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NOVEL TREATMENT STRATEGIES FOR SUPERFICIAL MYCOSES

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Optimal growth conditions for the determination of the antifungal susceptibility of three species of dermatophytes with the use of a microdilution method

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As a prerequisite to standardization of dermatophyte susceptibility testing, conditions that support optimal growth of different dermatophyte species must be established. Eighteen isolates of *Trichophyton* spp. *(T rubrum, T mentagrophytes, T tonsurans)* were grown in 4 different media: RPMI 1640 with L-glutamine, without sodium bicarbonate and buffered at pH = 7.0; antibiotic medium #3 (Penassay); yeast nitrogen base with 0.5% dextrose buffered at pH = 7.0; and Sabouraud dextrose broth. Incubation for 6 days at 35° C produced the following results: RPMI and Sabouraud dextrose supported equally sufficient growth for all strains tested; Penassay supported growth of only 33% of the isolates tested, and buffered yeast nitrogen base did not support growth of any isolates. RPMI was selected as the optimal medium, and organisms were tested at both 30° C and 35° C with a standardized inoculum density of 10^3 conidia/mL. No temperature differences were noted in the amount of growth of the dermatophytes tested. With RPMI at an incubation temperature of 35° C, 3 inoculum sizes (10^3, 10^4, and $l0^5$ conidia/mL) were tested against 4 antifungal agents: griseofulvin, itraconazole, terbinafine, and fluconazole. Inoculum size did not affect minimum inhibitory concentration (MIC) results for itraconazole or terbinafine, but a larger inoculum produced a slightly higher MIC for griseofulvin and a noticeably higher MIC for fluconazole. Our data support the use of RPMI 1640, 35° C, and 4 days as an incubation temperature and time, respectively, and an inoculum of 10^3 conidia/mL as optimal conditions for the determination of the antifungal susceptibility of dermatophytes. (J Am Acad Dermatol 1999;40:S9-13.)

Data related to susceptibility testing of dermatophytes are largely lacking. Efforts by the National Committee for Clinical Laboratory Standards (NCCLS) have focused primarily on testing yeast, and those efforts have resulted in the publication of a *Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts,* M27-A.[1] However, with the increasing variety of drugs available to treat dermatophytosis, the need for a reference method for dermatophyte testing has become apparent.[2]

Many variables need to be studied when a testing method is being developed, because the minimal inhibitory concentration (MIC) can be greatly affected by changes in inoculum size, length and temperature of incubation, media, and endpoint definition.[3] Our aim was therefore to select a group of organisms responsible for causing most dermatophytic infections and determine their growth under various conditions. Eighteen isolates of *Trichophyton* spp. were tested in 4 types of media at both 30° C and 35° C. Once the media and temperatures were selected, we studied the effect of inoculum

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Table I. Components of the 4 culture media used

Component	RPMI 1640*	Yeast nitrogen broth with 5% dextrose*	Sabouraud dextrose broth†	Penassay (antibiotic medium #3)†
Ammonium sulfate		5.0 g		
p-Aminobenzoic acid	1.0 mg	0.2 mg		
L-Arginine	200 mg			
L-Asparagine	50 mg			
L-Aspartic acid	20 mg			
Beef extract				1.5 g
Biotin	200 µg	2.0 µg		
Boric acid		500 µg		
Calcium chloride		0.1 g		
Calcium nitrate	100 mg			
Calcium pantothenate		400 µg		
Chlorine chloride	3.0 mg			
Copper sulfate		40 µg		
L-Cystine 2HCl	65.2 mg			
Dextrose	2.0 g	5.0 g	20 g	1.0 g
Dipotassium phosphate				3.68 g
Ferric chloride		200 µg		
Folic acid	1000 µg	2.0 µg		
L-Glutamic acid	20 mg			
L-Glutamine	300 mg			
Glutathione, reduced	1.0 mg			
Glycine	10 mg			
L-Histidine	15 mg			
L-Histidine monohydrochloride		10 mg		
L-Hydrosyproline	20 mg			
Inositol		2.0 mg		
myo-Inositol	35 mg			
L-Isoleucine	50 mg			
L-Leucine	50 mg			
L-Lysine HC1	40 mg			
Magnesium sulfate	48.8 mg	500 mg		
Manganese sulfate		400 µg		

concentration (10^3,10^4, and 10^5 conidia/mL) against 4 antifungal agents: griseofulvin (Sigma Chemical Company, St Louis, Mo), itraconazole (Janssen Research Foundation, Beerse, Belgium), terbinafine (Novartis, East Hanover, NJ), and fluconazole (Pfizer Inc, New York, NY). The results of this study serve as the foundation for the development of a standardized susceptibility testing method for dermatophytes.

