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Isolation, Identification and Antifungal Susceptibility of Dermatophytes Isolated from Clinically Suspected Cases of Tinea Infections in Pakistan

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Authors' contributions

This work was carried out in collaboration among all authors. Author Shumaila Shakir designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Author Sidrah Saleem managed the analyses of the study. Authors WA and JI managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Dermatophytosis or tinea is a type of cutaneous infection caused by keratinophilic fungi, infecting the skin, nails and hair. A correct diagnosis is important for epidemiological purposes and initiating appropriate treatment. An increase in the prevalence of fungal infection worldwide is due to abuse of antibiotics, immunosuppressive treatments and numerous medical conditions.

Aim: To isolate, identify, and examine the in-vitro antifungal susceptibility of dermatophytes in clinically suspected cases of tinea infections.

Methodology: After taking informed consent, we took 65 patients suspected of tinea infection and obtained samples from skin, hair and nail, under aseptic precautions, at Department of Microbiology, University of Health Sciences (UHS), Lahore, Pakistan. The identification of dermatophytes was performed using potassium hydroxide (KOH) mounts and culture on Sabouraud Dextrose Agar (SDA) and Dermatophyte Test Medium (DTM). The cultures were incubated at 30ºC for up to 4 weeks in case of SDA and 2 weeks in case of DTM. Lactophenol cotton blue (LCB) stain was used

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to identify the species morphologically. Susceptibility test was done by agar diffusion method using antifungal disks and zones of inhibition were measured.
Results: More females (55.38%) than males (44.61%) were observed in the study. Most of the cases belonged to the age categories of 1-10 years and 21-30 years. Tinea corporis was the most common clinical type found (27.69%) followed by Tinea capitis (21.53%) and Tinea cruris (12.30%).*Trichophyton mentagrophytes* was the commonest species isolated (32%) followed by *Trichophyton violaceum* (28%) and *Trichophyton rubrum* (12%). Terbinafine was seen to be the most effective drug against the isolates, followed by clotrimazole. Fluconazole showed least activity.
Conclusion: Fungal culture remains the gold standard in identifying the causative species. Terbinafine promises to be a potent antifungal, whereas fluconazole has low efficacy against such organisms. Disk diffusion method adopted for antifungal susceptibility is cost effective and easily performable in small laboratories not having an established mycology bench.

Keywords: Dermatophytes; disk diffusion method; antifungal drugs; susceptibility; tinea infections; Pakistan.

ABBREVIATIONS

KOH : Potassium hydroxide;
LCB : Lacto phenol cotton blue;
SDA : Sabouraud Dextrose Agar;
DTM : Dermatophyte Test Medium;
UHS : University of Health Sciences

1. INTRODUCTION

Dermatophytes are a group of filamentous fungi prone to infect keratin-rich tissues, i.e., skin, nail and hair; this feature designates them as keratinolytic fungi [1,2]. Tinea infection ranks among the most common and rampant infectious diseases worldwide [3]. Living in close communities, poor personal hygiene, low socioeconomic status, humidity, contact with animals, unnecessary use of antibiotics, corticosteroids and tumour suppressing drugs are a few risk factors for such disease [4,5,6].

The three genera of dermatophytes [7] are further differentiated by their classical spores (microconidia and macroconidia) into a total of 40 species [8,9]. *Trichophyton* species infect all three body sites; *Epidermophyton* having the prefix epiderm focuses on skin and nails. *Microsporum* is the opposite of *Epidermophyton* and infects hair [10,11]. Medically, the disease can be categorized on the basis of the site involved. The clinical manifestations include tinea barbae (beard), tinea capitis (scalp), tinea corporis (hairless body skin), tinea cruris (groin), tinea manuum (hand), tinea pedis (foot) and tinea unguium (nails) [12,13]. Dermatophytes can either opt for human (anthropophilic), animal (zoophilic) or soil (geophilic) as their host [14,15].

