

Evaluation of the disk-diffusion method to determine the in vitro efficacy of terbinafine against subcutaneous and superficial mycoses agents

Avaliação do método de disco-difusão para determinação da eficácia da terbinafina in vitro em agentes de micoses superficiais e subcutâneas*

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Abstract: BACKGROUND: Superficial and subcutaneous mycoses have a high prevalence and, often, chronic evolution. Therefore, they need extensive treatment with topic and/or systemic antifungal agents. Azoles and allylamines (terbinafine) are first-choice drugs to treat human and animal infections. Thus, evaluation of the efficacy of these drugs is important for a successful treatment. However, there are few studies that evaluate the in vitro activity of antifungal agents.

OBJECTIVE - To evaluate the in vitro efficacy of terbinafine activity against filamentous fungi and yeasts that cause mycoses. METHOD - The in vitro activity of terbinafine (0.125-100 μ g) against 10 fungi species was evaluated by the disk-diffusion and microdilution/reference methods to determine the Minimum Inhibitory Concentration (MIC).

RESULTS - We found a high susceptibility to terbinafine in: *T. rubrum*, *M. gypseum*, *T. mentagrophytes*, *T. tonsurans*, *M. canis*, *C. carrionii* and *E. floccosum* (halo \geq 40mm with 0.125 μ g disk). *S. hyalinum* and *C. parapsilosis* were considered susceptible, but less than the others. *Fusarium* spp. showed the lowest susceptibility (halo=12mm with 2 μ g disk; MIC 8 μ g/mL).

CONCLUSIONS - The results of this research confirm previous findings about the efficacy of terbinafine. The drug was shown to be highly effective to treat dermatophyte infections. The disk-diffusion method was easy to use and is a suitable technique for routine use in clinical laboratories.

Keywords: Antifungal agents; Arthrodermataceae; Fungi; Mycosis

Resumo: FUNDAMENTOS: As micoses superficiais e subcutâneas têm alta prevalência e, muitas vezes, caráter crônico, necessitando tratamentos tópicos e/ou sistêmicos com antifúngicos. As drogas de escolha são azóis e alilaminas (terbinafina). É necessário avaliar a eficácia das drogas para tratamento em humanos e em animais. Estudos para avaliar in vitro a ação dos antimicóticos são raros, especialmente, contra fungos filamentosos.

OBJETIVO - Avaliar a eficácia in vitro da terbinafina pelo método de disco-difusão contra fungos filamentosos e leveduras agentes de micoses.

MÉTODOS - Avaliou-se a ação da terbinafina (0,125 μ g-100 μ g) contra dez espécies fúngicas pelos métodos disco-difusão e microdiluição/referência, para determinar a concentração inibitória mínima (MIC).

RESULTADOS - Observou-se alta sensibilidade à terbinafina em: *T. rubrum*, *M. gypseum*, *T. mentagrophytes*, *T. tonsurans*, *M. canis*, *C. carrionii* e *E. floccosum* (halo \geq 40mm com disco de 0,125 μ g). *S. hyalinum* e *C. parapsilosis* foram considerados sensíveis, mas com halos menores. *Fusarium* spp. apresentou menor sensibilidade (halo=12mm com disco de 2 μ g; MIC 8 μ g/mL).

CONCLUSÕES - Os resultados reiteram estudos anteriores quanto à alta eficácia da terbinafina em relação a dermatófitos. A técnica de disco-difusão foi de fácil aplicação e adequada na rotina de laboratórios clínicos.

Palavras-chave: Antimicóticos; Arthrodermataceae; Fungos; Micoses

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INTRODUCTION

Superficial mycoses are frequent in routine medical practice and are mostly caused by filamentous fungi and, less often, yeasts.¹ Oftentimes these infections are not adequately treated with topical antimycotic drugs, which makes systemic treatment necessary.² The discovery of azole antifungal agents, such as ketoconazole, itraconazole, and fluconazole, is a considerable advancement in the treatment of these mycoses.² However, the appearance of resistant fungi established the need to research different therapy alternatives and action sites.³ Terbinafine is among the drugs that have an action mechanism different from that of azoles. It belongs to the family of alilamines, acts on the epoxidase enzyme of the fungal cell and is especially indicated to treat skin infections caused by dermatophytes.⁴ Contrary to imidazoles, terbinafine has an antifungal action.^{1,2,3} Terbinafine is more selective for the fungal cell than amphotericin B, a standard antifungal agent, due to a difference between the epoxidase enzymes of mammals and fungi.⁵ Terbinafine can be administered in combination with other drugs and presents mild toxic effects or adverse reactions.⁶ The most common are gastrointestinal disorders and taste alterations.¹ Rare cases of a positive antinuclear factor have been reported.¹

