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A Recent Study on The Determination of Antifungal Susceptibility of Fungal Isolates of Dermatophytes Using Micro Broth Dilution Method

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ABSTRACT

Dematophytoses are the most common types of cutaneous fungal infections seen in human and animals. It is caused by a group of keratinophilic fungi, which are capable of invading keratinized tissues of skin, hair and nail. They belong to the genus Trichophyton, Microsporum, and Epidermophyton, collectively known as dermatophytes. Isolation and antifungal susceptibility pattern aids to select the appropriate antifungal agent for the management of dermatophytes infection. To isolate and speciate the dermatophytes from clinical specimens like skin, nail and hair obtained from patients attending the Department of Dermatology. To determine the antifungal susceptibility of the isolates by Micro broth dilution method was carried out. A total of 122 samples are collected from the patient and examined in 10% and 40% KOH to identify the fungal hyphae and inoculated on to SDA and incubated at 25^{0}C and 37^{0}C for 10-14 days. Based on the colony characteristics and microscopic morphology in LPCB mount, identifications are done. To detect the MIC of antifungals, microbroth dilution method is used. All the 122 clinically suspected specimen of dermatophytes are subjected to mycological study. Most of the cases are seen between the age group of 21-30 years. Trichophyton species are the predominant isolate (96.70%). Trichophyton mentagrophytes is the most common isolate (58.24%). The minimum inhibitory concentration (MIC) was performed by using Micro broth dilution the modern antifungal drugs. Results showed that the minimum inhibitory concentration of Griseofulvin is 0.03-0.5µg/ml, Fluconazole is 1-8µg/ml, Ketoconazole is 0.03-0.5µg/ml and Itraconazole is 0.015-0.25µg/ml. Fluconazole seems to beless effective and Itraconazole is found to be the most effective. Among the five species isolated by culture, *T. mentagrophytes* is the predominant isolate followed by *T. rubrum*. Study revealed that Itraconazole is the most effective antifungal drug. Early appropriate identification of the dermatophytes and the timely giving therapy will be of greater value.

INTRODUCTION

Dermatophytoses are the common types of superficial cutaneous fungal infections seen in human and animals. These are caused by group of closely related keratinophilic fungi, which are capable of invading keratinized tissues of skin and its appendages like hair and nail. They belong to three mycelial fungal genera. Trichophyton, Microsporum, and Epidermophyton and are collectively known as dermatophytes.[1,2,3.] Skin, hair and nail are infected by Trichophyton species. Skin and hair are infected by Microsporum species. Skin and nail are infected by Epidermophyton species. The diseases caused by non-dermatophytic fungi infecting skin are called dermatomycoses, whereas those of hair and nail are known as piedra and onychomycosis respectively. The other frequently used terms like tinea and ringworm infections are synonym of dermatophytoses. Depending on the usual habitat (Humans, Animals or soil) dermatophytes are classified as Anthropophilic the fungal species exclusively infecting humans, Zoophilic infecting animals as well as birds, Geophilic the fungal species frequently isolated from soil [4,5,6.] Dermatophytosis is more common in tropical countries like India due to hot and humid climate. Poverty, overcrowding, poor peripheral circulation, poor personal hygiene, cancer chemotherapy, immunosuppressive therapy and immunocompromised conditions are the risk factors for Dermatophytoses[7]. Contact with the infected person/pet animals, fomites or auto inoculation from another body site are the mode of transmission.

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Predisposing factors include moist humid skin and tight-ill fitted underclothing. Fungus growth in skin showed in a centrifugal pattern in the stratum corneum leading Lesions are not produced by the tissue invasion by the fungi per se; but to the formation of characteristic well-demarcated annular or ring shaped. Hair may become brittle and areas of alopecia may appear [8, 9,10] in response to the host inflammatory reaction elicited by fungal antigen [11]. In 1958, Griseofulvin became available after breakthrough experimental works of Gentles in guinea pigs. Dermatophytosis is cured with azole derivatives and allied group of antifungal drugs which was discovered in 1980's[12, 13]. To select a specific antifungal agent by the clinicians, Antifungal susceptibility test is very much helpful. Micro broth dilution method of the filamentous fungi established the interpretive break points for the newer triazoles like Ravuconazole, Voriconazole, Posaconazole, Fluconazole and Itraconazole[14, 15, 16]. To reduce the emergence of resistant strains and morbidity, early diagnosis and initiation of treatment is essential. [17,18,19.]

