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In-vitro susceptibility testing of dermatophytes isolated in Cairo, Egypt against eight antifungal agents by broth microdilution and disk diffusion methods

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Abstract: Nine dermatophytic strains belonging to 7 species, namely *Microsporum canis* (1), *Trichophyton tonsurans* (1), *Trichophyton rubrum* (2), *Trichophyton mentagrophytes* (1), *Trichophyton violaceaum* (1), *Arthroderma* sp. (1), and *Epidermophyton floccosum* (2) isolated from clinical specimens in Cairo, Egypt were tested for their susceptibility toward eight antifungal drugs using broth microdilution and disk diffusion methods. The antifungals used were Amphotericin B, Itraconazole, Voriconazole, Fluconazole, Terbinafine, Ketoconazole, Griseofulvin and Caspofungin. Results of this study demonstrate that the broth-microdilution method is still the preferable method since it is reliable and helps in quantitative MICs determination. The *in-vitro* testing revealed also that Voriconazole, Itraconazole, Ketoconazole, Griseofulvin as well as Caspofungin (echinocandin) were the most active antifungal drugs against the dermatophytic strains tested.

Keywords: antifungal susceptibility, dermatophytes, broth microdilution, disk diffusion, Egypt.

Introduction

Dermatophytes are filamentous keratinophilic fungi commonly involved in cutaneous infections of human. They are classified into three genera, *Epidermophyton*; *Microsporum*, and *Trichophyton* (Perez *et al.* 2010)).

Dermatophytoses are spreading easily and rapidly, especially in lower economic classes of populations. Topical formulations of antifungal drugs such as Ketoconazole, Clotrimazole, and Terbinafine were often used for their treatment (Singh *et al*. 2007).

Oral antifungal drugs such as Itraconazole, Griseofulvin, and Terbinafine are often used to treat severe cases of dermatophytoses such as tinea capitis and tinea ungium. Some other compounds, such as Posaconazole, Voriconazole and Ravuconazole are also effective (Fernandez-Torres *et al*. 2002).

In the last two decades, numerous studies discussed the development of standardized antifungal susceptibility testing methods for dermatophytes (Jessup *et al*. 2000, Ghannoum *et al*. 2004, Esteban *et al*. 2005, Fernandez – Torres *et al*. 2002, 2006, and Singh *et al*. 2007). The Clinical and Laboratory Standards Institute (CLSI) had approved the microdilution method (M38A2) as a reference technique for antifungal susceptibility testing of moulds and dermatophytes (CLSI 2008).

Broth-microdilution, disk-diffusion, and E-test methods are the most frequently techniques used for *in vitro* antifungal susceptibility of dermatophytes (Fernandez – Torres *et al*. 2003, Singh *et al*. 2007, and Nweze *et al*. 2010).

Although, the incidence of dermatophytoses among Egyptian populations was considerably high, studies devoted to the antifungal susceptibility testing of dermatophytes are rare (Girgis *et al*. 2006, Abdel Aal *et al* 2007, and Zaki *et al*. 2009).

The present study aimed at testing the susceptibility of some strains of dermatophytes isolated in Cairo, Egypt toward eight antifungal drugs by using broth-microdilution and disk-diffusion methods.

Materials and Methods

Dermatophytic strains

Nine strains of clinical origin were tested for their susceptibility toward some selected antifungal agents. These strains were identified by traditional and molecular methods (Zaki *et al*. 2009). They were deposited at Assuit University Mycological Center (AUMC), Assuit, Egypt. The test strains included: *Microsporum canis* AUMC 4328, *Trichophyton tonsurans* AUMC 4330, *T. rubrum* AUMC 4332, *T. rubrum* AUMC 9063, *T. mentagrophytes* AUMC 4333, *T. violaceum* AUMC 9064, *Arthroderma* sp. AUMC 9065, *Epidermophyton floccosum* AUMC 4329, and *E. floccosum* AUMC 5497.

