A comparative evaluation of Etest and broth microdilution methods for fluconazole and itraconazole susceptibility testing of *Candida* spp.

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The Etest strip is a promising tool of broad application in clinical microbiology. The method provides MIC readings and is easier to perform than broth microdilution. We carried out a study to compare the MICs of fluconazole and itraconazole obtained by the Etest with those obtained by broth microdilution, performed according to the guidelines of the NCCLS document M27-A, with 402 clinical isolates (360 *Candida albicans*, 17 *Candida tropicalis*, nine *Candida krusei*, nine *Candida glabrata* and seven *Candida parapsilosis*) and seven control isolates. The agreement between MICs by the two methods (at \(\pm 2\) dilutions) was 74.5\% for fluconazole and 61.4\% for itraconazole. These results suggest that further development is necessary to standardize the medium and incubation conditions before introduction of the Etest as a routine method in the clinical microbiology laboratory for fluconazole and itraconazole susceptibility testing.

**Introduction**

Fungal infections cause significant morbidity and mortality in immunocompromised patients. Oral candidosis is the most common opportunistic infection in human patients infected with HIV and AIDS.\textsuperscript{1,2} Paralleling the increased prevalence of fungal infections has been the introduction of new antifungal agents and the recognition of resistance.\textsuperscript{3} The chronic use of azoles for prophylaxis in systemic mycoses in bone marrow transplant patients and as long-term suppressive therapy in AIDS patients are factors in the selection of isolates that are more resistant to azole therapy.\textsuperscript{4-6} Consequently, there is a greater need for a reproducible susceptibility testing method as a guide to selecting and monitoring antifungal therapy.\textsuperscript{3,7,8} In recent years, numerous susceptibility test methods have been evaluated for testing of *Candida* spp. and other yeast isolates.\textsuperscript{9,10}

In 1992, the National Committee for Clinical Laboratory Standards (NCCLS) proposed a standardized reference broth microdilution method for antifungal susceptibility testing of yeast (M 27-P).\textsuperscript{11} A microdilution method following the NCCLS criteria has shown good correlation with these macrodilution methods and was incorporated in the NCCLS M 27-T document.\textsuperscript{12} The initially proposed version (M 27-P) has been revised to the approved level (M-27A).\textsuperscript{13} Irrespective of these advances the methods have some limitations, and further efforts are necessary for the development of simpler and more economical methods.\textsuperscript{3,14}

The Etest (AB Biodisk, Solna, Sweden) is a novel susceptibility testing method that involves placing a plastic strip carrying a continuous gradient of an antifungal agent on the surface of an inoculated agar plate. The Etest and the broth microdilution method, in accordance with the NCCLS recommendations,\textsuperscript{13} were used to determine the MICs of fluconazole and of itraconazole for 402 yeast isolates representing five *Candida* spp. isolated from oropharyngeal infections in AIDS patients. The purpose of this study was to compare the results obtained by the Etest and broth microdilution methods.

**Materials and methods**

**Yeast isolates**

A total of 402 yeast isolates recovered from oropharyngeal specimens from 145 AIDS patients attending the Valme
University Hospital of Seville and the Department of Microbiology, País Vasco University, Bilbao, and seven quality control and reference isolates (Candida albicans ATCC 90028, C. albicans ATCC 64548, C. albicans ATCC 64540, Candida glabrata ATCC 2238, Candida krusei ATCC 6258, Candida tropicalis ATCC 750, Candida parapsilosis ATCC 90018),15,16 were studied. The clinical isolates consisted of 360 C. albicans, 17 C. tropicalis, nine C. krusei, nine C. glabrata and seven C. parapsilosis. Fungal identification was based on standard methods17 as well as biochemical characterization with the YBC card in the Vitek system (API-bioMérieux, Montalieu Vercieux, France).

