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Research Article

Qualitative Assessment of *In Vitro* Proteolytic Activity and Antifungal Susceptibility of Dermatophytes Recovered from *Tinea capitis* Patients

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ABSTRACT

Background: Tinea capitis is a global health related issues affecting both children and adolescent and necessitates characterization of causative agents, understanding of their pathogenesis and better drug of choice. Transmission of tinea capitis is well contributed by poor stander of living and hygiene, climatic conditions, and overcrowding as some of the predisposing factors. *Objective:* isolation and phenotypic characterization as well as qualitative assessment of proteolytic activity and antifungal susceptibility of recovered dermatophytes from the patients suffering from tinea capitis. *Methodology:* recovery of dermatophytes using SDA supplemented with chloramphenicol and cycloheximidine and DTM. Phenotypic characterization was accomplished by using morphological examination and biochemical tests like urease test and growth characters on Bromocresol Purple agar. Proteolytic activity was determined qualitatively using three different media such as keratin medium, casein agar and gelatin peptone agar. Antifungal susceptibility of dermatophytes was assessed by disk diffusion methods using four most commonly used antifungal agents (GRI-10 microgram/disk, TERB-01 microgram/disk, FLUC-2 microgram/disk and ITRA-10 microgram/disk). *Results:* total of 41 dermatophytes recovered. The most prevalent pathogen was found to be Microsporum canis (n=14 or 34.1%, male/female: 9/5) followed by Trichophyton mentagrophytes (n=10 or 24.3%, male/female: 6/4), Trichophyton violaceum (n=09 or 21.9%, male/female: 7/2), Trichophyton verrucosum (n=05 or 12.1%, male/female: 3/2) and Trichophyton rubrum (n=3 or 7.3%, male/female: 2/1) which is the least prevalent one. The male infection was found to be dominant over female infection. Infection in children less than 10 years of age was more the most prevalent. Terbinafine was found to be the most active one followed by griseofulvin while fluconazole was least active or inactive in case of most of the isolates. The most of the isolates exhibited good activity on keratin medium. *Conclusion:* The most prevalent dermatophytes causing tinea capitis infection was found to be *Microsporum canis followed by Trichophyton mentagrophytes, Trichophyton violaceum*, *Trichophyton verrucosum* and *Trichophyton rubrum***.** Male and advanced age people are higher risk of tinea capitis. Keratin was the most suitable protein source for most of the dermatophytes which confirmed their pathogenesis and virulence. Terbinafine could be one of the best options of antifungal drugs for the treatment of tinea capitis followed by griseofulvin.

Keywords: *Antifungal, Dermatophytes, Proteolytic and Tinea Capitis*

INTRODUCTION

Dermatophytosis is a global health related problems and a most common type of communicable cutaneous mycosis[1]. Tinea capitis is a type of superficial mycosis[2]. *Tinea capitis* is a cutaneous infection involving scalp, eye lashes and eye brows. The infection is described in different terms such as ringworm of the scalp and *Tinea tonsures*. *Tinea capitis* is a global health related issues affecting both children and adolescent and necessitates characterization of causative agents, understanding of their pathogenesis and better drug of choice. Tinea capitis is one of the most common dermatophytosis occurring in children under the age of 12 years[3-5]. Adult cases mostly involve women due to hormonal disorders which consequently carryover the infection from childhood and patients with severe immunodepression such as leukemia, lymphoma, or treatment with immunosuppreive drugs[6]. It is caused by species of genera *Trichophyton* and *Microsporum*. There are various sources of infection such as humans, animals, or soil. Transmission of tinea capitis is well contributed by poor stander of living and hygiene, climatic conditions, and overcrowding as some of the predisposing factors. Being adaptable to non-living keratinized cutaneous layers and its appendages such as hairs they usually causes features like scaling, erythema, itching, hair loss and lesions similar to impetigo[7,8]. Phenotypic characterization of the dermatophytes isolated from tinea capitis patients is accomplished by observing morphological and physiological characteristics[9,10] as well as cultural characteristics[11]. Ability of dermatophytes to invade non-living cutaneous layer and its appendages such as hair nails[12] and digest the proteinaceous substrate including keratin substrates for its growth and multiplication which contribute to their pathogenesis. They secrete a variety of extracellular proteinases which help them to invade and penetrate the host tissues[13,14]. Proteolytic including keratinolytic

