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Evaluation of Gradient Concentration Strips for Detection of Terbinafine Resistance in *Trichophyton* spp.

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ABSTRACT The number of dermatophytosis cases resistant to terbinafine is increasing all over the world. Therefore, there is a need for antifungal susceptibility testing of dermatophytes for better management of the patients. In the present study, we have evaluated a gradient test (GT) method for testing the susceptibility of dermatophytes to terbinafine. MIC values to terbinafine determined by the EUCAST reference technique and by gradient test were compared for 79 *Trichophyton* spp. isolates. Overall, MICs were lower with gradient test (MIC₅₀ of 0.002 μ g/mL) than with EUCAST (MIC₅₀ of 0.016 μ g/mL). Good categorical agreement (>90%) between the 2 techniques was obtained but the essential agreement was variable depending on the batch of gradient test.

KEYWORDS EUCAST, terbinafine, *Trichophyton*, antifungals, Etest, antifungal susceptibility testing

Dermatophytosis is the most common superficial fungal infection with an estimated 1 billion cases in the world (1). *Trichophyton rubrum* and *Trichophyton mentagrophytes/Trichophyton interdigitale* are the major etiological agents of dermatophytosis of skin and nails in humans. Their incidence varies according to geographical regions. Terbinafine is the main molecule used to treat this type of infection (2). In recent years, a high incidence of chronic infections, reinfections, and treatment failures due to a newly described species, *Trichophyton indotineae*, has been reported in India (3, 4). These infections represent a public health problem in this country (5, 6) where an important proportion (up to 72%) of *T. indotineae* isolates are terbinafine-resistant (3, 7). More recently, terbinafine resistance has also been reported in Europe and other parts of the world both in *T. indotineae* (8–11) as well as in other dermatophyte species (3, 12–15).

Until now, the monitoring of dermatophyte susceptibility to antifungals was rarely performed due to the lack of standardized *in vitro* tests. Since then, an *in vitro* technique, specific for dermatophytes, has been standardized by the European Committee for

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Antimicrobial Susceptibility Testing (EUCAST) to test terbinafine and other antifungals (16, 17). Nevertheless, the EUCAST reference techniques are time-consuming and there is a need for ready-to-use techniques more adapted to the routine in clinical microbiology laboratories. For terbinafine, a gradient concentration strip method is commercially available (Terbinafine Ezy MIC Strip, HiMedia), but, to our knowledge, it has never been evaluated.

Therefore, we conducted a study to compare terbinafine susceptibility of *Trichophyton* spp. testing by the gradient test (GT) method and the EUCAST standardized method.

RESULTS

Terbinafine MIC results against the 79 *Trichophyton* isolates are summarized in Table 1. EUCAST MIC values ranged from 0.008 to 0.0625 $\mu\text{g}/\text{mL}$ (geometric means [GM] of 0.015 $\mu\text{g}/\text{mL}$) and from 4 to 16 $\mu\text{g}/\text{mL}$ (GM of 12.4 $\mu\text{g}/\text{mL}$) for susceptible and resistant isolates, respectively. Gradient test MIC values ranged from 0.002 to 0.03 $\mu\text{g}/\text{mL}$ (GM of 0.003 $\mu\text{g}/\text{mL}$) and from 0.125 to 64 (GM of 4 $\mu\text{g}/\text{mL}$) for susceptible and resistant isolates, respectively. Overall, MICs were lower with the gradient test than with EUCAST. Examples of gradient test inhibition pattern for susceptible and resistant isolates are presented in Fig. 1.

Agreement analysis between gradient test and EUCAST is presented in Table 2. There were 45/79 (57.0%) isolates with $>2 \log_2$ dilutions difference between EUCAST and the gradient test. The number of isolates with differences of 3 and 4 \log_2 dilutions were 33/79 (41.8%) and 9/79 (11.4%), respectively (Table S1). The results obtained were different depending on the gradient test batch used. The essential agreement (EA) (at $\pm 2 \log_2$ dilutions) for the first batch of gradient test was lower (43.0%) than for the second (97.1%). When comparing results from both batches, MICs obtained with batch A were lower than those obtained with batch B. These results are presented in Fig. 2. Nevertheless, results from both batches were within $\pm 2 \log_2$ dilutions in 85.5% of the cases.

