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Agreement of Direct Antifungal Susceptibility Testing from Positive Blood Culture Bottles with the Conventional Method for Candida Species

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Early availability of antifungal susceptibilities can ensure timely institution of targeted therapy in candidemia, which can improve patient outcomes. This study prospectively determines the agreement between the results of direct testing of antifungal susceptibilities from blood culture bottles by disk diffusion and Etest and the results of standardized susceptibility testing methods; direct testing would allow susceptibility results to be available 1 to 2 days earlier. A total of 104 blood cultures with different Candida species (28% C. albicans, 27% C. parapsilosis, 26% C. tropicalis, etc.) were evaluated between January 2012 and May 2013 for agreement of fluconazole, voriconazole, and amphotericin B susceptibility results by disk diffusion. Agreement in MICs obtained by Etest was determined for fluconazole (21 isolates), voriconazole (28 isolates), amphotericin B (29 isolates), and caspofungin (29 isolates). The kappa scores for categorical agreement were highest for fluconazole by disk diffusion (0.902, standard error [SE] = 0.076) and Etest (1.00, SE = 0.218) and for amphotericin B by disk diffusion (1.00, SE = 0.098). The Pearson correlation (r) of zone diameters was strongest for fluconazole (0.69) and amphotericin (0.70) and moderate for voriconazole (0.60), and the Pearson correlation of MICs was strongest for fluconazole (0.94) and caspofungin (0.88). However, the moderate correlation of amphotericin MICs with zone diameters (−0.42) precludes the use of amphotericin B disk diffusion for susceptibility testing. There were no very major errors; however, there were 1 (1%) major and 5 (4.8%) minor errors with disk diffusion and 4 (13.3%) minor errors with Etest. Thus, antifungal disk diffusion directly from blood culture bottles is a rapid and easy method for fluconazole and voriconazole susceptibility testing for timely tailoring of candidemia therapy.
MATERIALS AND METHODS

Study background. This prospective study was conducted in the clinical laboratory of the Aga Khan University Hospital (AKUH), Karachi, Pakistan, from January 2012 to May 2013. The laboratory has a national specimen collection network with more than 200 collection points in major cities and towns across the country.

Specimens. A total of 104 blood culture specimens submitted to the laboratory and smear positive for yeasts were included in this study. When the incubated BacTec 9420 aerobic and/or anaerobic blood culture bottles signaled positive, an aliquot was examined under the microscope for budding yeast cells. Blood cultures positive for yeast mixed with bacteria were excluded (data not recorded) from the study.

Organism identification. During the study period, Candida species were identified using the standard protocol, as follows: germ tube production, colony morphology on BigGgy agar (Becton Dickinson), urease test (Oxoid), cycloheximide sensitivity test (Becton Dickinson), and presence of pseudohyphae on corn meal agar with Tween 80. Isolated colonies were also evaluated for sugar assimilation on the biochemical test panel API 20C AUX (bioMérieux).

Direct antifungal susceptibility testing. The procedure for direct susceptibility testing from positive blood cultures was optimized as published previously (19). The concentration of Candida species in a positive blood culture bottle was not determined in this study, as a previous study has shown that Candida cell counts in positive blood culture bottles were in the range of 10^6 to 10^7 CFU/ml (exactly the count in 0.5 McFarland standards) (13).

A hundred-microliter amounts of uncentrifuged broth from smear-positive blood culture bottles were used to make lawns on Mueller-Hinton agar supplemented with 2% dextrose and 0.5% g/ml methylene blue dye (MHA-MB). Neo-Sensitab (Rosco) disks with 25 µg fluconazole, 25 µg voriconazole, or 10 µg amphotericin B were placed on the plates, and the plates were incubated for 20 to 24 h at 35 ± 2°C. After 20 to 24 h of incubation, the zones of inhibition around each disk were measured using a millimeter scale and interpreted according to the manufacturer’s recommendations (20). For voriconazole, for all species, the susceptible (S) inhibition zone diameter was ≥17 mm, the susceptible–dose–dependent (S-DD) inhibition zone diameter was 14 to 16 mm, and the resistant (R) inhibition zone diameter was ≥13 mm. For fluconazole, ≥19 mm was interpreted as S, ≥15 to 18 mm as S-DD, and ≤14 mm as R for C. albicans, Candida parapsilosis, and C. tropicalis and ≥15 mm was interpreted as S-DD and ≤14 mm as R for Candida glabrata (18). For amphotericin B, a ≥15-mm zone of complete inhibition was interpreted as S, 10 to 14 mm as intermediate susceptibility, and <10 mm as R according to the manufacturer’s recommendations (Neo-Sensitabs; Rosco), although there are no established interpretive criteria for amphotericin B (20).

