Antifungal Susceptibility Testing For Dermatophytes Isolated From Human and Animal Dermatophytosis

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Abstract

This study was designed to characterize antifungal susceptibility pattern of different species of dermatophytes isolated from human and animal dermatophytosis against six commercially available antifungal agents recommended for treatment. Broth microdilution method was used to determine minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of different drugs against the tested dermatophyte species. MIC was done against fluconazole, itraconazole, terbinafine, tioconazole, miconazole and griseofulvin. Of the antifungal agents tested, the best results in terms of sensitivity were found with terbinafine for all isolates and tioconazole, miconazole for *M. canis* and *T. verrucosum* while the antifungal activity of fluconazole was found to be weak. Routine detection of MIC for antifungal agents serves as a rapid and reliable method for treatment of human and animal dermatophytosis.

Introduction

Dermatophytes are a group of fungi that infect keratinized tissues such as hair, skin, and nail. In recent years, the incidence of infections caused by dermatophytes has increased considerably, especially in immunocompromised patients. These fungal infections establish an important public health problem because of prolonged treatment of the disease and its refractivity to therapy ***(Yang et al., 2007).*** The selection of proper therapeutic options is usually decided by location and extent of infection. Topical therapies with azoles are used for localized dermatophyte infections. For the treatment of extensive or resistant dermatophytosis, systemic drugs are required ***(Fernandez-Torres et al., 2002).*** Although many topical and oral antifungal agents are available for the treatment of dermatophytosis, some patients have a poor clinical response to these drugs. *In vitro* antifungal susceptibility testing has become important for choosing an effective antifungal therapy ***(Chadeganipour et al., 2004).*** *In vitro* susceptibility testing also provides monitoring of the development of antifungal resistance.

Clinical and Laboratory Standards Institute (CLSI) had developed the standard broth micro dilution M38-

A2 method for antifungal susceptibility of some filamentous fungi, including the dermatophytes in 2008 ***(CLSI, 2008 and Fernandez-Torres et al., 2002).*** Various studies with variable results were reported for antifungal susceptibility testing of dermatophytes. In developing a standardized method for antifungal susceptibility testing of dermatophytes several variables need to be considered, like the medium for conidiation, the size of inoculum, temperature and duration of incubation, medium of inoculation, and endpoint determination.

Antifungal susceptibility test by broth microdilution technique is method of choice where disc diffusion is not applicable. Terbinafine is the drug of choice for dermatophytes ***(Jha et al., 2015).***

Dermatophyte species are closely related to each other phylogenetically and drugs that are effective against one species are generally effective against others ***(Gupta et al., 1999).*** There are a few exceptions to this generality: for example, *Trichophyton verrucosum* and ‘‘*Trichophyton mentagrophytes* have limited susceptibility to fluconazole ***(Rippon and Fromting 1993),*** a drug that is by no means the most commonly used in treating dermatophytosis.

In this study, we aimed to establish the *in vitro* antifungal susceptibilities of fluconazole, terbinafine, miconazole, itraconazole, griseofulvin, and tioconazole against dermatophyte isolates by broth microdilution method.

Material and Methods

Dermatophyte isolates:

Dermatophyte species (n = 12) were isolated from human and animal dermatophytosis and identified as: *Trichophyton violaceum*, *Trichophyton verrucosum*, *Trichophyton mentagrophytes* and *Microsporum canis.* The cultures were freshly growing on Sabouraud dextrose agar slants supplemented with cycloheximide, chloramphenicol then transferred to oatmeal agar slants two weeks prior to the study to enhance conidial production.

Antifungal susceptibility testing:

Antifungal susceptibility testing was conducted in accordance with the broth microdilution method proposed in protocol M38-A of the Clinical and Laboratory Standards Institute ***(CLSI, 2002)*** and adapted for dermatophytes. six commercially available antifungal agents recommended for the treatment of dermatophytosis were used: grisofulvin 125 mg tablet (Ultragrisofulvin),

terbinafine 250 mg tablet (Terbin, GNP), fluconazole 150 mg tablet (Diflucan, Pfizer), itraconazole 100 mg tablet (Sporanox, Janssen-Belgica),

tioconazole 100 mg tablet (Gyno-Trosyd, Pfizer) and miconazole 20

mg/ml spray (Micoban spray, Amriya Pharm).

