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SPECIATION AND IN VITRO ACTIVITY OF FOUR ANTIFUNGAL DRUGS AGAINST CLINICAL ISOLATES OF DERMATOPHYTES BY E-TEST METHOD

Microbiology

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ABSTRACT

Dermatophytes majorly a saprophytic fungus, have a capacity to invade keratinized tissue particularly skin, hair and nails of humans and other animals to produce infection. According to world health organization (WHO), it is estimated that about 25% of the world's population are being effected by dermatophytes. Prolonged and inappropriate usage of antifungals has led to genetic mutations and emergence of antifungal resistance strains. Hence this study aimed to species specific identification and determine the MIC values of four antifungal agents against dermatophytes using E-test method. Hence, this laboratory based prospective study was carried out in department of Microbiology, JSS medical college and hospital, Mysore for a period of 1 year. All the samples received in the laboratory with clinical suspicion of dermatophytoses were included and subjected to microscopic examination (KOH wetmount), followed by culture and antifungal susceptibility testing for 4 antifungal agents and the present study helped in determining the activity of four antifungal agents against clinical isolates of dermatophytes using E-strips and we conclude that this method is easy to perform, less laborious and would help in identification of antifungal resistance and also helps in choosing appropriate antifungal agent

KEYWORDS

Dermatophytosis; Dermatophyte test medium ; E-Strips; Antifungal agents.

INTRODUCTION:

Dermatophytoses a general public health problem that can be seen in all age groups and both sexes, forms over 16–75% of all the mycological infections. This is the most common infection seen in heat and humid areas such as tropical and sub-tropical countries including India. Infection caused by dermatophytes are usually non-threatening but causes great discomfort to the patients. The infection can be more severe during immunosuppression. Hence, prompt laboratory diagnosis is very much indicated as the clinical features of dermatophytosis vary and may closely resemble other skin infections particularly caused by dermatomycosis and secondary bacterial infections[1]. Laboratory investigations for dermatophytes generally include potassium hydroxide (KOH) wet mount examination and skin scrapings or biopsy for fungal culture. Identification of the agents causing dermatophytes is very much necessary which can reduce the risk of further transmission of the infection[2]. Factors such as inappropriate usage of antifungals, application of topical steroids, environmental changes, socio-economic conditions and occupational exposure are majorly responsible for increased incidence of dermatophytoses. The ultimate choice for treating these infections would be topical or systemic antifungals. Newer antifungal agents have also been introduced into clinical practice which were found to be safe and effective against dermatophytosis. Genetic mutations due to inappropriate usage of antifungals has already led to the emergence of antifungal resistant strains. Hence, the invitro activity of antifungal susceptibility testing of dermatophytes may helpful in understanding the resistance patterns and helps the clinician to select the appropriate therapy for the successful treatment of infections caused by dermatophytic fungi[3]. Dermatophyte Test Medium (DTM) is an alternative culture medium that can be used for isolation and laboratory confirmation of pathogenic dermatophytic fungi. DTM is less expensive when compared to other fungal culture media and can also be used at limited resource settings. The disadvantage of this culture medium is doesn't identify the specific organisms, a positive culture and colour change indicates the presence of pathogenic dermatophytic fungi[4]. Determination of antifungal resistance using E-test method is a good alternative for broth dilution which can also be used to determine minimum inhibitory concentration(MIC) of filamentous fungi. The E-strip contains a thin, calibrated plastic strip with a predefined, exponential and continuous gradient of antifungal agent across 15 twofold dilutions. This study is mainly aimed to isolate and determine the MIC of four antifungal agents (Fluconazole, Itraconazole, Terbinafiine and Griseofulvin) against clinical isolates of dermatophytes using the E-test method[5].

MATERIALS & METHODS:

Patients with clinical suspicion of dermatohytoses were included and the samples from the infected sites were collected after obtaining the informed consent form from the patients. Clinical specimens were collected from the patients under the supervision of clinicians following asceptic precautions and the specimens which were labelled with name, age, sex of the patient along with site of infection and were transported to the laboratory by sealing them in a sterile paper for further laboratory investigations such as, direct microscopic examination and fungal culture for isolation of dermatophytes.

Microscopic examination and fungal culture

The samples that were received in the laboratory were divided into two portions among which one was processed for the KOH wet mount microscopy to observe the presence of fungal elements and the other portion was used for fungal culture for isolation of dermatophytes. Wet mount was thoroughly examined under low power (10X) and high power(40X) magnification for the presence of hyphae or arthocondidia **(FIG 1)**. Irrespective of the demonstration of fungal elements, the samples were inoculated on to Sabouraud dextrose agar (SDA), and Dermatophyte test medium and incubated at 22^{o}C for 2-4 weeks. The identification of the isolated fungi was mainly done based on the macroscopic (growth, texture and pigmentation) and microscopic characteristics using lacto-phenol cotton blue (macroconidia and microconidia) for species identification (**FIG 2)**. Absence of fungal growth after 4 weeks, the sample was considered as culture negative.

