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Use of the Sensititre Colorimetric Microdilution Panel for Antifungal Susceptibility Testing of Dermatophytes

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The Sensititre YeastOne antifungal panel was used to test 49 dermatophytes belonging to the species *Epidermophyton floccosum*, *Microsporum gypseum*, *Microsporum canis*, *Trichophyton tonsurans*, *Trichophyton rubrum*, and *Trichophyton mentagrophytes*. The MICs of four antifungals obtained with the Sensititre YeastOne antifungal panel were compared with those obtained by the reference NCCLS microdilution method. The levels of agreement between the two methods (≤2 dilutions) were 81.6% with amphotericin B, 87.7% with itraconazole, 67.3% with fluconazole, and 69.4% with ketoconazole.

Infections caused by dermatophytes are probably the most common communicable fungal diseases affecting humans. Although a wide variety of both topically and systemically administered compounds with activities against these fungi are available, some of these infections are still difficult to resolve completely and remissions and relapses are often observed (3, 9). Remissions and relapses are more likely due to the inability of the antifungal drug to penetrate the site of infection rather than to the intrinsic resistance of the fungus. In recent years there has been growing interest in the development of a reference method for in vitro antifungal susceptibility testing of dermatophytes. The studies that have been described in the literature (1, 8, 11, 12, 14) are based on slight modifications of the broth macro- and microdilution techniques for molds recommended by the NCCLS (document M38-P) (13). However, these methods may not be the most practical procedures for use in the routine clinical laboratory, mainly due to the need for the subjective determination of endpoints.

The addition of an oxidation-reduction colorimetric indicator, such as Alamar Blue, which changes from blue to red in the presence of metabolically active growing organisms, has been shown to facilitate the reading of MIC endpoints (4, 7) and could be an alternative to broth macro- and microdilution techniques for molds for use in a general laboratory. The Sensititre YeastOne Colorimetric Antifungal panel (Trek Diagnostic Systems Ltd., East Grinstead, United Kingdom) is a commercial microdilution plate that is already available and that contains dried serial dilutions of five antifungal agents (amphotericin B, flucytosine, fluconazole, itraconazole, and ketoconazole) in a diluent with Alamar Blue. The aim of this study was to compare the in vitro activities of four antifungals both by tests with the Sensititre YeastOne panel and by an adaptation of the NCCLS broth microdilution method, performed independently.

Sensititre method. The Sensititre YeastOne test panels were provided by IZASA S.A. (Barcelona, Spain). A total of 49 clinical isolates belonging to six of the most common species of dermatophytes were tested (Table 1). *Paecilomyces variotii* ATCC 36257 was included as a reference strain. The fungi were subcultured on potato dextrose agar, and stock inoculum suspensions were prepared according to the recommendations of the NCCLS (13). This suspension was then adjusted with a spectrophotometer to 65 to 70% transmittance for dermatophytes and to 74 to 76% transmittance for *P. variotii* at a wavelength of 530 nm. The working suspension was made by dilution of the suspensions 1:100 in RPMI 1640 to produce the final test concentration of the inoculum. Aliquots of 100 μl of the diluted suspension were inoculated into the wells with antifungals and the growth control well (containing only diluent and colorimetric indicator) with a multichannel pipette. The concentrations of the amphotericin B, itraconazole, and ketoconazole dilutions ranged from 0.008 to 16 μg/ml, and the concentrations of the fluconazole dilutions ranged from 0.12 to 256 μg/ml. Flucytosine was not evaluated, as in previous studies the MICs of this drug for these fungi were very high. The panels were incubated at 28°C until a change in color from blue (indicative of no growth) to red (indicative of growth) was observed in the growth control well. The MIC of amphotericin B was defined as the lowest drug concentration which prevented the development of a red color (the first blue well). For the rest of the antifungals tested, the same criterion was applied, but when a purple color remained during a change from red to blue (indicative of partial growth inhibition), the MIC was defined as the lowest drug concentration which resulted in a purple color.

