

## IN VITRO ANTIFUNGAL SUSCEPTIBILITY OF TRICHOPHYTON SPECIES AGAINST ITRACONAZOLE, KETACONAZOLE AND TERBINAFINE.

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### ABSTRACT

**OBJECTIVE:** This study was to evaluate the antifungal susceptibility of *Trichophyton rubrum*(n-75), *Trichophyton mentagrophyte*(n-50) and *Trichophyton tonsurans*(n-25) against three antifungal drugs namely Itraconazole, Ketoconazole and Terbinafine. **METHOD:** Antifungal susceptibility was evaluated by CLSI M38 (A) method with minor modifications. **RESULT:** All tested organisms produced detectable growth after 7 days of incubation at room temperature. Buffered RPMI1640 medium yielded adequate growth of dermatophyte. When all the strains were considered together, the geometric means of the MICs of terbinafine was lowest, which is 0.025 for *T. rubrum*, 0.03 for

*T. mentagrophyte* and 0.012 for *T. tonsurans*. The comparison of the in vitro susceptibilities of all three tested antifungal agents revealed that they were highly effective, but terbinafine was the most effective. Ketoconazole showed highest MIC values to the tested isolates. Mean difference in susceptibility of each antibiotic to different species was significant at the 0.05 level (P>0.05). **Conclusion:** Drug resistance could possibly be due to the absence of a proper Antifungal bio gram. While it may seem that fungal infections are generally benign, a continued lack of adherence to protocols of antifungal susceptibility testing will eventually lead to a situation akin to that observed in the emergence of multi-resistant organisms. If clinical resistance to antifungals is suspected, *in vitro* antifungal susceptibility testing is to be utilized for better antibiotic choice in contrast to a blind misuse of antifungals.

**KEYWORDS:** Dermatophytes, Antifungal susceptibility testing, Trichophyton.

## INTRODUCTION

Dermatophytosis is a fungal infection commonly seen in humans and animals. It is caused by a group of fungi known as dermatophytes. These fungi have the capacity to invade the keratinized tissues (skin, hair, and nails) of humans and other animals to produce an infection, dermatophytosis, commonly referred to as ringworm and this group of fungus is closely related.<sup>[1]</sup> The dermatophyte fungi are ubiquitous and no people or geographical area spared by these organisms.<sup>[2]</sup> Fungal infections of the skin and nails have developed to become significant infectious diseases in the last few years. Many cases of uncommon infections were also reported.<sup>[3]</sup> The lack of accurate diagnosis, misleading antibiotic selection which leads to treatment failure and relapses in infection are often cited as reasons for this trend. Deep infections caused by dermatophytes were also reported in many studies.<sup>[4,5]</sup> The injudicious use of antifungal agents for the treatment of dermatophytosis has led to an upsurge in clinical resistance to dermatophytosis.<sup>[6,7]</sup> Many *in vitro* antifungal susceptibility studies have reported an increase in the MIC (Minimum Inhibitory Concentration) range in antifungal agents and the emergence of resistant strains.

The successful oral therapy with griseofulvin of experimental dermatophytosis in guinea pigs reported by Gentles in 1958<sup>[8]</sup> revolutionized the therapy of dermatophytosis and initiated the first major change in the therapy of tinea capitis since the work of Sabouraud. In recent years, the number of infections caused by these fungi has increased considerably<sup>1</sup> often exhibiting atypical manifestations when they infect immunocompromised patients resulting in more severe, extensive lesions.<sup>[9]</sup> The newer antifungal agents available to treat the dermatophytoses include orally active triazoles (fluconazole, itraconazole), the allylamines (naftifine and terbinafine) and the morphilines (amorolfine). Generally topical antifungals are used for the treatment of Dermatophytoses, but this local therapy may be inappropriate for extensive infections or for infections affecting the nails or scalp and requires the use of systemic antimycotics.<sup>[10]</sup> Recently, a number of safe and highly effective antifungal agents have been introduced for the treatment of fungal infections. Among them, terbinafine, itraconazole, ketaconazole are more effective against dermatophytes.<sup>[11,12]</sup> Over the past few decades the number of infections caused by dermatophyte fungi has increased considerably and such infections are often recalcitrant to therapy.<sup>[13]</sup> Of late, antifungal resistant strains of dermatophytes have been reported.<sup>[6,14]</sup>

