Prevalence and antifungal drug resistance of dermatophytes in the clinical samples from Pakistan

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ABSTRACT

Dermatophytosis is a major health problem all over the world including Pakistan. This is the first report of detection of dermatophytes and their antifungal drug resistance in the Northern and Western parts of Pakistan. A total of 154 samples were collected from different hospitals of Khyber Pakhtunkhwa, and out of them 136 samples were found positive. Tinea corporis (35%) was the most predominant type of infection followed by Tinea capitis (22%). The fungi identified in Tinea corporis infection types were identified as Trichophyton rubrum, Trichophyton mentagrophytes, Epidermophyton floccosum. The fungi identified in Tinea capitis included Trichophyton violaceum, T. mentagrophytes, Microsporum ferrugineum. The gender wise distribution showed both males (52%) and females (48%) were infected with the fungi. More cases belonged to the rural parts of the country. Age wise distribution showed that the infection was more prevalent in the children and the prevalence decreased with the increase in age. The positive samples were checked against two antifungal agents: fluconazole and nystatin. Among 136 positive samples, none of the isolates showed resistance to nystatin while 7% of the samples showed resistance to fluconazole. The resistant isolates were then identified by amplifying the 18S rRNA gene, using universal primers (ITS1, ITS4). Among the 9 resistant isolates, 5 isolates were identified as Trichophyton spp., 3 as Microsporum spp. and 1 as Epidermophyton spp.

KEYWORDS

fungal infection, gender wise distribution, age wise distribution, antifungal drug resistance, 18S rRNA gene sequencing

INTRODUCTION

Dermatophytes are a group of pathogenic fungi that cause mostly superficial diseases on humans and other mammals [1]. The diseases that result from a dermatophyte infection are known as Tinea infections. Dermatophytes are ascomycetes with septate hyphae, most closely related to Coccidioides immitis within the Onygenales [2–3]. There are three genera of dermatophytes i.e. Trichophyton, Microsporum, and Epidermophyton. Although the species were classically divided into these genera by morphology and physical attributes, the recent analysis by rRNA sequencing indicates that the dermatophytes as a whole are a cohesive group, with no clear distinction between the three genera. The closest relatives to any Microsporum species might be two Trichophyton species [3].

Dermatophytosis prevails all over the world but a great deal of variations has been seen in the epidemiology, distribution of infections, and host target. The variations are because of differences in various conditions of different geographic locations. Such conditions may be
climatic including temperature, humidity and wind [4]. Overpopulation, healthcare, and socioeconomic conditions are the other factors which may bring variations in the disease prevalence [5]. Superficial mycoses have been recorded as the most common form of human infections which effect a population of more than 25% all over the world [6].

Dermatophytosis is frequently associated with relapses following the interruption of antifungal therapy. Clinical resistance to antifungal agents was rare until the late 1990s, with only isolated cases in patients with chronic mucocutaneous candidiasis [7]. The incidence of fungal infections, including resistant infections, has increased during the last few years, and may be due to inadequate or irregular use of drugs or increased incidence of immunodeficiency states [8]. The increased use and over the counter sale of antifungal agents in recent years has also resulted in the development of resistance to these drugs. Drug resistance in fungi, especially to azoles, is becoming more prevalent clinically. After the appearance of resistance to griseofulvin, a case of clinical resistance to terbinafine was reported in 2003 [9].

In Pakistan, no extensive work has been carried out on dermatophytic diseases in human. However, some work has been done in Karachi [10], Quetta [11], Lahore [12], Chitral [13], Jamshoro [14], Rawalpindi [15], and Faisalabad [16] on different aspects of the dermatophytic infections in different target populations. In Karachi, skin infections were reported to be common problems. These diseases were more common in children as compared to adults and the fungal infections were dominated among these cases [17]. On one instance, the antifungal resistance has not been studied so far in Pakistani samples and on the other, there has been no report of the prevalence from the North Western parts of the country.