Antifungal susceptibility:

Preparation of inoculums: A loop full of fungal colonies were transferred into 0.5% saline and inoculum suspensions were gently mixed with the tip of a sterile pipette. The heavy particles containing tubes were allowed to settle down for 30 minutes at room temperature. The inoculum suspensions were mixed well with vortex mixer for 10-15 seconds and adjusted to match with 0.5 McFarland's standard.

E-test: The E-test was performed according to the manufacturer's protocol. All the isolates were sub-cultured onto potato dextrose agar and tubes were incubated at 25-30˚C for one week. Four Antifungal agents such as Itraconazole, Terbinafine, Griseofulvin and

Fluconazole were tested against the dermatophyte isolates. Inoculum suspensions are then lawn cultured onto Muller hinton agar (MHA) plates with a sterile cotton swab. Plates were allowed to dry then antifungal E-strips were placed onto the medium with a pair of forceps, the plates were then incubated at 28 °C and the results were read after 48-72 hours.

Determination of MIC endpoints: MIC is defined as the lower concentration of drug at where the ellipse intersects the zone of inhibition in the MIC scale on the E-test strip (**FIG 3)**

RESULTS:

In the present study 100 samples from clinically suspected cases of dermatophytosis were processed. Out of 100 samples, 56(56%)collected from male and 34 (34%) samples were from female. Of 100 samples collected, 85 were skin scrapings followed by 10 hair & 5 nail clippings. All the samples were processed for direct microscopic examination using KOH wet mount and simultaneously inoculated into fungal culture media for isolation of dermatophytes. Off 100 samples 28 (28%) were both KOH and culture positive for dermatophytes, 5 (5%) of the samples demonstrated the presence of fungal elements by KOH wet mount but were culture negative, 3 (3%) of the samples did not demonstrate the presence of fungal elements by KOH wet mount, but were culture positive for dermatophytes and remaining 64 (64%) of the samples were both KOH and culture negative (**table 1)**. The samples were simultaneously inoculated into Dermatophyte test media (DTM) and Sabarauds dextrose agar media for isolation of dermatophytes. Among 100 samples, 31 (31%) were isolated on DTM showing an alkaline (red) reaction on DTM within 2 weeks of time and in contrast SDA yielded growth of dermatophytes only in 28 (28%) of the samples (**table 2)** . Out of the 100 clinical cases, tinea corporis accounted for 64%, tinea cruris accounted for 6%, tinea pedis 10%, tinea faciei 5%, tinea capitis 10% and tinea unguium 5% and the dermatophytes isolated were 11 (35%) T.rubrum followed by 8 (25%) were T.mentagrophytes, 5 (16%) were T.tonsurans, 3 (10%) were M.gypseum, 2 (6%) were T.verrucosum and 1 (4%) were T.violaceum and M.audonii each (**table 3)**.

Antifungal susceptibility testing of 31 dermatophytes were done according to the manufacturers protocol which was discussed earlier in the methodology. Antifungal E-strips such as Flucanazole (0.016-256 ug/ml), Itracanazole (0.002-32ug/ml), Terbinafine (0.002-32ug/ml), and Griesofulvin (0.002-32ug/ml) was used. It was found that all the 31 (100%) dermatophytes were sensitive to Itracanazole and Greisofulvin. 4 (12.90%) of the dermatophytes (2 T.rubrum, 1 T.mentagrophytes and 1 M.gypseum) showed resistance to Flucazaole. 2(6.45%) of the isolates (T.rubrum and T.tonsurans) showed resistance to Terabinafine (**table 4)**.

DISCUSSION:

Dermatophytoses form over 50-75% of all the mycological infections. The diagnosis of a dermatophytic infection is mostly done clinically, but often confused with other skin infections due to the topical application of steroid ointments and creams, leading to further misdiagnosis and mismanagement [4]. Hence, there arises the need for the correct, efficient, and rapid laboratory diagnosis of dermatophytes. The establishment of a reference antifungal susceptibility testing method may allow the clinician to select the appropriate therapy for the treatment and also for studying mechanisms of drug resistance of dermatophytic fungi [5]. In vitro analysis of the antifungal activity of anti-fungal agents enables comparison between different antimycotics, which in turn may clarify the reasons for lack of clinical response and assist clinicians in choosing an effective th erapy for their patients. However, it is important that the methodologies used for in vitro testing be standardized to facilitate the establishment of quality control parameters and interpretive break points [12].

