available at www.sciencedirect.com

ScienceDirect

journal homepage: www.elsevier.com/locate/mycres

In vitro methods for antifungal susceptibility testing of *Trichophyton* spp.

*Maria Elisabete da Silva BARROS**, *Daniel de Assis SANTOS*, *Júnia Soares HAMDAN*

Department of Microbiology, Institute of Biological Sciences, Federal University of Minas Gerais, Av. Antônio Carlos, 6627 PO Box 486 CEP: 31.270-901, Belo Horizonte, Minas Gerais, Brazil

ARTICLE INFO

Article history:
Received 8 November 2005
Received in revised form
14 July 2006
Accepted 12 August 2006
Published online 25 October 2006
Corresponding Editor:
Mark Ramsdale

Keywords:
Antifungal drugs
Dermatophytes
Microdilution
Susceptibility testing

ABSTRACT

In general, methods to test the susceptibility of fungi to antifungal drugs require standardized techniques, but so far there is no methodology that is widely applicable to dermatophytes. Here we introduced modifications to the protocols from documents of the National Committee for Clinical Laboratory Standards (CLSI) M38-A and the Antifungal Susceptibility Testing Subcommittee of the European Committee on Antimicrobial Susceptibility Testing (EUCAST) that are usually applied to moulds and fermentative yeasts, in order to adjust the conditions for the growth of dermatophytes. The modifications included: growth on potato dextrose agar supplemented with 2 % in-house rice flour to encourage sporulation, the addition of 2 % glucose to the culture media (RPMI-1640), and an incubation temperature of 28 °C. In addition, the incubation period was 7 d, the minimum inhibitory concentration (MIC) was defined as 80 % growth inhibition endpoints for azole agents, and the inocula only contained microconidia. Results obtained by both tested methodologies were very similar to the ones reported by other researchers. MIC_{90} (MIC at which 90 % of isolates tested were inhibited) values were identical for four out of five antifungal drugs tested and there was only a difference of one or two dilutions when MIC_{50} values were compared. Although the modifications introduced did not interfere with the results, more studies are necessary to establish a standard technique to test susceptibility of dermatophytes to antifungal drugs.

Introduction

The approval of protocols M27-A2 (CLSI 2002a) and M38-A (CLSI 2002b), by the National Committee for Clinical Laboratory Standards (CLSI), motivated research on new methods for the standardization of susceptibility tests for yeasts and filamentous fungi. As an example, Meletiadis *et al.* (2002) have developed colorimetric and diffusion in agar methods. Protocol M27-A2 is specific for the determination of minimum inhibitory concentrations (MICs) for yeasts (*Cryptococcus neoformans* and *Candida* spp.), and protocol M38-A for filamentous, sporangiospore and conidium-forming fungi that cause invasive mycoses. Both protocols use the culture medium RPMI-1640 (without sodium bicarbonate and L-glutamine at pH 7.0) supplemented with 0.165 M morpholinepropanesulphonic acid (MOPS) and an incubation temperature of 35 °C. Inocula are 10^4 CFU ml^{-1} for *Cryptococcus neoformans* and 10^3 CFU ml^{-1} for *Candida* spp. The incubation period is of 24–48 h (document M27-A2) or up to 4 d (document M38-A). Visual readings are performed in both cases. The protocol of the Antifungal Susceptibility Testing Subcommittee of the European Committee on Antimicrobial Susceptibility Testing (AFST-EUCAST; approved in 2002) is used to determine MIC values for fermentative yeasts. This document recommends

* Corresponding author.
E-mail address: betebarros@ef.ufop.br
0953-7562/$ – see front matter
doi:10.1016/j.mycres.2006.08.006

