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Original Research Article

In vitro susceptibility testing of four antifungal drugs against fungal isolates in onychomycosis

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ABSTRACT

Background: Onychomycosis is chronic fungal infection of fingernails and toenails. Variety of fungi cause onychomycosis. Due to importance of high prevalence rate of onychomycosis this study was conducted.
Methods: In this study 100 patients suspected of onychomycosis were examined. Diagnosis of onychomycosis was based on the patient's history, physical examination, microscopy and culture of nail specimens.
Results: Direct microscopy of the nail clippings in 20% KOH solution was positive in 61% and culture was positive in 54% cases. The common etiological agent was dermatophytes (79.6% cases) followed by non dermatophyte moulds (11.1% cases) and yeasts (9.2% cases). Amongst dermatophytes, *T. rubrum* was found to be commonest etiological agent (57.6%) followed by *T. mentagrophytes*. We had performed the in vitro antifungal susceptibility testing of isolated fungal species against Amphotericin B, Fluconazole, Itraconazole and Terbinafine according to standard guidelines recommended by the CLSI. Antifungal susceptibility testing of dermatophytes and non-dermatophyte moulds was performed by broth macrodilution method. For *Candida* species we used broth macrodilution method as well as disk diffusion method. All three *Candida albicans* isolates were sensitive to amphotericin B, fluconazole and itraconazole. Two strains of *Candida krusei* were sensitive to amphotericin B and resistant to fluconazole and itraconazole. Two isolates of *T. rubrum* had MIC >64µg/ml and one *T. Mentagrophytes* isolate had MIC 32µg/ml for fluconazole. Among non dermatophyte moulds, *Aspergillus niger* and one isolate of *Fusarium oxysporum* showed high MICs against fluconazole.
Conclusions: Terbinafine exhibited the lowest MICs among all the tested antifungal drugs.

Keywords: Dermatophytes, Nails, Non dermatophyte moulds antifungal susceptibility testing, Onychomycosis

INTRODUCTION

Fungal infections of skin and its appendages are more prevalent in India due to favourable climatic conditions like temperature and humidity. Onychomycosis is a chronic fungal infection of fingernails and toenails. Fungal infections of the fingernails and toenails, in contrast to those at other body sites, are particularly difficult to eradicate with drug treatment. This is the consequence of factors intrinsic to the nail such as the hard nail plate, sequestration of pathogens between the nail bed and plate and slow growth of the nail.

The increased use of antifungal drugs, often for prolonged periods, has led to acquired antifungal resistance. Therefore, there is a need to determine the antifungal susceptibility of isolates to available drugs.[1] Despite good therapeutic response to newer modalities,

long-term outcome is unsatisfactory due to therapeutic failure, relapse and reinfection. In such cases antifungal susceptibility testing would obviously be beneficial. It is now possible to perform reliable in vitro antifungal susceptibility tests on a wide range of yeasts and moulds. The subcommittee for antifungal susceptibility testing on CLSI, USA has recommended standard broth dilution procedures to determine MIC of yeasts M27-A2 and filamentous fungi M38-A2.[2,3]

METHODS

The present study was carried out in the Department of Microbiology, Government Medical College, Aurangabad over the period of two years from January 2011 to December 2012. A total of 100 clinically suspected cases of onychomycosis were included. Patients who had received previous treatment for onychomycosis were excluded. Nail samples were collected by standard procedure and subjected to direct microscopy using 20% potassium hydroxide (KOH). Remaining nail sample was cultured on Sabouraud's dextrose agar (SDA) with and without cycloheximide. The media were incubated at room temperature and 37^0C for a minimum period of 4 weeks. Filamentous fungi were identified on the basis of cultural characteristic, pigment production, microscopic examination in lacto phenol cotton blue preparation and slide culture. The yeasts were identified by standard germ tube test, carbohydrate fermentation and assimilation tests.