MATERIALS AND METHODS

This Cross section study was conducted in the Department of Microbiology, Karpaga Vinayaga Institute of Medical Sciences and Research Center, Chinna kolambakkam, Kanchipuram District, Tamil Nadu, India. Over a period of one year between December 2016 and January 2018. A total number of 122 samples includes skin scrapings, hair and nail were collected from patients who were attended the Dermatology Outpatient department at Karpaga Vinayaga Institute of Medical Sciences and Research Centre. Chinna kolambakkam. Both males and females of all age groups of clinically diagnosed dermatophytosis cases were included in this study, however, patients with dermatophytic infection who are undergoing treatment and non dermatophytic skin infection patients are not included in this study. Details like age, sex, duration of complaint, distribution of lesion, history of previous similar complaints and treatment history, history for diabetes, tuberculosis, neoplasms, HIV and surgeries. Exposure to animals, known cases, pets at home or any other suspected sources history were also collected.

Specimen Collection

Skin Specimen: Disinfected the affected site with 70% alcohol to remove surface contaminants. After the alcohol is dried, with the help of sterilized blunt scalpel, scraped the lesion from center to edge crossing margins and collected.

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Hair Specimen: Taken basal root portion of hair by plucking and not by clipping, scraped scales and excavate hair for direct examination as well as culture.

Nail Specimen: Cleaned the affected nail with 70% alcohol and nail clippings are taken from an appropriate site depending on the type of nail infection. Collected samples are transported in sterile black paper packs to keep the specimen dry and to prevent bacterial contamination.

Fungal identification

Direct microscopic examination of KOH wet mounts of keratinous material collected were treated with 10 – 40% KOH in a clean glass slide covered with a coverslip (Skin - 10% of KOH, Hair - 10% of KOH and nail - 40% of KOH). Flamed the slide slightly to clear the materials within 5-20 minutes and examined under both low and high power objectives of light microscope for presence of hyphae. Fungal culture was carried out with the clinical specimens by inoculating on to fungal culture media Sabouraud's Dextrose Agar containing cycloheximide (0.05mg/ml) and chloramphenicol (0.05mg/ml) in duplicate. Irrespective of the

findings of direct examination to detect the dermatophytes in the clinical sample, slopes were incubated at 25ºC and 37ºC for 4 weeks and examined daily during the first week and twice a week thereafter for any fungal growth. Slopes not showing growth for 4 weeks were considered negative for growth**.** Fungal growth was identified using Lacto Phenol Cotton Blue (LPCB) mount preparation.

A bit of fungal colony was teased out from the culture tube and the LPCB mount was made on a slide and viewed under microscope to study the nature of the hyphae (such as septate or aseptate, hyaline or narrow or wide) and to study the Type of sporulation (conidia or sporangia etc) for the purpose of Identification. Dermatophyte test medium was also used for the cultivation. Dematophytes on incubation at 25^0c, the dermotophytes turn medium red due to change in color of indicator phenol red by increased pH through their metabolic activity while other fungi and bacteria do not. Similarly, the species differentiation was determined by using hair perforation test to differentiate between *T. rubrum* and *T. mentagrophytes*. It was also used to differentiate between *M. canis* and *M. equinum*. Test result showed positive in *Trichophyton mentagrophytes* and *Microsporum canis* but was negative in *Trichophyton rubrum* and *Microsporum equinum*. The other differential test that we did was urease test using Christensens medium. *T. mentagrophytes* strain, hydrolyse urea thereby medium turns bright pink color while *T. rubrum* shows negative results.

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Antifungal susceptibility testing for dermatophytes

Once the fungal species were identified Antifungal susceptibility test was performed using microbroth dilution method. Five mL stock solution for each drug was prepared and two fold dilutions were used for water soluble drugs. For water insoluble drugs DMSO was used as the diluent. Prepared 5ml of antifungal agent, pipetted 4.9 ml of RPMI 1640 medium into each of 10 sterile test tubes and added 0.1ml of DMSO alone to one 4.9ml lot of medium as control medium using a single pipette, then 0.1ml of lowest (3.13µg/ml) drug concentration in DMSO, then 0.1 ml of the 6.25µg/ml concentration likewise continued in sequence up the concentration series, each time adding 0.1ml to 4.9ml medium. These volumes were adjusted according to the total number of tests required. Because there will be 1:2 dilution of the drug when combined with the inoculums, the working antifungal solution were twofold more concentrated than the final concentration.

