

Intra- and Interlaboratory Study of a Method for Testing the Antifungal Susceptibilities of Dermatophytes

M. A. Ghannoum,^{1*} V. Chaturvedi,² A. Espinel-Ingroff,³ M. A. Pfaller,⁴
M. G. Rinaldi,⁵ W. Lee-Yang,⁶ and D. W. Warnock⁶

Center for Medical Mycology, University Hospitals of Cleveland/Case Western Reserve University, Cleveland, Ohio¹; Department of Health, State of New York, Albany, New York²; Medical College of Virginia/Virginia Commonwealth University, Richmond, Virginia³; Department of Pathology, University of Iowa College of Medicine, Iowa City, Iowa⁴; Laboratory Service, University of Texas Health Science Center, Audie L. Murphy Memorial Veterans Hospital, San Antonio, Texas⁵; and Mycotic Diseases Branch, Centers for Disease Control and Prevention, Atlanta, Georgia⁶

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The National Committee for Clinical Laboratory Standards (NCCLS) M38-A standard for the susceptibility testing of conidium-forming filamentous fungi does not explicitly address the testing of dermatophytes. This multicenter study, involving six laboratories, investigated the MIC reproducibility of seven antifungal agents tested against 25 dermatophyte isolates (5 blinded pairs of five dermatophyte species per site for a total of 300 tests), using the method of dermatophyte testing developed at the Center for Medical Mycology, Cleveland, Ohio. The dermatophytes tested included *Trichophyton rubrum*, *Trichophyton mentagrophytes*, *Trichophyton tonsurans*, *Epidermophyton floccosum*, and *Microsporum canis*. Seven antifungals with activity against dermatophytes were tested, including ciclopirox, fluconazole, griseofulvin, itraconazole, posaconazole, terbinafine, and voriconazole. Interlaboratory MICs for all isolates were in 92 to 100% agreement at a visual endpoint reading of 50% inhibition as compared to the growth control and 88 to 99% agreement at a visual endpoint reading of 80% inhibition as compared to the growth control. Intralaboratory MICs between blinded pairs were in 97% agreement at a visual endpoint reading of 50% inhibition as compared to the growth control and 96% agreement at a visual endpoint reading of 80% inhibition as compared to the growth control. Data from this study support consideration of this method as an amendment to the NCCLS M38-A standard for the testing of dermatophytes.

There have been several multicenter studies involving filamentous fungi which have been used in the development of the National Committee for Clinical Laboratory Standards (NCCLS) *Reference Method for Broth Dilution Antifungal Susceptibility Testing of Conidium-Forming Filamentous Fungi. Approved Standard M38-A* (8). However, the published document does not address the antifungal susceptibilities of dermatophytes such as *Trichophyton*, *Microsporum*, and *Epidermophyton* species. Earlier studies of dermatophyte susceptibility have compared various methods. Niewerth et al. (9) compared the agar macrodilution and broth microdilution methods, demonstrating consistently lower MICs with the microdilution method. These lower MICs were more in line with skin tissue levels achieved by usual treatment regimens. Granade and Artis (5) used standardized fragmented mycelia as an alternative to conidial suspensions to overcome the lack of conidiation in some dermatophyte strains. Fernandez-Torres et al. (4) compared two different conidial suspension concentrations in the broth microdilution method and found no difference in the resulting MICs. Clearly, development and standardization of a method to determine the susceptibility of dermatophytes are needed.

The purpose of this multicenter study was to determine the inter- and intralaboratory reproducibility of MIC testing of

common dermatophyte species such as *Trichophyton*, *Epidermophyton*, and *Microsporum*, using the microdilution method developed at the Center for Medical Mycology. In order to develop such a method, Norris et al. (10) initially established the optimal growth conditions for the most common dermatophyte strains. These included the use of RPMI 1640 as the growth medium and 35°C for 4 days as the optimal temperature and incubation time, respectively. However, one special problem posed by dermatophytes is that conidium formation by *Trichophyton rubrum* is very poor on standard fungal isolation media. Subsequently, Jessup et al. (6) established the use of oatmeal cereal agar as the optimal growth medium for inducing conidium formation in *T. rubrum* isolates.