the use of RPMI-1640 supplemented with 2 % glucose at pH 7.0 (buffered with MOPS 0.165 M), a temperature of 35 °C, an incubation period of 48 h, inocula of 10^5 CFU ml^{-1} and spectophotometric readings. Whilst these methods are reproducible (Cuenca-Estrella *et al.* 2002, 2003), so far there are no methods to determine the MIC values of dermatophyte fungi that cause infections of the skin, hair and nail in humans and animals. Among these infections, onychomycosis are the most difficult to treat, affecting 20 % of the world population under 40-years old (Bradley *et al.* 1999). These infections affect the nail bed causing dystrophy and sometimes result in complete nail loss (Roberts *et al.* 2003). Although there are many antifungal drugs available that can be taken orally, only terbinafine, itraconazole and fluconazole are effective in the treatment of onychomycosis. Several topical antifungal preparations like amorolfine and tioconazole are available as nail lacquer or solution form. These topical antifungal drugs can be combined with oral therapy and achieve variable results, producing cure rates ranging from 20–70 % (Roberts *et al.* 2003; Marty *et al.* 2005). Griseofulvin is an antifungal that has been used since 1959 for *tinea capitis*, and although it is not efficient in nail treatment, it is used for this purpose (Mock *et al.* 1998; Niewerth & Korting 2000). Gupta & Shear (2000), in an excellent review, discussed the percentage cure obtained by different investigators with these drugs using different treatments in patients with toenail onychomycosis.

The aim of our work was to compare the MIC values of five oral antifungals (fluconazole, ketoconazole, itraconazole, terbinafine and griseofulvin) using two methodologies (document M38-A from CLSI and document used by AFST-EUCAST). One hundred samples of *Trichophyton* spp. (50 strains of *T. mentagrophytes* and 50 strains of *T. rubrum*), isolated from adult nails were tested. The following modifications were introduced in the protocols: RPMI-1640 media was supplemented with 2 % glucose at pH 7.0, buffered with MOPS 0.165 M, incubation period of 7 d, and temperature of 28 °C, endpoints for fluconazole, itraconazole, ketoconazole and griseofulvin were set at 80 % growth inhibition and 100 % growth inhibition for terbinafine.

Materials and methods

Isolates

One hundred strains of *Trichophyton mentagrophytes* (50 strains) and *T. rubrum* (50 strains) isolated from different patients diagnosed with onchomycosis, were examined in this study. The clinical mycology laboratory, Mycoses Ltda., Belo Horizonte, Minas Gerais, Brazil, kindly donated these strains. Quality control isolates included *T. mentagrophytes* (ATCC 40004), *T. rubrum* (ATCC 40051), *Candida parapsilosis* (ATCC 22019), and *Candida krusei* (ATCC 6258). Isolates were cultured on Mycosel™ (Difco, Sparks, USA) for identification. The isolates were plated on Sabouraud dextrose agar (Difco) at 28 °C and maintained as a suspension in sterile distilled water (Gupta & Kohli 2003) at 4 °C (Pujol *et al.* 1996) until use.

Medium

Tests were performed in RPMI 1640 with L-glutamine, but without bicarbonate (Gibco BRL, Life technologies, Woerden, The Netherlands), pH 7.0, supplemented with 2 % glucose, buffered with MOPS (FisherBiotech, New Jersey). The medium was sterilized by filtration.

Antifungal agents

Three azole derivatives were used in this study: fluconazole (Pfizer São Paulo, Brazil), ketoconazole and itraconazole (Janssen-Cilag, São José dos Campos, São Paulo, Brazil). The allylamine terbinafine was obtained from Novartis (São Paulo, Brazil) and griseofulvin from Schering-Plough (Rio de Janeiro, Brazil). All drugs were dissolved in 100 % dimethylsulfoxide (DMSO) (Gibco, Belo Horizonte, Minas Gerais, Brazil) following the CLSI protocol and were prepared as stock solutions of 1 mg ml^{-1}. Serial two-fold dilutions were prepared according to document (M38-A) from the CLSI at 100 times the final concentration required, followed by further dilution (1:50) in RPMI 1640 to yield twice the final strength required for the test. The highest concentration of DMSO used in the tests corresponded to 1 % of the total volume and did not interfere with the growth of *Trichophyton* spp. studied.

