ORIGINAL ARTICLE

Sensitivity pattern of causative species of dermatophytoses to various antifungals – an *in vitro* hospital based study

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ABSTRACT

Background: Dermatophytoses is one of the most common cutaneous fungal infections worldwide. Antifungal drugs, such as the allylamines (terbinafine), and orally active triazoles (itraconazole) have been reported to have substantial activity in these cases.

Aim: Aim of this study is to ascertain the susceptibility pattern of dermatophytoses to fluconazole, itraconazole and terbinafine using agar based disk diffusion method which is a simple, inexpensive and does not need any specialized equipment.

Methodology: In the present study 139 KOH positive clinically diagnosed cases of dermatophytoses were included. Paper disks containing terbinafine, fluconazole and itraconazole of potency 1µg/disk, 25µg/disk and 8µg/disk were used respectively. The *in vitro* activity of these antifungal agents was evaluated by measuring the diameter of inhibition around these disks.

Results: Total 106 strains belonging to 2 genera and 6 species as: *Trichophyton mentagrophytes* (55%), *Trichophyton violaceum* (21%), *Trichophyton rubrum* (14%), *Trichophyton verrucosum* (2%), *Microsporum audouinii* (2%), *T. mentagrophytes* and *T.violaceum* (5%) and *T.violaceum* and *T.tonsurans* (1%) were isolated. Majority of cases showed resistance to fluconazole (25.5%) followed by terbinafine (7.5%). No strain showed resistance to itraconazole.

Conclusion: This study revealed that itraconazole has least resistance followed by terbinafine. Total 30 strains were resistant to one drug. 3 strains were resistant to two drugs and none of the strain was resistant to all three drugs.

Limitation: It is a single centre hospital based study. Correlation with broth dilution method could not be done and sample size of the study was small. A multicentre study with larger sample size and clinical correlation is required.

INTRODUCTION

Dermatophytoses is one of the most common cutaneous fungal infections worldwide.[1] The incidence of resistant superficial fungal infections has increased during the last few years.[2] It can be due to increased use and over the counter sale of antifungal agents, inadequate or irregular use of drugs or increased incidence of immunodeficiency states.[3] Broth macro and micro-dilution assays can be used to determine antifungal susceptibility of dermatophytes. But these methods are expensive and require specific media and equipment such as RPMI (Roswell Park Memorial Institute), MOPS (3-(N-morpholino)propanesulfonic acid) buffer and micro plate trays. In comparison to these, agar-based disk diffusion (ABDD) susceptibility method is simple, inexpensive and does not require specialized equipments.[4] Main purpose of the study is to determine the *in vitro* susceptibility of the fungal organisms to terbinafine, fluconazole and itraconazole.

MATERIALS AND METHODS

This hospital based observational study was con-

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ducted in the department of Dermatology and Microbiology in a tertiary level hospital attached to a medical college in Northern India from Jan 2014 to Dec 2015. One hundred clinically diagnosed KOH and fungal culture positive cases of superficial dermatophytic infection were included in the study after taking informed written consent. Exclusion criterion were patients not willing for investigations, isolation of fungi other than dermatophytes on culture growth and patients on antifungals were excluded from the study. The study was started after obtaining approval from the college ethical committee.

SAMPLE COLLECTION

Samples were taken from the erythematous, peripheral, actively growing margins of the lesions. First the skin was decontaminated with 70% alcohol to remove surface bacterial contamination. An open, sterile Petri dish was held immediately below the area to be sampled and skin scales were flaked into it by using the blunt edge of a sterile surgical blade or microscopic slide. Samples were collected in two parts, for culture sensitivity and KOH examination each. Only KOH positive and culture positive cases were included in the study. Fig. 1 describes the study design.

