for direct susceptibility testing of blood cultures positive for yeasts. The method is simple, and the results could be read between 24 and 48 h after direct inoculation, whenever the inhibition zones were discernible.**

With the increased incidence of systemic fungal infections and the growing number of antifungal agents, laboratory aids to guide in the selection of antifungal therapy have gained greater attention. There are two reasons for this: (i) amphotericin B resistance and therapeutic failure have been reported for several *Candida* species (12, 16, 27), and (ii) an expanding list of fluconazole-resistant fungi has also been reported (3, 21, 28, 29, 31). Antifungal resistance should, therefore, be a matter of concern, by analogy with the known emergence of antibacterial resistance.

Another factor compounding the resistance problem is that a variety of yeast species are emerging as important etiological agents of nosocomial bloodstream infections (24, 25), a complication associated with a high mortality rate (2, 5). In a survey from 1980 to 1989, Banerjee et al. (1) found that the rate of nosocomial candidemia in the United States increased by almost 500% in large teaching hospitals and by 219 to 370% in small teaching hospitals. Bloodstream fungal infections constitute a serious health problem because of the excessive hospital stay, added health care costs, and high morbidity and mortality attributed to the disease (36).

Once a positive blood culture containing yeasts is found, antifungal susceptibility testing normally is performed on colonies obtained on subculture plates. The steps of colony isolation and susceptibility testing require about 3 days to complete. For bacteremia, many clinical microbiologists may perform direct susceptibility testing based solely on the Gram stain information available at the time that a positive blood culture is detected. Direct susceptibility testing of positive blood cultures containing bacteria or yeasts has been performed by using the disk diffusion method (8, 18), broth microdilution method (15), Etest (13), electrical measurement (14), and capacitance method (4). These direct methods appear to be reliable and accurate under most circumstances.

The macrodilution (MD) method has been established by the National Committee for Clinical Laboratory Standards (NCCLS) through years of development and collaborative studies (20). Although the MD method serves to provide a standard basis from which other methods can be developed, it is cumbersome and labor-intensive for the following reasons: (i) several antifungal agents are water insoluble, and stock solutions must be dissolved in organic solvent; (ii) pure compounds of some antifungal agents (fluconazole and itraconazole) are not commercially available and must be obtained

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from their respective manufacturers; and (iii) the determination of 80% growth inhibition for azole compounds and flucytosine using a spectrophotometer is tedious and poses a biosafety problem. For those reasons, several alternative methods have been developed. One of these, the Etest, has proven to be a good alternative to the NCCLS reference method, mainly due to its simplicity and good correlation with the reference method (6, 26, 32). The purpose of this study was to evaluate the feasibility of extending the use of the Etest to direct antifungal susceptibility testing of blood cultures positive for yeasts.

## MATERIALS AND METHODS

**Clinical specimens.** Blood specimens were collected from the National Cheng Kung University Hospital (an 800-bed teaching hospital) and from Chang Gung Memorial Hospital. BACTEC blood culture bottles (Becton Dickinson Microbiology Systems, Cockeysville, Md.) were inoculated with 3 to 10 ml of blood from patients, inserted into the BACTEC NR9240 instrument (Becton Dickinson Microbiology Systems), and incubated at 35°C. Gram stain smears of positive bottles were prepared to check for the presence of yeasts. Mixed cultures containing yeasts and bacteria were not included for study, nor were blood samples from patients with breakthrough fungemia (i.e., fungemia detected during antifungal treatment or prophylaxis). A total of 141 positive blood cultures were analyzed in this study.

**Cell concentration of yeasts in positive blood bottles.** Thirty-nine randomly selected yeast-positive blood cultures were used for enumeration of cell numbers. Serial 10-fold dilutions of the culture broths were made in sterile saline, and the cell numbers (CFU per milliliter) of the diluted suspensions were determined in duplicate by the plate count method (11) using Sabouraud dextrose agar as the culture medium. Plates were incubated at 35°C for 48 h before enumeration.

**Media.** RPMI 1640 with L-glutamine but without bicarbonate was buffered with MOPS (3-[*N*-morpholino]propanesulfonic acid) to pH 7.0 and used in the MD assay (20). RPMI 1640 agar (1.5%) buffered to pH 7.0 and supplemented with 2% glucose was used as the medium for the Etest as recommended by the manufacturer; it was poured into 150-mm-diameter plates.

**Antifungal agents.** Five antifungal agents were used in this study. Amphotericin B (Sigma Chemicals, St. Louis, Mo.), flucytosine (United States Pharmacopoeia, Rockville, Md.), fluconazole (Pfizer, New York, N.Y.), itraconazole (Janssen Pharmaceutica, Beerse, Belgium), and ketoconazole (United States Pharmacopoeia) were used for MIC determination by the MD method (20). The lowest concentration of the five antifungal agents tested with the MD method was 0.0156 μg/ml, and the highest concentrations used were as follows: amphotericin B, 32 μg/ml; flucytosine, 64 μg/ml; fluconazole, 256 μg/ml; itraconazole, 32 μg/ml; ketoconazole, 64 μg/ml. For both the MD method and Etest, the off-scale-high MICs were converted to the next-highest concentrations and the off-scale-low MICs were left unchanged (23).

