Original Article

To determine antifungal susceptibility for the isolates using agar dilution method: A prospective cohort study

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ABSTRACT

The aim of the study was to standardize *in vitro* antifungal susceptibility testing by agar dilution method to find out the minimum inhibitory concentration (MIC) of fluconazole, ketoconazole, itraconazole, terbinafine, and griseofulvin fungal isolates of skin, hair, and nail. Various samples were collected from patients with clinically diagnosed dermatophytosis. Skin scrapings, hair, and nail were collected from one hundred and seventy patients. The antifungal drugs such as fluconazole (64–0.0625 µl/ml), ketoconazole (32–0.0313 µl/ml), itraconazole (32–0.0313 µl/ml), terbinafine (32–0.0313 µl/ml), and griseofulvin (32–0.0313 µl/ml) these water-insoluble drugs were incorporated in dissolved in dimethyl sulfoxide. The MIC was determined as the lowest concentration of the antifungal drug preventing the growth of macroscopically visible colonies on drug-containing plates when there was visible growth on the drug-free control plates. The MIC range, MIC 50, and MIC 90 for the drug griseofulvin were found to be 0.06–0.1, 0.25, and 1, respectively, for ketoconazole were found to be 0.06–0.1, 0.5, and 1, respectively, for the drug fluconazole were found to be 1–32, 8, and 16, respectively, itraconazole were found to be 0.06–4, 0.5, and 1, respectively, and for terbinafine were found to be 0.03–0.12, 0.06, and 0.12, respectively. This technique was found to be reliable, cost-effective and easy to perform with consistent results. Further results concluded that the fluconazole showed a higher MIC value when compared to other antifungal drugs.

Keywords: Agar dilution method, antifungal susceptibility testing, fluconazole, itraconazole, ketoconazole, minimum inhibitory concentration, terbinafine and griseofulvin

Introduction

The cutaneous infections of man include a wide variety of diseases in which the integuments and its appendages the hair and the nail are involved.[1] Infection is generally restricted to the non-lining cornified layer, but a variety of changes occur in the host due to the presence of the infectious agent and its metabolic products.[2] Majority of the infections are caused by a homogenous group of keratinophilic fungus called the dermatophytes. An overwhelming number of dermatophytes and species have been implicated as a cause of skin, hair, and nails and this number is steadily increasing.[3] Dermatophytosis remains a significant public health problem. Numerous antifungal agents have been developed since griseofulvin became available through a breakthrough experimental work of gentles in Guinea pig in 1958.[4]

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The need for antifungal susceptibility testing increases beyond testing dermatophytes species because resistance to antifungal drugs has been demonstrated against such diverse fungi as *Trichophyton rubrum*, *Trichophyton interdigitale*, *Trichophyton tonsurans*, *Trichophyton verrucosum*, *Tricholosporum violaceum*, *Trichophyton schoenleini*, *Epidermophyton floccosum*, *Microsporum gypseum*, and *Microsporum audouinii* species. Hence, it becomes evident that the need for meaningful susceptibility test result is very important for fungi as it is for bacteria.[5,6]

Although antifungal susceptibility testing remains less well-established and utilized than antibacterial testing, the scientific support for its validity has benefited greatly by extrapolation from antibacterial testing.[7] The methods for antifungal sensitivity testing include National Committee for Clinical Laboratory Standards new name Clinical Laboratory Standards Institute (CLSI), broth-based methodology (M 27-A), CLSI methodology for molds,[8] E-test agar based testing methods, and flow cytometry and use of viability dyes. The above methods are time-consuming and labor intensive; hence, a more economical method such as agar dilution have been described.[9] There are only a limited number of antifungal susceptibility testing reports on ocular fungal isolates from India.[9] The

present study is focused on standardization of the *in vitro* agar dilution method for determination of minimum inhibitory concentration (MIC) of fluconazole, ketoconazole, itraconazole, ter1binafine and griseofulvinonskin scrapings, hair, and nail isolated fungal isolates.

Materials and Methods

Research design

The present study follows the prospective cohort study and is especially focus on the susceptibility of isolates using agar medium, which gives the rational results and will help to understand the effect of various antifungal drugs. Such research design was chosen to aid in the attainment of the objectives stated in this study.