Antifungal agents

The Etest strips were provided by the manufacturer and had drug concentrations ranging from 0.016 to 256 mg/L for fluconazole and from 0.02 to 32 mg/L for itraconazole. The strips were stored at −20°C until used. Reference grade powders of fluconazole (Pfizer Inc., Sandwich, UK) and itraconazole (Janssen Pharmaceutical, Beerse, Belgium) were used to prepare drug dilutions ranging from 0.125 to 64 mg/L for fluconazole and from 0.015 to 8 mg/L for itraconazole. A fluconazole stock solution of 6400 mg/L was prepared in 2% dimethylsulphoxide and an itraconazole stock solution of 800 mg/L in 100% dimethylsulphoxide. Ten-fold drug dilutions were diluted 1:5 with liquid RPMI 1640 medium described below to achieve the 2 × 10^6 final concentrations.

Assay media

Liquid RPMI 1640 with L-glutamine and 2% glucose18 without bicarbonate was buffered with morpholine–propane sulphonic acid (MOPS) to pH 7, and used in broth microdilution tests in sterile flat-bottomed microtitre plates (Nunc InterMed ed, LabClinics, Barcelona, Spain). Solidified RPMI 1640 with L-glutamine and 2% glucose but without bicarbonate was buffered with potassium phosphate to pH 7.0, solidified with 1.5% Bacto-agar (Difco, Detroit, MI, USA) and used for Etest MICs.

Susceptibility testing

The broth microdilution method was used as the reference method, as described by the NCCLS.11–13 The inoculum was prepared from Sabouraud glucose agar subcultures incubated at 35°C for 24 h and the resulting suspension was adjusted spectrophotometrically to a density equivalent to a 0.5 McFarland standard at 530 nm (1.5 × 10^6 cfu/mL). A working suspension was made by 1:100 dilution of the suspension in RPMI medium and 100 µL of the diluted inoculum was added to each well. The final inoculum size was (0.5–2.5) × 10^3 cfu/mL. The broth dilution tests were incubated at 35°C, and MICs were determined after 48 h incubation by observation of the presence or absence of visible growth. The endpoint for azole MICs was defined as the lowest concentration where a prominent decrease in turbidity was observed.11–13

The Etest was performed according to the manufacturer’s instructions. The inoculum suspension was adjusted as described above. The solidified medium was inoculated by dipping a sterile cotton swab into the respective undiluted stock inoculum suspension and streaking evenly in three directions over the entire surface of a 150 mm diameter RPMI agar plate. The plate was permitted to dry for at least 15 min before the Etest strips with antifungal were placed on the medium surface. The incubation time was 48 h and the MIC was considered as the drug concentration at which the border of the elliptical inhibition zone intercepted the scale on the strip. A scale Etest scale has a continuous gradient of concentrations instead of the two-fold dilutions that are tested by the broth dilution method, when the MIC determined by the Etest was intermediate it was raised to the next two-fold dilution level of the reference method for the sake of comparison. No information about the MIC determinations by the reference method was available during the reading of Etest results and vice versa.

Analysis of the results

The MICs at which 50% and 90% of the isolates tested were inhibited were determined for each azole. A comparison of azole MICs as determined by both methods was also performed. Essential agreement (EA) occurred when the MIC results by the Etest and reference methods were in exact agreement or were within two two-fold dilutions. The MICs were read by two different readers. All isolates showing discrepancies were repeated by both methods. We also studied the impact of MIC disagreements on the categories of susceptibility for the organisms tested by both methods, with the susceptibility breakpoints for the azoles published in the NCCLS M 27-A document.13,19,20

Results

The MIC ranges and MIC90 values of fluconazole and itraconazole for 402 isolates, excluding the control and the reference microorganisms, tested by Etest and microdilution methods are illustrated in Tables I and II. Control isolates gave MIC results in agreement with NCCLS reference values. C. albicans and certain isolates of other Candida spp., e.g. C. tropicalis, gave diffuse endpoints by the Etest and the determination of MIC endpoints was difficult. Large to minute colonies or a decreasing intensity of growth (double halo) at the endpoint or within the inhibition ellipse were observed.
Susceptibility testing of *Candida* species

