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Comparison of *in vitro* activity of five antifungal agents against dermatophytes, using the agar dilution and broth microdilution methods

Comparação da atividade *in vitro* de cinco agentes antifúngicos para dermatófitos, usando os métodos de diluição em ágar e microdiluição em caldo

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ABSTRACT

The purpose of this study was to compare the agar dilution and broth microdilution methods for determining the minimum inhibitory concentration (MIC) of fluconazole, itraconazole, ketoconazole, griseofulvin and terbinafine for 60 dermatophyte samples belonging to the species *Trichophyton rubrum*, *Trichophyton mentagrophytes* and *Microsporum canis*. The percentage agreement between the two methods, for all the isolates with ≤ 2 dilutions that were tested was 91.6% for ketoconazole and griseofulvin, 88.3% for itraconazole, 81.6% for terbinafine and 73.3% for fluconazole. One hundred percent agreement was obtained for *Trichophyton mentagrophytes* isolates evaluated with ketoconazole and griseofulvin. Thus, until a reference method for testing the *in vitro* susceptibility of dermatophytes is standardized, the similarity of the results between the two methods means that the agar dilution method may be useful for susceptibility testing on these filamentous fungi.

Key-words: *In vitro* susceptibility. Agar dilution. Broth microdilution. Dermatophytes.

RESUMO

O propósito do presente trabalho foi comparar os métodos de diluição em ágar e diluição em caldo para a determinação de concentração inibitória mínima de fluconazol, itraconazol, cetoconazol, griseofulvina e terbinafina para 60 amostras de dermatófitos pertencentes às espécies, *Trichophyton rubrum*, *Trichophyton. mentagrophytes* e *Microsporum canis*. A porcentagem de acordo entre os dois métodos para todos os isolados testados considerando-se valores ≤ 2 diluições, foram de 91,6% para cetoconazol e para griseofulvina, de 88,3% para itraconazol, de 81,6% para terbinafina e de 73,3% para fluconazol. Uma concordância de 100% foi obtido para isolados de *Trichophyton mentagrophytes* avaliados com cetoconazol e griseofulvina. Desta forma, até que um método de referência seja padronizado para testar a suscetibilidade *in vitro* para os dermatófitos, os resultados semelhantes encontrados para os dois métodos fazem com que o método de diluição em ágar possa ser útil no teste de suscetibilidade para estes fungos filamentosos.

Palavras-chaves: Suscetibilidade *in vitro*. Diluição em ágar. Microdiluição em caldo. Dermatófitos.

Infections caused by dermatophytes are probably the most common cutaneous fungal diseases in humans and animals[4]. Increasing numbers of antifungal agents have been used for treating dermatophytosis[2,3]. However, not all species have the same susceptibility patterns, and relative or absolute microbial resistance may occur in relation to some dermatophytes[6]. Research to evaluate *in vitro* susceptibility has been hampered by the lack of reliable *in vitro* techniques for determining the minimum inhibitory concentration (MIC) of antifungal agents against dermatophytes.

Various methods, such as broth macro and microdilutions, agar dilution, Etest®, Sensititre® colorimetric microdilution panels and disk diffusion have been used for determining the susceptibility of dermatophytes to antifungal agents[7,12,13,14,15,16,19]. However, there is no reference method available for filamentous fungi. Dermatophytes were not included in the M38-A document, published by the Clinical and Laboratory Standards Institute in 2002[5], in which MICs for several antifungal agents against conidium-forming filamentous fungi are determined.

Standardization and the development of new methods for determining the *in vitro* susceptibility of dermatophytes to the antifungal activities of different drugs are needed. In order to

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compare such results, we used the agar dilution and broth microdilution methods for five antifungal agents against 60 dermatophyte strains belonging to three different species.

MATERIAL AND METHODS

Isolates. A total of 60 dermatophyte strains, including *Trichophyton rubrum* (27), *Trichophyton mentagrophytes* (14) and *Microsporum canis* (19) were tested. All of the microorganisms were collected from skin lesions and nails, from patients at the University Hospital of Goiania, Brazil, between March and July 2006. The fungi were maintained in sterile distilled water at room temperature and, prior to testing, the strains were subcultured onto potato dextrose agar (PDA) medium at 28°C for seven days, to ensure the viability and purity of the inoculum. *Candida parapsilosis* ATCC 22019 was included as a reference strain.