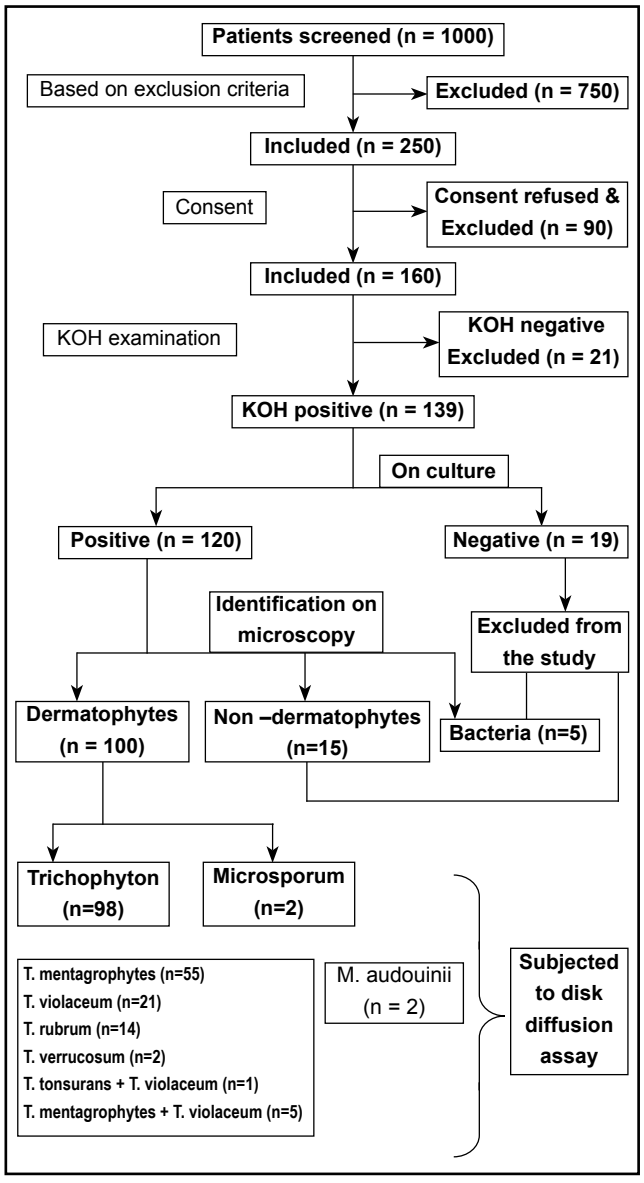

Fig. 1 Study design.

CULTURE AND SENSITIVITY

Isolation of dermatophytes

The samples were cultured under sterile conditions on the Sabouraud's dextrose agar (Himedia, India) and Sabouraud's dextrose agar containing cyclohexamide (0.05%) and chloramphenicol (0.004%) (Himedia, India). The colonies on the slants were examined for their morphology, texture and pigmentation (front and reverse) etc. upto 21 days. If growth came earlier the fungus was reported on the respective day. The confirmation was done by microscopic examination of the stained preparations.

Identification by microscopy

Colony of each isolate was stained in Lactophenol cotton blue (Himedia, India) and observed under low (10× lens) as well as high power (40× lens) of light microscope.

Subculture

Organisms were sub cultured on potato dextrose agar (PDA) and oatmeal agar (for T. rubrum)

(HI MEDIA, India) at 30°C upto 15 days and if results appeared earlier they were noted and reported.

Preparation of inoculum

Following growth, conidia were harvested in sterile saline using a haemocytometer; the conidial suspension was adjusted to 1.0 × 106 conidia/ml.

Inoculation on agar plate

Sterile cotton swab was dipped in inoculum rotated several times and pressed firmly against the side wall of tube above the fluid level which removes excess fluid. Mueller-Hinton (MH) agar plates measuring 90/100mm were evenly streaked in three different directions swabbing near the rim of plate with a swab dipped into the standardized inoculum suspension.

Preparation of disks

Fluconazole (batch no. FLP0I90214) and terbinafine (batch no. TBP0290414II) were procured from Synergene, Hyderabad and itraconazole (batch no. IT/09/13/010) from SMS Pharmaceuticals, Hyderabad in pure powdered form.