Therefore, the current study was based on collecting clinical samples of dermatophytosis from different hospitals situated in four different Districts of the Province Khyber Pakhtunkhwa, Pakistan. The samples after confirmation of disease and fungal isolation, were screened for the antifungal drug resistance against flucanazol and nystatin. The dermatophytes that showed resistance against any of the drugs were identified based on 18S rRNA gene sequencing.

### MATERIALS AND METHODS

#### Sampling and isolation

Clinical samples were collected from the District Headquarter (DHQ) hospitals of Kohat, Peshawar, Abbottabad and Dera Ismail Khan Districts in Khyber Pakhtunkhwa Province of Pakistan during October 2017 to March 2018. Prior data collection, the informed written consent and assented forms were obtained from the participants. Samples of nails, skin and hair were collected and grown on Sabouraud dextrose agar (SDA) and potato dextrose agar (PDA) media. Antibiotics and chloramphenicol were supplemented to inhibit the growth of bacteria and saprophytic fungi respectively. The cultures were incubated at 25 °C to 30 °C and the samples were examined frequently for 4 weeks.

#### Culture and microscopic characterization

Initially, the mold isolates were identified by observing the microscopic and macroscopic characteristics of the colony grown in Petri dishes. Microscopic identification of isolates was performed by placing pieces of a fresh colony from SDA or PDA on a clean microscopic slide followed by staining with lactophenol cotton blue using the mount or tape touch methods [18]. Moreover, both the front and reverse of the Petri dishes were observed with naked eye for the culture characteristics including colony shape, color and growth rate.

#### Antifungal drug susceptibility assay

For antifungal susceptibility test, two antifungal agents (flucanazole and nystatin) were tested against all the isolates of dermatophytes and the antifungal ready-made discs were used for this purpose. The isolates were sub-cultured on PDA for one week and the conidia were harvested and suspended in sterile saline. Plates of Mueller-Hinton agar

<table>
<thead>
<tr>
<th>Infection</th>
<th>Prevalence (%)</th>
<th>Fungal Species</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tinea corporis</td>
<td>35</td>
<td>Trichophyton rubrum,</td>
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<tr>
<td></td>
<td></td>
<td>Epidermophyton floccosum,</td>
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<tr>
<td></td>
<td></td>
<td>Trichophyton violaeum,</td>
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<tr>
<td></td>
<td></td>
<td>Trichophyton mentagrophytes,</td>
</tr>
<tr>
<td>Tinea capitis</td>
<td>22</td>
<td>Microsporum ferrugineum,</td>
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<tr>
<td></td>
<td></td>
<td>Microsporum canis,</td>
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<tr>
<td></td>
<td></td>
<td>Trichophyton tonsurans,</td>
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<td></td>
<td></td>
<td>Trichophyton schoenleini.</td>
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<tr>
<td>Tinea pedis</td>
<td>9</td>
<td>Trichophyton concentricum</td>
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<tr>
<td></td>
<td></td>
<td>Trichophyton rubrum,</td>
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<tr>
<td></td>
<td></td>
<td>Epidermophyton floccosum,</td>
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<td></td>
<td></td>
<td>Candida albicans</td>
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<tr>
<td>Tinea cruris</td>
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<td>Trichophyton mentagrophytes,</td>
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<td>Tinea faciei</td>
<td>6</td>
<td>Microsporum gypseum,</td>
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<td>Microsporum audionii</td>
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<td>Tinea manum</td>
<td>5</td>
<td>Trichophyton mentagrophytes,</td>
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<tr>
<td></td>
<td></td>
<td>Microsporum canis,</td>
</tr>
<tr>
<td>Tinea unguium</td>
<td>4</td>
<td>Trichophyton rubrum,</td>
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<tr>
<td>Other Infections</td>
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<td></td>
<td></td>
<td>Alternaria,</td>
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<td></td>
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<td>Penicillium,</td>
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<td></td>
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<td>Malassezia,</td>
</tr>
</tbody>
</table>

Table 1. Prevalence of dermatophytosis (Tinea infection) in clinical samples collected from Headquarter Hospitals of Kohat, Peshawar, Abbottabad and DI Khan Districts of Pakistan (n = 136)
(MHA) were prepared. The inoculum was streaked on MHA media in three directions and then the plates were allowed to dry. Two readymade antifungal discs: fluconazole (25 μg) and nystatin (100 U) were placed on MHA media. Plates were placed in the incubator at 28 °C for 3–7 days. The zone of inhibition was observed after growth [19].