Inoculum preparation and procedure

7-15 days old cultures grown on SDA at 25ºC was used. Mature colonies were covered with 10 ml of sterile saline (0.85%). Growth scraped by sterile Pasteur pipette. Heavy particles allowed to settle for 15-20 minutes at room temperature. Supernatant was mixed with vortex for 15 seconds. Supernatant turbidity was adjusted to 530nm 65-70% absorbance by spectrophotometer. Each suspension was diluted in the ratio 1:50 in RPMI 1640. After preparation of inoculum, in each well on the day of test, 0.1 ml of inoculum suspension was inoculated. It will dilute the drug concentration, solvent and inoculum densities used to the final desired test concentration. 0.1ml of the diluted inoculums suspension and 0.1ml of the drug diluents without antifungal agents will be in the growth control well in sterile microtitre plates, test is performed. In first row of 1-10 microtitre wells aliquot 100µl of drug dilution is inoculated.

Fluconazole concentration 0.01-64µg/ml is added into each well beginning from 1-10, to which 100µl of inoculums is added. Well number 11 with inoculum without antifungal drug as growth control. Well number 12 is medium control (RPMI 1640) and are incubated at 37ºc. Same procedure was repeated for other drugs like griseofulvin, ketoconazole and itraconazole.

RESULTS AND DISCUSSION

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The lowest concentration of antifungal agent that inhibits growth detected visually is taken as MIC. Each MIC well with growth is compared with that of the growth control and each microtitre well was given a numerical number. Reduction in growth- 4. Slight reduction in growth or approximately 80% of growth control (drug free medium)- 3. Prominent reduction in growth or approximately 50% of growth control- 2. Slight growth or approximately 25% of growth control- 1. Optically clear or absence of growth.

Data were entered in MS Excel spreadsheet. Data were analyzed Using Statistical Package for Social Sciences Software (SPSS). To compare the proportions Chi-square test, P value less than 0.05 is considered as statistical significance. Out of 122 isolates KOH positive is 98 (80.32%) and KOH negative is 24 (19.67%). Among the 122 samples, 78 (63.93%) are skin scrapings, 18 (14.75%) are hair samples and 26 (21.31%) are nail samples. Out of 122 tested cases, KOH positive is 98 (80.32%) and KOH negative is 24 (19.67%) (Table 8). Whereas the culture positive showed 91(74.59%) and culture negative is 31(25.40%). 72.13% are both KOH

and culture positive. In this study three of culture, positive samples showed no fungal filaments on direct KOH mount because of inactive form but able to grow in culture medium.

In culture negative cases 10 showed fungal elements on KOH mount but failed to grow in culture. Surenderan KAK *et al*[20] study showed similar results. It may be due to nonviability of the fungi prior to inoculation, self-medication and inappropriate use of antimycotic treatment. Age wise distribution of dermatophyte infection showed that the common age group is 21-30 years (31.96%) followed by 31-40 years showed in table 1. The higher incidence in young males could be due to greater physical activity and increased sweating. Same results were shown in the studies of Smita sarma *et al* [21] 39%, U. S. Agarwal *et al* [22] 30.3%, B V Peerapur *et al* [23], Clarissa J. Lyngdoh *et al* [24] 34.4%. Surendran KAK *et al* [20] showed 44% which is slightly higher than this study.

Table No. 1: Age wise distribution of dermatophyte infection

S. NO.	AGEGROUP	MALE	FEMALE	TOTAL	%
1	1-10	1	3	4	3.27
2	11-20	14	8	22	18.03
3	21-30	23	16	39	31.96
4	31-40	16	16	32	26.22
5	41-50	4	7	11	9.01
6	51-60	2	4	6	4.91
7	61-70	2	3	5	4.09
8	71-80	2	1	3	2.45

Male to female ratio almost similar which was 64 in male (52.45%) and 58 (47.54%) in female out of 122 cases.