Stock solution

Stock solution were prepared by dissolving the powders in their specific solvents (DMSO, distilled water) in concentration of 5mg/2.5ml, 250µg/5ml and 48mg/4.8ml for fluconazole, terbinafine and itraconazole respectively.

Potency of drugs

Using above prepared stock solution, discs of diameter 9mm, 9mm and 6mm were prepared of concentration 25µg/disk (fluconazole), 8µg/disk (itraconazole) and 1µg/disk (terbinafine) respectively.

Disk diffusion assay

Disk containing terbinafine was applied to the surface of one inoculated plate and disks containing fluconazole and itraconazole to the surface of other inoculated plate.

Plates were inverted and incubated at 30°C upto 7 days and if results came earlier those were noted and reported. Inhibition zone diameters (IZD) were measured in millimetres and sensitivity pattern with antifungals was analyzed based on criteria in Table 1. T. mentagrophytes MTCC 7687 obtained from Institute of Microbial Technology, Chandigarh was used as control strain.

Table 1 Criteria for sensitivity and resistance of antifungal drugs[1,5,6]

Drugs	Zone wise interpretation (mm)		
	Sensitive	Intermediate sensitive	Resistant
Fluconazole	≥22	15-21	≤14
Itraconazole	≥19	11-18	≤10
Terbinafine	≥27	20-26	≤19

RESULTS

Total 139 patients were included in the study on the basis of inclusion criteria after taking written and informed consent. According to growth on culture, 19 cases were excluded as there was no growth seen on culture. In 15 and 5 cases non-dermatophytes and bacterial contamination was seen respectively. In 6 out of 100 culture positive cases for dermatophytes 2 strains were isolated. These 106 dermatophytic strains were further subjected to disk diffusion assay.

In present study dermatophytes isolated belong to 2 genera (Trichophyton and Microsporum) and 6 species. No Epidermophyton species were isolated. Trichophyton species were the most common species isolated. Among Trichophyton, T.mentagrophytes (microscopic image shown in Fig. 2) was the most common species isolated in 55% patients followed by T. violaceum (Fig. 3) in 21%, T.rubrum (Fig. 4) in 14%, and

T.verrucosum in 2%. M.audouinii was isolated in 2% patients. In 6% patients 2 species were isolated from the same sample. In 5% of patients both T.mentagrophytes and T.violaceum were isolated. In 1% patient both T.violaceum and T.tonsurans were isolated. (Fig. 5)

Sensitivity pattern observed with fluconazole (Table 2) showed resistance in 27/106 (25.47%) strains. (Fig. 6) 79/106 (74.52%) strains were sensitive to fluconazole out of which 16.03% were intermediate sensitive.

Sensitivity pattern observed with itraconazole (Table 2) showed zero resistance and 13/106 (12.3%) cases were intermediate sensitive. Sensitivity pattern observed with terbinafine (Table 2) showed resistance in 8/106 (7.54%) strains. (Fig. 7) 4/106 (3.8%) cases were intermediate sensitive to terbinafine.

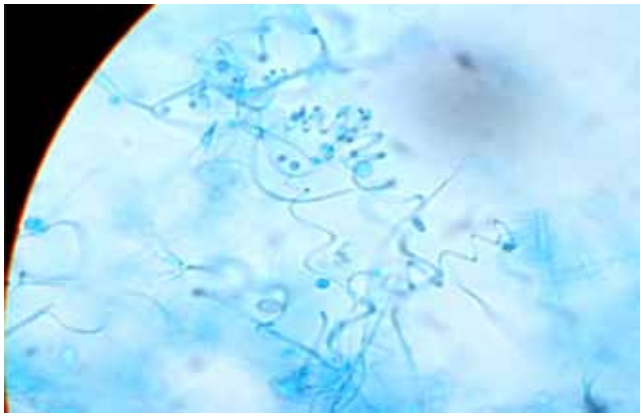

Fig. 2 Spiral hyphae of Trichophyton mentagrophytes on lactophenol cotton blue mount seen on 40x magnification.