MIC determinations using Etest (AB Biodisk, Solna, Sweden) for fluconazole, voriconazole, amphotericin B, and caspofungin were performed using RPMI agar as recommended by the manufacturer and read at 24 h as recommended by CLSI M27-A3 (21). Susceptibility cutoffs were determined using CLSI M27-S4 criteria (22). Although clinical breakpoints or epidemiological cutoff values (ECVs) for amphotericin have not been verified for MICs obtained by Etest, data suggest that broth microdilution cutoffs may be used (23). Hence, for amphotericin B, an ECV of 2 µg/ml was used (24), and for those Candida species for which CLSI cutoffs were not available, ECVs were used according to the recommendations of Pfäffer et al. (25).

Conventional antifungal susceptibility disk diffusion. The conventional disk diffusion method was used as the gold standard for comparison (18). A lawn was made on MHA-MB using a suspension of Candida species equal to 0.5 McFarland turbidity standard. Neo-Sensitab fluconazole (25 µg), voriconazole (25 µg), and amphotericin B (10 µg) disks were placed on the agar surface, and plates were incubated for 24 h at 35 ± 2°C. After 20 to 24 h of incubation, the zones of inhibition around each disk were measured and interpreted similarly to the direct testing method described above. For fluconazole and voriconazole, the zone of inhibition showing from 50 to 80% drop in growth was measured, while for amphotericin B, the zone diameter from the point showing complete inhibition of growth was measured. Etests against fluconazole, voriconazole, amphotericin B, and caspofungin were performed (AB Biodisk, Solna, Sweden) using standard inocula from colonies on RPMI agar. For quality control, Candida parapsilosis (ATCC 22019) and Candida krusei (ATCC 6258) were used with each new batch of medium prepared or new lot of antifungal disks or Etests used.

Statistical analysis. Descriptive statistics were calculated using Microsoft Excel. Statistical analysis was performed with Stata 12 statistical software. The frequencies of isolation of the different species and their rates of resistance were calculated. Kappa scores were generated for categorical agreement, and the Pearson correlation test was applied to assess whether the results of the direct disk diffusion and Etest methods of Candida susceptibility testing correlated with the results from the standard method. It was also applied to assess how well zone diameters correlated with MICs for amphotericin B. The following standards were used for the strength of agreement for the kappa coefficient: 0 to 0.0099, poor; 0.01 to 0.20, slight; 0.21 to 0.40, fair; 0.41 to 0.60, moderate; 0.61 to 0.80, substantial; and 0.81 to 1, almost perfect.

A correlation coefficient of >0.8 was considered very strong correlation, 0.6 to 0.79 strong, 0.4 to 0.59 moderate, 0.2 to 0.39 weak, and <0.19 as very weak. The Pearson chi-square test was used to evaluate whether the total number of errors in categorizing a strain as S, S-DD, or R was significantly associated with any species. For all statistical tests, a P value of <0.05 was considered significant.

RESULTS

One hundred six Candida species were isolated from 104 blood culture bottles. Two cases had polymicrobial candidemia, with C. albicans and C. krusei. Disk diffusion antifungal susceptibility testing using the direct method and the conventional method was performed on all 106 isolates, and Etest susceptibility testing was performed on 21 isolates for fluconazole, 28 for voriconazole, and 29 for amphotericin and caspofungin. A description of the isolates with their susceptibility patterns is shown in Table 1.

Comparison of the results of the conventional and the direct susceptibility testing methods by kappa score demonstrated excel-
TABLE 2 Agreement rates and kappa scores of the results of susceptibility testing performed directly from blood culture bottles and following standard methodology.