Evaluation of *in vitro* efficacy is done by reference method, broth dilution or Agar diffusion.^{7,8} In Brazil, Almeida et al. evaluated the susceptibility of fungi that cause superficial mycoses to antifungal agents through the microdilution technique.⁸ Nevertheless, there is no reference methodology for terbinafine and more studies are necessary for its validation. In addition, there is no technique that is easily employed in clinical laboratories to search for the ideal therapy in cases of mycoses caused by filamentous fungi, including dermatophytes, such as those that exist in cases of yeast infections. For the latter, the M44-A (NCCLS, 2004) method is used and is accepted as a reference only for yeasts of the *Candida* species, based on the disk-diffusion technique with fluconazole. The objective of this study was to suggest procedures for the disk-diffusion technique to determine and compare the *in vitro* efficacy of terbinafine and other agents in the treatment of fungi that cause superficial and subcutaneous mycoses.

METHOD

This study was conducted with culture samples of superficial or subcutaneous mycosis agents common in Brazil, obtained from lesions of patients at the Ambulatory of Dermatology. The following fungi were included in the experiment:

Dermatophytes- *Trichophyton rubrum*, *Trichophyton tonsurans*, *Microsporum gypseum*,

Microsporum canis, *Epidermophyton floccosum* (superficial and subcutaneous mycosis agents);

Non-dermatophyte fungi - - *Scytalidium hyalinum*, *Fusarium oxysporum* (onycosis agents) and *Cladophialophora carrionii* (subcutaneous mycosis agent).

Two standard fungal cultures of *Candida parapsilosis* (ATCC 22019) and *Trichophyton mentagrophytes* (ATCC 05533) were included. Clinical samples, isolated up to three years before the study, were stored in distilled water according to a technique standardized in our laboratory.¹⁰ After recovery, they were reidentified by means of modified microcultivation,¹¹ based on morphological analysis.¹²⁻¹³ Novartis Biociencias S.A. provided the terbinafine, in pro-analysis pure powder form (p.a.). Terbinafine disks were prepared in 13 different concentrations, with two-fold serial dilutions, from 0.125mg up to 16mg and from 25mg to 100mg. Disks (CECON – Centro de controle e produtos para diagnosticos Ltda, Brazil) with 9 antifungal agents were used to compare efficacy: 5-fluorocytosine (5 FC), amphotericin B (AB), nistatin (NY), econazole (EC), clotrimazole (CTR), miconazole (MCZ), ketoconazole (KET), fluconazole (FLU), and itraconazole (ICZ).

For sensitivity tests, an inoculant of each fungus sample was prepared by adding to the surface of cultures, incubated for different periods based on the growth demand of filamentous fungi, 5mL of saline solution at 0.85%, with a drop of polysorbate (*Tween* 20, Sigma).^{11,12,13} Inoculants of yeast samples were prepared by suspension of 1-5 colonies (<5 mm diameter) in saline solution at 0.85%. Each suspension of filamentous fungus or yeast was adjusted to contain 1×10^6 a $5,0 \times 10^6$ UFC/mL, initially through a comparison of the turbidity with the 0.5 tube of the McFarland scale.⁹ The concentration was then calculated by three methodologies, as follows:

Transmittance adjustment (68-70%) in spectrophotometer at 530 nm¹³

Hemocytometer counting¹⁴

Colony-forming units (CFUs) count on Sabouraud Agar¹⁵

The disk-diffusion technique was performed according to the recommendations described in the M44-A method.⁹ Two culture media were employed: Muller Hinton agar, containing 2% of methylene blue (MH-GMB) (Difco, USA),¹⁶ and yeast nitrogen agar (YNA)¹⁷ (Difco, USA), which were formulated following instructions from the manufacturers and divided (70 mL) into sealed containers, heated and placed in Petri plates at the time of use. The cultures were distributed in Petri plates (150 x 6 mm) and the inoculants, in the surface. After complete absorption of the

