

Original Research Article

Antifungal susceptibility testing for dermatophytes isolated from clinical samples by microbroth dilution method

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ABSTRACT

Background: The cases of dermatophytoses have increased over the past few decades. Dermatophytoses affect the outer layers of skin, nails and hairs without tissue invasion. These infections are mostly not dangerous but, are important as public health problem particularly in the immunocompromised. The increased use of antifungal drugs for prolonged periods may lead to acquired antifungal resistance among previously susceptible strains. With this background present study was conducted to know the susceptibility pattern of dermatophytes.

Methods: A total 35 isolates of dermatophytes isolated from clinically suspected cases of dermatophytoses were examined. Broth microdilution method M38-A2 approved protocol of CLSI (2008) for filamentous fungi was followed for determining the susceptibility of dermatophyte species to antifungal agents- itraconazole, fluconazole and ketoconazole.

Results: Itraconazole minimum inhibitory concentrations (MIC) varied from 0.0156 to 1 µg/ml for all dermatophytes. *T. rubrum* species showed higher MIC range for Ketoconazole than *T. mentagrophytes* and *T. tonsurans*. Fluconazole had poor susceptibility for all dermatophytes by having higher MIC values.

Conclusions: The MIC values observed in present study will help clinician to select an appropriate antifungal agent with minimal side effects. The data from present study can be useful as reference for future studies covering large no. of isolates and more drugs.

Keywords: Antifungal susceptibility testing, Minimum inhibitory concentrations, *T. rubrum*, *T. mentagrophytes*, *T. tonsurans*

INTRODUCTION

Dermatophytes are group of fungi causing infection of skin, hair and nail.¹ These infections are chronic and sometimes may be associated with secondary bacterial infections.^{2,3} More extensive or severe fungal infections require treatment with specific antifungal drugs. The various antifungal agents now available for clinical use against dermatophytes are terbinafine, itraconazole, fluconazole, ketoconazole, voriconazole. However, their activity against different species of dermatophyte has not

yet been fully investigated.⁴ As there is absence of antifungal policy in most of the hospitals across the world, it has increased the distribution of antifungal resistant strains of dermatophytes and poor infection control.⁵ For in vitro detection of resistance to antifungal agents there are guidelines given by CLSI document M38-A2 for broth microdilution method for filamentous fungi, in which MIC is calculated. Depending on that, a particular drug with higher MIC is considered relatively resistant.⁶ With this background present study was conducted.

METHODS

The study was conducted in Department of Microbiology at B. J. Govt. Medical College and Sassoon general hospital Pune. After getting a due permission from Institutional Ethical Committee, study was conducted. A total 35 isolates of dermatophytes comprising 20 *T. rubrum*, 8 *T. mentagrophytes* and 7 *T. tonsurans* were included in present study. The isolates were maintained in sterile distilled water till antifungal testing done. Broth micro dilution method M38-A2 approved protocol of CLSI (2008) for filamentous fungi was followed for determining the susceptibility of dermatophyte species.

Inoculum preparation

Stock inoculum suspensions of the fungi were prepared from 7 to 10 days old cultures grown on PDA at 28°C. Mature colonies were covered with approximately 5 mL of sterile saline (0.85%) by scraping the surface with the tip of a sterile swab. The resulting mixture of conidia and hyphal fragments was withdrawn and transferred to sterile tubes. Heavy particles were allowed to sediment for 15 to 20 min at room temperature. The upper suspension was transferred to another sterile tube and mixed with a vortex mixer for 15 sec. The turbidity of the supernatants was measured spectrophotometrically. Each suspension was diluted 1:50 to 1:100 in RPMI 1640 to obtain twice the final test inoculum concentration. The inocula corresponding to the strains were quantified by plating 5 µL of a 1:100 dilution of the adjusted inoculum on PDA plates and spread in 4 directions by a sterile loop. The plate contents were incubated at 30°C and observed daily for the presence of fungal colonies.^{7,8}

Drug preparation

Stock dilutions of itraconazole, fluconazole and Ketoconazole were prepared according to the standard protocol following CLSI M38-A2.