**Susceptibility tests using Etest.** Hereafter, direct susceptibility testing of positive blood cultures using Etest (AB BIODISK, Solna, Sweden) is designated DET whereas ET refers to the testing of pure cultures as normally performed. The MICs from DET were determined by inoculating positive culture broths (i.e., a blood-yeast-broth mixture) via swabs directly onto RPMI 1640 solid medium without any further pretreatment. Plates were allowed to dry for 10 to 15 min, and the five Etest strips (amphotericin B, flucytosine, fluconazole, itraconazole, and ketoconazole) were placed on the agar surface according to the manufacturer's recommendations. Plates were incubated at 35°C, and results were read at 24 (DET-24) and 48 (DET-48) h after inoculation. For comparison with the MD results, the MICs obtained by Etest were rounded up to the nearest doubling dilution values. Yeast isolates were identified by conventional procedures (35), and MICs of the five drugs for them were determined by ET after 48 (*Candida* spp.) and 72 (*Cryptococcus neoformans*) h of incubation as recommended by the manufacturer.

**Effect of inoculum concentration on the Etest MICs.** To determine the effect of inoculum concentration on the MICs determined by Etest, four strains (*Candida krusei* ATCC 6258, *Candida albicans* ATCC 18804, *Candida glabrata* ATCC 2001, and *Candida tropicalis* C2-1) were used for comparison. Colonies of these strains were suspended in saline to obtain McFarland turbidities of 2 and 0.5 at a wavelength of 530 nm. The McFarland 0.5-turbidity cell suspensions were further diluted 1:10 to obtain yeast suspensions with an estimated McFarland turbidity of 0.05. Etest strips were then used to determine the MICs for each strain of each of the five drugs at the three inoculum levels, and MICs were read in a blinded manner after 48 h of incubation.

**MD method and quality control.** The MICs of the five antifungal agents for each isolate were determined by the MD method after 48 h of incubation (20). In the beginning of this study, a quality control strain (*C. krusei* ATCC 6258) and a reference strain (*C. albicans* ATCC 24433) were included on each day of testing to check the drug dilution and the reproducibility of the results as recommended by NCCLS (20). At the later phase of this study, another quality control strain (*Candida parapsilosis* ATCC 22019) was also included.

**Data treatment.** Of the 141 positive blood cultures tested, three were mixed cultures as revealed on isolation plates followed by identification. Of the remaining 138 blood samples, a total of 690 (138 specimens times five drugs) MIC data points were obtained by each method (DET-24, DET-48, ET, and MD). The MICs at which 50% ($MIC_{50}$) and 90% ($MIC_{90}$) of the 138 isolates were inhibited by each antifungal agent tested were also determined by different methods. A head-to-head comparison of the MICs was made for the DET (or ET) and MD methods. Discrepancies among MIC end points within 1 $\log_2$ dilution were used to calculate the percent agreement.

**Statistical analysis.** A proportion test (17) adjusted to MICs was used to test the significance of the agreement and to obtain the lower limits of the 95% one-sided confidence interval (CI); the lower limits were the probabilities, with 95% confidence, that the DET (or ET) MICs were within 1 $\log_2$ dilution of those found by the MD method. A large-sample *t* test (17) was used to test the hypothesis that a difference between MICs obtained by the DET (or ET) and MD methods was $>1$ $\log_2$ dilution. To accurately measure the difference in MICs between methods, a 95% CI in the scale of $\log_2$ dilution was established. For comparison of results of the different methods among the four major species of yeasts infecting blood (i.e., *C. albicans, C. tropicalis, C. glabrata*, and *C. parapsilosis*), Tukey's multiple comparisons (19) were performed to analyze differences between the DET-48 and MD methods.

**Definitions of test errors.** Based on the interpretive breakpoints defined by NCCLS for flucytosine, fluconazole, and itraconazole (20), the MICs obtained by the different methods were translated into susceptibility categories (resistant, intermediate/susceptible-dose dependent, and susceptible). The results of interpretive susceptibilities obtained from DET (or ET) were compared with those obtained from the MD method, and discrepancies were classified as very major (false susceptibility by DET or ET), major (false resistance by DET or ET), or minor (9). A minor error was defined as any change involving a dose dependently or intermediately susceptible result.

## RESULTS

**Effect of inoculum concentration on the Etest MICs.** *C. krusei* ATCC 6258 served as the quality control strain for this study, and all five Etest MICs fell within the NCCLS-established ranges (20) (Table 1), irrespective of different inoculum concentrations. The Etest MICs seemed to be "tolerant" of the changes in inoculum concentration for all yeast-drug combinations. Although lower inoculum concentrations tended to produce lawns of lighter growth, the Etest MICs seemed not to be influenced. If the manufacturer's recommended McFarland turbidity of 0.5 was used as a standard concentration for the Etest, all MICs obtained by the other inoculum densities were within 1 $\log_2$ dilution.