The stock solution of antifungal agents was prepared in 100% dimethyl sulfoxide (DMSO; Vetec, Brazil) except fluconazole was dissolved in RPMI1640 and dilutions were later made in RPMI 1640 medium (Sigma, St. Louis, MO, USA) buffered at pH 7.0 to obtain concentrations of 0.25 to 128 g/ml for fluconazole and 0.03 to 16 µg/ml for the other antifungal agents.

Stock inoculum suspensions were obtained from each strain by covering the fungal colonies with 1 ml of sterile saline and gently probing the surface with the tip of a transfer pipette. The resulting mixture of conidia was transferred to sterile tubes and allowed to settle for 10–15 minutes. Then, conidia were counted with a hemocytometer and diluted with RPMI 1640 medium (Sigma) buffered with MOPS (3-[N-morpholino] propanesulfonic acid) (Sigma) to obtain the final inoculum size of approximately 1–3 x 10^3 conidia/ml.

The assay was performed using sterile, 96-well plates with a U-shaped base into which 100 µL were added of each antifungal concentration to be tested.

Next, 100 µl aliquots of the 1:50 dilution of the inoculum were added to each one of the wells. An antifungal-free control (growth control) and a control containing no organisms (sterility control) were included in these tests. The plates were incubated at 28ºC for three days.

Minimum inhibitory concentration (MIC) was determined visually by comparing the test with the growth of the drug-free control. MIC was defined as the lowest concentration of the drug capable of completely inhibiting fungal growth in the case of itraconazole and terbinafine and capable of inhibiting 80% of growth in the case of the other antifungal agents ***(Gupta and Kohli 2003).*** All the experiments were performed in triplicate.

After reading the MIC, the minimum fungicidal concentration (MFC) was determined. A 100 µl aliquot from the wells in which no growth was observed was transferred to test tubes containing 2 ml of Sabouraud-dextrose broth (Difco, Detroit, MI, USA). A positive control (growth control) and a negative control (sterility control) were included in the test. The tubes were incubated for 7 days at 28ºC and growth was observed visually. MFC was defined as the minimum concentration at which no fungal growth occurred ***(Favre et al., 2003).*** These assays were performed in duplicate.

Results

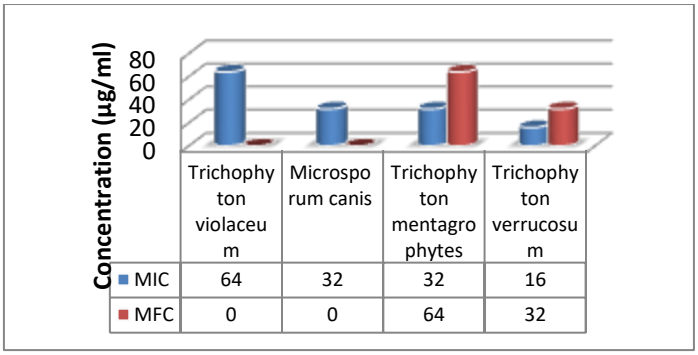
The *in vitro* sensitivity profile of six commercially available antifungal agents against different species of dermatophytes isolated from human and animal dermatophytosis was evaluated using the broth

microdilution method. Of the azole antifungal agents, the best results in terms of MIC values were found with tioconazole, miconazole, itraconazole (Table 1). The results obtained with tioconazole were significantly better in case of *T. mentagrophytes* and *T. verrocosum*, since this drug had the lowest geometric mean MIC (Table 2). On the other hand, the activity of fluconazole was weak against all the species with the exception of *T. verrucosum*, one isolate of *M.canis* and one isolate of *T. mentagrophytes*. Of the non-azole antifungal agents tested, terbinafine was found to be the most effective, followed by griseofulvin.