We also compared MICs obtained on MH methylene blue agar and RPMI 1640 agar for 24 isolates. Overall, the results were similar with the 2 media: 100% of the MICs were within ± 2 dilutions and 96% were within ± 1 dilution. For the few strains for which there was a difference between the 2 media, MICs determined in RPMI were higher than in MH.

In contrast, EUCAST results were reproducible with 100% agreement at $\pm 2 \log_2$ dilutions between results obtained for the 2 runs. Categorical agreement (CA) between EUCAST and gradient test was 98.7% (Table 2). Only 1 out of the 11 resistant strains was missed by the gradient test (Table 1). The batch had only a marginal effect on the CA as it was 98.7% and 98.5% for batch A and batch B, respectively.

DISCUSSION

The emergence of terbinafine resistance all over the world has complicated the treatment of dermatophytosis. Because of this, it has become necessary to perform antifungal susceptibility testing of dermatophytes isolates particularly in case of extensive dermatophytosis and/or in patients coming from regions with high rate of resistance (1).

The recommended technique for antifungal susceptibility testing of dermatophytes is the standardized technique of EUCAST microdilution broth method. Nevertheless, EUCAST is not well adapted for routine laboratories because the technique is time-consuming and requires significant training of qualified personnel. An easier usable method would be of great interest. Recently, a screening method with agar supplemented with terbinafine has been proposed but is not yet commercially available (3, 18).

To our knowledge, only 1 commercialized technique is available (gradient test HiMedia) but has never been evaluated. Therefore, we performed the evaluation of this method with a collection of well characterized isolates of *T. interdigitale*, *T. mentagrophytes*, and *T. indotineae*. In the present study, lower MICs were obtained with gradient test compared to EUCAST although the incubation time was the same for the 2 techniques. Initially, a poor EA of 43.0% was obtained with the first batch of the gradient test. Nevertheless, with the second batch, the EA was higher at 97.1%. Despite these discrepancies, resistance was

