Original Article

In vitro susceptibility of dermatophytes to oral antifungal drugs and amphotericin B in Uttar Pradesh, India

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Abstract

Background: Dermatophytosis is a major public health problem in our country. Although resistance to conventional oral and topical antifungal agents is being increasingly encountered, the sensitivity pattern of dermatophytes has not been systematically analysed.

Aims: We aimed to determine the sensitivity pattern of dermatophyte isolates to amphotericin B and six oral antifungal drugs.

Materials and Methods: Patients with dermatophytosis attending the outpatient department of dermatology were enrolled in the study. Samples were collected for mycological examination and *in vitro* antifungal sensitivity testing was done by broth microdilution as per the Clinical and Laboratory Standard Institute M38-A standards.

Results: A total of 804 patients were enrolled. Specimens from 185 patients (23%) were both KOH and culture positive, and 44 of these isolates (41 *Trichophyton mentagrophytes* and 3 *Trichophyton rubrum)* were subjected to sensitivity testing. Minimum inhibitory concentrations (MIC) of itraconazole, ketoconazole, voriconazole and amphotericin B were comparable. The median MIC to fluconazole was higher than the other tested drugs. Dermatophytes were most susceptible to ketoconazole and voriconazole, followed by itraconazole, amphotericin B, fluconazole and griseofulvin. A high incidence of resistance was found to terbinafine and the difference was statistically significant in comparison to fluconazole, itraconazole, voriconazole, ketoconazole ($P = 0.001$) and griseofulvin ($P = 0.003$). The strains were more sensitive to amphotericin B as compared to griseofulvin ($P = 0.02$) and terbinafine ($P < 0.001$).

Limitations: This was a hospital-based study and may not reflect the true pattern in the community. Only a few of the isolates were selected for study. The clinical response of patients, whose isolates were studied for *in vitro* sensitivity of the antifungals, was not studied.

Conclusions: The sensitivity pattern of dermatophytes to various antifungals including amphotericin B, ketoconazole, voriconazole and itraconazole were determined. The studied isolates were least susceptible to terbinafine.

Key words: Amphotericin-B, antifungal, dermatophytes, *in vitro*, resistance, voriconazole

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Introduction

Dermatophytoses are a major public health problem[1] and are commonly encountered in the dermatology clinics.[2] These infections are frequently ignored, and rampant self-medication with inappropriate drug combinations has lead to resistance and frequent failure of treatment. For many years, griseofulvin was the only approved systemic antidermatophytic agent.[3] But today, it is not widely used due to griseofulvin-resistant isolates of dermatophytes and existence of strains with elevated minimum inhibitory concentration levels to griseofulvin.[4,5] Resistance to griseofulvin was first reported in 1969[6] and terbinafine resistance was documented in 2003.[7] Decreased clinical responses to currently available oral and topical antifungal agents with frequent clinical failures and relapses has necessitated updosing of antifungals and finding any other antifungal agent, which is not generally used for dermatophytoses.

Materials and Methods

The study was conducted on patients with dermatophytosis attending the dermatology outpatient department at S.S. Hospital, Institute of Medical Sciences, Banaras Hindu University, Varanasi, a tertiary health-care system, from January 2016 to June 2017. Ethical clearance was obtained from the Institutional Ethical Committee. Samples were collected after informed consent and subjected to microscopy and culture. Only samples that were both KOH and culture-positive were selected for study.

Microscopy

Potassium hydroxide preparation (KOH mount)

The specimen was placed on a slide and a few drops of 30% KOH solution was added. A cover slip was placed and the slide was warmed over a flame and left for 15 min (longer in the case of nails) for clearing of the specimen. The slide was then examined for the presence of fungal elements.

Culture

Culture media (Sabouraud dextrose agar) with cycloheximide (0.05 g/L) and chloramphenicol (0.005 g/L) were used for culturing the specimens. All cultures were incubated at 28°C in a bio-oxygen demand (BOD) incubator for 4 weeks. Plates and tubes were examined every day during first week and every 2 days thereafter. Re-inoculation was done if any bacterial or saprophytic fungal contamination was detected. The day of appearance of the dermatophyte colony was noted for assessing the rate of growth of the isolates.