Table (1): *In vitro* susceptibility testing of four species of dermatophytes against six commercially available antifungal agents

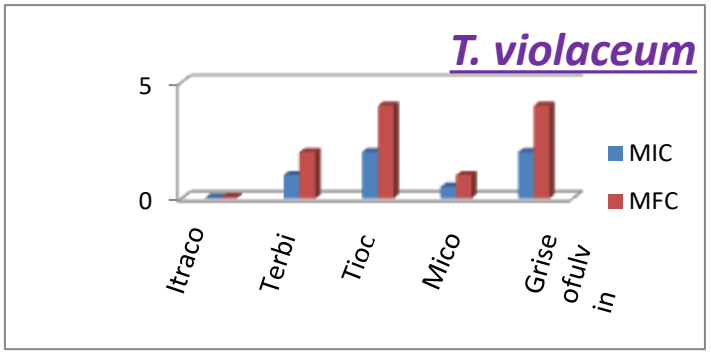
Isolates	Antifungal agents (µg/ml)											
	Fluconazole		Itraconazole		Terbinafine		Tioconazole		Miconazole		Griseofulvin	
	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC
T. violaceum												
1	64	1	0.03	0.06	1	2	2	4	0.5	1	2	4
2	128	0	2	4	4	8	4	8	2	4	4	8
3	128	0	1	2	2	4	4	8	4	8	2	4
GM	101.59		0.39		2.00		3.17		1.59		2.00	
range	64-128		0.03-1		1-4		2-4		0.5-4		2-4	
Mean±SE	106.67 ±21.33		1.01 ±0.57		2.33 ±0.88		3.33 ±0.67		2.17 ±1.01		2.67±0.67	
M. canis												
1	128	0	4	8	2	4	4	8	2	4	1	2
2	128	0	4	8	0.5	1	2	4	2	4	0.25	0.5
3	128	0	2	4	4	8	2	4	0.5	1	8	16
4	64	128	2	4	2	4	0.5	1	0.06	0.125	0.125	0.25
5	128	0	4	8	0.25	0.5	4	8	0.03	0.06	0.25	0.5
6	32	64	4	8	2	4	0.5	1	0.25	0.5	1	2
GM	128.00		3.17		1.59		2.52		1.26		1.26	
range	32-164		2-4		0.24-4		0.5-4		0.06-2		0.125-8	
Mean±SE	128.00± 0.00		3.33 ±0.67		2.17 ±1.01		2.67 ±0.67		1.50±0.50		3.08±2.47	
T. mentagrophytes												
1	32	64	2	4	0.25	0.5	0.25	0.5	2	4	0.5	1
2	64	128	1	2	0.25	0.5	1	2	2	4	0.5	1
3	64	128	4	8	0.5	1	2	4	4	8	2	4
GM	31.99		1.82		0.25		0.25		1.82		0.48	
range	32-64		1-4		0.25-0.5		0.25-2		2-4		0.5-2	
Mean±SE	32.00± 0.58		2.00 ±0.58		0.25 ±0.01		0.25 ±0.01		2.00 ±0.58		0.50±0.10	
T. verrucosum												
1	16	32	2	4	1	2	0.25	0.5	0.125	0.25	4	8
2	32	64	4	8	1	2	0.03	0.06	0.03	0.06	4	8
GM	22.63		2.83		1.00		0.09		0.06		4.00	
range	16-32		2-4		1		0.03-0.25		0.03-0.125		4	
Mean±SE	24.00± 8.00		3.00 ±1.00		1.00 ±0.00		0.14 ±0.11		0.08 ±0.05		4.00±0.00	

MIC: minimum inhibitory concentration; MFC: minimum fungicidal concentration. GM, geometric mean; SE, standard error.

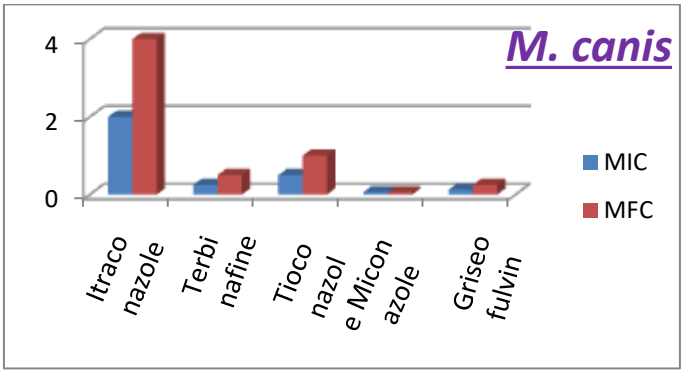


	Trichophyton violaceum	Microsporum canis	Trichophyton mentagrophytes	Trichophyton verrucosum
MIC	64	32	32	16
MFC	0	0	64	32