The laboratory diagnosis of dermatophytes is of prime importance since it is difficult to diagnose such infections simply based on clinical presentation. In routine, direct microscopy of a clinical specimen is done, followed by culture [16]. After this, the antifungal therapy is started, which, sometimes can be quite complicated because of the long treatment duration, high cost, and possible side effects [17]. As a result, resistant strains have emerged, leading to poor treatment outcomes [18]. Therefore, it is of utmost importance to check the drug sensitivity, not only for directing the treatment protocols, but also for studying mechanisms of drug resistance in these fungi [19]. Several commercial methods like disk diffusion, agar dilution, micro/macro broth dilution, sensititre and E-test are available for this purpose [20]. The disk diffusion method is the simplest one and doesn't require any specialized equipment. Therefore, it can be performed in a clinical laboratory on routine basis [21,22,23,24]. In 1996, Barry and Brown whereas in 2000, Meis, et al. found out the disk diffusion method as an accurate, economical, easily performable and reproducible test for antifungal sensitivity [25,26,27].

In the present study, isolation of the dermatophytes was carried out from suspected cases of tinea infections, identified to species level, and antifungal susceptibility was evaluated against common antifungal drugs using disk diffusion method.

2. MATERIALS AND METHODS

2.1 Study Design and Patients

The study was carried out at the Department of Microbiology, University of Health Sciences, Lahore, Pakistan. Sample collection was done after obtaining informed consent, from clinically suspected patients, irrespective of their age or

gender, attending outpatient/inpatient department of Dermatology at Mayo Hospital, Lahore, Pakistan. The study was approved by the ethical committee of UHS, Lahore.

2.2 Specimen Collection

The specimens were taken from all three sites after cleaning with 70% ethyl alcohol. This was done to remove any surface bacterial contaminants. Skin scrapings were taken from the periphery of the erythematous inflamed margins of the wound with the help of a sterile blunt scalpel blade (size 22). The nail clippings were taken from the affected part of the nail with the help of a nail clipper. The area was scraped, which could not be clipped. Infected hair was epilated with the help of epilating forceps. Samples were collected from different sites if more than one area of the scalp was involved. All the specimens obtained were sealed in sterile dry petri dishes. These samples were labelled with the name, sex, age and date of collection and subsequently brought to the lab for mycological examination.

2.3 Microscopic Examination and Fungal Culture

Direct microscopy under low power (10X) and high power (40X) was done on all the collected samples using 10% and 40% KOH to check for the presence of fungal elements. Dermatophytes were recognized by their typical narrow, regular septate hyphae. Branching was also seen (Fig. 1). In some cases, instead of hyphae, arthrospores were seen in chains or scattered at various places. This procedure was adopted for both skin and nail specimens.In cases of hair specimen, the dermatophytes were confirmed by observing the arrangement of arthrospores with respect to the hair shaft. In case they were present inside the hair shaft, they were noted to be endothrix whereas their presence outside the shaft confirmed the infection to be ectothrix.

After a direct microscopic examination, irrespective of the demonstration of fungal elements, specimens were inoculated on two sets of petri dishes; Sabouraud Dextrose Agar (SDA) base (Oxoid,UK) and Dermatophyte Test Medium (DTM) base (Hi-Media, India), both incorporated with chloramphenicol (acts as a broad spectrum antibiotic) and cycloheximide (to inhibit saprophytic fungi). All the plates were incubated aerobically at 28-30ºC up to 4 weeks for SDA and 2 weeks for DTM after placing them in polythene zipper bags to prevent drying and contamination [9,3]. The SDA plates were checked twice weekly for a maximum of 30 days. DTM plates were checked daily for 2 weeks. SDA plates showing no growth after 4 weeks were considered negative. DTM plates showing no growth after 14 days were considered negative. Plates showing any growth after above mentioned time period on any of the above mentioned culture media were also discarded. Dermatophyte species were identified by observing the colonial morphology. Colonies of dermatophytes were recognized as being light coloured, often brown and white as shown in Figs. 2-4.

