Section: Mycology

Original Research Article

ANTIFUNGAL SUSCEPTIBILITY TESTING USING AGAR BASED DISC DIFFUSION METHOD OF DERMATOPHYTIC ISOLATES IN A TERTIARY TEACHING INSTITUTE

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Abstract

Background: Dermatophytes comprise of filamentous pathogenic fungi including three important genera of Epidermophyton, Microsporum, and Trichophyton which may lead to superficial infections in both humans and animals. Their clinical presentation, typical of ringworm infection is very often confused with other skin disorders particularly due to overuse of broad-spectrum steroid containing skin ointments and creams leading to misdiagnosis and further mismanagement. Despite the availability of the wide range of antifungal drugs for dermatophytosis, the treatment failure has been reported worldwide. Aim & Objective: Antifungal susceptibility testing was performed to provide information to allow clinicians to select appropriate antifungal agents useful for treating a particular fungal infection. **Materials and Methods:** Total 90 samples were collected from skin O.P.D. Appropriate samples were taken in sterile containers and sent our laboratory for KOH mount, fungal culture, species identification, and sensitivity determination using agar based disk diffusion method and result. Trichophyton mentagrophyte was most frequently isolated dermatophyte in our study. **Result:** Overall trichophytons were most sensitive to Griseofulvin, 43 (87.7%) followed by terbinafine, 36 (73.5%), itraconazole ,30 (60%) and fluconazole, 17 (34%). **Conclusion:** The agar-based susceptibility testing methods for dermatophytes is simple, inexpensive & does not require specialized equipment & can be adapted for routine assessment of dermatophyte resistance to antifungal agents.

INTRODUCTION

The incidence of dermatophytosis is increasing in recent times especially in extremes of ages. Over the past few years, it has been documented that dermatophyte infections have increased by many folds in India.[1,2] Dermatophytes comprise of filamentous pathogenic fungi including three important genera of Epidermophyton, Microsporum, and Trichophyton which may lead to superficial infections in both humans and animals.[3] They produce keratinases which degrade the keratin and thus, invade the superficial skin tissue4. Dermatophytes a group of keratinophilic fungi that require long incubation period to grow. Their clinical presentation, typical of ringworm infection is very often confused with other skin disorders particularly due to overuse of broad-spectrum steroid containing skin ointments and creams leading to misdiagnosis and further mismanagement.[4,5] However, Pityriasis versicolor, Saccharomyces cerevisiae, and Candida spp. as opportunistic pathogenic fungi are also capable of causing superficial mycotic infections in human beings.[6,7] These organisms are assuming greater significance due to the excessive use of immunosuppressive drugs for controlling serious infectious as well as non-infectious conditions.

Dermatophytes are also associated with secondary bacterial infections leading to systemic skin infections.[8] Despite the availability of the wide range of antifungal drugs for dermatophytosis, the treatment failure has been reported worldwide.[9,10] Antifungal susceptibility testing is performed to provide information to allow clinicians to select appropriate antifungal agents useful for treating a particular fungal infection. For a definitive therapy, it is essential to evaluate the resistant dermatophytes using a standardized, simple and reproducible in vitro assay to determine the antifungal activity of drugs against isolates. A study conducted by Singal et al.,

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2001, in North India showed that there was treatment failure to griseofulvin among the Tinea capitis patients. Many studies suggest that trichophyton mentagrophytes had emerged as principal causative organism and high terbinafine resistance could be one of the reasons.[11,12] Various methods such as broth micro & macro dilution, agar dilution, E test, sensititre, colorimetric micro dilution & disk diffusion have been available (Karaca et al., 2004, Niewerth et al., 1998; Pujol et al., 2002).[13-15] For the foregoing reasons we conducted this study to identify the dermatophytic isolates and observe their antibiotic susceptibility pattern using the conventional agar-based disk diffusion method (ABDD). This study determines the in-vitro activity of two azole derivatives (Fluconazole & Itraconazole), Griseofulvin & Terbinafine antifungal derivatives that are commonly used to treat dermatophyte infection.

MATERIALS AND METHODS

The descriptive study was conducted over a period of one year from Nov. 2018to May 2019 in Department of Microbiology, Shri Ram Murti Smarak Institute of Medical Sciences, Bhojipura, Bareilly

After obtaining ethical clearance from the Institutional Ethics Committee, samples from skin, nail and hair were collected from the clinically suspected cases of dermatophytosis coming to skin O.P.D. and were processed in the Department of Microbiology as soon as possible following the laboratory protocols.