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

Broth microdilution method. The broth microdilution assay for antifungal susceptibility testing on dermatophytes was performed in accordance with the Clinical and Laboratory Standards Institute guidelines in the M38-A document on filamentous fungi[5], with some modifications. The inoculum suspensions of dermatophytes were prepared from seven-day cultures grown on potato dextrose agar at 28°C. The fungal colonies were covered with approximately 10 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 removed and transferred to sterile tubes. Heavy particles in the suspension were allowed to settle for 15 to 20 minutes at room temperature. The optical density of the suspensions containing conidia and hyphal fragments was read at 530nm and adjusted to transmittance of 65 to 70%. The concentration of colony-forming units (CFU/ml) was quantified by plating 10ml of suspension in Sabouraud dextrose agar. The plates were incubated at 28°C and the colonies were counted when the growth became visible. Each suspension was diluted (1:50) with RPMI 1640 medium (Sigma Chemical Co, St. Louis, MO, USA) to obtain the final inoculum concentration of 0.4 to 5 X 10^4 cells/ml. Aliquots of 100µl of these suspensions were inoculated into microtiter plate wells containing 100µl of specific antifungal drug concentrations. The microdilution plates were incubated at 28°C and readings were made every 24h until growth in the drug-free control well was shown. Each assay was carried out in duplicate.

Endpoint criteria. 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, it was defined as the lowest concentration showing 100% growth inhibition[18].

Agar dilution method. The agar dilution method was performed as described by Souza et al[20], with slight modification. The antifungal agents were serially two-fold diluted in RPMI 1640 agar broth medium, to obtain concentrations ranging from 1.25 to 640µg/ml for fluconazole, 0.3 to 160µg/ml for ketoconazole, itraconazole and terbinafine, and 0.3 to 80µg/ml for griseofulvin. These antifungal agents were diluted 1:10 in plates containing melted RPMI agar medium. Steel perforators were inserted into these plates after the medium had solidified, in order to produce 37 holes of 3mm diameter, as shown in **Figure 1**. The holes were filled with 10µl of inoculum containing 0.4 to 5 X 10^4 cells/ml

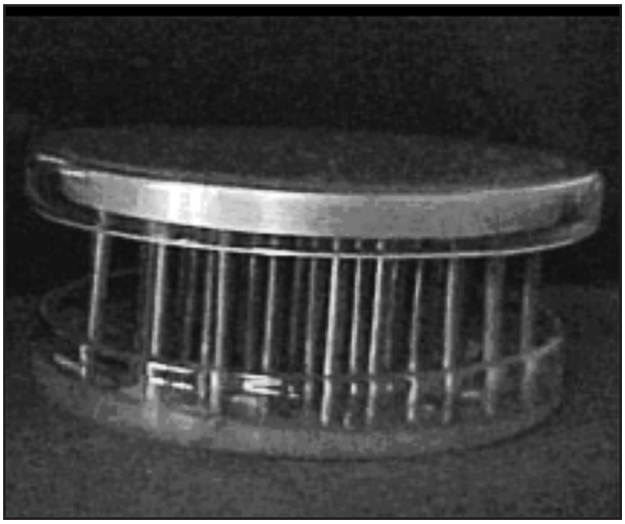

FIGURE 1

Formation of holes of 3mm in diameter in melted RPMI agar medium by using steel perforators, in the agar dilution method.

(as described for the broth microdilution method). The plates were incubated at 28°C and the growth was read every 24h, until growth in the drug-free control hole was shown. For all the antifungal agents tested, the MIC was read as the lowest drug concentration that prevented any discernible growth.

Quality control. One CLSI quality control strain (*Candida parapsilosis* ATCC 22019), which was incubated at 28°C for 48h was included on each day of testing in order to check the accuracy of drug dilutions (fluconazole, ketoconazole and itraconazole) and the reproducibility of the results.

Data analysis. All tests were performed in duplicate. MIC_{50} and MIC_{90}, at which respectively 50% and 90% of the isolates were inhibited, along with MIC ranges, were determined to facilitate comparisons between drug activity levels (**Table 1**). For each isolate, the two methods were considered to be in agreement if the difference in MIC was no more than two dilutions, as described in **Table 2**.

TABLE 1

In vitro antifungal susceptibility of 60 strains of dermatophytes as determined by broth dilution and agar dilution.

