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Original Article

Agar-Based Disc Diffusion Assay for Susceptibility Testing of Dermatophytes in the Rajshahi Region

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Abstract

Background: Dermatophytosis is considered one of the most common public health problems in the world and is the most commonly diagnosed skin disease in Bangladesh. The prevalence and types of dermatophyte infections vary with climate conditions, age, lifestyle, and population migration patterns. Depending upon the anatomical site of the lesion, dermatophytes are classified into different varieties.

Objective: To isolate dermatophytes and their antifungal susceptibility pattern by agar-based disc diffusion method in the Rajshahi region.

Materials and Methods: Clinical samples (e.g., skin scrapings, nail clipping, and hair plucking) were collected under aseptic precautions. The identification of dermatophytes was performed through a microscopic examination using 10% KOH mount, mycological culture, and species identification by lactophenol cotton blue mount from culture. In addition, all dermatophytes isolates were subjected to antifungal susceptibility testing using the agar-based disc diffusion method.

Results: Out of 171 samples, *Trichophyton rubrum* was the predominant dermatophyte species with 76(71.7%), followed by *T.mentagrophyte* were 15(14.2%), *E. floccosum* were 12(11.3%), and *M. canis* were 03(2.8%). Voriconazole and Itraconazole were more effective drugs. Griseofulvin was the least effective drug, followed by Fluconazole.

Conclusion: Despite several treatment options being available for cutaneous fungal infections, Due to the increasing trend of antifungal drug resistance among dermatophytes, treatment should be based on antifungal sensitivity testing. The disc diffusion method is a simple and valuable method for the evaluation of antifungal susceptibility of dermatophytes.

Keywords: Dermatophytes, Dermatophytosis, Antifungal agents, Antifungal susceptibility test, Disc diffusion method.

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Introduction

Dermatophytes are a group of closely related fungi that has a predilection for keratinized tissues. It can invade keratinized human and animal tissues such as skin, hair, and nail, causing dermatophytosis. They are an essential cause of

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cutaneous fungal infections. The hot and humid climate in tropical and subtropical countries and the crowded living conditions make dermatophytosis a very common cutaneous fungal skin infection.[1]

Cutaneous fungal infections are common skin diseases, affecting millions of people worldwide. These infections occur in both healthy and immunocompromised patients, and aetiologic agents consist of dermatophytes, yeasts, and non dermatophyte molds. Dermatophytes are responsible for most cutaneous fungal infections, and the estimated lifetime risk of acquiring a dermatophyte infection is 10-20%.[2] According to World Health Organization, dermatophytes affect about 25% 0f the normal immunocompetent world population.[3]

Dermatophytes consist of three genera. *Trichophyton, Microsporum,* and *Epidermophyton*. Worldwide the most common cause of tinea pedis, tinea unguium, tinea cruris, tinea corporis, tinea faciei, and tinea capitis is *Trichophyton rubrum*. Other frequently implicated agents include *Trichophyton mentagrophytes, Microsporum canis, Microsporum gypseum,* and *Epidermophyton floccosum*.[4]

The laboratory diagnosis of dermatophytosis routinely involves direct microscopic examination of clinical specimens followed by in vitro culture techniques. The microscopic examination is usually done by KOH and Lactophenol cotton blue mount.

There are many antifungal agents that are used to treat dermatophytosis. However, not all species of dermatophytes have the same susceptibility pattern, and relative or absolute resistance may occur.[5] The reason for treatment failure and development of resistance is attributed to decreased drug uptake, phenotypic or genotypic alterations, or an increase in drug efflux.[6] Therefore, it is essential to evaluate the antifungal susceptibility test of dermatophytes using a standardized, simple, and reproducible in vitro assay. In vitro susceptibility test is helpful in selecting an effective antifungal agent to treat dermatophytes. A susceptibility test can also help to distinguish relapse or reinfection.[7]

So, the aim of this study was to detect dermatophytes by direct microscopic examination with KOH mount and Lactophenol cotton blue mount, to isolate and identify the different species of dermatophytes by mycological culture from skin, hair, and nail specimens and in vitro susceptibility of antifungal drugs by agar based disc diffusion method.

