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Evaluation of susceptibility of *Trichophyton mentagrophytes* and *Trichophyton rubrum* clinical isolates to antifungal drugs using a modified CLSI microdilution method (M38-A)

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Onychomycosis is a common adult human mycosis, and dermatophytes of the *Trichophyton* genera are the most common causative agent. Many antimycotic agents are safe and highly effective for the treatment of dermatophytosis, and are available for clinical practice. Successful treatment depends on the ability of antifungal drugs to eradicate the fungal isolates. The aim of this work was to determine the MICs of four antifungal drugs (fluconazole, itraconazole, terbinafine and griseofulvin) recognized for ungual dermatophytosis treatment caused by *Trichophyton* species, especially *Trichophyton mentagrophytes* and *Trichophyton rubrum*. MICs were determined using a broth microdilution method in accordance with Clinical and Laboratory Standards Institute approved standard M38-A with some modifications, such as an incubation temperature of 28 °C, an incubation time of 7 days and inocula constituted of only microconidia. The results showed that the activities of terbinafine and itraconazole were significantly higher (MICs of $<$0.007–0.031 and 0.015–0.25 μg ml^{-1}, respectively) than other tested agents. All isolates had reduced susceptibility to fluconazole (1–64 μg ml^{-1}). The MIC of griseofulvin varied among strains (MICs of 0.062–1 μg ml^{-1}). The parameters adopted to perform susceptibility testing of *T. rubrum* and *T. mentagrophytes* to antifungal agents appeared to be suitable and reliable, and could contribute to the possible development of a standard protocol.

INTRODUCTION

Dermatophytes have the capacity to invade keratinized tissues (skin, hair and nails), producing dermatophytosis (Makimura *et al.*, 1998). Dermatophytosis ranks among the most common and widespread infectious diseases worldwide, and *Trichophyton rubrum* and *Trichophyton mentagrophytes*, which cause infections of skin and nails, are two of the most frequently isolated dermatophytes (El Fari *et al.*, 1999). Onychomycosis is a common infection of toenails, causing disease in up to 20 % of the population over 40 years of age and especially in the elderly. This infection is caused by dermatophytes, especially *T. rubrum* (Bradley *et al.*, 1999; Goldstein *et al.*, 2000; Gupta, 2000). A great number of antifungal agents have become available for the treatment of dermatophytosis, and in clinical practice, many antimycotic agents are safe and highly effective (Favre *et al.*, 2003). However, dermatophytes that cause lesions in nails do not respond well to treatment (Roberts *et al.*, 2003), suggesting that factors such as age, nail growth, extent of nail involvement, peripheral vascular disease, fungal growth patterns and the presence of dormant fungal spores (arthrospores) in the nail may adversely affect a successful outcome (Evans, 2001; Sigurgeirsson *et al.*, 2002). Successful treatment depends on the ability of a given antimycotic to eradicate the fungal isolate (Santos *et al.*, 2006). In order to predict this ability, *in vitro* susceptibility testing becomes helpful because it can help clinicians to choose the correct treatment for their patients. The Clinical and Laboratory Standards Institute (CLSI, formerly the NCCLS) approved standard protocol M38-A (Clinical and Laboratory Standards Institute, 2002) does not provide a methodology for testing susceptibility of dermatophytes to antifungal drugs. The protocol has generated a great number of methodologies proposed by many researchers, which makes comparison of results difficult. However, some conditions for performing the tests have been evaluated and have demonstrated reproducibility and reliability (Santos & Hamdan, 2005; Santos *et al.*, 2006).

The aim of this study was to determine the MICs of antifungal agents on 50 clinical isolates each of *T. mentagrophytes* and *T. rubrum* obtained from patients with

Abbreviation: CLSI, Clinical and Laboratory Standards Institute.

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toenail onychomycosis. We used the broth microdilution antifungal susceptibility guidelines of the approved standard M38-A, adapted for dermatophytes, which are producers of microconidia. This important methodology used for *in vitro* testing of dermatophytes could provide information for the development of a standard assay for testing dermatophytic fungi.