<table>
<thead>
<tr>
<th>Antifungal</th>
<th>Test modality</th>
<th>No. of isolates tested</th>
<th>Categorical agreement (%)</th>
<th>Kappa score (SE)</th>
<th>Kappa P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Voriconazole</td>
<td>Disk diffusion</td>
<td>104</td>
<td>97.12</td>
<td>−0.0130 (0.0923)*</td>
<td>0.5559</td>
</tr>
<tr>
<td></td>
<td>Etest</td>
<td>28</td>
<td>100</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Fluconazole</td>
<td>Disk diffusion</td>
<td>104</td>
<td>97.12</td>
<td>0.9019 (0.0755)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>Etest</td>
<td>21</td>
<td>100</td>
<td>1.000 (0.2182)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Amphotericin B</td>
<td>Disk diffusion</td>
<td>104</td>
<td>100</td>
<td>1.000 (0.0981)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>Etest</td>
<td>29</td>
<td>100</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Caspofungin</td>
<td>Etest</td>
<td>29</td>
<td>86.21</td>
<td>0.4844 (0.1675)</td>
<td>0.0019</td>
</tr>
</tbody>
</table>

*The kappa score is negative because there was only one isolate in the nonsusceptible category and the expected agreement rate is higher than the detected agreement.

DISCUSSION

We determined the agreement between the results of conventional and direct antifungal susceptibility disk diffusion testing of fluconazole, voriconazole, and amphotericin B for Candida species isolated from positive blood culture bottles. Additionally, the agreement between conventional and direct susceptibility testing using Etest was also assessed for fluconazole, voriconazole, amphotericin B, and caspofungin.

Using disks, excellent agreement was observed for fluconazole and amphotericin, confirmed by high kappa scores of 0.90 and 1.00, respectively. However, despite good reproducibility, the zone diameters of amphotericin B correlated moderately well with the amphotericin B MICs using Etest. All correlation results were found to be statistically significant (P < 0.05) except for the Etest results for amphotericin B, which appears to be more inoculum dependent than other agents.

Analysis of major and minor errors in disk diffusion results (Table 4) revealed 1 major error (for voriconazole against a C. parapsilosis isolate) and 5 minor errors (for 2 C. tropicalis isolates, 2 C. parapsilosis isolates, and 1 C. albicans isolate) and no very major errors. These errors were not found to be significantly associated with species. There were no major or very major errors identified for the test method using Etest. However, four minor errors were identified, including 2 cases of C. glabrata and 2 cases of mixed C. krusei and C. albicans, showing that the probability of error in direct caspofungin MICs for these species was greater than by chance alone (P < 0.001).

TABLE 3 Pearson correlation of zone diameters and Etest mean results and standard deviations.

<table>
<thead>
<tr>
<th>Antifungal</th>
<th>Test modality</th>
<th>No. of isolates tested</th>
<th>Mean value (SD) for disk diffusion (mm) or Etest (µg/ml) using:</th>
<th>Pearson correlation (r) (strength)*</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Voriconazole</td>
<td>Disk diffusion</td>
<td>104</td>
<td>28.55 (5.45)</td>
<td>29.34 (5.64)</td>
<td>0.6040 (M)</td>
</tr>
<tr>
<td></td>
<td>Etest</td>
<td>28</td>
<td>0.11 (0.12)</td>
<td>0.12 (0.16)</td>
<td>0.5808 (M)</td>
</tr>
<tr>
<td>Fluconazole</td>
<td>Disk diffusion</td>
<td>104</td>
<td>26.49 (6.12)</td>
<td>26.94 (6.38)</td>
<td>0.6907 (S)</td>
</tr>
<tr>
<td></td>
<td>Etest</td>
<td>21</td>
<td>2.76 (5.59)</td>
<td>3.66 (10.37)</td>
<td>0.9476 (VS)</td>
</tr>
<tr>
<td>Amphotericin B</td>
<td>Disk diffusion</td>
<td>104</td>
<td>21.78 (3.55)</td>
<td>21.72 (3.23)</td>
<td>0.7017 (S)</td>
</tr>
<tr>
<td></td>
<td>Etest</td>
<td>29</td>
<td>0.24 (0.26)</td>
<td>0.14 (0.13)</td>
<td>0.1637 (VW)</td>
</tr>
<tr>
<td>Caspofungin</td>
<td>Etest</td>
<td>29</td>
<td>0.32 (0.36)</td>
<td>0.24 (0.27)</td>
<td>0.8884 (VS)</td>
</tr>
<tr>
<td>Amphotericin B</td>
<td>Disk diffusion</td>
<td>29</td>
<td>23.37 (2.04)</td>
<td>0.14 (0.13)</td>
<td>−0.4216 (M)*</td>
</tr>
</tbody>
</table>

*VW, very weak; M, moderate; S, strong; VS, very strong.