inoculants, 10 disks with various terbinafine concentration levels (0.125 up to 16 mg) were placed in equidistant points. Disks with a higher concentration of terbinafine (25mg, 50mg and 100mg) were also analyzed due to the lower sensitivity of some samples (lack of inhibition with the original lower concentrations). Disks with the other antifungal agents were evaluated based on the same methodology.⁹ Next, plates were incubated upside down at $30 \pm 2^\circ\text{C}$.¹¹ The level of sensitivity to terbinafine and other antifungal agents of each fungus sample was measured by the diameter (mm) of the inhibition zone formed around the disk after 24 to 120 hours of inoculation. Each isolate was classified into: sensitive, intermediate sensitivity, or resistant to the drugs, based on the size of the inhibition zone.¹⁷ With the exception of terbinafine, the classification criteria followed the manufacturer's instructions (CECON Ltda, Brazil). The inhibition zones for terbinafine were expressed in millimeters and the classification of the samples was done only after comparison with the results of the microdilution technique.¹⁸ ATCC standard strains were included in all tests.¹⁹ The criteria to interpret inhibition zones to indicate resistant strains were: 5 FC < 10 mm, AB \leq 10 mm, NY \leq 10 mm, EC10 mm, CTR 10 mm, MCZ 10 mm, KET10 mm, FLU 14mm and ICZ \leq 11mm.

For the microdilution technique, stock solutions ($1600 \mu\text{g/mL}$)²⁰⁻²¹ of terbinafine were prepared in dimethylsulphoxide (DMSO, Sigma, USA). Ten different concentration levels were prepared from this solution, with two-fold dilutions, in RPMI-1640 medium¹⁸ (CULTILAB, Brazil). Tests were prepared in 96 well, flat-bottomed microtitulation plates, according to the M38-A document and recommendations described by Rodriguez-Tudela (2003).¹⁴ Positive and negative growth controls, in addition to standard strains, were included in all tests to assure quality. Test plates were homogenized for 15 minutes at 65 rpm/minute (Klime agitator, Marconi, Brazil) and incubated at $35 \pm 2^\circ\text{C}$ for up to 72 h. After the first 24 hours, visual readings were done to determine the minimum inhibitory concentration (MIC) of terbinafine in relation to each one of the isolates. In cases of unsatisfactory growth, the plate was reincubated and read after 48 and 72 h. Reading of the endpoint in tests with filamentous fungi was done under a mirror, and the minimum inhibitory concentration was the one that resulted in total growth inhibition (IC₁₀₀) of the isolate. For tests with yeasts, a spectrophotometer with a 492 nm filter for an automated reading of the endpoint, established to be 50% (IC₅₀) growth inhibition of the isolate, was used. All tests were duplicated.

RESULTS

The fungi cultures used to evaluate the efficacy of terbinafine showed a high production of spores, being thus suitable to the preparation of inoculants.²² Figure 1 illustrates the aspect of the prepared fungi.

The three different methodologies used for the inoculants were equivalent, with suspensions containing 1 to 5×10^6 UFC/mL. The inhibition zones in MH-GMB agar were larger, on average, than those obtained in YMA, regardless of the isolate. The inhibition zones indicated that 70% of the samples, including all species of dermatophytes, were inhibited with the lowest evaluated concentration (0,125mg) of terbinafine (Figure 2, Table 1). Only three isolates (*Candida parapsilosis*, *Fusarium oxysporum* e *Scytalidium hyalinum*) did not show an inhibition zone under these conditions.

In the experiment with a $2 \mu\text{g/mL}$ disk, we were able to separate isolates with distinct levels of sensitivity, based on inhibition zones:

Sensitive (zone > 40 mm), category in which dermatophytes are included;

Intermediate sensitivity (zone of 24 to 35 mm), which includes *S. hyalinum* and *C. Parapsilosis*;

Resistant (12 mm zone), category to which *Fusarium oxysporum* belongs.

Inhibition zones for the isolates classified as having intermediate sensitivity and being resistant are shown in table 2. MIC results through the broth microdilution method for the three samples least sus-

