A disc test of antifungal susceptibility

Joanna Cheng, Jeanette Pham & Sydney Bell

Department of Microbiology South Eastern Area Laboratory Services
Prince of Wales Hospital Randwick NSW 2031

Diagnostic laboratories are frequently requested to perform antifungal susceptibility tests on isolates recovered from systemic infections. A standard reference procedure of broth dilution was proposed by the Clinical and Laboratory Standards Institute (CLSI) [1], but it is too labour-intensive for most clinical laboratories. CLSI also suggested a disc diffusion method [2], which is a cost-effective alternative method and simple to use in diagnostic microbiology laboratories. However, the method does not deal satisfactorily with the difficulty in interpretation of inhibitory zones on disc testing of those strains that show a 'trailing effect' on minimum inhibitory concentration (MIC) determination of the azole group of antifungals [3]. Although disconcerting, trailing is regarded as an *in vitro* phenomenon and these strains are generally regarded as susceptible [4]. We present a disc diffusion test for use with available antifungals, based on the Calibrated Dichotomous Sensitivity (CDS) method, used in antibiotic susceptibility testing of bacteria [5]. The disc testing was done under predetermined optimal condition of growth [6], that overcame the problem of the ill-defined inhibitory zones of the azoles.

Development of the disc test

One hundred clinical isolates of yeasts including *Candida* species and *Cryptococcus neoformans* were used in the development of the test. *Candida parapsilosis* ATCC 22019 was used as a control. Amphotericin B, itraconazole, fluconazole, voriconazole and caspofungin were calibrated for testing, by comparing the size of the zone of inhibition around a 6mm disc containing the antifungal drug with the MIC of each agent.

Casitone Complex agar (Difco, Michigan, USA) was used in both disc testing and MIC determination. Paper discs 6mm in size, containing the five antifungals with potencies as shown in Table 1 were obtained from Oxoid (Thermofisher Scientific, UK).

The MICs of amphotericin B, fluconazole, voriconazole and caspofungin were performed by agar dilution in duplicate with each of the 100 clinical isolates [7]. Itraconazole MIC was determined by E test [8]. The inoculum was prepared by suspending 24-hour culture in isotonic saline to obtain an absorbance of 0.15 for *Candida* species or 0.30 for *Cryptococcus neoformans* at a wave length of 640nm, using a spectrophotometer (Bausch & Lomb, Germany). A ten-fold dilution of each suspension was used to inoculate the MIC plates, using a Steers replicator [9], which delivered 4μL per spot. The plates were incubated at 35°C for 24 hours in air and *Cryptococcus neoformans* was incubated at 35°C for 48 hours. For amphotericin B and caspofungin, the MIC was defined as the lowest concentration of the antifungal that completely inhibited growth. For fluconazole and voriconazole

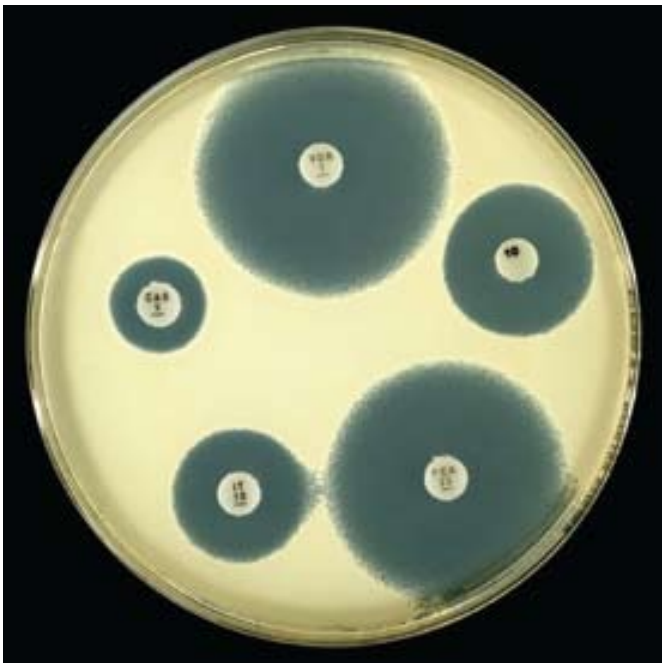

Figure 1: The CDS test of the reference strain *Candida parapsilosis* ATCC 22019 susceptible to all five antifungals tested, voriconazole (VOR 1), itraconazole (IT 10), fluconazole (FCA 25), amphotericin B (10) and caspofungin (CAS 5).

the MIC was defined as the lowest level that inhibited 80% or more of the growth [3].

