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Standardization of a Hyphal Inoculum of Aspergilli for Amphotericin B Susceptibility Testing

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Standardized, evenly dispersed hyphal suspensions served as the inoculum in a microtiter technique for amphotericin B antifungal susceptibility testing. Preliminary testing with six strains of *Aspergillus fumigatus* and *A. flavus* produced consistent and reproducible results at 30°C over 24 h. The observed amphotericin B MICs required for hyphae (0.3 to 0.6 μg/ml) were comparable to MICs required for conidia (0.16 to 0.6 μg/ml). The results were evaluated and compared with previously published information.

Several different methods exist to test the susceptibility of fungi against antimycotic drugs, including broth dilution (17), agar dilution (18), semisolid agar dilution (22), disk diffusion (8, 20), radiometric methods (14), and microtiter (5, 6).

The currently available in vitro methods for testing the activity of antimicrobial agents against filamentous fungi provide variable results in different laboratories because of the lack of standardization. They are, therefore, of generally limited value to the clinician. As a consequence, a number of microbiology laboratories refrain from testing the susceptibility of mycelial fungi to the principal antifungal agents. The clinical application of MICs can be also questioned because the current techniques for moulds, using inocula consisting of conidia, measure the MIC necessary to inhibit cells which are often not the morphological form of the causative fungus seen in vivo.

Unfortunately, few studies have been conducted on the actual susceptibility of fungal hyphae to antifungal agents (11, 16). A major reason for this is the lack of standardized techniques to obtain a pure and fully viable hyphal suspension. Attempts to standardize a hyphal inoculum for dermatophytes (7) have been suggested with reproducible results. These techniques, nevertheless, leave a variable number of broken hyphae, and therefore, even an identical optical density of such hyphal suspensions could lead to a considerable variation in the number of viable cells; this would obviously prevent proper standardization of the inoculum.

MATERIALS AND METHODS

Aspergilli. Preliminary studies were done with *Aspergillus fumigatus* NCMH 785, a strain maintained in the fungus collection at the North Carolina Memorial Hospital, Chapel Hill. After standardizing the basic technique for microtiter susceptibility testing, additional strains of aspergilli were studied, including *A. fumigatus* strains NCMH 77 and NCMH 1473 and *A. flavus* strains NCMH 768 and NCMH 8195.

Culture media. Liquid Czapek broth (Difco Laboratories, Detroit, Mich.) supplemented with 2% Tween 80 and 0.07% agar (LCTA) was used as the medium to obtain the hyphal inoculum needed for susceptibility testing. The liquid medium was gently heated and sterilized by membrane filtration before sterile agar was aseptically added.

M-3 medium for susceptibility testing (Difco Bacto-Antibiotic Medium 3) in double strength (35 g/liter) was used to prepare amphotericin B concentrations for testing in the microtiter system.

Preparation of conidial suspension. Isolates of aspergilli growing on Sabouraud agar slant cultures for 5 to 7 days were harvested by flooding the growth with a few milliliters of sterile 2% Tween 80-water to provide a conidial suspension. The conidial suspensions were adjusted to 50% transmittance at 530 nm in a spectrophotometer and used to inoculate the LCTA (see above). Estimation of the number of conidia in the conidial suspensions was made by: (i) microscopy of the suspension (×400 magnification), (ii) hemacytometer, and (iii) Sabouraud agar plate dilution (surface). We showed that 50% T at 530 nm corresponded to 150 to 200 cells per field in method (i), 1×10^7 to 3×10^7/ml in method (ii), and 2×10^6 to 4×10^6 CFU/ml in method (iii). The number of conidia after inoculation into 10 ml of LCTA resulted in approximately 0.5×10^5 to 1×10^5 CFU/ml.

Preparation of hyphal inocula. Erlenmeyer flasks (125 ml) containing 10 ml of LCTA were each inoculated with 0.25 ml of the conidial suspension. After the inoculum was mixed with the medium, the flasks were incubated without shaking at 30°C for 16 h. Afterwards, the cultures were measured for density in a spectrophotometer at 530 nm. If necessary, they were adjusted to 90% transmittance by adding distilled water.