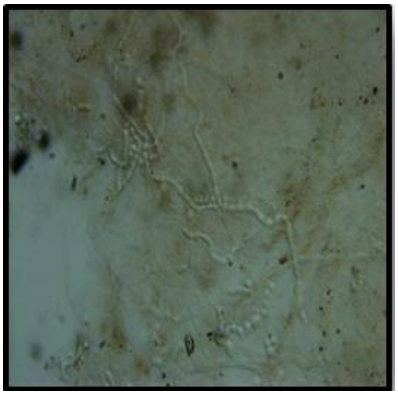

Fig. 1. KOH (40%) mount of skin scraping showing septate hyphae

To obtain a sample for microscopic identification, a small sample from the mature spore-producing area of the colony was picked up with the help of sterile loop or straight wire. It was then teased out in a drop of lactophenol cotton blue stain on a clean microscope glass slide. A cover slip was applied. Care was taken as to avoid any air bubbles. Microscopic examination revealed the hyphae, spore arrangement and the presence of macroconidia and microconidia as shown in Figs. 5-7 [28,29].

2.4 Antifungal Susceptibility

The samples were transferred to sterile distilled water in tubes and stocked at 25ºC until required [21,20]. When needed, they were sub-cultured on PDA at 28ºC to augment sporulation. 7 days old cultures were mixed with 1ml distilled water. The colonies were explored with the tip of a sterile Pasteur pipette to get an assortment of mycelium and conidia. The suspensions were allowed to sediment for 30 minutes. Suspensions were adjusted by using 0.5 McFarland standard. [20,30]. The adjusted inocula was evenly

streaked on the surface of petri dishes containing SDA [30]. Commercially available neo-sensitabs disks (Rosco Diagnostica, Germany) fluconazole (25 µg), clotrimazole (10 µg), ketoconazole (15 µg), miconazole (10 µg) and terbinafine (30 µg) were applied onto the inoculated agar plates and incubated at 28ºC to 30ºC. The zones of inhibition around the disks were measured and recorded. The criteria of susceptibility and resistance to antifungal agents were measured, according to Pakshir et al. [31] which is presented in Table 1.

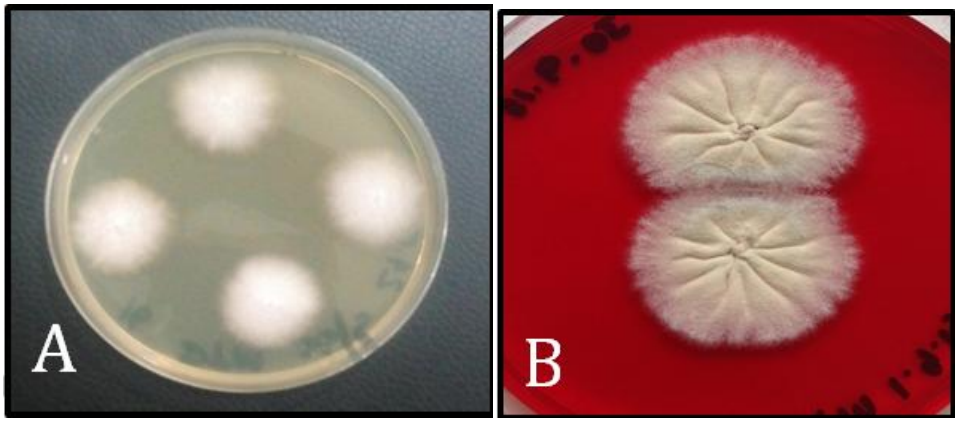

Fig. 2. *Trichophyton mentagrophytes* on SDA (A) and DTM (B)

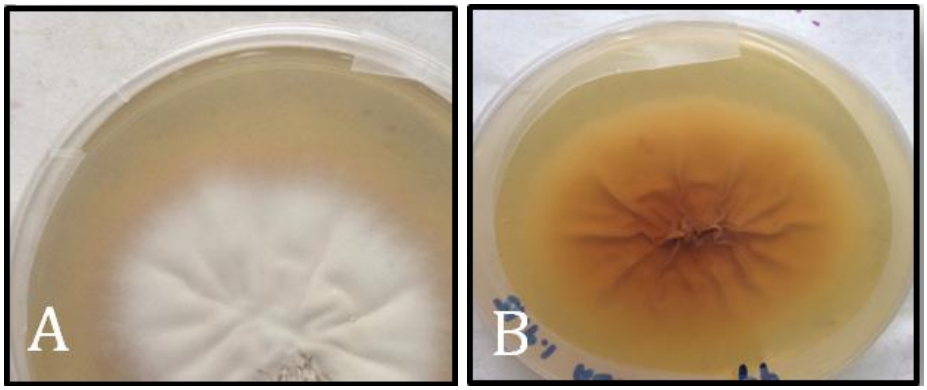

Fig. 3. *Trichophyton tonsurans* on SDA; Surface view (A) and Reverse view (B)

