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In vitro antifungal Susceptibility testing of Dermatophytes isolated from clinical samples in tertiary care hospital

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Abstract
Background: Dermatophytosis is colonization by dermatophytic fungus of the keratinized tissues like hair, nails and skin. They are considered important as a public health problem as well. The term 'tinea' is synonyms of word dermatophytosis. Depending upon the anatomical site of lesion, dermatophytes are classified into different varieties.
Objective: To isolate, identify, and detect the in-vitro antifungal sensitivity pattern of various dermatophytes isolates, from clinically suspected cases of dermatophytosis
Material and Methods: To find out antifungal resistance pattern among dermatophytes, 240 patients of all age group with clinical diagnosis of dermatophytosis were included under study. Identification and isolation was done by various test like macroscopic, microscopic, biochemical test and culture. Antifungal susceptibility testing was done by Agar Based Disc Diffusion (ABDD)
Result: Out of 240 samples 100 isolates of dermatophytes were grown on fungal culture. Tinea ungium followed by tinea cruris and tinea corporis were common types. *Trichophyton mentagrophytes* (52%) and *T. rubrum* (28%) were most common species. Out of six antifungal used in our study fluconazole and clotrimazole were found most resistant while voriconazole was most effective drug.
Conclusion: The epidemiology of dermatophyte infections may change with time. The Agar-Based Disk Diffusion method of antifungal susceptibility testing will aid the clinician in initiating prompt and appropriate antifungal therapy and prevent emergence of resistance.

Keywords: agar based disc diffusion (ABDD), antifungal sensitivity, Dermatophytosis, Tinea infection, trichophyton

Introduction
Dermatophytes are a group of closely related fungal species that have the capacity to invade keratinized tissue of human and other vertebrates and produce dermatophytosis. They are able to damage and utilize keratin found in the skin, hair, and nails [1].
Infections caused by these fungi are among the most prevalent cutaneous infections globally and affect 20% to 25% of the world's population [2]. Dermatophytes are classified into three groups: *Trichophyton, Epidermophyton*, and *Microsporum*. Consequently, dermatophyte species are divided into anthropophilic, zoophilic and geophilic species, respectively, on the basis of their primary habitat association [3, 4]. Dermatophytosis are influenced by multiple factors which include the virulence of the infected strain or species, anatomic location of the infection, environmental factors and the hosts reaction to fungal metabolic products[5]. Dermatophytes are more prevalent in India, due to favorable climatic conditions like temperature and humidity. The other factors which influence dermatophytosis are poverty, poor personal hygiene and social conditions like overcrowding [6].
The term 'tinea' is synonyms of word dermatophytosis. Depending upon the anatomical site of lesion, dermatophytes are classified into different varieties. The first part of name is 'tinea' that denotes dermatophytosis and the second part of the name of the disease identifies the part of the body infected, i.e.in case of Tinea corporis, "corporis " refers to the body, Tinea capitis, "capitis " refers to scalp and so on [7]. Over the past few years, there is an increased usage of antifungal drugs in the treatment, which has led to increased chance of antifungal resistance [8]. For a definitive therapy, it is essential to evaluate the resistant dermatophytes using standardize, simple and reproducible *In vitro* assay to determine the antifungal activity of drugs against isolates.
The agar-based disk diffusion (ABDD) susceptibility method for dermatophytes is simple, inexpensive, and does not require specialized equipment. The disk diffusion method has a good correlation with the reference dilution assay [9].
Present study was undertaken to isolate, identify, and detect the in-vitro antifungal sensitivity pattern of various dermatophytes isolates, from clinically suspected cases of dermatophytosis to the most commonly used antifungal agents, using the agar-based disk diffusion method.

Material and Methods
The present study was conducted from April 2017 to March 2018 in the Department of Microbiology, S.M.S Medical College, Jaipur after approval by ethical committee. Clinically suspected 240 cases of dermatophytosis attending the Out Patient Department of Dermatology &VD at SMS hospital were studied. After taking detailed case history, informed written consent, respective samples like skin scrapping, hair and nails were collected in sterile petri dish or black paper with proper sterilization and aseptic conditions. Specimen collected was subjected to potassium

hydroxide (KOH) wet preparation (10% KOH for skin and hair; 30% KOH for nail) for the presence of fungal elements.