The following inoculum densities were used in preliminary susceptibility investigations: undiluted and diluted 1:5, 1:10, 1:15, and 1:20. A dilution of 1:20 (3,000 CFU/ml) was chosen because it provided the most consistent MICs when read at 24 and 48 h.

Microtiter technique. Microtiter plates with the following amphotericin B concentrations in M-3 broth (0.1-ml volume per well) were prepared: 10, 5, 2.5, 1.25, 0.6, 0.3, 0.16, 0.8, 0.4, and 0.2 μg/ml. Freshly made plates or those kept frozen at −70°C for 2 to 3 weeks were used for susceptibility testing.

After adding 0.1 ml of the hyphal inoculum to each antimicrobial agent-containing well, the final amphotericin B concentrations were halved and ranged from 0.1 to 5 μg/ml. Each inoculum dilution was tested in duplicate. After inoculation, the plates were shaken, sealed, and incubated at 30°C for 18 to 72 h, during which period they were read four times. Control wells included growth controls (in M-3 medium) and inoculum controls (without M-3). The endpoint, i.e., the lowest drug concentration allowing the growth of aspergilli, was determined by visual observation of turbidity

TABLE 1. MICs of amphotericin B obtained in five experiments for a hyphal inoculum of *A. fumigatus* NCMH 785

Inoculum		MIC (μg/ml) after incubation at 30°C for:			
Dilution	CFU per well	18 h	24 h	48 h	72 h
1:5	1,200	0.3	0.3–0.6	0.6	0.6–1.25
1:10	600	0.3	0.3	0.6	0.6
1:15	400	0.16–0.3	0.16–0.3	0.3–0.6	0.6
1:20	300	0.16–0.3	0.16–0.3	0.3–0.6	0.6

when the microtiter plate bottom was observed with the aid of an illuminated mirror reader. The lowest drug concentration inhibiting growth was considered the MIC.

RESULTS

Effect of hyphal inoculum density. In five experiments conducted with a hyphal inoculum of *A. fumigatus* NCMH 785, consistent MICs, either identical or differing only in one concentration of amphotericin B, were obtained (Table 1). Depending on the length of incubation, the lowest MICs were 0.16 μg/ml (at 18 to 24 h), and the highest MIC was 1.25 μg/ml (in one experiment). There were no differences in the MICs at 18 and 24 h of incubation, and only a slight change in MICs was observed between 48 and 72 h.

With two strains of *A. flavus*, only two inocula of varying density were tried. We showed that both strains required identical MICs of 0.3 μg/ml at 18, 24, and 48 h, with the inoculum diluted 1:20.

On the basis of these results, further amphotericin B susceptibility testing was conducted with an inoculum diluted 1:20.

Effect of incubation period on MICs. Six strains of aspergilli were tested for susceptibility to amphotericin B, and the results of a single experiment (except in the case of *A. fumigatus* NCMH 785) are presented in Table 2.

There was essentially no difference in MICs when read at 18 and 24 h, and therefore, in subsequent experiments, 18-h readings were omitted. MICs at 24 h were 0.16 to 0.3 μg/ml for *A. fumigatus* and 0.3 to 0.6 μg/ml for *A. flavus*; the corresponding data at 48 h were 0.3 to 0.6 μg/ml for both *A. fumigatus* and *A. flavus* strains tested. In some strains there was a one dilution increase of MIC at 72 h, whereas in other strains the MIC remained unchanged.

Amphotericin B susceptibility of conidia and comparison with hyphal MICs. *A. fumigatus* NCMH 785 and three strains of *A. flavus* were investigated to determine the effect of the number of conidia on the outcome of testing (Table 3). The data presented show that the MICs were directly proportional to the inoculum density and the length of incubation, i.e., the lower the inoculum or the shorter the incubation, the lower the MIC.

Most of the MICs obtained with *Aspergillus* conidia were in a range of 0.3 to 1.25 μg/ml, which was almost identical to the hyphal MICs presented in Tables 1 and 2. To compare the susceptibility of conidia and hyphae, only those experiments from Table 3 which would match those obtained with the hyphal inoculum of a similar size were taken into consideration (Table 4). (Although it was not possible to count directly the number of hyphae in the inoculum, hyphal numbers have nevertheless been estimated based on quantitatively known conidial inocula and the fact that all conidia did germinate to produce hyphae [as judged by light microscopy].) The few one tube dilution variations noted (0.16 versus 0.3 or 0.3 versus 0.6 μg/ml) between conidial and hyphal MICs did not represent a significant difference in susceptibility of these two types of fungal elements.

