JOURNAL OF CLINICAL MICROBIOLOGY, Nov. 2002, p. 3999–4003 Vol. 40, No. 11
0095-1137/02/$04.00+0 DOI: 10.1128/JCM.40.11.3999–4003.2002

Collaborative Evaluation of Optimal Antifungal Susceptibility Testing Conditions for Dermatophytes

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Received 13 March 2002/Returned for modification 5 April 2002/Accepted 18 August 2002

A multicenter study was conducted to define the most suitable testing conditions for antifungal susceptibility of dermatophytes. Broth microdilution MICs of clotrimazole, itraconazole, and terbinafine were determined in three centers against 60 strains of dermatophytes. The effects of inoculum density (ca. 10^3 and 10^4 CFU/ml), incubation time (3, 7, and 14 days), endpoint criteria for MIC determination (complete [MIC-0] and prominent [MIC-2] growth inhibition), and incubation temperature (28 and 37°C) on intra- and interlaboratory agreement were analyzed. The optimal testing conditions identified were an inoculum of 10^4 CFU/ml, a temperature of incubation of 28°C, an incubation period of 7 days, and MIC-0.

Dermatophytes are a group of morphologically and physiologically related molds that cause well-defined infections in vertebrates. The incidence of dermatophytoses has increased over recent years, particularly in immunocompromised patients (29, 30, 32, 33). The choice of the proper treatment is determined by the site and extent of the infection and the species involved, as well as by the efficacy, safety profile, and kinetics of the available drugs. For localized nonextensive lesions, topical therapies with clotrimazole (CLT) are generally used. For tinea unguium, scalp ringworm, extensive infections, or skin lesions with folliculitis, systemic antifungal treatment is necessary (1, 4, 23, 26). Oral drugs such as itraconazole (ITC) and terbinafine (TRB) are the antifungal agents currently most used to treat severe infections (4, 23). Some novel compounds, such as UR-9825, posaconazole, voriconazole, or ravuconazole, also appear to be promising candidates for the treatment of dermatophytosis (2, 10, 28).

In the in vitro method proposed by the National Committee for Clinical Laboratory Standards (NCCLS) for testing molds (25), the dermatophytes were not included. Therefore, it is necessary to develop a reproducible standardized method for these important fungi that would lead to protocols for proper treatment. In recent years, some authors, possibly encouraged by the development of the above-mentioned reference method, have published various articles wherein several species of dermatophytes have been tested (11, 27, 28, 34). In these works, different adaptations or modifications of the NCCLS methods have been assayed, although other techniques have also been used (3, 12, 15). The results obtained have been clearly contradictory in some aspects, which makes evident the need for standardization and the development of reference methods. Recently, we evaluated the activity of 11 antifungal drugs against an important number of strains of dermatophytes (n = 508) by using a microdilution method (10). In that study the testing conditions adopted were an inoculum size of 10^4 CFU/ml, a temperature and time of incubation of 28°C and 7 days, respectively, and the MIC endpoint determination was 50% growth inhibition for azoles and 100% for the rest. It is unknown whether varying the conditions would have changed the results significantly. We have therefore conducted a multicenter study in order to determine the most reproducible conditions for testing the antifungal activity of three of the most commonly used drugs—ITC, CLT, and TRB—against six frequent species of dermatophytes.

MATERIALS AND METHODS

Study design. Three laboratories participated in the present study. Each one received the same panel of 60 coded isolates, belonging to six species of dermatophytes, and two reference strains. Each isolate was tested three times in each laboratory. The reference strains were tested each time that a set of isolates was tested in each laboratory. The antifungal agents tested were ITC, CLT, and TRB. We used a broth microdilution method according to a standard protocol. The protocol included a detailed description of the parameters to be evaluated, which were, briefly: (i) two inoculum sizes (ca. 10^3 and 10^4 CFU/ml); (ii) two temperatures of incubation (28 and 37°C); (iii) three incubation times (3, 7, and 14 days); and (iv) two MIC endpoints (50 and 100% growth inhibition).