Study period

The present study was conducted in the Department of Microbiology at Government Stanley Medical College and Hospital, Chennai, and over a period of 1 year from May 2008 to June 2009.

Sample specifications

The various samples were collected from patients with clinically diagnosed dermatophytosis. Skin scrapings, hair, and nail were collected from 170 patients who attended the mycology section in the Dermatology Outpatient Department at Stanley Medical College and hospital Chennai.

Inclusion criteria

All patients with clinically diagnosed dermatophytosis irrespective of age and sex who are not undergoing treatment for the same were included in the study.

Exclusion criteria

All patients with ringworm infection and who were on pharmacological treatment were excluded from the study.

Respondents

The subjects of this study were first diagnosed clinically for present and confirmation of dermatophytosis. Further, history from patients is collected and all other relevant details such as age, sex, duration of complaint, distribution of lesion and history of previous similar complaints, and treatment history. All details about general health and treatment history for diabetes tuberculosis neoplasms, HIV, and surgeries, etc., a detailed history of exposure to animals, known cases, pets at home, or any other suspected sources.

Specimen

The specimens were collected from skin scrapings, nail clippings, and hair.

Antifungal susceptibility testing evaluated using agar dilution method and selected antifungal drugs (fluconazole, ketoconazole, itraconazole, griseofulvin, and terbinafine).

Requirements

Sterile test tubes for drug dilution/inoculum preparation/agar slopes with drug dilution/micropipette/sterile tips/gloves/disposable face masks.

Medium

Nutrient agar.

Preparation of standard inoculum

7–15 days old cultures grown on sabouraud dextrose agar at 25°C was taken. Mature colonies were covered with 10 ml of sterile saline (0.85%) growth scraped by sterile Pasteur pipette. Heavy particles were allowed to settle for 15–20 min at room temperature. Supernatant mixed with a vortex for 15 s. Turbidity of supernatant was adjusted spectrophotometrically to 530 nm 65–70% absorbance.[8,10]

Antifungal drugs

Fluconazole (64–0.0625 µl), ketoconazole (32–0.0313 µl), itraconazole (32–0.0313 µl), terbinafine (32–0.0313 µl), and griseofulvin (32–0.0313 µl) were used as antifungal drugs.[8,10]

Preparation of stock solutions of antifungal agents

Stock solutions of each drug were prepared at an initial concentration of 1000 µg/ml. Water insoluble drugs such as griseofulvin, ketoconazole, itraconazole, and terbinafine dissolved in dimethyl sulfoxide. Fluconazole dissolved in sterile distilled water. Further dilutions to get the required dilutions for each drug are made in distilled water.[8,10]

Test procedure

About 1.8 ml of molten nutrient agar poured into sterile test tubes. Allowed to cool to 50°C. 0.2 ml of drug dilutions from stock solutions added in descending concentration to NA slopes. 10 µl of standardized inoculum added to all tubes except sterility control tube. Tubes incubated at 35°C for 7 days visualized macroscopically for growth. The lowest concentration of the drug which permitted no macroscopically visible growth after 7 days is taken as MIC.

Interpretation of results

The MIC was the lowest concentration of drug preventing the growth of macroscopically visible colonies on drug-containing plates when there was visible growth on the drug-free control plates. The MIC readings were taken at the end of 7 days of incubation when growth appeared on the control plate.

Results and Discussion

The present study was designed to develop a simple, cost-effective procedure of agar dilution method to determine the MICs of antifungal drugs. Eight different strains were included in the study for correlation of the results with agar dilution method standardized

in this study with the published reports of other standard methods of susceptibility testing. The antifungal susceptibility test was performed by agar dilution method. The results reveal the MIC and comparative effects of various species with the selected antifungal drugs. The drugs taken for antifungal susceptibility testing were griseofulvin, ketoconazole, fluconazole, itraconazole, and terbinafine. Antifungal susceptibility testing was done by agar dilution and micro-broth dilution methods.