Inocula preparation

Stock suspensions of dermatophytes were prepared from sporulating 7-d-old cultures grown on potato dextrose agar (Acumedia, Baltimore, USA) with 2 % in-house rice flour (Heinlaid *et al.* 2003; Jessup *et al.* 2000) at 28 °C. Colonies were covered with 5 ml sterile distilled water and the surface scraped with a sterile loop. The mixture of conidia and hyphal fragments was filtered (Sartorius AG, Goettingen) through an 8 μm (Whatman 40, São Paulo, Brazil) sterile filter and collected in a sterile tube. This procedure removed the majority of the hyphae, producing inocula composed mainly of spores (Petrikkou *et al.* 2001; Santos & Hamdan 2005; Santos *et al.* 2006). Turbidity of the final inocula was adjusted to 0.5×10^6–5.0×10^6 spores ml^{-1}, at a wavelength of 520 nm, and transmission adjusted to 70 % in a spectrophotometer (Micronal B542, São Paulo). Quantification was made by plating 0.01 ml of a 1:100 dilution of the adjusted inocula (varying between 0.5 to 2.5×10^6 CFU ml^{-1}) on Sabouraud dextrose agar plates. Plates were incubated at 28 °C and observed daily. Colonies were counted as soon as growth became visible. All inocula were adjusted to a final dilution recommended by both methodologies, in RPMI-1640 supplemented with 2 % glucose.

Test procedure

Tests were performed in sterile 96-well flat bottom polystyrene plates; 100 μl of each drug (in two-fold dilutions) were added to the plates that were then stored at −70 C until use. For the tests, 100 μl of diluted cell suspension was added to each well so that the final concentration was 0.5×10^4–5×10^4 spores ml^{-1} for protocol M38-A (CLSI) and 0.5×10^5–5×10^5 spores ml^{-1} for protocol AFST-EUCAST. For fluconazole, the concentrations were 64–0.125 μg ml^{-1}, for ketoconazole and griseofulvin 8–0.015 μg ml^{-1}, for itraconazole 4–0.007 μg ml^{-1} and for terbinafine 4–0.007 μg ml^{-1}. Control wells (growth and sterility) were included for each assay performed alongside a duplicate series of drug dilutions. After 7 d at 28 °C, plates were read

visually (CLSI method) or with a spectrophotometer (AFST-EUCAST method). MICs were determined as the lowest concentration of drug that gave approximately 80 % inhibition (Ghannoum *et al.* 2004) of the growth control for fluconazole, ketoconazole, itraconazole and griseofulvin. For terbinafine, MICs were the lowest drug concentration that showed 100 % growth inhibition.

Data analysis

Determination of all MICs was repeated twice. Statistical analyses were performed with Wilcoxon (Mann–Whitney) and Kruskal–Wallis tests. $P < 0.05$ was considered significant.

Results

MIC values for 100 isolates of *Trichophyton* spp. are summarized in Tables 1 and 2. Itraconazole and terbinafine had the highest inhibitory activities with both methodologies; 90 % of the isolates had MIC values of 0.25 $\mu g\,ml^{-1}$ and 0.015 $\mu g\,ml^{-1}$, respectively (Table 2). When the EUCAST method was used, seven isolates of *T. mentagrophytes* had MICs of 0.5 $\mu g\,ml^{-1}$ for itraconazole and one had an MIC of 0.031 $\mu g\,ml^{-1}$ for terbinafine (Table 1).

For the drugs ketoconazole and griseofulvin, the MIC_{90} values were the same using either methodology (1 $\mu g\,ml^{-1}$; Table 2). Using the AFST-EUCAST method, one sample of *T. mentagrophytes* had a MIC of 4 $\mu g\,ml^{-1}$ for griseofulvin and none of the *T. rubrum* isolates had MICs less than 0.5 $\mu g\,ml^{-1}$. The same method detected five samples of *T. mentagrophytes* and 13 of *T. rubrum* with MICs of 2 $\mu g\,ml^{-1}$ for the same drug (Table 2).

Fluconazole was the drug with the lowest activity against the isolates. Using both methods the same MIC_{50} and MIC_{90} values were observed (Table 2). None of the *T. mentagrophytes* isolates were susceptible to 4 $\mu g\,ml^{-1}$, nevertheless five samples of *T. rubrum* were susceptible to $\leq$4 $\mu g\,ml^{-1}$ when protocols M38-A of CLSI were used and eight samples of *T. mentagrophytes* had MICs $\geq$64 $\mu g\,ml^{-1}$ when the AFST-EUCAST protocol was used (Table 1).