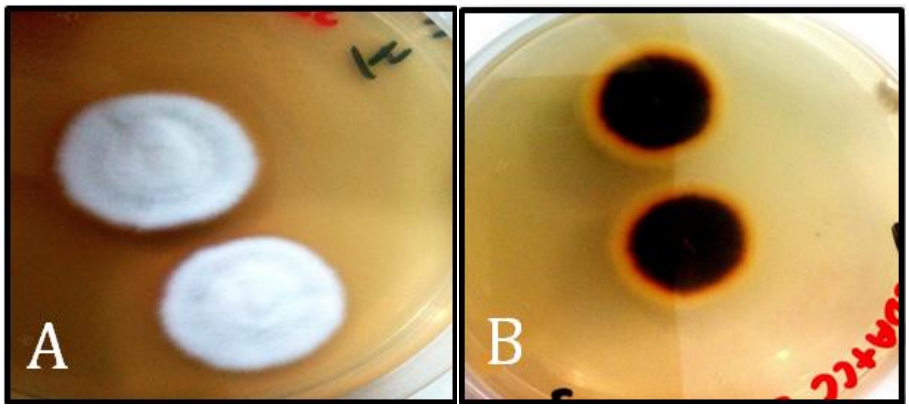

Fig. 4. *Epidermophyton floccosum* on SDA; Surface view (A) and Reverse view (B)

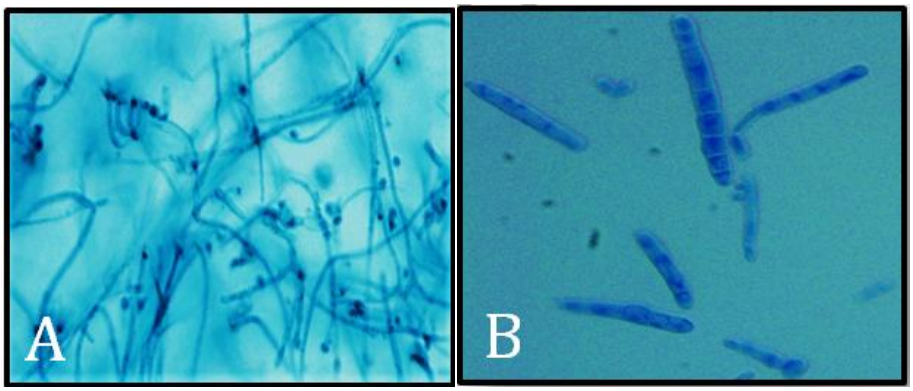

Fig. 5. LCB preparation under microscope; (A) *T. mentagrophytes* (B) *E. floccosum*