**Distribution of species in blood cultures.** *C. albicans* accounted for 52.2% of all yeasts isolated from the 138 blood cultures, followed by *C. tropicalis* (22.5%), *C. glabrata* (10.1%), and *C. parapsilosis* (8%). The remainder consisted of *C. krusei* and *Cryptococcus neoformans* (2.2% each) and *Candida famata, Candida guilliermondii*, a *Candida* sp., and *Trichosporon beigelii* (0.7 each).

**Yeast cell numbers in positive blood cultures.** The cell counts of yeasts in 39 randomly selected positive blood bottles ranged from $10^5$ to $10^8$ CFU/ml, with 34 samples (87%) being in the range of $10^6$ to $10^7$ CFU/ml. Three of the remaining five blood cultures had cell densities of $10^8$ CFU/ml, and two had cell counts of $10^5$ CFU/ml.

TABLE 1. Effect of inoculum concentrations on the MICs determined by Etest

| Microorganism and McFarland turbidity | MIC (μg/ml) of: | | | | |
|---|---|---|---|---|---|
| | Amphotericin B | Flucytosine | Fluconazole | Itraconazole | Ketoconazole |
| *C. krusei* ATCC 6258 | | | | | |
| 2 | 0.5 | 16 | 32 | 0.25 | 0.125 |
| 0.5 | 0.5 | 16 | 32 | 0.25 | 0.125 |
| 0.05 | 0.5 | 16 | 16 | 0.25 | 0.125 |
| *C. albicans* ATCC 18804 | | | | | |
| 2 | 0.125 | 0.062 | 0.25 | 0.016 | 0.008 |
| 0.5 | 0.125 | 0.031 | 0.125 | 0.016 | 0.008 |
| 0.05 | 0.125 | 0.016 | 0.25 | 0.016 | 0.008 |
| *C. glabrata* ATCC 2001 | | | | | |
| 2 | 0.25 | 0.016 | 8 | 4 | 0.5 |
| 0.5 | 0.25 | 0.016 | 8 | 4 | 0.5 |
| 0.05 | 0.125 | 0.016 | 8 | 2 | 0.25 |
| *C. tropicalis* C2-1 | | | | | |
| 2 | 0.125 | 0.016 | 0.5 | 0.032 | 0.008 |
| 0.5 | 0.125 | 0.016 | 0.5 | 0.016 | 0.008 |
| 0.05 | 0.125 | 0.016 | 0.25 | 0.016 | 0.008 |

**MIC ranges, $MIC_{50}$s, and $MIC_{90}$s.** The MIC ranges, $MIC_{50}$s, and $MIC_{90}$s determined by the DET, ET, and MD methods are summarized in Table 2. The MICs of all drugs except amphotericin B covered a broad range. For DET, *Cryptococcus neoformans* (3 strains) and 3 of the 11 strains of *C. parapsilosis* produced very fine lawns of growth on RPMI agar plates at 24 h so that determining the MICs for these strains was difficult. For this reason, the MIC data from DET-24 did not include these six strains. For each of the five drugs tested, $MIC_{50}$s determined by the different methods were identical except that the $MIC_{50}$ (0.25 μg/ml) of amphotericin B obtained by DET-24 was 1 $\log_2$ dilution lower than that (0.5 μg/ml) determined by the DET-48 and MD methods. $MIC_{90}$s of each drug by different methods were either identical or different by only 1 $\log_2$ dilution (Table 2), with the exception of those of itraconazole. The $MIC_{90}$ (0.5 μg/ml) of itraconazole obtained by the MD method was 2 and 3 $\log_2$ dilutions lower than those obtained by the DET-24 (2 μg/ml) and DET-48 (4 μg/ml) methods, respectively.

Based on the interpretive breakpoints established by NCCLS (20), the MICs of flucytosine, fluconazole, and itraconazole obtained for each strain were translated into interpretive susceptibility (resistant, dose dependently or intermediately susceptible or susceptible). There were 1 strain (0.7%), 8 strains (5.8%), and 16 (11.6%) strains, respectively, which were intermediately (or dose dependently) susceptible to flucytosine, fluconazole, and itraconazole (20). However, there were 6 (4.3%), 3 (3.2%), and 12 (8.7%) strains resistant to flucytosine, fluconazole, and itraconazole, respectively.

**Agreement between the DET and MD methods.** If 1 $\log_2$ dilution was allowed for method discrepancy, the agreement rates between the DET-24 and -48 and MD methods for all strains tested were, in general, ≥80% (Table 2). The only exception was itraconazole, which yielded agreement rates ranging from 63.8 to 72.5%. For amphotericin B and flucytosine, the agreement rates increased from 81.8 and 84.8%, respectively, to 93.5 and 87.7% if the DET incubation time was increased from 24 to 48 h (Table 2). However, for fluconazole, itraconazole, and ketoconazole, the agreement rates decreased