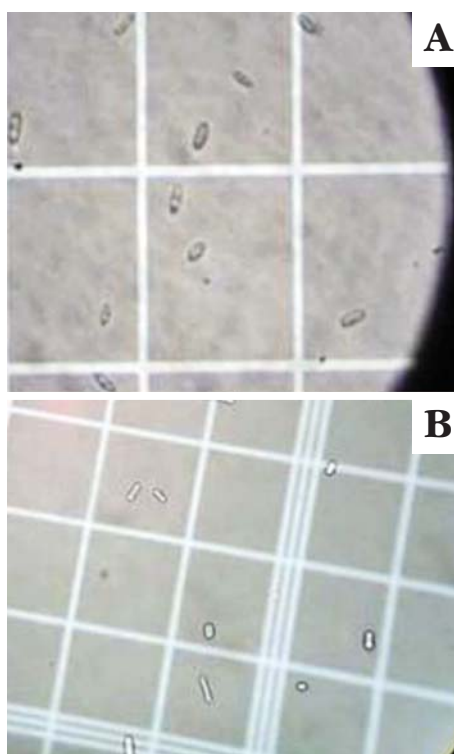


FIGURE 1: A e B. Microscopic aspect of spores (400x) of *Cladophialophora carrionii* and *Trichophyton tonsurans* - Preparation of the inoculant and spore counting on Neubauer chamber

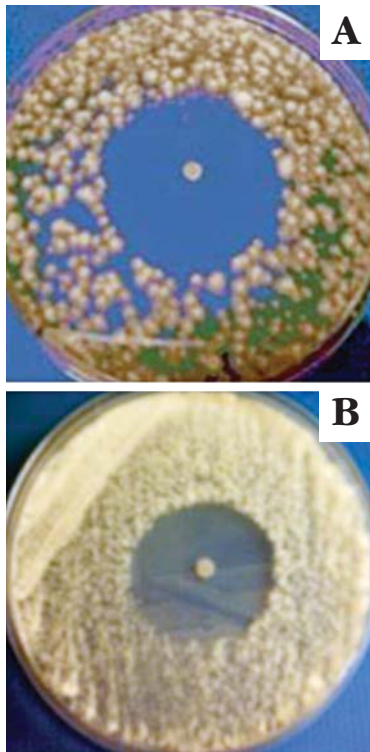


FIGURA 2: A e B. A and B. Petri plates containing dermatophyte cultures showing exaggerated inhibition halos with a lower concentration (0,125 µg) of terbinafine

ceptible to terbinafine were: 0.25mg/mL (*Scytalidium hyalinum*); >8mg/mL (*Fusarium oxysporum*) and 0.5mg/mL for *Candida parapsilosis*. The MIC of terbinafine for *Trichophyton mentagrophytes* was 0.015 mg/mL.

A comparison between the efficacy of terbinafine and nine other antifungal agents by the microdilution and disk-diffusion methods is shown on tables 3 and 4.

DISCUSSION

Clinical isolates were stored in distilled water for three years⁹ with no need for periodic maintenance, thus avoiding contamination. A high production of hyphae-free spores, as recommended for sensitivity tests, was obtained in this manner.²² Inoculants prepared through three different methods also yielded similar results. Hemocytometer spore count was considered the best method because it reduced the biological risk of the passage of the content from the test tube to the glass cuvette of the spectrophotometer, and guaranteed the desired concentration of the filamentous fungi inoculant without interference from color, form, and size of the spores,²³ contrary to what may happen in the spectrophotometer technique.¹⁴ Moreover, the method that uses a hemocytometer is more practical and faster in comparison with the colony forming unit count method, which needs an additional culture procedure.

Another parameter evaluated by the disk-diffusion method was culture medium. The MH-GMB was better because it made the diffusion of terbinafine possible and allowed a larger inhibition zone, as compared with the YMA. Another advantage of the MH-GMB is its availability in microbiology laboratories that perform tests of sensitivity to bacteria. The incubation temperature adopted in this study (30 ± 2°C) resulted in a visible and homogeneous growth; therefore, it was considered suitable to the species studied. Inhibition zones were evident and easily measurable after 24 hours for *C. Parapsilosis* and from 72 to 120 h for other agents, considering the growth time.

Regarding the concentration in terbinafine disks, we noticed that values ≥ 2mg resulted in inhibition zones for all the species. A 2mg disk exposes the

TABLE 1: Diameter of inhibition zones (mm) caused by terbinafine activity against superficial and subcutaneous mycoses agents in the disk-diffusion method