Zone diameter with MIC (both by standard method).
TABLE 4 Distribution of major and minor errors among the Candida species isolates tested by disk diffusion and Etest directly from blood culture bottles compared to the results following the standard method.

<table>
<thead>
<tr>
<th>Organism</th>
<th>No. (%) of isolates with errors</th>
<th>No. of isolates</th>
<th>Minor errors</th>
<th>Major errors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Disk diffusion</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C. albicans</td>
<td></td>
<td>28</td>
<td>1 (3.4)</td>
<td>0</td>
</tr>
<tr>
<td>C. tropicalis</td>
<td></td>
<td>25</td>
<td>2 (7.4)</td>
<td>0</td>
</tr>
<tr>
<td>C. parapsilosis</td>
<td></td>
<td>25</td>
<td>2 (7.1)</td>
<td>1 (3.6)</td>
</tr>
<tr>
<td>C. glabrata</td>
<td></td>
<td>8</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Non-albicans Candida</td>
<td></td>
<td>4</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>C. lusitaniae</td>
<td></td>
<td>4</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>C. pelliculosa</td>
<td></td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>C. albicans + C. kruisent</td>
<td></td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total for disk diffusion</td>
<td></td>
<td>98 (94.2)</td>
<td>5* (4.8)</td>
<td>1# (1.0)</td>
</tr>
<tr>
<td>Etest</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C. albicans</td>
<td></td>
<td>7</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>C. tropicalis</td>
<td></td>
<td>5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>C. parapsilosis</td>
<td></td>
<td>9</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>C. glabrata</td>
<td></td>
<td>2</td>
<td>2 (50)</td>
<td>0</td>
</tr>
<tr>
<td>Non-albicans Candida</td>
<td></td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>C. pelliculosa</td>
<td></td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>C. albicans + C. kruisent</td>
<td></td>
<td>0</td>
<td>2 (100)</td>
<td>0</td>
</tr>
<tr>
<td>Total for Etest</td>
<td></td>
<td>26 (86.7)</td>
<td>4# (13.3)</td>
<td>0</td>
</tr>
</tbody>
</table>

* The antifungals tested by Etest were fluconazole, voriconazole, amphotericin B, and caspofungin. Caspofungin was not tested by the disk diffusion method.
# One hundred four strains were tested by disk diffusion. The Pearson chi-square value was 4.652, and the P value was 0.0349 (errors were significantly associated with the species C. albicans).
# Twenty strains were tested against all agents using the Etest, and 10 additional isolates against at least one agent. The Pearson chi-square value was 63.44, and the P value was <0.001 (errors were significantly associated with the species C. glabrata and the mixed species C. kruisent and C. albicans).
# Direct susceptibilities were compared with standard susceptibilities of the most resistant strain of the two isolates (C. kruisent in both cases).
# There were five minor errors in determining azole susceptibilities by disk diffusion, as follows: for voriconazole, 2 C. tropicalis isolates were reported as S-DD when they were S and one C. parapsilosis isolate was reported as S when it was S-DD, and for fluconazole, 1 C. albicans isolate was reported as S when it was S-DD and a C. parapsilosis isolate as R when it was S-DD.
# There was 1 major error for fluconazole by disk diffusion (one C. parapsilosis isolate was labeled R when it was S).
# All four cases of errors by Etest showed a minor discrepancy in caspofungin susceptibility results (2 C. glabrata isolates were reported as S when they were I and vice versa, and the 2 mixed cultures were reported as R and I while being I and S, respectively). Thus, there is perfect correlation by Etest if caspofungin is not tested directly.
# Minor errors included the reporting of S as S-DD or I or vice versa, and the major error was the reporting of S as R.

The Pearson correlation test also showed a moderate to strong correlation for azoles using standard and direct disk diffusion testing.

Similar results (100% agreement) were obtained for direct susceptibility testing of fluconazole, voriconazole, and amphotericin B using Etest. A slightly lower agreement (86%) with a moderate kappa score of 0.4844 was observed for caspofungin using Etest for direct susceptibility testing, in spite of very strong correlation, primarily due to minor errors (one dilution) at clinical breakpoints for mixed cultures and for C. glabrata.

