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Agar-Based Disk Diffusion Assay for Susceptibility Testing of Dermatophytes▽

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Currently, no agar-based susceptibility testing method has been standardized for testing dermatophytes. We describe a newly developed agar-based method employing disk diffusion assay to test the susceptibility of 47 isolates of dermatophytes against 8 antifungals. Our results show that the method is reproducible, is simple, and could be used to determine the antifungal susceptibility of dermatophytes.

The incidence of dermatophytosis (caused by *Trichophyton*, *Epidermophyton*, or *Microsporum* spp. [23]) has increased considerably, especially among immunocompromised patients (2, 20). Relapse reported for some dermatophyte species and primary resistance of *Trichophyton rubrum* strains to terbinafine (18) underscore the need for determination of their *in vitro* antifungal susceptibilities. A reference microdilution method (M38-A2) is approved by the Clinical and Laboratory Standards Institute (CLSI) for antifungal susceptibility testing of molds and dermatophytes (7, 19). However, no agar-based susceptibility testing method has been standardized for the testing of dermatophytes. Advantages of a standardized disk diffusion-based assay for evaluating the antifungal susceptibility of dermatophytes include the ease of use, reproducibility, accuracy, and low cost (1, 3, 10, 15, 16, 22).

In the current study, we optimized an agar-based disk diffusion method to determine the susceptibility of dermatophytes to various antifungals. We tested 47 clinical isolates (*Trichophyton tonsurans* [$n = 12$], *T. rubrum* [$n = 10$, including 5 terbinafine-resistant isolates] [18], *Trichophyton mentagrophytes* [$n = 9$], *Microsporum canis* [$n = 8$], and *Epidermophyton floccosum* [$n = 8$]). Two isolates (*T. rubrum* ATCC MYA 4438 and *T. mentagrophytes* ATCC MYA 4439) served as CLSI-recommended quality control (QC) strains (7), while *Candida albicans* ATCC 9028, *Candida parapsilosis* ATCC 22019, and *Candida krusei* ATCC 6258 served as additional QC strains.

Commercially available discs (9 mm diameter) preloaded with ciclopirox (50 μg/disk), fluconazole (25 μg/disk), itraconazole (8 μg/disk), ketoconazole (15 μg/disk), miconazole (10 μg/disk), and voriconazole (1 μg/disk) were used (Rosco Neo-Sensitabs; Key Scientific, TX). Discs containing griseofulvin (10 μg/disk) and terbinafine (1 μg/disk) were not commercially available and were prepared in our laboratory as part of this study. The concentrations of the drugs to be loaded in griseofulvin and terbinafine disks were determined by first performing preliminary experiments to determine the optimal concentration that produced inhibition zones which can be conveniently measured on the 100- by 15-mm plate (Fisher Scientific Co., KY). For these two antifungals, the drugs terbinafine (Novartis, NJ) and griseofulvin (Acros Organics, NJ) were obtained in powdered form. A stock solution of each drug was prepared using dimethyl sulfoxide, as follows: griseofulvin, 1.25 mg/ml; terbinafine, 50 μg/ml. Blank paper discs (6 mm diameter) were loaded with 20 μl of the prepared stock solutions to obtain the desired drug concentration per disk (1 μg and 25 μg for terbinafine and griseofulvin, respectively) and allowed to air dry at room temperature. The air-dried disks were stored at 4°C in a refrigerator.

Organisms were subcultured on potato dextrose agar (PDA) or oatmeal agar (for *T. rubrum*) at 30°C for 4 to 15 days. Following growth, conidia were harvested in sterile saline, and using a hemacytometer, the conidial suspension was adjusted to 1.0×10^6 conidia/ml. Mueller-Hinton (MH) agar (Remel, KS) plates were streaked evenly with a swab dipped into the standardized inoculum suspension. Lids were left ajar for 3 min in a laminar flow cabinet to allow for any excess surface moisture to be absorbed into the agar before the drug-impregnated disks were applied. Disks containing the test agents were applied to the surfaces of inoculated plates. Plates were inverted and incubated at 30°C for 4 to 7 days to allow for fungal growth. Inhibition zone diameters (IZD) were measured in millimeters. To evaluate the reproducibility of our method, a new inoculum was prepared for each replicate, all isolates were run in triplicate, and the standard deviations were determined. The CLSI-approved microdilution method (M38-A2) (7) was used to determine the MIC of terbinafine against a subset of dermatophyte isolates that were terbinafine susceptible and resistant.