Comparison of fluconazole MICs

The MICs determined by both methods for the 402 isolates tested demonstrated a broad range of susceptibility, as shown in Table I. It can be seen that the MIC\textsubscript{90} values of fluconazole by the Etest were one or two dilutions higher than the MICs determined by the reference method for all species studied. The details of the agreement between the MICs determined by the two methods are given in Table I. The overall EA was 74.5%, with agreement rates among five species of yeast tested covering a range from 66.6% (C. *glabrata*) to 88.2% (C. *tropicalis*). When we studied the impact of MIC disagreement between methods on the susceptibility interpretation for the yeasts tested, we found that 80% of *C. albicans* isolates were susceptible or resistant by both methods, whereas 14 isolates (3.8%) showed very major discrepancies (resistant by the reference method but susceptible by the Etest). Thirteen isolates (3.6%) showed major discrepancies (susceptible by the reference method but resistant by Etest) and 45 (12.5%) showed minor discrepancies. No species other than *C. albicans* showed very major discrepancies.

**Table I.** Fluconazole susceptibility of 402 *Candida* spp. clinical isolates determined by the broth microdilution and Etest methods

<table>
<thead>
<tr>
<th>Species (no. of isolates)</th>
<th>Broth microdilution</th>
<th>Etest</th>
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<tbody>
<tr>
<td></td>
<td>MIC range (mg/L)</td>
<td>MIC\textsubscript{50} (mg/L)</td>
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<td></td>
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<tr>
<td><em>C. albicans</em> (360)</td>
<td>0.12–64</td>
<td>1</td>
</tr>
<tr>
<td><em>C. tropicalis</em> (17)</td>
<td>0.16–64</td>
<td>1</td>
</tr>
<tr>
<td><em>C. glabrata</em> (9)</td>
<td>2–64</td>
<td>8</td>
</tr>
<tr>
<td><em>C. krusei</em> (9)</td>
<td>0.12–64</td>
<td>0.5</td>
</tr>
<tr>
<td><em>C. parapsilosis</em> (7)</td>
<td>0.12–2</td>
<td>0.5</td>
</tr>
</tbody>
</table>

Comparison of itraconazole MICs

The MICs determined by the two methods for the 402 isolates tested covered a broad range, as shown in Table II. The MIC\textsubscript{90} values were identical or ±1 dilution by the two methods, except for *C. tropicalis* (four dilutions higher by Etest). However, with this antifungal agent all the species except *C. parapsilosis* showed MIC\textsubscript{90} values ≥0.5 mg/L by both methods. The EA between the methods for itraconazole is shown in Table II. The overall agreement was 61.7%, covering a range from 42.9% (C. *parapsilosis*) to 88.8% (C. *glabrata*). The problems regarding itraconazole MIC interpretation by Etest were fewer than for fluconazole. On the other hand, the MIC disagreement between the methods produced a change in susceptibility interpretation with 45% of *C. albicans* tested, 27 (7.5%) isolates showing very major discrepancies, 20 (5.6%) showing major discrepancies and 129 (35.8%) showing minor discrepancies. No species other than *C. albicans* showed very major discrepancies.

**Table II.** Itraconazole susceptibility of 402 *Candida* spp. clinical isolates determined by broth microdilution and Etest methods

<table>
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<td></td>
<td></td>
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<tr>
<td><em>C. albicans</em> (360)</td>
<td>0.015–16</td>
<td>0.25</td>
</tr>
<tr>
<td><em>C. tropicalis</em> (17)</td>
<td>0.03–8</td>
<td>0.12</td>
</tr>
<tr>
<td><em>C. glabrata</em> (9)</td>
<td>1–2</td>
<td>1</td>
</tr>
<tr>
<td><em>C. krusei</em> (9)</td>
<td>0.015–8</td>
<td>1</td>
</tr>
<tr>
<td><em>C. parapsilosis</em> (7)</td>
<td>0.12</td>
<td>0.12</td>
</tr>
</tbody>
</table>

