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In vitro SUSCEPTIBILITY TESTING OF DERMATOPHYTES ISOLATED IN GOIANIA, BRAZIL, AGAINST FIVE ANTIFUNGAL AGENTS BY BROTH MICRODILUTION METHOD

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SUMMARY

The antifungal activities of fluconazole, itraconazole, ketoconazole, terbinafine and griseofulvin were tested by broth microdilution technique, against 60 dermatophytes isolated from nail or skin specimens from Goiania city patients, Brazil. In this study, the microtiter plates were incubated at 28 ºC allowing a reading of the minimal inhibitory concentration (MIC) after four days of incubation for *Trichophyton mentagrophytes* and five days for *T. rubrum* and *Microsporum canis*. Most of the dermatophytes had uniform patterns of susceptibility to the antifungal agents tested. Low MIC values as 0.03 µg/mL were found for 33.3%, 31.6% and 15% of isolates for itraconazole, ketoconazole and terbinafine, respectively.

KEYWORDS: Dermatophytes; Broth microdilution method; Minimal Inhibitory Concentration.

INTRODUCTION

Dermatophytosis, mycotic infections caused by dermatophytes, are commonly related in tropical countries and represent an important public health problem yet unresolved[4]. *Trychophyton rubrum*, *T. mentagrophytes* and *Microsporum canis* are the most common species isolated in Brazil as causative agents of dermatophytosis[1,6,7,8,21]. In Goiania city, Brazil, these same etiological agents have been recovered in studies of COSTA *et al*.[6,7]. Antifungal agents such as triazoles (itraconazole, fluconazole), imidazole (ketoconazole), allylamine (terbinafine) and griseofulvin have been reported to have substantial activity in dermatophytosis[16,17]. However, infections due to dermatophytes are often associated with relapses after cessation of therapy[20]. By the way, *in vitro* antifungal susceptibility tests could help to optimize the therapy and to select an effective antifungal agent for this mycosis[3]. A standard method for susceptibility testing of dermatophytes is lacking, but good results of MIC using either broth macrodilution or broth microdilution tests have been obtained in several reports[9,10,12,18]. The purpose of this work was to establish the *in vitro* antifungal susceptibility of fluconazole, itraconazole, ketoconazole, terbinafine and griseofulvin against clinical dermatophytes isolated in Goiania-GO, Brazil, using the broth microdilution method.

MATERIALS AND METHODS

Organisms: A total of 60 dermatophyte strains, including *Trichophyton rubrum* (n = 27), *T. mentagrophytes* (n = 14) and *Microsporum canis* (n = 19) were tested. All microorganisms were clinical isolates obtained from nail or skin specimens recovered from patients from Goiania University Hospital from March to July 2006. The fungi were maintained in sterile distilled water at room temperature and prior to testing, the strains were subcultured on potato dextrose agar (PDA) at 28 ºC for seven to 15 days to ensure the viability and the purity of the inoculum. *Candida parapsilosis* ATCC 22019 was included as reference strain.

***In vitro* susceptibility testing:** The broth microdilution assay for antifungal susceptibility testing of dermatophytes was performed, when possible according to the CLSI guidelines in the document M38-A of filamentous fungi[5].

Antifungal drugs dilution: The drugs were obtained from their respective manufacturers: fluconazole (Pfizer International, New York, NY), ketoconazole, itraconazole (Jansen Pharmaceuticals, Beerse, Belgium), terbinafine (Novartis Research Institute, Vienna, Austria) and griseofulvin (Sigma Chemical Company, St. Louis, Mo). Fluconazole was dissolved in distilled water while the other drugs were dissolved in 100% dimethyl sulfoxide (Sigma-Aldrich). They were subsequently prepared as stock solution and serial twofold dilutions were performed. Final concentrations ranged from 0.125 to 64 µg/mL for fluconazole, 0.03 to 16 µg/mL for ketoconazole, itraconazole and terbinafine, and 0.03 to 8 µg/mL for griseofulvin.