Antifungal agents

The following drugs were obtained in powder form, Amphotericin B (Bristol Myers Squibb Woerden, The Netherlands), Itraconazole (Apex pharma., Egypt), Voriconazole (Pfizer,

Egypt), Fluconazole and Terbinafine (Novartis, Egypt), Ketoconazole (Ramedia, Egypt), Griseofulvin (Kahira pharm & Chem. Ind. Co., Egypt), and Caspofungin (Merck, Rahway, NJ, USA). A starting dose of 32 µg/ml for each of Amphotericin B, Itraconazole, Voriconazole, Ketoconazole and Terbinafine antifungals was prepared by weighing 3.2 mg powder and dissolving in 1 ml dimethyl sulfoxide (DMSO). In case of Griseofulvin and fluconazole, 6.4 mg powder were weighed and dissolved individually in 1 ml DMSO to get 64 µg/ml starting dose. Caspofungin was prepared at a higher dose where a total of 25.6 mg powder was dissolved in 1 ml sterile water for 256 µg/ml. All prepared stock solutions were maintained at -20^{o}C until needed. A working solution of each drug was prepared by making 1:10 dilution in DMSO or sterile water as appropriate.

For disk-diffusion method, four antifungal drugs were obtained as ready to use as disks, these were Amphotericin B (100 µg), Voriconazole (1 µg), Fluconazole (25 µg), and Ketoconazole (50 µg) (Biorad, USA). For the other four drugs a stock solution of each drug was prepared according to antifungal disks potency standard of Rosco Diagnostica Company (Neo-Sensitabs, Denmark) using DMSO or sterile water for Caspofungin, as follows: Griseofulvin, 0.8 mg/ml; Caspofungin 0.25 mg/ml, Terbinafine, 50 µg/ml, Itraconazole, 0.4 mg/ml. Taxo™ Blank paper disks (6 mm diameter) were loaded with 20 µl of the prepared stock solutions to obtain the desired drug concentration per disk (16 µg/disk for Griseofulvin, 5 µg/disk for Caspofungin, 1 µg/disk for Terbinafine and 8 µg/disk for Itraconazole). Disks were allowed to dry up at room temperature and then stored at 4°C in a refrigerator.

Medium

Broth microdilution method was performed in RPMI 1640 medium (Sigma-Aldrich, Mississauga, Ontario, Canada) with L-glutamine but without sodium bicarbonate and buffered with 0.165M morpholinepropanesulfonic acid (MOPS) (Sigma-Aldrich) at pH 7.0 (CLSI, 2008). The disk diffusion method was performed on Mueller-Hinton (MH) agar medium (Remel, KS) (Espinel-Ingroff *et al*. 2007).

Test procedure

Broth-microdilution method: The CLSI M38-A2 guidelines (CLSI, 2008) were followed. Fungal strains were grown on Sabouraud dextrose agar for 7-14 days at 35°C while the quality control strain (QC) *Candida albicans* NCPF 8154 was grown for 24h before testing. Each culture was gently swabbed with a cotton tip applicator to dislodge the conidia from the hyphal mat. The spore suspension was transferred to a sterile tube where the volume was adjusted to 5 ml with sterile normal saline and shaked well. Spores were counted with a hemocytometer and then diluted into RPMI 1640 broth medium to a concentration of 2x 10^3 CFU /ml. The QC strain cell suspension was prepared by taking a swab from 24 h culture into 5 ml of sterile distilled water and then adjusting the concentration using the 0.5 McFarland standard tubes to give a yeast suspension of 10^6 CFU/ml. A working suspension was then prepared in sterile distilled water to yield 10^3 CFU/ml. A sterile microdilution plate (96-flat bottomed wells) was used for each strain so that 100 µl of RPMI medium were added to each well then another 100 µl of the working solution of each antifungal drug were pipetted to a well in the 1st row. Two-fold serial dilutions were made using the multichannel pipette so that rows 1-10 contain series of drug dilutions in 100 µl volumes. The final concentrations of the antifungal agents were 0.06-64 mg/l for Griseofulvin and Fluconazole, 0.25–256 mg/l for Caspofungin and 0.03–32 mg/l for the rest of the drugs. Aliquots of fungal spore suspension (100 ml each) were then added to each well. The 11th row which served as the positive control contained 100 µl of inoculum suspension and 100 µl of drug free medium whereas the 12th row which served as negative control contained only 200 µl of RPMI broth. Microdilution trays were incubated at 35^{o}C. Minimum inhibitory concentrations (MICs) were determined after 4-7 days. MIC was defined as the concentration at which there was 100 % inhibition of fungal growth as compared with control when read visually in the microtitre plates.

