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Identification of Dermatophytes Isolated from Tinea Capitis Patients and Their in vitro Susceptibility to Terbinafine

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ABSTRACT

Background: Tinea capitis is an infection caused by dermatophyte fungi of scalp hair follicles and the neighboring skin. Antifungal drugs as terbinafine that are used to treat dermatophytosis direct the ergosterol synthesis pathway
Aim of the study: The current study aimed to identify species and *in vitro* antifungal susceptibility profile of terbinafine on dermatophytes clinical isolates accompanied by sequencing of the highest minimal inhibitory concentration (MIC) isolates.
Patients and Methods: The present study is a descriptive cross-sectional study was conducted at Al-Azhar Hospital outpatient clinic, in the period from the 1st of July 2019 to the 15th of July 2020. Samples from 50 patients diagnosed clinically as tinea capitis were taken for microscopic examination by KOH and lactophenol cotton blue and culture on Sabouraud's dextrose agar followed by *in vitro* susceptibility testing to terbinafine then sequencing of the highest MIC isolates.
Results: The isolated dermatophyte species were 16 (32%) isolates of *M. canis* and 23(46%) isolates of *T.violaceum*. The MIC varied from 0.003-.092 µg/ml for *M.canis* and from 0.005-0.116 µg/ml for *T.violaceum*. It was found in *M. canis* strain has two base variations with type strain from GenBank whereas *T.violaceum* strain has ten base variations in the two representative isolates with the highest MIC.
Conclusion: *T. violaceum* was detected as the most common fungal species followed by *M. canis*. The MIC results to terbinafine showed sensitivity to the antifungal agents. By comparing the *M. canis* and *T. violaceum* isolates with type strain on GenBank, some base-pair substitutions were found.

Keywords: *tinea capitis; dermatophytes; terbinafine*

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INTRODUCTION

Tinea capitis is a common dermatophyte infection of the scalp hair and mainly happening at a young age[1]. Its clinical manifestations are determined through hair invasion by the dermatophyte and the host's immune response, ranging from mild scaling with little hair loss to severe inflammatory and pustular presentations with scarring alopecia[2]. Tinea capitis caused by *Trichophyton* (*T*), and *Microsporum* (*M*) dermatophytes genera can dissolve keratinized tissue [1].

Antifungal drugs that are used to treat dermatophytosis direct the ergosterol synthesis pathway. Azoles inhibit lanosterol 14-α-demethylase, leading to the aggregation of sterol precursors, resulting in disruption of plasma membrane structure and function[3]. In contrast, terbinafine belongs to allylamines inhibits squalene epoxidase enzyme, which is involved in the initial steps of ergosterol biosynthesis [4]. This inhibition results in the aggregation of squalene and fungal growth inhibition [5].

However, the effect of the drug is different according to the infectious agent. Terbinafine was reported to be higher in treating *T. tonsurans* and *T. violaceum*, while griseofulvin was higher in treating *M. canis* and other *Microsporum* species [6, 7]. Lipozencic et al. [8] showed that the use of terbinafine for a long duration does not enhance its activity against *Microsporum* species, concluding that terbinafine not recommended as a first- line in treating tinea capitis caused by *Microsporum* species

Therefore, the importance of early identification of dermatophyte species and their antifungal susceptibility profiles is mandatory for establishing ways to control the spread of dermatophytosis [9].

This study was conducted to identify species and *in vitro* antifungal susceptibility profile of terbinafine on dermatophytes, accompanied by sequencing of the highest minimal inhibitory concentration (MIC) isolates.

PATIENTS AND METHODS

A descriptive cross-sectional study was conducted at Al-Azhar Hospital outpatient clinic, Al Azhar University (Assiut), and Assiut University Mycology Center (AUMC), in the period from the 1st of July 2019 to the 15th of July 2020. The study protocol was reviewed and approved by the Ethical Review Committee of Al Azhar Faculty of Medicine (Assiut). All participants have signed informed consent.

Study Participants

Fifty patients suspected clinically as tinea capitis infection were asked to participate in the study. Data was taken from the patients, including age, sex, presenting symptoms, and signs. Patients who received antifungal therapies in the previous two weeks were kept out from the study.