Inclusion criteria were clinically diagnosed KOH positive cases of fungal infection and patient and their attendants should be willing for investigations. Exclusion criterions were patients not willing for investigation, KOH negative samples and isolated fungi was other than Dermatophytes on culture growth. Samples from all age both males and females patients were collected.

Sample collection

Total 90 samples were collected from skin O.P.D. Skin scrapings were collected from the peripheral, actively growing margins of the lesions after decontaminated with 70% alcohol Nail clippings and hair samples were also collected with proper asepsis. The samples were taken in sterile containers and sent our laboratory for fungal culture, species identification, and sensitivity determination.

Direct microscopic examination, Fungal cultivation and Species identification:

All the specimens received in the Mycology laboratory were subjected to KOH mount. Positive KOH specimens (73) were subjected to culture on the Sabouraud's Dextrose Agar (SDA, Himedia) containing Cycloheximide (0.05%) and chloramphenicol (0.004%) under sterile conditions. and incubated at 25°C and 37°C for a period of 4 weeks along with Trichophyton rubrum ATCC 28188 and Trichophyton mentagrophytes ATCC 9533 as control. The colonies on the slants were examined for their morphology, texture and pigmentation (front and reverse) Species identification was further done by macroscopic and microscopic features on the basis of slide culture, teased mount by using lactophenol cotton blue stain, , and urea hydrolysis test. The isolated dermatophytes were then subcultured on potato dextrose agar (PDA) and oatmeal agar (for T.rubrum) (HI MEDIA, India) at 28°C upto7-15 days.

The antifungal susceptibility testing was performed according to Agarwal et al., 201516. Inhibition zone diameter was measured in millimeters and recorded as sensitive, intermediate or resistant. [Table 1].[17,18] Commercially available disks 9mm diameter preloaded with fluconazole 25 µg and itraconazole 10 µg were used (HI MEDIA, India). Disks containing griseofulvin 10µg and terbinafine 2µg were not commercially available and were prepared in our laboratory (Synergene active ingredients).

Statistical Analysis: The interpretation and analysis of the data were done by using Microsoft Excel. The quantitative data were expressed as numbers and percentages in a tabular form.

RESULTS

Out of 90 samples received 73(81.1%) samples showed fungal element in KOH and were processed further for fungal culture and 17(18.9%) samples were KOH negative and excluded [Figure 1]. Among 73 samples that were cultured and incubated, 15 samples showed non dermatophytes, 8 samples got contaminated with bacterial growth and laboratory contaminants and in 50 samples dermatophytic isolates were identified. Out of 50 samples 40 were from skin and 10 were from nail site [Table 2]. The isolates belonged to 2 genera and five species. Trichophyton mentagrophyte was most frequently isolated dermatophyte in our study [Table 3].

Antifungal susceptibility of Dermatophytes species

Dermatophyte sensitivity was tested to four Antifungals and sensitivity was recorded [Table 4] Overall trichophytons were most sensitive to Griseofulvin, 43 (87.7%) followed by terbinafine ,36 (73.5%), itraconazole ,30 (60%) and fluconazole, 17 (34%). [Table 5].

Table 1: Criteria for IZD for sensitivity and resistance of antifungal drugs IZD-inhibition zone diameter.

Drugs	Potency	Zone wise interpretation (mm)		
		Sensitive	Intermediate sensitive	Resistant
Fluconazole	25µg	≥21	15-22	≤14
Itraconazole	10µg	≥19	11-18	≤10
Terbinafine	2µg	≥20	12-19	≤11
Griseofulvin	10µg	≥10	0	No Zone

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Table 2: Distribution of samples according to site

Sample Site	Dermatophyte Isolated (N=50)	%
SKIN	40	80%
HAIR	0	0%
NAIL	10	20%

Table 3: Isolated Dermatophytes

Species isolated	Number of cases (n=50)	Percentage
Trichophyton mentagrophyte	22	44%
Trichophyton rubrum	16	32%
Trichophyton violaceum	9	18%
Trichophyton verrucosum	2	4%
Epidermophyton flocossum	1	2%

Table 4: Disc diffusion sensitivity-species wise

DERMATOPHYTES	DRUGS			
	FLU	ITRA	TER	GRI
Trichophyton mentagrophyte (N=22)	7 (31.8%)	13 (59%)	18 (81.8%)	19 (86.4%)
Trichophyton rubrum (N=16)	5 (31.3%)	8 (50%)	12 (75%)	13 (81.25%)
Trichophyton violaceum (N=9)	4 (44.4%)	6 (66.7%)	4 (44.4%)	9 (100%)
Trichophyton verrucosum (N=2)	0	2 (100%)	2 (100%)	2 (100%)
Epidermophyton flocossum (N=1)	1 (100%)	1 (100%)	0	0

Trichophyton mentagrophyte MTCC 7687 obtained from Institute of Microbial Technology, Chandigarh was used as control strain.