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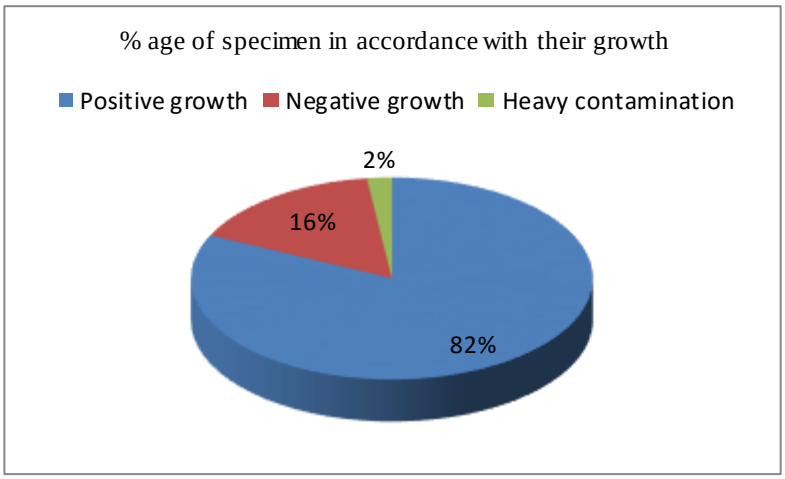

Figure 1: Response of clinical specimens on inoculation into culture medium for recovery of isolates.

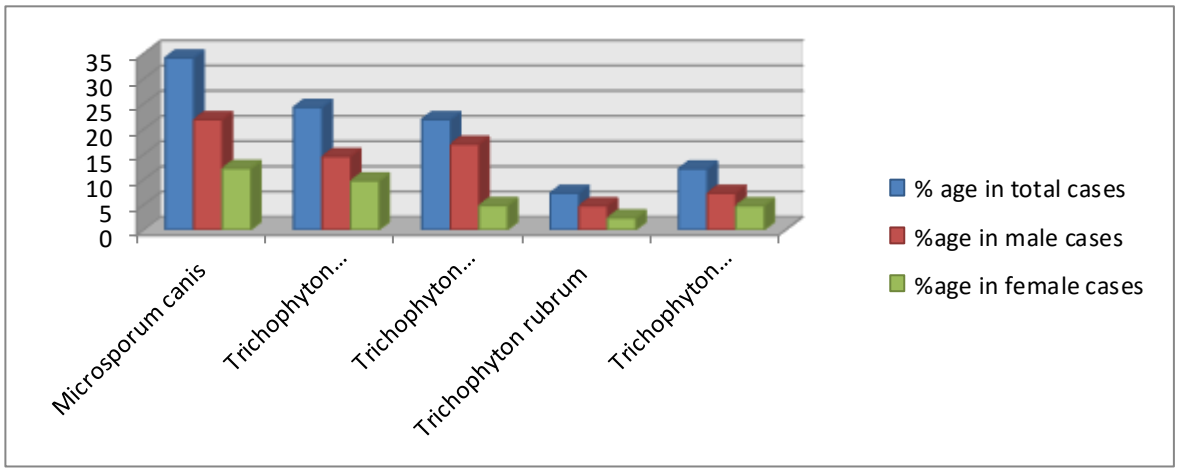

Figure 2: Prevalence of different dermatophytes in male and female cases against total cases of tinea capitis.

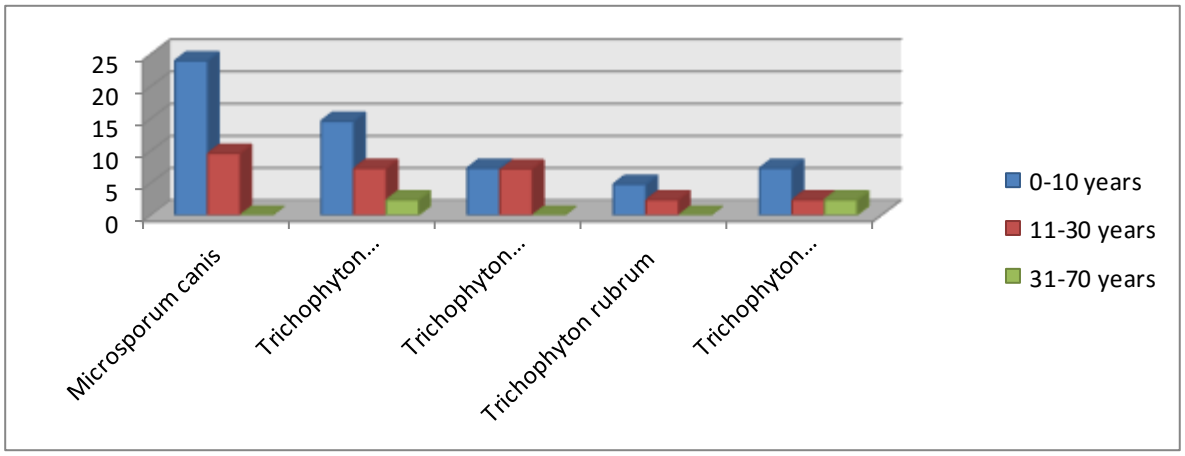

Figure 3: Depiction of different pathogens according to their frequency of occurrence among different age group patients with tinea capitis.