TABLE 1 MIC results of terbinafine against the 79 *Trichophyton* isolates^a

Isolate	Species	SQLE	MIC values ($\mu\text{g/mL}$)		Interpretation EUCAST/GT
			EUCAST ^a	GT ^{a,b}	
AVC 49	<i>T. interdigitale</i>	ND ^d	0.016	0.002	S/S ^c
AVC 56	<i>T. interdigitale</i>	ND	0.03	0.002	S/S
AVC 92	<i>T. indotineae</i>	ND	0.03	0.008	S/S
AVC 95	<i>T. interdigitale</i>	ND	0.03	0.004	S/S
AVC 97	<i>T. interdigitale</i>	ND	0.008	0.002	S/S
BCL 101	<i>T. interdigitale</i>	ND	0.03	0.004	S/S
BCL 103	<i>T. interdigitale</i>	ND	0.016	0.002	S/S
BCL 105	<i>T. interdigitale</i>	ND	0.06	0.002	S/S
BCL 106	<i>T. interdigitale</i>	ND	0.016	0.002	S/S
BCL 108	<i>T. interdigitale</i>	ND	0.008	0.002	S/S
BCL 109	<i>T. interdigitale</i>	ND	0.03	0.002	S/S
BCL 110	<i>T. interdigitale</i>	ND	0.03	0.002	S/S
BCL 113	<i>T. interdigitale</i>	ND	0.03	0.016	S/S
BCL 117	<i>T. interdigitale</i>	ND	0.016	0.002	S/S
BCL 120	<i>T. indotineae</i>	ND	0.03	0.002	S/S
BCL 129	<i>T. interdigitale</i>	ND	0.016	0.002	S/S
BCL 136	<i>T. interdigitale</i>	ND	0.008	0.002	S/S
BCL 140	<i>T. interdigitale</i>	ND	0.016	0.002	S/S
BCL 141	<i>T. interdigitale</i>	ND	0.016	0.002	S/S
BCL 146	<i>T. interdigitale</i>	ND	0.016	0.002	S/S
BCL 170	<i>T. interdigitale</i>	ND	0.016	0.004	S/S
BCL 173	<i>T. interdigitale</i>	ND	0.06	0.03	S/S
BCL 22	<i>T. interdigitale</i>	ND	0.016	0.002	S/S
BCL 38	<i>T. interdigitale</i>	ND	0.008	0.002	S/S
BCL 51	<i>T. interdigitale</i>	ND	0.016	0.002	S/S
BCL 53	<i>T. interdigitale</i>	ND	0.016	0.002	S/S
BCL 63	<i>T. interdigitale</i>	ND	0.016	0.002	S/S
BCL 85	<i>T. interdigitale</i>	ND	0.016	0.002	S/S
BCL 98	<i>T. interdigitale</i>	ND	0.016	0.002	S/S
BCL 99	<i>T. interdigitale</i>	ND	0.008	0.002	S/S
HMD 124	<i>T. interdigitale</i>	ND	0.016	0.002	S/S
HMD 75	<i>T. mentagrophytes</i>	ND	0.03	0.016	S/S
HMD 79	<i>T. interdigitale</i>	ND	0.03	0.002	S/S
PSL 14	<i>T. interdigitale</i>	ND	0.016	0.002	S/S
PSL 20	<i>T. interdigitale</i>	ND	0.016	0.002	S/S
PSL 22	<i>T. interdigitale</i>	ND	0.016	0.002	S/S
PSL 41	<i>T. indotineae</i>	ND	0.008	0.002	S/S
PSL 42	<i>T. interdigitale</i>	ND	0.016	0.002	S/S
VPCI 1979/P/16	<i>T. indotineae</i>	ND	0.03	0.002	S/S
BCL 115	<i>T. interdigitale</i>	ND	0.008	0.002	S/S
SAT 67	<i>T. interdigitale</i>	ND	0.016	0.002	S/S
PSL 53	<i>T. interdigitale</i>	ND	0.016	0.002	S/S
SAT 14	<i>T. interdigitale</i>	ND	0.03	0.002	S/S
SAT 28	<i>T. interdigitale</i>	ND	0.008	0.002	S/S
SAT 43	<i>T. interdigitale</i>	ND	0.008	0.002	S/S
SAT 51	<i>T. interdigitale</i>	ND	0.016	0.002	S/S
SAT 52	<i>T. interdigitale</i>	ND	0.06	0.002	S/S
SAT 54	<i>T. interdigitale</i>	ND	0.03	0.002	S/S
SAT 56	<i>T. interdigitale</i>	ND	0.016	0.002	S/S
BCL 99	<i>T. interdigitale</i>	ND	0.008	0.002	S/S
AVC 45	<i>T. interdigitale</i>	ND	0.008	0.002	S/S
BCL 12	<i>T. interdigitale</i>	ND	0.008	0.002	S/S
BCL 75	<i>T. indotineae</i>	ND	0.06	0.008	S/S
HMD 117	<i>T. interdigitale</i>	ND	0.03	0.002	S/S
BCL 40	<i>T. interdigitale</i>	ND	0.016	0.002	S/S
AVC 23	<i>T. interdigitale</i>	ND	0.008	0.002	S/S
HMD 44	<i>T. interdigitale</i>	ND	0.008	0.002	S/S
BCL 125	<i>T. interdigitale</i>	ND	0.008	0.002	S/S
AVC 28	<i>T. interdigitale</i>	ND	0.008	0.002	S/S
BCL 44	<i>T. interdigitale</i>	ND	0.008	0.002	S/S
BCL 143	<i>T. interdigitale</i>	ND	0.008	0.002	S/S

(Continued on next page)

TABLE 1 (Continued)