The two fold dilutions of the stock solution were further prepared in RPMI 1640 with L-glutamine and without sodium bicarbonate. These dilutions were used in the test at a pH of 7.0±0.1 with 3-(N-morpholino) propanesulfonic buffer along with 1N NaOH. The Final concentrations of antifungal drug s ranged between 0.0625 and 256 µg/mL for fluconazole, 0.0312 and 32 µg/mL for ketoconazole, 0.0078 and 8µg/mL for itraconazole.^{7,8}

Test procedure

All the tests were performed in sterile, flat-bottomed, 96-well micro plates. 100 µL of the drug dilutions were inoculated into the wells with a multichannel pipette. For performing the susceptibility testing, 100 µL of the diluted inoculum suspensions was added to each well to bring the drug dilutions to the final test concentrations. Growth and sterility control wells were also prepared for

each isolate tested. The micro plate contents were incubated at 35 °C, avoiding desiccation of the wells, and were read visually with the aid of an inverted reading mirror after 4-5 days of incubation.⁸

Quality control reference strains

Candida parapsilopsis strain ATCC-22019 was used as Quality control reference strain as approved by the CLSI.⁸

Determination of minimum inhibitory concentration values

For Fluconazole the MIC was defined as approximately 80% growth inhibition compared to the growth control well.^{8,9} For itraconazole and ketoconazole the MIC was defined as the lowest concentration showing 100% growth inhibition.⁸

Statistical analysis

MIC range, MIC50, MIC90 were obtained for all the isolates tested. MIC value of antifungal drugs for different species were compared by one-way ANOVA using SPSS version 16 software and a p value <0.05 was considered statistically significant.

RESULTS

The MIC ranges, minimum concentration that inhibited 50% of the isolates (MIC50) and minimum concentration that inhibited 90% of the isolates (MIC90) of each antifungal drug shown in Table 1.

The MIC range, MIC50, MIC90 values of Itraconazole against *T. rubrum* and *T. tonsurans* were 0.0156 to 1 µg/ml, 0.375 µg/ml and 0.50 µg/ml respectively, while these values were 0.0156 to 1 µg/ml, 0.125 µg/ml and 0.50 µg/ml in case of *T. mentagrophytes*.

The MIC range, MIC50, MIC90 values of Ketoconazole against *T. rubrum* were 0.0313 to 8 µg/ml, 1 µg/ml and 4 µg/ml respectively, while these values were 0.0313 to 2 µg/ml, 0.50 µg/ml and 1 µg/ml against *T. mentagrophytes*. However, 2 isolates of *T. rubrum* had higher MIC values of ketoconazole as 4 µg/ml (sd 15, sd 19).

Lower values of MIC range, MIC50, MIC90 were seen against *T. tonsurans* as 0.0313 to 1 µg/ml, 0.50 µg/ml and 1 µg/ml respectively.

The MIC range, MIC50, MIC90 values of fluconazole against *T. mentagrophytes* and *T. tonsurans* were 1 to 4 µg/ml, 2 µg/ml and 4 µg/ml respectively while these values were 1 to 8 µg/ml, 4 µg/ml and 8 µg/ml against *T. rubrum*.

Higher MIC values were observed against 2 isolates of *T. rubrum* (sd 11=8 µg/ml, sd 19=8 µg/ml) and one isolate

of each *T. mentagrophyte* (sd 5=4 µg/ml) and *T. tonsurans* (sd 4 =4 µg/ml).

Table 1: Determination of MIC values of antifungal drugs against dermatophyte species (broth microdilution method).

