Review

Standardization of antifungal susceptibility testing

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The application of in-vitro antifungal susceptibility testing to clinical research and to the guidance of antifungal therapy has been limited by a lack of reproducibility and uncertain clinical relevance. As a result of studies of the identified variables impacting on in-vitro susceptibility results, the National Committee for Clinical Laboratory Standards have proposed a standardized antifungal susceptibility test method M27-P. The degree of intra- and inter-laboratory reproducibility which can be achieved with this method have been defined in multi-laboratory collaborative studies. More convenient methods (microdilution broth and stable gradient technology) have been evaluated relative to the proposed standard method and the potential for a similar process with a disc diffusion method is apparent. A modification of this standard method for susceptibility testing of filamentous fungi appears promising. The existence of a standardized method and of alternative methods with a defined relationship to the proposed standard, facilitates meaningful analysis of published studies addressing the issue of clinical relevance of antifungal susceptibility testing. As a result of this process, correlation of MICs determined \textit{in vitro} with clinical response to therapy is beginning to emerge, most notably in relation to fluconazole therapy for oropharyngeal candidosis associated with infection with the human immunodeficiency virus.

Introduction

The central objective of essentially all in-vitro susceptibility testing is to facilitate prediction of the likely impact of administration of the tested compound on the outcome of disease caused by the tested organism or similar organisms. In the clinical diagnostic laboratory, the focus is on a specific isolate from a specific patient. In drug development the focus may be on selection of the most potent of a series of compounds for further development. In clinical epidemiological research the issue may be the tendency of resistance to emerge in initially susceptible isolates or species and to establish the local pattern of resistance. In each of these situations it is appropriate to bear in mind that the prediction of outcome in a complex and dynamic biological system (clinical infection) from results obtained in a relatively simple, well defined matrix is an inherently error-prone process and that only modest degrees of correlation can be anticipated. Certainly decades of experience with standardized susceptibility test
methods for antibacterial agents confirm the limited degree of correlation which is achievable (McCabe & Treadwell, 1985).

Standardization of antifungal susceptibility testing is clearly decades behind that for antibacterial susceptibility testing for a variety of reasons. Until recent years fungal infection accounted for a numerically minor element of invasive life threatening infectious disease in humans. In addition, the number of available agents was limited and the potential for emergence of resistance to antifungals was not well recognized. Fungal infection is now a major component of nosocomial infection. Data from the United States National Nosocomial Infection Surveillance System indicate that 8% of blood stream infection in USA hospitals is caused by *Candida* species (Schaberg, Culver & Gaynes, 1991). Invasive infection with filamentous fungi is also a significant cause of morbidity and mortality in the profoundly immunocompromised patient (Pfaller & Wenzel, 1992). The increase in the frequency of fungal infection has coincided with an increase in the number of antifungals available for therapy of infection (fluconazole, itraconazole and others in advanced stages of development) so that there now exist options in antifungal therapy whereas, formerly, amphotericin B with or without flucytosine was the only therapy available. An additional relatively recent development in antifungal therapy is the necessity to provide chronic suppressive therapy for profoundly immunocompromised HIV infected patients, a situation with clear potential for development of resistance in formerly susceptible strains (Redding et al., 1994; Barchiesi et al., 1995). All these factors have combined to increase the need for reproducible, clinically-relevant susceptibility testing, in particular for yeasts, but also for the filamentous fungi. Inevitably the lack of interlaboratory agreement among susceptibility test results resulted in confusion in the area of development of clinical correlates. Because of these developments and the primitive stage of antifungal susceptibility testing relative to antibacterial testing, the area of antifungal susceptibility testing is now rapidly evolving with simultaneous (rather than sequential) development of standardized reference methods (broth macrodilution), of more readily applicable methods (broth microdilution) and of commercial systems (Alamar, Sensititre, and Etest).

Development of a proposed standardized method for yeast isolates

The principal methodological issues which were considered in development of the NCCLS proposed standardized method have been comprehensively reviewed recently and are only briefly summarized here (Galgiani, 1993).

Antifungal susceptibility testing is not new, but has been hampered by poor interlaboratory agreement and uncertain clinical relevance. Using a variety of methods there was marked variation (up to 50,000-fold) in the measured MIC of a given antifungal tested against the same strains in a series of laboratories. Interestingly, however, the interlaboratory agreement as judged by the rank order of the susceptibility of the members of a series of strains to a specific antifungal was much better (Galgiani et al., 1987).

Much of the interlaboratory variation in antifungal MICs can be explained on the basis of variations in methodology. As with antibacterial susceptibility testing, the MIC is dependent on the inoculum preparation and size, the composition and pH of the medium, and the duration and temperature of incubation (Rex et al., 1993). The standardized macrodilution broth method for susceptibility testing of yeasts proposed
Antifungal susceptibility testing

Table I. Principal elements of the National Committie for Clinical Laboratory Standards proposed standard macrobroth susceptibility test procedure (cf. Galgiani et al. (1993))