Antifungal susceptibility testing[2-5]

Finally, antifungal susceptibility testing of each of the isolated species was done according to standard guidelines recommended by the CLSI. Antifungal susceptibility testing of dermatophytes and non-dermatophyte moulds was performed by broth macrodilution method. For *Candida* species we used both broth macrodilution method and disk diffusion method.

Antifungal agents

The powder forms of following antifungal drugs were used for broth macrodilution method.

- Amphotericin B (Himedia Laboratories Pvt Ltd, Mumbai),
- Fluconazole (Himedia Lab Pvt Ltd, Mumbai),
- Terbinafine (Tetramed Pharmaceuticals India),
- Itraconazole (Glenmark Pharmaceuticals India).

A total of 54 well-characterized fungal isolates were tested. They included 43 dermatophytes, 5 *Candida* species, and 6 non-dermatophyte moulds. *C. krusei* ATCC 6258 and *Aspergillus flavus* ATCC 204304 and *Tichophyton rubrum* ATCC 40051 were used as quality control organisms.

Stock solutions of antifungal agents

Stock solutions of fluconazole were prepared in sterile distilled water. Stock solutions of water insoluble drugs, amphotericin B, terbinafine and itraconazole were prepared in 100% dimethyl sulfoxide (DMSO).

Working solutions of antifungal agents

Working antifungal solutions were prepared at ten times the highest concentration to be tested for water soluble drugs. When DMSO was used as solvent a series of dilutions at 100 times the final concentration was prepared from the antifungal stock solution in the same solvent. Each of the intermediate concentration was then further diluted 1:10 in RPMI 1640 medium. Dilution procedures for water- soluble and water- insoluble drugs are shown in Table 1 and Table 2 respectively.

Storage

Stock solutions were dispensed into sterile plastic vials, sealed and stored at -30^0C until used.

Table 1: Scheme for preparing dilutions of water- soluble antifungal agents.

Step	Concentration (µg/ml)	Source	Vol Medium (ml)	Intermediate (ml)	Final concentration (µg/ml)	concentration at 1:10 (µg/ml)
1	5120	Stock	1	7	640	64
2	640	Step 1	1	1	320	32
3	640	Step 1	1	3	160	16
4	160	Step 3	1	1	80	8
5	160	Step 3	0.5	1.5	40	4
6	160	Step 3	0.5	3.5	20	2
7	20	Step 6	1	1	10	1
8	20	Step 6	0.5	1.5	5	0.5
9	20	Step 6	0.5	3.5	2.5	0.25
10	2.5	Step 9	1	1	1.25	0.125
11	2.5	Step 9	0.5	1.5	0.625	0.0625
12	2.5	Step 9	0.5	3.5	0.3125	0.03125

Table 2: Scheme for preparing dilutions of water- insoluble antifungal agents.

Step	Concentration (µg/ml)	Source	Vol Solvent (ml) + (DMSO) =	Intermediate concentration =	Final concentration at (µg/ ml)	1:100 (µg/ ml)
1	1600	Stock			1600	16
2	1600	Stock	0.5	0.5	800	8
3	1600	Stock	0.5	1.5	400	4
4	1600	Stock	0.5	3.5	200	2
5	200	Step 4	0.5	0.5	100	1
6	200	Step 4	0.5	1.5	50	0.5
7	200	Step 4	0.5	3.5	25	0.25
8	25	Step 7	0.5	0.5	12.5	0.125
9	25	Step 7	0.5	1.5	6.25	0.0625
10	25	Step 7	0.5	3.5	3.13	0.0313

Number of concentrations tested

Based on previous studies, the following drug concentration ranges were tested.

- Fluconazole 0.125 to 64µg/ml,
- Itraconazole 0.015 to 8µg/ml,
- Terbinafine 0.007 to 4µg/ml,
- Amphotericin B 0.03 to16µg/ml.