METHODS

Isolates. The strains of *T. mentagrophytes* and *T. rubrum* (50 of each) used in this study were obtained from nails of patients with toenail onychomycosis. Quality control strains of *T. mentagrophytes* (ATCC 40004), *T. rubrum* (ATCC 40051), *Candida parapsilosis* (ATCC 22019) and *Candida krusei* (ATCC 6258) were included. The isolates were identified by routine mycological procedures and were maintained in sterile distilled water (Gupta & Kohli, 2003) at 4 °C (Pujol *et al.*, 1996) until tests were performed.

Medium. Tests were performed in RPMI 1640 with L-glutamine but without bicarbonate (Gibco). The medium was buffered to pH 7.0 and included 0.165 M MOPS, and was prepared and sterilized by filtration.

Antifungal agents. Two azole derivatives were used in this study: fluconazole (Pfizer) and itraconazole (Janssen-Cilag). The alylamine terbinafine was obtained from Novartis and the griseofulvin from Schering-Plough. All drugs were dissolved in 100 % DMSO (Gibco) following approved standard M38-A and were prepared as stock solutions of 1000 μg ml^{-1}. Serial twofold dilutions were prepared according to M38-A at 100 times the strength of the final concentration, followed by further dilution (1 : 50) in RPMI 1640 to yield twice the final strength required for the test. Concentrations ranged from 4 to 64 μg ml^{-1} for fluconazole, from 0.125 to 4 μg ml^{-1} for griseofulvin, from 0.062 to 2 μg ml^{-1} for itraconazole and from 0.007 to 0.25 μg ml^{-1} for terbinafine.

Inoculum preparation. Stock inoculum suspensions of the dermatophytes were prepared from 7 day cultures grown on potato dextrose agar (Santos & Hamdan, 2005) at 28 °C to induce sporulation. The 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 transferred to a sterile syringe attached to a sterile filter holder with a sterile filter, pore diameter 8 μm (Whatman no. 40), filtered and collected in a sterile tube as recommended by Santos *et al.* (2006). This procedure removed the majority of the hyphae, producing an inoculum composed mainly of spores. The turbidity of the final inoculum was adjusted to 0.5×10^6–5.0×10^6 spores ml^{-1} at a wavelength of 520 nm and transmission was adjusted to 65–70 % in a spectrophotometer. The inocula were quantified by plating on Sabouraud glucose agar plates, using 0.01 ml adjusted inoculum. The plates were incubated at 28 °C and observed daily for the presence of growth. All inocula were adjusted to a final dilution of 1 : 50 in RPMI 1640.

Test procedure. The tests were performed in polystyrene microtitre plates with 96 flat-bottomed wells. Aliquots of 100 μl of the twofold drug dilutions were inoculated into the wells with a multichannel pipette. The microplates were stored at −70 °C until use. Each microplate was inoculated with 100 μl of the diluted inoculum suspensions to bring the dilutions of the inoculum to 0.5×10^4–5×10^4 spores ml^{-1}. Growth and sterility controls were included for each assay and tests were performed in duplicate. The microplates were incubated at 28 °C and read visually after 7 days of incubation, as recommended by Santos & Hamdan (2005). MICs for fluconazole, itraconazole and griseofulvin were the lowest drug concentration that showed approximately 80 % growth inhibition (Ghannoum *et al.*, 2004). For terbinafine, the MIC was the lowest drug concentration that showed 100 % growth inhibition.

Statistical analyses. Comparison of MIC values of antifungal drugs for the two tested species was performed using Kruskal–Wallis and Wilcoxon (Mann–Whitney) tests. A P value of <0.05 was considered to be significant.