<table>
<thead>
<tr>
<th>Variable</th>
<th>Standard</th>
</tr>
</thead>
<tbody>
<tr>
<td>Culture medium</td>
<td>RPMI 1640</td>
</tr>
<tr>
<td>Medium pH</td>
<td>7.0</td>
</tr>
<tr>
<td>pH buffer</td>
<td>Morpholinepropane sulphonic acid (0.165 M)</td>
</tr>
<tr>
<td>Inoculum size</td>
<td>0.5–2.5 x 10^3 cells/mL</td>
</tr>
<tr>
<td>Inoculum preparation</td>
<td>Subculture yeast on Sabouraud’s agar</td>
</tr>
<tr>
<td></td>
<td>Suspend yeast in saline</td>
</tr>
<tr>
<td></td>
<td>Spectrophotometrically standardized relative</td>
</tr>
<tr>
<td></td>
<td>to a 0.5 McFarland standard</td>
</tr>
<tr>
<td></td>
<td>Dilute 1:2000 in test medium</td>
</tr>
<tr>
<td>Incubation temperature</td>
<td>35°C</td>
</tr>
<tr>
<td>Incubation time</td>
<td>2 days for most yeasts</td>
</tr>
<tr>
<td></td>
<td>3 days for Cryptococcus neoformans</td>
</tr>
<tr>
<td>Endpoint definition</td>
<td>Amphotericin B: No visible turbidity</td>
</tr>
<tr>
<td></td>
<td>Flucytosine/Azoles 80% inhibition</td>
</tr>
</tbody>
</table>

by the National Committee for Clinical Laboratory Standards (NCCLS M27-P) addresses these variables by use of a chemically defined medium (RPMI 1640), and by definition of the inoculum preparation and incubation temperature (see Table I) (NCCLS, 1992). Antifungal susceptibility testing presents a few specific problems which have been addressed in the standardization process. The first of these is that determination of the endpoint for a number of antifungals, specifically the azoles and flucytosine, is complicated by the phenomenon of trailing. This phenomenon is related to the fungistatic action of these agents which may result in a lag period of some hours before complete inhibition of growth is established. As a result, some growth may be seen in wells containing high concentrations of the antifungal although a “prominent decrease in turbidity from control” is observed at modest concentrations of the antifungal agent. This is analogous to the similar phenomenon observed against bacteria with the bacteriostatic sulphonamides. The term “prominent decrease in turbidity from control” may be quantified as 80% inhibition of growth relative to the growth observed in the control tube. A 1:5 dilution of the growth in the control well in sterile media,

Table II. Recommended MIC limits of five antifungal agents for two quality control strains when tested by the National Committee for Clinical Laboratory Standards M27-P proposed standard method (Pfaller et al., 1995a; Rex et al., 1996)

<table>
<thead>
<tr>
<th>Organism</th>
<th>Antifungal agent</th>
<th>MIC range</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Candida parapsilosis</em> ATCC 22019</td>
<td>amphotericin B</td>
<td>0.25–1.0</td>
</tr>
<tr>
<td></td>
<td>ketoconazole</td>
<td>0.064–0.25</td>
</tr>
<tr>
<td></td>
<td>itraconazole</td>
<td>0.064–0.25</td>
</tr>
<tr>
<td></td>
<td>fluconazole</td>
<td>2.0–8.0</td>
</tr>
<tr>
<td></td>
<td>5-fluorocytosine</td>
<td>0.12–0.5</td>
</tr>
<tr>
<td><em>C. krusei</em> ATCC 6258</td>
<td>amphotericin B</td>
<td>0.5–2.0</td>
</tr>
<tr>
<td></td>
<td>ketoconazole</td>
<td>0.125–0.5</td>
</tr>
<tr>
<td></td>
<td>itraconazole</td>
<td>0.125–0.5</td>
</tr>
<tr>
<td></td>
<td>fluconazole</td>
<td>16–64</td>
</tr>
<tr>
<td></td>
<td>5-fluorocytosine</td>
<td>4.0–16</td>
</tr>
</tbody>
</table>
may be performed to assist in definition of the endpoint (Espinel-Ingroff, Steele-Moore, & Galgiani, 1994).

Much of the methodology of the proposed standard was adopted to minimize intra- and interlaboratory variation in results. The current NCCLS method performs well for flucytosine and the azoles, itraconazole, ketoconazole, and fluconazole (Tables II and III), but there is concern that the current proposed method may mask clinically relevant variations in amphotericin B susceptibility (Rex et al., 1993). Rex et al. (1995a) have evaluated a variety of media for susceptibility testing of Candida species including Candida lusitaniae strains proven resistant to amphotericin B in animal models. By the NCCLS M27-P method, the MICs for all tested strains lay in the range 0.125–2 mg/L. Although the MICs for the resistant strains were generally higher (2 mg/L) than those of the susceptible strains (< 1 mg/L), the limitations of reproducibility are such that in approximately one-third of a series of replicate MIC determinations, the MIC for the “resistant” isolates was 1 mg/L. Using an alternative non-defined medium (antibiotic medium 3) at pH 7.0, a broader range of amphotericin B MICs (0.0625–32 mg/L) was obtained with somewhat better, though incomplete, separation of susceptible and resistant strains. A similar degree of separation of amphotericin susceptible and resistant Candida species has been reported using the Etest method on RPMI 1640 agar (Wanger et al., 1995). Further research is necessary to refine the available proposed standard method for susceptibility testing of amphotericin B.

The proposed standard macrodilution broth method for yeast has formed the basis for development of a variety of antifungal susceptibility test methods which are more suited for use in clinical laboratories and in studies of large numbers of isolates. These include a standardized microdilution broth method and the Alamar, Sensititre, and Etest commercial systems. The NCCLS proposed standard for yeast susceptibility testing has also acted as a starting point for development of a standard method for susceptibility testing of the filamentous fungi.