Medium used for broth macrodilution method

A complete synthetic medium RPMI-1640 medium (with glutamine and phenol red, without bicarbonate) supplemented with 0.2% glucose and buffered to a pH of 7.0 with 0.165mol/L MOPS (3-[N-morpholino] propanesulfonic acid) was used for susceptibility testing of yeasts, dermatophytes as well as non dermatophytes moulds by broth macrodilution method.

Antifungal susceptibility testing of Candida species

Broth macrodilution method

Antifungal susceptibility testing of *Candida* species was determined for three drugs, amphotericin B, fluconazole and itraconazole.

Inoculum preparation

All the isolates of *Candida* were subcultured on Sabouraud's dextrose agar and incubated at 35^0C for 24 hours. Inoculum suspensions were prepared by picking five colonies of approximately1mm diameter from 24 hours old cultures and suspended in 5ml of sterile normal saline (0.85%). The resulting suspension was vortexed for 15 seconds. The cell density was adjusted with spectrophotometer by adding sufficient sterile saline to increase the transmittance to that produced by a 0.5 McFarland standard at 530nm wavelength. A working suspension was made by 1:100 dilution followed by 1:20 dilution of the stock suspension with RPMI1640 broth medium.

Procedure

On the day of test, 0.1ml of each antifungal (at the intermediate concentration for water- soluble drugs and the intermediate concentration diluted 1:10 for water-insoluble drugs) was dispensed into 12x75mm sterile tubes. Each tube was inoculated with 0.9ml of the corresponding diluted inoculum suspension. This step resulted in dilution of each drug to the final test concentrations. The growth control received 0.1ml of 10-fold of the drug diluents without antifungal agent and was inoculated with 0.9ml of the corresponding diluted inoculums suspension. Sterility control included RPMI medium only, with no organisms or drug added. The quality control organisms were tested in the same manner and were included each time an isolate was tested. Tubes were incubated at 35^0C for 24 hours and observed for presence or absence of visible growth.

The amount of growth in the tubes containing the antifungal agent was compared to that of the growth in the growth-control tubes. MICs were determined visually. For amphotericin B, the MIC was read as the lowest concentration of drug that resulted in 100% reduction in turbidity as compared to drug-free control tubes. For azoles the MIC was read as the lowest concentration of drug that exhibited 80% reduction in turbidity.

Disk Diffusion method[6]

- Following antifungal disks were used
- Fluconazole (Himedia Lab Pvt Ltd, Mumbai) 10µg / disk
- Itraconazole (Himedia Lab Pvt Ltd, Mumbai) 10µg / disk
- Amphotericin B (Himedia Lab Pvt Ltd, Mumbai) 20µg / disk

Media used for disk diffusion test

Mueller-Hinton agar supplemented with 2% glucose and 0.5ug/ml methylene blue dye was used.

Procedure

The inoculum was prepared similar to that of inoculum preparation for broth dilution method.

Inoculation of test plates

The plates were inoculated as per standard CLSI guidelines and incubated at 35^0C.

Zone diameters (in millimetres) for the zone of complete inhibition were determined after 24 hours of incubation. When insufficient growth was encountered at the 24-hour reading, the plates were re-evaluated after a further 24 hours.

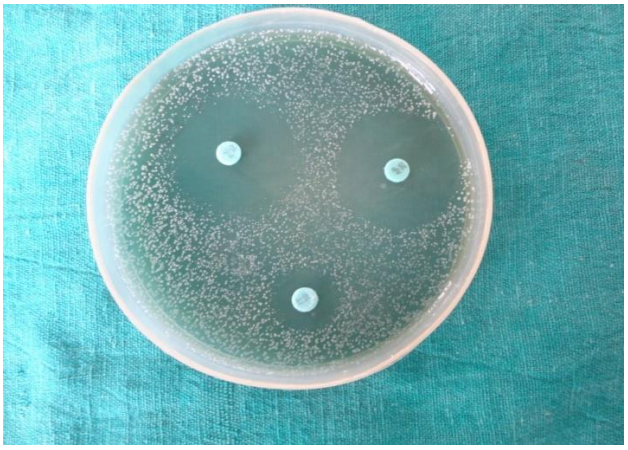

Figure 1: Antifungal susceptibility pattern of *Candida albicans* by Disk diffusion method.

