

Tablet sensitivity testing of pathogenic fungi

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SUMMARY A diffusion method for determining the sensitivity of pathogenic fungi to therapeutic agents is described using tablets containing the following antibiotics: amphotericin B, clotrimazole, econazole, fluorocytosine, and miconazole. The composition of the media used, standardisation of inocula, incubation time, and temperature are detailed.

Fungus infections in man range from those involving only the most superficial areas of the body, through those in which subcutaneous tissue is attacked, to those deep and systemic infections that often involve most of the major organs of the body. The last decade has seen a marked increase in systemic mycoses, an increase that has been matched by the recent introduction of new systemic antifungal agents. Previously almost all effective systemic agents carried prohibitive toxic hazards. The sensitivity of fungi is rarely tested on a routine basis in hospitals. However, the appearance in the future of resistant strains of organisms, such as candida, may necessitate more sensitivity testing than has been done in the past. This paper outlines a procedure (an agar diffusion method using tablets) for testing antifungal drugs, which may prove easier to perform than those used in the past.

Range of antifungal drugs

Neo-Sensitabs® tablets, containing different antibiotics, have shown an increased stability at room temperature compared with paper discs (Brown and Kothari, 1975), mainly because the tablets, due to the manufacturing process, contain the antibiotics in a crystalline form, while commercially available paper discs contain amorphous antibiotics (Pfeiffer *et al.*, 1976). Grendahl and Sung (1978) have found a loss of activity of low-concentration miconazole discs after one week's storage at -15°C . The lability of the amphotericins is also well known (Hoeprich and Huston, 1978).

The following range of antifungal agents in tablet form has been studied:

Tablet	Diffusible amount of antibiotic
Amphotericin B	20 IU
Clotrimazole	10 μg
Econazole	10 μg
Fluorocytosine	10 μg
Miconazole	10 μg

There is cross-resistance between amphotericin B, amphotericin B methyl ester, and nystatin, and only one needs to be tested routinely.

Culture media

It is important to control the composition of the medium. The antifungal activity of 5-fluorocytosine is antagonised by peptones, yeast, and beef extracts (containing pyrimidines and purines). Culture media containing such ingredients—for example, Sabouraud's agar—are inappropriate for sensitivity testing with this drug (Hoeprich and Finn, 1972).

Miconazole and clotrimazole are antagonised by Sabouraud's glucose and brain-heart infusion agars, while the antifungal activity of amphotericin B is not currently affected by the culture medium used for testing (Hoeprich and Huston, 1976).

Those most highly recommended, therefore, are chemically well-defined culture media containing ammonia and/or asparagine as nitrogen sources, for example, modified Bacto Yeast Nitrogen Base and Bacto Yeast Morphology Agar. Nevertheless, it should be noted that the last-mentioned medium has a pH value of 5.0, and acidic pH values have an adverse effect on the activity of amphotericin B and other polyene compounds (Shadomy, 1974). Differences in pH may influence the zone sizes, either by affecting the growth of the organism or by altering the activity of the drug itself.

The medium recommended by Shadomy (1969)

for tests with 5-fluorocytosine may be used as routine for all antifungal agents. It is prepared by taking:

Bacto-Yeast-Nitrogen Base (code 0392)	6.7 g
L-asparagine	1.5 g
Glucose (dextrose)	10.0 g
Distilled water	to 100 ml

After dissolution and sterilisation by membrane filtration, a $\times 10$ concentrate (YNB concentrated sterile solution) remains. To prepare 1 litre of solid culture medium for sensitivity testing take:

NaH ₂ PO ₄	0.33 g
K ₂ HPO ₄	0.92 g
Ion agar (low content of ions)	11.00 g
Distilled water	to 900 ml

The phosphates, agar, and water are melted by heating to 90-100°C for 10 minutes; thereafter the pH is adjusted to 7.0. Now sterilisation in glass bottles follows by heating in an autoclave to 120°C for 20 minutes. After cooling to 45°C, 100 ml of the above-mentioned YNB concentrated sterile solution is added under aseptic conditions. The medium is then ready to be poured into petri dishes. The agar medium should have a pH of 6.9-7.1 at room temperature and must therefore be checked after gelling. When bacterial contamination is likely, gentamicin or chloramphenicol may be incorporated into the medium at a final concentration of 100 µg/ml. These agents do not appear to affect the activity of antifungal drugs.

As recommended by Holt (1975), pH 7.0 has been adopted as reflecting biological conditions. The addition of glucose to fungal media greatly enhances growth but undoubtedly causes a lowering of pH, because acid is produced by carbohydrate fermentation. Nevertheless, the inclusion of phosphate buffer in the medium should avoid a drastic lowering of the pH during incubation.

Inoculum, seeding, and incubation

When the primary cultures appear homogeneous, samples of several identical colonies are mixed; when even small differences in colonial morphology are apparent, sensitivity tests are performed individually on each variety.

A suspension of the fungus is made directly into 0.01 M phosphate buffer (0.33 g NaH₂PO₄ and 0.92 g K₂HPO₄ in 1 litre distilled water) containing a non-ionic surfactant (0.1% Triton X-100). The cell density of suspensions of yeast-like organisms or fungal spores is counted (for example, in a Fuchs-Rosenthal chamber), and the suspension is further diluted in surfactant buffer to a final inoculum density of approximately 10⁵ cells per ml.