Disk-diffusion method: Fungal cell suspensions were prepared as mentioned above and adjusted to a concentration of 10^6 CFU/ml. Sterile cotton swabs were loaded with the fungal inocula were individually streaked on the surface of MH agar plates in 4 different directions (at 90 degree angles) to cover the entire surface. Using a flamed sterilized forceps, disks loaded with the antifungal agents were applied separately onto the surface of the inoculated agar and pressed lightly to ensure complete contact with the culture which were then incubated at 35^{o}C for 4-7 days. The inhibition zone diameters (IZDs) were around disks measured to the nearest millimeter (Espinel-Ingroff *et al*. 2007).

Quality control: The quality control strain *Candida albicans* NCPF 8154 was included in each set of experiments for comparison.

Data analysis: MICs and IZDs breakpoints were analyzed according to the values presented in

Tables 1 and 2. The categorical agreement between the results of broth microdilution method as a reference method and the disk diffusion assay was calculated, within the same pattern of susceptibility. Errors were ranked as very major (false-susceptible result by disk diffusion), major (false-resistance by disk diffusion) and minor (intermediate by disk diffusion), resistant or susceptible in broth microdilution method) (Espinel-Ingroff *et al.* 2007).

Table 1: The minimum inhibitory (MIC) or minimum effective concentrations (MEC) breakpoints of tested antifungal agents.

Antifungal agent	MIC or MEC			Reference
	S	I	R	
Amphotericin B	≤ 1 µg/ml	2 µg/ml	≥ 4 µg/ml	Espinel-Ingroff *et al*. (2007)
Itraconazole	≤ 1 µg/ml	2 µg/ml	≥ 4 µg/ml	Espinel-Ingroff *et al*. (2007)
Voriconazole	≤ 1 µg/ml	2 µg/ml	≥ 4 µg/ml	Espinel-Ingroff *et al*. (2007)
Caspofungin	≤ 1 µg/ml	2 µg/ml	≥ 4 µg/ml	Espinel-Ingroff *et al*. (2007)
Ketoconazole	< 4 µg/ml	-	4-16 µg/ml	Ellis (2012)
Fluconazole	< 8 µg/ml	16-32 µg/ml	> 64 µg/ml	Ellis (2012)
Terbinafine	< 1 µg/ml	1 µg/ml	> 1 µg/ml	Ghannoum *et al.* (2011)
Griseofulvin	< 2 µg/ml	2 µg/ml	> 2 µg/ml	Fachin *et al.* (1996)

S=Susceptible; I=Intermediate; R=Resistant

Table 2: The inhibition zone diameters (IZDs) breakpoints of tested antifungal agents

Antifungal agent	IZDs			Reference
	S	I	R	
Amphotericin B	> 15 mm	13-14 mm	≤ 12 mm	Espinel-Ingroff *et al*. (2007)
Itraconazole	≥ 17 mm	14-16 mm	≤ 13 mm	Espinel-Ingroff *et al*. (2007)
Voriconazole	≥ 17 mm	14-16 mm	≤ 13 mm	Espinel-Ingroff *et al*. (2007)
Caspofungin	≥ 17 mm	14-16 mm	≤ 13 mm	Espinel-Ingroff *et al*. (2007)
Ketoconazole	≥ 30 mm	23-29 mm	≤ 22 mm	Pakshir *et al*. (2009)
Fluconazole	≥ 21 mm	15-22 mm	≤ 14 mm	Pakshir *et al*. (2009)
Terbinafine	≥ 20 mm	12-19 mm	≤ 11 mm	Pakshir *et al*. (2009)
Griseofulvin	≥ 10 mm	-	0 mm	Pakshir *et al*. (2009)

