AKADÉMIAI KIADÓ

Acta Microbiologica et Immunologica Hungarica

68 (2021) 4, 291–296

DOI:
10.1556/030.2021.01461
© 2021 Akadémiai Kiadó, Budapest

RESEARCH ARTICLE

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

BAKHTAWAR USMAN[1], ABDUL REHMAN[1], IFFAT NAZ[2] and MUHAMMAD ANEES[1*]

[1] Department of Microbiology, Kohat University of Science & Technology (KUST), Kohat, Khyber Pakhtunkhwa, Pakistan

[2] Department of Biology, Scientific Unit, Deanship of Educational Services, Qassim University, Buraidah, 51452, Qassim, Kingdom of Saudi Arabia (KSA)

Received: April 4, 2021 • Accepted: July 19, 2021
Published online: August 11, 2021

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

*Corresponding author.
Tel.: +92 92252914445.
E-mail: dr.anees@kust.edu.pk

AKJournals

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 dermatophtic 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 (fluconazole 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 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)

Infection	Prevalence (%)	Fungal Species
Tinea corporis	35	• *Trichophyton rubrum,* • *Trichophyton mentagrophytes,* • *Epidermophyton floccosum*
Tinea capitis	22	• *Trichophyton violaceum,* • *Trichophyton mentagrophytes,* • *Microsporum ferrugineum*
Tinea pedis	9	• *Microsporum canis,* • *Trichophyton tonsurans,* • *Trichophyton schoenleinii.* • *Trichophyton concentricum*
Tinea cruris	7	• *Trichophyton rubrum,* • *Epidermophyton floccosum,* • *Candida albicans*
Tinea faciei	6	• *Trichophyton mentagrophytes,* • *Microsporum gypseum,* • *Microsporum audionii*
Tinea manum	5	• *Trichophyton mentagrophytes,* • *Microsporum canis*
Tinea unguium	4	• *Trichophyton rubrum,* • *Trichophyton mentagrophytes*
Other Infections	12	• *Candida albicans,* • *Alternaria,* • *Penicillium,* • *Malassezia*

(MHA) were prepared. The inoculum was streaked on MHA media in three directions and then the plates was allowed to dry. Two readymade antifungal discs: fluconazole (25 ug) 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, 10× PCR buffer with 2.0 mM $MgCl_2$, 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.

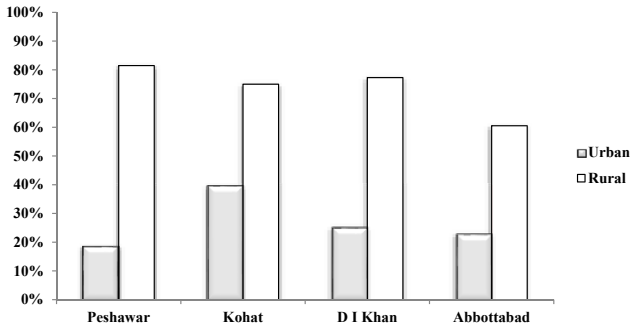

Fig. 1. Prevalence of dermatophytes in urban and rural areas of the four districts i.e. Peshawar, Kohat, DI Khan and Abbottabad from where the clinical samples were collected

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 predominant 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

Table 2. Age-wise frequency of Tinea infections

Infection	Age groups						Total
	1–10	11–20	21–30	31–40	41–50	≥50	
Tinea corporis	21	13	8	6	6	0	54
Tinea capitis	11	7	9	5	0	0	34
Tinea pedis	5	4	3	0	1	1	14
Tinea cruris	6	3	0	0	1	0	10
Tinea faciei	2	3	2	2	1	0	10
Tinea manum	1	0	1	3	2	1	8
Onychomycosis	1	2	1	1	1	0	6
Others	5	6	2	3	1	1	18

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 Gebreabiezgi 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, Iitraconazole 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 Habeb 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 5. Resistant species of dermatophytes and range of their zones of inhibition