Materials and Methods

A cross-sectional type of descriptive study was conducted from January 2019 to December 2019 at the Department of Microbiology, Rajshahi Medical College, and the outpatient department of Dermatology and Venerology. A questionnaire and a checklist were the tools for data collection. Before collecting the specimen, each patient was interviewed, and relevant information was recorded systematically in a pre-designed standard data sheet. The samples from the patients were collected in aseptic precautions from infected areas such as skin, nails, and hair. Specimens were processed at the department of Microbiology for direct microscopic examination and fungal culture per standard protocol. Culturing organisms from skin, nails, and hair was done on a selective medium such as Sabouraud's chloramphenicol agar with supplements for identifying dermatophytes species. The identified fungi were subcultured on Potato dextrose agar media to enhance sporulation and processed for drug susceptibility tests. Isolation and identification of dermatophytes were made based on macroscopic observation of fungal colonies and lactophenol cotton blue mount microscopic examination. Antifungal susceptibility testing was performed after identifying dermatophytes on morphological, microscopic, cultural, and biochemical characteristics.[7,8]

Disk diffusion assay:

Inocula adjusted to 65% were streaked on the surface of 10 cm SDA plates in four different directions, rotating the plate at approximately 60° and rotating the swab stick around the margin of the petri dish to cover the entire surface. Using a flamed sterilized middle 5 ml syringe, antifungal disks of fluconazole, miconazole, Itraconazole, ketoconazole, voriconazole, and griseofulvin were evenly distributed on the inoculated plate. Within

30 minutes of applying the disks, the plate was inverted and incubated aerobically at 25°C for five days. After the colonies grew, the inhibition zones around the disks were measured and recorded. Criteria of susceptibility of antifungal disks were measured.[7,9,10]

Results

The present study was conducted on 171 samples; 112 were skin, 39 were nails, and 20 were hair.

Table 1: Distribution of age group of study population according to the clinical diagnosis. (N=171)

	Age group (in years)					
Clinical type	**<11**	**11-20**	**21-30**	**31-40**	**41-50**	**>50**
T. corporis **(n=62)**	2(3.2)	8(12.9)	21(33.9)	20(32.3)	4(6.5)	7(11.3)
T. cruris **(n=18**)	00	3(16.7)	10(55.6)	2(11.1)	2(11.1)	1(5.6)
T. pedis **(n=22)**	00	4(18.2)	7(31.9)	3(13.7)	2(9.1)	6(27.3)
T. faciei **(n=4)**	00	1(25)	1(25)	2(50)	00	00
T. manuum **(n=4)**	00	1(25)	2(50)	00	1(25)	00
T. barbae **(n=2)**	00	00	00	00	1(50)	1(50)
T. unguium **(n=39)**	00	6(15.4)	7(17.9)	4(10.2)	6(15.4)	16(41)
T. capitis **(n=20)**	2(10)	2(10)	2(10)	12(60)	2(10)	00
Total (N=171)	04(2.4)	25(14.6)	50(29.2)	43(25.2)	18(10.5)	31(18.1)

Table 1- shows the distribution of the age group of the study population according to clinical diagnosis. The age of the study population was divided into six groups. The most predominant age group was 21-30 years with 50 cases (29.2%), followed by the 31-40 years age group with 43 cases (25.2%). According to the clinical diagnosis, Tinea corporis was the most common type of dermatophytosis with 62 cases (36.3%), followed by tinea unguium in 39 patients (22.8%).

Table 2: Distribution of gender according to the site involved (N=171).

Site of lesion	**Name of the clinical lesion (n)**	**Percentage %**	**Gender**	
			Male (%)	**Female (%)**
	T. corporis **(n=62)**	36.3	24(38.7)	38(61.3)
	T. cruris **(n=18**)	10.5	16(88.9)	02(11.1)
	T. pedis	12.9	14(63.6)	8(34.4)
	(n=22)			
	T. faciei	2.3	02(50)	02(50)
	(n=4)			
Skin (n=112)	T. manuum **(n=4)**	2.3	01(25)	03(75)
	T. barbae	1.2	02(100)	00
	(n=2)			
Nail (n=39)	T. unguium **(n=39)**	22.8	13(33.3)	26(66.7)
Hair (n=20)	T. capitis **(n=20)**	11.7	6(30)	14(70)
Total (N=171)		100	78(45.6)	93(54.4)

Table 2: shows the gender-wise distribution of the study population according to clinical diagnosis. In the current study, among the 171 study population, 93(54.4%) were female, and 78(45.6%) were male, with a male and female ratio of 1:1.2. Tinea corporis 62(36.3%) was the most common type of dermatophytosis males were 24(38.7%), and females were 38(61.3%).

