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Evaluation of susceptibility and response in the surface of agents of surface mycoses (*Trichophyton mentagrophytes*; *T. tonsurans*) to antifungal drugs of interest in a medical clinic

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ABSTRACT

Introduction: The resistance of fungal species to drugs usually used in clinics is of great interest in the medical field. **Objective:** To evaluate susceptibility and in vitro response of species of *Trichophyton spp*. to antifungal drugs of interest in clinical medicine. **Methods:** 12 samples of clinical isolates from humans were used, nine of *T. mentagrophytes* and three of *T. tonsurans*. Susceptibility tests were performed according to the agar diffusion (AD) and broth microdilution (BM) methods. **Results:** In the AD method, the species *T. tonsurans* presented a percentage of sensitivity of 33% in relation to amphotericin B and 66% to itraconazole, with 100% resistance to ketoconazole and fluconazole. *T. mentagrophytes* also showed 100% resistance to ketoconazole in this technique, with 11% sensitivity to ketoconazole, 22% to itraconazole and 22% of samples classified as sensitive dose dependent. In the MC method, the species *T. tonsurans* presented a sensitivity percentage of 66%, 55% and 33% in relation to ketoconazole, fluconazole and itraconazole, respectively. The *T. mentagrophytes* species presented sensitivity percentages of 11%, 11%, 33% and 55% for amphotericin B, itraconazole, ketoconazole and fluconazole, respectively. **Conclusion:** There was resistance in vitro of the species of *T. mentagrophytes* and *T. tonsurans* against the antifungal fluconazole and relative resistance against ketoconazole in the AD method. In BM, however, important percentages of sensitivity were observed for the two species analyzed in relation to the antifungals fluconazole and ketoconazole when compared to itraconazole and amphotericin B.

Keywords: antifungal agents; disease susceptibility; microbial sensitivity tests; *Trichophyton*.

INTRODUCTION

The term dermatophytes are used to designate a group of fungi that invade keratinized tissues, such as the skin, nails, and hair of humans and animals, producing superficial mycoses called dermatophytoses[1]. These fungi comprise several species distributed in seven

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genera: *Trichophyton*, *Epidermophyton*, *Nannizzia*, *Paraphyton*, *Lophophyton*, *Microsporum* and *Arthroderma*[2]. Such microorganisms are differentiated in macroscopic, microscopic, physiological and molecular aspects, having as criteria in vitro morphological characters and combination of clinical pictures[2]. They are classified as geophilic, zoophilic and anthropophilic microorganisms and can be transmitted by direct and/or indirect contact with infected animals and humans[3].

Infection by dermatophytes can assume a wide clinical-morphological variety, depending on the anatomical structure affected and the species involved. The characteristic lesions in skin infections are generally circular, erythematous, pruritic, with regular or irregular edges and result from the direct action of the fungus or from hypersensitivity reactions to the microorganism and/or its metabolic products. In onychomycosis, for example, there may be removal of edges, thickening, the appearance of white spots and total dystrophy of the nails[3].

It is estimated that 20 to 25% of the world population is affected by dermatophytoses and that 30 to 75% of adult individuals are asymptomatic carriers of these pathogens. These numbers make dermatophyte infections one of the most common types of infectious diseases in the world[4].

The South and Southeast regions of Brazil have shown an increase in the number of infections caused by the dermatophytes Trichophyton rubrum, accompanied by *Microsporum canis* and *Trichophyton mentagrophytes*. In the Northeast region, on the other hand, there is a higher prevalence of *Trichophyton tonsurans*, *T. rubrum* and *M. canis*[5].

The selection of the most appropriate therapy for dermatophytosis is determined by the anatomical site, the size of the infection, the etiologic agent, the effectiveness of the treatment, the safety and availability of drugs, with the option of choosing topical, systemic forms, or even associating these two treatment strategies[6].