S=Susceptible; I=Intermediate; R=Resistant

Results

The minimum inhibitory concentrations (MICs) of the *in vitro* susceptibility testing of nine dermatophytic strains toward eight antifungal drugs are shown in Table 3. Results indicated that both Voriconazole and Ketoconazole showed antifungal activities against all tested strains except *Trichophyton mentagrophytes* AUMC 4333. The MICs of Voriconazole against susceptible strains ranged from 0.25–1 µg/ml while those of Ketoconazole ranged from 0.25-2 µg/ml. Terbinafine showed high activity against all tested strains except *Trichophyton violaceum*. The MIC for Terbinafine against *T. mentagrophytes* AUMC 4333, *T. tonsurans* AUMC 4330 and *T. rubrum* AUMC 4332 was 0.125 µg/ml which means that these fungal strains are susceptible to this antifungal agent. In case of *Microsporum canis* AUMC 4328, *Arthroderma* sp. AUMC 9065, *Trichophyton rubrum* AUMC 9063 and the two *Epidermophyton floccosum* strains the MIC of Terbinafine (1 µg/ml) was in the intermediate susceptibility range. Itraconazole, Griseofulvin and Caspofungin showed antifungal activities against seven strains but they were not active against *T. rubrum* AUMC 9063 and *T. violaceaum* AUMC 9064. Amphotericin B and Fluconazole exhibited a narrow spectrum of antifungal activity where Amphotericin B had activities against four strains at 0.5 µg/ml while, Fluconazole was active against two strains at 16 µg/ml.

As shown in Table (3), *Arthroderma* strain AUMC 9065 showed sensitivity toward all drugs used whereas the two strains of *Epidermophyton floccosum* and *T. rubrum* (AUMC 4332) were sensitive to seven drugs but resistant only to Fluconazole. Both *M. canis* and *T. tonsurans* showed sensitivity towards the tested antifungal agents except Amphotericin B and Fluconazole. *T. mentagrophytes* showed sensitivity to 4 drugs whereas, *T. rubrum* AUMC 9063 and *T. violaceum* were only sensitivite to Voriconazole, Ketoconazole and Terbinafine.

The inhibition zone diameters (IZDs) of the *in-vitro* susceptibility testing of the nine dermatophytic strains to eight antifungal drugs are shown in Table 4. The results indicated that Voriconazole and Ketoconazole exhibited high activities against all tested strains except *T. mentagrophytes* AUMC 4333. Itraconazole, Griseofulvin and Caspofungin showed almost high antifungal activities against seven strains but they were not effective against *T. rubrum* AUMC 9063 and *T. violaceum* AUMC 9064. Terbinafine exhibited high to intermediate activity against six strains but it displayed no visible activity towards *M. canis* AUMC 4328, *T. violaceum* AUMC 9064 and *Arthroderma* sp. AUMC 9065. Amphotericin B demonstrated activity against five fungal strains only, whereas Fluconazole (25 µg/disk) was not effective against all tested strains (Table 4).

T. rubrum AUMC 4332 and the two tested strains of *E. floccosum* showed high sensitivity to all antifungal drugs except Fluconazole. *T. tonsurans* and *Arthroderma* sp. came next showing sensitivity towards six drugs and resistance to two drugs. *M. canis* and *T. mentagrophytes* were susceptible to five and four drugs respectively. Both *T. rubrum* AUMC 9063 and *T. violaceum* AUMC 9064 responded to only three drugs but it displayed no visible activity towards *M. canis* AUMC 4328, *T. violaceum* AUMC 9064 and *Arthroderma* sp. AUMC 9065 (Table 4).