Isolate	Species	SQLE	MIC values ($\mu\text{g/mL}$)		Interpretation EUCAST/GT
			EUCAST ^a	GT ^{a,b}	
BCL 145	<i>T. interdigitale</i>	ND	0.008	0.002	S/S
BCL 93	<i>T. interdigitale</i>	ND	0.008	0.002	S/S
HMD 22	<i>T. interdigitale</i>	ND	0.008	0.002	S/S
BCL 81	<i>T. interdigitale</i>	ND	0.008	0.016	S/S
BCL 55	<i>T. interdigitale</i>	ND	0.008	0.002	S/S
BCL 23	<i>T. interdigitale</i>	ND	0.008	0.004	S/S
PSL 40	<i>T. indotineae</i>	ND	0.016	0.002	S/S
HMD 38	<i>T. indotineae</i>	L393S	4	1	R/R ^e
BOD 1	<i>T. indotineae</i>	F397L/A448T	16	4	R/R
BCL 90	<i>T. interdigitale</i>	F397L	4	0.125	R/S
SAT 005	<i>T. indotineae</i>	F397L	16	2	R/R
VPCI 1032/P/14	<i>T. indotineae</i>	L393F	16	2	R/R
VPCI 1242/P/16	<i>T. indotineae</i>	L393F	16	64	R/R
VPCI 976/P/15	<i>T. indotineae</i>	L393F	16	2	R/R
VPCI 2110/P/16	<i>T. indotineae</i>	L393F	16	2	R/R
VPCI 2004/P/16	<i>T. indotineae</i>	F397L	16	64	R/R
VPCI 1983/P/16	<i>T. indotineae</i>	F397L	16	2	R/R
VPCI 2452/P/16	<i>T. indotineae</i>	ND	16	64	R/R

^aMICs were determined twice, the results in the Table are from run 2 for EUCAST and from batch A for gradient test. SQLE, Squalene Epoxidase gene

^bGT: Gradient test.

^cS: Susceptible.

^dND: not determined.

^eR: Resistant.

correctly detected by both batches of the gradient test as demonstrated by a good CA of > 98%.

The major limitation of our study is the limited number of batches for the gradient test that were tested compared with the reference EUCAST method. Variations between batches highlights the necessity to systematically include quality control strains. Further studies are needed to test a larger data set of isolates and it will be important to perform a multicenter study including more isolates with low level of resistance. We choose to