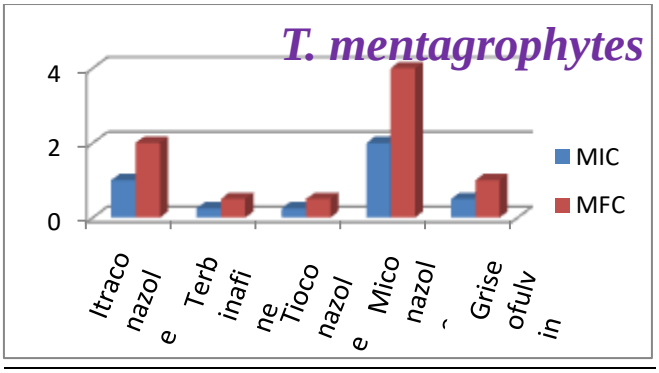
GRAPH 1: *Sensitivity profile of dermatophytes to Fluconazole, based on the geometric mean minimum inhibitory concentration (MIC) and on the geometric mean minimum fungicidal concentration (MFC).*



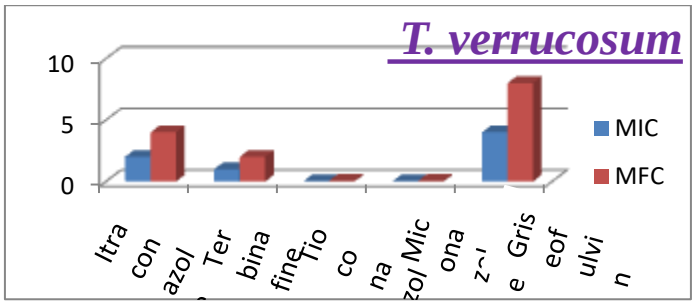
GRAPH 2: *Sensitivity profile of T. violaceum to the antifungal agents: Itraconazole, Terbinafine, Tioconazole, Miconazole and Griseofulvin*



GRAPH 3: *Sensitivity profile of M. canis to the antifungal agents: Itraconazole, Terbinafine, Tioconazole, Miconazole and Griseofulvin*



GRAPH 4: *Sensitivity profile of T. mentagrophytes to the antifungal agents: Itraconazole, Terbinafine, Tioconazole, Miconazole and Griseofulvin*



GRAPH 5: *Sensitivity profile of T. verrucosum to the antifungal agents: Itraconazole, Terbinafine, Tioconazole, Miconazole and Griseofulvin*

Discussion

Dermatophyte infections are probably the most common cutaneous fungal infections in humans and animals ***(Chinelli et al., 2003).*** Over the past few decades, the number of antifungal agents used in clinical practice for the treatment of dermatophytoses has increased ***(Barchiesi et al., 2001).*** Nevertheless, not all species have the same susceptibility pattern and there is evidence that dermatophytes have become resistant to certain antimycotics ***(Fernández-Torres et al., 2002).***

In this study the parameters established in the Clinical and Laboratory Standards Institute (CLSI) M38-A document for filamentous fungi were taken into consideration, which establish MIC resistance ≥ 64 µg/ml for fluconazole and MIC ≥ 8 µg/ml for itraconazole ***(CLSI, 2002)***.

Fluconazole (FCZ) was found to be the least active of all the antifungal agents evaluated and this is in agreement with results published from other studies ***(Da Silva-Barros and Hamdan, 2005).*** Furthermore, *Trichophyton violaceum*, 17% of

Microsporum canis showed resistance to fluconazole, findings that were in agreement with the results published by ***Da Silva Barros and Hamdan (2005).***

As regards *Trichophyton verrucosum* and *Trichophyton mentagrophytes* have limited susceptibility to fluconazole and this result in agreement with ***(Rippon and Fromting 1993)*** who found that fluconazole a drug that is by no means the most commonly used in treating dermatophytosis.

Terbinafine is the most sensitive drug against *Microsporum canis* and *Trichophyton mentagrophytes* as mentioned by ***Lupi et al. (2005) and Santos and Hamadan (2005)*** who found that Terbinafine is the most sensitive drug against *Microsporum canis* and *Trichophyton mentagrophytes* and it provides long term clinical efficacy and lower relapse.

