Antimicrobial Agents and Chemotherapy, July 1975, p. 107-109
Vol. 8, No. 1
Copyright © 1975 American Society for Microbiology
Printed in U.S.A.

New Method for Susceptibility Testing with Antifungal Agents

GERALD WAGNER, SMITH SHADOMY,* LARRY D. PAXTON, AND ANA ESPINEL-INGROFF

Department of Medicine, Medical College of Virginia, Virginia Commonwealth University, Richmond, Virginia 23298

Received for publication 28 February 1975

A new method for the determination of minimal inhibitory and fungicidal concentrations of antifungal agents for filamentous fungi is described. 5-Fluorocytosine (5-FC) was used as a representative agent and was tested against the following genera: *Allescheria*, *Aspergillus*, *Cladosporium*, *Fonsecaea*, and *Phialophora*. 5-FC was found to be inhibitory but not fungicidal for many of the fungi tested, including eight of clinical origin.

Methods used for the determination of minimal inhibitory concentrations (MIC) of a drug for the filamentous fungi include broth, semisolid agar, and agar dilution techniques (1, 3). These techniques are both time-consuming and expensive; furthermore, they do not lend themselves well to use in the clinical laboratory. One major problem with broth and semisolid agar dilution procedures is preparing and inoculating 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. The main advantage of the semisolid agar dilution technique is the convenience in determining minimal fungicidal concentrations (MFC) of the drug being studied. Such end points cannot be measured with conventional agar dilution techniques. In this communication we report the use of a modification of the Ericsson and Sherris agar dilution technique (2), which provides for determining both the MIC and MFC of antifungal agents such as 5-fluorocytosine (5-FC) for various filamentous fungi.

Serial dilutions of 5-FC were prepared in sterile ×10 yeast nitrogen base (Difco) supplemented with 5% glucose as a carbon source. Concentrations of 5-FC ranged from 0.63 to 1,280 μg/ml. The solutions of yeast nitrogen base and drug were then diluted 1:10 with sterile 2% agar and dispensed in 20-ml volumes in square Integrid petri plates (15 by 15 cm, Falcon Plastics). After solidification of the media, sterile membrane filters (0.45-μm pore size, 13-mm diameter, Millipore Corp.) were aseptically placed on the agar surfaces. Up to eight membrane filters were placed on each plate.

Inocula were prepared by dispersing spores and cells scraped from the surface of Sabouraud agar (Difco) slants or plates in a small volume of physiological saline. These preparations were then homogenized in Tenbroeck tissue grinders (Corning Glass Works). Large pieces of cellular material were allowed to settle out and suspended cells were adjusted to an absorbancy of 0.05 at 530 nm with physiological saline. The inocula were applied to the surfaces of the filters with a device similar to Steers replicator (Fig. 1). Identical plates without filters also were inoculated. Plates were incubated at 30 ± 1 C in an upright position for 24 h, after which time they were inverted and incubated until growth appeared on the drug-free control plates. The MIC was defined as the lowest concentration of drug which inhibited vegetative growth as determined visually. MIC values determined simultaneously with and without membrane filters were identical.

To determine the MFC, the filters were removed from the surface of the media, placed in a membrane filter holder, washed once by filtration with a small volume of sterile physiological saline, and immersed in culture tubes containing 5.0 ml of yeast nitrogen base. They also could have been placed on surfaces of drug-free agar. The tubes were incubated at 30 ± 1 C and the MFC was read after a suitable period of time. The MFC was defined as the lowest concentration of drug which proved fungicidal to the organism as determined by absence of growth.

In determining MFC values by broth and semisolid agar dilutions techniques, a calibrated loop was used to remove material from the negative growth tubes for inoculation on antibiotic-free media. Thus, only a portion of the inoculum originally introduced in the tubes was examined for fungicidal effects of the drug. In contrast, the membrane method for determination of the MFC examines the entire inocu-