After direct microscopic examination, irrespective of demonstration of fungal elements, the specimen was inoculated into 2 sets of test tubes containing Sabouraud's dextrose agar with 0.004% chloramphenicol and 0.05% cycloheximide. One set was incubated at 25°C and other set at 37°C for up to 4 weeks. If no growth was found after 4 weeks, it was taken as negative for growth of fungi. Fungal isolates were identified based on colony morphology, pigmentation, growth rate, microscopy (Lactophenol Cotton Blue mount), and slide culture. Special tests were done when necessary viz: hair perforation test and urease test for species identification. Fungal growth was also subculture on DTM (dermatophyte test media) for the confirmation of dermatophyte.

Antifungal susceptibility test

Anti-fungal susceptibility test was carried out for 100 isolates of dermatophytes by Agar based disc diffusion (ABDD) susceptibility method as per the methodology described by Nwaze *et al.* [10] for Fluconazole (25µg/disc), Itraconazole (10µg/disc), Clotrimazole (10µg/disc), Voriconazole (1µg/disc), Griseofulvin (10µg/disc) and Terbinafine (1µg/disc). Fluconazole, Itraconazole, Clotrimazole, Voriconazole were available commercially and purchased from Himedia, Mumbai India. Griseofulvin (10 µg) &Terbinafine (1µg/disk) were prepared in lab by dissolving the pure powders in DMSO& then diluting it to give a final concentration of 1mg/ml & 100 µg/ml for Griseofulvin & Terbinafine respectively & then delivering 10 µl onto each sterile disc. The disks were air dried at room temperature then stored in refrigerator at 4℃.

The conidial suspension was prepared from seven day old cultures and adjusted to1.0 x 10^6 conidia/ml. The inocula were evenly spread with a swab on the surface of Petri dishes containing Mueller-Hinton (MH) agar medium. The lids were left ajar for 3 min in a laminar flow to allow for absorption of extra moisture to be absorbed. The antifungal disks were applied to the inoculated plates, after which the plates were inverted and incubated at 30°C for 4-7 days. Inhibition zone diameter (IZD) around the disks were measured in mm and recorded. Two reference strains *Trichophyton rubrum* ATCC MYA 4438 and *Trichophyton mentagrophytes* ATCC MYA 4439 were used as control.

Statistical Analysis

Descriptive and Inferential statistical analysis has been carried out in the present study using computer software (SPSS Trial version 23 and primer). The qualitative data were expressed in proportion and percentages, and the quantitative data expressed as mean and standard deviations. The difference in proportion was analyzed by using chi square test. Significance level for tests was determined as 95% ($P< 0.05$)

Table 1: Inhibition zone diameter criteria for susceptibility and resistance of antifungal discs [11, 12]

Antifungal drugs	Potency	Zone diameter in mm		
		S	I	R
Fluconazole	25 µg	≥22 mm	21-15mm	≤14 mm
Voriconazole	1µg	>14mm		<14
Itraconazole	10 µg	≥22	21-15	<15
Terbinafine	1 µg	≥20	12-19	≤11
Clotrimazole	10 µg	≥20	19-12	≤11
Griseofulvin	10 µg	>31	31-26	<26

S= Sensitive, I= Intermediate, R= Resistance

Results

All the samples were cultured on two sets of SDA slants for isolation of fungal species. Out of 240 samples of suspected cases 212 (88.34) were culture positive while no fungal growth was found in 28 samples (11.66%). Out of these 212 samples, dermatophytes were identified in 100 samples while Candida and non dermatophytes were identified in 112samples.

A total of 100 isolates of dermatophytes out of 240 samples were grown on culture. 56% of dermatophytes were isolated from patients of 16-30 years age group. 81% of dermatophytes were isolated from male and 19% from female. Male to female ratio was 4.26:1

Maximum numbers of dermatophytes were isolated from skin samples (86.64%). We received hair from only one patient and dermatophyte was isolated from this. Rate of isolation of dermatophyte was less in nails (28.80%)

Dermatophytes were isolated from six type of clinical presentation. Tinea ungium was the most common presentation (53%) followed by Tinea cruris (26%) and Tinea corporis (17%). No dermatophyte was isolated from patients presented with Tinea fascie. There is a significant association was observed between clinical diagnosis and specimen of fungi with P=0.002

The most common dermatophytes isolated was *Trichophyton mentagrophytes (*52%) followed by *Trichophyton rubrum (28*%), *Trichophyton verrucosum (*3%), *Trichophyton Tonsurans (*15%), *Microsporum gypseum (*1%) *and* Microsporum *nanum(1%)* in our study.