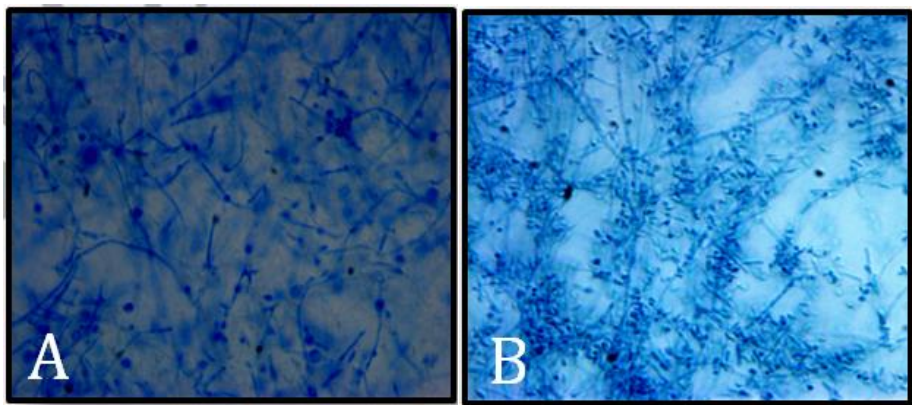

Fig. 6. LCB preparation under microscope; (A) *T. tonsurans* (B) *T. rubrum*

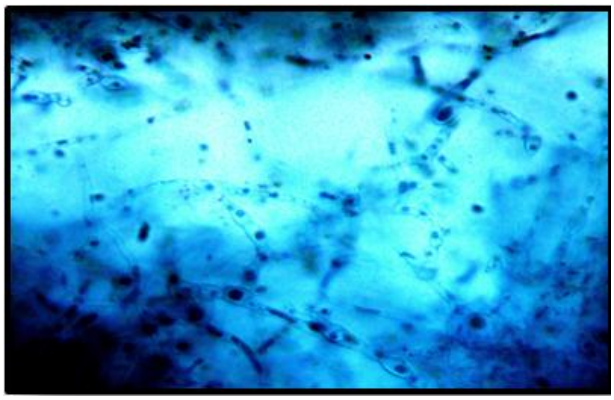

Fig. 7. LCB preparation under microscope of *T. violaceum*

Table 1. Criteria of susceptibility and resistance of antifungal disks

Antifungal drugs	Potency	Zone diameter in mm		
		Sensitive	Intermediate	Resistance
Fluconazole	25 µg	≥22	21-15	≤14
Ketoconazole	15 µg	≥30	29-23	≤22
Clotrimazole	10 µg	≥20	19-12	≤11
Miconazole	10 µg	≥20	19-12	≤11
Terbinafine	30 µg	≥20	19-12	≤11

3. RESULTS

Sixty-five patients with clinically suspected disease were included in this study. Out of them, twenty-nine were males and thirty-six females. Of the seven age groups made, the groups most commonly affected were 1-10 years and 21-30 years, both with 17 cases each.

In accordance with the clinical types, Tinea corporis was predominant, followed by Tinea capitis, Tinea cruris, Tinea facium, Tinea unguium and Tinea pedis (Fig. 8). The males were seen to have high incident of Tinea corporis whereas, females showed more cases of Tinea capitis (Table 2). The most common species isolated was *Trichophyton mentagrophytes.* It was followed by *Trichophyton violaceum, Trichophyton rubrum, Trichophyton tonsurans, Epidermophyton floccosum, Trichophyton verrucosum* and *Microsporum ferrugenium* (Fig. 9).

The tests for the susceptibility to antifungal drugs showed following results; fluconazole: 4 (16%) sensitive, 21 (84%) resistant. ketoconazole: 14 (56%) sensitive, 2 (8%) intermediate and 5 (20%) resistant. clotrimazole: 24 (96%) sensitive and 1 (4%) resistant. miconazole: 20 (80%) sensitive and 4 (16%) resistant. terbinafine: 25 (100%) sensitive. Regarding the data, it was revealed that terbinafine and clotrimazole were the most effective antifungal drugs and fluconazole had the poorest activity (Figs. 10-13).

4. DISCUSSION

The fungus that causes Tinea is very common all over the world and affects all. In our study, a higher incidence of dermatophytosis was seen in females as compared to males, which supports findings in other studies as well [32-36]. It may be attributed to the fact that more patients of this gender attended the outpatient department. Women, being more involved in house chores mostly tend to ignore their hygiene, leading to having Tinea infections.

Similarly, children and young adults pose a greater risk to contract Tinea infections, as