Table 1 – *In vitro* antifungal activities of five drugs tested using two methods against 100 strains of *Trichophyton* spp.

Antifungal drugs	Minimum inhibitory concentration ($\mu g\,ml^{-1}$)	*Trichophyton mentagrophytes* ($n = 50$)[c]		*Trichophyton rubrum* ($n = 50$)[c]	
		CLSI[a]	EUCAST[b]	CLSI	EUCAST
Fluconazole	>64	0	8	0	0
	64	7	14	1	21
	32	32	19	12	20
	16	8	8	27	8
	8	3	1	5	1
	4	0	0	3	0
	2	0	0	1	0
	1	0	0	1	0
Ketoconazole	2	2	2	0	3
	1	8	8	4	19
	0.5	16	31	10	18
	0.25	18	5	28	7
	0.125	5	3	5	2
	0.062	1	1	3	1
Itraconazole	0.5	0	7	0	0
	0.25	4	14	7	32
	0.125	20	27	15	17
	0.062	15	2	14	1
	0.031	4	0	14	0
	0.015	7	0	0	0
Griseofulvin	4	0	1	0	0
	2	0	5	0	13
	1	3	8	9	25
	0.5	10	33	12	12
	0.25	16	3	24	0
	0.125	21	0	4	0
	0.062	0	0	1	0
Terbinafine	0.031	0	1	3	1
	0.015	22	11	0	16
	0.007	13	9	14	33
	<0.007	15	29	33	0

a Document M38-A of the National Committee for Clinical Laboratory Standards (CLSI 2002b).
b Document of the Antifungal Susceptibility Testing Subcommittee of the European Committee on Antimicrobial Susceptibility Testing (AFST-EUCAST 2002).
c Number of tested strains.

Table 2 – MICs of five drugs against *Trichophyton* species assessed by National Committee for Clinical Laboratory Standards (CLSI)/European Committee on Antimicrobial Susceptibility Testing (EUCAST) methods

Antifungal drugs	MIC_{50} ($\mu g\,ml^{-1}$)[a]		MIC_{90} ($\mu g\,ml^{-1}$)[a]	
	NCCLS[b]	EUCAST[c]	NCCLS	EUCAST
Fluconazole	32	64	32	64
Ketoconazole	0.25	0.5	1	1
Itraconazole	0.125	0.25	0.25	0.25
Griseofulvin	0.25	0.5	1	1
Terbinafine	0.007	0.007	0.015	0.015

a - MICs at which 50 % and 90 % of the isolates tested were inhibited, respectively.
b Document M38-A (2002).
c Document AFST-EUCAST (2002).

For all drugs tested, both methodologies gave similar MIC_{50} values (Table 2). Table 3 shows that more than 92 % of the strains varied by less than three dilutions in both methods (CLSI and EUCAST). There was no significant difference ($P < 0.05$) between MIC values of both tested species with the two tested methods, considering no dilution interval.

Discussion

Currently, there are published data comparing the *in vitro* susceptibility of yeasts, especially *Candida* spp., using documents CLSI M27-A2, AFST-EUCAST and E-test (Chryssanthou & Cuenca-Estrella 2002; Cuenca-Estrella *et al.* 2002, 2005; Romero *et al.* 2004), but there is a scarcity of studies including dermatophytic fungi. In this study we compared two methods of microdilution in liquid media to determine the susceptibility of dermatophytes to antifungal drugs. Five drugs currently used in the treatment of dermatophytosis were tested (Mukherjee *et al.* 2003).