TABLE 2. Antifungal susceptibilities of yeasts from 138 positive blood cultures: MIC results, agreement (±1 $\log_2$ dilution) rates, and distribution of differences between the DET and ET methods and the NCCLS standard MD method

| Antifungal agent | Method | *n* | MIC (μg/ml)[a] | | | Agreement rate (%) | *P*[b] | Lower limit of 95% CI (one sided) |
|---|---|---|---|---|---|---|---|---|
| | | | Range | 50% | 90% | | | |
| Amphotericin B | DET-24 | 132 | 0.063–2 | 0.25 | 0.5 | 81.8 | 0.003 | 0.76 |
| | DET-48 | 138 | 0.063–2 | 0.5 | 0.5 | 93.5 | 0.000 | 0.90 |
| | ET | 138 | 0.063–2 | 0.5 | 0.5 | 89.9 | 0.004 | 0.86 |
| | MD | 138 | 0.031–2 | 0.5 | 1 | | | |
| Flucytosine | DET-24 | 132 | 0.016–64 | 0.063 | 0.25 | 84.8 | 0.000 | 0.80 |
| | DET-48 | 138 | 0.016–64 | 0.063 | 0.5 | 87.7 | 0.000 | 0.83 |
| | ET | 138 | 0.016–64 | 0.063 | 0.5 | 87.0 | 0.000 | 0.82 |
| | MD | 138 | 0.016–128 | 0.063 | 0.5 | | | |
| Fluconazole | DET-24 | 132 | 0.063–512 | 0.5 | 8 | 89.4 | 0.000 | 0.85 |
| | DET-48 | 138 | 0.063–512 | 0.5 | 16 | 85.5 | 0.000 | 0.81 |
| | ET | 138 | 0.063–512 | 0.5 | 16 | 78.3 | 0.036 | 0.73 |
| | MD | 138 | 0.016–256 | 0.5 | 8 | | | |
| Itraconazole | DET-24 | 132 | 0.016–64 | 0.063 | 2 | 69.7 | 0.001 | 0.64 |
| | DET-48 | 138 | 0.016–64 | 0.063 | 4 | 63.8 | 0.014 | 0.58 |
| | ET | 138 | 0.016–64 | 0.063 | 2 | 72.5 | 0.000 | 0.67 |
| | MD | 138 | 0.016–16 | 0.063 | 0.5 | | | |
| Ketoconazole | DET-24 | 132 | 0.016–64 | 0.063 | 0.5 | 87.9 | 0.000 | 0.85 |
| | DET-48 | 138 | 0.016–64 | 0.063 | 1 | 79.0 | 0.000 | 0.75 |
| | ET | 138 | 0.016–64 | 0.063 | 1 | 84.1 | 0.000 | 0.81 |
| | MD | 138 | 0.016–16 | 0.063 | 1 | | | |

[a] 50% and 90%, $MIC_{50}$ and $MIC_{90}$, respectively.
[b] *P* values of <0.05 indicate significant agreement.

TABLE 3. Analysis of differences between methods for MIC determination for 138 positive blood cultures containing yeasts

| Antifungal agent and test methods compared | *n* | Mean difference between methods ($log_2$ dilutions) | *P*[a] | Limit of 95% CI ($log_2$ dilution) | | No. (%) of indicated type of error | |
|---|---|---|---|---|---|---|---|
| | | | | Lower | Upper | Minor | Major |
| Amphotericin B | | | | | | | |
| DET-24/MD | 132 | −0.753 | 0.016 | −0.978 | −0.528 | NA[b] | NA |
| DET-48/MD | 138 | −0.138 | 0.000 | −0.352 | 0.076 | NA | NA |
| ET/MD | 138 | −0.297 | 0.000 | −0.514 | −0.080 | NA | NA |
| Flucytosine | | | | | | | |
| DET-24/MD | 132 | −0.258 | 0.003 | −0.791 | 0.275 | 3 (2.3) | 0 (0) |
| DET-48/MD | 138 | −0.065 | 0.001 | −0.604 | 0.474 | 2 (1.4) | 0 (0) |
| ET/MD | 138 | −0.181 | 0.001 | −0.719 | 0.357 | 3 (2.2) | 0 (0) |
| Fluconazole | | | | | | | |
| DET-24/MD | 132 | −0.356 | 0.018 | −0.957 | 0.245 | 4 (3.0) | 0 (0) |
| DET-48/MD | 138 | 0.174 | 0.003 | −0.419 | 0.767 | 5 (3.6) | 0 (0) |
| ET/MD | 138 | −0.167 | 0.004 | −0.772 | 0.439 | | |
| Itraconazole | | | | | | | |
| DET-24/MD | 132 | 0.662 | 0.130 | 0.074 | 1.249 | 24 (18.2) | 4 (3.0) |
| DET-48/MD | 138 | 0.957 | 0.442 | 0.373 | 1.540 | 23 (16.7) | 5 (3.6) |
| ET/MD | 138 | 0.703 | 0.150 | 0.141 | 1.265 | 22 (15.9) | 6 (4.3) |
| Ketoconazole | | | | | | | |
| DET-24/MD | 132 | −0.042 | 0.001 | −0.591 | 0.506 | NA | NA |
| DET-48/MD | 138 | 0.529 | 0.053 | −0.043 | 1.101 | NA | NA |
| ET/MD | 138 | 0.065 | 0.001 | −0.502 | 0.632 | NA | NA |

[a] A *P* value of <0.05 indicates that the difference was small.
[b] NA, not applicable.

from 89.4, 69.7, and 87.9%, respectively, to 85.5, 63.8, and 79.0% if the MICs found by DET were read at a second time 24 h later. For the azole compounds tested with DET, extending the 24-h incubation seemed to decrease the rates of agreement with the MD method. The majority of MIC disagreements were associated with lower MIC results obtained by both the DET and MD methods.