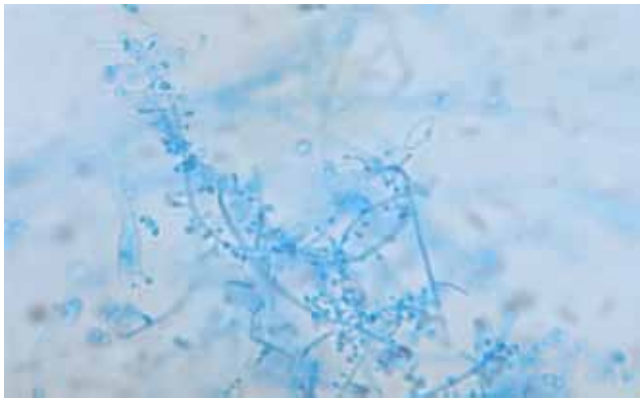

Fig. 4 Lactophenol cotton blue mount of T.rubrum showing clavate to tear drop shaped microconidia arranged along the sides of the hyphae on 40x magnification.

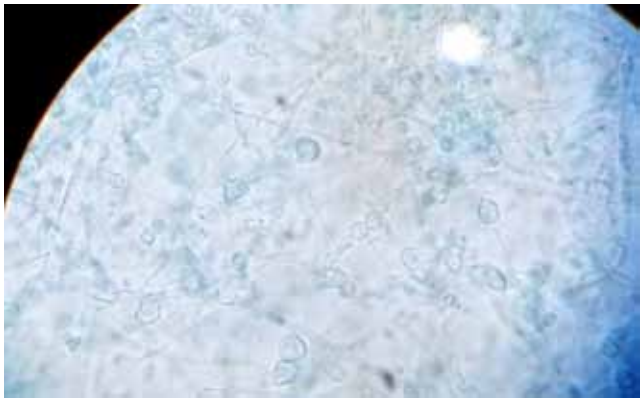

Fig. 3 Lactophenol cotton blue mount of T.violaceum showing long chains of chlamydoconidia on 40x magnification.

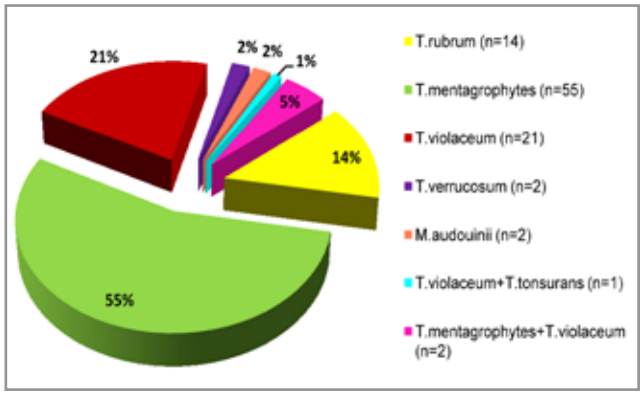

Fig. 5 Details of different dermatophytic species isolated.

Table 2 Showing the observed inhibitory zone diameters for different antifungals

	Fluconazole (n=106)	Terbinafine (n=106)	Itraconazole (n=106)
Range of IZD (mm)	0-60	0-88	11-70
Mean ± SD IZD (mm)	23.04 ± 15.64	54.40 ± 21.50	40.64 ± 12.72
Resistant	27 (25.5%)	8 (7.5%)	0
Intermediate sensitive	17 (16%)	4 (3.8%)	13 (12.3%)
Sensitive	62 (58.5%)	94 (88.7%)	93 (87.7%)

Fig. 6 Disk diffusion assay of a dermatophyte strain showing resistance with fluconazole with IZD 9mm.