The IZD produced by the two reference strains, *T. rubrum* ATCC MYA 4438 and *T. mentagrophytes* ATCC MYA 4439, were included as part of the data under the respective species listed in Table 1 and Table 2. The results for the reference QC strains (*C. albicans* ATCC 90028, *C. krusei* ATCC 6258, and *C. parapsilosis* ATCC 22019), which served as internal controls, were within the acceptable ranges recommended by the CLSI (data not shown).

Initially, we compared three different inoculum sizes ($1.0 \times$

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TABLE 1. *In vitro* activities of 8 antifungal agents against 37 strains of dermatophytes[a]

Species (no. of strains tested)	Antifungal agent	Mean IZD ± SE (mm)	IZD range (mm)
T. tonsurans (12)	Ketoconazole	28.65 ± 7.53	15–45
	Miconazole	28.70 ± 5.55	20–40
	Itraconazole	39.40 ± 5.05	28–45
	Terbinafine	53.10 ± 10.90	25–70
	Fluconazole	8.70 ± 9.71	0–30
	Griseofulvin	49.14 ± 7.63	36–65
	Ciclopirox olamine	38.00 ± 2.36	34–40
	Voriconazole	49.00 ± 8.09	40–68
T. mentagrophytes (9)	Ketoconazole	21.38 ± 4.24	15–30
	Miconazole	23.75 ± 4.13	15–30
	Itraconazole	32.22 ± 6.35	22–55
	Terbinafine	69.54 ± 2.52	61–73
	Fluconazole	4.71 ± 8.80	0–25
	Griseofulvin	57.75 ± 5.08	45–65
	Ciclopirox olamine	33.38 ± 3.05	29–39
	Voriconazole	38.96 ± 11.35	20–60
M. canis (8)	Ketoconazole	26.67 ± 7.49	15–40
	Miconazole	30.04 ± 6.70	15–40
	Itraconazole	33.17 ± 3.84	25–40
	Terbinafine	53.25 ± 7.12	36–67
	Fluconazole	0	
	Griseofulvin	52.29 ± 9.08	35–68
	Ciclopirox olamine	37.75 ± 3.54	32–45
	Voriconazole	55.50 ± 5.43	45–68
E. floccosum (8)	Ketoconazole	51.96 ± 7.51	40–72
	Miconazole	44.58 ± 7.17	33–60
	Itraconazole	44.88 ± 5.33	35–53
	Terbinafine	55.58 ± 5.97	45–65
	Fluconazole	38.67 ± 10.09	15–60
	Griseofulvin	68.33 ± 3.19	60–76
	Ciclopirox olamine	38.04 ± 5.36	30–46
	Voriconazole	66.67 ± 6.83	50–78

[a] Each drug testing was performed with fresh inoculum in triplicate on three different days.

10^4, 1.0×10^5, and 1.0×10^6 cells/ml) to identify the one that shows enough coverage on the plate for all the species tested (data not shown). Based on this, we identified 1.0×10^6 cells/ml as the optimum inoculum size and used it to seed agar plates in all subsequent experiments. Furthermore, we tested a range of concentrations of terbinafine (0.0156, 0.03125, 0.0625, and 1.0 μg/disk) and observed that terbinafine concentrations of 0.0156, 0.03125, and 0.0625 μg/disk produced inhibition zones with diameters ranging from 5 to 15 mm, 7 to 18 mm, and 10 to 22 mm, respectively, for both *T. mentagrophytes* and *T. tonsurans* isolates. However, 1 μg/disk of terbinafine produced IZD of 0 to 73 mm for all the isolates tested in the study. We also optimized the best growth medium to use in the disk diffusion assay and tested Mueller-Hinton (MH) medium alone or MH medium supplemented with 2% glucose and 0.5 μg/ml methylene blue. Our data showed that use of MH medium alone resulted in clear inhibition zones for all the strains tested in the study and that supplementation with 2% glucose and 0.5 μg/ml methylene blue did not enhance the clarity in zone edge definition. Based on these data, we identified nonsupplemented MH medium as an optimal medium. Finally, we optimized the time of incubation needed to produce reproducible inhibition zones and found that the optimum incubation time was 3 days for *T. mentagrophytes* isolates but was up to 7 days for *T. rubrum*, *T. tonsurans*, *E. floccosum*, and *Microsporum canis*. Therefore, we recommend an optimal incubation time of 7 days for the disk diffusion assay.