T. mentagrophyte and *T. rubrum* were most common species found in all types of clinical lesions of Dermatophytosis. No significant association of dermatophytes was observed with different clinical types.

Out of 100 isolates 90% were sensitive to Griseofulvin while 10% were resistant. Among that out of 52 isolates of *T. mentagrophytes* 47(90.3%) were sensitive. Similarly out of 28 isolates of *T. rubrum* 25(89.28%) were sensitive. *T. verrucosum, T.nanum and M gypseum* were 100% sensitive to Griseofulvin.

Out of 100 isolates 47 % were sensitive to Fluconazole while 52% were resistant. Among that, out of 52 isolates of *T.mentagrophytes* 22(42.3%) were sensitive. Similarly out of 28 isolates of *T.rubrum* 16(57.14%) were sensitive. *T. tonsurans,*and *T. verrucosum,* were 53.3%,& 33% sensitive

to Fluconazole respectively. *T.nanum and M gypseum* were 100% resistant to this.

Out of 100 isolates 74% isolates were sensitive to Clotrimazole while 26% were resistant. Among that, out of 52 isolates of *T.mentagrophyte* 41(78.8%) were sensitive. Similarly out of 28 isolates of *T.rubrum* 21(75%) were sensitive. *T.tonsurans,* and *T. verrucosum,* was 73.3%, 33%, sensitive to Clotrimazole respectively.

Out of 100 isolates of dermatophytes all were sensitive to Voriconazole. 95 % isolates were sensitive to Itraconazole, 2% were intermediate while 3% were resistant. All the strains of *T.mentagrophytes, T.rubrum, T. verrucosum, T.nanum and M.gypseum* were sensitive to Itraconazole while 10 strains (66%) of *T. tonsurans* were sensitive.

Out of 100 dermatophytes 75 % were sensitive to Terbinafine while 25% were resistant. Among that out of 52 isolates of *T. mentagrophytes* 31(59.6%) were sensitive. Similarly out of 28 isolates of *T. rubrum* 25(89.2%) were sensitive. *T. tonsurans, T. nanum and M gypseum* were 100% sensitive while *T. verrucosum* were 66.6% sensitive.

Table 2: Distribution of dermatophytes according to age group (n=100)

Age group in years	No of cases	Percentage %
0-15years	1	1.00
16-30 years	56	56.00
31-45 years	25	25.00
46-60 years	12	12.00
>60 years	6	6.00
Total	100	100.00

Table 3: Isolation of dermatophytes from different clinical samples (n=100)

Specimen	Isolation of dermatophytes	Number of sample Collected	Percentage %
Skin	46	55	86.64%
Hair	01	01	100%
Nail	53	184	28.80%
Total	100	240	41.67%

Table 4: Association of dermatophytes with different clinical types

Isolated Dermatophytes	Tinea corporis	Tinea cruris	Tinea ungium	Tinea barbae	Total isolates	P Value LS
T. mentagrophyte	8	14	28	2	52	1.0NS
T. rubrum	6	5	16	1	28	0.95NS
T. tonsurans	4	5	6	-	15	0.75NS
T. verrucosum	-	1	2	-	03	1.0NS
Microsporum gypseum	1	-	-	-	01	0.39NS
Microsporum nanum	-	1	-	-	01	0.56 NS
Total	19	26	52	03	100	

Table 5: Association of Dermatophytes with different antifungal drugs

Dermatophytes	FLU			VOR			ITR			TER			CLO			GRI		
	S	I	R	S	I	R	S	I	R	S	I	R	S	I	R	S	I	R
T. mentagrophyte (n=52)	22	0	30	52	0	0	52	0	0	31	0	21	41	0	11	47	0	5
T.rubrum (n=28)	16	0	12	28	0	0	28	0	0	25	0	3	21	0	7	25	0	3
T. tonsurans (n=15)	8	0	7	15	0	0	10	2	3	15	0	0	11	0	4	13	0	2
T. verrucosum (n=3)	1	0	2	3	0	0	3	0	0	2	0	1	1	0	2	3	0	0
M. gypsium (n=1)	0	0	1	1	0	0	1	0	0	1	0	0	0	0	1	1	0	0
T. nanum (n=1)	0	1	0	1	0	0	1	0	0	1	0	0	0	0	1	1	0	0
Total(n=100)	47	1	52	100	0	0	95	2	3	75	0	25	74	0	26	90	0	10