Test procedure: Inoculum suspensions of dermatophytes were prepared from the seven days cultures grown on potato dextrose agar at 28 ºC. The fungal colonies were covered with approximately 10

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mL of distilled water, and the suspensions were made by scraping the surface with the tip of a sterile loop. The resulting mixture of conidia and hyphal fragments was withdrawn and transferred to sterile tubes and left for 15 to 20 minutes at room temperature to sediment the heavy particles. The optical density of the suspensions containing conidia and hyphal fragments was read at 530 nm, adjusted to transmittance of 65 to 70% (~ 2 to 4 X 10^6 cells/mL) and diluted with RPMI 1640 medium (Sigma Chemical Co., St. Louis, Mo) to obtain the final inoculum size of approximately 0.4 to 5 X 10^4 cells/mL. Aliquots of 100 µL of these suspensions were inoculated in well of microtiter plate containing 100 µL of specific antifungal drug concentration and incubated at 28 °C. Each assay was carried out in duplicate.

Endpoint determination: Endpoint determination values were performed visually every 24 h until the indication of growth in control well drug-free. For azole agents and griseofulvin, the MIC was defined as the lowest concentration that produced prominent inhibition of growth (approximately 80% inhibition) while for terbinafine, was defined as lowest concentration showing 100% growth inhibition[23].

RESULTS

MICs of antifungal agents for 60 dermatophytes isolates could be determined after four days for *T. mentagrophytes* and five days for *T. rubrum* and *M. canis* when incubated at 28 °C.

In general, the species of dermatophytes showed similar patterns of susceptibility to each antifungal agent tested. The determination of the isolates as susceptible or resistant is complex and not yet been established for dermatophytes, but high MIC values were found for some isolates. Seven (11.6%) dermatophytes strains (five *T. rubrum* and two *M. canis*) had MICs of fluconazole of 32 µg/mL, five (8.3%) strains (four *T. rubrum* and one *M. canis*) had MICs of ketoconazole of 4 µg/mL and one *M. canis* isolate had MIC of 8 µg/mL for griseofulvin. However most isolates showed low MIC values, being that 33.3%, 31.6% and 15% of isolates had MIC of 0.03 µg/mL for itraconazole, ketoconazole and terbinafine, respectively.

Table 1 summarizes the MIC ranges, concentrations inhibiting 50% (MIC_{50}) and 90% (MIC_{90}) of the isolates and geometric mean of the MICs of the five antifungal drugs against 60 strains of dermatophytes.

The MIC ranges of fluconazole, itraconazole and ketoconazole for *C. parapsilosis* ATCC 22019 were within the values standardized by CLSI document M-38-A.

DISCUSSION

The determination of the *in vitro* susceptibility may prove helpful to predict the ability of a given antifungal agent to eradicate dermatophytes. Although a reference method for dermatophytes, is not available, a good correlation between the *in vitro* data, using broth microdilution method, and clinical outcome has been demonstrated[19].

Some recommendations, as temperature and duration of incubation used in the broth microdilution method according with the document M

Table 1
In vitro activities of five antifungal agents against 60 strains of dermatophytes isolated from patients from Goiania University Hospital from March to July 2006

Species (No. of isolates)	Antifungal agents	MIC (µg/mL)			
		Range	MIC_{50}	MIC_{90}	Geometric mean
T. rubrum	Fluconazole	2-32	8	32	7.60
(27)	Itraconazole	0.03-4	0.125	0.5	0.10
	Ketoconazole	0.03-4	0.06	4	0.13
	Terbinafine	0.03-0.5	0.125	0.25	0.11
	Griseofulvin	0.25-2	0.5	1	0.65
T. mentagrophytes	Fluconazole	4-16	16	16	11.31
(14)	Itraconazole	0.03-0.25	0.125	0.25	0.09
	Ketoconazole	0.03-1	0.125	0.25	0.12
	Terbinafine	0.03-0.5	0.06	0.25	0.08
	Griseofulvin	0.25-1	0.5	0.5	0.45
M. canis	Fluconazole	2-32	8	16	9.96
(19)	Itraconazole	0.03-0.25	0.125	0.25	0.08
	Ketoconazole	0.03-4	0.125	0.25	0.12
	Terbinafine	0.03-1	0.125	0.25	0.11
	Griseofulvin	0.06-8	0.25	0.5	0.31

