Fluconazole disc diffusion testing for the routine laboratory

Joanne L. May, Anna King and Christine A. Warren

Department of Microbiology, UMDS, St Thomas's Campus, London SE1 7EH, UK

The increasing incidence of resistance to the antifungal agent, fluconazole, prompted the need for a rapid, reliable and easy-to-use susceptibility test. We have developed a disc diffusion test for fluconazole against Candida spp. suitable for a clinical laboratory. Disc diffusion tests on six different media were compared with MIC values. On the basis of correlation coefficient with MICs ($r = -0.95$), quality of growth and zone edge definition, Yeast Nitrogen Base agar with glucose (YNBG) produced the best results. Further studies on YNBG showed that the method is reliable for Candida albicans and for resistant isolates with no zone of inhibition, but results for the slower growing and uncommon species must be interpreted with some caution. Implementation of this test in the clinical laboratory has provided a much needed therapeutic service for clinicians within the hospital. It has also reduced the reliance on the reference laboratory for susceptibility results and the consequent costs involved.

**Introduction**

The increasingly frequent use of cytotoxic, antibacterial and immunosuppressive drugs required to treat both malignant and non-malignant disease has recently been associated with a rise in the incidence of serious fungal infection. Serious fungal infection carries considerable morbidity and mortality. Patients infected with HIV contribute to the majority of cases associated with this increase and the use of long-term antifungal therapy in these patients and others gives rise to an increase in the frequency of resistance to antifungals. This has highlighted the need for easy, rapid and reliable antifungal susceptibility tests, which at present are generally performed in a reference laboratory.

Azoles play an important role in the treatment of fungal infections. Methods for the in-vitro testing of these drugs are generally not available in most microbiology laboratories. Susceptibility testing for the azoles is dramatically influenced by both methodology and technique. Precisely defined techniques are required for accurate results and to reduce both intra- and inter-laboratory discrepancies.

We have developed a disc diffusion method that enables a routine laboratory to perform a sensitivity test for fluconazole, an antifungal drug commonly used in the treatment of infections with Candida spp. This test can be performed in the same time as a routine bacterial sensitivity test.

**Materials and methods**

**Organisms and identification**

One hundred patient isolates of Candida spp. were collected for the initial study to determine the best medium for disc diffusion susceptibility testing. These were isolated in the clinical microbiology laboratory at St Thomas's Hospital during a 6 month period. The majority of isolates were obtained from patients in the intensive therapy unit and those attending a specialist HIV unit. They were chosen to represent as many different species, specimen types and medical specialities as possible. These included: Candida albicans ($n = 64$), Candida glabrata ($n = 21$), Candida parapsilosis ($n = 7$), Candida tropicalis ($n = 6$), Candida guilliermondii ($n = 1$) and Candida famata ($n = 1$).

After choosing the medium for disc testing, a second set of clinical strains was collected for further susceptibility testing and included isolates chosen to represent different species and, on the basis of MICs, categories of sensitivity (Table).

Strains were isolated on Sabouraud agar (bioMérieux, Basingstoke, U K) and were maintained on Sabouraud agar slopes at room temperature. All isolates were speciated by routine testing methods. C. albicans was identified by the production of a germ tube within 2 h in sterile horse serum after incubation at $37^\circ$C. Germ tube-
negative isolates were identified with the API 20C AUX (bioMérieux).

Controls

Control candida strains were obtained from two sources, Pfizer (Sandwich, U K), who donated three C. albicans control strains representing resistant (Y O1.17), intermediate (Y O1.108) and sensitive (Y O1.09) organisms, and the American Type Culture Collection (ATCC), which provided C. albicans ATCC 90028 (fluconazole-sensitive), C. albicans ATCC 90029 (fluconazole-sensitive) and C. glabrata ATCC 90030 (fluconazole-resistant).

Antifungal drug

Fluconazole powder, provided by Pfizer, was dissolved and diluted in sterile distilled water for use in MIC determinations. Fluconazole discs (25 μg) were obtained from Mast Laboratories (Bootle, U K).

Media

The disc diffusion procedure was performed for each isolate simultaneously on the following six media. (i) Sabouraud dextrose agar (bioMérieux), chosen because it is the routine medium for primary isolation and a familiar medium for laboratory staff; (ii) Yeast Nitrogen Base agar (Difco, West Molesey, U K) with 0.5% glucose (Y N B G), which is used by the PHLS Mycology Reference Laboratory, Bristol, U K, for determining fluconazole MICs; (iii) RPMI 1640 (Gibco, Paisley, U K), which is the medium specified by the NCCLS for determining MICs; (iv) High Resolution Medium (Oxoid), buffered with 0.165 M M O P S (3-(N-morpholino)propanesulphonic acid) pH 7 (BDH), is recommended by Pfizer for determining fluconazole MICs; (v) an alternative High Resolution Medium produced by Mast Laboratories and buffered with 0.2 M phosphate buffer pH 7.5; (vi) casitone agar (Difco), one of the media recommended for determining fluconazole MICs by E test (A B Biodisk, Solna, Sweden).