activities of various dermatophytes have become subject of research recently understand the pathogenicity of dermatophytosis[15]. The incidence of dermatophytosis including tinea capitis has grown up significantly with endemic and epidemic fashion of existence. Some cases are found to be recalcitrant to antifungal therapeutic options[16,17]. These infections can easily and rapidly spread among the people with poor standard of living, practicing poor sanitation and with lower socioeconomic conditions so they need economic and safe therapeutic options for the clinical management of the infections. Topical antifungal agents like, ketoconazole, econazole, terbinafine, tolnaftate and many others are most commonly used which exhibit in vitro antifungal activity[18,19]. Severe and chronic dermatophytosis such as tinea capitis and tinea unguium are often clinically

Table 1: Different doses of antifungal agents applied to determine *in vitro* antifungal activity against the dermatophytes.

S. No.	Name of the of antifungal agents	Abbreviations	Amount of drug loaded (in microgram)
1	Griseofulvin	*GRI*	10
2	Terbinafine	*TERB*	01
3	Fluconazole	FLUC	02
4	Itraconazole	ITRA	10

managed by administration of systemic antifungal agents like griseofulvin, terbinafine and itraconazole[20-23]. There are many new systemic antifungal agents are under trial and are found to be the significantly effective in vitro that could be one of the best therapeutic options for the management of the dermatophytosis. Although there is an increasing collection of potential drugs or antifungal agents for the clinical management of the tinea capitis infections however there is need for investigation of in vitro antifungal susceptibility of dermatophytes to find out the most effective and economic therapeutic options. The present study has been focusing on the characterization of dermatophytes, indirect determination of their pathogenesis and evaluation antifungal susceptibility.

MATERIAL AND METHODS

Sample collection

Scraping Scrapings of scale were collected from the leading edge of the lesion of patients having tinea capitis infections using surgical blade after being wiped with antiseptic (70% alcohol) to minimize the chances of bacterial and heavy mould contamination. Hair stumps were also collected by pulling them out with help of sterile forceps. Both the types of specimen were placed in a sterile container or a black paper envelope to avoid moisture exposure and contaminations.

Direct microscopic examination

Each sample was placed on a clean glass slide followed by addition of 1-2 drops of potassium hydroxide solution (20% w/v). Slide was allowed to be heated briefly and gently over flame. The preparation was examined under microscope after placing a cover slip over it. Fungal hyphae and spores were observed to get the preliminary idea of tinea capitis infections.

Isolation and phenotypic characterization of the dermatophytes

The clinical specimens collected were inoculated in duplicate into media Sabouraud Dextrose Agar (SDA) supplemented with antibiotics- Chloramphenicol and Cycloheximide and Dermatophytes Test Medium (DTM). Following inoculation the plates were incubated for 3 weeks at 25°C and 37°C. SDA as standard medium for isolation and DTM as dual purpose medium were used. Following incubation plates were observed for growth and change in the color of the medium to red (shift of pH of the medium from acidic to alkaline) on DTM. The isolates were preserved for their characterization. Phenotypic characterization of dermatophytes was confirmed by cultural characterization (morphology and texture of growth), microscopic characterization (typical microscopic characteristics, supplemented with slide culture), and biochemical characterization (urease test and growth characters on Bromocresol Purple agar (inoculated and incubated at 30°C for 7 days). Presence or absence of micro or macro conidia, cell wall arrangements, characteristics of their hyphae were observed as identification features. Bromocresol purple agar were inoculated and incubated at 30°C for 7 days. Rate of growth and pH of the medium were recorded on Bromocresol purple agar.