Clinical presentation of dermatophytes infection showed in Table 2. It is observed that incidence of *Tinea corporis* 46 (37.70%) and *Tinea unguium* 26 (21.31%) is high followed by *Tinea cruris* 19 (15.57%) and Tinea capitis 13 (10.65%) high followed by *Tinea cruris* 19 (15.57%) and *Tinea capitis* 13 (10.65%). The common clinical types of dermatophytoses are *Tinea corporis* (Table 2) is comparable with Suman Singh *et al*[25] study (6.92%) which is lesser than this study.

Tinea capitis is less common in India than in other countries, this may be attributable to the use of hair oils used by Indians. Hair oils have been shown to have an inhibitory effect on dermatophytosis.

The prevalence of *Tinea pedis* (4.91%) (Table 7) in this study is comparable with G. Venkatesan *et al* [26] (5.6%). The predominance of *T. pedis* could be because of the regular use of shoes and socks, resulting in conditions like dampness and warmth of the body thereby facilitating the skin surface for the growth of dermatophytes.

Table No. 2: DERMATOPHYTOSIS CLINICAL PRESENTATION (n=122)

S. NO.	SPECIMEN	FUNGAL LESION	NO OF CASES	%
1	SKIN (n=78)	*Tinea corporis* *Tinea cruris* *Tinea mannum* *Tinea pedis* *Tinea facei*	46 19 3 5 5	37.70 15.57 2.45 4.09 4.09
2	HAIR (n=18)	*Tinea capitis* *Tinea barbae*	13 5	10.65 4.09
3	NAILS (n=26)	*Tinea unguium*	26	21.31

Among the 91 culture positive isolates, 78 from skin scrapings, 18 from hair and 26 from nail clippings (Table 2). 11 *T. rubrum* are isolated from skin samples, 13 from scalp hair and 11 from nail. 43 *Trichophyton mentagrophytes* from skin (47.25%) and 10 from nail clippings are isolated. (Table 4) *T. mentagrophytes* is the most common isolate from skin samples almost similar to Sundar Khadka et al [6] (39.6%) study. *T. rubrum* is the second common isolate.

Table No. 3: DERMATOPHYTIC SPECIES FREQUENCIES (n=91)

ISOLATES	NO OF CASES	%
T. mentagrophytes	53	58.24
T. rubrum	35	38.46
T. tonsurans	1	1.09
E. floccosum	1	1.09
M. gypseum	1	1.09

T. mentagrophytes are found to be the commonest etiological agent (58.24%) (Table 10) isolated from *Tinea pedis*, *Tinea corporis*, *Tinea manuum*, *Tinea barbae*, *Tinea facei*, *Tinea capitis*, *Tinea unguium* and *Tinea cruris*, followed by *T. rubrum* (38.46%), *Epidermophyton floccosum* (1.09%), *T. tonsurans* (1.09%) and *Microsporum gypseum* (1.09%). A study by Sundar Khadka et al[6] showed *T. mentagrophytes* 39.6%, but Suman Singh *et al*[25]showed *Trichophyton rubrum* as the common isolate. *Trichophyton mentagrophytes* is the predominant dermatophyte isolated from *Tinea corporis* in this study. Similar results are seen in Sundar Khadka *et al*[6] study.

Table No. 4: DISTRIBUTION OF DERMATOPHYTES IN CLINICAL SPECIMENS (n=91)

SPECIMEN	ISOLATES	NO OF CASES	%
SKIN	*T. mentagrophytes*	43	47.25
	T. rubrum	11	12.08
HAIR	*T. rubrum*	13	14.28
	T. tonsuran	1	1.09
	M. gypseum	1	1.09
NAIL			
	T. rubrum	11	12.08
	T. mentagrophytes	10	10.98
	E. floccosum	1	1.09

Among the 91 culture positive isolates, 78 from skin scrapings, 18 from hair and 26 from nail clippings. 11 *T. rubrum* are isolated from skin samples, 13 from scalp hair and 11 from nail. 43

Trichophyton mentagrophytes from skin (47.25%) and 10 from nail clippings are isolated (Table 11). *T. mentagrophytes* is the most common isolate from skin samples almost similar to Sundar Khadka *et al* [6] (39.6%) study. *T. rubrum* is the second common isolate.