Broth microdilution method. The broth microdilution method described previously (8) was an adaptation of the method recommended by the NCCLS (13) for the testing of dermatophytes. The inoculum was prepared and standardized spectrophotometrically as described above for the method with the Sensititre YeastOne panel. Eleven dilutions of each drug were tested, i.e., concentrations of 0.01 to 16 μg/ml for amphotericin B, itraconazole, and ketoconazole and concentrations of 0.12 to 128 μg/ml for fluconazole. The microdilution plates were incubated at 28°C. The amphotericin B MIC was defined as the lowest drug concentration at which there was no growth. The azole MICs were defined as the lowest drug con-

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TABLE 1. Agreement between MICs of four antifungals for 49 strains of dermatophytes obtained with the Sensititre YeastOne panel and a microdilution method

Species (no. of strains tested)	Antifungal agent[a]	MIC (μg/ml) range		No. of isolates for which MICs determined with the Sensititre YeastOne panel differed from MICs determined by microdilution method by the following dilution:							% Agreement[b]
		Sensititre YeastOne panel	Microdilution method	<−2	−2	−1	0	+1	+2	>+2	
E. flocossum (8)	AMB	0.12–0.5	0.03–1	0	0	2	2	1	2	1	87.5
	FLC	0.5–32	0.25–64	1	0	2	2	1	1	1	75
	ITC	≤ 0.008–0.06	≤ 0.01–0.5	1	0	4	1	2	0	0	87.5
	KTC	≤ 0.008–0.25	0.03–2	2	1	1	1	1	0	2	50
M. canis (8)	AMB	0.25–2	0.25–8	1	0	1	4	0	2	0	87.5
	FLC	8–64	0.5–16	0	0	0	2	1	3	2	75
	ITC	0.01–0.12	0.03–0.12	0	1	3	1	2	1	0	100
	KTC	0.25–0.5	0.06–0.25	0	0	0	0	5	1	2	75
M. gypseum (9)	AMB	2–8	0.12–8	0	1	0	0	2	0	6	33.3
	FLC	1–64	8–32	1	2	1	2	2	1	0	88.9
	ITC	≤ 0.008–0.25	0.03–0.25	0	2	0	4	3	0	0	100
	KTC	0.06–1	0.12–2	1	1	3	1	3	0	0	88.8
T. mentagrophytes (8)	AMB	0.12–1	0.25–0.5	0	1	0	4	3	0	0	100
	FLC	4–64	8–128	4	0	2	1	0	1	0	50
	ITC	0.01–0.12	0.03–0.5	3	2	0	3	0	0	0	62.5
	KTC	0.03–1	0.12–2	2	1	2	2	0	1	0	75
T. rubrum (8)	AMB	0.25–1	0.12–1	0	0	2	2	3	1	0	100
	FLC	0.25–64	4–8	3	1	0	0	1	1	2	37.5
	ITC	≤ 0.008–0.25	≤ 0.01–1	1	3	1	1	1	0	1	75
	KTC	≤ 0.008–2	0.5–2	4	0	3	0	0	1	0	50
T. tonsurans (8)	AMB	0.12–1	0.25–1	1	1	2	2	2	0	0	87.5
	FLC	1–32	≤ 0.12–64	0	2	1	1	1	1	2	75
	ITC	≤ 0.008–0.06	≤ 0.01–0.03	0	1	2	3	1	1	0	100
	KTC	≤ 0.008–0.12	0.06–0.5	2	3	3	0	0	0	0	75
Overall (49)	AMB	0.012–8	0.03–8	2	3	7	14	11	5	7	81.6
	FLC	0.25–64	≤ 0.12–128	9	5	6	8	6	8	7	67.3
	ITC	≤ 0.008–0.25	≤ 0.01–1	5	9	10	13	9	2	1	87.7
	KTC	≤ 0.008–2	0.03–2	11	6	12	4	9	3	4	69.4

[a] AMB, amphotericin B; FLC, fluconazole; ITC, itraconazole; KTC, ketoconazole.
[b] Percent agreement defined as discrepancies in MIC endpoints of no more than 2 dilutions.

centrations which produced a ≥50% reduction in growth compared to the growth of the corresponding growth control. Differences in MICs of no more than 2 dilutions between the two methods were used to calculate the percent agreement.

The majority of the isolates tested by the colorimetric method produced detectable growth (a red color in the growth control well) between 72 and 96 h of incubation. The change of color in the growth control well was observed early, i.e., after 48 h of incubation, in only five of the isolates (two strains of *Trichophyton mentagrophytes* and one strain each of the species *Epidermophyton floccosum*, *Trichophyton rubrum*, and *Trichophyton tonsurans*). In another two isolates (one isolate each of *T. tonsurans* and *Microsporum canis*), the color changed after 5 and 6 days, respectively. MICs were determined by the broth microdilution method after 4 days of incubation for all isolates of *Microsporum gypseum* and *T. mentagrophytes* and after 7 days of incubation for the rest of the isolates tested.