Test organisms. A total of 60 well-characterized clinical isolates were tested. They included 10 strains of each of the following species: *Trichophyton rubrum*, *Trichophyton mentagrophytes*, *Trichophyton tonsurans*, *Microsporum gypseum*, *Microsporum canis*, and *Epidermophyton floccosum*. Each isolate was maintained as a water suspension at room temperature at each center, until testing was performed. *Aspergillus fumigatus* NCPF 7099, which is resistant to ITC (6, 31), and *Paecilomyces variotii* ATCC 36257, were included as reference strains. The MICs for these isolates were as follows: CLT (1 to 2 μg/ml), ITC (>32 μg/ml), and TRB (4 to 8 μg/ml) for *A. fumigatus* NCPF 7099 and CLT (0.01 to 0.06 μg/ml), ITC (0.01 to 0.03 μg/ml), and TRB (0.03 to 0.06 μg/ml) for *P. variotii* ATCC 36257.

Medium. A single lot of RPMI 1640 broth (Gibco-BRL, Izasa, Barcelona, Spain) with L-glutamine and without sodium bicarbonate was used. The medium was buffered to a pH 7.0 at 25°C with 0.165 M morpholinepropanesulfonic acid (Sigma, Barcelona, Spain). Sterility control of each bottle was performed prior to use in each laboratory.

Antifungal agents. ITC (Janssen Research Foundation, Beerse, Belgium), CLT (Química Farmaceútica, Bayer, Barcelona, Spain), and TRB (Novartis, Basel, Switzerland) were provided by the manufacturers as standard powders. All drugs were dissolved in 100% dimethyl sulfoxide to obtain stock solutions of 1,600 μg/ml for ITC and CLT and of 4,000 μg/ml for TRB. Drug dilutions were

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performed by an additive twofold drug dilution scheme described in the NCCLS reference method (25) at 100 times the strength of the final test concentration, followed by further dilutions (1:50) in RPMI medium to yield twice the final strength required for the test. The final drug concentrations were 16 to 0.01 μg/ml for ITC and CLT and 4 to 0.0039 μg/ml for TRB.

Preparation of inocula. Preparation of inoculum suspensions was based mainly on the NCCLS guidelines (25) and described previously (10). The isolates were subcultured onto potato dextrose agar (PDA) plates at 28°C. Stock inoculum suspensions of each isolate were prepared for each experiment from 7- to 14-day-old cultures grown on PDA. The fungal colonies were covered with ca. 10 ml of distilled water, and suspensions were made by gently probing the surface with the tip of a Pasteur pipette. The resulting mixture of conidia and hyphal fragments was withdrawn and transferred to a sterile tube. Heavy particles were allowed to settle for 5 to 20 min, and the upper homogeneous suspensions were collected and mixed with a vortex mixer. The densities of these suspensions were adjusted with a spectrophotometer at a wavelength of 530 nm to obtain two types of standardized inocula: (i) 80 to 85% transmission (T) and (ii) 65 to 70% T. These stock suspensions were diluted 1:50 in RPMI medium to obtain the final inoculum sizes, which ranged from 2×10^3 to 6.8×10^3 CFU/ml (low inoculum) and from 1.2×10^4 to 6×10^4 CFU/ml (high inoculum), respectively. Inoculum quantification was performed in each laboratory by plating 0.01 ml of a 1:100 dilution of the adjusted inoculum on PDA plates. The plates were incubated at 28°C and were examined daily for the presence of fungal colonies. Colonies were counted as CFU/milliliter when growth became visible.

Test procedure. The broth microdilution tests were performed according mainly to the NCCLS guidelines for testing filamentous fungi (25), and as we described in a previous study (10). Microdilution plates (96 U-shaped wells; Izasa, Barcelona, Spain) were prepared and frozen at −70°C in each laboratory until needed. Rows 2 to 12 contained the series of drug dilutions in 100-μl volumes, and row 1 contained 100 μl of drug-free medium, which served as the growth control. Each well was inoculated on the day of the test with 100 μl of the corresponding inoculum. This step brought the drug dilutions and inoculum size to the final test concentrations given above.