Species	Drugs	Broth dilution (μg/ml)			Agar dilution (μg/ml)		
		range	MIC_{50}	MIC_{90}	range	MIC_{50}	MIC_{90}
Trichophyton. rubrum (n=27)	Fluconazole	2-32	8	32	1-64	8	64
	Itraconazole	0.03-4	0.125	0.5	0.03-4	0.06	0.25
	Ketoconazole	0.03-4	0.125	4	0.03-8	0.125	1
	Terbinafine	0.03-0.5	0.125	0.25	0.03-0.5	0.03	0.125
	Griseofulvin	0.25-2	0.5	1	0.06-4	0.5	1
Trichophyton mentagrophytes (n=14)	Fluconazole	4-16	16	16	2-64	8	64
	Itraconazole	0.03-0.25	0.125	0.25	0.03-0.125	0.06	0.125
	Ketoconazole	0.03-1	0.125	0.25	0.06-4	0.5	1
	Terbinafine	0.03-0.5	0.06	0.25	0.03-0.06	0.03	0.03
	Griseofulvin	0.25-1	0.5	0.5	0.25-1	0.5	1
Microsporum Canis (n=19)	Fluconazole	2-32	8	16	2-64	8	64
	Itraconazole	0.03-0.25	0.125	0.25	0.03-4	0.125	0.5
	Ketoconazole	0.03-4	0.125	0.25	0.125-8	0.25	1
	Terbinafine	0.03-1	0.125	0.25	0.03-1	0.03	0.03
	Griseofulvin	0.06-8	0.25	0.5	0.06-8	0.25	1
Over all (n=60)	Fluconazole	2-32	8	32	1-64	16	64
	Itraconazole	0.03-4	0.125	0.25	0.03-4	0.06	0.25
	Ketoconazole	0.03-4	0.125	1	0.03-8	0.25	1
	Terbinafine	0.03-1	0.125	0.25	0.03-1	0.03	0.125
	Griseofulvin	0.06-8	0.5	1	0.06-8	0.5	1

MIC: minimal inhibitory concentration, μg/ml: microgramа/milliliter.

TABLE 2

Agreement between minimal inhibition concentrations (MICs) of five antifungal agents for *Trichophyton rubrum* (n=27), *Trichophyton mentagrophytes* (n=14) and *Microsporum canis* (n=19) obtained using the broth microdilution and agar dilution methods.

Species	Antifungals	Number of isolates with differences in MICs, from the broth microdilution and agar dilution methods, within the following dilutions*							Percentage agreement**
		< -2	-2	-1	0	+1	+2	>+2	
Trichophyton rubrum	Fluconazole	3	1	7	1	6	6	3	77.7
	Itraconazole	2	6	5	1	9	3	1	88.8
	Ketoconazole	4	0	1	5	12	5	0	85.2
	Terbinafine	3	12	8	3	0	0	1	85.2
	Griseofulvin	3	6	5	7	6	0	0	88.8
Trichophyton mentagrophytes	Fluconazole	3	1	2	1	3	1	3	57.1
	Itraconazole	2	1	5	2	0	4	0	85.7
	Ketoconazole	0	0	0	1	4	9	0	100
	Terbinafine	3	2	5	4	0	0	0	78.5
	Griseofulvin	0	0	3	3	6	2	0	100
Microsporum canis	Fluconazole	0	2	3	3	5	2	4	78.9
	Itraconazole	1	1	2	2	8	4	1	89.5
	Ketoconazole	0	1	0	2	4	11	1	94.7
	Terbinafine	3	5	5	4	1	0	1	78.9
	Griseofulvin	1	0	3	10	3	1	1	89.5
Overall	Fluconazole	6	4	12	5	14	9	10	73.3
	Itraconazole	5	8	12	5	17	11	2	88.3
	Ketoconazole	4	1	1	8	20	25	1	91.6
	Terbinafine	9	19	18	11	1	0	2	81.6
	Griseofulvin	4	6	11	19	16	3	1	91.6

*The differences in dilutions between the two methods were determined by taking the broth microdilution method as the reference. **Agreement between the two methods for each isolate was taken to be no difference in MIC of more than two dilutions.

RESULTS

The isolates tested produced detectable growth over the period between 72 and 120h of incubation through both the broth microdilution and the agar dilution method. MICs were determined by means of the broth microdilution method after four days of incubation, for all the isolates of *Trichophyton mentagrophytes*, and after five days for *Trichophyton rubrum* and *Microsporum canis* isolates. By using agar dilution, detectable growth was observed after five days of incubation for all the isolates (**Figure 2**).