It was found that miconazole, terbinafine, and griseofulvin were the most ideal antifungal drugs for the treatment of dermatophytosis and this result in agreement with ***Agarwal et al., (2015) and Afshari et al., (2016).***

The present study resulted that isolates of dermatophytes were resistant for fluconazole dermatophytosis and this result in agreement with ***Araújo et al., (2009)*** who showed that the antifungal drugs of fluconazole, itraconazole, ketoconazole, terbinafine and griseofulvin, with exception of fluconazole, displayed good activity against the dermatophytes. The resistance for fluconazole may be attributed to fluconazole is the drug which is more frequently used in the hospital so it is acquiring resistance.

Regarding the data obtained for *T. violaceum*, it was revealed that it was highly sensitive for griseofulvin, moderately sensitive for itraconazole and less sensitive for fluconazole these data agreed with ***Pakshir et al. (2009).***

This study showed that fluconazole had the lowest activity against dermatophytes and this result in agreement with ***Favre et al., 2003, Singh et al., 2007 and Pakshir et al., 2009.***

The MIC for terbinafine from 0.25 - 4 μg/ml, itraconazole ranged from 0.03 -4 μg/ml, fluconazole from 16 - 128 μg/ml and griseofulvin from 0.125- 8 μg/ml. Our results were significantly higher than the result obtained by ***Santos and Hamadan (2005)*** where MICs were, 0.007-0.0015 for terbinafine, 0.062-1.0 for itraconazole and 0.025-2.0 for griseofulvin. Our reports were also higher than the MIC obtained by Jha ***et al. (2015)*** where MICS were, 0.03-0.5 for terbinafine, 0.03-4 for itraconazole, 4-64 for fluconazole and 0.25-1 for griseofulvin. The higher MIC than those of other research, possibly because of increased use and misuse of antifungal agents, partial dose of the drug for shorter period and over the counter medication directly by patient remains chronic infection.

The obtained result on terbinafine as the most active agent agrees with the observation from previous authors ***Fernandez-Torres et al., (2001)*** *and* ***Santos and Hamdan (2005)***. This antimycotic showed an excellent in vitro potency and broad-spectrum activity against all the tested species. This suggests that terbinafine can be used to treat a majority of dermatophytic infections especially those showing high MIC values on the azoles.

In this study itraconazole minimal inhibitory concentrations (MIC) varied from 0.03 to 4 μg/mL in the microdilution method; for fluconazole, MICs were in the range of 16 to 128 μg/mL and these results in disagreement with ***Siqueira et al. (2008)*** who found that itraconazole minimal inhibitory concentrations (MIC) varied from 0.03 to 0.5 μg/mL in the microdilution method; for fluconazole, MICs were in the range of 0.125 to 16 μg/mL. This rise in MIC in our study may be due to the misuse of antifungal agents.

The most effective antifungal agents were miconazole and tioconazole against *T. verrucosum* and terbinafine for the other species including *M. canis* as reported *by* ***Nweze et al., (2007) and Araújo et al., (2009).***

Conclusion:

It was concluded that terbinafine was the most effective drug against all isolates and tioconazole, miconazole for *M. canis* and *T. verrucosum* while the antifungal activity of fluconazole was found to be weak. Routine detection of MIC for antifungal agents serves as a rapid and reliable method for treatment of human and animal dermatophytosis.

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اختبار حساسية الفطريات الجلدية المعزولة من الإنسان والحيوان

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هذا البحث يهدف الى عمل اختبار حساسية الفصائل المختلفة من الفطريات الجلدية المعزولة من الإنسان والحيوان ضد ستة أدويه للفطريات.حيث تم إستخدام طريقة التخفيف الدقيق جدا للسوائل لتحديد التركيز الاقل فى منع النمو والتركيز القاتل من الادوية الذى يمنع نمو الفطريات الجلدية للعزلات تحت الدراسة . وكانت الادوية المستخدمة كالآتى فلوكونازول و إتراكونازول و تربينافين و تيوكونازول و ميكونازول و جريزوفلفين.وأظهرت النتائج أن كل العزلات كانت ذات حساسيه عالية الى تربينافين وكانت الميكروسبورم كانز و ترايكوفايتون فريكوزم ذات حساسية عالية الى التيوكونازول و الميكونازول. يمكننا القول أن طريقة تحديد اقل الادوية تركيزا فى منع نمو الفطريات الجلدية تعتبر من اسرع واكثر الطرق المعتمد عليها فى معالجة الفطريات الجلدية فى الانسان والحيوان.