Species	TBF concentration (µg)									
	0,125	0,25	0,5	1,0	2,0	4,0	5,0	8,0	10,0	16,0
<i>C. parapsilosis</i>	Ø	Ø	10	20	24	30	32	38	40	42
<i>C. carrionii</i>	40	≥ 40	≥40	≥40	≥40	≥40	≥40	≥40	≥40	≥40
<i>T. mentagrophytes</i>	40	≥ 40	≥40	≥40	≥40	≥40	≥40	≥40	≥ 40	≥ 40
<i>T. tonsurans</i>	62	≥62	≥62	≥62	≥62	≥62	≥62	≥62	≥62	≥62
<i>T. rubrum</i>	55	≥55	≥55	≥55	≥55	≥55	≥55	≥55	≥55	≥55
<i>M. canis</i>	47	≥47	≥47	≥47	≥47	≥47	≥47	≥47	≥47	≥47
<i>M. gypseum</i>	50	≥50	≥50	≥50	≥50	≥50	≥50	≥50	≥50	≥50
<i>E. floccosum</i>	55	≥55	≥55	≥55	≥55	≥55	≥55	≥55	≥55	≥55
<i>F. oxysporum</i>	Ø	Ø	Ø	8	12	18	20	22	25	27
<i>S. hyalinum</i>	Ø	16	20	29	35	40	41	43	46	48

TABLE 2: Diameter of inhibition zones for resistant isolates or those with intermediate sensitivity to 2µg/mL of terbinafine in two distinct media: MH-GMB (Mueller Hinton Agar + 2% glucose and methylene blue) and YMA (yeast nitrogen Agar)

Agent	Additional TBF concentrations					
	25mg		50mg		100mg	
	MH-GMB	YMA*	MH-GMB	YMA	MH-GMB	YMA
<i>S. byalinum</i>	51	50	56	54	61	59
<i>C. parapsilosis</i>	44	41	46	44	48	46
<i>F. oxysporum</i>	29	25	33	31	38	35

Inhibition zones (mm) of three agents less sensitive to TBF

isolate to concentrations that are equivalent to those found in human serum, thus partly mimicking what occurs *in vivo*.

The 2 mg concentration was considered adequate for the disk-diffusion test since it isolated distinct profiles of susceptibility to terbinafine. In tests with dermatophytes, a fungi group with high sensitivity to terbinafine which produces inhibition zones ≥ 61 mm, it is recommended that only one 2mg disk per Petri plate be used.

This study corroborated previous findings that showed the high efficiency of terbinafine in relation to dermatophytes.²⁴ Inhibition zones ≥ 35 mm were obtained with 2 µg disks, which corresponds to a MIC of 0.03µg/mL in the microdilution method. This MIC value is well below the serum (0.8 to 1.5µg/mL⁶) and tissue dosage established in the developmental stage of the drug.²⁵ This allowed classification of the samples into three categories: sensitive, intermediate sensitivity,

and resistant to terbinafine. Tests with most agents showed large growth inhibition zones, which is in agreement with data from the literature studied.²⁶ It is worth mentioning that, so far, no breakpoint, or interpretation criterion, has been established to designate strains that are resistant to terbinafine. There are no clinical studies in the literature researched that support the *in vitro-in vivo* correlation of fungi resistance to antimycotic agents. Only these types of studies, associated with knowledge about the pharmacodynamics and pharmacokinetics (PK-PD) of the drug, would allow the determination of sensitivity breakpoints.

The results we obtained cannot be compared with those from previous studies in which filamentous fungi with inhibition zones of 25 to 28 mm resulting from disks with 30 µg²⁶ of terbinafine were classified as sensitive. In our study, we observed that disks with concentrations higher than 10 µg would not allow the discrimination of isolates with distinct sensitivity profiles (Table 1).

TABLE 3: Efficacy of TBF and other nine drugs [inhibition halo - mm, (S) Sensitive (R) Resistant]

ANTIFUNGAL AGENTS (µg)	<i>S. byalinum</i>		<i>C. parapsilosis</i>		<i>F. oxysporum</i>	
	MH-GMB	YMA	MH-GMB	YMA	MH-GMB	YMA
AB (100)	55 (S)*	40 (S)	26 (S)	13 (S)	13 (S)	12 (S)
5 FC (1)	Ø (R)*	Ø (R)	Ø	Ø	Ø (R)	Ø (R)
NY (100 U.I.)	61 (S)	55 (S)	36 (S)	23 (S)	20 (S)	13 (S)
CTR (50)	40 (S)	45 (S)	41 (S)	41 (S)	11 (R)	11 (R)
MCZ (50)	42 (S)	43 (S)	28 (S)	32 (S)	Ø (R)	Ø (R)
KET (50)	30 (S)	26 (S)	55 (S)	53 (S)	Ø (R)	Ø (R)
EC (50)	60 (S)	61 (S)	34 (S)	35 (S)	25 (S)	21 (S)
ICZ (10)	Ø (R)	Ø (R)	Ø (R)	Ø (R)	Ø (R)	Ø (R)
5 FC(10)	Ø (R)	Ø (R)	Ø (R)	Ø (R)	Ø (R)	Ø (R)
FLU (25)	24 (S)	33 (S)	28 (S)	22 (S)	Ø (R)	Ø (R)
TBF (25)	51	50	44	41	29	25
TBF (50)	56	54	46	44	33	31
TBF (100)	61	59	48	46	38	35