The MICs of amphotericin B determined by DET-24 were skewed to lower levels than those obtained by the MD method. Ninety-one percent (79 of 87) of the amphotericin B disagreements in the MIC results involved MICs determined by DET-24 that were 1 or more dilutions lower than MICs determined by the MD method. The values decreased to 59% (43 of 73) when the DET incubation time was increased to 48 h. The same phenomenon was observed for flucytosine; 76% (53 of 70) of the disagreements in MIC results involved MICs obtained by DET-24 that were 1 or more dilutions lower than the MICs obtained by the MD method. In contrast, there was a consistent tendency for itraconazole MICs determined by both DET-24 and DET-48 to be higher than those obtained by the MD method. For example, 72% (68 of 94) of the disagreements in the MIC results involved MICs obtained by DET-24 that were 1 or more dilutions higher than the MICs obtained by MD. The value increased to 83% (78 of 94) if the DET incubation time for itraconazole was 48 h.

Table 2 also includes the lower limits of one-sided 95% CIs for different test methods. The lower limits indicate the probabilities of DET (and ET) yielding MICs within 1 $log_2$ dilution of the reference MICs. For amphotericin B, this probability was determined to be at least 0.76 at a confidence of 95%. The lower limits of the CI ranged from 0.58 (DET-48 result for itraconazole) to 0.90 (DET-48 result for amphotericin B). All *P* values in Table 2 were <0.05, and hence there was association between DET (and ET) and the MD method.

Statistical analysis of difference between methods revealed that the difference between the DET (or ET) and MD methods was >1 $log_2$ dilution (Table 3). For itraconazole, the difference of >1 $log_2$ dilution among the DET-24 ($P = 0.13$), DET-48 ($P = 0.442$), ET ($P = 0.15$), and MD methods was quite large. The 95% CIs for the difference were 0.074 to 1.249, 0.373 to 1.540, and 0.141 to 1.2650 $log_2$ dilution for intervals which contained 1 $log_2$ dilution, as was expected. Another difference ($P = 0.053$) between the DET-48 and MD methods was found when testing ketoconazole (95% CI, −0.043 to 1.101 $log_2$ dilution) (Table 3). It is interesting that the performance of DET was comparable to that of ET. Through Tukey's multiple comparisons, generally, no difference between the DET-48 and MD methods was found when *C. albicans, C. tropicalis, C. glabrata*, and *C. parapsilosis* were tested (Table 4). But an excep-

TABLE 4. Differences between the DET and MD methods among yeast species

| Species | 95% CI[a] for antifungal agent: | | | | |
|---|---|---|---|---|---|
| | Amphotericin B | Flucytosine | Fluconazole | Itraconazole | Ketoconazole |
| *C. albicans* | −0.33–0.16 | −0.59–0.48 | −0.63–0.13 | 0.36–1.39 | −0.01–0.96 |
| *C. tropicalis* | −0.83–0.10 | −0.69–2.03 | −0.54–0.48 | −0.17–1.43 | −0.44–1.77 |
| *C. glabrata* | −0.86–0.43 | −0.63–0.63 | −0.22–2.08 | −0.54–2.39 | −0.59–1.97 |
| *C. parapsilosis* | −0.65–0.80 | −0.82–1.13 | −1.98–1.21 | 0.69–4.02 | −0.38–2.38 |

[a] CI for Tukey's 95% multiple comparisons. The values are for differences ($log_2$ dilution) between the DET and MD methods.

tion was noted for itraconazole when testing *C. albicans* and *C. parapsilosis*. However, the difference was less than 1 $\log_2$ dilution.

**Test errors of DET and ET.** No very major errors were found for the DET and ET methods compared to the MD method. The minor error rates for flucytosine ranged from 1.4 to 2.3%, and those for fluconazole ranged from 2.9 to 3.6% (Table 3). No major errors were found for flucytosine and fluconazole. The minor errors produced by DET-24 (18.2%), DET-48 (16.7%), and ET (15.9%) for itraconazole were unacceptable. In addition, the DET and ET methods produced major-error rates of 3.0 to 3.6% and 4.3%, respectively, for itraconazole.

## DISCUSSION

Fungal susceptibility testing has proven notoriously difficult to introduce into the routine clinical laboratory for a variety of reasons. The chief reasons have been that the reference method is laborious and cost-inefficient and that there have not been enough studies of correlations between laboratory results and clinical outcome. Although the NCCLS M27-A reference method remains the standard by which all other methods are judged, it is impossible for a modest-size laboratory to perform the test on a routine basis. There have been many alternatives to the MD method developed over the past several years, including the broth colorimetric microdilution technique (7, 23, 33), flow cytometry (37), and MIC diffusion strips (Etest) (6, 26, 32). Of these, the Etest seems more adaptable for the routine workload, and, in several reports, results have been comparable to those by the MD method.