The various isolates of dermatophytes were tested for its MIC values with different concentrations of antifungal drugs. Antifungal susceptibility testing was performed by microbroth dilution method for Griseofulvin, Ketoconazole, Fluconazole and Itraconazole. The MIC range for Griseofulvin by microbroth dilution method is 0.03µg/ml-0.50µg/ml. The MIC range for Ketoconazole by microbroth dilution method is 0.03µg/ml-0.50µg/ml. The MIC range for fluconazole by microbroth dilution method is 1µg/ml-8µg/ml. The MIC range for Itraconazole by microbroth dilution method is 0.015µg/ml-0.25µg/ml. The results were shown in table nos. 5, 6, 7 and 8 respectively.

Table No. 5: DRUG GRISEOFULVIN'S MINIMAL INHIBITORY CONCENTRATION

DRUG CONCENTRATION IN µg/ml

SPECIES	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	MIC 50	MIC 90
T. mentagrophytes (n=53)	1	2	12	37	1	.	.	.	.	.	0.12	0.25
T. rubrum (n=35)	.	5	8	21	1	.	.	.	.	.	0.12	0.25
T. tonsurans (n=1)	.	.	1	.	.	.	.	.	.	.	0.12	0.25
E. floccosum (n=1)	.	.	.	1	.	.	.	.	.	.	0.12	0.25
M. gypseum (n=1)	.	.	1	.	.	.	.	.	.	.	0.12	0.25

MIC 50 and MIC 90 of griseofulvin for the species isolated.

Table No. 6: DRUG KETACONAZOLE'S MINIMAL INHIBITORY CONCENTRATION

DRUG CONCENTRATION IN µg/ml

SPECIES	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	MIC 50	MIC 90
T. mentagrophytes n=53	2	1	12	35	3	.	.	.	.	.	0.12	0.25
T. rubrum n=35	.	2	21	8	4	.	.	.	.	.	0.12	0.25
T. tonsurans n=1	.	.	1	.	.	.	.	.	.	.	0.12	0.12
E. floccosum n=1	.	1	.	.	.	.	.	.	.	.	0.06	0.06
M. gypseum n=1	.	.	1	.	.	.	.	.	.	.	0.12	0.12

MIC 50 and MIC 90 of Ketoconazole for the species isolated.

Table No. 7: DRUG FLUCONAZOLE'S MINIMAL INHIBITORY CONCENTRATION

DRUG CONCENTRATION IN µg/ml

SPECIES	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	MIC 50	MIC 90
T. mentagrophytes n=53	.	.	.		.	8	39	3	3	.	2	8
T. rubrum n=35	.	.	.	.	.	22	11	2	.	.	1	4
T. tonsurans n=1	.	.	.	.	.	.	.	1	.	.	4	4
E. floccosum n=1	.	.	.	.	.	.	.	1	.	.	4	2
M. gypseum n=1	.	.	.	.	..	.	1	.	.	.	2	2

MIC 50 and MIC 90 of fluconazole for the species isolated.

Table No. 8: DRUG ITRACONAZOLE'S MINIMAL INHIBITORY CONCENTRATION

DRUG CONCENTRATION IN µg/ml

SPECIES	0.0075	0.015	0.03	0.06	0.12	0.25	0.5	1	2	MIC 50	MIC 90
T. mentagrophytes n=53	.	20	17	7	9	.	.	.	.	0.015	0.12
T. rubrum n=35	.	.	3	16	12	4	.	.	.		
T. tonsurans n=1	.	.	.	.	1	.	.	.	.		
E. floccosum n=1	.	.	.	1	.	.	.	.	.		
M. gypseum n=1	.	.	.	.	1	.	.	.	.		

MIC 50 and MIC 90 of itraconazole for the species isolated

MIC testing by micro broth dilution method by C. J. Jessup *et al*[27], shows an increase in the MIC values of Griseofulvin and Fluconazole. The present study correlates with the studies conducted by C. J. Jessup *et al* [27]. For this study, the micro broth dilution method was chosen because of its reproducibility, convenience and greater ease of performance.

CONCLUSION

The study result concludes that Fluconazole showed a higher MIC values in the range of 1-8µg/ml. Itraconazole showed the lowest MIC values by micro broth dilution method and found to be the most potent drug. The increased incidence and availability of various new drugs for dermatophytic infection in the last two decades emphasize a reference susceptibility testing method, which aids the clinician to select the appropriate drugs for the management of dermatophytic infection.

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