Table 5: Disc diffusion sensitivity genus wise

	DRUGS			
	FLU	ITRA	TER	GRI
TRICHOPHTYON (N=49)	16 (32.6%)	29 (59.2%)	36 (73.5%)	43(87.7%)
EPIDERMOPHYTON (N=1)	1 (100%)	1 (100%)	0	0

Griseofulvin, (GRI) terbinafine, (TER), itraconazole, (ITRA) and fluconazole, (FLU).

DISCUSSION

Dermatophytosis is a major health problem in tropical and subtropical countries, yet remains unresolved. Therefore, it is essential that good laboratory methods should be available for the rapid and precise identification of the Dermatophytes involved in order to apply appropriate treatment and prevention measures. In the present study, a higher incidence of dermatophytosis was seen in males than in females i.e., 4:1, which was supported by Agarwal E et al,[19] findings. Male predominance due to increased outdoor physical activities and increased sweating. Most of the infections were seen in the younger age group between 15-30 years of age due to working culture which predisposes them to the hot and humid climatic conditions. In addition, personal hygiene and the nature of the job also act as an additive factor in the occurrence of dermatophytosis in young adults. Similar findings were observed by Peerapur et al,[20] reported higher incidence in 21-30 years age group.

In present study dermatophytes isolated belong to 2 genera (Trichophyton and Epidermophyton) and 5 species. No Microsporum species were isolated. Among Trichophyton, *T.mentagrophytes* was the most common species isolated in 44% patients, which is in accordance with the study done by Shrestha et al.[21] Similar findings was observed by Pakshir et al,[17] and Bhatia and Sharma Springer Plus 2014, 3:134.[22] The other studies like Gadangi Indra et al,[23] showed the higher incidence of Trichophyton rubrum. The reverse trend in our study is due to the fact that T.rubrum is usually linked to chronic dermatophytoses. In our study most cases were reported within 6 months of onset of disease. Besides, extended use of antifungal therapy to treat patients might have also reduced the occurrence of this species in the region.

In this study we used four antifungal agents including Fluconazole, Itraconazole, Terbinafine, and Griseofulvin which were tested against 50 dermatophytic strains of clinical origin using disk diffusion. On analysing the results by disk diffusion method, in Trichophyton maximum number of cases were found sensitive to griseofulvin i.e., 87.7%, followed by terbinafine 73.5%, still followed by itraconazole i.e., 60% and least for fluconazole i.e., 34%. Similar findings observed by Zaki et al,[24] found griseofulvin and itraconazole have good activity and fluconazole displayed no visible inhibition zone. Pakshir et al,[17] found griseofulvin was sensitive in 92.5% cases.

Disk strength & inhibition zone diameters (IZDs) are two very important variables. Variable IZDs have been reported by various workers employing different disk strength of antifungal agents. Pakshir et al. (2009) used terbinafine disks of 30 g & griseofulvin of 25 g & reported that IZD of more than 20 mm & 10 mm should be regarded as sensitive. On the other hand, most of the workers have used a disk strength of 10 g for griseofulvin & 1 2 g for

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terbinafine (Venugopal et al., 1995; Nweze et al.,2010),[25,26] & have found much wider IZD usually >35 mm. (Agrawal R.K. et al). One more variable which affects the IZD is the type of inoculum preparation; lots of workers including CLSI guidelines recommend the use of microconidia. for susceptibility to antifungal drugs; (Nweze et al., 2010; Barros et al 2007).[26,27] Studies also show that microconidia of the tested species present higher susceptibility to antifungal drugs than hyphal preparations (Santos et al., 2001),[28] and it may be the reason for getting low MIC or very large IZD by several of them. However, we used mixture of both in our inoculum preparation in accordance with Agrawal R.K et al.[16]

CONCLUSION

The agar-based susceptibility testing methods for dermatophytes is simple, inexpensive & does not require specialized equipment & can be adapted for routine assessment of dermatophyte resistance to antifungal agents.

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