The NCCLS M38-A standard for susceptibility testing of filamentous fungi describes both macro- and microdilution methodologies, and according to results achieved in the multicenter study of filamentous fungi by Espinel-Ingroff, (1), interlaboratory agreement was demonstrated by both macro- and microdilution methods. Therefore, for this study the microdilution method was chosen because of its greater ease of performance. The aim of this study was to validate this method as a prelude to inclusion as a reference method for determining the antifungal susceptibility of dermatophyte isolates.

* Corresponding author. Mailing address: Center for Medical Mycology, University Hospitals of Cleveland/Case Western Reserve University, 11100 Euclid Ave., Cleveland, OH 44106-5028. Phone: (216) 844-8580. Fax: (216) 844-1076. E-mail: mag3@cwru.edu.

MATERIALS AND METHODS

Study participants. The following laboratories participated in this interlaboratory study: Department of Health, State of New York, Albany; Medical College of Virginia/Virginia Commonwealth University, Richmond; Center for

Medical Mycology, University Hospitals of Cleveland/Case Western Reserve University, Cleveland, Ohio; University of Iowa College of Medicine, Iowa City; University of Texas Health Science Center, Audie L. Murphy Memorial Veterans Hospital, San Antonio; and Centers for Disease Control and Prevention, Atlanta, Ga.

Study design. The six participating laboratories were supplied with frozen microtiter plates, prepared by Trek Diagnostics, Westlake, Ohio. These plates contained 10 serial dilutions of the antifungals used, prepared in RPMI 1640, as well as two rows of RPMI 1640 without antifungal agents, which acted as growth and sterility controls. The individual antifungal ranges used were as follows: ciclopirox, 0.06 to 32 $\mu\text{g/ml}$; fluconazole, 0.125 to 64 $\mu\text{g/ml}$; griseofulvin, 0.125 to 64 $\mu\text{g/ml}$; itraconazole, 0.001 to 0.5 $\mu\text{g/ml}$; posaconazole, 0.015 to 8.0 $\mu\text{g/ml}$; terbinafine, 0.001 to 0.5 $\mu\text{g/ml}$; and voriconazole, 0.001 to 0.5 $\mu\text{g/ml}$. Each well contained 100 μl of drug or RPMI 1640 alone. Ten-milliliter tubes of RPMI 1640 were also supplied for inoculum preparation.

Isolates. Isolates were chosen from the culture collection of clinical isolates maintained at the Center for Medical Mycology, University Hospitals of Cleveland/Case Western Reserve University. These isolates had originally been subcultured onto potato dextrose agar (PDA) slants, incubated at 30°C until luxuriant, and frozen at -80°C. Stored isolates were thawed and subcultured onto PDA plates, from which sets of new slants were prepared. Each laboratory was sent a set of five blinded pairs of five dermatophyte strains, including *T. rubrum*, *Trichophyton mentagrophytes*, *Trichophyton tonsurans*, *Epidermophyton floccosum*, and *Microsporum canis*. Three *T. rubrum* strains for which terbinafine MICs were low were included, corresponding to the published MIC data by Osborne et al. (11) and Mukherjee et al. (7) that the majority of terbinafine MICs for *T. rubrum* wild-type strains were 0.004 and 0.002 $\mu\text{g/ml}$, respectively. Two *T. rubrum* strains for which MICs were elevated were also included.

Antifungal agents. Standard powders of seven antifungals were obtained from the respective manufacturers, including ciclopirox (Dermik/Aventis, Berwyn, Pa.), fluconazole (Pfizer, New York, N.Y.), griseofulvin (Sigma), itraconazole (Janssen, Titusville, N.J.), posaconazole (Schering-Plough Research Institute, Kenilworth, N.J.), terbinafine (Novartis, Basel, Switzerland), and voriconazole (Pfizer).