Table 3: Correlation between findings of KOH mount and culture:

Results of culture	**Results of KOH mount**		
	KOH mount positive	**KOH mount negative**	**Total**
Culture positive	79	27	106
Culture negative	13	52	65
Total	92	79	171

Table 3- shows the results of KOH mount microscopy with culture in diagnosing dermatophytosis. Of 171 cases, 92 (53.8%) were positive by KOH mount microscopy, and 106 (62.1%) were culture-positive. 27(15.8%) patients were positive by culture but negative by KOH mount microscopy. 13(7.6%) cases were positive by KOH mount microscopy but negative by culture. Both microscopy and culture-positive cases were 79 (46.2%), and total negative cases were 52 (30.4%).

Table 4: Detection of dermatophyte species by lactophenol cotton blue mount microscopy from culture.

Identified species	Skin	Nail	Hair	Total
T. rubrum	52(71.2)	16(69.6)	08(80)	76(71.7)
T.mentagrophyte	11(15.1)	03(13)	01(10)	15(14.2)
E. floccosum	08(11)	04(17.4)	00	12(11.3)
M. canis	02(2.7)	00	01(10)	03(2.8)
Total	73(68.7)	23(21.7)	10(9.4)	106(100)

Table 4: showed isolated dermatophyte species from positive culture by lactophenol cotton blue mount microscopy of different types of clinical lesions. Among 73(68.7%) isolated dermatophytes from the skin, 52(71.2%) were identified as *Trichophyton rubrum*, 11(15.1%) as *T. mentagrophyte* and 8(11%) as *Epidermophyton floccosum* and 02(2.7%) as *M. canis*. Out of 23(21.7%) isolated dermatophytes from the nail, *T. rubrum* was 16(69.6%), *T.mentagrophyte* was 03(13), and *E. floccosum* was 04(17.4%). Among 10(9.4%) isolated dermatophytes from hair, *T. rubrum* was 08(80%), *T.mentagrophyte* were 01(10%), and *M. canis* were 01(10%). *Trichophyton rubrum* was the predominant dermatophyte 76(71.7%), followed by *T.mentagrophyte* 15(14.2%), *E. floccosum* 12(11.3%), and *M. canis* 03(2.8%).

Table 5: Antifungal susceptibility pattern of the isolated dermatophytes species:

Dermatophytes	FLU		ITC		KCA		MCL				FOR AGE	
	S	R	S	R	S	R	S	R	S	R	S	R
T. rubrum n=76	04	72	63	13	55	21	59	17	62	14	03	73
T.mentagrophyte n=15	03	12	13	02	11	04	09	06	12	03	01	14
E. floccosum n=12	02	10	10	02	09	03	07	05	11	01	01	11
M. canis	00	03	03	00	02	01	03	00	03	00	00	03

n=3

S= Sensitive, R= Resistant

FLU= Fluconazole, ITC= Itraconazole, KCA= Ketoconazole, MCL= Miconazole, VOR= Voriconazole, AGF= Griseofulvin

Table 5- shows the susceptibility pattern of antifungal drugs against different species of dermatophytes. Highest sensitivity was shown to Itraconazole (84%), Voriconazole (83%) followed by Miconazole (73.6%) and Ketoconazole (72.6%). The highest resistance was shown against Griseofulvin (95.3%), followed by Fluconazole (91.5%).

Discussion

The current study showed most commonly affected age group was 21-30 years 50(29.2%). Santosh *et al*. in India and Rahim *et al.* in Bangladesh showed that the highest incidence of dermatophytosis was seen in the age group of 21

to 30 years.[11,12] Due to increased participation in outdoor physical activity, increased sweating, increased exposure to wet work, and shoe-wearing habits among this age group could be some of the contributing factors to the increased prevalence within the 21-30 age group.