Definition of quality control strains

The process of development of standardized reproducible methods for antifungal susceptibility testing has at every stage benefited from the prior experience with development of standardized methods for antibacterial susceptibility testing. Quality control (QC) strains with well defined MICs which consistently fall within a relatively narrow range of values when the test is properly performed have proven of critical importance in ensuring the accuracy of bacterial susceptibility testing whether for clinical or research purposes. Recently quality control strains for antifungal susceptibility testing with similar properties have been defined (Pfaller et al., 1995a; Rex et al., 1996). The properties of these strains are summarized in Table II. The QC parameters specified have been developed by multi-laboratory studies using multiple lots of RPMI media from different manufacturers in accordance with the recommendations of the NCCLS document M23-A (NCCLS, 1994). Quality control strains are invaluable for routine quality control of susceptibility testing in clinical laboratories. They are also of value in the initial evaluation of the performance of methods other than the NCCLS standard and in studying the factors which influence antifungal MIC results. It is important to emphasize however, that proposed or established QC strains are in part selected for the consistent reproducibility of their MICs in the particular test system. It is possible therefore that the MIC for these strains
are less subject to variation in response to minor methodological variations than is the case for yeasts in general. For this reason equivalent MICs for a small group of QC strains following modification of the standard method should not be regarded as conclusive evidence that the standard and modified methods are equivalent for all purposes.

**Alternatives to the reference macrodilution broth method**

In developing a proposed standardized susceptibility test method, the NCCLS subcommittee on antifungal susceptibility testing chose a macrodilution broth format because it is widely used and because this format facilitates analysis of all parameters, including end point determination (Rex *et al.*, 1993). The macrodilution broth method is cumbersome, however, and clearly more convenient and less expensive methods would be preferable for clinical laboratories and for studies of large numbers of isolates. The proposed standard method has been used as a benchmark against which to evaluate possible alternative methods such as microdilution broth, disc diffusion, and stable gradient agar diffusion.

**Microdilution broth susceptibility testing**

The most direct approach to development of a microdilution broth method is the minaturization of the broth macrodilution method. A preliminary study by Espinel-Ingroff *et al.* (1991) using RPMI 1640 medium buffered with morpholine-propane sulphonic acid (MOPS) at 0.165 M and pH 7.0 (as per M27-P) and the same inoculum (0.5–2.5 × 10^3 cfu/mL) and incubation conditions (35°C) as for M27-P, reported that MICs determined by broth microdilution did not, in general, differ in a statistically significant degree from those determined by the M27-P method. This encouraging initial report resulted in refinement of the microdilution broth method in subsequent studies (Espinel-Ingroff *et al.*, 1992; Pfaller *et al.*, 1994; Sewell, Pfaller & Barry, 1994).

An important factor impacting on the MIC is the period of incubation prior to reading. In a series of studies (Tables III) overall agreement of the microdilution broth method with the M27-P method is a little better at 48 h of incubation than at 24 h. The differences are small however and are mostly attributable to a small number of strains which exhibit markedly higher microdilution broth MICs at 48 h than at 24 h. At the present time, it is not apparent if 24 or 48 h MICs correlate best with clinical outcome for these strains.

A variety of modifications to the microdilution method have been presented with a view to improved ease of interpretation of endpoints or improved precision of endpoint determination. The most simple of these modifications is the agitation of the microtitre plate before reading to achieve a homogenous suspension of yeast. Anaissie, Paetznick, & Bodey (1991) reported improved reproducibility and lower MICs of fluconazole with reading after agitation of plates. A more recent study included five antifungal agents and used the current “prominent decrease in turbidity” criterion for endpoint determination (Pfaller, Messer & Coffman, 1995b). Reproducibility of results in that study was essentially the same (97–99%) for results read with or without agitation at 24 or 48 h. Agreement between MICs determined by both methods was 99%. Thus it appears that agitation of plates before visual reading may increase ease of reading, but
in experienced laboratories does not significantly alter the MIC using the current criteria for endpoint determination.

A further modification with implications for future automation of antifungal susceptibility testing is spectrophotometric determination of endpoints. For fluconazole, Anaissie et al. (1991) demonstrated that spectrophotometric determination of endpoints following agitation of microtitre plates was equivalent to visual reading. In that study, the lowest drug concentration which reduced turbidity to \( \leq 50\% \) of that in the control well was the MIC. Pfaller et al. (1995b) studied five antifungals (including amphotericin B and flucytosine) using both visual and spectrophotometric readings and \( \leq 50\% \) of turbidity in the control as the MIC endpoint. Reproducibility of the spectrophotometric determination was higher at 48 h (99%) than at 24 h (89%) and agreement with visual endpoint determination at 48 h was 97–99%. Other studies also support the use of a spectrophotometer to provide a highly reproducible and objective means of determining MIC endpoints for antifungal susceptibility testing (Rodriguez-Tudela & Martinez-Suarez, 1995; Odds, Vranckx & Woestenborghs, 1995; Wardle et al., 1995).

In addition to studies addressing methods of endpoint determination, modifications to the proposed RPMI 1640 medium have also been investigated. Sugar & Liu (1994) have demonstrated that RPMI 1640 without phenol red is equivalent to standard RPMI 1640 for this purpose. The equivalence of three different formulations of RPMI 1640 medium has also been demonstrated (Espinel-Ingroff & Steele-Moore, 1994). The use of RPMI 1640 medium supplemented with additional glucose has also been advocated (Polanco et al., 1995; Rodriguez-Tudela & Martinez-Suarez, 1995). Better growth of Candida species (as judged by increased turbidity in the growth control well) can be achieved in RPMI 1640 supplemented with glucose to a final concentration of 2%. The glucose supplementation is reported to enhance the ease of endpoint determination without altering significantly the MIC determined.