The MICs of *Candida albicans* NCPF 8154 (control strain) ranged from 0.06 to 16 µg/ml where the lowest concentration was observed with Itraconazole and Voriconazole and the highest with Griseofulvum. Considering the overall range of inhibition zone diameters (IZDs) exihibited by the different antifungal agents, the present data in Table 4 revealed that Voriconazole, followed by Ketokonazole and Caspofungin were the most effective against the tested dermatophytes showing IZDs ranging from 10-55 mm. Similarly, Griseofulvin, Itraconazole and Amphotericin-B exhibited IZDs ranging from 9-45 mm. The lowest range of IZD was observed with Terbinafine (13-21 mm) whereas Fluconazole displayed no visible inhibition as mentioned before.

In this study, a categorical agreement between the broth microdilution and disk diffusion methods was found for all antifungal agents tested. No major errors were detected, but minor errors were observed, ranging from 1 (11.1%) for Amphotericin B, to 2 (22.2%) for each of Fluconazole and Terbinafine. Minor errors shown between the results of the two methods for amphotericin B were due to the categorization of *T. violaceum* as resistant by the broth microdilution method and its categorization within the intermediate range by disk diffusion method. For fluconazole, *T. violaceum* and *Arthroderma* sp. were categorized as susceptible (dose- dependent) by broth microdilution method while they were within the resistance range by the disk diffusion method. The two minor errors detected for Terbinafine included the *M. canis* and *Arthroderma* sp. strain that were categorized as susceptible (dose-dependent by broth microdilution method) while they were within the resistance range by the disk diffusion method. The overall levels of agreement between the results of broth microdilution and disk diffusion methods came in the following sequence: 100% for each of Itraconazole, Voriconazole, Ketoconazole, Griseofulvin and Caspofungin, 89% for Amphotericin B and 78 % for Fluconazole, and Terbinafine.

Table 3: *In-vitro* susceptibility of dermatophytes strains toward 8 antifungal drugs using broth microdilution method.

Strain	MIC (µg/ml) for antifungal agent							
	AMB	ITC	VRC	FLC	KTC	TRB	GRS	CAS
M. canis AUMC 4328	16	0.5	0.5	64	0.5	1	1	1
T. tonsurans AUMC 4330	8	0.125	0.5	64	0.5	0.125	0.5	1
T. rubrum AUMC 4332	0.5	0.125	0.25	64	0.25	0.125	0.25	0.5
T. rubrum AUMC 9063	16	16	1	64	1	1	32	128
T. mentagrophytes AUMC 4333	8	0.25	8	64	8	0.125	0.5	2
T. violaceum AUMC 9064	4	16	1	16	2	8	16	128
Arthroderma sp. AUMC 9065	0.5	0.125	0.25	16	0.25	1	0.25	0.5
E. floccosum AUMC 4329	1	0.25	1	64	0.5	1	0.5	2
E. floccosum AUMC 5497	1	0.25	1	64	0.5	1	0.5	2
MIC range	0.5-16	0.125-16	0.25-8	16-64	0.25-8	0.125-8	0.25-32	0.5-128
Candida albicans NCPF 8154	0.5	0.06	0.06	0.125	16	0.5	16	0.5

MIC: Minimum Inhibitory Concentration; AMB, amphotericin B; ITC, itraconazole; VRC, voriconazole; FLC, fluconazole; KTC, ketoconazole; TRB, terbinafine; GRS, griseofulvin; CAS, caspofungin. AUMC, Assuit University Mycological Center. NCPF: National Center for Pathogenic Fungi, England.

Table 4: *In-vitro* susceptibility of dermatophytes strains toward 8 antifungal drugs using disk diffusion method.

Strain	Mean value of inhibition zone diameter, IZDs (mm)							
	AMB 100 µg	**ITC 8 µg**	**VRC 1 µg**	**FLC 25 µg**	**KTC 50 µg**	**TRB 1 µg**	**GRS 16 µg**	**CAS 5 µg**
M. canis AUMC 4328	0	17	22	0	36	0	21	30
T. tonsurans AUMC 4330	10	25	27	0	36	21	38	38
T. rubrum AUMC 4332	32	41	55	0	50	20	45	49
T. rubrum AUMC 9063	0	0	17	0	24	13	0	0
T. mentagrophytes AUMC 4333	9	21	0	0	15	20	31	18
T. violaceum AUMC 9064	14	12	25	0	40	0	0	10
Arthroderma sp. AUMC 9065	20	35	55	0	50	0	45	52
E. floccosum AUMC 4329	28	35	50	0	55	15	42	55
E. floccosum AUMC 5497	19	37	55	0	50	13	40	55
Range of inhibition zone in resported strains	0-32	0-41	0-55	0	15-55	0-21	0-45	0-55
Candida albicans NCPF 8154	16	25	35	35	40	0	0	23