TABLE 2. *In vitro* activities of 8 antifungal agents against terbinafine-resistant and -sensitive strains of *T. rubrum*[a]

Type of *T. rubrum* strain (no. of strains tested)	Antifungal agent	Mean IZD ± SE (mm)	IZD range (mm)
Terbinafine resistant (5)	Ketoconazole	50.53 ± 10.85	20–74
	Miconazole	41.53 ± 10.88	25–60
	Itraconazole	41.67 ± 8.99	34–60
	Terbinafine	0	0
	Fluconazole	51.13 ± 11.61	35–70
	Griseofulvin	49.07 ± 8.92	33–60
	Ciclopirox olamine	37.27 ± 4.45	32–48
	Voriconazole	63.00 ± 9.43	52–80
Terbinafine sensitive (5)	Ketoconazole	30.13 ± 10.09	20–50
	Miconazole	29.80 ± 7.34	20–45
	Itraconazole	34.80 ± 5.32	30–45
	Terbinafine	64.33 ± 7.01	47–70
	Fluconazole	19.40 ± 17.55	0–50
	Griseofulvin	41.00 ± 8.68	30–55
	Ciclopirox olamine	35.47 ± 1.96	32–40
	Voriconazole	51.67 ± 8.24	35–65

[a] Each drug testing was performed with fresh inoculum in triplicate on three different days.

Next, we used the optimized testing conditions and evaluated the antifungal susceptibility of 47 clinical isolates of dermatophytes against eight antifungals. Our analysis using five *T. rubrum* isolates that are known to be resistant to terbinafine using the CLSI microdilution testing method (7, 18) showed that our disk diffusion method is able to differentiate terbinafine-susceptible and -resistant (IZD = 0 mm) strains (Table 2). Interestingly, most of the terbinafine-resistant strains showed good *in vitro* susceptibility to other antifungal agents tested in the study using the IZD, including fluconazole, indicating no cross-resistance with the azoles. The same observation was noted using the microdilution method (18). For comparative purposes, Table 3 presents terbinafine IZD and MICs determined by CLSI M38-A2 for 19 clinical dermatophyte isolates (7). These data indicate that there is some correlation between the two methods.

Development of a standardized disk diffusion-based assay for determining the antifungal susceptibility of dermatophytes is desirable and provides a number of advantages. For instance, Macura (15) found the disk susceptibility assay to be very simple and advocated its use in routine clinical testing. Similarly, Meis et al. (16) and Barry and Brown (1) separately found that the disk diffusion method is not only reproducible and accurate but also economical and very easy to perform. Other authors have also successfully used disk diffusion to test the susceptibility of *Candida* species to azole antifungal agents (13, 21) and various kinds of molds such as *Fusarium* (4), *Scedosporium* (5), and other dematiaceous fungi (6).

In summary, we have successfully developed an agar-based assay for susceptibility testing of dermatophytes. The optimal conditions for performing this assay involve the use of 1×10^6

TABLE 3. Inhibition zone diameters and terbinafine MICs of 19 strains tested in the study[a]

Species	Isolate	Mean IZD ± SE (mm)	MIC (μg/ml)
T. mentagrophytes	MRL 12523	70.33 ± 0.52	0.016
	MRL 12528	70.33 ± 2.74	0.008
	MRL 12539	67.67 ± 1.37	0.008
	MRL 12680	69.67 ± 2.25	0.016
	MRL 12692	70.67 ± 1.03	0.004
	MRL 12719	70.00 ± 1.79	0.004
	MRL 12860	67.00 ± 4.65	0.008
	MRL 12870	70.67 ± 1.03	0.004
	MYA 4439[b]	71.00 ± 0.89	0.004
T. rubrum	MRL 475	0	4
	MRL 476	0	4
	MRL 479	0	4
	MRL 11200	0	32
	MYA 4438[b]	0	4
	MRL 17960	68.00 ± 2.37	0.008
	MRL 17964	51.33 ± 3.61	0.008
	MRL 17966	66.67 ± 1.86	0.004
	MRL 17967	67.67 ± 2.25	0.004
	MRL 17968	68.00 ± 1.79	0.008

[a] Each drug testing was performed with fresh inoculum in triplicate on three different days.
[b] ATCC designation.

cells/ml as an inoculum and Mueller-Hinton agar in 100- by 15-mm plates incubated at 30°C for 7 days. Inter- and intralaboratory studies to determine the reproducibility of the developed method are warranted.

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