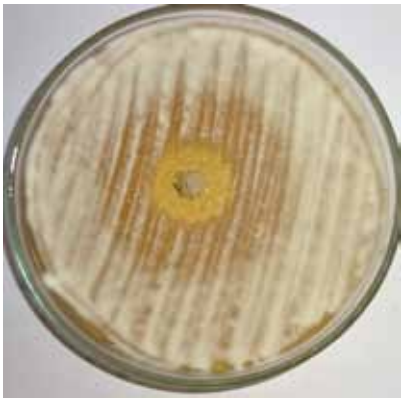

Fig. 7 Disk diffusion assay of a dermatophyte strain showing resistance with terbinafine with IZD 18mm.

DISCUSSION

Extensive research on antifungal susceptibility is done by only a handful of studies from Indian subcontinent.[5,6,7] Worldwide few studies have determined antifungal susceptibility among dermatophytes by agar based disk diffusion method and broth micro dilution method. Singh et al[8] in Canada (2007) used terbinafine 1µg/disk, fluconazole 25µg/disk and itraconazole 10µg/disk. Pakshir et al[1] conducted a study in Iran in year 2009 with terbinafine 30µg/disk, fluconazole 25µg/disk and Nweze et al[9] in Nigeria in 2010 using terbinafine 1µg/disk, fluconazole 25µg/disk, itraconazole 10µg/disk. Recently couple of studies from India have been reported. Mishra et al[5] in Rajasthan (2015) used fluconazole 10µg/disk, itraconazole 10µg/disk and Agarwal et al[6] at Dehradun (2015) used terbinafine 2µg/disk, fluconazole 25µg/disk, itraconazole 10µg/disk.

The data in present study correlates with study by Bhatia et al[10] in 2014 conducted in Himachal Pradesh in which Trichophyton sp. was isolated in 98.6% and no Epidermophyton sp. was isolated. In this study among Trichophyton species, T. mentagrophytes was the most common isolate i.e. in 63.5% followed by T.rubrum in 34.6% patients. In Putta et al[11] in 2016 conducted at Kohlapur. T.mentagrophytes was isolated in 37.74% and T.rubrum in 24.53% patients. In Nasimuddin et al[12] in 2014 T.mentagrophytes and T.rubrum were isolated in 38.5% and 27.13% patients respectively. Similar findings were reported by Pakshir et al[1] in which T.mentagrophytes (32.5%), T.rubrum (20%), T.violaceum (10 %) and T.verrucosum (5%) were isolated.

In present study T.violaceum was isolated in 21% cases. Similar high percentage was reported by Karmakar et al[13] conducted in Rajasthan in 1995 which T.violaceum was isolated in 55.6% and

Table 3 Sensitivity pattern of dermatophytes to various antifungals in past and present studies

Study Author	Place	Year	Antifungal resistance		
			Terbinafine	Fluconazole	Itraconazole
Present study	**Rohilkhand**	**2016**	**7.5%**	**25.7%**	**nil**
Mishra et al[5]	Rajasthan	2015	-	26.8%	14.9%
Agarwal et al[6]	Dehradun	2015	9%	10.9%	nil
Pakshir et al[1]	Iran	2009	2.5%	97.5%	-

Teklebirhan et al[14] conducted in Ethiopia in 2015 who reported isolation of T.violaceum in 37.7% cases. Borman et al[15] found that contributions of T.violaceum to total dermatophytic isolation has increased by 1000% from 1980 to 2005 in the British Isles. Grover et al[16] conducted in 2003 reported T.tonsurans as the commonest species followed by T.rubrum, explaining that the variation was due to different geographical locations harbouring different dermatophytic species.