Due to the success of the Etest in fungal susceptibility testing and in light of its success in direct antibacterial susceptibility testing (9, 15, 18), we felt that a similar procedure for the direct antifungal testing of fungi could be developed. The major question at the outset appeared to be whether fungi grew to a level in blood culture medium significant enough to serve as a direct inoculum for the test. To answer that question, we determined that yeast cell densities in randomly selected positive blood culture bottles ranged from $10^5$ to $10^8$ CFU/ml. We further determined in a controlled experiment that varying the inoculum within this range had no appreciable effect on the MICs for common clinical yeast isolates (Table 1), supporting the hypothesis that direct fungal susceptibility testing was feasible. The advantages of rapid antimicrobial susceptibility testing have already been recognized (8, 34).

The second obstacle (and the most obvious one) to contend with in direct antifungal testing was the possibility that mixed cultures, either bacteria and fungus or two types of fungi, would significantly affect results. Since bacteria tend to overgrow fungi on agar plates, we found that it was important to do a Gram stain prior to direct testing. Although mixed cultures containing yeasts and bacteria were purposely screened out (by Gram stain) for study, three mixed cultures (2%) were detected upon subculturing followed by identification. Since bacteria tend to overgrow the agar plates used for Etest, it is important to prepare smears for Gram staining before performing direct susceptibility tests of positive blood cultures. Normally, mixed cultures containing yeasts and bacteria could be easily detected by Gram stain. Mixed cultures containing two strains of yeast would be difficult to detect on routine subculture agar plates (e.g., Sabouraud dextrose agar). However, the use of CHROMagar *Candida* (Hardy Diagnostics, Santa Monica, Calif.), a differential medium containing chromogenic substrates, would make it easier to detect mixed cultures of yeasts (22). Compared with bacteremia, however, fungemia caused by multiple strains of yeasts is very rare.

After testing 138 positive blood cultures, the rates of agreement ($\pm 1$ $\log_2$ dilution) between the DET and MD methods covered a range of 63.8 to 93.5% (Table 2). Except for those for itraconazole, the agreement rates were generally $\geq$80%. Of the three azoles tested in this study, itraconazole seemed to be a difficult drug for MIC determination by using Etest, confirming a similar observation by Ruhnke et al. (30). It is interesting that the DET results in this study were comparable to those of ET reported in this study (Tables 2 and 3). In addition, there was no difference between the DET-48 and MD methods among the four major yeast species recovered from blood cultures, again with the exception of itraconazole when testing *C. albicans* and *C. parapsilosis*. However, the difference was less than 1 $\log_2$ dilution (Table 4).

For amphotericin B, the agreement rate increased from 81.8 to 93.5% if the DET incubation time was increased from 24 to 48 h (Table 2). However, a decrease in agreement rates accompanied increased incubation time when the azole compounds were tested. Some authors reported that the MICs by Etest after a 24-h incubation showed agreements comparable to (6, 30) or even better than (10) those obtained after a 48-h incubation. However, the problem with a 24-h incubation for DET was that some strains of *C. parapsilosis* and *Cryptococcus neoformans* were unable to develop visible inhibition zones on RPMI agar plates within that time period. Colombo et al. (6) also found that a 24-h incubation was not enough for Etest MICs when some strains of *C. parapsilosis* were tested. The difficulties in determining the in vitro susceptibility of *Cryptococcus neoformans* were possibly related to suboptimal growth of the organism in RPMI 1640 medium. *C. parapsilosis* and *Cryptococcus neoformans* represented about 10% of the total isolates of yeasts from blood. Since species information is not available at the time a positive blood culture is found, it is recommended that the Etest MICs be read between 24 and 48 h after direct inoculation.

Although the $MIC_{50}$s of each of the five drugs tested by different methods were almost identical, the $MIC_{90}$s of itraconazole determined by the MD method were 2 to 3 $\log_2$ dilutions lower than those obtained by the DET method (Table 2). This might be due to the difficulty in determining MICs when trailing end points or a fine lawn of growth within the zone of inhibition occurred. We usually found inhibition zones like "bottlenecks" at the base of the eclipse inhibition zones produced by the itraconazole strips. As the growth of yeast proceeded, the yeast cells tended to "fill in" the bottlenecks, and this produced higher MICs.

In conclusion, except for itraconazole, the DET showed a good correlation with the NCCLS-proposed MD method. The DET method is simple and less labor-intensive, and the MIC results are available within 24 to 48 h after a positive blood culture containing yeast is found. The method can save up to 2 days compared to the standard procedures encompassing strain isolation followed by susceptibility testing. However, direct testing provides rapid presumptive MIC results only, and

these results should be confirmed by broth dilution or by a standard Etest.