The NCCLS standard method specifies the use of an inoculum of 0.5 to 2.5 \( \times \) 10 \( \text{cfu/mL} \). This low inoculum was chosen to maximize interlaboratory agreement. A number of recent studies have utilized a somewhat higher inoculum (\( \geq 10^4 \) cfu/mL) (Odds et al., 1995; St. Germain et al., 1995; Wardle et al., 1995). Based on the MICs for the control strains of yeast it appears that this increase in inoculum does not significantly alter results in these laboratories. Further multicentre studies of larger groups of isolates are needed to determine if this degree of inoculum modification has any impact on interlaboratory agreement.

Other modifications to the broth microdilution method have also been proposed and studied, in particular the development of a colourimetric broth microdilution method. The use of a pH indicator dye (Fournier et al., 1995) and of an oxidation/reduction indicator dye, XTT, have been reported (Sugar & Liu, 1995); however, the best studied and developed of these approaches uses the oxidation/reduction indicator dye Alamar blue (Alamar Biosciences, Inc., Sacramento, CA, USA). MICs determined by this method are equivalent to those obtained by the standard broth microdilution method (Pfaller et al., 1994a,e; Tiballi et al., 1995; To, Fothergill & Rinaldi, 1995). A dried commercially prepared broth microdilution tray (Sensititre) has also been evaluated and its performance is compared to the standard broth macrodilution method. Agreement (\( \pm 2 \) dilutions) was 83 to 92%, with agreement lowest for itraconazole and highest for flucytosine (Messer & Pfaller, 1996). Additional variations on the microdilution broth method reported in the literature include the use of lower concentrations of the MOPS buffer and alternative incubation temperatures (30°C) (Hacek et al., 1995). A number
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of laboratories use alternative media for broth microdilution susceptibility testing. The alternatives include defined media such as Eagles minimal essential medium (Hacek et al., 1995) and high resolution medium (Pfaller et al., 1990; Ruhnke et al., 1994) and the undefined medium yeast nitrogen base (Johnson et al., 1995; Law et al., 1994). Results with high resolution medium have been reported as similar to results with RPMI in the broth macrodilution method (Pfaller et al., 1990). The lot-to-lot variability of undefined media is clearly a limiting factor in the definition of a standard method although such media may be useful in individual laboratories and in specific circumstances.

In summary, a microdilution broth method, which is a miniaturization of the macrodilution broth M27-P method, is equivalent to the macrodilution broth method and is likely to be recommended as a tentative standard method by the NCCLS subcommittee on antifungal susceptibility testing. The limitations of the standard macrodilution broth method in detection of amphotericin resistance discussed previously apply equally to the microdilution method (Rex et al., 19956).

Disc test and Etest modifications of agar diffusion methodology

One of the more widely used methods for antibacterial susceptibility testing is the disc diffusion method. The sustained popularity of this method is related to its convenience and relatively low cost. Disc diffusion interpretive criteria for bacteria are developed by regression analysis of the diameters of zones of inhibition relative to MICs determined by a reference methodology. The applicability of this process to fungal susceptibility testing has been complicated by the absence of a standardized method before the M27-P proposal. In addition the absence of clinically relevant interpretive breakpoints for MIC methods for antifungals makes the definition of the categories resistant, intermediate, and susceptible impractical for disc diffusion methods. Despite these problems, there is evidence that disc diffusion susceptibility testing has potential applications. Troillet et al. (1993) have demonstrated a good correlation ($r = 0.85$) between MICs determined in yeast nitrogen broth with the diameter of the zones of inhibition of growth of strains around a 50 μg fluconazole disc on yeast nitrogen agar. They proposed interpretive breakpoints of $> 25$ mm indicating susceptibility (clinical response; MIC $< 4$ mg/L) and $\leq 25$ mm indicating resistance (failure of response). No intermediate category was defined. Pfaffer et al. (1992a), using high resolution antifungal assay medium (HR) and a 25 μg fluconazole disk, reported good intra-laboratory reproducibility (replicate test results with 4 mm of each other) of 91% but interlaboratory agreement of only 58%. Taken together, these studies indicate that disc diffusion susceptibility testing may be useful. Studies correlating disc diffusion methodologies with the NCCLS proposed standard method is a logical next step in the standardization process.

A relatively recent development in the field of antibacterial susceptibility testing is the stable gradient technology marketed as the Etest. The Etest is a plastic strip with the relevant antimicrobial on one side and a scale on the other side. When applied to an agar plate, a continuous gradient of antimicrobial is established in the surrounding agar. An ellipse of growth inhibition occurs and the intersection of the ellipse with the numeric scale of the strip allows reading of the MIC. This technology is being applied also to antifungal susceptibility testing. The degree of agreement of MICs determined by Etest with the reference macrodilution broth M27-P method in three independent
Table III. Drug specific agreement between micro- and M27-P macrodilution methods (±2 dilutions) for susceptibility testing of yeast isolates in five independent studies (Espinel-Ingroff et al., 1992; Barchiesi et al., 1994; Pfaller et al., 1994c; Sewell et al., 1994; Rex et al., 1995)