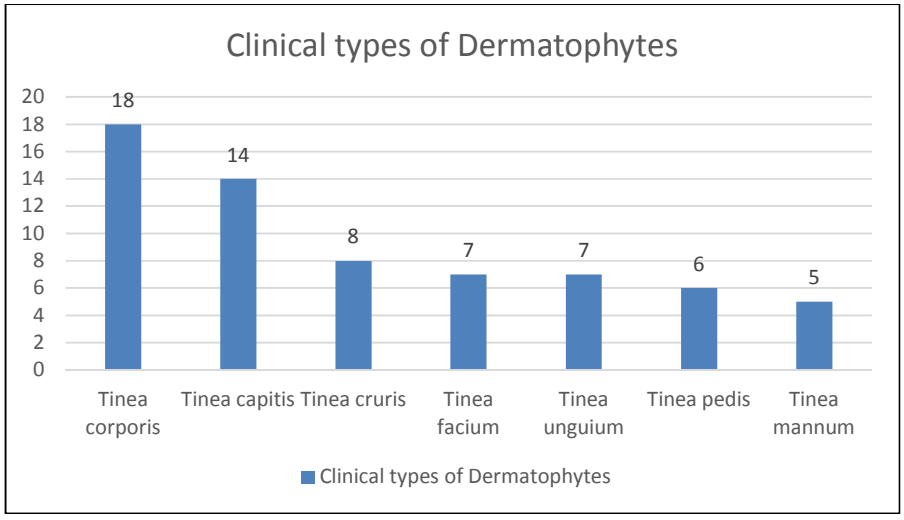

Fig. 8. Distribution of patients with tinea infection according to their clinical types

Table 2. Age and sex wise distribution in relation to clinical types

Clinical types	Age groups in years							Sex		Total
	1-10	11-20	21-30	31-40	41-50	51-60	>60	Male	Female	
Tinea corporis	4	3	6	1	22	1	1	12	6	18
Tinea capitis	9	2	2	-	-	-	1	5	9	14
Tinea cruris	3	1	1	2	-	1	-	4	4	8
Tinea facium	1	2	1	-	2	1	-	1	6	7
Tinea unguium	-	1	2	1	-	2	1	3	4	7
Tinea pedis	-	1	3	1	-	-	1	2	4	6
Tinea mannum	-	3	2	-	-	-	-	2	3	5
Total	17	13	17	5	4	5	4	29	36	65