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## REFERENCES

1. **Banerjee, S. N., T. G. Emori, D. H. Culver, R. P. Gaynes, W. R. Jarvis, T. Horan, J. R. Edwards, J. Tolson, T. Henderson, and W. J. Martone.** 1991. Secular trends in nosocomial primary bloodstream infections in the United States, 1980–1989. National Nosocomial Infections Surveillance System. Am. J. Med. **91**(Suppl. 3B)**:**86S–89S.
2. **Beck-Sague, C., and W. R. Jarvis.** 1993. Secular trends in the epidemiology of nosocomial fungal infections in the United States, 1980–1990. National Nosocomial Infections Surveillance System. J. Infect. Dis. **167:**1247–1251.
3. **Boschman, C. R., U. R. Bodnar, M. A. Tornatore, A. A. Obias, G. A. Noskin, K. Englund, M. A. Postelnick, T. Sariano, and L. R. Peterson.** 1998. Thirteen-year evolution of azole resistance in yeast isolates and prevalence of resistant strains carried by cancer patients at a large medical center. Antimicrob. Agents Chemother. **42:**734–738.
4. **Chang, H. C., J. J. Chang, A. H. Huang, and T. C. Chang.** 2000. Evaluation of a capacitance method for direct antifungal susceptibility testing of yeasts in positive blood cultures. J. Clin. Microbiol. **38:**971–976.
5. **Chen, Y. C., S. C. Chang, C. C. Sun, L. S. Yang, W. C. Hseih, and K. T. Luh.** 1997. Secular trends in the epidemiology of nosocomial fungal infections at a teaching hospital in Taiwan, 1981 to 1993. Infect. Control Hosp. Epidemiol. **18:**369–375.
6. **Colombo, A. L., F. Barchiesi, D. A. McGough, and M. G. Rinaldi.** 1995. Comparison of Etest and National Committee for Clinical Laboratory Standards broth macrodilution method for azole antifungal susceptibility testing. J. Clin. Microbiol. **33:**535–540.
7. **Davey, K. G., A. Szekely, E. M. Johnson, and D. W. Warnock.** 1998. Comparison of a new commercial colorimetric microdilution method with a standard method for in-vitro susceptibility testing of *Candida* spp. and *Cryptococcus neoformans*. J. Antimicrob. Chemother. **42:**439–444.
8. **Doern, G. V., D. R. Scott, and A. L. Rashad.** 1982. Clinical impact of rapid antimicrobial susceptibility testing of blood culture isolates. Antimicrob. Agents Chemother. **21:**1023–1024.
9. **Doern, G. V., D. R. Scott, A. L. Rashad, and K. S. Kim.** 1981. Evaluation of a direct blood culture disk diffusion antimicrobial susceptibility test. Antimicrob. Agents Chemother. **20:**696–698.
10. **Espinel-Ingroff, A.** 1994. Etest for antifungal susceptibility testing of yeasts. Diagn. Microbiol. Infect. Dis. **19:**217–220.
11. **Espinel-Ingroff, A., C. W. Kish, Jr., T. M. Kerkering, R. A. Fromtling, K. Bartizal, J. N. Galgiani, K. Villareal, M. A. Pfaller, T. Gerarden, M. G. Rinaldi, and A. Fothergill.** 1992. Collaborative comparison of broth macrodilution and microdilution antifungal susceptibility tests. J. Clin. Microbiol. **30:**3138–3145.
12. **Goldman, M., J. C. Pottage, Jr., and D. C. Weaver.** 1993. *Candida krusei* fungemia: report of 4 cases and review of the literature. Medicine **72:**143–150.
13. **Hong, T., and J. Ndamukong.** 1996. Direct application of Etest to gram-positive cocci from blood cultures: quick and reliable minimum inhibitory concentration data. Diagn. Microbiol. Infect. Dis. **25:**21–25.
14. **Huang, A. H., J. J. Wu, Y. M. Weng, H. C. Ding, and T. C. Chang.** 1998. Direct antimicrobial susceptibility testing of gram-negative bacilli in blood cultures by an electrochemical method. J. Clin. Microbiol. **36:**2882–2886.
15. **Kiehn, T. E., C. Capitolo, and D. Armstrong.** 1982. Comparison of direct and standard microtiter broth dilution susceptibility testing of blood culture isolates. J. Clin. Microbiol. **16:**96–98.
16. **Law, D., C. B. Moore, and D. W. Denning.** 1997. Amphotericin B resistance testing of *Candida* spp.: a comparison of methods. J. Antimicrob. Chemother. **40:**109–112.
17. **Lehmann, E. L.** 1994. Testing statistical hypothesis, p. 135–151. Chapman & Hall, New York, N.Y.
18. **Mirrett, S., and L. B. Reller.** 1979. Comparison of direct and standard antimicrobial disk susceptibility testing for bacteria isolated from blood. J. Clin. Microbiol. **10:**482–487.
19. **Montgomery, D. C.** 1984. Design and analysis of experiments, 2nd ed., p. 43–78. John Wiley & Sons, Inc., New York, N.Y.