Korting *et al.* (1995) using the method described by Granade & Artis (1980), reported an MIC_{90} for griseofulvin of 10 $\mu g\,ml^{-1}$ for *T. mentagrophytes* isolates and of 3 $\mu g\,ml^{-1}$ for *T. rubrum*. Here, we observed using both protocols (M38-A of CLSI and AFST-EUCAST) lower MIC_{90} values of 1 $\mu g\,ml^{-1}$, for both species (Table 2). They also obtained very high MIC values for fluconazole (1024 $\mu g\,ml^{-1}$). In this study values obtained for fluconazole were MIC $\geq$ 64 $\mu g\,ml^{-1}$. An MIC_{90} of 2 $\mu g\,ml^{-1}$ was found for fluconazole against dermatophytes by both Jessup *et al.* (2000) and Ghannoum *et al.* (2004), which is very different from the MIC found in the present study ($MIC_{90} = 32\ \mu g\,ml^{-1}$). This is possibly due to differences in the incubation period. In the case of terbinafine, very low values were observed by Korting *et al.* (MIC = 0.05 $\mu g\,ml^{-1}$) and similar results were obtained in our study (0.015 $\mu g\,ml^{-1}$).

Our data, by using both methods, were similar to the ones obtained by Fernández-Torres *et al.* (2000), Fernández-Torres *et al.* (2001) and Serrano-Martino *et al.* (2003). MIC_{50} values for ketoconazole and itraconazole varied by just for one dilution (plus or minus), while for terbinafine both methods were the same.

Gupta & Kohli (2003), working with 68 samples of *T. rubrum* and 14 of *T. mentagrophytes* and method M27-A (CLSI), found the $MICs_{90}$ for ketoconazole, itraconazole and terbinafine very similar to the ones reported here. Using a different method, and other species of dermatophytes such as *Epidermophyton floccosum*, *Microsporum canis* and *Microsporum gypseum*, Favre *et al.* (2003) also found the MIC_{90} for fluconazole similar to the ones reported here, leading us to conclude that fluconazole should not chosen for the treatment of dermatophyte infections.

Overall, our data demonstrate the reproducibility of the methods employed to perform susceptibility tests in dermatophytes and confirm the *in vitro* inefficacy of fluconazole, as well as the excellent antifungal activity of terbinafine, and good activity of itraconazole. The methods suggested by the CLSI (document M38-A) and by the AFST-EUCAST can be considered very good protocols to determine MIC values in dermatophytes. Our modifications to adapt the susceptibility tests to this group of fungi did not affect the accuracy of the techniques when applied to this specific group. Moreover, the results we have obtained provide greater reproducibility and reliability of susceptibility/resistance

Table 3 – Agreement of the National Committee for Clinical Laboratory Standards (CLSI) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) methods at the same dilutions for 100 isolates of *Trichophyton* species

Drugs	Dilutions[a]	*Trichophyton mentagrophytes*		*Trichophyton rubrum*	
		$n = 50$[b]	%[c]	$n = 50$	%
Fluconazole	0	15	30	16	32
	1	22	44	14	28
	2	9	18	13	26
	3	3	6	4	8
	4	1	2	1	2
	5	0	0	1	2
	6	0	0	1	2
Ketoconazole	0	18	36	7	14
	1	24	48	17	34
	2	4	8	19	38
	3	3	6	5	10
	4	1	2	2	4
Itraconazole	0	11	22	12	24
	1	20	40	14	28
	2	9	18	16	32
	3	6	12	8	16
	4	2	4	0	0
	5	2	4	0	0
Griseofulvin	0	9	18	8	16
	1	18	36	14	28
	2	16	32	16	32
	3	4	8	12	24
	4	2	4	0	0
	5	1	2	0	0
Terbinafine	0	12	24	5	10
	1	21	42	31	32
	2	17	34	14	28

a 0 – at same dilution; 1, 2, 3, 4, 5, 6 – number of dilutions for up or down, between CLSI and EUCAST methods.
b Number of isolates.
c Percentage in agreement.

determination than previous methods. In the future, we hope that standardization of these methodologies will allow clinical-laboratory studies to better correlate MIC values with clinical outcomes.

Acknowledgements

We are grateful to Walquíria Lopes Borges, Bernardo Drumond Matias, and Tânia Mara de Gomes Pinho for excellent technical assistance.

REFERENCES

AFST-EUCAST, 2002. *Reference Method for Determination of Minimal Inhibitory Concentration (MIC) by Broth Dilution of Fermentative Yeasts. Discussion document E*. Dis. 7.1 ESCAMID. Subcommittee of Antifungal Susceptibility Testing of the European Committee on Antibiotic Susceptibility Testing of the European Society of Clinical Microbiology and Infectious Diseases. Taufkirchen.