This may be impossible with filamentous fungi, and 1:100 dilutions of well-mixed overnight cultures in YNB broth are used in such cases. These dilutions may be homogenised by shaking with small sterile glass beads. Whenever possible the spores are harvested separately and counted (Holt, 1975). When testing against 5-fluorocytosine, it is necessary to subculture first into YNB broth and to incubate for 6 to 8 hours to deplete the cultures for traces of cytosine and pyrimidine bases. Further dilutions are made as mentioned above.

An even lawn of 0.2 ml diluted suspension is sown over YNB agar plates; within a few minutes the fluid has been absorbed into the agar (if necessary, drying for 5-10 minutes at 37°C) and the tablets are placed on the surface. Inocula may also be prepared by dispersing spores and cells scraped from the surface of Sabouraud agar slants or plates in a small volume of physiological saline. These preparations are now homogenised in Tenbroeck tissue or similar grinders. Large pieces of cellular material are allowed to settle, and suspended cells are adjusted to a transmittance of 95-97% measured at 530 nm in a photometer. A sterile cotton swab is dipped into the suspension, the dried agar plate is inoculated by streaking the swab over the entire surface, and thereafter the tablets are placed onto the agar medium.

Incubation is for 24-48 hours at 37°C (for candida and fungi responsible for systemic invasion). This temperature is chosen in an attempt to simulate body conditions. For dermatophytes the incubation temperature should be 30°C. Most sensitivity tests, including those for aspergilli, can be reliably read after only 18-24 hours (Holt, 1975).

Zone diameter interpretative standards

The sizes of the zones of inhibition are interpreted as indicated in Table 1.

Our studies with 80 strains of fungi, including *Candida (albicans, tropicalis)*, *Cryptococcus neoformans*, *Torulopsis glabrata*, and *Aspergillus fumigatus*, indicate that strains of normal susceptibility will currently show inhibition zones of 15-20 mm with

Table 1 Interpretation of results: Diameters of inhibition zones (tablet included)

	Diameter (mm)		
	Fluorocytosine	Clotrimazole Econazole Miconazole	Amphotericin B
Sensitive	≥30	≥20	≥15
Intermediate	23-29	12-19	10-14
Resistant	≤22	≤11	≤9

Table 2 Correlation between zone size and MIC

Tablets	Zone of inhibition (diam. mm)	MIC ($\mu\text{g/ml}$)
Amphotericin B	≥ 15	≤ 1
	14-10	1-4
	≤ 9	> 4
Clotrimazole	≥ 20	≤ 1
	Miconazole and Econazole	1-4
Fluorocytosine	≤ 11	> 4
	≥ 30	≤ 4
	29-23	4-16
	≤ 22	> 16

Amphotericin B, 20-40 mm with Clotrimazole, Econazole, and Miconazole Neo-Sensitabs, and 35-55 mm with Fluorocytosine Neo-Sensitabs.

Correlation between the agar diffusion sensitivity test with tablets and minimal inhibitory concentration by agar dilution was attempted, and the results are shown in Table 2.

When testing with clotrimazole and miconazole, a zone of gradually decreasing growth consisting of partially inhibited colonies often appears; the inhibition zone should be measured up to colonies of normal size.

Interpretation of the different degrees of sensitivity is as follows:

Sensitive The infection due to the strain tested may be expected to respond to a normal dosage of this antifungal drug.

Intermediate The infection may be treated, in some cases, by using a high dosage of this drug (if possible because of toxicity), or may respond to concentrations attainable in areas (such as the urinary tract, etc) where the antifungal drug is concentrated (including local application).

Resistant The drug cannot be used for treatment in this case.

Discussion

Dilution methods used for the determination of minimum inhibitory concentration (MIC) values of a drug against fungi include broth, semisolid agar, and agar dilution techniques.

One major problem with broth and semisolid agar dilution procedures is to prepare and inoculate the tubes so that growth does not occur on the sides above the main portion of the media and out of contact with the drug. Also some difficulty may be encountered in determining exact end-points since traces of growth may not be clearly visible (Wagner *et al.*, 1975).

Antifungal agents are rarely soluble in water, fluorocytosine being one of the few exceptions. All the polyenes and imidazoles are freely soluble in organic solvents, but on dilution into aqueous

nutrient media, these solutions give a definite turbidity, which slowly precipitates on standing. Reynolds *et al.* (1977) found major differences between results obtained by the broth dilution and agar dilution methods when testing clotrimazole against *Bacteroides fragilis*. The discrepancies were explicable in terms of the relative insolubility of clotrimazole in aqueous solution (broth dilution), while, by the agar dilution method, clotrimazole remaining evenly distributed gave a more reliable index of MIC values.

The diffusion method described in this paper has evolved from those already in use for the study of antibacterial substances. When the most exacting accuracy is required, the agar dilution method is generally preferred. Nevertheless, diffusion methods based on a standardised methodology (medium, inoculum, interpretation) can also be accurate and reliable. Quantitative results may be obtained by means of regression lines relating diameter of inhibition zones to MIC values (Casals and Gylling Pedersen, 1972; Casals, 1977). As a general recommendation, no topical or systemic antimycotic therapy should be started without reliable sensitivity tests, and fungal reisolates during therapy should be tested for increased resistance to the drug in use.

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