Discussion

The Etest strip for antimicrobial susceptibility testing is a method with broad applications in microbiology.\textsuperscript{21} Several studies have reported good agreement between the Etest and the NCCLS reference method.\textsuperscript{7–10} The present study was undertaken to evaluate the potential use of the Etest for obtaining MICs of antifungal agents for five different species of yeast. Fluconazole MIC\textsubscript{90} values were higher (1–2 dilutions) by
the E test than by broth microdilution for all species studied. These data coincide with those obtained by other workers.\textsuperscript{7,8,22} For itraconazole, the MIC\textsubscript{90} values by E test were equal to those obtained by broth microdilution, and these data also coincided with those obtained by Colombo et al.\textsuperscript{7,8} for most of the species studied. For C. albicans, MIC\textsubscript{90} values by the E test were higher than those obtained by broth microdilution in the studies by Colombo et al.\textsuperscript{7,8} and for C. tropicalis our MIC\textsubscript{90} values by E test were four times higher than those by broth microdilution. Our MIC\textsubscript{90} results for both antifungal agents are in agreement with those obtained by Runhke et al.\textsuperscript{22} In spite of these discrepancies, all the species studied except C. parapsilosis and C. tropicalis showed an MIC\textsubscript{90} > 32 mg/L for fluconazole by both methods. With C. glabrata and C. krusei the MIC\textsubscript{90} was > 64 mg/L, and 64 mg/L is the resistance breakpoint for fluconazole. For itraconazole all the species except C. parapsilosis showed an MIC\textsubscript{90} > 0.5 mg/L by both methods.

The overall percentage agreement between the two methods, based on MICs within ±2 dilutions, was higher for fluconazole (74.5%) than for itraconazole (61.4%). These values were lower than those obtained by others,\textsuperscript{7,8,23–25} who observed an EA > 80% with both antifungal agents. On the other hand, Sewell et al.\textsuperscript{9} obtained similar results to ours (EA < 80%), whereas Runhke et al.\textsuperscript{22} obtained lower percentage agreement than in our study for fluconazole and similar results for itraconazole. On studying the species separately, we observed that for fluconazole, C. tropicalis showed the highest percentage of agreement (88.2%), whereas for itraconazole, C. glabrata showed the highest percentage of agreement (88.8%).

The NCCLS document M 27-A\textsuperscript{13} includes susceptibility breakpoints for the azoles. When we analysed the impact of MIC disagreements between the two methods on susceptibility interpretation of the isolates tested, we found a high correlation for fluconazole. With 80% of isolates the MIC discrepancies did not produce a change in their susceptibility interpretation. For itraconazole the correlation was very poor (< 60%) with a high percentage of isolates showing very major discrepancies. These discrepancies could be attributed to the difficulty in determining the endpoints for the azoles, because partial inhibition in an E test ellipse was frequently observed. This difficulty in reading of the E test plates for azole antifungals has been observed by other authors,\textsuperscript{26–28} who noted in most cases an inhibition zone with diffuse edges and growth of microcolonies within the inhibition zone on RPMI agar. Sharper edges were observed on Casitone agar. C. albicans was the species that gave greatest problems in reading, but our data do not coincide with those published by Colombo et al.,\textsuperscript{7,8} who found greater problems with C. tropicalis. Pfaffer et al.\textsuperscript{26} found for C. krusei ATCC 6258 that MIC values by the E test were higher than by the broth microdilution method, probably because the composition of the culture medium was different (a solidified medium with 2% glucose, which according to some workers\textsuperscript{29} would allow the yeast to grow in the presence of fluconazole). A study of MICs for the control isolates included in this study were within the established ranges, and our essential agreement for the isolates from clinical specimens was lower than found by other workers.\textsuperscript{29–31} These discrepancies could exist because the isolates we studied were from HIV patients with oropharyngeal candidosis or candida colonization. As stated by Odds,\textsuperscript{30} with antifungal susceptibility tests the published data show that the results are of scientifically proven worth only in a limited set of circumstances and that clinical correlation with MICs is weaker than ideal.

We can conclude that the E test could be a useful alternative method for study of the susceptibility of yeasts to antifungal agents, because it is technically an easier method than broth dilution. However, because of the difficulty in reading the endpoint, further studies are necessary to determine the culture medium and incubation conditions which give clearer endpoints.

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References

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