Bradley MC, Leidich S, Isham N, Elewski BE, Ghannoum MA, 1999. Antifungal susceptibilities and genetic relatedness of serial *Trichophyton rubrum* isolates from patients with onychomycosis of the toenail. *Mycoses* **42**: 105–110.

Chryssanthou E, Cuenca-Estrella M, 2002. Comparison of the antifungal susceptibility testing subcommittee of the European committee on antibiotic susceptibility testing proposed standard and E-test with the CLSI broth microdilution method for voriconazole and caspofungin susceptibility testing of yeast species. *Journal of Clinical Microbiology* **40**: 3841–3844.

CLSI (NCCLS), 2002a. *Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeast; Approved Standard document M27-A2*, 2nd edn. National Committee for Clinical Laboratory Standards, Wayne, PA.

CLSI (NCCLS), 2002b. *Reference Method for Broth Dilution Antifungal Susceptibility Testing of Filamentous Fungi; Approved Standard document M38-A*. National Committee for Clinical Laboratory Standards, Wayne, PA.

Cuenca-Estrella M, Gomez-Lopez A, Mellado E, Rodriguez-Tudela JL, 2005. Correlation between the procedure for antifungal susceptibility testing for *Candida* spp. of the european committee on antifungal susceptibility testing (EUCAST) and four commercial techniques. *Clinical Microbiology and Infection* **11**: 486–492.

Cuenca-Estrella M, Lee-Yang W, Ciblak MA, Arthington-Skaggs BA, Mellado E, Warnak OW, Rodriguez-Tudela JL, 2002. Comparative evaluation of CLSI M27-A and EUCAST broth microdilution procedures for antifungal susceptibility testing of *Candida* species. *Antimicrobial Agents and Chemotherapy* **46**: 3644–3647.

Cuenca-Estrella M, Moore CB, Barchiese F, Bille J, Chryssanthou E, Denning DW, Donnelly JP, Dromer F, Dupont B, Rex JH, Richardson MD, Sancak B, Verweij PE, Rodriguez-Tudela JL, AFST Subcommittee of the European Committee on Antimicrobial Susceptibility Testing, 2003. Multicenter evaluation of the reproducibility of the proposed antifungal susceptibility testing method for fermentative yeasts of the Antifungal Susceptibility Testing Subcommittee of the European Committee on Antimicrobial Susceptibility Testing (AFST-EUCAST). *Clinical Microbiology and Infection* **9**: 467–474.

Favre B, Hofbauer B, Hildering KS, Ryder NS, 2003. Comparisson of *in vitro* activities of 17 antifungal drugs against a panel of 20 dermatophytes by using a microdilution. *Journal of Clinical Microbiology* **41**: 4817–4819.

Fernández-Torres B, Vázquez-Veiga H, Llovo X, Pereiro Jr M, Guarro J, 2000. *In vitro* susceptibility to itraconazole, clotrimazole, ketoconazole and terbinafine of 100 isolates of *Trichophyton rubrum*. *Chemotherapy* **46**: 390–394.

Fernández-Torres B, Carrillo AJ, Martín E, Del Palacio A, Moore MK, Valverde A, Serrano M, Guarro J, 2001. *In vitro* activities of ten antifungal drugs against 508 dermatophyte strains. *Antimicrobial Agents and Chemotherapy* **45**: 2524–2528.

Ghannoum MA, Chatuverdi V, Espinel-Ingroff A, Pfaller MA, Rinaldi MG, Lee-Yang W, Warnock DW, 2004. Intra- and interlaboratory study of a method for testing the antifungal susceptibilities of dermatophytes. *Journal of Clinical Microbiology* **42**: 2977–2979.

Granade TC, Artis WM, 1980. Antimycotic susceptibility testing of dermatophytes in microcultures with a standardized fragmented mycelial inoculum. *Antimicrobial Agents and Chemotherapy* **17**: 725–729.

Gupta AK, Kohli Y, 2003. *In vitro* susceptibility testing of ciclopirox, terbinafine, ketoconazole and itraconazole against dermatophytes and nondermatophytes, and *in vitro* evalution of combination antifungal activity. *British Journal of Dermatology* **149**: 296–305.