## MATERIALS AND METHODS

A total of 150 strains of dermatophytes belonging to 3 species namely *Trichophyton rubrum* (n-75), *Trichophyton mentagrophyte* (n-50), *T. tonsurans* (n-25) were tested. These were isolated from skin, hair and nail of clinically suspected Dermatophytosis patients attending the out patients clinics of dermatology department of a tertiary care hospital in north Kerala. All strains were identified by standard methods, which included the macroscopic and microscopic characteristics of the culture strains, urease test, rice grain test and hair perforation test. Evaluated the antifungal susceptibility by CLSI M38 (A) method, which is a standard method for the evaluation of antifungal susceptibility of filamentous fungi, was used with certain modifications. The adaptations involved, as mentioned in previous studies, a 10 day old PDA subculture growth for inoculum preparation and incubation of the micro titre plates at room temperature for 7 days **Medium.** The tests were performed in RPMI 1640 medium, with L-glutamine and without sodium bicarbonate, buffered at pH 7.0 with 0.165 M morpholinepropanesulfonic acid (MOPS).

**Method.** Tests were performed using a broth microdilution technique as described in the M 38(A) method<sup>19</sup> of CLSI procedures.

**Inoculum preparation.** 10-day-old cultures of fungus grown on PDA (Potato Dextrose Agar) at room temperature were used for the preparation of stock Inoculum suspensions. Approximately 10 ml of sterile saline (0.85%) was poured over the mature colonies and the surface of these colonies was scraped with the tip of a Pasteur pipette. The resulting mixture of conidia and hyphal fragments were transferred to sterile tubes. Heavy particles were allowed to settle for 15 to 20 min at room temperature; the upper suspension was mixed with a vortex mixer for 15s. The turbidity of the volume was made visually according to 0.5 McFarland Standard tube. Each suspension was diluted 1: 100 in RPMI 1640 to obtain the final Inoculum size approximately  $10^4$  CFU ml. Inoculum quantization can be performed by plating 0.01  $\mu$ l of a 1:100 dilution of the adjusted Inoculum on Sabouraud glucose (dextrose) agar to determine the viable number of CFU per milliliter. The plates were incubated at 28<sup>0</sup> to 30<sup>0</sup> C and observed daily for the presence of fungal colonies. Colonies were counted as soon as growth became visible.

**Test procedure.** The tests were performed in sterile, round-bottomed, 96-well micro plates. Each well was inoculated on the day of the test with 0.1 ml of the inoculum suspension. This step diluted the drug concentrations, inoculum densities and solvent as required achieving the

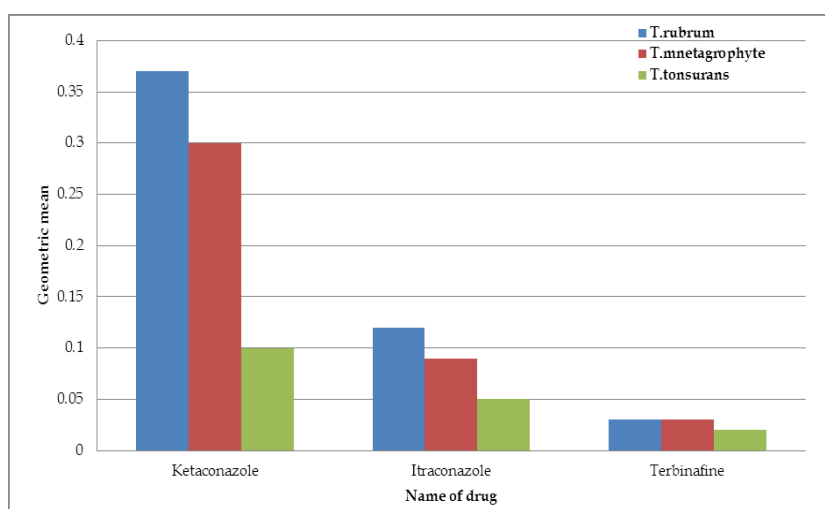
final desired test concentrations. The growth control wells contained 0.1 ml of the corresponding diluted inoculum suspension and 0.1 ml of the drug diluents (2%) without antifungal agent. All agents were tested at 0.001 to 16 mg/ml. Growth and sterility control wells were included for each test. The strain *Candida albicans* ATCC 90028 was included as the quality control. The microplates were incubated at room temperature and were read after 7 days of incubation. The minimum inhibitory concentrations (MICs) endpoints for antifungal drugs were determined visually by comparing the growth inhibition of each well to that of the drug free growth control well. For azole derivatives, the MIC was the lowest concentration showing prominent growth inhibition (approximately 50% of the growth control). For terbinafine, the MIC was defined as the lowest concentration showing 100% growth inhibition.