Sample Taking and Transport

The suspected tinea capitis lesions were cleaned with 70% ethyl alcohol, then hair, including the roots, was plucked for hair samples, a sterile scalpel was used to collect scales from the scalp. Samples were transported in sterile plastic petri dishes and were divided between microscopic examination and culture.

Microscopic Examination and Culture of Collected Samples

Direct microscopic examination was done to all samples using 20% potassium hydroxide (KOH) and lactophenol cotton blue (LCB) followed by gentle heating for 3-4 seconds. Every sample was inoculated on Sabouraud's dextrose agar **(**SDA) plates (Himedia Company, India). Chloramphenicol (0.5 gm/L) was added to prevent bacterial contamination and to prevent saprophytic fungi cycloheximide (0.5 gm/L) was added. Incubation of plates at 28ºC for at least six weeks was done [10].

In vitro Susceptibility Testing to terbinafine

Antifungal activity study was performed *in vitro* against six isolates of *Microsporum canis* and six of *T. violaceum* using modified agar cup diffusion method. The isolates were subcultured on SDA at 28°C, and dermatophyte suspension was prepared in Sabouraud's broth by smoothly scraping the colonies' surface with a sterile cotton swab then vortex and permitted to settle. The final inoculum concentration was adjusted to 1×10^3 to 3×10^3 CFU/mL. SDA was poured into Petri dishes at 50–70 °C, and it was left to solidify under ultraviolet rays in sterile category II laminar flow for 15 min. Standardized inoculum 300ul was streaked by cotton swab over the agar medium. Cylindrical plugs were removed from the agar by a sterile cork borer. Terbinafine was obtained as a powder (Sigma-Aldrich), a stock was made by dilution with Dimethyl sulfoxide (DMSO) kept at -70 °C. 50ul of final concentrations in distilled water ranged from 4ug/ml to 0.001ug/ml were added to the wells. The plates were incubated at 28 °C, and then the inhibition zones were measured in millimeters[11].

Molecular Identification

DNA extraction*:* following the manufacturer's instructions, DNA was extracted by QIAamp DNA Mini Kit extraction kit (QIAGEN, Germany).

Sequencing

The extracted product was sent to SolGent lab (Daejeon, South Korea). Before sequencing, using two universal fungal primers, Internal transcribed spacer (ITS1) (forward) and ITS4 (reverse), the ribosomal RNA (rRNA) gene was amplified by PCR reaction. Primers used have the following composition: ITS1 (5' - TCC GTA GGT GAA CCT GCG G - 3'), and ITS4 (5'- TCC TCC GCT TAT TGA TAT GC -3') [12]**.** The PCR reaction was prepared using Solgent EF-Taq (SolGent, Daejeon, South Korea).

RESULTS

The current work was a descriptive cross-sectional study of 50 patients having clinically suspected tinea capitis infections. Out of these 50 patients, 21 were diagnosed with tinea capitis scaly type while 15 patients were having tinea capitis black dot type with scales. Black dot without scales was noted in 10 patients, and four patients were having kerion.

Data analysis showed that 43 were males (86%) and 7 were female (14%). The youngest was 1.4 years old, and the eldest was 14 years old male patients with mean age 6.1± 2.67 years old. Tinea capitis infections were found to be more prevalent in males than in females.

Out of 50 samples, fungi were demonstrated in 68 % of cases by direct microscopy and 78% by culture. Both of them were positive in 48% of the samples. Dermatophyte species isolated from tinea capitis infection were 16 (32%) isolates of *M. canis* and 23(46%) isolates of *T.violaceum*, as shown in (Table 1).

	Number and (%) of tested patients	Number and (%) of Positive cases
KOH smear	50 (100%)	34 (68 %)
Culture	50(100%)	23(46%) *T.violaceum* 16 (32%) *M. canis*

Table 1: Showing the number and (%) of tested and positive patients with fungal growth (KOH:

potassium hydroxide, *T:Trichophyton, M:Microsporium).*

On examination, it was observed that scales and +ve hair pulling were the most common signs in tinea capitis. Also, 100% of kerion cases were presented with a secondary bacterial infection. Most of the patients were presented with a single lesion (n=32, 64%) whereas multiple lesions were noted in 36%. The type of the lesions was mainly black dot in 50% (n= 25); however, scales were common in both types as shown in (Table 2).