Standardization of *in vitro* susceptibility testing by agar dilution method

The results of MIC 50 and MIC 90 of griseofulvin, ketoconazole, fluconazole, itraconazole, and terbinafine, for all the isolates of this study, are mentioned in Tables 1-5, respectively.

In the present study, the MIC range, MIC 50, and MIC 90 for the drug griseofulvin were found to be 0.06–0.1, 0.25, and 1, respectively. The MIC range, MIC 50, and MIC 90 for the drug ketoconazole were found to be 0.06–0.1, 0.5, and 1, respectively. The MIC range, MIC 50, and MIC 90 for the drug fluconazole were found to be 1–32, 8, and 16, respectively. The MIC range, MIC 50, and MIC 90 for the drug itraconazole were found to be 0.06–4, 0.5, and 1, respectively. The MIC range, MIC 50, and MIC 90 for the drug terbinafine were found to be 0.03–0.12, 0.06, and 0.12, respectively.[11]

In Dr. Pankajalaxsmi's study MIC range, MIC 50, and MIC 90 were found to be higher for griseofulvin and ketoconazole than the present study. The MIC range, MIC 50, and MIC 90 were equal for itraconazole and lower for terbinafine. Favre *et al.* (2003) conducted a comparison of *in vitro* activities of 17 antifungal drugs against a panel of the 20 dermatophytes using a microdilution method have shown griseofulvin, ketoconazole, itraconazole, and terbinafine were the most potent agents [Table 6].

The microdilution assay for dermatophytes is convenient and reproducible. A marked reduction in the MIC values was seen in the microdilution method when compared to the agar dilution method in all drugs tested.

Table 1: Antifungal susceptibility testing by agar dilution method of antifungal drug-griseofulvin

Species	Drug concentrations (in μg/ml)											
	0.03	0.06 (%)	0.12 (%)	0.25 (%)	0.5 (%)	1 (%)	2	4	8	16	MIC 50	MIC 90
T. rubrum (*n*=16)	0	3 (18.7)	0	2 (12.5)	2 (12.5)	9 (56.2)	–	–	–	–	0.5	1
T. mentagrophytes (*n*=13)	0	0	2 (15.3)	2 (15.3)	6 (46.1)	3 (23)	–	–	–	–	0.5	1
T. tonsurans (*n*=10)	0	3 (30)	5 (50)	2 (20)		–	–	–	–	–	0.12	0.25
T. verrucosum (*n*=8)	0	0	2 (25)	2 (25)	2 (25)	2 (25)	–	–	–	–	0.25	1
T. violaceum (*n*=6)	0	0	1 (16.6)	2 (33.3)	2 (33.3)	1 (16.6)	–	–	–	–	0.5	1
T. schoenleinii (*n*=2)	0	0	1 (50)	0	1 (50)	–	–	–	–	–	0.12	0.5
E. floccosum (*n*=2)	0	0	1 (50)	1 (50)	–	–	–	–	–	–	0.12	0.25
M. gypseum (*n*=2)	0	0	0	1 (50)	0	1 (50)	–	–	–	–	0.25	1
M. audouinii (*n*=1)	0	0	0	1 (100)	–	–	–	–	–	–		0.25

MIC 50 and MIC 90 of griseofulvin for all the isolates of this study are as follows *T. rubrum*–0.5 and 1 μg/ml, respectively, *T. mentagrophytes*–0.5 and 1 μg/ml, respectively, *T. tonsurans*–0.12 and 0.25 μg/ml, respectively, T. verrucosum–0.25 and 1 μg/ml, respectively, *T. vioaceum*–0.5 and 1 μg/ml, respectively, *T. schoenleinii*–0.12 and 0.5 μg/ml, respectively, *E. floccosum*–0.12 and 0.25 μg/ml, respectively, *M. gypseum*–0.25 and 1 μg/ml, respectively, *M. audouinii*–0.25, respectively (MIC 90). MIC: Minimum inhibitory concentration, *T. rubrum: Trichophyton rubrum, T. mentagrophytes: Trichophyton mentagrophytes, T. tonsurans: Trichophyton tonsurans, T. verrucosum: Trichophyton verrucosum, T. violaceum: Tricholosporum violaceum, T. schoenleinii: Trichophyton schoenleinii, E. floccosum: Epidermophyton floccosum, M. gypseum: Microsporum gypseum, M. audouinii: Microsporum audouinii*