Only one study has previously evaluated the use of fluconazole disks for direct susceptibility testing of Candida species; however, that study used CHROMagar, which is not a recommended susceptibility testing medium. Another approach has been the direct inoculation of Vitek antifungal cards for susceptibility testing. However, the results of that study were suboptimal, with a high number of errors. A recent study also evaluated direct inoculation of Sensititre YeastOne from blood cultures for a limited number of cases. Although the results were good, this approach is expensive and would not be practical in many resource-limited settings due to high shipping costs and shipment delays. MHA with methylene blue and 2% glucose is a much cheaper medium than RPMI broth for disc diffusion, and swabbing a lawn on a medium plate is easier and less technically demanding, resulting in less waste than in broth-based susceptibility methods.

As previously reported for direct susceptibility testing using Etest, variations in the inoculum concentrations did not affect the susceptibility results using disks, as all results were interpretable after 24 h. This was also reflected by finding no significant differences between the mean zone diameters and mean MICs by conventional and direct method using either disks or Etest.

There were no major errors in direct susceptibility testing using either disks or Etest. Major errors using disks were noted with only one strain of C. parapsilosis, where the result for fluconazole was reported as resistant. No major errors were noted in direct testing using Etest.

A high proportion of very major and major errors in direct sensitivity testing using Etest has not been reported previously for fluconazole, voriconazole, and caspofungin. Due to a high rate of very major (3%) and major errors (23%) reported in amphotericin B testing previously, direct susceptibility testing using Etest for amphotericin B has not been recommended. We noted no major errors in amphotericin B testing using disks and no errors using Etest for direct susceptibility. However, as there were no resistant isolates in our sample, the discriminatory ability of this technique to detect resistance could not be assessed.

Amphotericin B deoxycholate is the first-line agent in Pakistan for invasive candidiasis due to nonavailability of lipid preparations and echinocandins, and thus, amphotericin susceptibilities are relevant in this setting. There has also been an upsurge in less common Candida species in our region in the last 5 years, and therefore, it is important to document the susceptibility profiles of naive and treated Candida strains. The clinical benefit usually lies in whether the patient is currently on amphotericin therapy and whether the patient would benefit from a higher dose than 0.7 μg/ml.

Although a low percentage of C. krusei strains as agents of candidemia from Pakistan has been reported previously, the frequency is recent years has increased. Additionally, voriconazole treatment is also considered in patients at a high risk of concomitant invasive mold infection, for example, in patients with hematologic malignancies receiving chemotherapy or bone marrow transplant. As modification of therapy from voriconazole to fluconazole is difficult in such patient populations, the availability of voriconazole susceptibility results is relevant for these patients.

No errors were noted in direct susceptibility testing of C. glabrata, C. albicans, and C. tropicalis. Very major errors have been reported previously in direct testing of C. glabrata and C. tropicalis but were not found in our study. On the other hand, previous
studies have not reported such testing errors in *C. parapsilosis* as were noted in our study (14).

Antifungal susceptibility testing is not yet widely used for directing treatment of invasive candidiasis, despite the fact that antifungal resistance has emerged globally (28). A rapid, easy method of drug susceptibility testing can be expected to encourage laboratories with limited financial and technical resources to start monitoring antifungal susceptibilities with meaningful clinical implications, choosing at least those agents which are most commonly used in their center.

**Limitations.** There were only four fluconazole-resistant isolates and no amphotericin B- or caspofungin-resistant isolates in our collection. Due to the small sample size and rarity of resistance among *Candida* species in our study, we cannot say how the technique will perform in a setting with more resistant isolates. Therefore, we suggest that any unexpected results in terms of resistance must be confirmed using standard methodology until more data are available. Etest could not be performed for all isolates due to financial limitations. Another limitation of this study was that neither clinical outcomes nor tailoring of empirical antifungal therapy in accordance with the direct drug susceptibility testing results was recorded, as the study was designed as an in vitro study.

**Conclusion.** The use of Etest for direct susceptibility testing for *Candida* species has already been reported as a rapid antifungal susceptibility testing tool that could provide results in 24 to 48 h. The results of our study demonstrate that, as an alternative to Etest, antifungal disks could also be used for direct susceptibility testing for azoles. This approach will be very useful in settings with limited resources and expertise to allow early reporting of susceptibilities that will result in prompt administration of appropriate antifungal agents. However, the use of this technique for amphotericin B testing cannot be recommended.

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The findings and conclusions of this article are those of the authors and do not necessarily represent the views of the Centers of Disease Control and Prevention.

The study was approved by the Aga Khan University Ethical Review Committee, 1373-Path-ERC-09.

There is no conflict of interest for any author.

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