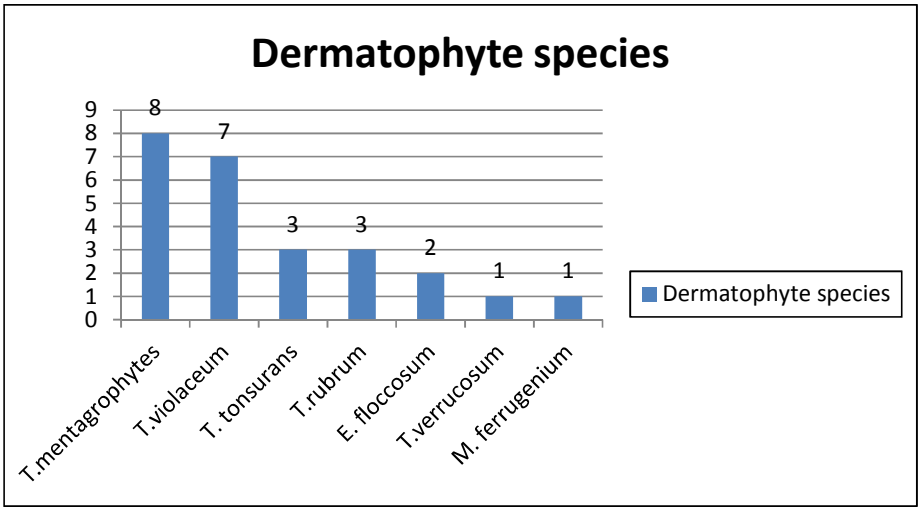

Fig. 9. Isolation of dermatophyte species on culture

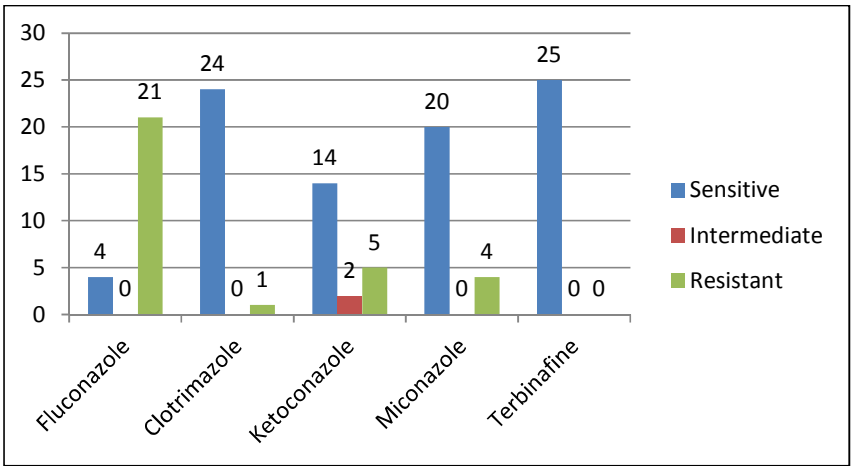

Fig. 10. Susceptibility of antifungal drugs

shown in this study. Staying out for longer periods of time (parks etc.) increases their chance of exposure. Increased transmission, particularly in school going children is due to increased contact, overcrowding in classrooms (a major cause in Pakistan) and lack of awareness. Having pets is another cause. Cats, dog and goats in such cases, are infected with dermatophytes and transmit the condition to humans through saliva, infected urine/feces or close contact. A population whose immune function is compromised for any reason is most susceptible to the infection [33, 37, 38]. Tinea corporis and Tinea capitis were the most common clinical types found in patients under study. Tinea corporis affected predominantly the male population, while females showed more cases of Tinea capitis. The risk factors include strenuous physical activity outside, warm climate, contact sports such as wrestling, use of communal baths or locker rooms, poor nutrition, unhygienic lifestyle, poor socio-economic conditions, sharing of infected materials among family members (combs, clothing, towels and bed linen) and occupational contact like gardeners and farmers [39-41].

The distribution of the dermatophytosis and their etiological agents has unusual frequencies, with variations of their prevelance according to the countries and even the regions of the same country. In the present study, various dermatophytes isolated were *T. mentagrophytes* (32%), *T.violaceum* (28%), *T.rubrum* (12%), *T.tonsurans* (12%), *E.floccosum* (8%) and *T.verrucosum* (4%), *M.ferrugenium* (4%). The

commonest isolate was *T.mentagrophytes,* showing concurrence with other studies [30,42].

For the ability to eradicate pathogenic dermatophytes, in vitro antifungal susceptibility need to be determined [43]. In recent years, there has been an astonishing development in the standardization of antifungal susceptibility tests throughout the world. Unfortunately, in Pakistan, the field of mycology has been neglected for quite a long time. There is a lack of a reference mycology lab, skilled personnel, and resources that deprive us of making efforts in doing research in this field. Although, studies have been done on various fungi like Candida, Aspergillus, and non-dermatophytes, very less work has been done related to dermatophytes and little or no data is available on their antifungal susceptibility. Regardless of the many guidelines that NCCLS have published for susceptibility tests of molds (such as M-27A, M28A), there is no exact method and a routine test for the screening of dermatophyte antifungal activity [44].

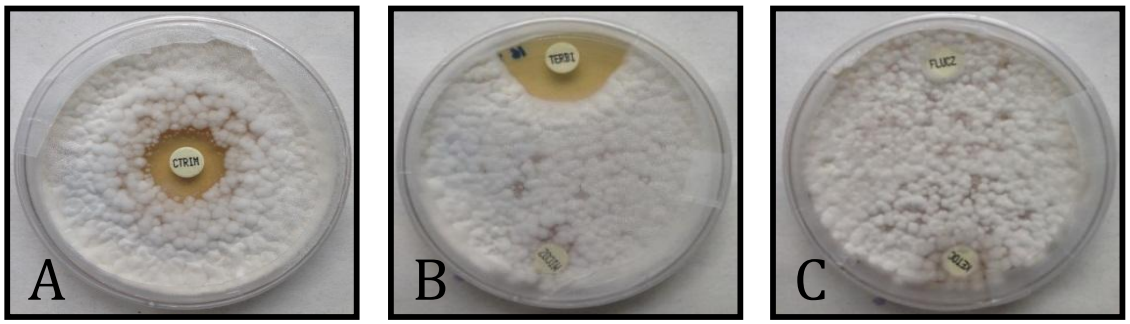

Fig. 11. Antifungal susceptibility plates for *T. mentagrophytes* showing inhibitory zones of the drugs