TABLE 2. MIC of amphotericin B for different *Aspergillus* strains tested with a hyphal inoculum diluted 1:20[a]

Strain	MIC (μg/ml) after incubation at 30°C for:			
	18 h	24 h	48 h	72 h
A. fumigatus				
NCMH 785	0.16–0.3[b]	0.16–0.3[b]	0.3–0.6[b]	0.6
NCMH 77	0.16	0.3	0.3	0.6
NCMH 1473	0.16	0.16	0.3	0.3
A. flavus				
NCMH 768	0.3	0.3	0.3	0.3
NCMH 7844	0.3	0.6	0.6	0.6
NCMH 8195	0.3	0.3	0.3	0.6

[a] Average of 300 CFU or 5,000 hyphal cells per well.
[b] Range of results of five experiments.

TABLE 3. MIC of amphotericin B with conidial inocula of aspergilli

Strain and no. of conidia (CFU/ml)	MIC (μg/ml) after incubation at 30°C for:		
	24 h	48 h	72 h
A. fumigatus NCMH 785			
100,000	1.25	2.5	5.0
25,000	0.6	0.6	1.25
10,000	0.3	0.6	0.6
2,500	0.3	0.3	0.6
A. flavus NCMH 768			
30,000	0.6	1.25	1.25
6,000	0.3	0.6	0.6
3,000	0.3	0.6	0.6
600	0.16	0.3	0.6
A. flavus NCMH 7844			
40,000	1.25	1.25	2.5
8,000	0.6	1.25	1.25
4,000	0.6	0.6	1.25
800	0.3	0.6	0.6
A. flavus NCMH 8195			
20,000	0.6	1.25	1.25
4,000	0.3	0.6	0.6
2,000	0.3	0.6	0.6
400	0.3	0.3	0.3

DISCUSSION

Our novel approach towards the antimicrobic susceptibility testing of aspergilli was based on the following premises:

TABLE 4. Comparison of MICs obtained with conidial and hyphal inocula of aspergilli

Strain	No. of CFU per well	MIC (μg/ml) after incubation at 30°C (conidia, hyphae) for:		
		24 h	48 h	72 h
A. fumigatus NCMH 785	1,500	0.3, 0.6	0.3, 0.6	0.6, 0.6
A. flavus NCMH 768	500	0.16, 0.3	0.3, 0.3	0.6, 0.3
A. flavus NCMH 7844	600	0.3, 0.6	0.6, 0.6	0.6, 0.6
A. flavus NCMH 8195	300	0.3, 0.3	0.3, 0.3	0.3, 0.6

(i) testing the tissue forms, i.e., hyphae, instead of conidia or mixed inocula; (ii) developing a technique to obtain a good, standardizable hyphal inoculum; and (iii) standardizing the microtiter technique.

When testing conidial inocula to determine MICs, one is actually measuring primarily the inhibition of conidial germination. This may not necessarily mean that identical drug concentrations would also inhibit the growth of hyphae. Unreliable results are likely to be obtained when mixed inocula, composed of both hyphae and conidia, are tested.

Our main concern was to prepare pure hyphal inocula, in which fully viable hyphae were uniformly dispersed without any mycelial mats, to enable correct density measurements, and in addition, the hyphae were stabilized in suspension to enable accurate dilution.

Attempts to obtain such an inoculum from suspensions prepared from cultures grown on solid media, even when very young, or by growing aspergilli in liquid media under normal conditions were unsuccessful. In the first case, suspensions were not either uniformly dispersed or in instances when the grinding procedure was employed, not fully viable. In the second case, the suspensions contained mycelial mats or fungal microcolonies.

Therefore, to obtain a satisfactory hyphal suspension of aspergilli, several different media that were maintained in various containers or tubes and incubation temperatures with variable lengths of incubation, were tested before optimal parameters were ascertained. We found the following to be essential: a modified Czapek medium (LCTA) in a standardized volume, flask type, and size, a standardized conidial inoculum, and a 16-h incubation at 30°C, as described above.