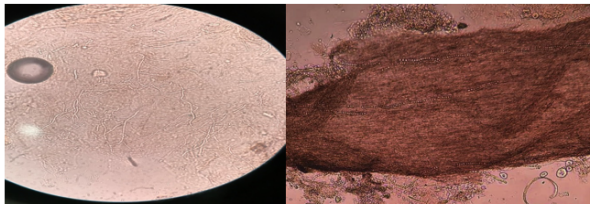
In the present study a total of 100 specimens (skin scrapings, hair fragments and nail clippings) were collected from clinically suspected cases of dermatophytosis. Table:1shows Direct Microscopy and Culture, Out of a 100 samples, 64% were KOH negative and 36% were KOH positive and 69 (69%) were culture negative,31(31%) were culture positive. A study conducted by Anupama.A *et al* (2017) also reported similar proportions whereby out of 100 clinical samples 58% samples were culture positive and KOH positive, 19% samples were Culture positive and KOH negative, 18% samples were Culture as well as KOH negative. 5% samples were KOH positive and culture negative. In our study *T.corporis* is the most common clinical condition and *Trichophyton rubrum* was the most commonest dermatophyte isolated.Table:3 shows isolated dermatophyte strains in relation to localization. In culture, the isolated dermatophyte isolates were identified by macroscopic morphological characteristics (pigmentation, growth rate and texture etc.) and followed by microscopic examination. The isolated organisms were identified as *Trichophyton rubrum* 11(35%), *Trichophyton mentagrophyte* 8(25%), *Trichophyton tonsurans* 5(16%), Microsporum gypseum 3(10%), *Trichophyton verrucosum* 2(6%), *Trichophyton violaceum* 1(3%) and *Microsporum audouinii* 1(3%). In a study done by Keyvan Pakshir *et al* in (2009), similar findings were reported but in their study *T. rubrum* accounted for 13(32.5%), *Trichophyton mentagrophytes* 8(20%), *T.violaceum* 4(10%), *Microsporum gypseum* 3(7.5%), *T. tonsurans* 2(5%), *T. verucosum* 2(5%) respectively.

The isolated dermatophytes were further subjected to antifungal susceptibility testing by E-test method with four antifungal agents namely Fluconazole (0.016-256 mcg/ml), Itraconazole (0.002-32 mcg/ml), Terbinafine (0.002-32 mcg/ml), and Griseofulvin (0.002-32 mcg/ml). In our study the antifungal susceptibility of dermatophyte isolates may contribute to a choice of antifungal treatment to ringworm infections. Itraconazole is the most active agent against dermatophytosis with an MIC range (µg/ml) (0.5-0.125) and followed by Griseofulvin with an MIC range (µg/ml) (0.16-2) and Terbinafine with an MIC range(0.125-2 µgm/ml),(0.256-4 µgm/ml). The least active agent was Fluconazole with an MIC range (0.5-2 µgm/ml),(0.25-2µgm/ml),(8-64µgm/ml),(64-256µgm/ml).

CONCLUSION:

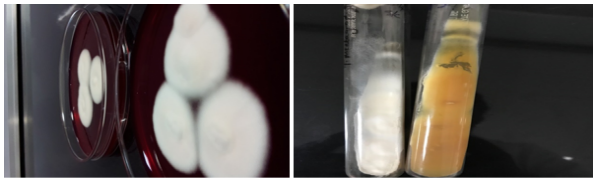
In conclusion, The isolation, identification and antifungal susceptibility testing plays an important role in treatment and prevention of dermatophytoses. Dermatophyte test medium is a good screening medium for the selective primary isolation and early detection of dermatophytes from clinical specimens. The study shows Itraconazole is most active antifungal agent against dermatophytes and followed by Griseofulvin,Terbinafine. The least active agent was Fluconazole. The drug resistance of dermatophytes has increased tremendously in the last few decades due to inproper usage of antifungal agents.Thus it is important to perform antifungal susceptibility testing along with routine culture which is useful to choose proper antifungal agent.

Fig 1 showing the KOH wet-mount Positive for branching septate hyphae and Endothrix type of infection



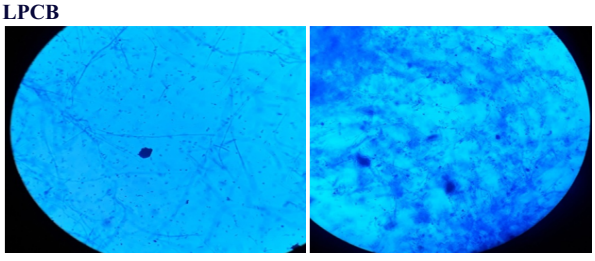
Branched septate hyphae **Endothrix type infection**

FIG 2 showing the culture Positive isolates from DTM and SDAac



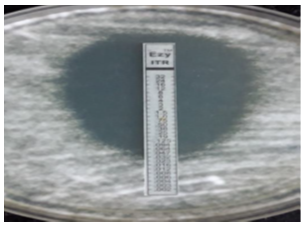
Dermatophytes on DTM **Dermatophytes on SDA**