Fluconazole

In present study range of inhibition zone of diameters (mean ± SD) varied from 0- 60 (23.15 ± 15.48) mm with fluconazole (25µg/disk). (Table 2) In study by Singh J et al,[8] range (mean ± SD) of inhibition zone diameters varied from 0-24 (3.37 ± 0.85) mm with fluconazole (25µg/disk). Agarwal et al[6] tested 55 strains with fluconazole (25µg/disk) and observed a range (mean ± SD) of 10-32 (22.6 ± 4.2) mm. Further, on analysis of results in terms of sensitivity, intermediate sensitive and resistant by taking into consideration the defined IZD for particular drugs 27/106 (25.5%) strains showed resistance and 62/106 (58.5%) strains were sensitive to fluconazole in present study. This may be due to misuse and overuse besides rampant over the counter sale of fluconazole. Moreover majority of patients in our study also gave history of prior usage of fluconazole in improper dose and/or duration. This is similar to the findings reported by Mishra U et al.[5] Agarwal et al[6] found 10.9% strains resistant and 9% intermediate sensitive to fluconazole (25µg/disk). Mishra U et al[5] used fluconazole (10µg/disk) and showed resistance in 26.8%. Pakshir et al[1] showed a high resistance i.e. 97.5% with fluconazole (25µg/disk). (Table 3 shows resistance pattern in various studies)

Terbinafine

In present study for terbinafine (1µg/disk) range of inhibition zone diameters (mean ± SD) observed were 0- 88 (53.67± 21.78)mm. In study by Singh J et al,[8] range (mean ± SD) inhibition zone diameters varied from 56-82 (72.8 ± 0.61) mm and in Agarwal et al[6] range (mean ± SD) inhibition zone diameters varied from 0-44 (32.1 ± 6.1)mm. In study conducted by Nweze et al,[9] a range of concentrations of terbinafine (0.0156, 0.03125, 0.0625 and 1.0 µg/disk) was used and observed that terbinafine concentrations of 0.0156, 0.03125, and 0.0625 µg/disk produced inhibition zones with diameters ranging from 5-15 mm, 7-18 mm and 10-22 mm, respectively, However, 1µg/disk of terbinafine produced IZD of 0-73 mm for all the isolates tested in that study. In study by Venugopal et al,[17] 0.25ug/disk was used and range (mean) IZD observed was 32-40 (36.5) mm. In our study terbinafine showed 8/106 (7.6%) resistance and 4/106 (3.8%) strains were intermediate sensitive. This is similar to the data reported by Agarwal et al,[6] who found 9% resistance and 91% sensitivity to terbinafine (2µg/disk). Pakshir et al[1] reported very less resistance with terbinafine (30µg/disk) i.e. 2.5%.

Itraconazole

Present study observed range of inhibition zone diameters (mean ± SD) for itraconazole (8µg/disk) as 11-70 (40.64 ± 12.72) mm. Singh J et al[8] used itraconazole 10µg/disk and range (mean ± SD) inhibition zone diameters in this study varied from 12-50 (21.7 ± 0.92) mm. Similarly, Agarwal et al[6] used 10µg/disk concentration and the range (mean ± SD) IZD varied from 17-36 (27.3 ± 6.2) mm. In present study no strain was resistant to itraconazole while 13/106 (12.26%) were intermediate sensitive. Past studies have

reported varied results while Agarwal et al[6] observed 7.3% intermediate sensitivity in their study and no resistance similar to present study. Mishra U et al[5] showed 14.9% resistance with itraconazole (10µg/disk).

Limitation

It is a single centre hospital based study with a relatively small sample size. Correlation with broth dilution method could not be done due to financial and logistic constraints.

CONCLUSION

We concluded that fluconazole showed maximum resistance followed by terbinafine. Noteworthy, itraconazole showed no resistance. Alarmingly 30/106 (28.3%) strains were resistant to one drug, 3/106 (2.8%) strains were resistant to two drugs and none of the strain was resistant to three drugs. In view of above results it can be drawn that usage of fluconazole should be limited and usage of terbinafine or itraconazole should be promoted. Our hypothesis for using combination of terbinafine and itraconazole is open to debate due to various issues associated with their pharmacokinetic properties. Further research should be done to find the efficacy of different combinations of antifungals and/or to evaluate the response of increased concentration of drugs in-vitro and their clinical co-relation. The results of this study will add upon the existing data regarding antifungal susceptibility of various dermatophytic infections.

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