All media were prepared according to the manufacturers’ instructions and the agar was dispensed into 90 mm Petri dishes to a depth of 4 mm (25 mL) ready for use. Batch-to-batch variation of the agar was monitored with control strains.

Fluconazole MICs were measured in YNBG broth (Difco).

Preparation of inoculum

Stock strains were inoculated on Sabouraud agar and incubated for 18–24 h at 35°C. A repeat subculture on Sabouraud agar was made to ensure purity and viability. The inoculum was then prepared for susceptibility testing.

Disc diffusion

A suspension equivalent to a McFarland No. 2 standard was prepared in 2 mL sterile distilled water. A sterile swab was dipped in the suspension and the excess fluid drained by rotating the swab against the inside of the container. Each of the six media was inoculated from the same suspension, with a fresh swab with a rotary plater. A disc containing 25 μg fluconazole was placed slightly off-centre on the agar. The plates were incubated at 35°C for 18–24 h, read, and then incubated for a further 18–24 h to determine the optimal incubation time. The quality of growth was noted as was the presence and quantity of microcolonies within the zone. Zone sizes were measured in millimetres with vernier callipers, from the edge of the disc to the ‘edge’ of the zone. The edge of the zone was defined as a marked increase to semiconfluent or confluent growth.

<table>
<thead>
<tr>
<th>Species</th>
<th>n</th>
<th>Sensitive</th>
<th>Intermediate</th>
<th>Resistant</th>
</tr>
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<tbody>
<tr>
<td>C. albicans</td>
<td>96</td>
<td>51</td>
<td>27</td>
<td>18</td>
</tr>
<tr>
<td>C. famata</td>
<td>4</td>
<td>2</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>C. glabrata</td>
<td>42</td>
<td>3</td>
<td>39</td>
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<tr>
<td>Candida kefyr</td>
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<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>C. krusei</td>
<td>11</td>
<td>1</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>Candida lusitaniae</td>
<td>6</td>
<td>1</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>C. parapsilosis</td>
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<td>15</td>
<td>9</td>
</tr>
<tr>
<td>C. tropicalis</td>
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<td>5</td>
<td>9</td>
<td>6</td>
</tr>
<tr>
<td>Saccharomyces cerevisiae</td>
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<td>1</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>Total</td>
<td>216</td>
<td>61</td>
<td>66</td>
<td>89</td>
</tr>
</tbody>
</table>

Table. Clinical isolates included in the second collection
In determining the annular radius, the microcolonies, if present within the zone, were disregarded as a trailing effect is known to occur with yeast–azole interaction. Growth of microcolonies within the zone was given a score of 0, 1, 2 or 3, where 0 represents a clear zone with no microcolonies, 1 a few colonies, 2 moderate numbers of colonies and 3 a large amount of growth within the zone.

Determination of MICs

The MICs were determined by broth microdilution based on the UK reference method. Fluconazole was diluted in YNBG broth and the dilutions prepared in sterile 96-well microtitre trays. Growth controls were included in each tray. Trays were stored at −70°C until required.

A spectrophotometric method for measuring the inoculum was used according to the guidelines of the NCCLS. The inoculum was prepared by suspending five colonies of at least 1 mm diameter in 5 mL of sterile 0.85% saline and adjusting the turbidity of the suspension, as measured spectrophotometrically, to give a final transmission of 85% at 530 nm. The working suspension was made by making a further 1:20 dilution in YNBG broth and inoculated with an MIC 2000 (Dynatech, Basingstoke, UK) to give a final inoculum of 10^5 cfu/mL. The microtitre trays were incubated at 35°C for 48 h. Plates were agitated immediately before manual reading and the endpoint was determined according to the NCCLS recommendation of an 80% reduction in turbidity. MICs for the fluconazole susceptibility categories are defined as follows: sensitive, <8 mg/L; intermediate, 8–32 mg/L; resistant, >32 mg/L. The control strains were included with each batch of MIC plates and agar media.

All results were recorded in a microcomputer and a computer program designed by Dr K. P. Shannon was used to analyse the data. Pearson's method was used to calculate the correlation between MICs and zone sizes; the MIC values were converted to \( \log_2 \) MIC + 21 for this calculation.

Results

The initial collection of candida isolates was tested on all six media. All isolates grew, but the quality of growth after 24 h incubation was variable. Confluent growth was evident, in order of density, on Sabouraud, casitone and YNBG, whereas RPMI and both high resolution media gave the poorest growth leading to difficulties in the measurement of zone sizes. There was no significant difference between zone radii measured at 24 or 48 h. A photograph of C. albicans on the six media is shown in Figure 1.

MICs and zone sizes were compared for all six media. The best correlation was achieved with YNBG \((r = 0.95)\) followed closely by Oxoid's High Resolution Medium \((r = -0.87)\), whereas Sabouraud's agar \((r = -0.54)\) gave the poorest correlation (Figure 2). Correlation coefficients for the three categories of susceptibility, based on MICs, were also distinguishable by zone size.