Identification

The rate of growth, colony morphology and pigment on the obverse and reverse were noted. A lactophenol cotton blue mount was prepared from suspect colonies and examined microscopically. Presence of any conidia, type, size, shape and any other special structures such as spiral or racquet hyphae was noted. Dermatophyte identification was as per standard procedure. The urease test was performed when needed. A portion of the specimen was saved for re-examination or re-inoculation.

Sensitivity was performed using the broth microdilution method as per CLSI (Clinical and Laboratory Standard Institute) M38-A standards (see appendix 1 for details).[8]

Results

A total of 804 patients were enrolled. Of the 804 specimens, 678 (84.3%) were KOH positive, 194 (24.1%) culture positive and 185 (23%) were both KOH and culture positive. These 185 strains were stored for further study. Forty four of these 185 isolates (41 strains of *Trichophyton mentagrophytes* and 3 strains of *Trichophyton rubrum*) were revived after repeated culture and minimum inhibitory concentration testing was done by broth microdilution on these pure isolates only. The details are summarized in the flowchart in Figure 1.

MIC range and median MICs for *T. mentagrophytes* and *T. rubrum* are shown in Table 1. Resistance patterns of *Trichophyton mentagrophytes* isolates are presented in Table 2. The MIC levels above which strains are considered resistant for the tested drugs is also displayed in Table 2.

Most strains of *T. mentagrophytes* were resistant to terbinafine (33/41, 65.9%) and griseofulvin (20/41, 48.8%). There was a statistically significant difference in the susceptibility of

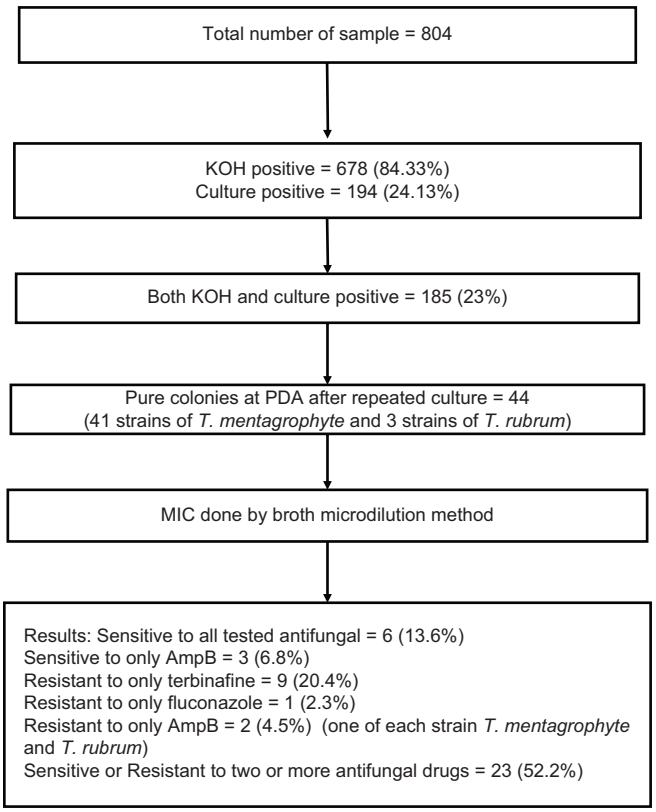

Figure 1: Details of sample, isolates and susceptibility

T. mentagrophytes isolates to amphotericin B compared with griseofulvin ($P = 0.02$) and terbinafine ($P < 0.001$). However the susceptibility of *T. mentagrophytes* to amphotericin B was similar to that of fluconazole, itraconazole, voriconazole and ketoconazole ($P = 0.80$, 0.41, 0.27 and 0.25, respectively). Although dermatophytes were more sensitive to voriconazole and ketoconazole than itraconazole, this difference was not statistically significant ($P = 0.76$ and $P = 0.29$, respectively). A large number of strains were resistant to terbinafine and was statistically significant in comparison to fluconazole, itraconazole, voriconazole, ketoconazole ($P = 0.001$) and griseofulvin ($P = 0.003$).