* Inhibition Zone in mm

TABLE 4: Comparison between the efficacy of terbinafine and nine other antifungal drugs in relation to mycosis-inducing fungi by the disk-diffusion method

Antifungal agents (concentration, °g)	<i>S. hyalinum</i>		<i>C. parapsilosis</i>		<i>F. oxysporum</i>	
	MH-GMB	YMA	MH-GMB	YMA	MH-GMB	YMA
Amphotericin B (100)	55* (S)**	40 (S)	26(S)	13 (S)	13(S)	12
5 fluorocytosine(1)	Ø (R)	Ø (R)	Ø (R)	Ø (R)	Ø (R)	Ø (R)
Nistatin (100 UI)	61 (S)	55 (S)	36(S)	23(S)	20(S)	13 (S)
Clotrimazole (50)	40 (S)	45 (S)	41(S)	41(S)	11(R)	11(R)
Miconazole (50)	42 (S)	43 (S)	28(S)	32(S)	Ø (R)	Ø (R)
ketoconazole (50)	30 (S)	26 (S)	55(S)	53(S)	Ø (R)	Ø (R)
Econazole (50)	60 (S)	61 (S)	34(S)	35(S)	25(S)	21(S)
Itraconazole (10)	Ø (R)	Ø (R)	Ø (R)	Ø (R)	Ø (R)	Ø (R)
5-fluorocytosine (10)	Ø (R)	Ø (R)	Ø (R)	Ø (R)	Ø (R)	Ø (R)
Fluconazole (25)	24 (S)	33 (S)	28(S)	22(S)	Ø (R)	Ø (R)
TBF (25)	51	50	44	41	29	25
TBF (50)	56	54	46	44	33	31
TBF (100)	61	59	48	46	38	35
TBF (100)	61	59	48	46	38	35

*Inhibition halo diameter (mm)

**S: sensitive; I: intermediate sensitivity; R: resistant, UI: international unit

S. hyalinum and *C. parapsilosis* in 2mg of terbinafine showed, respectively, 35 mm and 24 mm inhibition zones, which correspond to a MIC of 0.25 µg/mL and 0.5 µg/mL. These species were classified as having intermediate sensitivity due to the proximity of the MIC to serum values of terbinafine. *Fusarium oxysporum* was considered resistant (zone ≤ 12 mm with a 2 µg/mL disk), based on the results of a MIC of 8µg/mL in microdilution, a value well above the one found in the serum dose. In fact, this species shows low susceptibility to terbinafine, as previously described.²⁷ Concentrations higher than 32µg/mL are necessary to inhibit the growth of *Fusarium* species.²⁸⁻²⁹⁻³⁰ *Fusarium oxysporum* was used in this study as a model to compare the efficacy of the investigated drugs. This species, although resistant, showed larger inhibition zones caused by terbinafine, as compared to the other nine drugs evaluated, when the same concentrations were used.

Species of *Candida*, and other non-dermatophyte agents of interest in Dermatology, have variable sensitivity to terbinafine. Therefore, its susceptibility

profile is not predictable. In these cases, the implementation of trial tests in routine laboratories is recommended to orient treatment.

We conclude that the disk-diffusion method, with the proposed parameters, was easily employed in routine laboratory testing and allowed the identification of clinical isolates with low sensitivity to terbinafine. The concentration of 2mg of terbinafine is indicated for disk-diffusion tests, Mueller-Hinton medium with 2% glucose and methylene blue, incubation at 30°C for a period of 24 to 120 h, according to the fungal species; for tests with dermatophytes, the placement of only one disk per plate and the adoption of *Fusarium oxysporum* as quality control-strain are recommended. Terbinafine *in vitro* was good against dermatophytes, but its action was weaker against yeasts. Future studies are necessary to correlate strains with lower sensitivity to terbinafine *in vitro* and the clinical evolution of cases treated with this drug. The validation of breakpoints for terbinafine and its importance in the clinical prognosis are essential and require additional investigation. □

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