Table 2: Drug ketoconazole

Species	Drug concentrations (in μg/ml)											
	0.03	0.06 (%)	0.12 (%)	0.25 (%)	0.5 (%)	1 (%)	2	4	8	16	MIC 50	MIC 90
T. rubrum (*n*=16)	0	0	7 (43.7)	2 (12.5)	3 (18.7)	4 25	–	–	–	–	0.12	1
T. mentagrophytes (*n*=13)	0	1 (7.6)	1 (7.6)	2 (15.3)	5 (38.4)	4 (30.7)	–	–	–	–	0.5	1
T. tonsurans (*n*=10)	0	0	2 (20)	2 (20)	5 (50)	1 (10)	–	–	–	–	0.5	1
T. verrucosum (*n*=8)	0	0	2 (25)	1 (12.5)	2 (25)	3 (37.5)	–	–	–	–	0.5	1
T. violaceum (*n*=6)	0	0	0	0	3 (50)	3 (50)	–	–	–	–	0.5	1
T. schoenleinii (*n*=2)	0	0	1 (50)	0	1 (50)	–	–	–	–	–	0.12	0.5
E. floccosum (*n*=2)	0	0	0	1 (50)	0	1 (50)	–	–	–	–	0.25	1
M. gypseum (*n*=2)	0	0	1 (50)	0	1 (50)	–	–	–	–	–	0.12	0.5
M. audouinii (*n*=1)	0	0	0	0	1 (100)	–	–	–	–	–		0.5

MIC 50 and MIC 90 of ketoconazole for all the isolates of this study are as follows-*T. rubrum*–0.12 and 1 μg/ml/0.12 and 0.5 μg/m, respectively, T. mentagrophytes–0.5 and 1 μg/ml, respectively, *T. tonsurans*–0.05 and 1 μg/ml, respectively, *T. verrucosum*–0.5 and 1 μg/ml, respectively, *T. violaceum*–0.5 and 1 μg/ml, respectively, *T. schoenleinii*–0.12 and 0.5 μg/ml, respectively, *E. floccosum*–0.25 and 1 μg/ml, respectively, M. gypseum–0.12 and 0.5 μg/ml, respectively, *M. audouinii*–0.5 μg/ml (MIC 90). MIC: Minimum inhibitory concentration, *T. rubrum: Trichophyton rubrum, T. mentagrophytes: Trichophyton mentagrophytes, T. tonsurans: Trichophyton tonsurans, T. verrucosum: Trichophyton verrucosum, T. violaceum: Tricholosporum violaceum, T. schoenleinii: Trichophyton schoenleinii, E. floccosum: Epidermophyton floccosum, M. gypseum: Microsporum gypseum, M. audouinii: Microsporum audouinii*

Table 3: Drug fluconazole

Species	Drug concentrations (in μg/ml)											
	0.12	0.25	0.5	1 (%)	2 (%)	4 (%)	8 (%)	16 (%)	32 (%)	64	MIC 50	MIC 90
T. rubrum (*n*=16)	0	0	0	5 (31.2)	5 (31.2)	3 (18.7)	3 (18.7)	–	–	–	2	8
T. mentagrophytes (*n*=13)	0	0	0	0	0	3 (23)	2 (15.3)	3 (23)	5 (38.4)	–	16	32
T. tonsurans (*n*=10)	0	0	0	0	0	3 (30)	3 (30)	4 (40)	–	–	8	16
T. verrucosum (*n*=8)	0	0	0	0	0	0	2 (25)	5 (62.5)	2 (25)	–	16	32
T. violaceum (n=6)	0	0	0	0	2 (33.3)	1 (16.6)	2 (33.3)	1 (16.6)	–	–	4	16
T. schoenleinii (*n*=2)	0	0	0	0	0	0	1 (50)	–	1 (50)	–	8	32
E. floccosum (*n*=2)	0	0	0	0	0	1 (50)	0	1 (50)	–	–	4	16
M. gypseum (*n*=2)	0	0	0	0	0	0	1 (50)	–	1 (50)	–	8	32
M. audouinii (n=1)	0	0	0	0	0	0	–	1 (100)	–	–		16