Assessment of proteolytic activities of the isolates

Only phenotypically identified pure cultures were used in the present investigation. 41 isolates (*Microsporum canis*-n=14, *Trichophyton mentagrophytes*-n=10, *Trichophyton violaceum*-n=09, *Trichophyton verrucosum*-n=05 and *Trichophyton rubrum*-n=3) were qualitatively assessed for protease activity following standard methods. It was assessed in the dermatophytes using different media such as casein agar medium (in gram/liter of deionized water - peptic digest of animal tissue 5, beef extract 1.5, yeast extract1.5, sodium chloride 5, casein 10, agar agar 15 together with bromophenol blue-0.0015% (w/v), pH 6.8), gelatin peptone agar (in gram/ liter of deionized water-gelatin peptone 5, agar 15, pH 7.0 ± 0.2) and solid keratin agar media(in gram/ liter of deionized water-$MgSO_4.H_2O$ 0.5, KH_2PO_4 0.1, $FeSO_4.7H_2O$ 0.01, $ZnSO_4.7H_2O$ 0.005, agar 1.5, supplemented with keratin substrate-0.06% as the sole source of carbon and nitrogen). Pure cultures were inoculated separately into three media and incubated at 25-28ºC for one week. Following incubation protease activities were recorded as color change in case of casein agar[24], zone of clearance in case of keratin medium[26] while uncolored zone was observed after the plates were flooded with mercuric chloride solution in case of gelatin peptone agar[25].

Determination of susceptibility of dermatophytes

Inoculums were prepared in sterile normal saline. Hyphal fragments and spores were added to saline using sterile cotton swabs followed by vortexing for 30 seconds and then allowed to settle down bigger fragments and conidia. Homogenous inoculums suspensions were collected in another sterile tube and diluted to achieve inoculums size of 1 x 10^6 cells/ml in sterile saline. The concentrations of the inoculums suspension were confirmed by recording the colony counts after plating serially diluted suspensions on Sabouraud's dextrose agar (SDA) medium. The antifungal drugs used were griseofulvin, terbinafine, fluconazole and itraconazole. The solvent used for all the drugs was 100% dimethyl sulfoxide (DMSO) except fluconazole was dissolved in sterile distilled water. The drugs were loaded on to the 6 mm sterile blank paper disk to achieve the concentration presented in Table-1. Antifungal susceptibility was determined by disk diffusion method using Dermasel agar medium. The plates were inoculated with the help of sterile cotton swab by streaking all across the surface of the medium. Drug loaded disks were placed on to the

Table 2: Activity of antifungal agents against four dermatophytes evaluated by disk diffusion method (diameter inhibition zone ranges in mm).

Name of and no. of isolates	GRI	TERB	FLUC	ITRA
Microsporum canis (n=14)	39-48	52-69	0-1	14-21
Trichophyton mentagrophyte (n=10)	23-41	60-70	0-0	14-21
Trichophyton violaceum(n=09)	50-54	73-75	0-1	26-31
Trichophyton rubrum (n=03)	38-56	62-75	0-24	16-34
Trichophyton verrucosum (n=05)	20- 35	33-49	0-6	15-29

Table 3: Proteolytic activities of dermatophytes on three different protein substrates containing media.

Name of and no. of isolates	On solid keratin medium		On gelatin peptone agar		On casein agar medium	
	Good activity	Poor /no activity	Good activity	Poor /no activity	Good activity	Poor /no activity
Microsporum canis (n=14)	78.5%	21.4%	85.7%	14.2%	42%	58%
Trichophyton mentagrophyte (n=10)	90%	10%	60%	40%	70%	30%
Trichophyton violaceum(n=09)	88%	12%	22.2%	77%	92.8%	6.2%
Trichophyton rubrum (n=03)	33%	66%	33.3%	66.3%	66.3%	33.3%
Trichophyton verrucosum (n=05)	80%	20%	20%	80%	60%	40%

streaked, dried plate. Following incubation for 72 hours to a week at 28-30ºC the diameter of the zone of inhibition was measured in mm and well documented.

RESULTS

Causative agents (n=41) recovered from clinical specimens collected from 50 Tinea capitis patients (both male and female). Eight specimens did not exhibit any growth while one sample became heavily contaminated so they were excluded from the study (Figure-1). The most prevalent pathogen was found to be *Microsporum canis* (n=14 or 34.1%, male/female: 9/5*) followed by Trichophyton mentagrophytes* (n=10 or 24.3%, male/female: 6/4), *Trichophyton violaceum* (n=09 or 21.9%, male/female: 7/2), *Trichophyton verrucosum* (n=05 or 12.1%, male/female: 3/2) and *Trichophyton rubrum* (n=3 or 7.3%, male/female: 2/1) which is the least prevalent one (Figure-2). The male infection was found to be dominant over female infection. Infection in children less than 10 years of age was more the most prevalent. *Microsporum canis Trichophyton mentagrophyte, Trichophyton violaceum* were found to be usual causative agents in case of patients with age group 0-10 years (Figure 3). Assessment of activities of four antifungal agents was carried out against five different groups of dermatophytes isolated from the patients of tinea capitis with the size of inoculum ($1x10^6$ cells/ml) and the amount of different antifungal drugs loaded on the disks has been presented in table-1. Clear and well defined zones of inhibition (diameter in mm) produced were recorded that has been presented in the Table-2. Terbinafine (1 microgram/disk) was found to be the most active one producing largest zone of inhibition which was followed by griseofulvin (10 microgram/disk), itraconazole(10 microgram/disk) while fluconazole was found to be least active or inactive in case of most of the isolates investigated even with the load of 25 microgram/disk. The Proteolytic activities of all the isolates were evaluated using various protein substrates containing media (CAM, GPA and KM) which revealed that most of the isolates across the genus exhibited good activity on keratin medium. Microsporum canis showed good activity on both CAM and GPA too. Trichophyton mentagrophytes showed greatest activity on CAM. Trichophyton violaceum showed greatest activity on CAM. Trichophyton verrucosum exhibited poor activity on GPA while moderate activity on CAM. The detailed results of Proteolytic activities have been well described in Table-3. Keratin medium was found to be the most suitable medium for the evaluation of the proteolytic activities of various isolates of dermatophytes that specifically given the idea of keratinolytic activity of dermatophytes.