FLU= Fluconazole, VOR= Voriconazole, ITR= Itraconazole, TER= Terbinafine, CLO = Clotrimazole, GRI = Griseofulvin

Discussion

Dermatophytes possess the affinity for parasitizing keratin rich tissues and produce dermal inflammatory response. This leads to redness, intense itching/burning in turn causes cosmetically poor appearance [13]. The severity of the infection depends on various factors like immune reactions of the host to the fungal metabolic products, virulence of infecting strain, anatomical location of the infection and environmental factors [14].

In this study 56% of dermatophytes were isolated from patients who were in 16-30 years of age. Our results are in accordance with various studies [11, 15, 16] who also reported higher infections rate in young adults. The higher incidence may be due to increased physical activity, increased sweating because of environmental conditions such as hot and humid weather and increased opportunity for exposure.

In this study 81% of dermatophytes were isolated from male patients and 19% from female. Male to female ratio was 4.26:1. Our results are in accordance with B Janardan *et al.* [17] who reported male to female ratio as 3.86:1. Male preponderance was reported by many studies conducted all over India [18-21]. High incidence of dermatopytosis in male may be due to increased physical activity and frequent interaction with different people of the society.

The predominant clinical manifestations of dermatophytosis vary considerably in different studies reported in literature. In this study tinea unguium was the dominant clinical manifestation involving 53% of the total cases of dermatophytosis from nail followed by tinea cruris 26% from skin. Our findings are in concordance with various studies [22, 23] who also reported tinea unguium as the dominant clinical manifestation. Infection of the nail was most common, as majority of this group of patients consisted of either agricultural workers or house-wives engaged more often in activities that involved soaking of hands in water [24]. However there are studies conducted in India who reported tinea corporis (35.4%) as the predominant clinical condition followed by tinea cruris (16.8%) and tinea capitis (16.7%) [25]. Similar study conducted in Iran by Rassai *et al.* [26] revealed that tinea cruris and tinea corporis were the most common clinical manifestation. A study carried out by Devliotou-

Panagiotidou *et al.* [27] in Greece depicted that tinea pedis was the most frequent clinical manifestation. Adefemi *et al.* [28] reported tinea capitis as a predominant clinical manifestation. The variations observed in the clinical type of dermatophytosis could be due to varied climatic conditions, migration of population to earn a livelihood, type of occupation, pathogen and host relationship [29].

In this study genus *Trichophyton* represented 98% of the isolates of dermatophytes, with *Trichophyton mentagrophyte* as the predominant isolate (52%) followed by *Trichophyton rubrum* (28%) and *Trichophyton tonsurans* (15%). The other species isolated was *Microsporum gypseum* and *Microsporum nannun* isolated from one patient each. Our findings are in concordance with Ravika K *et al.* [30] who also reported genus *Trichophyton* isolated from 97.6% of patients. Soniya Mahajan *et al.* [31] also reported *Trichophyton mentagrophytes* as most common species isolated from 75.9% of patients followed by *Trichophyton rubrum*(11.3%). Similar findings were also observed by Sahai and Mishra [32] and Bhatia and Sharma [33]. However there are various studies [6, 9, 3, 4, 35] who reported *Trichophyton rubrum* as the predominant isolate. Ajello, in 1960, said "species not only differ from region to region but may change with the passage of time [36].

The determination of *In vitro* antifungal susceptibility has been reported to be important for the ability to eradicate pathogenic Dermatophytes [19]. In this study we studied an Agar-Based Disk Diffusion method to determine the susceptibility of dermatophytes of six commonly used antifungal drugs Griseofulvin (10 µg), Fluconazole (25µg), Terbinafine (1 µg), Itraconazole (10 µg), Clotrimazole (10 µg) and Voriconazole (1 µg).