## RESULT

All tested organisms produced detectable growth after 7 days of incubation at room temperature. Adequate growth of dermatophytes was obtained on Buffered RPMI1640 medium, When all the strains were considered together, the geometric means of the MICs of terbinafine was lowest, which is 0.025 for *T.rubrum*, 0.03 for *T.mentagrophyte* and 0.012 for *T.tonsurans*. MIC ranges and geometric means of other antifungal agents have been shown in table-1 and figure-1. The comparison of the in vitro susceptibilities of all three tested antifungal agents revealed that they were highly effective, but terbinafine was the most effective. Ketoconazole showed highest MIC values to the tested isolates. Mean difference in susceptibility of each antibiotic to different species was significant at the 0.05 level ( $P > 0.05$ ). *T.rubrum* was highly susceptible to terbinafine and the MIC range was 1- 0.004 µg/ml while the geometric mean of MIC was 0.025 µg/ml. The MIC of other antifungal agents was higher than terbinafine. The MIC range of Itraconazole was between 2 – 0.008 µg/ml and the geometric mean was 0.118 µg/ml. For Ketoconazole, the MIC range was 4 – 0.03 µg/ml whereas the geometric mean was 0.369 µg/ml. *T.rubrum* showed higher MIC than *T.mentagrophytes* and *T.tonsurans* against the antifungals tested. *T.tonsurans* demonstrated the lowest MIC against the antifungals tested when compared to both *T.rubrum* and *T.mentagrophyte*.

**Table-1: Anti-fungal susceptibility pattern of dermatophytes.**

Species (no. of strains tested)	Antifungal Agents	MIC ranges ( $\mu\text{g/ml}$ )	Geometric means( $\mu\text{g/ml}$ )	Std deviation
T.rubrum (75)	Ketoconazole E	4 – 0.03	0.369	0.554
	Itraconazole	2 – 0.008	0.118	0.226
	Terbinafine	1 – 0.004	0.025	0.096
T.mentagrophytes (50)	Ketoconazole	2 - 0.03	0.301	0.702
	Itraconazole	1 – 0.016	0.090	0.324
	Terbinafine	0.5 - 0.004	0.031	0.105
T.tonsurans (25)	Ketoconazole	1 – 0.016	0.102	0.160
	Itraconazole	0.5 – 0.008	0.048	0.110
	Terbinafine	0.125 – 0.002	0.016	0.031

**Figure 1: MIC of dermatophytes**

## DISCUSSION

Antifungal resistance has often been described as an ‘emerging problem’ in recent years. The past few decades have witnessed a considerable increase in the number of infections caused by dermatophyte fungi and such infections are often unaffected by conventional therapy.<sup>[13]</sup> Recently antifungal resistance strains of dermatophytes have been reported.<sup>[6,14]</sup> *T. rubrum*, *T. mentagrophytes*, *T. tonsurans* are the common dermatophytes causing skin, nail and hair infections<sup>[16,2,17]</sup>, and have begun to exhibit clinical resistance and increased range of MIC values.<sup>[16,14,13,12,18]</sup> Three commonly employed antimycotic agents for the treatment of dermatophytosis; Ketoconazole, itraconazole, terbinafine were used for antifungal susceptibility testing in this study in accordance with CLSI protocols<sup>[19]</sup> (standard M38-A) with some modifications. This modification was previously adapted by Santos and Hamdan<sup>[16]</sup>, Norris *et al*<sup>[20]</sup> and Ozkutuk *et al*.<sup>[21]</sup> The modifications included incubation at room temperature, an incubation time of 7 days and inocula consisting mostly of conidia to