IZD: Inhibition Zone Diameter; AMB, amphotericin B; ITC, itraconazole; VRC, voriconazole; FLC, fluconazole; KTC, ketoconazole; TRB, terbinafine; GRS, griseofulvin; CAS, caspofungin. AUMC, Assuit University Mycological Center. NCPF: National Center for Pathogenic Fungi, England.

Discussion

Dermatophyte infections are frequently recurrent or chronic and mandate long-term treatment with antifungal agents. However, poor compliance and emergence of antifungal drug resistance account for the increasing disease prevalence and rates of treatment failures. This makes it imperative to conduct in-vitro antifungal susceptibility testing for the commonly used and newly introduced drugs (Pfaller, 2000). Many antifungal agents have been recently introduced but their susceptibility patterns and imperative criteria are lacking (Espinel-Ingroff et al. 2007). In addition to treatment failure problems, selection of an appropriate antifungal drug is extremely necessary to evade liver and cardiac side effects as well as possible drug interactions following prolonged administration, as in the case of Terbinafine and azole-like compounds (Bao et al. 2012).

In this study, eight antifungal agents including Amphotericin B (a member of polyene antibiotcs), Itraconazole, Voriconazole, Fluconazole Itriazoles), Ketoconazole (imidazole), Terbinafine (allylamines), Caspofungin (echinocandin), and Griseofulvin were tested against nine dermatophytic strains of clinical origin using broth microdilution and disk diffusion methods.

The MIC and IZD results using the broth microdilution and disk diffusion methods support and extend the findings presented in previous reports of the in-vitro activity of Voriconazole, Ketoconazole, Itraconazole, and Griseofulvin (Perea et al. 2001, Fernandez-Torres et al. 2002, and Esteban et al. 2005).

The newly introduced echinocandin drug (Caspofungin) in the Egyptian market showed good activity against dermatophytic strains, similar to that obtained obtained by Bao et al. (2012).

In the present study, a range of MICs for Terbinafine (0.125-8 µg/ml) which was slightly higher than by those reported by Perea *et al.* (2001). *Arthroderma* sp. and *M. canis* were intermediately affected by Terbinafine in the broth microdilution, but they showed no inhibition zones upon using disk diffusion method. This error might be attributed to the use of Terbinafine at a low concentration of 1µg/disk instead of 30µg/disk used in the other studies.

Five of the 9 strains tested showed resistance to Amphotericin B and this is in agreement with the findings of Abdel Aal *et al.* (2007) whereas *T. rubrum* AUMC 4332, *Arthroderma* sp. AUMC 9065, and *E. floccosum* AUMC 4329 and AUMC 5497 were susceptible to this drug. *T. violaceum* demonstrated resistance in broth microdilution, but its response was within the intermediate range when disk diffusion method was used. This was regarded as a minor error, possibly because of the high Amphotericin B disk content in disk diffusion test (100 µg/disk).

Resistance of dermatophytic strains to Fluconazole was also reported previously by Pakshir *et al*. (2009).

In conclusion: the current results demonstrated that the broth-microdilution method employed for antifungal susceptibility of dermatophytes is preferred because it is simple, cheap and reliable. Moreover, it helps in quantitative MICs determination. It is also concluded that *in-vitro* testing showed that Voriconazole, Itraconazole, Ketoconazole and Caspofungin were the most active drugs against dermatophytes tested. It is also highly recommended to encourage dermatologists to cosult mycologists for isolation, identification of pathogenic fungi and performing *in-vitro* sensitivity tests to choose the most effective antifungal drugs needed for successful treatment of fungal infections.

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