The overall levels of agreement between the results of the two methods were 81.6% with amphotericin B, 87.7% with itraconazole, 67.3% with fluconazole, and 69.4% with ketoconazole. Variabilities in the levels of agreement were observed and depended on the species and antifungal tested (Table 1). Agreements of 100% were observed for *M. canis*, *M. gypseum*, and *T. tonsurans* with itraconazole and for *T. mentagrophytes* and *T. rubrum* with amphotericin B. The lowest levels of agreement were observed for *M. gypseum* with amphotericin B (33.3%), for *E. floccosum* with ketoconazole (50%), for *T. mentagrophytes* with fluconazole (50%) and itraconazole (62.5%), and for *T. rubrum* with fluconazole (37.5%) and ketoconazole (50%).

The reference strain was tested six times by each method with each drug on different days, and the results were highly reproducible. The modal MICs obtained with the Sensititre YeastOne panel and by the microdilution method were identical for amphotericin B (0.25 μg/ml) and fluconazole (4 μg/ml) but differed by 1 dilution for itraconazole (0.06 and 0.03 μg/ml, respectively) and 2 dilutions for ketoconazole (0.03 and 0.12 μg/ml, respectively).

In recent years the usefulness of Alamar Blue and the Sensititre YeastOne panel have been repeatedly evaluated for the testing of yeasts, with good correlations with the reference macro- and microdilution methods (2, 6, 7, 15–20; L. Alcalá, T.

Peláez, M. S. Díaz-Infantes, and M. Marín, Abstr. 40th Intersci. Conf. Antimicrob. Agents Chemother., abstr. 925, p. 366, 2000). In contrast, colorimetric methods for susceptibility testing of filamentous fungi have rarely been used, and the results have been controversial (4, 10; J. Meletiadis, B. A. Bouman, J. W. Mouton, and J. F. G. Meis, Abstr. 40th Intersci. Conf. Antimicrob. Agents Chemother., abstr. 211, p. 355, 2000).

In this study, we have shown greater than 80% concordances between the results of the standard method and a method with a commercial product for determination of the susceptibilities of dermatophytes to amphotericin B and itraconazole; the concordance rates were 67 and 69% in tests with fluconazole and ketoconazole, respectively. There are two possible reasons for the low levels of correlation between the two methods: (i) the small number of strains of each taxon tested and (ii) difficulty obtaining standardized inocula with the dermatophytes, mainly those with low levels of sporulation or high levels of pleomorphism, such as *E. floccosum* and *T. rubrum*. In general, we observed profuse sporulation with potato dextrose agar, the medium recommended by the NCCLS (13). Other investigators have successfully used other media such as oatmeal or rice agar (11). A third possible reason for the low levels of correlation between the two methods may be the effect of the incubation temperature on susceptibility. It has previously been reported that there is no observable difference in the amount of growth in microdilution tests at incubation temperatures of 30 and 35°C (14). However, in establishing the parameters for our study, we chose an incubation temperature of 28°C. We discovered that at this temperature the MICs were more highly reproducible between test runs and that, when the isolates were grown on solid media, had more typical growth and morphology. On the basis of the preliminary results that we have obtained, it appears that a large multicenter study for standardization of dermatophyte susceptibility testing is warranted. The two most noticeable shortcomings in the use of Alamar Blue for antifungal susceptibility testing were, first, its low level of stability after long incubation periods, which can be an inconvenience for the testing of slowly growing organisms. Although in our study practically all isolates tested provided detectable growth within the first 4 days of incubation, we observed that the color of the majority of the noninoculated wells had changed after 7 days of incubation (data not shown). Second, up to now the commercial product (the Sensititre YeastOne panel) has not included some of the antifungals commonly used in the treatment of infections caused by dermatophytes, such as griseofulvin, terbinafine, and clotrimazole. On the positive side, the MICs were easier to read and interpretation of the MICs was more objective, although the evaluation of partial inhibition (purple color) in tests with the azoles still requires some subjective interpretation. Another important advantage is that in this colorimetric method the test endpoint is not growth but metabolic inhibition. Use of this endpoint therefore reduces the incubation time of the test, as seen by other investigators for yeasts (5, 17). In this study, we observed that use of the Sensititre YeastOne panel allowed faster determination of MIC endpoints than the microdilution method for the majority of the isolates tested. Finally, this commercial colorimetric method has additional advantages: the test panel contains dried serial dilutions of drugs with the colorimetric indicator ready to use and can be stored at room temperature, and no special equipment is needed to perform the procedure. All these aspects make the product especially useful for a busy clinical laboratory. However, until a reference method with a high predictive value for testing the susceptibilities of dermatophytes is developed, the real usefulness of the Sensititre YeastOne panel cannot be proven.

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