S. No	Isolates	Species identified	Resistance range ≤14 mm
1	B 1	*Microsporum audouinii*	8
2	B 2	*Trichophyton terrestre*	10
3	B 3	*Epidermophyton floccosum*	6
4	B 4	*Trichophyton schoenleinii*	9
5	B 5	*Microsporum gypseum*	6
6	B 6	*Trichophyton concentricum*	7
7	B 7	*Trichophyton rubrum*	4
8	B 8	*Trichophyton terrestre*	11
9	B 9	*Microsporum ferrugineum*	8

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%)*

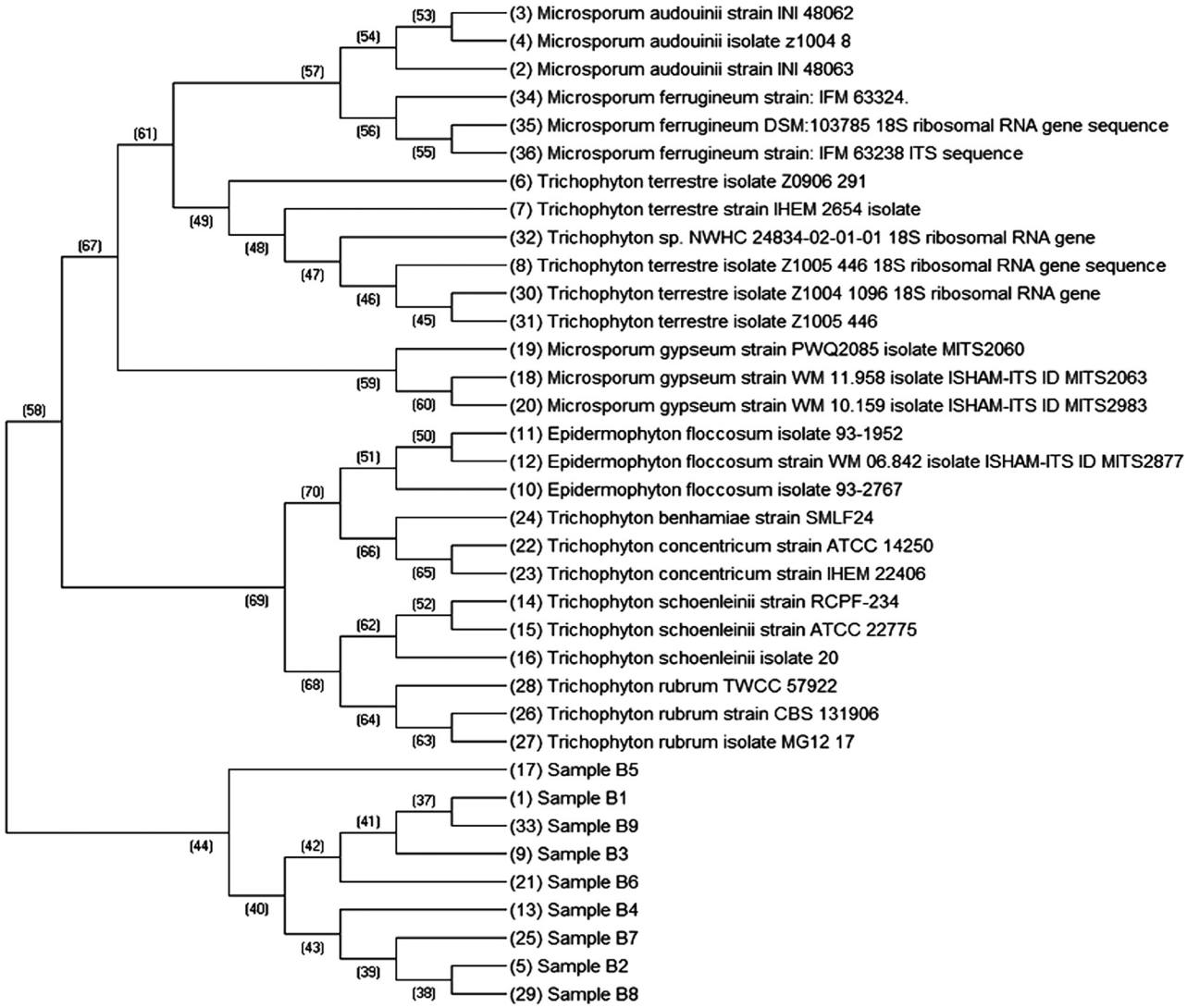

Fig. 2. Evolutionary analysis for the identification of resistant fungal isolates using 18S rRNA gene sequencing

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.

Declaration of interest statement: No potential conflict of interest was reported by the authors.

Funding: This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

ACKNOWLEDGMENTS

The authors acknowledged Department of Microbiology, Kohat University of Science & Technology (KUST), Kohat for providing all possible facilities to accomplish this research work.

REFERENCES

1. White TC., Findley K, Dawson TL, Scheynius A, Boekhout T, Cuomo CA, Saunders CW. Fungi on the skin: dermatophytes and Malassezia. Cold Spring Harbor Perspect Med 2014; 4(8): a019802.
2. Oliveira LDQD. Influence of the essential oils of *Cinnamomum cassia* and *Cymbopogon flexuosus* on susceptibility and virulence factors in yeasts of the *Cryptococcus neoformans* complex; 2017.