Susceptibility method. The method used to determine the antifungal susceptibilities of dermatophytes to these agents was based on the publications of Norris (10) and Jessup (6). Each dermatophyte isolate was subcultured onto PDA and incubated at 30°C for 4 to 5 days or until good conidiation was produced.

T. rubrum isolates were subcultured onto cereal (oatmeal) agar instead of PDA in order to induce conidium production. A suspension of conidia in sterile saline was made by gently swabbing the colony surface with a sterile swab. The suspension was allowed to settle for 5 to 10 min, and conidia were counted with a hemacytometer. (For *T. rubrum* strains, turbidity produced by transference of oatmeal agar precludes the use of McFarland standards.) Working suspensions of conidia were prepared in 10 ml of RPMI 1640 to a final concentration of 1×10^3 to 3×10^3 CFU/ml. Yeast controls, which included *Candida parapsilosis* ATCC 22019 and *Candida krusei* ATCC 6258, were subcultured onto PDA and incubated at 35°C for 24 h. Yeast inocula were prepared to a final concentration of 0.5×10^3 to 2.5×10^3 CFU/ml. Microtiter plates were removed from the freezer and allow to thaw. Each drug concentration well and growth control well was inoculated with 100 μl of cell suspension by using a multichannel pipette. The final volume in each well was 200 μl . Dermatophyte plates were incubated at 35°C for 4 days (yeast controls were incubated for 48 h.) Plates were examined visually for 50 and 80% growth inhibition as compared to the growth control. MIC results were recorded in micrograms per milliliter.

Data analysis. All reported results were included in the data analysis. The measurement of interlaboratory agreement for each isolate (*T. rubrum*, *T. mentagrophytes*, etc.) was determined as a percentage of endpoints within 1, 2, and 3 dilutions (e.g., 0.5, 1.0, and 2.0 $\mu\text{g/ml}$). Interlaboratory agreement for the entire group of isolates, as well as intralaboratory agreement of blinded pair test results, was similarly determined. Results were considered in agreement if they fell within 3 dilutions.

RESULTS

Table 1 shows the cumulative MIC data from all sites reported by individual drug. All six participants detected two pairs of terbinafine-resistant *T. rubrum* isolates (one site had an additional outlier). Two sites did not report MICs for four

TABLE 1. Cumulative MIC parameter data from all sites

Endpoint and antifungal	MIC ($\mu\text{g/ml}$) ^a			
	Range	50%	90%	Mean
50% inhibition				
Ciclopirox	0.06–2.0	1.0	1.0	0.96
Fluconazole	0.125– \geq 64	1.0	8.0	4.09
Griseofulvin	0.125–64	0.125	0.5	0.86
Itraconazole	0.001–0.125	0.008	0.06	0.02
Posaconazole	0.015–0.125	0.015	0.03	0.03
Terbinafine	0.001– \geq 0.5	0.008	0.03	0.05
Voriconazole	0.001–0.25	0.008	0.06	0.02
80% inhibition				
Ciclopirox	0.06–2.0	1.0	1.0	1.0
Fluconazole	0.125– \geq 64	2.0	16.0	6.99
Griseofulvin	0.125– \geq 64	0.125	0.5	0.93
Itraconazole	0.001–0.5	0.015	0.125	0.04
Posaconazole	0.015–0.25	0.015	0.06	0.04
Terbinafine	0.001– \geq 0.5	0.008	0.03	0.06
Voriconazole	0.001– \geq 0.5	0.015	0.06	0.03

^a 50% and 90%, MIC₅₀ and MIC₉₀, respectively.

isolates, either because of lack of conidiation, which occurred with *M. canis* subcultures in particular, or contamination.

When read at the 50% endpoint, the MICs at which 50 and 90% of the isolates tested are inhibited (MIC₅₀ and MIC₉₀, respectively) for all antifungals were within 1 dilution of those read at the 80% endpoint.