***Antifungal susceptibility testing of dermatophytes and moulds*[3,5,7]**

Inoculum preparation

Dermatophytes and non-dermatophyte moulds were subcultured on potato dextrose agar and incubated at 28^0C for 7 days or until good conidiation was produced. The fungal colonies were covered with approximately 10ml of distilled water, and the suspensions were made by scraping the surface with the tip of a pasteur pipette. One to two drops of Tween-20 were added to facilitate the preparation of *Aspergillus* inoculum. The resulting mixture of conidia and hyphal fragments was withdrawn and transferred to a sterile tube. Heavy particles were allowed to settle for 15 to 20 minutes, the upper homogeneous suspension was transferred to a sterile tube and vortexed for 15 seconds.

The optical density of the suspensions containing conidia and hyphal fragments was read at 530 nm. The optical density of the suspensions of dermatophytes was adjusted to transmittance (T) of 65% to 70%. The optical density for *Aspergillus* species was adjusted to 80 to 82% transmittance. The adjusted inoculum suspensions were diluted 1:100 with RPMI 1640 medium to obtain the final inoculum size.

Broth macrodilution procedure

On the day of test, 0.1ml of each antifungal (at the intermediate concentration for water- soluble drugs and the intermediate concentration diluted 1:10 for water-insoluble drugs) was dispensed into 12x75mm sterile tubes. Each tube was inoculated with 0.9ml of the corresponding diluted inoculums suspension. The growth control received 0.1ml of 10-fold of the drug diluents without antifungal agent and was inoculated with 0.9ml of the corresponding diluted inoculums suspension. A sterility control was run in parallel by including a 1ml volume of uninoculated, drug-free medium. The quality control organisms were tested in the same manner and were included each time an isolate was tested. All isolates were run in duplicate and the results were read visually.

Incubation

For dermatophytes, tubes were incubated at 35^0C for 4 to 5 days, depending on the growth in control tubes without drug. *Aspergillus* species, *Fusarium* species and *Scopulariopsis* species were incubated at 35^0C for 48 hours and *Scedosporium apiospermum* for 72 hours.

The amount of growth in each tube containing the antifungal agent was compared to that of the growth in the growth-control tubes. Numerical score from 0 to 4 was given to each tube.

- 4-no reduction in growth
- 3-slight reduction in growth or approximately 75% of the growth control
- 2-prominent reduction in growth or approximately 50% of the growth control
- 1-slight growth or approximately 25% of the growth control
- 0-optically clear or absence of growth

Endpoint determination

For itraconazole and terbinafine the MIC was defined as the lowest concentration that produced 100% growth inhibition or numerical score 0. For fluconazole the MIC was defined as the lowest concentration showing 50% growth inhibition or numerical score 2. MIC ranges for each antifungal agent were obtained for each species. We also determined the $MIC_{50,}$ the MIC at which 50% of the isolates were inhibited and MIC_{90}, the MIC at which 90% of the isolates were inhibited.

RESULTS

Antifungal susceptibility pattern of Candida species by disc diffusion method

The Table 3 shows that all three *Candida albicans* isolates were sensitive to amphotericin B, fluconazole and itraconazole. Two strains of *Candida krusei* were sensitive to amphotericin B and resistant to fluconazole and itraconazole.

Table 3: Antifungal susceptibility pattern of *Candida spp.* by disc diffusion method.

Candida spp.	Amphotericin B	Fluconazole	Itraconazole
	SIR	SIR	SIR
Candida albicans (3 isolates)	3 0 0	3 0 0	3 0 0
Candida krusei (2 isolates)	2 0 0	0 0 2	0 0 2

S= susceptible, I= intermediate, R= resistant

Antifungal susceptibility pattern of Candida species by broth dilution method

The broth macrodilution antifungal susceptibility test was performed to confirm the results of disk diffusion method.