<table>
<thead>
<tr>
<th>Drug</th>
<th>Reading time (h)</th>
<th>Number of strains</th>
<th>No. determinations</th>
<th>No. in agreement with M27-P</th>
<th>% agreement with M27-P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amphotericin B</td>
<td>24</td>
<td>600</td>
<td>980</td>
<td>908</td>
<td>93%</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>600</td>
<td>980</td>
<td>932</td>
<td>95%</td>
</tr>
<tr>
<td>Fluconazole</td>
<td>24</td>
<td>957</td>
<td>1337</td>
<td>1150</td>
<td>86%</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>957</td>
<td>1337</td>
<td>1158</td>
<td>87%</td>
</tr>
<tr>
<td>Flucytosine</td>
<td>24</td>
<td>368</td>
<td>748</td>
<td>668</td>
<td>88%</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>368</td>
<td>748</td>
<td>729</td>
<td>97%</td>
</tr>
<tr>
<td>Itraconazole</td>
<td>24</td>
<td>273</td>
<td>273</td>
<td>144</td>
<td>53%</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>273</td>
<td>273</td>
<td>234</td>
<td>86%</td>
</tr>
<tr>
<td>Ketoconazole</td>
<td>24</td>
<td>95</td>
<td>475</td>
<td>385</td>
<td>81%</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>95</td>
<td>475</td>
<td>332</td>
<td>71%</td>
</tr>
</tbody>
</table>

Table IV. Agreement of MIC determined by Etest with MIC determined by the M27-P macrodilution broth method in three independent studies

<table>
<thead>
<tr>
<th>Study</th>
<th>No. isolates</th>
<th>Time (h)</th>
<th>% agreement with M27-P for:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>fluconazole</td>
</tr>
<tr>
<td>Espinel-Ingroff (1994)</td>
<td>78 (± 1 dilution)</td>
<td>24</td>
<td>90</td>
</tr>
<tr>
<td></td>
<td></td>
<td>48</td>
<td>94</td>
</tr>
<tr>
<td>Sewell et al. (1994)</td>
<td>238 (± 2 dilutions)</td>
<td>24</td>
<td>76</td>
</tr>
<tr>
<td></td>
<td></td>
<td>48</td>
<td>71</td>
</tr>
<tr>
<td>Colombo et al. (1995)</td>
<td>100 (± 1 dilution)</td>
<td>24</td>
<td>80</td>
</tr>
<tr>
<td></td>
<td></td>
<td>24 (± 2 dilutions)</td>
<td>97</td>
</tr>
</tbody>
</table>

Table V. In-vitro susceptibility of 597 clinical yeast isolates to three antifungal agents determined by an NCCLS standardized microdilution broth method (cf. Pfaller & Barry, 1995)

<table>
<thead>
<tr>
<th>Antifungal agent</th>
<th>Incubation time (h)</th>
<th>≤0.016</th>
<th>0.03</th>
<th>0.06</th>
<th>0.12</th>
<th>0.25</th>
<th>0.5</th>
<th>0.1</th>
<th>2.0</th>
<th>4.0</th>
<th>8.0</th>
<th>16</th>
<th>32</th>
<th>64</th>
<th>128</th>
<th>256</th>
<th>512</th>
<th>&gt; 512</th>
<th>Cumulative % of isolates inhibited at concentrations (mg/L) cf:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amphotericin B</td>
<td>24</td>
<td>0.5</td>
<td>2</td>
<td>9</td>
<td>37</td>
<td>89</td>
<td>99</td>
<td>&gt;99</td>
<td>100</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
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</tr>
<tr>
<td></td>
<td>48</td>
<td>0.2</td>
<td>0.7</td>
<td>1</td>
<td>4</td>
<td>39</td>
<td>94</td>
<td>99</td>
<td>100</td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fluconazole</td>
<td>24</td>
<td>0.2</td>
<td>0.3</td>
<td>3</td>
<td>32</td>
<td>64</td>
<td>74</td>
<td>80</td>
<td>92</td>
<td>95</td>
<td>98</td>
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<td>100</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>0.2</td>
<td>0.2</td>
<td>0.8</td>
<td>13</td>
<td>42</td>
<td>60</td>
<td>69</td>
<td>73</td>
<td>78</td>
<td>82</td>
<td>86</td>
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<tr>
<td>Flucytosine</td>
<td>24</td>
<td>0.3</td>
<td>2</td>
<td>25</td>
<td>36</td>
<td>77</td>
<td>87</td>
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Antifungal susceptibility testing studies is summarized in Table IV. There was marked variation in the level of agreement noted in these three published reports. It should be noted that the relatively poor agreement (76% ± 2 dilutions) between Etest and M27-P methods for determination of fluconazole MICs in the study of Sewell et al. (1994) is mainly attributed to very poor agreement for two specific species, *Candida glabrata* (34%) and *Candida tropicalis* (67%). Agreement for other species of *Candida* was superior. A more recent study evaluated the Etest strip on RPMI 1640 agar with 2% glucose, and on casitone agar for four antifungals amphotericin B, fluconazole, itraconazole, and ketoconazole (Espinel-Ingroff et al., 1996). Interlaboratory agreement (±2 dilutions) was superior with casitone medium when compared with RPMI for amphotericin B, ketoconazole, and itraconazole. Interlaboratory agreement for fluconazole was superior on RPMI 1640. Agreement of MICs with the M27-P method was not assessed. Based on these early studies it appears that the Etest is a promising, but not perfect, method for performing antifungal susceptibility testing. Additional development of Etest for antifungal testing is warranted.