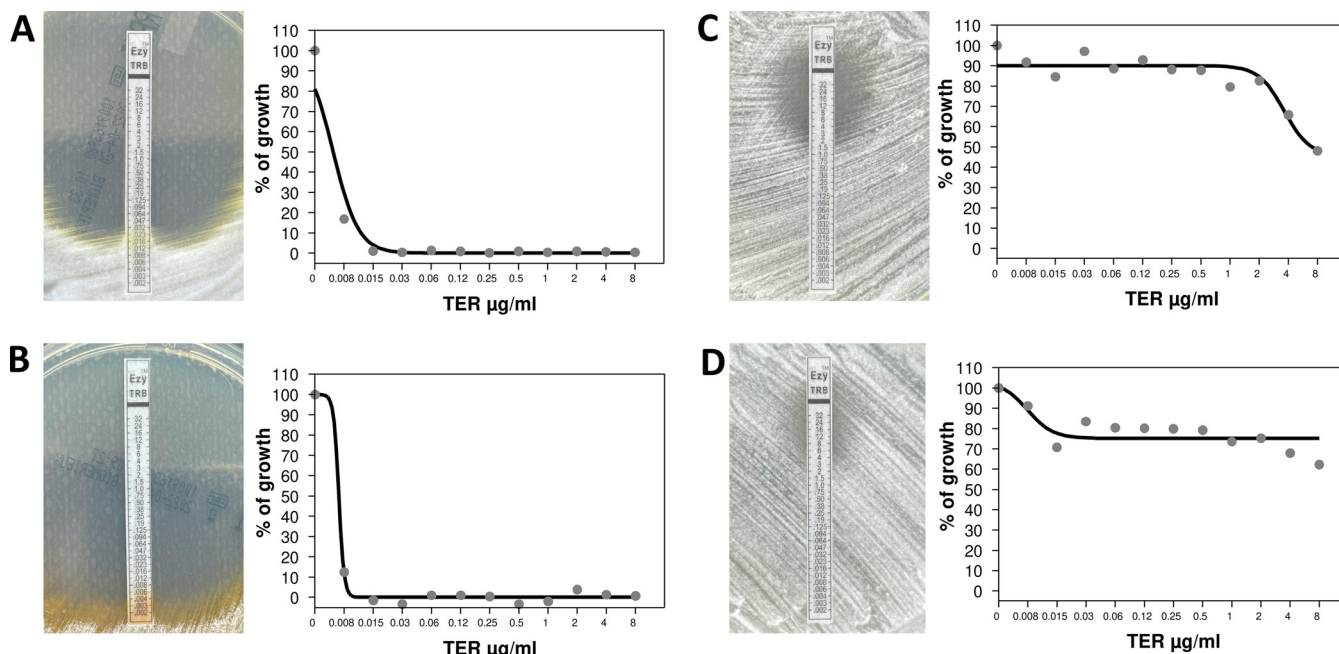


FIG 1 Examples of inhibition patterns obtained with gradient test (left panels) and EUCAST (right panels) for terbinafine-susceptible isolates BCL 173 (A) and PSL 42 (B) and for terbinafine-resistant isolates VPCI 1983/P/16 (C) and BOD 1 (D). Inhibition curves were obtained by non-linear curve fitting. Dots represent experimental values.

TABLE 2 Essential agreement (EA) and categorical agreement (CA) between EUCAST and the two different batches of gradient test (GT)

GT batch	EA (%)		CA (%)
	± 1 dilution	± 2 dilutions	
A	6.3%	43.0%	98.7%
B	70.6%	97.1%	98.5%

use RPMI to be consistent with the EUCAST methodology. As the medium could be an important parameter, we compared both media (RPMI and MH) for the gradient test. The results showed that the MICs were similar in both media with even a trend to lower MICs in MH. Then, the use of RPMI instead of MH could not explain the difference observed between the gradient test and EUCAST.

Conclusion. The gradient test can detect resistance to terbinafine and a good CA between the gradient test and EUCAST was obtained. Nevertheless, there were variations of MIC results depending on the batch of gradient test. The gradient test could be used as a screening method, but the results must be confirmed with the EUCAST reference method.

MATERIALS AND METHODS

Isolates. A panel of 79 molecularly identified isolates of *T. interdigitale* ($n = 62$), *T. mentagrophytes* ($n = 1$), and *T. indotineae* ($n = 16$) were used. Their identifications at the species and genotype levels were confirmed by sequencing of the ITS gene (19). The panel included 68 terbinafine-susceptible isolates and 11 terbinafine-resistant isolates for which the squalene epoxidase gene was sequenced (19). Among the resistant isolates, 7 were from India and 4 from France, and the amino acid substitutions, performed in 10 isolates, identified Leu393Phe ($n = 4$), Leu393Ser ($n = 1$), Phe397Leu ($n = 4$), and Phe397Leu/Ala448Thr ($n = 1$) as a source of terbinafine resistance.

The isolates were subcultured from frozen stocks on Sabouraud dextrose agar slants supplemented with chloramphenicol and cycloheximide (Bio-Rad) for 5 days at 25°C to ensure purity and viability.

The reference strains *Aspergillus flavus* ATCC 204304 and *Trichophyton interdigitale* SSI-9363 were included as quality controls.

EUCAST method. MICs of terbinafine were determined following the EUCAST microdilution broth method for dermatophytes with minor modifications (17).