METHODS

Organisms

We tested 18 clinical isolates of dermatophytes: 6 strains each of *T rubrum*, *T mentagrophytes* var *mentagrophytes,* and *T tonsurans.* All strains were isolated from nail or hair and were identified at the Center for Medical Mycology, University Hospitals of Cleveland, by conventional methods.[4] Isolates were kept frozen at –80° C on potato dextrose agar slants until time of use.

Two quality control organisms were used, *Candida parapsilosis* ATCC 22019 and *Paecilomyces variotii* ATCC 22319 (American Type Culture Collection, Rockville, Md).

Antifungal drugs

Four antifungal drugs were used: griseofulvin (Sigma Chemical Company, St. Louis, Mo), itraconazole (Janssen Research Foundation, Beerse, Belgium), terbinafine (Novartis, East Hanover, NJ), and fluconazole (Pfizer Inc, New York, NY).

Media

Four types of media were used (Table I): RPMI 1640 (American Biorganics, Niagara Falls, NY) with L-glutamine, without sodium bicarbonate and buffered at pH = 7.0; antibiotic medium #3 (Penassay; Difco Laboratories, Detroit, Mich); yeast nitrogen base (Difco Laboratories) with 0.5% dextrose buffered at pH = 7.0; and Sabouraud dextrose broth (Difco Laboratories).

Table I. Continued

Component	RPMI 1640*	Yeast nitrogen broth with 5% dextrose*	Sabouraud dextrose broth†	Penassay (antibiotic medium #3†
D,L-Methionine		20 mg		
L-Methionine	15 mg			
Monopotassium phosphate				1.32 g
Neopeptone			10 g	
Niacin		400 µg		
Niacinamide	1.0 mg			
D-Pantothenic acid	0.25 mg			
Peptone				5.0 g
pH	7.0	7.0	5.6	7.0
Phenol red	5.3mg			
L-Phenylalanine	15 mg			
Potassium chloride	400 mg			
Potassium iodide		100 µg		
Potassium sulfate		1.0 g		
L-Proline	20 mg			
Pyridoxine HCl	1000 µg	400 µg		
Riboflavin	200 mg	200 mg		
L-Serine	30 mg			
Sodium chloride	6.0 g	0.1 g		3.5 g
Sodium molybdate		200 µg		
Sodium phosphate, dibasic	800 mg			
Thiamine HCl	100 µg	400 µg		
L-Threonine	20 mg			
D,L-Tryptophan		20 mg		
L-Tryptophan	5.0 mg			
L-Tyrosine	28.8 mg			
L-Valine	20 mg			
Vitamin B_{12}	0.005 mg			
Yeast extract				1.5 g
Zinc sulfate		400 µg		

*Chemically defined media.
†Chemically nondefined media.

Drug dilutions

Serial 2-fold dilutions were prepared according to the NCCLS M27-A approved standard.[1] Fluconazole and griseofulvin were diluted in water and 100% dimethyl sulfoxide, respectively, at 64 µg/mL to 0.13 µg/mL. Itraconazole and terbinafine were diluted in 100% dimethyl sulfoxide at 32 µg/mL to 0.06 µg/mL.

Inoculum preparation

We prepared a standardized inoculum by counting microconidia. Cultures were grown on a potato dextrose agar slant for 6 to 10 days at 30° C. Sterile saline solution (0.85%) was added to the slant, and the culture was gently swabbed with a cotton-tipped applicator to dislodge conidia from the hyphal mat. The suspension was transferred to a sterile tube, and the volume was adjusted to 5 mL with sterile saline solution. The resulting suspension was counted with a hemocytometer and diluted in RPMI 1640 to the desired concentration.

Incubation

Microdilution plates were incubated at 30° C and 35° C and were read on day 1 through day 6 for growth analysis and temperature comparison. Microdilution plates for testing MIC values were read on days 3, 4, and 5.

Broth microdilution testing

Microdilution plates were set up in accordance with the NCCLS M-27A reference method,[1] with the exception of inoculum preparation, described earlier. Briefly, column 1 was filled with 200 µL of RPMI and served as a sterility control. Columns 2 through 11 were filled with 100 µL of the inoculum and 100 µL of the serially diluted antifungal agent. Column 12 was filled with 200 µL of the inoculum and served as a growth control. All isolates were run in duplicate and read visually. Results of growth in the 4 media at both 30° C and 35° C were recorded as follows: (1+) growth barely visible, (2+) moderate growth, and (3+) heavy growth. The

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