DISCUSSION

Present study was focused on the isolation and characterization of dermatophytes from the clinical samples collected from the patients suffering from tinea capitis infections and on qualitative determination of proteolytic activities of isolated organism together with antifungal susceptibility. The most prevalent dermatophytes causing tinea capitis infection was found to be *Microsporum canis followed by Trichophyton mentagrophytes, Trichophyton violaceum, Trichophyton verrucosum* and *Trichophyton rubrum***.** The male infection was found to be dominant over female infection. Infection in children less than 10 years of age was more the most prevalent. *Microsporum canis Trichophyton mentagrophyte, Trichophyton violaceum* were found to be usual causative agents in case of patients with age group 0-10 years[4,5] *Trichophyton violaceum and Tinea rubrum* was not found to be infective agent in case of patients with age group 0-10 years. *In vitro* exhibition of proteolytic activities by dermatophytes play a key role in pathogenesis which enables dermatophytes to easily parasitize the tissues like the stratum corneum, hair and

nails[27] and penetrating keratin substrates of mycelium by degrading it [4,5,27]. 41 isolates (*Microsporum canis*-n=14, *Trichophyton mentagrophytes*-n=10, *Trichophyton violaceum*-n=09, *Trichophyton verrucosum*-n=05 and *Trichophyton rubrum*-n=3) were qualitatively assessed for protease activity using casein, gelatin, and keratin as protein source. Keratin was observed to be the most suitable protein source for most of the dermatophytes under investigation. But a very few investigations suggested casein, gelatin, peptone and ammonium nitrate as preferred source for protease production[27-29]. Microsporum canis showed good activity on both CAM and GPA too along with the high activity on keratin agar[27-29]. In vitro susceptibility of dermatophytes to four different antifungal agents have exhibited variations that may be because of nature of medium used, size of inoculum applied, amount of drugs loaded per disk and duration of incubation period which is why it is difficult to compare the results of disk diffusion methods with that of other authors[30-35]. In the present study four most commonly used antifungal agents for the treatment of dermatophytoses were investigated. Good zone of inhibition was recorded. Terbinafine (1 microgram/disk) was found to be the most active one producing largest zone of inhibition which was followed by griseofulvin (10 microgram/disk), itraconazole (10 microgram/disk) while fluconazole was found to be least active or inactive in case of most of the isolates investigated even with the load of 25 microgram/disk which is confirmed by other studies carried out on dermatophytes[30-35]. Furthermore, antifungal susceptibility ought to be determined using different methods to make a correlation of the results.

CONCLUSION

The most prevalent dermatophytes causing tinea capitis infection was found to be *Microsporum canis followed by Trichophyton mentagrophytes, Trichophyton violaceum*, *Trichophyton verrucosum* and *Trichophyton rubrum***.** The male infection was found to be dominant over female infection. Infection in children less than 10 years of age was more the most prevalent. *Microsporum canis Trichophyton mentagrophyte, Trichophyton violaceum* were found to be usual causative agents in case of patients with age group 0-10 years. Terbinafine (1 microgram/disk) was found to be the most active while fluconazole was found to be least active or inactive in case of most of the isolates investigated. Furthermore, antifungal susceptibility should be determined using different methods to make a correlation of the results which would give more precise idea of drug of choice for the treatment of tinea capitis infections. *In vitro* exhibition of proteolytic activities by dermatophytes play a significant role in understanding pathogenesis and degree of virulence of the dermatophytes.

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Conflict of Interest

The author has no conflict of interest

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