Our technique to prepare a hyphal inoculum has functioned well not only with old laboratory strains of *A. fumigatus* and *A. flavus* but also with several fresh isolates of *A. flavus* and *A. niger*. Only in the cases of a stock culture of *A. fumigatus* NCMH 77 and a fresh *A. niger* strain was an additional 24-h incubation required for germination of all conidia in LCTA.

After we had developed a technique to obtain a good hyphal suspension, we conducted a series of experiments to obtain data which would allow standardization of the hyphal inoculum to be used in the microtiter technique to test amphotericin B susceptibility of aspergilli. The experiences of various authors (2, 4, 6, 13) with the microtiter method of susceptibility testing of fungi are somewhat conflicting, with the possible exception of *Cryptococcus neoformans*; better correlation is seen when testing yeasts (5, 12, 13, 15). It has recently been shown that *C. neoformans* can be tested by this method (B. E. Robinson, P. Goldson, and M. R. McGinnis, unpublished data), and M. R. McGinnis, North Carolina Memorial Hospital, Chapel Hill, has had success with this method (personal communication). Our proposed microtiter method differs from the method of Ellis et al. (5), in that a different culture medium was substituted for brain heart infusion broth, the incubation temperature was 30°C instead of 35°C, and turbidity was used rather than change of an indicator color. Thus, in our method, Bacto-Antibiotic Medium 3, which has been established as a standard medium for testing amphotericin B (21), and readings at 24 and 48 h, which are often used as a standard in the microtiter technique in many laboratories, were the substitutions introduced.

Our results, using amphotericin B and the hyphal inoculum of a standard strain of *A. fumigatus* NCMH 785, are consistent, as shown in repeatedly steady MICs. Such consistency was observed with a lower or higher density inoculum or a shorter or longer incubation. The hyphal inoculum prepared by our technique was reproducible and sufficiently stable to indicate standardization of this parameter.

The results of amphotericin B susceptibility studies presented in this paper, although restricted as to the number of strains investigated, indicate that there was no difference in MICs between conidia and hyphae of aspergilli. Furthermore, there was no significant difference in amphotericin B susceptibility between *A. fumigatus* and *A. flavus*. Published reports, based on different techniques, indicate MICs for conidia ranging between 0.14 and >25 μg/ml for *A. fumigatus*, and 0.12 to 12.5 μg/ml for *A. flavus* (1, 3, 9, 11, 18). The MIC results of Kitahara et al. (10) for three strains each of *A. fumigatus* (MIC = 0.1 μg/ml) and *A. flavus* (MIC = 0.2 to 0.5 μg/ml), although obtained with a higher inoculum (2×10^5), were similar or somewhat lower than those in our study; the same authors have concluded that there was no difference in the susceptibility of young hyphae and conidia.

In view of some slight variation of MICs at 18 and 24 h, as well as between 24 and 48 h, we recommend that readings be made at 24 and 48 h, and both MICs should be reported to the clinician. Nevertheless, one should have in mind that the MIC at 24 h, at least of amphotericin B (which rapidly deteriorates at elevated temperatures) is more likely to represent a true value, as already stressed by Kitahara et al. (10).

In our experience, with 18- to 24-h readings, there has been occasional difficulty in determining the MIC; in such cases, the endpoint was the lowest drug concentration showing any trace of turbidity. In some wells, at a sub-MIC concentration, individual fungal colonies (one to six per well) developed after 48 to 72 h, whereas at lower concentrations on such plates abundant growth was present.

More experimental data from the parallel susceptibility testing of pure hyphae and conidia of a number of freshly isolated aspergilli are needed, before a decision on which technique to adopt for general use can be made.

Recently, Trinci (19) has reported on *A. niger* grown as a dispersed, filamentous mycelium in the presence of acrylic resin (0.2%) containing Vogel's liquid medium and incubated at 30°C for 24 h on a rotary shaker. It is questionable whether an inoculum prepared in this manner would be appropriate for testing antimicrobial agents because of its anionic polymer content. In addition, the apparatus for shaking cultures to obtain such an inoculum of aspergilli may not be available in all laboratories wishing to perform the susceptibility testing.

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