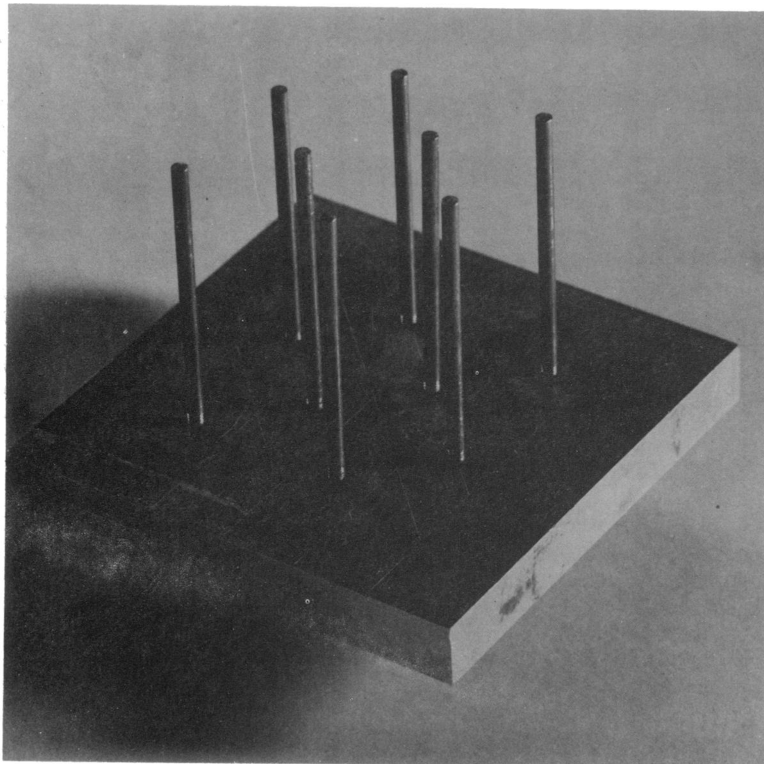

FIG. 1. *Replica inoculator used to inoculate agar plates containing graded concentrations of drug. Depending on the anticipated size of colonies, one inoculator only or two matching inoculators may be used to deliver 8 or 16 inocula.*

TABLE 1. *MIC and MFC of 5-FC for various filamentous fungi as measured by an agar dilution-transfer membrane technique*

Organism	Source[a]	MIC (μg/ml)	MFC (μg/ml)
Allescheria boydii 2.5[b]	Mycetoma	>128	>128
Aspergillus fumigatus 4.6	Pulmonary aspergillosis	>128	>128
A. fumigatus 4.19	Disseminated aspergillosis, Penguin	0.50	>128
A. fumigatus 4.28	NIH	>128	>128
A. fumigatus 4.33	NIH	0.50	>128
A. fumigatus 4.41	Endocarditis	0.50	>128
Aspergillus niger 4.55	Aspergillosis	0.50	>128
A. niger 4.60	Endocarditis	0.06	>128
Cladosporium sp 53.10	CDC	32	>128
Cladosporium sp. 53.11	Chromomycosis	64	>128
Cladosporium sp. 53.13	Contaminant	128	>128
Cladosporium sp. 53.15	CDC	64	>128
Cladosporium sp. 53.16	Cladosporiosis	2.0	>128
Cladosporium sp. 19.6	Contaminant	64	>128
Cladosproium sp. 19.4	Contaminant	2.0	>128
Cladosporium trichoides 53.12	Cladosporiosis	8.0	>128
C. trichoides 19.3	Chromomycosis	2.0	>128
Cladosporium gougerotii 53.14	Chromomycosis	>128	>128
Fonsecaea pedrosoi 19.1	Contaminant	>128	>128
F. pedrosoi 19.5	CDC	>128	>128
F. pedrosoi 19.7	Chromomycosis	2.0	>128
Philalophora verrucosa 29.5	CDC	128	>128
Saccharomyces cerevisiae ATCC 9763		0.06	0.06

[a] NIH, National Institutes of Health; CDC, Center for Disease Control.
[b] Accession numbers, MCV-VCU medical mycology culture collection.

lum. Such a complete evaluation of the fungicidal effect of the drug may be of clinical significance.

The method described here has been successfully employed in determination of MIC and MFC values of 5-FC for species of *Aspergillus*, *Phialophora*, *Fonsecaea*, *Allescheria*, and *Cladosporium* (Table 1). Results obtained in this study indicate that, although 5-FC may be inhibitory for many filamentous fungal pathogens, it is not fungicidal. Equally important is the fact that eight of 11 clinical isolates were found to be susceptible. This included four of five isolates of *Aspergillus fumigatus* and *A. niger*, and four of six isolates of dematiaceous species isolated from cases of chromomycosis and cladosporiosis. Data for *Saccharomyces cerevisiae* ATCC 9763, the quality control organism used in our laboratory for susceptibility testing with 5-FC, were within normal limits.

Several additional and intriguing possibilities for the membrane-bound colony of a filamentous organism can be envisioned. One such application is the ability to introduce an entire growing colony into another growth environment simply by transferring the filter from one plate to another.

This study was supported in part by a grant from the Brown-Hazen Fund, Research Corporation of America.

The 5-FC used in this study was supplied by Hoffman La Roche, Inc., Nutley, New Jersey.

LITERATURE CITED

1. Anderson, T. G. 1970. Testing of susceptibility to antimicrobial agents and assay of antimicrobial agents in body fluids, p. 299-310. *In* J. E. Blair, E. H. Lennette, and J. P. Truant (ed.), Manual of clinical microbiology, 1st ed. American Society for Microbiology, Bethesda, Md.
2. Ericsson, H. M., and H. C. Sherris. 1971. Antibiotic sensitivity testing. Report of an international collaborative study. Acta Pathol. Microbiol. Scand. Sect. B **217**(Suppl.):67-68.
3. Shadomy, S., and A. Espinel-Ingroff. 1974. Susceptibility testing of antifungal agents, p. 569-574. *In* E. H. Lennette, E. H. Spaulding, and J. P. Truant (ed.), Manual of clinical microbiology, 2nd ed. American Society for Microbiology, Washington, D.C.