**Molecular identification**

The DNA from the fungal mycelia was extracted using Chelex 100 method. Fungal mycelia were taken in the 1.5 ml of Eppendorf tubes. Then 200 μl of 5% Chelex-100 resin (Bio-Rad) and 4ul of proteinase K was added to each sample. The mixture was incubated for 45 min at 56 °C in the incubator. After incubation, the tubes were suspended in water bath for 8 min at 100 °C and then vortexed for 5–10 s followed by centrifugation at 11,000 rpm for 2 min. The supernatant containing DNA was stored at −20 °C [20].

The genomic DNA, after extraction, was subjected to the polymerase chain reaction (PCR) for the amplification of 18S rRNA gene according to the protocol given by Feghali et al. [21]. Briefly, the PCR was performed using primers ITS1 forward (TCCGTAGGTGAACCTGCGG) and ITS4 reverse (TCCTCCGCTTATTGATATGC). The 25 μl reaction mixture contained 100 uM DNTPs, 0.1 uM each primer, 10³ PCR buffer with 2.0 mM MgCl₂, 5 μl of template DNA sample, and 1U of Taq polymerase. The PCR conditions were set on initial denaturation at 96 °C for 10 min, followed by 30 cycles in series of denaturation at 95 °C for 1 min, annealing at 57 °C for 1 min, and extension at 72 °C for 1 min, with a final step of one cycle at 72 °C for 10 min to final extension.

**RESULTS**

**Prevalence of dermatophytes in the clinical samples**

Total of 154 samples were collected from hospitals out of which 136 samples were positive. The prevalence data showed that Tinea corporis (35%) was the most prominent clinical conditions followed by Tinea capitis (22%) and then Tinea pedis (9%) as shown in Table 1. In addition, frequency of dermatophytosis was higher in rural areas as compared to the urban areas as shown in Fig. 1. In Peshawar, 81% of the infection originated from the rural areas. The rural infected samples were 77, 75 and 60% of the total in Kohat, DI Khan and Abbottabad respectively. Age wise distribution of infection showed highest incidence in lower age groups as compared to the higher age groups as shown in Table 2 while, gender wise distribution of the infection showed 52% males and 48% females infected with the disease as shown in Table 3.

**Antifungal drug susceptibility pattern of dermatophytes**

All the fungal isolates were tested against two antifungal agents: fluconazole and nystatin. 75.73% of isolates were sensitive to nystatin, 24.26% were in intermediate group and none of the isolate was resistant to nystatin. In case of fluconazole, none of the isolate was sensitive, 127 isolates (93.38%) were in intermediate group while 7% of the isolates were resistant. The zones of inhibition were varied for nystatin i.e. 12–38 mm while that of fluconazole was 0–20 mm as shown in Table 4.

**Identification of resistant fungal isolates using 18S rRNA gene sequencing**

The resistant isolates as shown in Table 5 were identified by amplification of 18S rRNA gene sequencing using universal primers. The PCR products showed different variations in the banding pattern. The band sizes of the isolates ranged from 600 bps to 740 bps. The sequencing of the PCR product showed that five isolates have 97–98% similarity with different species of genus *Trichophyton* while three isolates

![Fig. 1. Prevalence of dermatophytes in urban and rural areas of the four districts i.e. Peshawar, Kohat, DI Khan and Abbottabad from where the clinical samples were collected](image-url)
were identified as from genus *Microsporum* as shown in Fig. 2. The evolutionary history was inferred using the neighbor-joining method. The optimal tree with the sum of branch length = 2.88018154 is shown. The evolutionary distances were computed using the maximum composite likelihood method and are in the units of the number of base substitutions per site. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 3,416 positions in the final dataset. Evolutionary analysis was conducted in MEGA X.