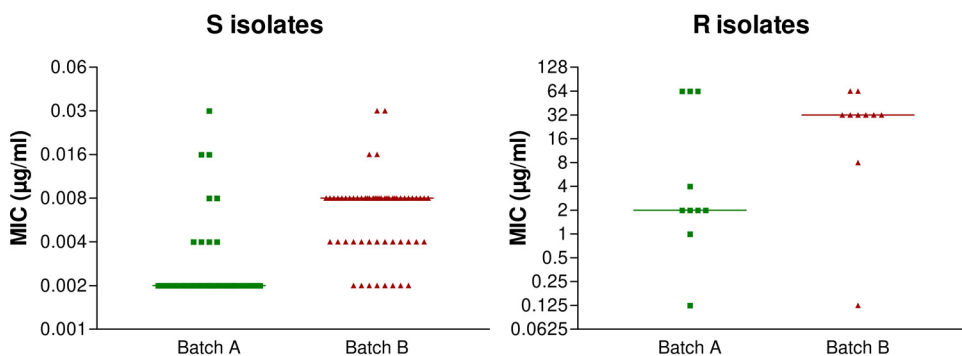
Medium preparation. For this study, Roswell Park Memorial Institute 1640 (RPMI) (with L-glutamine, with pH indicator, but without bicarbonate) (Sigma) prepared in double strength was used as the test medium. It contained 2% of D-glucose and was buffered with 3-(N-morpholino) propanesulfonic acid (VWR) at a final concentration of 0.165 mol/L. The final pH of 7.0 was adjusted with 1 molar sodium hydroxide (NaOH).

Drugs and microplate preparation. Terbinafine (Sigma) stock solution was prepared at 1600 µg/mL in dimethyl sulfoxide (DMSO).

The final concentration was 8 to 0.008 µg/mL.

Inoculum preparation and inoculation of microplates. Before the experiments, isolates were subcultured a second time on Sabouraud agar slants supplemented with chloramphenicol and cycloheximide (Bio-Rad) for 5 days at 25°C. Conidia suspension was counted in a hemocytometer and adjusted to 2 to 5 × 10⁶ conidia/mL. After a 1/10 dilution in water, inoculum was supplemented with cycloheximide (100 mg/mL) and chloramphenicol (50 mg/mL), and each well of the plate was inoculated with 100 µL of the spore suspension resulting in a final inoculum size of 1 to 2.5 × 10⁵ CFU/mL.

Incubation of microdilution plates and reading results. Plates were incubated at 25°C for 5 days and read spectrophotometrically at 550 nm with a 90% growth inhibition endpoint instead of the 50%

**FIG 2** Comparison of MICs obtained with batch A and batch B of the gradient test for susceptible and resistant isolates.

inhibition endpoint recommended by EUCAST. It was shown that both endpoints were comparable except for itraconazole for which trailing complicated 90% spectrophotometric inhibition readings. Experiments were performed twice.

Gradient test method. Terbinafine MICs were also determined using gradient test (Terbinafine Ezy MIC Strip, HiMedia) with a MIC range of 0.002 $\mu\text{g}/\text{mL}$ to 32 $\mu\text{g}/\text{mL}$. The medium used was RPMI instead of Mueller-Hinton (MH) supplemented with methylene blue which is the medium recommended by the manufacturer (Hi Media). RPMI agar plates (bioMérieux) were inoculated with the same conidia suspension prepared for the EUCAST method, and strips of terbinafine were placed on the agar. MICs were determined after 5 days of incubation at 25°C. After incubation, MICs were read by using a complete inhibition endpoint. Overgrowth into the ellipse was ignored.

Experiments were performed twice. A comparison between 2 different batches of the gradient test was performed for 68 isolates. A comparison of the 2 media (RPMI and MH) was also performed for 24 isolates with the same inoculum and same batch of gradient test.

Interpretation of results. For analysis, MICs obtained with gradient test were rounded up to the next 2-fold dilution of the EUCAST concentration scale. Results of the 2 methods were analyzed by providing EA values within ± 2 dilution steps. EA within ± 1 dilution step was also calculated. CA was calculated. Isolates were considered susceptible when MIC was $\leq 0.125 \mu\text{g}/\text{mL}$ which is the tentative ECOFF from EUCAST.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, DOCX file, 0.01 MB.

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