MICs were not unique to one set of media or candida species. Growth within the zone was observed with most isolates, resulting in a wide spectrum of scores. Over-all, RPMI 1640 had the highest score for microcolonies within the zone. The smallest score was achieved on Mast Laboratories' High Resolution Medium (HR (Mast)).

Discussion

Six different agar media were examined for their suitability for susceptibility testing. Sabouraud medium,
although excellent for quality of growth and definition of zone sizes, gave poor correlation with the MICs as previously reported by Rex; this was confirmed by our results. Casitone, like Sabouraud agar, gave high quality growth, a good visual zone edge and an unsatisfactory correlation with the MICs. YNBG agar provided the easiest visual reading of the zone edge and gave the best correlation with the MICs. Both high resolution media provided only adequate growth. Mast Laboratories' High Resolution Medium exhibited the poorest quality of growth of the all media tested. RPMI 1640, like the high resolution media, demonstrated poor quality of growth, zone edge determination was difficult and this medium produced the highest percentage microcolony score within the zone.

With strict adherence to the method and preparation of the media there was no evidence of significant batch-to-batch variation with any of the media tested. On the basis of all data, both visual and statistical, we chose YNBG medium for assessing susceptibility by disc diffusion.

We have observed that the depth of the medium is critical and erroneous results, particularly with the intermediate strains, are produced if the medium is less than 4 mm in depth.

All isolates in the initial study that were classified as susceptible on the basis of MICs had an annular zone radius of >12 mm and all isolates classified as resistant on the basis of MICs had zone radii of <7 mm. The isolates in the intermediate range, based on MICs, had zone sizes in the range of 0–10 mm. Thus, in the laboratory a zone size of >12 mm can be reported as sensitive. Isolates with a zone size of 7–12 mm can be reported as of intermediate sensitivity. However, a zone size of <7 mm may not distinguish between resistant isolates and those with intermediate sensitivity.

For the isolates tested only on YNBG the correlation of zone size and MIC was very good for the most commonly isolated species, C. albicans and C. glabrata, as it was for C. krusei with >95% agreement. However, the correlation for the less common species was not as good and there were some aberrant results. One isolate of C. tropicalis produced large numbers of microcolonies within the zone, but a defined edge was evident and, according to the criteria of the test, the strain was categorized as sensitive, although the MIC, read at 80% reduction in turbidity, categorized the organism as resistant. It is difficult to determine which of the results is correct for isolates such as this as the growth in the liquid medium may well be similarly affected and the 80% reduction equally difficult to recognize.

C. parapsilosis grew poorly on the media tested and zones were difficult to interpret at 24 h but they did
Fluconazole disc diffusion testing

Figure 4. Correlation of MICs with zones of inhibition on YNBG agar for (a) C. albicans, C. glabrata and C. krusei and (b) other Candida spp. The dotted vertical lines indicate the breakpoints between susceptibility, intermediate resistance and resistance. The dotted horizontal lines indicate the zone size criteria that gave the best discrimination between these three categories.

correlate with the MICs. Reading of the zone size at 48 h did not alleviate the problem of interpretation and the correlation with MICs was poor. The overall agreement for disc diffusion and MICs was 89%.

The need for a reproducible and reliable susceptibility test for fluconazole against candida species was recognized. The test needed to be readily available for use in a routine laboratory and with these factors in mind a disc diffusion technique was developed. The disc diffusion test for fluconazole may not be read as easily and defined as clearly as the majority of antibacterial disc diffusion susceptibility tests performed in routine laboratories. The concept of ignoring colonies within the zone is against usual practice in microbiology but this study indicates that this may be acceptable when testing yeasts against fluconazole.

The disc diffusion method has been implemented as a routine test in the Department of Microbiology at St Thomas's Hospital for candida isolates requiring fluconazole sensitivity testing. However, the technique has its limitations and these are taken into account in the reporting of yeasts and their susceptibility patterns. All germ tube-positive isolates (C. albicans) are reported confidently, given that a small number categorized as of intermediate sensitivity by MIC will be reported as resistant, as are germ-tube-negative yeasts producing no zone which are reported as resistant. These two groups cover most of the isolates in our patient population. Germ-tube-negative isolates that grow slowly or have large numbers of colonies within the zone must be interpreted with caution and we refer these cultures to the PHLS Mycology Reference Laboratory.

In the clinical laboratory the possibility of mixed cultures of Candida spp. arises, especially in HIV-positive patients. The use of CHROMagar (Mast Laboratories), a differential medium for yeasts, as a primary medium for selected specimens, provides added confidence in determining if mixtures are present. The need for a relatively heavy inoculum for the disc diffusion test makes the detection of mixed cultures imperative.

In conclusion, we recommend YNBG agar for disc testing of fluconazole against candida. This method is rapid and can be read after overnight incubation, and the results correlate well with the standard MIC method. Thus turnaround time and cost of processing samples for referral are minimized. Laboratories processing significant numbers of isolates requiring sensitivity testing may consider this method advantageous as results can be reported more promptly to the clinician, allowing amendment of the treatment regime if required, and thus reducing morbidity.

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References


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