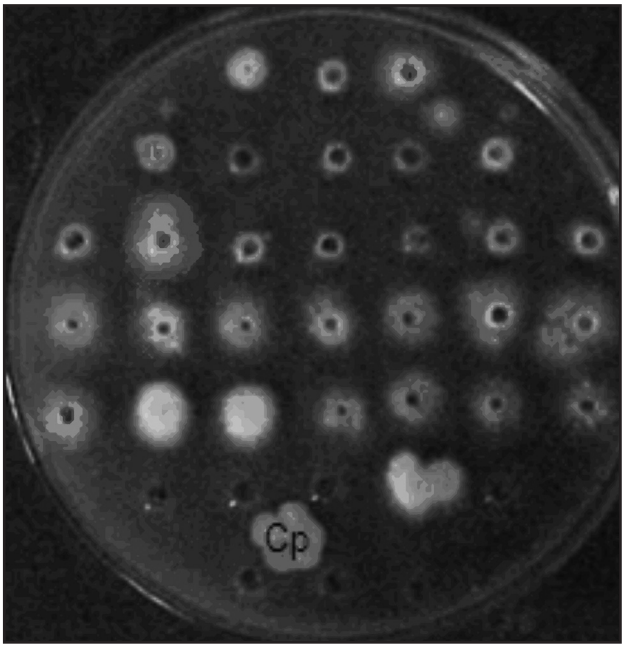

FIGURE 2

Detectable growth of dermatophyte isolates (fluconazole MIC = 2µg/ml), from the agar dilution method, after five days of incubation at 28°C. **Cp**: *Candida parapsilosis* ATCC 22019.

Table 1 shows the MIC ranges, MIC_{50} and MIC_{90} that were determined using the microdilution and agar dilution methods. In general, the ranges between these values were very narrow.

The percentage agreement between the two methods, taken such there were no differences in MICs greater than two dilutions, is summarized in **Table 2**. The highest levels of agreement were noted with ketoconazole (91.6%) and griseofulvin (91.6%), for all the isolates tested. Excellent agreement (100%) was obtained with ketoconazole and griseofulvin for *Trichophyton mentagrophytes* isolates. The lowest concordance was observed for fluconazole. The agreement rate was 73.3% for all dermatophytes, while it was only 57.1% for *Trichophyton mentagrophytes*. Fluconazole MIC values were higher using the agar dilution method than using the broth microdilution method.

Using the broth microdilution method, the MICs shown by fluconazole, ketoconazole and itraconazole in relation to *Candida parapsilosis* (ATCC 22019) were within the established ranges. The agar dilution method showed MICs for this strain that were slightly higher than those shown by broth microdilution (taking the limit of no more than two dilutions).

DISCUSSION

Although the Clinical and Laboratory Standards Institute has published a document regarding tests for filamentous fungi[5], no reference method has been established for testing the drug susceptibility of dermatophytes. Some parameters like temperature, incubation time and endpoint are difficult to standardize for *in vitro* susceptibility testing relating to filamentous fungi[1 11].

Despite the incubation temperature of 35°C that was established by the Clinical and Laboratory Standards Institute in relation to filamentous fungi, we performed the incubation at 28°C. In a preliminary experiment carried out in our laboratory, the dermatophyte strains presented better growth at 28°C than at 35°C (data not shown). According to Pujol et al[16], MICs are more reproducible at 28°C and the dermatophyte growth is more characteristic.

The MIC endpoints for our study were determined after five days for the agar dilution and after four to five days for the broth microdilution. The readings of MIC values for each method that were made every day made it possible to define this incubation time. Our results are similar to those of Ghannoun et al[10], who incubated dermatophytes for four days by using the broth microdilution method.

In the present study, similar results were obtained for MIC ranges, MIC_{50} and MIC_{90} for fluconazole, itraconazole, ketoconazole, terbinafine and griseofulvin in relation to 60 isolates of dermatophytes, investigated using both the agar dilution and the broth dilution method. The comparison between the two methods performed by Yoshida et al[21] presented similar MICs for fluconazole, amphotericin B and flucytosine, in relation to yeast isolates.

For all the isolates, we observed more than 73% concordance between the results by using these two methods for determining MICs for dermatophyte strains tested with fluconazole, ketoconazole, itraconazole, terbinafine and griseofulvin. It is interesting to note that the agreement between the two methods varied according to the dermatophyte species and the drug tested. For *Trichophyton mentagrophytes*, the agreement was 57.1% with fluconazole and 100% with ketoconazole and griseofulvin (**Table 2**).

As shown in **Table 2,** the greatest disagreement found in this study was in relation to fluconazole. Fernández-Torres et al[8] also found poor agreement for fluconazole, between the methods of broth microdilution and Etest (a method based on diffusion of the antifungal agent into an agar medium). However, there are no reasons for these disagreements between pairs of methods that have been found with fluconazole. A greater number of strains may be needed to explain these results.

Confirming the validity of our results, we found that the MIC ranges for dermatophytes in relation to different drugs were similar to those previously found by several researchers using the broth microdilution method[6 7 9 16 17]. Although high MIC values were found for some dermatophytes in our results, it was not possible to determine whether the isolates were susceptible or

resistant to the antifungal agents because no breakpoints have yet been established. Until a reference method for testing the antifungal susceptibilities of dermatophytes has been standardized, we can merely suggest that the broth microdilution and agar dilution methods may be useful for testing the susceptibility of these fungi.

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