MIC 50 and MIC 90 of fluconazole for all the isolates of this study are as follows: *T. rubrum*−0.2 and 8 μg/ml respectively, *T. mentagrophytes*−16 and 32 μg/ml, respectively, *T. tonsurans*−8 and 16 μg/ml, respectively, *T. verrucosum*−16 and 32 μg/ml, respectively, *T. violaceum*−4 and 16 μg/ml, respectively, *T. schoenleinii*−8 and 32 μg/ml, respectively, *E. floccosum*−4 and 16 μg/ml, respectively, *M. gypseum*−8 and 32 μg/ml, respectively, *M. audouinii*−16 μg/ml (MIC 90). MIC: Minimum inhibitory concentration, *T. rubrum: Trichophyton rubrum, T. mentagrophytes: Trichophyton mentagrophytes, T. tonsurans: Trichophyton tonsurans, T. verrucosum: Trichophyton verrucosum, T. violaceum: Tricholosporum violaceum, T. schoenleinii: Trichophyton schoenleinii, E. floccosum: Epidermophyton floccosum, M. gypseum: Microsporum gypseum, M. audouinii: Microsporum audouinii*

Table 4: Drug-itraconazole

Species	Itraconazole-drug concentrations (in μg/ml)											
	0.03	0.06 (%)	0.12 (%)	0.25 (%)	0.5 (%)	1 (%)	2 (%)	4 (%)	8	16	MIC 50	MIC 90
T. rubrum (*n*=16)	0	0	2 (6.25)	6 (37.5)	5 (31.2)	3 (18.7)	–	–	–	–	0.5	1
T. mentagrophytes (*n*=13)	0	2 (15.3)	4 (30.7)	3 (23)	4 (30.7)	–	–	–	–	–	0.12	0.5
T. tonsurans (*n*=10)	0	1 (10)	1 (10)	0	3 (30)	0	5 (50)	–	–	–	0.5	2
T. verrucosum (*n*=8)	0	0	4 (50)	0	2 (25)	0	2 (25)	–	–	–	0.12	2
T. violaceum (*n*=6)	0	0	0	1 (16.6)	5 (83.4)	–	–	–	–	–	0	0.5
T. schoenleinii (*n*=2)	0	0	0	1 (50)	0	0	1 (50)	–	–	–	0.5	2
E. floccosum (*n*=2)	0	0	0	0	1 (50)	1 (50)	–	–	–	–	0.5	1
M. gypseum (n=2)	0	0	1 (50)	0	0	0	0	1 (50)	–	–	0.12	4
M. audouinii (*n*=1)	0	0	0	0	0	1 (100)	–	–	–	–		1

MIC 50 and MIC 90 of itraconazole for all the isolates of this study are as follows: *T. rubrum*−0.5 and 1 μg/ml, respectively, *T. mentagrophytes*−0.12 and 0.5 μg/ml, respectively, *T. tonsurans*−0.5 and 2 μg/ml. 03, respectively, *T. verrucosum*−0.12 and 2 μg/ml, respectively, *T. violaceum*−0 and 0.5 μg/ml, respectively, *T. schoenleinii*−0.5 and 2 μg/ml, respectively, *E. floccosum*−0.5 and 1 μg/ml, respectively, *M. gypseum*−0.12 and 4 μg/ml, respectively, M. audouinii−1, respectively (MIC 90). MIC: Minimum inhibitory concentration, *T. rubrum: Trichophyton rubrum, T. mentagrophytes: Trichophyton mentagrophytes, T. tonsurans: Trichophyton tonsurans, T. verrucosum: Trichophyton verrucosum, T. violaceum: Tricholosporum violaceum, T. schoenleinii: Trichophyton schoenleinii, E. floccosum: Epidermophyton floccosum, M. gypseum: Microsporum gypseum, M. audouinii: Microsporum audouinii*