RESULTS

MIC_{50} and MIC_{90}, the MIC values that inhibited 50 and 90 % of isolate growth, as well as the MIC range of the four tested antifungal agents are summarized in Table 1. The activities of terbinafine and itraconazole were significantly higher than the other drugs tested. For terbinafine, *T. mentagrophytes* was inhibited at $MIC_{90}=0.015$ and $MIC_{50}=0.007$ μg ml^{-1}, whilst *T. rubrum* was inhibited at $MIC_{90}=0.007$ and $MIC_{50}<0.007$ μg ml^{-1}. Itraconazole inhibited *T. mentagrophytes* and *T. rubrum* at $MIC_{90}=0.125$ and $MIC_{50}=0.062$ μg ml^{-1}, and $MIC_{90}=0.25$ and $MIC_{50}=0.062$ μg ml^{-1}, respectively. All isolates had reduced susceptibility to fluconazole, demonstrated by the fact that MIC values for this drug were higher than the other agents ($MIC_{90}=64$ and $MIC_{50}=16$ μg ml^{-1}). For griseofulvin, *T. mentagrophytes* was inhibited at $MIC_{90}=0.5$ and $MIC_{50}=0.25$ μg ml^{-1}, and *T. rubrum* was inhibited at $MIC_{90}=1$ and $MIC_{50}=0.25$ μg ml^{-1}.

No significant differences ($P<0.05$) were observed between *T. rubrum* and *T. mentagrophytes* for any of the tested drugs.

Table 1. Susceptibility data for *T. mentagrophytes* and *T. rubrum*

Antifungal drug	MIC range (μg ml^{-1})	MIC (μg ml^{-1})			
		T. mentagrophytes		*T. rubrum*	
		MIC_{90}	MIC_{50}	MIC_{90}	MIC_{50}
Fluconazole	1–64	64	32	32	16
Itraconazole	0.015–0.25	0.125	0.062	0.25	0.062
Griseofulvin	0.062–1	0.5	0.25	1	0.25
Terbinafine	<0.007–0.031	0.015	0.007	0.007	<0.007

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Table 2. Summary of susceptibility data of *T. mentagrophytes* and *T. rubrum* to antifungal drugs in different studies

Method	*T. mentagrophytes*					*T. rubrum*					TP	IN	RT	Reference
	MIC range (μg ml^{-1})				NI	MIC range (μg ml^{-1})				NI				
	FCZ	ITZ	TER	GRI		FCZ	ITZ	TER	GRI					
M38-A	1–64	0.015–0.25	<0.007–0.031	0.062–1	50	1–64	0.015–0.25	<0.007–0.031	0.062–1	50	28	10^4	7	This study
M38-P	0.06–>64	0.01–2	0.007–0.5	–	122	0.06–>64	0.01–8	0.003–>16	–	144	28	10^4	4–7	Fernández-Torres *et al.* (2001)
M38-P	1–⩾64	0.06–2	⩽0.03	–	61	2–⩾64	0.12–1	⩽0.03	–	12	28	10^4	4	Serrano-Martino *et al.* (2003)
M38-P	–	–	–	–	–	–	0.03–2	0.0039–0.25	–	100	28	10^4	6	Fernández-Torres *et al.* (2000)
M38-P	–	0.06–2	0.01–0.06	–	10	–	0.06–2	0.01–2	–	10	28	10^3	7	Fernández-Torres *et al.* (2002)
M38-P	2–64	0.063–1	0.004–0.008	0.25–1	4	1–4	0.25–0.5	0.002–0.004	0.5	5	35	10^3	4–5	Favre *et al.* (2003)
M38-A	0.125–64	0.001–0.5	0.001–0.5	0.125–64	10	0.125–64	0.001–0.5	0.001–0.5	0.125–64	10	35	10^3	4	Ghannoum *et al.* (2004)
M27-A	–	0.06–32	0.003–0.5	–	14	–	0.06–32	0.003–1	–	68	35	10^3	7	Gupta and Kohli (2003)
M27-A	–	–	–	–	–	0.13–64	0.06–32	0.06–32	0.13–64	6	35	10^3	4	Mukherjee *et al.* (2003)

FCZ, Fluconazole; ITZ, itraconazole; TER, terbinafine; GRI, griseofulvin; NI, no. of isolates; TP, incubation temperature (°C); IN, inoculum (c.f.u. ml^{-1}); RT, reading time (days).

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DISCUSSION

In this study, we followed a CLSI protocol (standard M38-A) adapted by Santos and Hamdan (2005) to determine the MIC values of four antimycotic agents currently employed in the treatment of dermatophytosis. The modifications included an incubation temperature of 28 °C, an incubation time of 7 days and inocula consisting only of microconidia. This method demonstrated high reproducibility and reliability. It was difficult to compare the results found here with those of other authors, as there are a number of studies in the literature using CLSI methodologies (M27-A, M38-P and M38-A) and all use different versions of the methodology to test dermatophytes. Several collaborative studies testing filamentous fungi have demonstrated that azole MICs are extremely variable, being influenced by testing conditions (Fernández-Torres *et al.*, 2002).