FIG 2 showing microscopic morphology of dermatophyes using LPCB

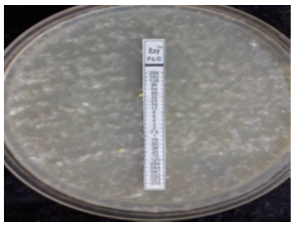


LPCB showing T.*rubrum* ***LPCB showing T.mentagrophytes***

FIG 3 showing antifungal sensitivity testing of dermatophytes using E-test:



T.*rubrum* showing sensitive to Itracanazole



T.rubrum showing resistance to Flucanazole

Table:1 showing Comparision of Direct Microscopy and Culture

Total samples (100)	KOH pos itive	KOH negative	Total
Culture positive	28 (28%)	3 (3%)	31 (31%)
Culture negative	5 (5%)	64 (64%)	69 (69%)

Table:2 showing comparision of SDA and DTM in primary isolation of dermatophytes

Total number of culture positive		31
Culture positive by SDA		28(90%)
Culture positive by DTM		31(100%)
SDA	DTM	Total number
+	+	28
-	+	03

Table:3 showing Isolated dermatophytes associated with clinical types

Clinicl types								
Isolates	No.	%	T.corporis	T.cruris	T.pedis	T.capitis	T.unguium	T.facei
T.rubrum	11	35	9	1	-	-	1	-
T.mentagrophytes	8	25	3	-	3	1	1	-
T.tonsurans	5	16	2	-	-	3	-	-
T.verucossum	2	6	-	-	-	1	-	1
T.violaceum	1	4	-	-	-	1	-	-
M.gypseum	3	10	3	-	-	-	-	-
M.audoinii	1	4	-	-	-	1	-	-
Total	**31**	**100%**	**17**	**1**	**3**	**7**	**2**	**1**

Table 4 showing antifungal susceptibility patterns of dermatophytes to four antifungal agents using E-test method.

Isolates (n)	Antifungal agents(mcg/ml)	Sensitive (mcg/ml)	Intermediate (mcg/ml)	Resistant (mcg/ml)
T.rubrum	FLUCONAZOLE-(0.016-256µgm) ITRACONAZOLE-(0.002-32µgm) TERBINAFINE-(0.002-32µgm) GRISEOFULVIN-(0.002-32µgm)	9(0.125-8) 11(0.15-1) 10 (0.125-1) 11(0.15-1)	0 0 0 0	2(64-256) 0 1(≥8) 0
T.mentagrophytes	FLUCONAZOLE-(0.016-256µgm) ITRACONAZOLE-(0.002-32µgm) TERBINAFINE-(0.002-32µgm) GRISEOFULVIN-(0.002-32µgm)	10(0.125-8) 11(0.15-1) 11(0.125-1) 11(0.15-1)	0 0 0 0	1(64-256) 0 0 0
T.tonsurans	FLUCONAZOLE-(0.016-256µgm) ITRACONAZOLE-(0.002-32µgm) TERBINAFINE-(0.002-32µgm) GRISEOFULVIN-(0.002-32µgm)	11 (0.125-8) 11(0.15-1) 10 (0.125-1) 11(0.15-1)	0 0 0 0	0 0 1(≥8) 0
T.violaceum	FLUCONAZOLE-(0.016-256µgm) ITRACONAZOLE-(0.002-32µgm) TERBINAFINE-(0.002-32µgm) GRISEOFULVIN-(0.002-32µgm)	11(0.125-8) 11(0.15-1) 11(0.125-1) 11(0.15-1)	0 0 0 0	0 0 0 0
T.verucossum	FLUCONAZOLE-(0.016-256µgm) ITRACONAZOLE-(0.002-32µgm) TERBINAFINE-(0.002-32µgm) GRISEOFULVIN-(0.002-32µgm)	11(0.125-8) 11(0.15-1) 11(0.125-1) 11(0.15-1)	0 0 0 0	0 0 0 0
M.gypseum	FLUCONAZOLE-(0.016-256µgm) ITRACONAZOLE-(0.002-32µgm) TERBINAFINE-(0.002-32µgm) GRISEOFULVIN-(0.002-32µgm)	10 (0.125-8) 11(0.15-1) 10 (0.125-1) 11(0.15-1)	0 0 1(4) 0	1(64-256) 0 0 0
M.audoinii	FLUCONAZOLE-(0.016-256µgm) ITRACONAZOLE-(0.002-32µgm) TERBINAFINE-(0.002-32µgm) GRISEOFULVIN-(0.002-32µgm)	11(0.125-8) 11(0.15-1) 11(0.125-1) 11(0.15-1)	0 0 0 0	0 0 0 0

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