MIC - minimal inhibitory concentration; MIC_{50} and MIC_{90} - MIC inhibiting 50% and 90% of the isolates

38-A of filamentous fungi were modified in this study. In this document, the temperature at 35 ºC is used for incubation; however we used for dermatophytes the temperature at 28 ºC according to BARROS *et al.*[2] and SANTOS *et al*.[23] which obtained success in their results. Studies of *in vitro* susceptibility testing for dermatophytes previously performed in our laboratory showed that the MIC values were easier to read and to interpret (a good visualization of the growth) when the microtiter plates were incubated at 28 ºC than at 35 ºC (data not shown). It is well known that an optimal growth on culture media is obtained by dermatophytes strains when incubated between 28 ºC and 30 ºC[9,11,22]. The document M38-A establish 24 to 72 h of incubation for filamentous fungi, however, in our work, a detectable growth was observed after four days for *T. mentagrophytes* and after five days for *T. rubrum* and *M. canis*. The ideal incubation time is still a matter of debate. FERNÁNDEZ-TORRES *et al.*[13] , SANTOS *et al.*[23,24] found that seven days was sufficient to observe prominent growth in control wells, while GHANNOUM *et al.*[14] and JESSUP *et al.*[18] verified growth in four days at 28 ºC.

In our work, the evaluation of *in vitro* susceptibility showed that the antifungal drugs tested, with exception of fluconazole, displayed good activity against the dermatophytes. It is worth mentioning that itraconazole, ketoconazole and terbinafine had the lowest MIC values and geometric means. Similar results have been verified by other authors that showed that these drugs had low MICs against dermatophytes[10,12,15]**.** These low MICs found for the three drugs can help to explain the promising results obtained for the treatment of dermatophytosis with these antifungal agents[19].

Although fluconazole has showed the highest MIC values of all the antifungal agents tested, we verified that *T. rubrum* strains, one specie that cause a recalcitrant chronic disease, were more susceptible to this drug than *T. mentagrophytes* and *M. canis* isolates (geometric mean of 7.60 for *T. rubrum*, 9.96 for *M. canis* and 11.31 for *T. mentagrophytes*). Our results are similar to FERNÁNDEZ-TORRES *et al.*[10] who demonstrated higher activity of fluconazole for *T. rubrum* than for *T. mentagrophytes*, *M. canis* and *M. gypseum*.

In summary, the parameters as temperature at 28 ºC, incubation time of five days and inoculum of 0.4 to 5 X 10^4 cells/mL allowed the determination of MIC for dermatophytes by using the microdilution method. Besides, our work demonstrated that *in vitro* different antifungal agents are active against dermatophytes independent of specie.

RESUMO

Teste de suscetibilidade *in vitro* de dermatófitos isolados em Goiânia, Brasil, contra cinco agentes antifúngicos pelo método de microdiluição em caldo

Atividades antifúngicas de fluconazol, itraconazol, cetoconazol, terbinafina e griseofulvina foram testadas pelo método de microdiluição em caldo contra 60 isolados de dermatófitos. Os resultados mostraram que todos os isolados produziram crescimento claramente detectável a 28 ºC e a concentração inibitória mínima (CIM) foi determinada após quatro dias de incubação para *Trichophyton mentagrophytes* e cinco dias para *T. rubrum* e *Microsporum canis.* A maioria dos isolados teve um padrão uniforme de suscetibilidade para os agentes antifúngicos testados. Baixos valores de CIM como 0,03 µg/mL foram encontrados para 33,3%, 31,6% e 15% dos isolados para itraconazol, cetoconazol e terbinafina, respectivamente.

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