As illustrated in Table 2, different experimental parameters have been used to determine MICs for dermatophytes. This is due to the absence of a standard protocol to perform susceptibility tests on these fungi. Published studies have demonstrated that buffered RPMI 1640 allows adequate growth of dermatophytes (Norris *et al.*, 1999; Fernández-Torres *et al.*, 2002; Santos & Hamdan, 2005). Discrepancies in the mode of inoculum preparation between this study and others might result in a lower degree of test reliability. In addition, inocula consisting of only microconidia at concentrations of 10^4 c.f.u. ml^{-1} are recommended by CLSI standard M38-A. As demonstrated by Santos *et al.* (2006), microconidia of the tested species present higher susceptibility to antifungal drugs than hyphal fragments. This explains the low MIC values obtained here for all of the tested agents (except fluconazole) in comparison with the studies outlined in Table 2, even when the inoculum size was 10^3 c.f.u. ml^{-1}. According to Fernández-Torres *et al.* (2002), the influence of inoculum size on MICs is dependent on the antifungal agent tested and may be related to the mechanism of action of the agent. It has also been demonstrated that inoculum size and consistency do not affect MIC values for terbinafine (Fernández-Torres *et al.*, 2002; Santos *et al.*, 2006).

Differences in MIC values cannot be attributed to the incubation temperature (28 or 35 °C), as Santos & Hamdan (2005) demonstrated that single parameters alone do not significantly influence MIC determination. According to Norris *et al.* (1999), a logistical advantage of using 35 °C is that dermatophyte plates can be incubated with plates set up for yeast testing, eliminating the need for a second incubator for susceptibility testing. The incubation period is another point of discrepancy among the studies mentioned. We used an incubation time of 7 days, as *T. rubrum* and *T. mentagrophytes* do not grow well in shorter periods (Santos & Hamdan, 2005). In addition, visualization of growth inhibition could be confused with poor growth of the fungi in microdilution wells, indicating a false susceptibility profile for a given agent.

With respect to inhibition end points, it is recommended in the literature to use 50 (Fernández-Torres *et al.*, 2000), 80 (Gupta & Kohli, 2003) and 100 % (Fernández-Torres *et al.*, 2002) growth inhibition as end points. A value of 80 % growth inhibition appears to be suitable for fungistatic agents and 100 % is suitable for fungicidal drugs.

Terbinafine was the most potent agent tested in this study, with MIC values for both tested species lower than the other agents tested, suggesting a possible correlation to data that support the use of terbinafine to treat onychomycosis or any dermatophytic infections. These data are in agreement with all of the studies mentioned except that of Mukherjee *et al.* (2003), which refers to serial isolates of *T. rubrum* exhibiting primary resistance to this drug. Among azole agents, itraconazole was the most active, followed by fluconazole. These data are similar to the studies presented in Table 2. Griseofulvin is an antifungal agent active only against filamentous fungi (Develoux, 2001). All of the tested isolates presented MIC values of $\leqslant 1$ μg ml^{-1}, which is generally lower than the MICs described by other researchers.

Fluconazole was the drug that presented the highest MIC values in comparison with the other tested agents, with an MIC of $\geqslant 32$ μg ml^{-1} for isolates of both species tested. Similar results were found by other authors (Table 2) independent of the experimental parameters. These data confirm the resistance of dermatophyte species to fluconazole, and are contradictory to cure rates achieved by patients who have used it to treat dermatophytosis (Korting *et al.*, 1995). Problems with interactions of fluconazole with particular media or problems with dilutions in high concentrations have been suggested as being responsible for its higher MIC values (Korting *et al.*, 1995).

In conclusion, the parameters for testing the susceptibility of *T. rubrum* and *T. mentagrophytes* to antifungal agents adopted here appear to be suitable and reliable, and could contribute to the possible development of a standard protocol.

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