Antifungal susceptibility testing of filamentous fungi

The filamentous fungi account for a minority of invasive fungal infection and the development of a standardized method for susceptibility testing of filamentous fungi was accorded lower priority than yeast by the NCCLS subcommittee on antifungal susceptibility testing. Despite their relative infrequency, however, invasive disease due to filamentous fungi is associated with a high mortality (Pannuti et al., 1991). The variety of filamentous fungi associated with invasive disease has increased in recent years and there is some evidence that some isolates and species may be resistant to amphotericin (Pfaller & Wenzel, 1992). In addition, with the introduction of itraconazole into clinical use and the likely introduction of additional agents in the next decade, clinicians will require laboratory guidance to optimize choice of therapy for a given individual. For these reasons, some progress towards standardization of in-vitro testing methods for filamentous fungi has been made.

Because the filamentous fungi do not grow as single cells, standardization of the test inoculum represents a significant challenge. Inoculum size is a more important factor for flucytosine than for amphotericin B, and its importance for itraconazole is somewhat species dependent (Gehrt et al., 1995). The only unicellular phase of the life cycle of the filamentous fungi is the conidia and therefore attention has focused on suspensions of conidia as the most practical inoculum. The susceptibility test therefore measures the inhibitory activity of the antifungal on germination of conidia which is believed to correlate with activity against the vegetative hyphae. Based on the experience with yeasts, a spectrophotometric method of inoculum preparation has been evaluated for a variety of filamentous fungi (Espinel-Ingroff & Kerkering, 1991). In this preliminary study, the ability to reproducibly prepare conidial suspensions yielding colony forming units within an acceptable range for six species of moulds of medical importance was demonstrated. A subsequent multicentre study addressed the interlaboratory variability in inoculum suspensions prepared spectrophotometrically and the variability in MICs determined with macrobroth and microdilution broth formats (Espinel-Ingroff et al., 1995). The method used in this study was very similar to the M27-P method. The medium for both macrodilution and microdilution methods are the same as for M27-P. The inoculum used was a spectrophotometrically standardized conidial suspension.
diluted to a final inoculum of 0.5 to 5 x 10^6 cfu/mL. Incubation was at 35°C until growth was visible in the antifungal free tube. Endpoints were determined by scoring of growth in the broth relative to a set of photographic standard with ≥ 75% inhibition of growth relative to the control tube taken as the MIC. This study documented the feasibility of achieving conidial suspensions within a relatively narrow range (0.4 x 10^6 to 3.3 x 10^6 cfu/mL) on 90% or greater of occasions. Better interlaboratory (94–97%) agreement was observed for Aspergillus fumigatus, Pseudallescheria boydii, and Rhizopus arrhizus than for Aspergillus flavus or Sporothrix schenckii (80–83%). Furthermore, interlaboratory agreement in MICs for both macrodilution and microdilution formats was excellent for amphotericin B, fluconazole, ketoconazole, and flucytosine (87–100%). For itraconazole interlaboratory agreement was less (74–80%) than for the other antifungals. This study also proposed tentative MIC ranges for two QC strains.

While the process of standardization of susceptibility testing for filamentous fungi is at a relatively early stage, the level of agreement achievable in this multicenter study with a method similar to M27-P is encouraging. It appears that as for yeasts, micro- and macrodilution broth methods may prove equivalent.

Clinical corelates of in-vitro susceptibility testing

For in-vitro susceptibility testing to be useful clinically, the results should provide a reliable prediction of in-vivo response to therapy in human infections. Unfortunately, the evidence for clinical correlation with in-vitro antifungal tests is limited at best (Pfaller & Rinaldi, 1993; Rex et al., 1993; Espinel-Ingroff & Pfaller, 1995; Rinaldi & Pfaller, 1995c). With the advent of standardized antifungal susceptibility testing methods, and microdilution adaptations thereof, it is now possible to perform large-scale surveys of clinical isolates (Pfaller & Barry, 1994). Such surveys may be used to develop population distribution profiles of MICs for antifungal agents against various species of fungi and to begin to correlate in-vitro data with clinical response.

A recent survey of 597 isolates of Candida spp. by Pfaller & Barry (1995) indicated that the majority of clinical isolates are inhibited by concentrations of amphotericin B, fluconazole, and flucytosine that are easily achievable in serum or other body fluids (Table V). It is clear from this and other studies that there are species-specific differences in susceptibility to the various agents (Wingard et al., 1991, 1993; Hitchcock et al., 1993; Pfaller et al., 1994a, 1995b). Notably, decreased susceptibility to both amphotericin B and flucytosine have been reported for C. lusitaniae and Candida krusei (Wingard et al., 1991; Pfaller, Messer & Hollis, 1994c; Pfaller & Barry, 1995). C. krusei and C. glabrata are also known to be relatively resistant to fluconazole and other azoles (Hitchcock et al., 1993; Wingard et al., 1991, 1993). Thus, the identification of fungal pathogens to the species level is a necessary first step in providing useful clinical and epidemiological markers for the presence of potentially resistant strains (Pfaller, 1996).