Among the 171 study population, 78(45.6%) were male, and 93(54.4%) were female, with predominance. The male and female ratio was 1: 1.2. This study is nearly similar to Ghosh *et al.* and Nahar *et al.* in Bangladesh.[13,14] The highest incidence in females may be due to prolonged exposure to water during household work, such as exposure to detergents while cooking and cleaning. It also may be the body of the female remains covered by wet clothes, which may help keep the body moist and provide a favorable environment for the growth of fungus. But the study of Islam *et al.* in Bangladesh and Dabas *et al.* in India showed that the prevalence of dermatophytosis was high among males.[15,16] The higher prevalence amongst males may be due to increased outdoor physical activity, increased sweating, and increased opportunity for exposure.[17]

In the present study, Tinea corporis was the most common dermatophytosis encountered (36.3%), followed by Tinea unguium (22.8%). A similar study was observed by Niranjan *et al.* and Janardgan *et al.* in India.[18,19]. Tinea corporis is the commonest clinical type of dermatophytosis in female due to patterns of clothing worn by the women in the catchment area- saree, salwar suits- acts as precipitating factors due to friction, maceration, high rate of sweating in the waist region, and collection of dust particles at belt line make this site more vulnerable to fungal growth. Tinea unguium (66.3%) and Tinea capitis (70%) are predominant in females due to prolonged contact with water and detergents, work associated with constant trauma to the nails, use of occlusive footwear resulting in hyperhidrosis, poor scalp hygienic condition, relative negligence in hairdressing and sharing of families like towels, combs, etc.[17]

In the present study, out of 171 clinically suspected dermatophytosis patients, 92(53.8%) cases were positive by direct microscopy with KOH, and 106(62.1%) points were positive by culture. The explicit microscopic finding is similar to the other studies by Niranjan *et al*. and Dass *et al*. in India.[18,20] But dissimilar to the survey of Afshar *et al*. and Rahim *et al*. were 36% and 32.8%, respectively.[21,12] The negative results of direct microscopic examination may be associated with an inadequate amount and preparation of specimens, skills of the observer, and a non-suitable temperature of the specimens.

In the present study, 106(62.1%) cases were positive by culture is nearly similar to the studies done by Rao *et al.* and Kakande *et al.* in India.[22,23] But dissimilar to the study of Islam et *al.* and Rahim *et al.*, their observations were 38.7% and 30.3%, respectively.[15,12] This variation may be due to the non-viability of fungal elements in some cases, and/or other reasons may be co-existing microbes that may inhibit the growth of pathogenic fungi.

Among the 106 culture-positive dermatophytes, *Trichophyton rubrum* was the most typical isolate, 76(71.7%), followed by *Trichophyton mentagrophyte* 15(14.2%). *Trichophyton rubrum* was found to be the main etiological dermatophyte species responsible for dermatophytosis in the present study, which is comparable with the study done by Santosh *et al.* and Singh *et al.* in India.[24,25] But dissimilar to the study of Sharma *et al.*and Sowmya *et al.* in India.[26,27] This variation may vary depending on the geographical area, social, cultural, environmental, and occupational factors.

In this study, 6 antifungal drugs named Fluconazole, Itraconazole, Miconazole, Ketoconazole, Griseofulvin and Voriconazole were tested by disc diffusion method against 106 isolates of dermatophytes. Antifungal test results revealed that Itraconazole and Voriconazole were the most effective antifungal drugs, followed by Miconazole and Ketoconazole. Griseofulvin and Fluconazole had the poorest antifungal activity. This result was comparable to the study by Sharma *et al.*, Khatri *et al.*, and Budhiraja *et al.* in

India.[26,28,29] According to the survey by Alim *et al.*, Rahim *et al.*, and Sabtharishi *et al.*, Griseofulvin and Fluconazole had the poorest antifungal activity.[30,12,31] Griseofulvin and Fluconazole showed the highest resistance because of universal usage due to their low cost and dosage and their widespread availability in all levels of healthcare centers, which in turn has turned up to increased resistance profile for that drugs.[30]

A standardized disc diffusion-based assay for determining the antifungal susceptibility testing of dermatophytes is desirable and has a number of advantages. In line with the availability of an increasing array of antifungal agents, both intrinsic and emergent antifungal drug resistance are encountered. There is a need for accurate, reproducible, and predictive susceptibility testing of fungal isolates in order to help physicians for choosing of antifungal drugs appropriately. The standard disc diffusion assay can be adapted for the assessment of dermatophyte resistance against antifungal drugs.[32]

Conclusion:

Despite several treatment options being available for cutaneous fungal infections, Due to the increasing trend of antifungal drug resistance among dermatophytes, treatment should be based on antifungal sensitivity testing. The disc diffusion method is a simple and valuable method for the evaluation of antifungal susceptibility of dermatophytes.

Conflict of interest: None declared

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