determine the MIC values. Antifungal susceptibilities of hyphae and conidia are probably different.<sup>[16]</sup> The hyphal fragments are separated from conidia during the sedimentation process. Generally *Trichophyton*sps do not produce macroconidia in the conventional culture media<sup>[18]</sup> and the inoculum mostly contains the microconidium which offers better results in antifungal susceptibility testing. Similar observations have been reported in other studies.<sup>[16,22,23,18]</sup>

Inoculum size exerts great influence on MIC's.<sup>[12,20,22,23]</sup> Fernadestorreset *al*<sup>[22]</sup> compared two inoculum size,  $10^3$ cfu/ml and  $10^4$ cfu/ml for antifungal testing of *Trichophyton*sps. The MIC's of azole agents showed clear detectable growth at highest inoculum size but for terbinafine no difference was observed. This shows that inoculum size can influence the MIC's of some drugs, but not others. In this study,  $10^4$ cfu/ml was shown to provide better results. Other authors also observed similar results.<sup>[12,22,23,21]</sup> The ideal incubation temperature and time for antifungal susceptibility testing of dermatophytes is open to debate. Some authors have proposed various times of incubation, ranging from 4 to 15 days at 26–30<sup>0</sup> C.<sup>[22]</sup> However, other authors have proposed higher temperatures, such as 35–37<sup>0</sup>C<sup>2</sup>. Fernadestorreset *al*<sup>[22]</sup> and Norris *et al*<sup>[12]</sup> observed a higher reproducibility achieved for azole agents at 7 days incubation ( rather than 14 days) and room temperature (rather than 37<sup>0</sup>C), whereas for terbinafine these factors did not significantly influence the MIC's. In this study, all isolates produced clearly detectable growth after 7 days of incubation. The majority of dermatophyte species showed an optimal growth at room temperature. Comparable results were published by other authors. Previous studies have demonstrated that buffered RPMI 1640 medium allows adequate growth of filamentous fungi, including dermatophytes.<sup>[20,21,22]</sup> This study has also supported those findings.

All the antifungal agents tested displayed good activity against dermatophytes. There is no ideal data concerning the breakpoint of dermatophytes which indicates their susceptibility.<sup>[21]</sup> In this study, antifungal susceptibility results of dermatophytes were evaluated according to MIC ranges and geometric means. The evaluation of *in vitro* activities of tested drugs revealed that terbinafine was the most potent active drug, confirming reports by Santos *et al*<sup>[16]</sup> and Fernandez-Torres *et al*.<sup>[11,12]</sup> The geometric means of MICs of itraconazole and terbinafine were apparently the lowest whereas ketoconazole presented high MIC range and Geometric mean to all tested isolates. Ozkutuket *al* conducted a study in Turkey and observed that ketoconazole showed increased MIC in dermatophytes when compared to

itraconazole and terbinafine.<sup>[23]</sup> Studies conducted in United States<sup>[24]</sup>, UK<sup>[11]</sup>, Brazil<sup>[15]</sup> and other countries<sup>[22,23]</sup> concur with our results. Santos *et al*<sup>[16]</sup> observed that among the azoles, itraconazole was the most active, followed by ketoconazole and fluconazole. These studies were supported our results.

## CONCLUSION

Our study pointed to an almost universal pattern in the development of fungal resistance. This suggests that resistance could possibly be due to the absence of a proper Antifungal bio gram. While it may seem that fungal infections are generally benign, a continued lack of adherence to protocols of antifungal susceptibility testing will eventually lead to a situation akin to that observed in the emergence of multi-resistant organisms.

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