Table 2 summarizes the interlaboratory agreement of individual drugs. Interlaboratory agreement within 3 dilutions ranged from 92% (itraconazole) to 100% (voriconazole and posaconazole) at the 50% inhibition endpoint and 87.8% (fluconazole) to 99.3% (posaconazole) at the 80% inhibition endpoint.

Table 3 summarizes the intralaboratory agreement or reproducibility of MIC results of blinded pairs by individual drug. Intralaboratory agreement within 3 dilutions ranged from 93.9% (fluconazole) to 100% (posaconazole) at the 50% inhibition endpoint and 89% (fluconazole) to 100% (ciclopirox and posaconazole) at the 80% inhibition endpoint.

TABLE 2. Interlaboratory agreement summary

Endpoint and antifungal	% of total isolates within:		
	1 dilution	2 dilutions	3 dilutions
50% inhibition			
Ciclopirox	94.6	96.0	99.0
Fluconazole	67.6	82.1	93.6
Griseofulvin	96.9	98.6	98.6
Itraconazole	63.9	79.8	92.0
Posaconazole	93.6	99.4	100
Terbinafine	84.5	92.9	98.0
Voriconazole	75.3	87.8	100
80% inhibition			
Ciclopirox	95.9	97.6	99.0
Fluconazole	64.9	85.8	87.8
Griseofulvin	88.9	97.7	98.4
Itraconazole	62.8	82.7	90.5
Posaconazole	88.8	95.6	99.3
Terbinafine	85.8	95.3	98.0
Voriconazole	73.6	89.1	97.5

TABLE 3. Intralaboratory agreement summary

Endpoint and antifungal	% of total isolates within:		
	1 dilution	2 dilutions	3 dilutions
50% inhibition			
Ciclopirox	91.8	96.6	99.3
Fluconazole	79.6	87.8	93.9
Griseofulvin	91.2	97.3	98.0
Itraconazole	73.5	86.4	95.2
Posaconazole	95.2	98.6	100
Terbinafine	84.4	91.2	97.3
Voriconazole	74.1	86.3	94.5
80% inhibition			
Ciclopirox	92.5	100	100
Fluconazole	74.8	83.6	89.0
Griseofulvin	91.8	96.6	98.3
Itraconazole	66.7	85.1	91.9
Posaconazole	89.8	97.3	100
Terbinafine	83.7	91.2	96.0
Voriconazole	74.1	87.4	96.2

DISCUSSION

Previously, griseofulvin had been the only antifungal available for the treatment of dermatophytoses. Recently, new antifungals have been developed that have activity against dermatophytes, and a reliable susceptibility testing method is needed to help physicians manage patients with dermatophyte infections. To this end, we designed a susceptibility method for dermatophyte isolates by first establishing the optimal growth conditions, including medium, temperature, and incubation time (6, 10). The current multicenter study was conducted as a prelude to the inclusion of this method as an NCCLS standard.

When establishing a susceptibility testing method, it is imperative to ensure reproducibility of endpoints and detection of resistance. Several previous multicenter studies have been conducted to establish guidelines for the susceptibility testing of yeasts and filamentous fungi (2, 3, 12, 13). The results from our study are similar to those obtained in those previous studies, which were subsequently used to establish approved standards.

In the present study, reproducibility of MIC endpoints was very high by both inter- and intralaboratory comparison. Lower percentages of agreement within 3 dilutions were achieved with fluconazole and itraconazole, although none were less than 87%. Since no significant differences were seen between 50 and 80% inhibition endpoints, we recommend the 80% inhibition endpoint for ease of interpretation. Importantly, all sites were able to detect resistance in the two pairs of terbinafine-resistant *T. rubrum* isolates provided.

Based upon the results of this multicenter study, adoption of this method as an amendment to the NCCLS M38-A standard for the testing of dermatophytes is warranted. Correlation of in vitro dermatophyte MICs with clinical outcomes, as well as establishing quality control strains, remains to be determined.

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