All three isolates of *T. rubrum* were sensitive to itraconazole, voriconazole and ketoconazole but resistant to both terbinafine and amphotericin B [Table 3].

Table 1: Results of *in vitro* sensitivity testing of 41 strains of *T. mentagrophytes* and 3 strains of *T. rubrum* (in µg/ml)

Drugs	MIC range	Median	
		T. mentagrophytes	*T. rubrum*
Fluconazole	0.25-64	16	32
Itraconazole	0.06-16	1	0.5
Voriconazole	0.06-16	0.5	0.5
Ketoconazole	0.06-16	1	1
Griseofulvin	0.06-16	2	1
Terbinafine	0.06-16	4	16
Amphotericin B	0.06-16	1	4

Table 2: Distribution of sensitive and resistant isolates of drugs for *T. mentagrophytes* (*n*=41)

Drugs	Breakpoint (µg/ml)	Sensitive strains, *n* (%)	Resistant strains, *n* (%)
Fluconazole	≥64	30 (73.2)	11 (26.8)
Itraconazole	≥8	34 (82.9)	7 (17.1)
Voriconazole	≥4*	35 (85.4)	6 (14.6)
Ketoconazole	≥8	35 (85.3)	6 (14.7)
Griseofulvin	≥3	21 (51.2)	20 (48.8)
Terbinafine	≥1	8 (34.1)	33 (65.9)
Amphotericin B	≥4*	31 (75.6)	10 (24.4)

*For filamentous *fungi*

Table 3: Distribution of sensitivity and resistance of drugs for *T. rubrum* (*n*=3)

Drugs	Breakpoint (µg/ml)	Sensitive strains, *n* (%)	Resistant strains, *n* (%)
Fluconazole	≥64	2 (66.6)	1 (33.3)
Itraconazole	≥8	3 (100)	_
Voriconazole	≥4*	3 (100)	_
Ketoconazole	≥8	3 (100)	_
Griseofulvin	≥3	2 (66.6)	1 (33.3)
Terbinafine	≥1	_	3 (100)
Amphotericin B	≥4*	_	3 (100)

*For filamentous *fungi*

Discussion

Dermatophyte infections have dramatically increased during this decade by misuse of topical corticosteroids cream alone or in combination with topical antibacterial and antifungal agents.[9] The rising trend of resistance among dermatophytes leading to poor response and frequent relapses is of serious concern and has been attributed to inappropriate treatments with steroid combination creams, improper dosages of antifungals and lifestyle changes. Resistance of dermatophytic infections to all antifungals (except voriconazole and amphotericin B) has been reported in the literature.[14-18]

Resistance to fluconazole is well documented[15,19,20] and was noted in 11 (26.8%) strains of *T. mentagrophytes* and 1 (33.3%) strain of *T. rubrum* in our study. The MICs of fluconazole ranged from 0.25 to >64 µg/ml which was similar to that reported in earlier studies.[10-14]

The MICs of itraconazole ranged from 0.06 to >16 µg/ml which was similar to that reported by Ataides *et al*.[21] Most other studies noted a narrow range of MIC (0.01–4 µg/ml)[4,11,16,20,22,23] but a wider range of MICs (0.06–32 µg/ml) has been observed by Gupta *et al*. in Canada.[13,25] All strains of *T. rubrum* were susceptible to itraconazole but 7/41 (17.1%) strains of *T. mentagrophytes* were resistant. An earlier study published from our institution reported a lower (6%) incidence of resistance to itraconazole[17] but Magagnin *et al*. observed resistance to itraconazole in 42.3% of the strains they studied.[21]

All strains of *T. rubrum* were susceptible to voriconazole. However, 14.6% strains of *T. mentagrophytes* were resistant to this drug. MICs of sensitive isolates ranged from 0.002 to 0.06 µg/ml,[25] similar to the reports of Deng *et al*.[26] but a wider ranges of 0.031–16 µg/ml have been previously reported.[24]

MICs of ketoconazole in our study was in the range of 0.06 to >16 µg/ml. Six strains (14.6%) of *T. mentagrophytes* were resistant to ketoconazole but all strain of *T. rubrum* were susceptible. A high incidence (53%) of resistance to ketoconazole was observed by Magagnin *et al*.[20]