Resistance to amphotericin B is clearly unusual (Rex et al., 1993, 1995a; Espinel-Ingroff & Pfaller, 1995); however, the application of in-vitro susceptibility testing has provided some interesting insights into the clinical response (or lack thereof) to amphotericin B in certain patient populations. Amphotericin B is both inhibitory and fungicidal for most yeasts at a concentration of ≤ 1.0 mg/L (Espinel-Ingroff & Pfaller, 1995). As shown by Pfaller & Barry (1995), 94–99% of clinical isolates of Candida spp. are inhibited by ≤ 1.0 mg/L of amphotericin B (Table V). An amphotericin B MIC
of ≥2.0 mg/L is distinctly unusual and suggests possible clinical resistance, since this MIC only approximates the concentration achievable in serum at high doses of amphotericin B (e.g. ≥1 mg/kg/day) and exceeds that achievable in CSF (Powderly et al., 1988; Espinel-Ingroff & Pfaller, 1995). Studies by both Bryce et al. (1992) and Powderly et al. (1988) reported that immunocompromised patients with fungaemia due to *Candida* spp. with amphotericin B MICs of >0.8 mg/L were significantly more likely to die because of that infection than were individuals infected with *Candida* spp. with amphotericin B MICs of ≤0.8 mg/L. Although these studies are interesting, some concern remains that the current NCCLS reference method may be insensitive to amphotericin B resistance (Rex et al., 1993). A recent study by Rex et al. (1995b) of candidaemia in non-neutropenic patients found a narrow range of amphotericin B MICs (0.125 mg/L to 1.0 mg/L) among blood stream isolates of *Candida* spp. This narrow range of amphotericin B MICs is a consistent feature of the M27-P method. Because the MICs were all ≤1.0 mg/L, the clinical failures were all concentrated among isolates with low MICs, resulting in an apparent lack of clinical correlation of the in-vitro test data. Although it is possible that a different test method may have provided a wider distribution of MICs and possibly a better correlation, an alternative explanation is that the organisms were indeed "susceptible" to amphotericin B but that factors other than the intrinsic antifungal susceptibility were important in these patients. Additional investigation will be necessary before a clinically significant MIC breakpoint for amphotericin B susceptibility can be recommended.

Due to low toxicity and ease of administration, fluconazole is used widely for the treatment of various forms of candidosis. Reports of fluconazole resistance have appeared and studies of some of these isolates have demonstrated relative resistance to fluconazole (Wingard et al., 1991; Cameron et al., 1993; Rex et al., 1993, 1995a; Sandven et al., 1993; Troillet et al., 1993; Redding et al., 1994). Fluconazole inhibits the majority of *Candida* spp. at concentrations of <8.0 mg/L; however, isolates of *C. glabrata* and *C. krusei* generally have MICs of 8.0–16 mg/L and 32–64 mg/L or greater, respectively (Table V) (Wingard et al., 1991, 1993; Pfaller & Barry, 1995; Rex et al., 1995a,b). In recent years, several studies have documented a trend toward decreased susceptibility to fluconazole among *C. albicans* isolates from patients with AIDS who have oropharyngeal candidosis (Cameron et al., 1993; Sandven et al., 1993; Troillet et al., 1993; Pfaller et al., 1994e; Redding et al., 1994; Rex et al., 1995c). It is increasingly clear that the NCCLS methodology and methods like it do detect intrinsic differences in susceptibility of candida isolates to fluconazole in both animal models (Anaissie et al., 1994; Graybill et al., 1995) and AIDS patients with oropharyngeal candidosis (Redding et al., 1994; Barchiesi et al., 1995; Rex et al., 1995c).

Anaissie et al. (1994) studied the correlation of MICs of three antifungal agents with response to therapy in a murine model of candidaemia. The MICs were determined by a modification of the M27-P method. Overall, a good correlation existed between in-vitro MICs of amphotericin B, fluconazole, and flucytosine and response to therapy as assessed by prolonged survival and clearance of candida from the kidneys of infected mice. In the case of fluconazole, higher doses were required to prolong survival in mice infected with strains with high fluconazole MICs. Similarly, Graybill et al. (1995), have demonstrated a good correlation between in-vivo response and fluconazole MICs in a murine model of candidosis. Mice infected with *C. albicans* strains with MICs ≤0.25 mg/L responded well to modest doses of fluconazole, whereas long term survival
of mice infected with strains with MICs of 16–64 mg/L was reduced even with large doses of fluconazole.

The clinical response of *C. albicans* to fluconazole in AIDS patients with oropharyngeal candidosis correlates nicely with the in-vitro susceptibility determined by NCCLS methods (Cameron et al., 1993; Rex et al., 1995c; Redding et al., 1994; Barchiesi et al., 1995). Macrodilution broth susceptibility testing by an alternative method using high resolution medium has also been reported to show a good correlation with clinical response in oropharyngeal candidosis in AIDS (Bailey et al., 1994). It is now apparent that oropharyngeal candidosis in AIDS patients infected with isolates of *C. albicans* with fluconazole MICs of ≤8.0 mg/L are likely to respond to doses of 100–200 mg/day of fluconazole (Redding et al., 1994; Barchiesi et al., 1995; Rex et al., 1995c). Response to therapy with higher fluconazole doses (400–800 mg/day) may be achieved in infections due to isolates with MICs of 16 or 32 mg/L and response is unlikely in patients infected with a *C. albicans* strain for which the MIC is ≥64 mg/L (Cameron et al., 1993; Redding et al., 1994; Rex et al., 1995c). Overall, these studies support the ability of in-vitro susceptibility testing of fluconazole to predict the clinical outcome of mucosal fungal infections.