The treatment of dermatophytoses has been the subject of discussions in the medical field due to the increase in the incidence of these diseases worldwide, as well as the expansion in the use of antifungals and the appearance of resistant strains, the main drugs used in clinical therapy[7]. On this subject, the scientific literature shows, however, that not all species have the same susceptibility pattern, with relative or absolute resistance concerning the various species and strains of fungi[2,7-10].

Studies that evaluate the in vitro susceptibility of these pathogens have described the techniques for determining the minimum inhibitory concentration (MIC) of antifungals against dermatophytes as laborious, time-consuming and difficult to apply in clinical practice[8-10]. This group of fungi is also not included in document M38-A, published by the Clinical and Laboratory Standards Institute (CLSI) in 2008, in which the MICs of various antifungal agents against the formation of filamentous conidia are determined[11].

Considering the clinical and epidemiological importance of dermatophytoses, especially those that originate from the species of *T. tonsurans* and *T. mentagrophytes*, it is relevant to carry out research that focuses on the use of antifungal drugs and compare the methods to determine susceptibility in of these agents and thus indicate a more effective treatment.

The aim of this study was to evaluate the susceptibility and *in vitro* response of species of *Trichophyton spp.* to usual antifungal drugs in the field of clinical medicine.

METHODS

Sampling

Twelve samples of *Trichophyton* species isolated from humans that presented a clinical picture of dermatophytosis were used, nine isolates of T. mentagrophytes and three of *T. tonsurans*. Such samples were provided by the Departamento de Micologia do Centro de Ciências Biológicas da Universidade Federal de Pernambuco (CCB-DM/UFPE) and were stored under mineral oil in the Coleção de Cultura Micoteca URM (Micoteca-URM) from UFPE.

Four commercially available antifungals were used for the treatment of dermatophytosis: ketoconazole (Attivos Magistrais, Brazil), fluconazole (Sigma, USA), itraconazole (Jansen-Cilag, USA) and amphotericin B (Inlab, Brazil).

Reactivation and purification

For reactivation, the samples were sown in glucose broth. After growth, it was carried out to the Sabouraud agar culture environment contained in a test tube. After the growth period (7 to 15 days), suspensions were prepared in sterile distilled water with the addition of chloramphenicol antibiotic (Inlab, Brazil) and sown on Sabouraud agar contained in Petri dishes, using the depletion technique (after 15 days). The samples were kept at room temperature (±28ºC) to confirm the pure culture and were later sown in tubes.

Procedures for assessing antifungal activity

The susceptibility of the isolates was performed using the agar diffusion method against itraconazole, ketoconazole and amphotericin B, which were dissolved in dimethylsulfoxide (DMSO; Vetec, Brazil), and fluconazole dissolved in distilled water. The methodology used was suggested by Barry and Brown[12], later being standardized by the CLSI document M44-P13 and M44-A1[4]. For the microdilution test, the method described in CLSI document M38-A11 was used and suitable for dermatophytes according to Fernández-Torres et al.[15] and Barros et al.[16]. The culture environment used for this procedure was RPMI 1640 (Sigma, USA) with L-glutamine without sodium bicarbonate, buffered to pH 7.0

using MOPS (3-(N-morpholino) propanesulfonic acid) at 0.165 M previously sterilized by membrane filtration with 0.22 µm porosity (Amresco, USA).

Agar diffusion

A suspension containing 107 Colony Forming Units (CFU)/mL of each species of dermatophyte was inoculated onto Petri dishes containing Müeller-Hinton Agar (Laborclin, USA) added with 2% glucose and 0.05 µg/mL blue methylene (Synth, Brazil), with the aid of a sterile swab, 30 minutes before adding antifungals. After that time, five 5 mm diameter wells were opened in the culture medium with the aid of sterile molds, where the four antifungals were tested: fluconazole at a concentration of 25 µg/mL, ketoconazole at a concentration of 15 µg/mL, itraconazole at concentration of 10 µg/mL, amphotericin B at a concentration of 10 µg/mL per plate, for each species of *Trichophyton spp.*, depositing 25 µL of the antifungal and negative dimethylsulfoxide control (DMSO; Vetec, Brazil) at a concentration of 100 µg/mL.