A high incidence of resistance to griseofulvin and terbinafine was noted in our study. Twenty (48.8%) strains of *T. mentagrophytes* and 1 (33.3%) of *T. rubrum* were resistant to griseofulvin and 33 strains (65.9%) of *T. mentagrophytes* and all the three strains (100%) of *T. rubrum* to terbinafine. Similar findings have been reported previously.[10,16-18,20,22,23,27]

MICs of amphotericin B ranged from 0.06 to >16 µg/ml, which is higher than that noted in a previous study.[27] Ten strains (24.4%) of *T. mentagrophytes* and all three strains (100%) of *T. rubrum* were resistant. However, Yenisehirli *et al*. found amphotericin B to be more active than itraconazole and ketoconazole.[4]

In our study, dermatophytes were most sensitive to ketoconazole and voriconazole and least to terbinafine. The sensitivity pattern of *T. mentagrophyte* in decreasing order was voriconazole and ketoconazole > itraconazole > amphotericin B > fluconazole > griseofulvin > terbinafine while that for *T. rubrum was* ketoconazole = itraconazole = voriconazole > fluconazole = griseofulvin > terbinafine = amphotericin B. *T. rubrum* was least sensitive to terbinafine and amphotericin B.

We did not attempt to correlate *in vitro* sensitivity to the clinical response of the patients. This hospital-based study may not reflect the true pattern in the community. These are some limitations of this study.

Conclusions

Knowledge of sensitivity to antifungals prevalent in the local area can help determine choice of drugs for dermatophytoses. With such a high degree of resistance to terbinafine and griseofulvin, these may not be appropriate choice for the treatmet of tinea in our area.

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Declaration of patient consent

The authors certify that they have obtained all appropriate patient consent forms. In the form the patients have given their consent for their images and other clinical information to be reported in the journal. The patients understand that their names and initials will not be published and due efforts will be made to conceal their identity, but anonymity cannot be guaranteed.

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Conflicts of interest

There are no conflicts of interest.

Appendix I

If any suspected dermatophyte colony was mixed with contaminating colonies, the colony was subcultured again on Sabouraud dextrose agar with gentamicin. Sensitivity was performed using the broth microdilution method as per CLSI (Clinical and Laboratory Standard Institute) M38-A standards. 8 Stock solutions of concentration 1 mg/ml were made in normal saline for fluconazole and in 100% dimethyl sulfoxide for the other drugs. Double dilutions (from 0.25–64 μg/ml for fluconazole and from 0.06–16 μg/ml for the others) were prepared in RPMI-1640 (HiMedia) with L-glutamine buffered at pH 7.0 with 3-(N-morpholino) propanesulfonic acid, monosodium salt (but without sodium bicarbonate). Fungal colonies grown on potato dextrose agar after 7 days were used for *in vitro* sensitivity testing. The slant was flooded with 1 ml of sterile normal saline. Colonies were scraped gently with the help of sterile loop. The heavy particles were allowed to settle for 3–5 min. The upper homogeneous suspension containing mixture of nongerminated conidial and hyphal fragments was mixed for 15s with vortex. The turbidity was measured using a spectrophotometer at 530 nm and adjusted to final optical density range of 0.09–0.11 or visually containing standard 1,000,000 cells/ml of fungi counted on Neubauer's chamber. Stock inoculums suspension was diluted at 1:50 in RPMI-1640 medium. This test was performed in round-bottomed 96-well microdilution trays. Columns 1–9 were filled with double dilutions of 100 μl of respective antifungal drugs in rows in each well. Column 10 was a sterility control, containing 200 μl of RPMI-1640 and column 11 acted as a growth control (drug-free), having 200 μl of pure conidial suspension. Now, 100 μl of conidial suspension was filled in 1–9 well of serially diluted drugs. This tray was incubated at 30°C for 48–96 h of incubation and minimum inhibitory concentrations were determined and read visually. The growth in each well was compared with that of drug-free growth control and negative control. For most of the drugs, the minimum inhibitory concentration end-point criterion for fungi was the lowest drug concentration, showing 80% inhibition for azole and 90% inhibition of growth for terbinafine and amphotericin B.

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