Presentation / Types Total=50	Scales only N (%)	+ve hair pulling only N (%)	Both signs N (%)	2ry infection N (%)	2ry Parasitic N (%)	Itching N (%)	Number of lesions per site
Tinea capitis (scaly type) N=21	7(33.3%)	negative	14(66.7)%	negative	negative	2(9.5%)	Single 14 (66.7%) Multiple 7(33.3%)
Tinea capitis (black dot type) N=25	negative	9(36%)	15(60%)	negative	1 (4%)	7(28%)	Single 14 (56%) Multiple 11(44%)
Kerion N=4	2(50%)	4 (100%)	negative	4(100%)	negative	1(25%)	Single 4(100%)

Table 2: Showing clinical profile in different clinical presentations (N=number, 2ry= secondary)

Identification of Isolated Dermatophyte Species

Identification of dermatophyte species from clinically suspected tinea capitis was dependent on direct microscopic examination from sample followed by inoculation of collected samples on culture media as shown in (figure 1, 2).

In vitro antifungal susceptibility test showed that all tested isolates were sensitive to terbinafine but at different concentrations. The MIC varied from 0.003-.092 µg/ml for *M.canis* and from 0.005-0.116 µg/ml for *T.violaceum* as shown in (Table 3).

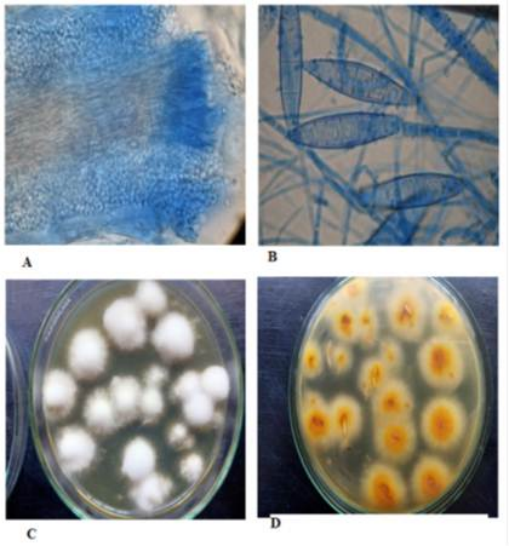

Fig 1: Microscopic and macroscopic appearance of *Microsporum canis*. (A) Ectothrix hair invasion pattern (B) Numerous spindle- shaped macroconidia, ,thick-walled (C) White fluffy, a fur-like colony with feathery border (D) reverse deep yellow.

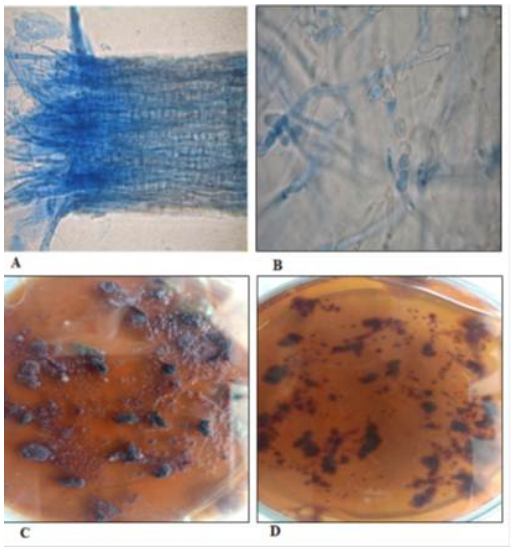

Fig 2: Microscopic and macroscopic appearance of *Trichophyton violaceum.* (A) Endothrix hair invasion pattern (B) Irregular hyphae with intercalary chlamydospores. (C)Waxy, cerebriform, heaped purple colony; (D) reverse deep port wine.

DNA sequencing

This was done only for two representative isolates with the highest MIC results. *M. canis* was identical by 99.28% with the type strain (NR_131265). *T. violaceum* was identical by 98.82% with the type material of *T. violaceum* (NR_144901). The query sequences were compared with those in the GenBank database by CLUSTALW analysis. It was found in *M. canis* strain two bases variations at positions 679, 680 and extra-base at position 678 in the alignment of ITS sequences of with type strain (NR_131265) from GenBank. Whereas *T.violaceum* strain has 10 base variations at positions 648, 645, 643, 642, 640,

638, 637, 636, 633, 631 in comparison with type strain (NR_144901).