Table 5: Drug-terbinafine

Species	Drug concentrations (in μg/ml)											
	0.03 (%)	0.06 (%)	0.12 (%)	0.25	0.5	1	2	4	8	16	MIC 50	MIC 90
T. rubrum (*n*=16)	2 (12.5)	5 (31.2)	9 (56.2)	–	–	–	–	–	–	–	0.06	0.12
T. mentagrophytes (*n*=13)	2 (15.3)	5 (35.7)	6 (42.8)	–	–	–	–	–	–	–	0.06	0.12
T. tonsurans (*n*=10)	0	7 (70)	3 (30)	–	–	–	–	–	–	–	0.06	0.12
T. verrucosum (*n*=8)	4 (50)	2 (25)	2 (25)	–	–	–	–	–	–	–	0.03	0.12
T. violaceum (*n*=6)	0	3 (50)	3 (50)	–	–	–	–	–	–	–	0.06	0.12
T. schoenleinii (*n*=2)	0	0	2 (100)	–	–	–	–	–	–	–	0	0.12
E. floccosum (*n*=2)	2 (100)	–	–	–	–	–	–	–	–	–	0	0.03
M. gypseum (*n*=2)	0	0	2 (100)	–	–	–	–	–	–	–	0	0.12
M. audouinii (*n*=1)	0	1 (100)	–	–	–	–	–	–	–	–		0.06

MIC 50 and MIC 90 of terbinafine for all the isolates of this study are as follows: *T. rubrum*−0.06 and 0.12 μg/ml, respectively, *T. mentagrophytes*−0.06 and 0.12 μg/ml, respectively, *T. tonsurans*−0.06 and 0.12 μg/ml, respectively, *T. verrucosum*−0.03 and 0.12 μg/ml, respectively, *T. violaceum*−0.06 and 0.12 μg/ml, respectively, *T. schoenleinii*−0 and 0.12 μg/ml, respectively, E. floccosum−0 and 0.25 μg/ml, respectively, M. gypseum−0 and 0.12 μg/ml, respectively, *M. audouinii*−0.06 μg/ml (MIC 90). MIC: Minimum inhibitory concentration, *T. rubrum: Trichophyton rubrum, T. mentagrophytes: Trichophyton mentagrophytes, T. tonsurans: Trichophyton tonsurans, T. verrucosum: Trichophyton verrucosum, T. violaceum: Tricholosporum violaceum, T. schoenleinii: Trichophyton schoenleinii, E. floccosum: Epidermophyton floccosum, M. gypseum: Microsporum gypseum, M. audouinii: Microsporum audouinii*

Antifungal susceptibility testing was performed by agar dilution method for griseofulvin, ketoconazole, fluconazole, itraconazole, and terbinafine.

The MIC range for terbinafine by agar dilution method was 0.03–0.12 μg/ml and by micro broth dilution method was 0.007–0.06 μg/ml.

Table 6: Comparison of *in vitro* activities of five antifungal drugs by agar dilution method

All dermatophytes	Drugs	Present study			Dr. Pankajalakshmi's study		
		MIC range (μg/ml)	MIC 50 (μg/ml)	MIC 90 (μg/ml)	MIC range (μg/ml)	MIC 50 (ζg/ml)	MIC 90 (μg/ml)
	Griseofulvin	0.06–0.1	0.25	1	0.1–10	1	5
	Ketoconazole	0.06–0.1	0.5	1	0.01–5	1	2.5
	Fluconazole	1–32	8	16	–	–	–
	Itraconazole	0.06–4	0.5	1	0.01–0.5	0.1	0.5
	Terbinafine	0.03–0.12	0.06	0.12	0.001–0.01	0.01	0.1

MIC: Minimum inhibitory concentration

Fluconazole showed a higher MIC value when compared to other antifungal drugs. Terbinafine recorded the lowest MIC values. The MIC values were much lower when tested by micro-broth dilution method. Terbinafine was found to be the most potent drug.

Conclusion

The present study indicates that the agar dilution method can be adopted for *in vitro* antifungal sensitivity testing, as it is a simple, reproducible, cost-effective and easy to perform the technique in a routine clinical microbiology laboratory. Further results concluded that the fluconazole showed a higher MIC value when compared to other antifungal drugs.

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