Table 4 summarizes the MIC ranges, MIC50 and MIC90 of the 5 strains of *Candida*.

Table 4: Antifungal susceptibility pattern of *Candida spp.* by broth dilution method.

Candida spp.	Antifungal Agent	MIC range (µg/ml)	MIC50 (µg/ml)	MIC90 (µg/ml)
Candida albicans (3 isolates)	Fluconazole	0.5-2	0.5	2
	Itraconazole	0.03-0.06	0.03	0.06
	Amphotericin B	0.06-0.5	0.06	0.5
Candida krusei (2 isolates)	Fluconazole	4-64	4	64
	Itraconazole	0.06-2	0.06	2
	Amphotericin B	0.25-0.5	0.25	0.5

According to CLSI breakpoints, we found that, all three isolates of the *Candida albicans* were susceptible to three drugs tested. Two isolate of *Candida krusei* were resistant to fluconazole (MIC 128µg/ml) and itraconazole (MIC 2µg/ml) while susceptible to amphotericin B (MIC 0.5µg/ml).

Antifungal susceptibility pattern of dermatophytes and moulds by broth dilution method

Table 5 summarizes the MIC ranges, MIC50 and MIC90 of the 43 strains of dermatophytes and six non dermatophytes against three antifungal drugs.

Table 5: Antifungal susceptibility pattern of dermatophytes and moulds by broth dilution method.

Species (No. of isolates)	Fluconazole	Itraconazole	Terbinafine
	MIC MIC50 MIC90 Range (µg/ml)	MIC MIC50 MIC90 Range (µg/ml)	MIC MIC50 MIC90 Range (µg/ml)
T. rubrum (26)	0.125-64 1 4	0.03-1 0.03 0.5	0.007-0.25 0.01 0.06
T. mentagrophytes (12)	0.5-32 1 2	0.01-2 0.03 1	0.007-0.125 0.03 0.06
T. verrucosum (03)	1-8 1 8	0.06-2 0.06 2	0.01-0.06 0.01 0.06
T. schoenlenii (01)	8 - -	0.03 - -	0.01 - -
T. tonsurans (01)	16 - -	0.25 - -	0.03 - -
Fusarium oxysporum (02)	4-64 - -	4-8 - -	0.03-0.125 - -
Aspergillus terreus (01)	0.5 - -	0.06 - -	0.06 - -
Aspergillus niger (01)	32 - -	0.25 - -	0.125 - -
Scopulariopsis brevicaulis (01)	0.5 - -	0.25 - -	0.06 - -
Scedosporium spp. (01)	2 - -	0.25 - -	0.06 - -

The observed MICs of all the drugs tested showed a broad range of variability against the different species. MICs of quality control strains were in agreement with the recommended CLSI range.

The highest MICs were measured for fluconazole. Two isolates of *T. rubrum* had MIC >64µg/ml and one *T. Mentagrophytes* isolate had MIC 32µg/ml for fluconazole. Among non dermatophyte moulds, *Aspergillus niger* and one isolate of *Fusarium oxysporum*

showed high MICs against fluconazole. Terbinafine exhibited the lowest MICs among all the tested antifungal drugs.

DISCUSSION

In this study, 54 fungal isolates were evaluated for antifungal susceptibility testing. Three antifungal agents; fluconazole, itraconazole and terbinafine were tested for dermatophytes and non dermatophyte moulds. *Candida* isolates were tested against fluconazole, itraconazole and amphotericin B.