Preliminary disc tests of *Candida* species to the azole group of antifungals were performed under different conditions of incubator temperature and atmosphere. These experiments demonstrated that incubation at 30°C in air was the optimal conditions of growth for demonstrating the susceptibility of Candida species to the azoles. However with *Candida tropicalis,* the addition of 5% CO_2 to the incubation atmosphere was required to demonstrate clearly the susceptibility to the azoles.

For disc diffusion testing, plates containing 20ml of Casitone Complex agar were surface-dried for an hour prior to inoculation. A volume of 2.5ml of undiluted saline suspensions of each isolate, prepared as described in the MIC determination, was used to flood each plate. The plates were incubated at 30°C in air for 24 hours. *Cryptococcus neoformans* plates were incubated for a further 24 hours, if necessary. After incubation, the annular radius of the zone of inhibition around each disc was measured, the annular radius being the distance from the edge of the disc to the edge of the lawn of confluent growth.

A calibration graph was prepared for each antifungal by plotting the annular radii of the inhibitory zones of the disc tests against the corresponding MIC.

Results

The disc potency of each antifungal, the breakpoint MIC for susceptible strains and the range of annular radii for susceptible and resistant strains are shown in Table 1. The table also contains the proposed cut-off radius for susceptible strains with each antifungal. The susceptible range of amphotericin with the seven strains of *Candida krusei* was recorded separately. The MIC to amphotericin of this species was four- to eight-fold higher than other susceptible *Candida* and yielded a zone size of 4mm or less on disc testing.

In MIC determination, a number of isolates of *C. albicans* demonstrated a trailing end-point with the azoles [6,7]. However, with disc testing of these antifungals, the plates incubated at 30°C had large zones of inhibition, which were either clear or contained only scanty growth, in contrast to plates incubated at 35°C, where overgrowth in the inhibitory zones created difficulty in measurement of the zone sizes. With *C. tropicalis* incubation at 30°C failed to improve the appearance of the zones but incubation in the presence of 5% CO_2 achieved clear-cut zones

Discussion

The distribution of susceptibilities with all agents generally fell into two distinct groups: a susceptible group with MICs below the breakpoint MIC and a resistant group with MICs above the breakpoint. The inhibitory zone sizes in disc testing correlated well with the MICs and this correlation was maintained when the disc test was performed at 30°C. Incubation at this temperature had the added advantage of markedly reducing the growth within the zones of inhibition observed on plates incubated at 35°C with itraconazole, fluconazole and voriconazole discs. This was consistent with previous observations on trailing end-point in MIC determinations [6]. The breakpoints used in this study were similar to those proposed in other methods including the CLSI method [10]. However, the exception was the breakpoint of amphotericin B, which in the present study was eight-fold lower

Table 1. Cut-off annular radius proposed for a susceptible strain (shown in bold) and range of annular radii for both susceptible and resistant isolates observed in the calibration of disc diffusion testing of 100 yeast isolates to the breakpoint MICs of 5 antifungal agents.

Antifungal agents breakpoint MIC	Cut-off radius and (disc potency) (mg/L)	Range of radii (mm)	
Amphotericin B (10µg)	≤0.125 >0.125*	**4**	4.5-9.0 1.5-3.5
Fluconazole (25µg)	≤16.0 >16.0	**6**	6.0-14.0 1.0-4.0
Itraconazole (10µg)	≤1.0 >1.0	**4**	4.0-7.0 1.0-3.0
Voriconazole (1µg)	≤1.0 >1.0	**6**	7.0-17.0 2.0-4.0
Caspofungin (5µg)	≤2.0 >2.0	**2**	2.5-7.0 0-2.0

*Includes seven isolates of *Candida krusei.*

than that proposed by CLSI. This arose because the susceptibilities of isolates we tested distributed into two distinct groups: a group with an MIC of 0.125mg/L or less that we regarded as susceptible and another group with an MIC of 0.25mg/L and above that we called resistant. As there is no published literature correlating the MIC of amphotericin to clinical response, while the breakpoint we set and CLSI breakpoint are both purely arbitrary, the natural distribution of MICs would support the choice of the lower breakpoint.

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Joanna Cheng is Senior Scientist in Microbiology, SEALS, Prince of Wales Hospital. Her interests are diagnostic mycology and clinical bacteriology.

Sydney Bell is Director of Microbiology (North and Central), SEALS, and Professor in Pathology, the University of NSW. He is the founder of the CDS method of antibiotic susceptibility testing (Pathology, 1975).

Jeanette Pham is Senior Scientist in charge of the CDS method reference laboratory, SEALS.