3. Gnat S, Łagowski D, Nowakiewicz A. Major challenges and perspectives in the diagnostics and treatment of dermatophyte infections. J Appl Microbiol 2020; 129(2): 212–32.
4. Martinez-Rossi NM, Bitencourt TA, Peres NT, Lang EA, Gomes EV, Quaresemin NR, Rossi A. Dermatophyte resistance to antifungal drugs: mechanisms and prospectus. Front Microbiol 2018; 9: 1108.
5. Teklebirhan G, Bitew A. Prevalence of dermatophytic infection and the spectrum of dermatophytes in patients attending a tertiary hospital in Addis Ababa, Ethiopia. Int J Microbiol 2015.
6. Kim SH, Cho SH, Youn SK, Park JS, Choi JT, Bak YS, Kim YK. Epidemiological characterization of skin fungal infections between the years 2006 and 2010 in Korea. Osong Public Health Res Perspect 2015; 6(6): 341–5.
7. Achterman RR, White TC. Dermatophyte virulence factors: identifying and analyzing genes that may contribute to chronic or acute skin infections. Int J Microbiol 2012.
8. Bassiri-Jahromi S. Epidemiological trends in zoophilic and geophilic fungi in Iran. Clin Exp Dermatol Clin Dermatol 2013; 38(1): 13–9.
9. Lockhart SR, Guarner J. Emerging and reemerging fungal infections. Semin Diagn Pathol 2019; 36(3): 177–81.
10. Attal RO, Deotale V, Yadav A. Tinea capitis among primary school children: a clinic mycological study in a rural hospital in central India. Int J Curr Res Rev 2017; 9(23): 25.
11. Husain MA, Alam MN, Joarder Y, Ferdous M. Correlation between clinical and mycological diagnosis of onychomycosis. J Pak Assoc Dermatol 2018; 27(3): 220–5.
12. Ahsan U, Zaman T, Rashid T, Jahangir M. Cutaneous manifestations in 1000 Pakistani newborns. J Pak Assoc Dermatol 2010; 20(4): 199–205.
13. Haroon TS, Qureshi AS, Alvi KH, Khan HZ, Lakhani S, Sherali A. A study of skin disease in Chitral. J Pak Med Assoc 1987; 37: 247–50.
14. Thebo NK, Abro H, Soomro AQ, Anwer J, Suhail M. Isolation and identification of dermatophytes from Sindh, Pakistan. Pak J Bot 2006; 38(2): 493.
15. Mirza SH, Gardezi AH, Khan Y, Wiqar MA. Subcutaneous facial mycosis in a child due to *Madurella mycetomatis*. J Pak Med Assoc 2007; 57(9): 466.
16. Abbas SQ, Maan A, Iqbal J, Niaz M, Waqas M, Ahmed H, Liaqat A. A report of *Paecilomyces variotii* on human from Pakistan. Pak J Bot 2009; 41(1): 467–72.
17. Yasmeen N, Khan MR. Spectrum of common childhood skin diseases: a single center experience. J Pak Med Assoc 2005; 55(1): 60–3.
18. Namboodiri MT, Pakshirajan K. Sustainable and green approach of chitosan production from *Penicillium citrinum* biomass using industrial wastewater as a cheap substrate. J Environ Manage 2019; 240: 431–40.