Incubation and determination of MIC endpoints. The microplates were incubated at 28 and 37°C and were read at 3, 7, and 14 days of incubation. The MICs were determined by visual inspection of the growth inhibition of each well compared with that of the growth control (drug-free) well. Two MIC endpoints were determined for each isolate and testing condition. The wells were given a numerical score as follows: (i) 0, optically clear or 100% inhibition of growth (designated MIC-0); and (ii) 2, ca. 50% reduction in growth (designated MIC-2).

Analysis of the results. All of the MICs were repeated three times in each laboratory, and the modal or median (when the three values were different) MIC of the three values was considered for agreement determination. Discrepancies of no more than two dilutions between the modal or median MICs obtained in the three laboratories were used to obtain the percent values of agreement. The percentage of MIC endpoints within two dilutions (e.g., 0.25, 0.5, and 1 μg/ml would be considered in agreement, and 0.25, 0.5, and 2 μg/ml would not) for each combination of isolate, drug and testing condition was determined. The differences were calculated between the two percentages of agreement for each variable (inoculum, temperature, and endpoint criteria) and with each drug-isolate pair. Comparisons of proportions were performed by chi-square test or Fisher exact test as appropriate. A two-tailed P value of < 0.05 was considered to be significant.

The intralaboratory agreement was evaluated for each combination of isolate, drug, and testing condition. A total of 60 triplicate MICs were obtained in each laboratory for each combination, i.e., 180 triplicate MICs (540 values in total). MICs were considered to be in agreement when the differences between the highest and the lowest values (of the three values) were not greater than two dilutions (example given above).

RESULTS

All isolates produced clearly detectable growth only after 7 days of incubation. Therefore, the first determination of MIC endpoints was only possible after that time for all testing conditions.

Inoculum reproducibility. A total of 540 inoculum preparations from the three laboratories, for each of the two inoculum sizes, were analyzed. The CFU/ml ranges for 90% of the inoculum size values were of 2×10^3 to 5.9×10^3 CFU/ml for the low inoculum (80 to 85% T) and of 2.5×10^4 to 6.8×10^4 CFU/ml for the high inoculum (65 to 70% T). The reproducibilities of the six species for the two inocula (low and high) were: *E. floccosum*, 93.4 and 81.1%; *M. canis*, 85.5% for both; *T. rubrum*, 92.1 and 85.5%; *M. gypseum*, 87 and 98.8%; *T. tonsurans*, 97.8 and 98.8%, and *T. mentagrophytes*, 83.4 and 96.1%, respectively.

TABLE 1. Interlaboratory agreement among three laboratories of results obtained by a broth microdilution method

Drug	Test condition[a]	% Agreement[b] at:			
		28°C		37°C	
		Low	High	Low	High
CLT	MIC-2/7	50	82	60	73
	MIC-2/14	77	82	68	75
	MIC-0/7	55	88	56	73
	MIC-0/14	75	87	67	75
ITC	MIC-2/7	12	60	58	63
	MIC-2/14	42	77	42	64
	MIC-0/7	67	88	20	57
	MIC-0/14	24	47	43	67
TRB	MIC-2/7	100	94	98	97
	MIC-2/14	100	95	93	90
	MIC-0/7	98	100	98	95
	MIC-0/14	100	98	93	93

[a] MIC-2 and MIC-0, 50 and 100% inhibition of growth, respectively; 7 and 14 refer to the days of incubation.
[b] Low, inoculum density of ca. 10^3 CFU/ml; high, inoculum density of ca. 10^4 CFU/ml.

Interlaboratory agreement. We analyzed a total of 8,640 modal or median MICs for the 60 isolates in each center or a total of 25,920 values across the species from the three laboratories. The percentages of interlaboratory agreement are summarized in Table 1. The results are stratified by antifungal agents and the three testing conditions.