The plates were incubated at 37ºC for a period of 15 days, with daily observations. The experiments were performed in duplicate and the sensitivity was determined by observing the presence or absence of growth and the size of the formed halo measured with the aid of a halometer.

Samples of *Trichopyton sp.* were interpreted according to the parameters of the inhibitory halos suggested by CLSI M44-A[14]. For fluconazole, halo ≥19 mm (sensitive), 18-15 mm (dose dependent sensitive) and ≤14 mm (resistant); ketoconazole, halo ≥28 mm (sensitive), 27-21 mm (dose dependent sensitive) and ≤20 mm (resistant); itraconazole, halo ≥23 mm (sensitive), 22-24 mm (dose dependent sensitive) and ≤13 mm (resistant); amphotericin B, halo ≥15 mm (sensitive), 14-20 mm (dose dependent sensitive) and <10 mm (resistant).

Microdilution in broth

The microdilution test was performed on sterile microdilution plates, with 96 U-shaped wells. Amphotericin B was solubilized in dimethylsulfoxide (DMSO) and the other antifungals in distilled water. Each antifungal drug was distributed in serial dilutions in columns 1 to 10 in 96-well microplates.

Antifungals were tested in 10 concentrations with the following limits: 0.031 to 16.0 µg/mL for amphotericin B, itraconazole and ketoconazole; for fluconazole, concentrations of 0.12 to 64 µg/mL were used. For the assay 100 µL of the measured inoculum was added and the plates were incubated at 25°C for seven days.

A positive control was included in each test plate, represented by the growth of each isolated fungus in the absence of drugs, and a negative control, which corresponds to the absence of drugs and fungi. The determination of the Minimum Inhibitory Concentration (MIC) of antifungals was performed visually by reducing the fungal growth, where the reading of the results was also carried out by observing the reduction in fungal growth.

Data analysis

The values suggested by CLSI M44-A[14] for the inhibitory halos formed in each of the microbiological isolates were used as parameters for the interpretation and analysis of antifungal sensitivity.

The data were grouped in tables, presented in terms of absolute and relative frequency, and the discussion of the findings was carried out in the light of specialized literature subjects.

RESULTS AND DISCUSSION

Results were obtained regarding the sensitivity to antifungals of 12 isolates belonging to the genus *Trichophyton spp.*, nine of the species *T. mentagrophytes* and three of the species *T. tonsurans*, from which the sensitivity test was carried out using agar diffusion techniques (Table 1) and the broth microdilution method (Table 2).

T. mentagrophytes isolates showed a high percentage of sensitivity to itraconazole (66.67%) and resistance to fluconazole (100%) in agar diffusion. The most important limitations of fluconazole, according to the literature, are related to its lack of activity against filamentous fungi[17,18]. Studies have pointed out that such an antifungal agent has been associated with the development of resistance among yeasts, molds and dermatophytes[16-20].

Table 1: Susceptibility profile to antifungal drugs from clinical isolates of *T. mentagrophytes* and *T. tonsurans* using the agar diffusion method (AD).

Antifungal	Species	Halo variation (mm)			Susceptibility		
		S	SDD	R	S (%)	SDD (%)	R (%)
Amphotericin B	*T. mentagrophytes* (n=9)	≥15	14-10	<10	0 (0%)	2 (22.22%)	7 (77.78%)
	T. tonsurans (n=3)				1 (33.33%)	1 (33.33%)	1 (33.33%)
Ketoconazole	*T. mentagrophytes* (n=9)	≥28	27-21	≤20	1 (11.11%)	0 (0%)	8 (88.89%)
	T. tonsurans (n=3)				0 (0%)	0 (0%)	3 (100%)
Itraconazole	*T. mentagrophytes* (n=9)	≥23	22-14	≤13	2 (22.22%)	2 (22.22%)	5 (55.56%)
	T. tonsurans (n=3)				2 (66.67%)	0 (0%)	1 (33.33%)
Fluconazole	*T. mentagrophytes* (n=9)	≥19	15-18	≤14	0 (0%)	0 (0%)	9 (100%)
	T. tonsurans (n=3)				0 (0%)	0 (0%)	3 (100%)

S: Sensible, SDD: Sensitive Dependent Dose, R: Resistant.