**DISCUSSION**

The distribution of superficial fungal infections has changed significantly because of changes in life style of peoples’ social and economic conditions, and migration in last century. There are few studies about the prevalence of superficial fungal infections in the developing world, and consequently, there is less knowledge of any changes to their epidemiology [22–23]. Superficial mycoses cause skin infections in various parts of the world, so in order to get reliable knowledge studies of one region of the country may not be a true representation of the overall disease pattern of that country; furthermore, incidence and prevalence figures may only be representative of the population sampled, which may have associated risk factors for infection [24]. The higher incidence of dermatophytosis could be attributed to environmental conditions.

In the present study, the clinical assessments of various types of dermatophytic infection and their causative agents were done in 154 patients aged between 1 and 60 years in the four districts of the KPK. Among all patients, Tinea corporis was much prevalent which coincide with the results of Olga Mukasia Mashedi and Srinivasan Balakumar [25–26]. The age group from 1 to 20 years were more affected (54) cases which were also found by Ali Naseri [27] and Gebreabiezi Teklebirhan [5]. The frequency of dermatophytosis in rural areas were more as compared to urban and the same results were found by Pontes et al. [28] and Akcaglar et al. [29].

The present study was focused on the antifungal drug susceptibility. Antifungal susceptibility was determined against two most commonly practicing antifungal drugs (fluconazole and nystatin) in order to see the resistant pattern of the isolates. The present study showed that resistance was seen against fluconazole. Among all isolates, only nine isolates showed resistance against fluconazole. This resistance against fluconazole was also found by other authors. These authors also found resistant against fluconazole [30–31]. However, there is no significant data available on susceptibility testing of nystatin. Nystatin was found to be sensitive against all isolates which make it a better drug against dermatophytes, while other antifungal agents were found resistant against antibiotics. Some of other antifungal agents like terbinafine, miconazole, Itraconazole were found to be resistant against dermatophytes [31]. The resistant isolates were also identified through PCR. Variant pattern was seen in the banding pattern of 18S rRNA gene amplification. The same variance in the banding pattern of dermatophytes was found by Habe et al. [32] The resistant isolates were mostly *Trichophyton* spp. which was also found by Agarwal et al. [30].

| Table 3. Gender-wise frequency and percentage distribution of Tinea infections |
|---|---|---|
| Clinical Manifestation | Gender | Total n (%) |
| | Male (%) | Female (%) | |
| Tinea corporis | 29 (54) | 25 (46) | 54 (40) |
| Tinea capitis | 24 (71) | 10 (29) | 34 (25) |
| Tinea pedis | 8 (57) | 6 (43) | 14 (10) |
| Tinea cruris | 8 (80) | 2 (20) | 10 (7) |
| Tinea faciei | 2 (20) | 8 (80) | 10 (7) |
| Tinea manum | 0 | 8 (100) | 8 (6) |
| Tinea unguium | 0 | 6 (100) | 6 (4) |
| Total | 71 (52) | 65 (48) | 136 (100) |

| Table 4. Antifungal drug susceptibility pattern of dermatophytes |
|---|---|---|---|
| Antifungal Agents | IZD Range of Isolates (mm) | Susceptibility pattern of dermatophytes (%) | Total n (%) |
| | | Sensitive (≥22 mm) | Intermediate (21-15 mm) | Resistant (≤14 mm) | |
| Nystatin | 12–38 mm | 103 (75.73) | 33 (24.26) | 0 (0) | 136 (100%) |
| Fluconazole | 0–20 mm | 0 (0) | 127 (93.38) | 9 (7) | 136 (100%) |
CONCLUSIONS

From the above the study it was concluded that dermatophytes prevail in the rural areas while males were more infected as compared to female. The children and teenagers were at high risk as compared to adults and older age individuals. Further, it was also concluded that dermatophytes were evolving and they were becoming resistant to antifungal drugs and among them, *Trichophyton* spp. were the most common resistant fungal species.

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