For CLT, the highest interlaboratory agreements (82 to 88%) were achieved when the high inoculum was used and the microplates were incubated at 28°C. In general, these conditions gave significantly better results than when the low inoculum or an incubation temperature of 37°C was used. At 37°C, no significant differences were observed when the inoculum density, the temperature, or the scoring criteria varied. The only exception to this was when the combination of 7 days and MIC-0 with high inoculum was compared with that at low inoculum (73 versus 56%). At 28°C, significantly higher reproducibility was observed when the higher inoculum was compared with the lower under these the three combination of conditions: MIC-2 and 7 days (82 versus 50%), MIC-0 and 7 days (88 versus 55%), and MIC-0 and 14 days (87 versus 75%). The results also improved at the two scoring criteria MIC-0 and MIC-2 when 14 days were compared with 7 days (77 versus 50% and 75 versus 55%, respectively).

In general the results obtained with ITC were not as good as with the other two drugs. The best reproducibility achieved (88%) was when the following testing conditions coincided: high inoculum, MIC-0, 7 days of incubation, and 28°C. With this drug the worst reproducibility (12%) was achieved, with the low inoculum, MIC-2 and 28°C. In general, MICs obtained with the high inoculum provided higher levels of agreement

TABLE 2. Intralaboratory agreement among three laboratories of results obtained by a broth microdilution method

Drug	Test condition[a]	% Agreement[b] at:			
		28°C		37°C	
		Low	High	Low	High
CLT	MIC-2/7	94	97	93	94
	MIC-2/14	95	97	88	96
	MIC-0/7	94	95	93	93
	MIC-0/14	94	97	89	96
ITC	MIC-2/7	87	93	88	90
	MIC-2/14	93	94	88	90
	MIC-0/7	91	94	87	92
	MIC-0/14	93	96	88	85
TRB	MIC-2/7	100	100	100	100
	MIC-2/14	99	100	100	100
	MIC-0/7	100	100	100	100
	MIC-0/14	100	100	100	98

[a] MIC-2 and MIC-0, 50 and 100% inhibition of growth, respectively; 7 and 14 refer to the days of incubation.
[b] Low, inoculum density of ca. 10^3 CFU/ml; high, inoculum density of ca. 10^4 CFU/ml.

(47 to 88%) than MICs obtained with the low inoculum (12 to 67%) for all testing conditions ($P < 0.05$). The only exception was for MIC-2, 7 days at 37°C, where agreement was similar when both inocula were compared.

Excellent reproducibility (90 to 100%) was observed among the three laboratories with TRB, regardless of the inoculum size, incubation time, temperature, or endpoint criteria. In general, there were no significant differences when the different parameters were evaluated. The only exceptions were the combination of low inoculum and 14 days of incubation, which agreed better at 28°C than at 37°C, and the combination high inoculum, 37°C, and MIC-2, which were more reproducible at 7 days than at 14 days of incubation ($P < 0.05$).

Intralaboratory agreement. The intralaboratory agreement for three drugs against the 60 strains of dermatophytes and for each testing condition are summarized in Table 2. In general, intralaboratory reproducibility was high for the three drugs under all testing conditions, ranging from 87 to 100% of agreement. TRB showed an excellent overall agreement (98 to 100%), whereas azoles showed slightly lower results, from 85 to 96% in the case of ITC and from 88 to 97% in the case of CLT.

Antifungal susceptibility by using optimized conditions. Table 3 summarizes the MICs of the three antifungal drugs against the 60 strains of dermatophytes obtained in the three laboratories under the best conditions (100% growth inhibition criterion [MIC-0], 7 days of incubation, a low inoculum, and a temperature of incubation of 28°C). In general, the three drugs were very active against all of the species tested. Overall, TRB was the most active, showing the lowest geometric mean MIC (0.04 μg/ml). CLT and ITC also showed good antifungal activity, their geometric mean MICs being similar (0.21 and 0.42 μg/ml, respectively).