Table 1 shows that the isolates of the genus *Trichophyton spp.*, both species *T. mentagrophytes* and *T. tonsurans*, were resistant to fluconazole in the agar diffusion method. These data corroborate the findings of the study by Silva et al.[21], in which *T. mentagrophytes* isolates also presented resistance to fluconazole. However, they differ from the results observed by other authors who demonstrated the susceptibility of species of the genus *Trichophyton spp.*[15] to fluconazole. This may occur, according to the literature consulted, due to the variability of the methods used, the lack of standardization of specific techniques for assessing the susceptibility of dermatophytes and the very imprecision in terms of cutoff points to determine the resistance of this genus to different antifungals[15,19,20].

From the nine samples of *T. mentagrophytes* analyzed using the agar diffusion method, four showed a halo of inhibition against amphotericin B, two classified as sensitive dose dependent (SDD), for having 12 mm diameter halos, and two classified as resistant because they have 8 mm diameter halos.

Regarding ketoconazole, two isolates of *T. mentagrophytes* presented an inhibition halo, one of which was classified as sensitive, with a 30 mm halo, and the other was resistant, for presenting a 20 mm inhibition halo.

Four samples of *T. mentagrophytes* showed growth inhibition compared to itraconazole, with three samples classified as sensitive dose dependent, for having a halo between 20-16 mm, one classified as sensitive, with a halo of 24 mm.

In relation to the three samples of *T. tonsurans*, two isolates presented an inhibition halo compared to amphotericin B, where one was classified as sensitive dose dependent for presenting a 12 mm halo and the other sensitive for presenting a 24 mm halo. For itraconazole, two isolates were classified as sensitive, with halos of 24 mm and 32 mm. All samples were resistant to ketoconazole and fluconazole, as they did not present a halo formation showing growth inhibition (Table 2).

The results obtained in the susceptibility tests using the broth microdilution method showed a percentage of *T. mentagrophytes* resistance of 88% for both amphotericin B and itraconazole in assessing the sensitivity of the isolates.

In this method, *T. tonsurans* was more sensitive to ketoconazole (66%), fluconazole (55%) and itraconazole (33%) in relation to the percentage of sensitivity obtained for T. mentagrophytes.

Both species had the same MIC_{50} and MIC_{90} values compared to itraconazole. The species *T. mentagrophytes* showed higher values of MIC_{90} for the drugs amphotericin B, fluconazole and ketoconazole in relation to the species *T. mentagrophytes*. In a study by Siqueira et al.[22] the minimum inhibitory concentration range (MIC_{50} and MIC_{90}) of species of the genus *Trichophyton spp*. was from 0.08 to 0.4 µg/mL for itraconazole, 0.09 to 1.1 µg/mL for ketoconazole and 16.2 to 24 µg/mL for fluconazole, these values being lower than those found in this work.

According to Araújo et al.[23] the in vitro determination of susceptibility has shown promising results in predicting the ability of a given antifungal agent to eradicate dermatophytes. For the authors, although there is no reference method for dermatophytes, a good correlation has been observed between in vitro and in vivo results in studies.

The sensitivity and resistance profile of the genus *Trichophyton spp*. compared to the antifungals used in this research, it was partially compatible with the results found by Magagnin et al.[19]. The authors obtained 53.8% of resistance of the genus *Trichophyton spp.*, compared to ketoconazole, 100% to fluconazole and 42.3% to itraconazole. In work developed by Grisolia[24], resistance to these drugs for the two species of *Trichophyton spp*. investigated. In their work, Gupta et al.[17] found a better response of itraconazole compared to fluconazole in the topical treatment of dermatophytoses.