Number of isolates (12)	MIC ug/ml
M.canis	0.092
M.canis	0.026
M.canis	0.026
M.canis	0.003
M.canis	0.014
M.canis	0.042
T.violaceum	0.025
T.violaceum	0.005
T.violaceum	0.022
T.violaceum	0.058
T.violaceum	0.032
T.violaceum	0.116

Table 3: Results of the Minimal Inhibitory Concentration of terbinafine antifungal in 12 isolates from tinea capitis patients. (MIC: Minimal Inhibitory Concentration, *M:Microsporum*, *T:Trichophyton*).

DISCUSSION

In developing countries, tinea capitis is a public health problem [13]. In the present study high occurrence of tinea capitis was in males than females. Bassyouni et al. [14] reported that males to females ratio was 5:1. They attributed their result to that girls apply vegetable oils on the scalp, described to have some antifungal effects against dermatophytes [15,16]. This study showed that tinea capitis predominate in children. This is in agreement with the previous study, showed that occurance of tinea capitis mainly in children less than ten years of age [17]. Despite the most common clinical type a black dot, scales were the main clinical presentation. This, following the study of El-Khalawany et al.[18] who reported that scaly scalp was the most clinical presentation in Egypt.

In this study, the commonest isolated fungal pathogen was *T. violaceum* (46%) and after that *M.canis* (32%), this was consistent with the previous multicenter study was done in Egypt by El-Khalawany et al. [18]. *T. violaceum* was also reported as the most common species in several countries. In Ethiopia, *T. violaceum* was detected as the most common fungal species (81.6%) [19]. Gargoom et al. [20] also reported that *T. violaceum* is the most common pathogen of Tinea capitis in Libya (50%). Despite in Kuwait, *T. violaceum* was not commonly isolated (19.3%) [21].

Conversely, a study was done in some Egyptian schools by Bassyouni et al. [14] found that *M.canis* was the most common isolated species. It may be due to contact with animals and sharing towels and caps among pupils. Positive cultures were obtained only in 78% of samples, which could be explained by applying homemade products before seeking medical advice.

The MIC results with terbinafine to *M. canis* were 0.003-.092 ug/ml. This is in agreement with the data published by Butty et al. [22] who found that MIC to *M. canis* was 0.01–0.08 ug/ml. Conversely, Mock et al. [23] reported higher MIC results to *M. canis* 0.05–0.5 ug/ml. *T.violaceum* MIC was 0.005-0.116 ug/ml. This consistent with the results of Badali et al. [24], who reported that MIC results to *T.violaceum* were 0.016–0.125 ug/ml.

The MIC results of terbinafine showed sensitivity to the antifungal. Terbinafine has been reported to be an effective antifungal agent in treating several dermatophytoses due to *Trichophyton* [24,25]. However, Yamada et al. [26] found that specific point mutations in squalene epoxidase genes were the cause of terbinafine resistance in *Trichophyton* clinical isolates. Insensitivity to terbinafine after treatment for 1.5-3 months in tinea capitis patients caused by *M. canis* has been reported [27]. Kano et al., [28] explained that by overexpression of genes encoding ABC transporter family in patients received long terbinafine treatment duration.

By comparing the *M. canis* and *T. violaceum* isolates with type strain on GenBank, some base-pair substitutions were found. Neither the phenotypic nor the molecular identification was affected by these substitutions. Also, it may be related to relatively high MIC detected in sequenced *trichophyton* strain. Osborne et al. [29] reported the silent nucleotide substitution of about 20 times common than non-silent nucleotide substitution rates. Singh et al.[30] estimated that the terbinafine resistance mechanism has been assigned to point mutations in the squalene epoxidase gene that leads to single amino acid substitutions. Squalene epoxidase is a key enzyme in the ergosterol synthesis pathway. Further sequencing to the squalene epoxidase gene will be required to fully elucidate this point.

Despite sequencing providing a highly accurate method for dermatophytes' characterization, it is very expensive to be implemented in routine identification. Therefore, it is suggested when atypical dermatophytes are isolated [31].

CONCLUSION

This study highlights the isolation of certain species, such as *M. canis* and *T. violaceum. T. violaceum* is prevalent in this region. Terbinafine exhibited good *in vitro* activity to *M. canis* and *T. violaceum* species.

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