TABLE 3. Antifungal susceptibility results for 60 strains of dermatophytes obtained in three laboratories under optimal testing conditions

Species	MIC parameter[a]	MIC (μg/ml) for antifungal agent:		
		CLT	ITC	TRB
E. floccosum	Range	0.01–0.5	0.03–1.0	0.01–0.125
	GM	0.14	0.26	0.04
	50%	0.25	0.25	0.03
	90%	0.25	0.5	0.06
M. canis	Range	0.03–0.5	0.06–2	0.01–0.5
	GM	0.11	0.37	0.06
	50%	0.125	0.25	0.06
	90%	0.25	1	0.125
M. gypseum	Range	0.125–2	0.03–2	0.03–0.125
	GM	0.55	0.54	0.06
	50%	0.5	0.5	0.06
	90%	1	1	0.06
T. tonsurans	Range	0.01–2	0.06–1	0.0078–0.06
	GM	0.27	0.46	0.03
	50%	0.25	0.5	0.03
	90%	1	1	0.06
T. mentagrophytes	Range	0.06–2	0.06–2	0.01–0.06
	GM	0.29	0.52	0.03
	50%	0.25	0.5	0.03
	90%	1	2	0.06
T. rubrum	Range	0.03–0.5	0.06–2	0.01–0.06
	GM	0.13	0.42	0.03
	50%	0.125	0.25	0.03
	90%	0.25	1	0.03
All organisms	Range	0.01–2	0.03–2	0.0078–0.5
	GM	0.21	0.42	0.04
	50%	0.25	0.5	0.03
	90%	1	1	0.06

[a] GM, geometric mean; 50 and 90%, MICs at which 50 and 90% of the isolates tested, respectively, are inhibited.

DISCUSSION

The performance of antifungal susceptibility testing for dermatophytes is still technically difficult due to the morphological characters of these fungi. Some of the species, especially the anthropophilic ones, sporulate with difficulty, which is more accentuated after repeated subcultures. When this occurs it is very difficult to prepare conidial inocula. It is important to use culture media that induce abundant fungal sporulation. Recently, Jessup et al. (17) demonstrated that oatmeal and rice agar are good media to enhance sporulation of *T. rubrum.* In the present study we used PDA, also recommended by the NCCLS reference method (25), which in preliminary studies has proven to work better than Sabouraud dextrose agar (data not shown), and in general we have obtained good results. Additionally, these fungi can develop two types of conidia, macro- and microconidia, whose sizes are considerably different. The different proportions of both that can be included in a mixture at a given transmittance can influence enormously the number of CFU/milliliter resulting in complicated inoculum standardization. Because of this, we demonstrated in previous works the lack of correspondence between density and number of particles in different filamentous fungi (16). Here, we have used two inocula measured spectrophotometrically, which allowed us to obtain suspensions with no particularly significant dispersion in the number of particles.

Various procedures for testing dermatophytes have been evaluated by different authors (3, 15, 24), but their results have showed great variability. This is likely due to the lack of standardization of different parameters that can influence MICs determination, such as inoculum (14), length and temperature of incubation (7, 20, 21), and endpoint criteria (9). After a general agreement has been achieved in the optimal conditions for in vitro testing of yeasts and some important filamentous fungi (8, 13), those for dermatophytes need to be defined. In spite of the numerous studies devoted to these fungi, there are no data reported as a result of collaborative studies. To our knowledge, the present study represents the first interlaboratory evaluation of dermatophytes testing. In general, we have followed the recommendations of the NCCLS for testing filamentous fungi (25). These include the use of a broth microdilution method, mainly the same drug dilution schema, buffer, and culture medium; the inocula were also standardized, as was mentioned earlier, by a spectrophotometric method, and the same visual evaluation of MIC readings were used. However, in the present work we have also evaluated other drugs (such as TRB and CLT), other parameters (such as incubation temperatures of 28 and 37°C), and two endpoint criteria to define MIC azoles. Previous studies have demonstrated that buffered RPMI 1640 medium allows adequate growth of filamentous fungi, including dermatophytes (9, 22, 27). Our study has also confirmed this because this medium produced a suitable visible growth for all strains tested. It is well known that the inoculum size exerts a great influence on the MICs (14). In the present study, we compared two inoculum densities, i.e., that proposed by the NCCLS method (10^4 CFU/ml) and a lower one (10^3 CFU/ml) that we used in a previous study, testing an important number of strains of *T. rubrum* (11). The MICs of ITC and CLT obtained in the present study with the highest inoculum clearly showed a better reproducibility than those obtained with the low inoculum, whereas with TRB no differences were observed. Our results agree with those of Norris et al. (27), who, also testing dermatophytes, demonstrated that inoculum size did not affect TRB MICs. The fact that the inoculum size can influence the MICs of some drugs but not others has also been reported by other authors, e.g., Gehrt et al. (14), testing filamentous fungi, observed a significant effect of inoculum density on ITC and flucytosine MICs but not on amphotericin B and miconazole MICs. It seems that the effect of inoculum size MICs for filamentous fungi is dependent upon the antifungal agent tested, and it may be related to the mechanisms of the action of the antifungal agent.