We followed CLSI protocol M27-A2 and M44-A for antifungal susceptibility testing of yeasts by broth dilution method and disk diffusion method respectively.[2,6]

Several multicentre studies have been conducted previously to establish guidelines for the susceptibility testing of yeasts and filamentous fungi.[8-10]

In this study authors found that, all the isolated strains of *C. albicans* were sensitive amphotericin B, fluconazole and itraconazole by disk diffusion method as well as broth macrodilution method. Two strains of *C. krusei* one strain were resistant to fluconazole and itraconazole while sensitive to amphotericin B by both these methods.

Hill et al, conducted the antifungal susceptibility of 375 *Candida* species isolated from skin and nail.[11] They observed that most isolates, with the exception of *C. krusei* and *C. glabrata* were susceptible or dose-dependent susceptible to the triazole antifungals and fluconazole.

Authors observed that, results obtained by disk diffusion method were in acceptable concordance with results obtained by the broth macrodilution method. Disc diffusion methods for antifungal susceptibility testing of yeasts are simple to perform and can suitably be standardized in the laboratories as it showed a good correlation with the reproducible broth dilution method. However, when the results are equivocal, especially for azoles, the broth dilution test should be performed.

In recent years several studies on the in vitro susceptibility of dermatophytes to antifungal drugs have been done and the results have shown considerable variations.[5,7,12,13] This variability is probably due to important methodological differences among the laboratories.

Norris et al, were the first to identify the optimal parameters to be used in performing antifungal susceptibility testing of dermatophytes.[14] In their study, variables such as growth medium (RPMI 1640), inoculum size (10^3 conidia/ml), temperature of incubation (35^0C) and duration of incubation (4 days) were addressed. Some of these conditions are very different from those recommended for the CLSI method for filamentous fungi.

Fernández-Torres et al, and Santos et al, found that seven days of incubation at 28^0C were sufficient to observe prominent growth of dermatophytes.[15,16] Ghannoum et al, and Jessup et al, verified growth in four days at 35^0C.[5,12]

In this study, CLSI M38-A2 protocol for filamentous fungi has been adapted for testing 43 strains of dermatophytes.

The size of inoculum is considered to be one of the most striking factors in performing antifungal susceptibility testing.[17] In this work we have adjusted the inoculum to 65 to 70% transmission by spectrophotometer for antifungal susceptibility testing of dermatophytes. An inoculum density used in this study for antifungal susceptibility testing of dermatophytes was corresponded to 10^4CFU/ml, which agrees with that recommended by the CLSI method for filamentous fungi.

Authors found that various parameters such as, use of RPMI 1640 medium, incubation temperature of 35^0C, incubation time of 4 to 5 days and an inoculum of 10^4 CFU/ml were optimal for determination of the antifungal susceptibility of dermatophytes.

In this study, the MIC readings of 43 strains of dermatophytes by broth macrodilution method were ranged from, 0.125 to 64ug/ml for fluconazole, 0.01 to 2ug/ml for itraconazole and 0.007 to 0.25ug/ml for terbinafine. Perea et al, found that the MIC readings of 100 strains of dermatophytes by broth macrodilution method were ranged from 0.5 to 16ug/ml for fluconazole and 0.015 to 8ug/ml for itraconazole.[18]

In this study, the evaluation of in vitro antifungal susceptibility of dermatophytes showed that the terbinafine (0.007ug/ml) and Itraconazole (0.01ug/ml) had the lowest MIC values. Similar results have been demonstrated by other authors.[12,19] These low MICs found for the two drugs can help to explain the high in vitro and in vivo activities of both drugs against dermatophytes.[13] Authors observed that terbinafine was more active than fluconazole and itraconazole. Ryder NS et al, also demonstrated that, terbinafine was the most active drug against dermatophytes.[20] Fluconazole showed the lowest activity of all the antifungals tested.

CONCLUSION

Authors have found that terbinafine was the best antimycotic agent against dermatophytes followed by itraconazole. The disk diffusion method for yeasts is a simple, reliable, inexpensive and easily adaptable assay as compared to broth dilution assays. The increase in number of resistant fungal strains associated with treatment failure indicates the need of antifungal sensitivity testing.

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