In this study out of 100 isolates 90% were sensitive to Griseofulvin while 10% were resistant. Among that out of 52 isolates of *T.mentagrophyte* 47(90.3%) were sensitive. Similarly out of 28 isolates of *T.rubrum* 25(89.28%) were sensitive. *T. verrucosum, T.nannum and M gypseum* were 100% sensitive to Griseofulvin. Our findings are in accordance with Pakshir [37] and colleagues who also reported 92.5% sensitivity to Griseofulvin.

In this study out of 100 isolates 47 % were sensitive to Fluconazole while 52% were resistant. Among that, out of 52 isolates of *T.mentagrophyte* 22(42.3%) were sensitive. Similarly out of 28 isolates of *T.rubrum* 16(57.14%) were sensitive. *T.tonsurans,*and *T. verrucosum,* were 53.3%,& 33% sensitive to Fluconazole respectively. *T.nannum and M gypseum* were 100% resistant to this. Our findings about poor susceptibility of dermatophytes to Fluconazole is compatible with the studies conducted by Favre *et al*., Santos *et al*., Barros *et al.* and Sarifakioglu *et al.* [38-41] The higher resistance to Flucanozole may be due to its easy availability at pharmacies, self medication by patients due to its over the counter (OTC) availability and a rampant practice of its irrational prescription by quacks.

In this study out of 100 isolates 74 % were sensitive to Clotrimazole while 26% were resistant. Among that, out of 52 isolates of *T.mentagrophyte* 41(78.8%) were sensitive. Similarly out of 28 isolates of *T.rubrum* 21(75%) were sensitive. *T.tonsurans,* and *T. verrucosum,* were 73.3%, 33%, sensitive to Clotrimazole respectively. Mona Fattouh[42] reported Clotrimazole as the most potent antifungal agent. Clotrimazole is one of the oldest antifungal drugs formulated for topical use against dermatophytosis.

In this study all the isolates of dermatophytes were sensitive to Voriconazole. Our results are in accordance with various authors [12, 30, 43] who all reported high sensitivity to Voriconazole. The high sensitivity of dermatophytes to Voriconazole observed in our study can be attributed to the fewer use by quacks and chemists and also to its high cost. Therefore, it can be interpreted that Voriconazole being the most sensitive antifungal drug for dermatophytes is a more suitable treatment option but it must be reserved for resistant and difficult to treat cases so as to prevent rapid development of resistance.

In this study out of 100 isolates 95 % were sensitive to Itraconazole, 2% were intermediate while 3% were resistant. All the strains of *T.mentagrophyte, T.rubrum, T. verrucosum, T.nannum and M gypseum* were sensitive to Itraconazole while 10 strains (66%) of *T. tonsurans* were sensitive. Itraconazole is a much more affordable antifungal drug that closely follows Voriconazole in its effectiveness against dermatophytes; hence, it must be a preferred treatment option for better outcome in patients suffering from dermatophytosis.

In this study out of 100 isolates 75 % were sensitive to Terbinafine while 25% were resistant. Among that out of 52 isolates of *T. mentagrophyte* 31(59.6%) were sensitive. Similarly out of 28 isolates of *T. rubrum* 25(89.2%) were sensitive. *T. tonsurans, T. nannum and M gypseum* were 100% sensitive while *T. verrucosum* were 66.6% sensitive. We observed high sensitivity to Terbinafine which is in agreement with various authors[44-46] who reported Terbinafine as a good choice for the treatment of dermatophytosis.

Our work suggests that in-vitro ABDD antifungal susceptibility testing is simple, inexpensive, and does not require specialized equipment; thus, it can be used for the routine assessment of dermatophyte susceptibility to antifungal agents in the routine clinical laboratory. It allows for a comparison between different antifungal agents and may help optimize the therapy for treating patients with dermatophytosis.

Conclusion

Dermatophytosis is a common cutaneous manifestation distributed worldwide. The epidemiology of dermatophyte infections may change with time and in a particular geographical region so studies should be conducted to provide information on the current status of the disease in that particular geographic area. Clinical suspicion, early laboratory examination to confirm diagnosis and appropriate treatment is very crucial. The Agar-Based Disk Diffusion method of antifungal susceptibility testing is simple, inexpensive, and does not require specialized equipment; thus, it can be used for the routine assessment of dermatophyte susceptibility to antifungal agents in the routine clinical laboratory. This will aid the clinician in initiating prompt and appropriate antifungal therapy and prevent emergence of resistance.

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