It is well known that the majority of dermatophyte species show an optimal growth between 4 and 15 days of incubation at 28 to 30°C (5). However, various authors have proposed higher temperatures such as 35 or 37°C and different times of incubation, ranging from 3 to 20 days, for testing these fungi (2, 3, 27, 28). In one of these studies, it was proven that the optimal time of incubation depended on the species tested (2), (from 6 to 10 days for *Microsporum* spp., *E. floccosum*, and *T. mentagrophytes* and from 15 to 20 days for *T. rubrum* and *T. verrucosum*). Interestingly, Perea et al. (28) also demonstrated, by using a macrodilution method for testing dermatophytes, that the time of incubation required to obtain adequate growth depended on the dissolvent of the drugs. For example, when distilled water was used, the time of incubation was shorter (48 to 72 h) than when polyethylene glycol was used (10 to 14 days). In our study, a higher reproducibility was achieved for CLT and ITC at 7 days rather than at 14 days and at 28°C rather than at 37°C, whereas for TRB the temperature and the time of incubation did not significantly influence MICs. The NCCLS document (25) recommends a 50% inhibition to define azole MICs. However, recently, Espinel-Ingroff et al. (9) demonstrated that a more stringent MIC determination criterion (MIC-0 or 100% inhibition) can differentiate between susceptible and resistant *Aspergillus* isolates to ITC or to other investigational drugs, i.e., posaconazole, ravuconazole, and voriconazole. Also, other collaborative studies have showed an excellent interlaboratory agreement with this criterion of reading (6, 7, 22). In our case, higher reproducibility was obtained when MICs were scored as MIC-0 than as MIC-2. This was more evident with azoles, and especially with ITC, when a high inoculum at 28°C and 7 days of incubation was assayed. In the present study, the reproducibility of azoles, under certain conditions, was low. This agrees with several collaborative studies testing yeasts and filamentous fungi, which demonstrated that azole MICs were extremely variable, being enormously influenced by testing conditions (13). Similar results were obtained in a multicenter study performed by Espinel-Ingroff et al. (8), who, testing filamentous fungi, found the lowest intra- and interlaboratory agreement for ITC (59 to 79% and 59 to 91%, respectively). Although several studies have been published on the in vitro susceptibility of the three drugs tested here (15, 17, 18, 24, 27), it is difficult to compare results due to variability in the different methods and conditions under which they were performed. We observed in general that TRB was more active than CLT and ITC. This agrees with previous reports, which showed the efficacy in vitro of this allylamine (3, 17, 18). However, for ITC we obtained MICs that were within the range of expected concentrations in nail with daily oral doses of 100 to 200 mg (4, 19). Similar results were obtained by Korting et al. (18), who tested numerous isolates of *T. rubrum* and *T. mentagrophytes* from patients with tinea unguium, also by a microdilution method.

In conclusion, we propose as optimal conditions for evaluating the in vitro antifungal susceptibility of dermatophytes an incubation time of 7 days, a temperature of 28°C, an inoculum of ca. 10^4 CFU/ml, and an MIC endpoint of 100% growth inhibition. However, further studies are needed in order to better define these conditions, especially those of the azoles. Additionally, MICs obtained need to be correlated with clinical outcome to demonstrate the true value of these data.

ACKNOWLEDGMENTS

We thank Aranzazu Moreno for her outstanding technical assistance.

This study was supported by the Fundació Ciència i Salut.

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