20. **National Committee for Clinical Laboratory Standards.** 1997. Reference method for broth dilution antifungal susceptibility testing of yeasts. Approved standard M27-A. National Committee for Clinical Laboratory Standards, Wayne, Pa.
21. **Newman, S. L., T. P. Flanigan, A. Fisher, M. G. Rinaldi, M. Stein, and K. Vigilante.** 1994. Clinically significant mucosal candidiasis resistant to fluconazole treatment in patients with AIDS. Clin. Infect. Dis. **19:**684–686.
22. **Odds, F. C., and R. Bernaerts.** 1994. CHROMagar *Candida*, a new differential isolation medium for presumptive identification of clinically important *Candida* species. J. Clin. Microbiol. **32:**1923–1929.
23. **Pfaller, M. A., and A. L. Barry.** 1994. Evaluation of a novel colorimetric broth microdilution method for antifungal susceptibility testing of yeast isolates. J. Clin. Microbiol. **32:**1992–1996.
24. **Pfaller, M. A., R. N. Jones, S. A. Messer, M. B. Edmond, and R. P. Wenzel.** 1998. National surveillance of nosocomial bloodstream infection due to species of *Candida* other than *Candida albicans*: frequency of occurrence and antifungal susceptibility in the SCOPE Program. Diagn. Microbiol. Infect. Dis. **30:**121–129.
25. **Pfaller, M. A., S. A. Messer, R. J. Hollis, R. N. Jones, G. V. Doern, M. E. Brandt, and R. A. Hajjeh.** 1999. Trends in species distribution and susceptibility to fluconazole among bloodstream isolates of *Candida* species in the United States. Mycology **33:**217–222.
26. **Pfaller, M. A., S. A. Messer, A. Karlsson, and A. Bolmstrom.** 1998. Evaluation of the Etest method for determining fluconazole susceptibilities of 402 clinical yeast isolates by using three different agar media. J. Clin. Microbiol. **36:**2586–2589.
27. **Rex, J. H., C. R. Cooper, Jr., W. G. Merz, J. N. Galgiani, and E. J. Anaissie.** 1995. Detection of amphotericin B-resistant *Candida* isolates in a broth-based system. Antimicrob. Agents Chemother. **39:**906–909.
28. **Rex, J. H., M. G. Rinaldi, and M. A. Pfaller.** 1995. Resistance of *Candida* species to fluconazole. Antimicrob. Agents Chemother. **39:**1–8.
29. **Ruhnke, M., A. Eigler, I. Tennagen, B. Geiseler, E. Engelmann, and M. Trautmann.** 1994. Emergence of fluconazole-resistant strains of *Candida albicans* in patients with recurrent oropharyngeal candidosis and human immunodeficiency virus infection. J. Clin. Microbiol. **32:**2092–2098.
30. **Ruhnke, M., A. Schmidt-Westhausen, E. Engelmann, and M. Trautmann.** 1996. Comparative evaluation of three antifungal susceptibility test methods for *Candida albicans* isolates and correlation with response to fluconazole therapy. J. Clin. Microbiol. **34:**3208–3211.
31. **Sanguineti, A., J. K. Carmichael, and K. Campbell.** 1993. Fluconazole-resistant *Candida albicans* after long-term suppressive therapy. Arch. Intern. Med. **153:**1122–1124.
32. **Simor, A. E., G. Goswell, L. Louie, M. Lee, and M. Louie.** 1997. Antifungal susceptibility testing of yeast isolates from blood cultures by microbroth dilution and the E test. Eur. J. Clin. Microbiol. Infect. Dis. **16:**693–697.
33. **Tiballi, R. N., X. He, L. T. Zarins, S. G. Revankar, and C. A. Kauffman.** 1995. Use of a colorimetric system for yeast susceptibility testing. J. Clin. Microbiol. **33:**915–917.
34. **Trenholme, G. M., R. L. Kaplan, P. H. Karakusis, T. Stine, J. Fuhrer, W. Landan, and S. Levin.** 1989. Clinical impact of rapid identification and susceptibility testing of bacterial blood culture isolates. J. Clin. Microbiol. **27:** 1342–1345.
35. **Warren, N. G., and K. C. Hazen.** 1995. *Candida, Cryptococcus*, and other yeasts of medical importance, p. 727–737. *In* P. R. Murray, E. J. Baron, M. A. Pfaller, F. C. Tenover, and R. H. Yolken (ed.), Manual of clinical microbiology, 6th ed. American Society for Microbiology, Washington, D.C.
36. **Weinstein, M. P., M. L. Towns, S. M. Quartey, S. Mirrett, L. G. Reimer, G. Parmigiani, and L. B. Reller.** 1997. The clinical significance of positive blood cultures in the 1990s: a prospective comprehensive evaluation of microbiology, epidemiology, and outcome of bacteremia and fungemia in adults. Clin. Infect. Dis. **24:**584–602.
37. **Wenisch, C., K. F. Linnau, B. Parschalk, K. Zedtwitz-Liebenstein, and A. Georgopoulos.** 1997. Rapid susceptibility testing of fungi by flow cytometry using vital staining. J. Clin. Microbiol. **35:**5–10.