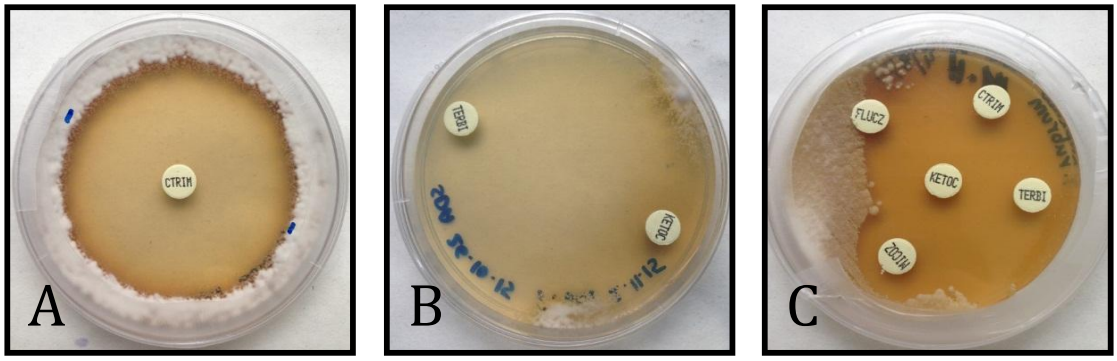

Fig. 12. Antifungal susceptibility plates for *T. rubrum* showing inhibitory zones of the drugs

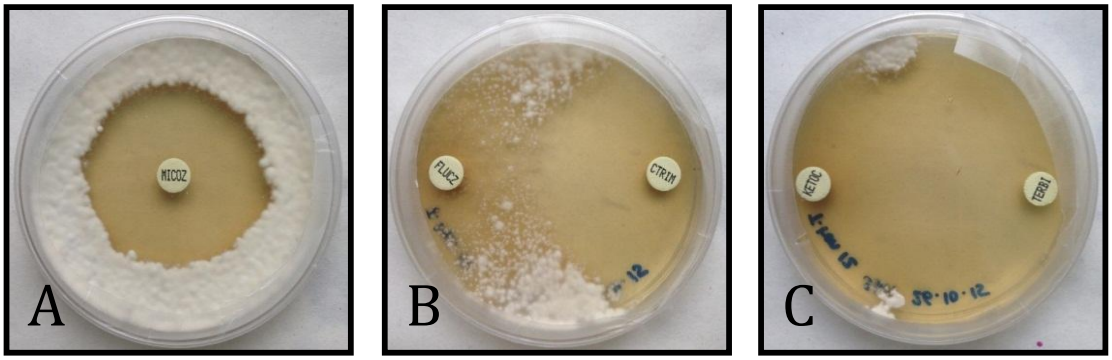

Fig. 13. Antifungal susceptibility plates for *T. tonsurans* showing inhibitory zones of the drugs

Macro and micro-dilution methods both can be used to determine antifungal susceptibility of dermatophytes, but these methods are costly and require specific media and equipment such as RPMI, MOPS buffer, and micro plate trays. The agar diffusion method, on the other hand, has a practical approach which assists in the determination of the activity of antifungal drugs against dermatophytes. For countries like Pakistan, the disk diffusion method is a good model to be used for investigation purposes. This method can be adapted for routine diagnosis in the laboratory and for assessment of dermatophyte resistance against antifungal drugs. There are studies which focused on the comparison of the disk diffusion method with the reference micro-dilution method. These studies suggest that disk diffusion is a reproducible method which in general shows good correlation with the reference method for micro-dilution antifungal susceptibility test [24, 26]. Our study showed that terbinafine and clotrimazole had large inhibition zones around the disks; terbinafine had the best activity against all the isolates. Fluconazole, on the other hand, had the least activity and in most isolates, no inhibition zone at all. Results of this study are in line with other studies conducted on dermatophytes [21, 25,26,30,45,46].

5. CONCLUSION

Dermatophyte infections are very common in our country, where the hot and humid climate in association with poor hygienic conditions play an important role in the growth of these fungi. Our study signified the importance of the mycological examination in the diagnosis of various Tinea infections for their effective management. Through this study, terbinafine and clotrimazole have been proved to be the most effective antifungal drugs against dermatophytes. Necessary changes in antifungal therapy can be made according to the antifungal susceptibility tests to prevent resistant strains and recurrent infections. Genetic basis of antifungal resistance can be explored in future to make accurate and prompt diagnosis, thus treating such infections.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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