Comparing the results of the agar diffusion and broth microdilution methods, it was possible to observe the sensitivity profile of samples of the genus *Trichophyton spp.* against the antifungals used. In general, it was verified: greater susceptibility of species of the genus *Trichophyton spp.* through the broth microdilution method in relation to the agar diffusion method; greater sensitivity to *T. tonsurans* samples using the agar diffusion method; greater resistance to both methods of *T. mentagrophytes* samples. Such findings are compatible with the studies by Mota et al.[25], Johnson et al.[26], Balouiri et al.[27] and Dogra et al.[10] who also evaluated the

Table 2: Minimum inhibitory concentration and susceptibility profile to antifungal drugs from clinical isolates of *T. mentagrophytes* and *T. tonsurans* using the broth microdilution (BM) method.

Antifungal	Species	Interval (µg/mL)	CIM 50 (µg/mL)	CIM 90 (µg/mL)	Susceptibility*	
					%S	%R
Amphotericin B	*T. mentagrophytes* (n=9)	0,125-4	16	>64	11.11	88.88
	T. tonsurans (n=3)	4-64	8	32	0.00	100.00
Ketoconazole	*T. mentagrophytes* (n=9)	0,5->64	32	64	33.33	66.66
	T. tonsurans (n=3)	2-32	8	32	66.66	33.33
Itraconazole	*T. mentagrophytes* (n=9)	0,125->64	16	64	11.11	88.88
	T. tonsurans (n=3)	0,125->64	16	64	33.33	66.66
Fluconazole	*T. mentagrophytes* (n=9)	16->64	32	64	55.55	44.44
	T. tonsurans (n=3)	4-32	8	32	100.00	0.00

* cutoff point based on CLSI (Clinical and Laboratory Standards Institute).
S: susceptibility, R: resistance.

susceptibility of filamentous microorganisms using the agar and microdilution dilution methods in broth and identified that the latter is the most sensitive for research of dermatophyte fungi.

Another antifungal drug, not used in this work for operational reasons, but which has also been used in clinical practice against superficial and subcutaneous mycoses is terbinafine hydrochloride. In a study carried out by Diogo et al.[28], the action of terbinafine (0.125-100 μg) against *T. mentagrophytes* and *T. tonsurans* was evaluated using the methods of disc diffusion and microdilution, with a high degree of sensitivity of species in the genus *Trichophyton spp.* at all evaluated terbinafine concentrations, with the formation of halos greater than ≥40 mm and inhibitory concentrations calculated at 0.015 mg/mL. Other studies[29,30] corroborate the findings of Diogo et al.[28] and report excellent results of sensitivity of dermatophyte species to terbinafine.

It is concluded, therefore, that the present study allowed to observe that there was resistance in vitro of the species of *T. mentagrophytes* and *T. tonsurans* against the antifungal fluconazole and relative resistance against ketoconazole through the agar diffusion method. Furthermore, for this same technique, there was a variable and discontinuous sensitivity profile of the species studied to the antifungals amphotericin B and itraconazole.

In the broth microdilution method, in turn, there was an important percentage of sensitivity of the species of *T. mentagrophytes* and *T. tonsurans* to the antifungals fluconazole and ketoconazole when compared to itraconazole and amphotericin B.

This study evaluated the susceptibility, in vitro, of *T. tonsurans* and *T. mentagrophytes* to different antifungals used in medical practice. Its design included a lineage of species of *Trichophyton spp.* isolated from humans, it is impossible to extend the results obtained to the entire set of fungi of the genus and species studied. Also, research planning included a limited number of drugs. One of the contributions of this work is that it highlights the profile of the selected clinical samples from a region and how the microorganisms responded, in vitro, to the selected antifungals. The importance of the topic in the treatment and clinical prognosis of dermatophytoses requires additional investigations in the area, and studies aimed at correlating data in vitro and in vivo.

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