Dermatophyte species	MIC values	Itraconazole (µg/ml)	Ketoconazole (µg/ml)	Fluconazole (µg/ml)
<i>T. rubrum</i>	MIC range	0.0156 to 1	0.0313 to 8	1 to 8
	MIC ₅₀	0.375	1	4
	MIC ₉₀	0.50	4	8
<i>T. mentagrophyte</i>	MIC range	0.0156 to 1	0.0313 to 2	1 to 4
	MIC ₅₀	0.125	0.50	2
	MIC ₉₀	0.50	1	4
<i>T. tonsurans</i>	MIC range	0.0156 to 1	0.0313 to 1	1 to 4
	MIC ₅₀	0.375	0.50	2
	MIC ₉₀	0.50	1	4

DISCUSSION

Dermatophytosis is the most common superficial fungal infection affecting humans and animals.² A vast range of antifungal has been used to treat dermatophytosis. Different dermatophyte strains have different antifungal susceptibility pattern.⁶ Several studies testing filamentous fungi have demonstrated that azole MICs are extremely variable.⁷ In present study we have evaluated the susceptibility pattern of dermatophytes by using itraconazole, ketoconazole and fluconazole.

In present study *T. rubrum* isolates found to be more susceptible to both Itraconazole and ketoconazole with MIC range values 0.0156 to 1 µg/ml and 0.313 to 8 µg/ml. This finding is comparable with similar study done by Bhatia VK et al.⁹ However, for fluconazole MIC values were higher with MIC₅₀ value 4 µg/ml, which is much smaller than the study done by Budhiraja RK et al.⁶ Budhiraja RK et al showed MIC₅₀ value 64 µg/ml.⁶ Rabiye A et al showed similar MIC range values (0.06 to 4 µg/ml) as seen in, present study for fluconazole but Sowmya N et al, in their similar study got lesser values of MIC range for fluconazole (0.125 to 2 µg/ml) comparing with present study (1 to 8 µg/ml).^{1,10} Korting et al, suggested that high values of MIC for fluconazole may be due to technical problems, such as interference with some ingredients of the culture media or insolubility at high concentrations, taking medications by consulting internet instead of doctors, self medication, and prescription by quacks and easy availability of over the counter drugs.¹¹

T. mentagrophytes found to be highly susceptible to all antifungal agents used in present study. MIC range values of fluconazole, ketoconazole and itraconazole against *T. mentagrophytes* in present study were 1 to 4 µg/ml, 0.0313 to 2 µg/ml and 0.0156 to 1 µg/ml respectively,

which are comparable with MIC range values obtained by Siqueira ER et al.¹² Similar studies by Bhatia VK et al, and Sowmya N et al, reported same MIC range values for fluconazole and itraconazole.^{1,9} For ketoconazole Sowmya N et al, showed higher values and Bhatia VK et al, showed lesser values.^{1,9} Ketoconazole MIC values higher may be due to different conditions for testing procedure. Ketoconazole was very active against all dermatophytes as shown by MIC determinations, which justify its wide therapeutic application not only because of activity but also in terms of price when compared to fluconazole and itraconazole.

Seven isolates of *T. tonsurans* in present study were examined for antifungal susceptibility testing. MIC range value obtained was 1 to 4 µg/ml for fluconazole, 0.0313 to 1 µg/ml for ketoconazole and 0.0156 to 1 µg/ml for Itraconazole. These findings are comparable with other similar studies done by Sowmya N et al, Parvaneh A et al, and Gadangi I et al.^{1,7,13}

Itraconazole found to be having lower MIC values against all the dermatophyte isolates studied in present study. Itraconazole is much more affordable antifungal drug. It must be preferred treatment options for better outcome in patients suffering from dermatophytosis.

In present study isolates were obtained from patients previously not on any antifungal agents. That may be the reason, that we did not encountered resistance to any antifungal drugs used in this study, but higher MIC values were seen with few isolates. So, author need to correlate this data with patients who showed resistance clinically. In future, study involving large no. of isolates and maximum no. of antifungal drugs along with clinical resistance need to be evaluated for better implication of treatment against dermatophytosis.

CONCLUSION

Present study gave important data regarding susceptibility pattern of dermatophyte which can be used as reference for future studies. It will be very useful to clinician for management of cases requiring longer treatment.

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Ethical approval: The study was approved by the Institutional Ethics Committee

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