19. Bitew A. Dermatophytosis: prevalence of dermatophytes and non-dermatophyte fungi from patients attending Arsho advanced medical laboratory. Addis Ababa, Ethiopia: Dermatol Res Pract; 2018.
20. Suárez-Villota EY, Carmignotto AP, Brandão MV, Percequillo AR, Silva MJDJ. Systematics of the genus Oecomys (Sigmodontinae: Oryzomyini): molecular phylogenetic, cytogenetic and morphological approaches reveal cryptic species. Zool J Linn Soc 2018; 184(1): 182–210.
21. Feghali N, Albertin W, Tabet E, Rizk Z, Bianco A, Zara G, Budroni M. Genetic and phenotypic characterization of a *Saccharomyces cerevisiae* population of 'Merwah' white wine. Microorganisms 2019; 7(11): 492.
22. Hasan TSS, Aleem S, Tabassum L. Clinical evaluation of efficacy of Majoon Ushba and Marham Gulabi in Qooba. Indian J Traditional Knowledge 2011; 10: 702–5.
23. Zhan P, Liu W. The changing face of dermatophytic infections worldwide. Mycopathologia 2017; 182(1–2): 77–86.
24. Chen M, Xu Y, Hong N, Yang Y, Lei W, Du L, Liao W. Epidemiology of fungal infections in China. Front Med-Prc 2018; 12(1): 58–75.
25. Mashedi OM. Characterization and antifungal drug profile of Dermatophytes from clinical sources in Nairobi. Doctoral dissertation, Kenya: University of Nairobi; 2010.
26. Balakumar S, Rajan S, Thirunalasundari T, Jeeva S. Epidemiology of dermatophytosis in and around Tiruchirapalli, Tamilnadu, India. Asian Pac J Trop Dis 2012; 2(4): 286–9.
27. Naseri A, Fata A, Najafzadeh MJ, Shokri H. Surveillance of dermatophytosis in northeast of Iran (Mashhad) and review of published studies. Mycopathologia 2013; 176(3–4): 247–53.
28. Pontes ZBVDS, Oliveira ACD, Guerra FQS, Pontes LRDA, Santos JPD. Distribution of dermatophytes from soils of urban and rural areas of cities of Paraiba State, Brazil. Rev Do Inst Med Trop SP 2013; 55(6): 377–83.
29. Akcaglar S, Ener B, Toker SC, Ediz B, Tunali S, Tore O. A comparative study of dermatophyte infections in Bursa, Turkey. Med Mycol 2011; 49(6): 602–7.
30. Agarwal RK, Gupta S, Mittal G, Khan F, Roy S, Agarwal A. Antifungal susceptibility testing of dermatophytes by agar-based disk diffusion method. Int J Curr Microbiol Appl Sci 2015; 4(3): 430–6.
31. Khatri PK, Kachhawa D, Maurya V, Meena S, Bora A, Rathore L, Khullar S. Antifungal resistance pattern among dermatophytes in Western Rajasthan. Int J Curr Microbiol Appl Sci 2017; 6: 499–509.
32. Habeb KA, Maikhan HK, Rachid SK. Molecular identification of dermatophytes among clinical isolates. Asian J Nat Appl Sci 2016; 5(2): 1–11.