The relationship between fluconazole MICs and clinical outcome is less clear when one considers more serious infections such as candidaemia (Rex et al., 1995b,c). Rex et al. (1995b,c) have demonstrated that although the distribution of fluconazole MICs among isolates of *Candida* spp. from blood stream infections is similar to that seen with isolates from oropharyngeal candidosis, the relationship between MIC and therapeutic outcome may be somewhat different. In the study of Rex et al. (1995b), 64 fluconazole-treated patients had candidaemia due to isolates for which fluconazole MICs were ≤16 mg/L and 19 (29%) of these patients failed fluconazole therapy. Conversely, there were four patients infected with *Candida* spp. (*C. glabrata*, two patients; *C. krusei*, one patient; *Candida lipolytica*, one patient) for which fluconazole MICs were ≥32 mg/L and all four responded to initial fluconazole therapy. Thus, the discrepancies between in-vitro results and clinical outcome were manifest both as clinical failures among isolates requiring low MICs and as clinical responses to fluconazole despite infection due to organisms with high fluconazole MICs. Although disappointing, this inexact correlation between MICs and outcome parallels that seen with bacterial infections and antibiotic susceptibility tests (Greenwood, 1981; Sanders & Sanders, 1982; Stratton, 1991). Numerous clinical studies of bacterial infection have demonstrated that the use of an antibiotic judged to be effective *in vitro* (low MIC) does not ensure recovery. Conversely, the use of an agent that appears relatively ineffective *in vitro* (high MIC) does not preclude recovery (Greenwood, 1981; Sanders & Sanders, 1982; Stratton, 1991; Rex et al., 1995b). It must be realized that in more complex clinical situations all tests of antimicrobial activity, whether they involve fungi or bacteria, merely provide data that must be interpreted in terms of the pharmacological properties of the drug and the condition of the patient (Greenwood, 1981; Stratton, 1991; Rex et al., 1995b). Certainly in the setting of candidaemia, patient management factors such as the removal (or lack thereof) of intravascular catheters can overshadow intrinsic antifungal activity (Rex et al., 1995b).

Evidence to support the clinical relevance of antifungal susceptibility testing is accumulating at a slow but steady pace. Ideally, the selection of interpretive breakpoints for antimicrobial agents is based on a combination of clinical outcomes, pharmacokinetic data, and the in-vitro susceptibility profiles of the organisms of
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interest. Unfortunately, the clinical data is frequently suboptimal or lacking altogether and tentative breakpoints for antibacterial agents have occasionally been established based on pharmacological data and the distribution of MICs (Pfaller et al., 1992b; Barry, Pfaller & Fuchs, 1993). Regarding antifungal agents, there is a large body of information on the human pharmacokinetics of existing agents and rapidly emerging in-vitro susceptibility data for certain organism groups such as Candida spp. (Powderly et al., 1988; Bryce et al., 1992; Cameron et al., 1993; Hitchcock et al., 1993; Pfaller et al., 1994d; Redding et al., 1994; Rex et al., 1993, 1995b; Espinel-Ingroff & Pfaller, 1995). Unfortunately, the clinical outcomes data are extremely limited. Although it is tempting to assign interpretive breakpoint values based upon pharmacokinetic data and the distribution of MICs, the complexity of patients with candidal infections is such that an overly simplistic approach to this issue is likely to create more problems than it solves (Pfaller & Barry, 1995). In the case of fluconazole, interpretation of results must also take into account the wide range of dosage (50–800 mg/L) which can be administered safely. Additional factors such as site of infection, presence of foreign devices, and other patient specific factors may influence the outcome of antifungal therapy and confound the interpretation of antifungal susceptibility tests.

Widely accepted interpretive MIC breakpoints have not been established for any antifungal agent at the present time. Interpretive breakpoints for fluconazole and ketoconazole have been proposed by some authors based on the limited available data (Bailey et al., 1994; Rodriguez-Tudela et al., 1995). More data correlating clinical outcome with in-vitro MICs are needed for both these agents and for itraconazole. Because of the uncertainty in relation to interpretation it is not recommended that antifungal susceptibility testing be performed routinely at this time. Nevertheless, the result of in-vitro testing may be useful as an adjunct to other clinical and experimental data in determining the course of action in a difficult case (Galgiani, 1993). Bearing in mind the limitations outlined, it may be reasonable to suppose that for isolates with low fluconazole MICs (perhaps ≤8 mg/L by the reference M27-P method) standard doses (100–200 mg/day) are likely to be adequate antifungal therapy in association with attention to other contributory factors. For isolates with moderate MICs (16–32 mg/L) higher doses of fluconazole may be justified. For isolates with high MICs (≥64 mg/L) an alternative agent may be preferable.

Conclusions

The past decade has been a period of rapid change in clinical mycology. Due to the increased numbers of profoundly immunocompromised patients, the frequency of fungal infection and the variety of fungi recognized as human pathogens has increased dramatically. In response to the increased incidence of fungal infection, the pharmaceutical industry has developed a number of newer less toxic antifungals for clinical use. The increased use of antifungals, often for prolonged periods, has lead to the recognition of the phenomenon of acquired antifungal resistance amongst previously susceptible strains or species and to the increased incidence of infection with less common species with intrinsic resistance to one or more of the available antifungals. For all of these reasons, and to facilitate the development of further antifungal agents, interlaboratory standardization of susceptibility testing is important. Considerable progress has been made in this area; however, further progress is urgently needed and a considerable degree of interlaboratory and international cooperation will be a critical
factor in collecting the data to further evaluate clinical correlates. Standardization of susceptibility testing is an evolving process and modifications to a standard may be necessary as new data emerge. The essential value of a reproducible standard is to provide a mechanism by which laboratory methods and clinical observations from diverse sources may be analyzed in a coherent framework. Progress in antifungal susceptibility test standardization and ultimately in therapy may be enhanced by further systematic investigation of variables which impact on in-vitro susceptibility results and prospective studies of clinical outcome.

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