Gupta AK, Shear NH, 2000. A risk–benefit assessment of the newer oral antifungal agents used to treat onychomycosis. *Drugs Safety* **22**: 33–52.

Heinlaid K, Naaber P, Jarv H, 2003. Evaluation of media to enhance conidial production of dermatophytes. *Clinical Microbiology and Infection* **9**: 335.

Jessup CJ, Warner J, Isham N, Hasan I, Ghannoum MA, 2000. Antifungal susceptibility testing of dermatophytes: establishing a medium for inducing conidial growth and evaluation of susceptibility of clinical isolates. *Journal of Clinical Microbiology* **38**: 341–344.

Korting HC, Ollert M, Abeck D, German Collaborative Dermatophyte Drug Susceptibility Study Group, 1995. Results of german multicenter study of antimicrobial susceptibilities of *Trichophyton rubrum* and *Trichophyton mentagrophytes* strains causing *tinea unguium*. *Antimicrobial Agents and Chemotherapy* **39**: 1206–1208.

Marty JP, Lambert J, Jackel A, Adjadj L, 2005. Treatment costs of three nail lacquers used in onychomycosis. *Journal of Dermatological Treatment* **16**: 299–307.

Meletiadis J, Mouton JW, Meis JFGM, Bouman BA, Verweij PE, EUROFUNG N, 2002. Comparison of the Etest and Sensititre colorimetric methods with the NCCLS proposed standard for antifungal susceptibility testing of *Aspergillus* species. *Journal of Clinical Microbiology* **40**: 2876–2885.

Mock M, Monod M, Baudraz-Rosselet F, Panizzon RG, 1998. *Tinea capitis* dermatophytes: susceptibility to antifungal drugs tested *in vitro* and *in vivo*. *Dermatology* **197**: 361–367.

Mukherjee PK, Leidich SD, Isham N, Leitner I, Ryder NS, Ghannoum MA, 2003. Clinical *Trichophyton rubrum* strain exhibiting primary resistance to terbinafine. *Antimicrobial Agents and Chemotherapy* **47**: 82–86.

Niewerth M, Korting HC, 2000. The use of systemic antimycotics in dermatotherapy. *European Journal of Dermatology* **10**: 155–160.

Petrikkou E, Rodríguez-Tudela JL, Cuenca-Estrella M, Gómez A, Molleja A, Mellado E, 2001. Inoculum standardization for antifungal susceptibility testing of filamentous fungi pathogenic for humans. *Journal of Clinical Microbiology* **39**: 1345–1347.

Pujol I, Guarro J, Llop C, Soler L, Fernández-Ballart J, 1996. Comparison study of broth macrodilution and microdilution antifungal susceptibility tests for the filamentous fungi. *Antimicrobial Agents and Chemotherapy* **40**: 2106–2110.

Roberts DT, Taylor WD, Boyle J, 2003. Guidelines for treatment of onychomycosis. *British Journal of Dermatology* **148**: 402–410.

Romero M, Cánton E, Péman J, Gobernado M, 2004. Estudio de la actividad *in vitro* de caspofungin sobre especies de levaduras diferentes de *Candida albicans*, determinada por dos métodos: M27–A2 y EUCAST. *Revista Española de Quimioterapia* **17**: 257–262.

Santos DA, Hamdan JS, 2005. Evaluation of broth microdilution antifungal susceptibility testing conditions for *Trichophyton rubrum*. *Journal of Clinical Microbiology* **43**: 1917–1920.

Santos DA, Barros MES, Hamdan JS, 2006. Establishing a method of inoculum preparation for susceptibility testing of *Trichophyton rubrum* and *Trichophyton mentagrophytes*. *Journal of Clinical Microbiology* **44**: 98–101.

Serrano-Martino MC, Chávez-Caballero M, Valverde-Conde A, Clar RM, Peman J, Martin-Mazuelos E, 2003. Actividad *in vitro* de voriconazol y otros tres antifúngicos frente a dermatofitos. *Enfermedades Infecciosas y Microbiología Clínica* **21**: 484–487.