



A Molecular Epidemiological Survey of Clinically Important Dermatophytes in Iran Based on Specific RFLP Profiles of Beta-tubulin Gene

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Abstract

Background: Surveillance of dermatophytosis is essential to determine the likely changes in etiological trends and distribution profile of this infection. In this study beta tubulin gene (BT2), was used as the first time in a PCR-RFLP format to clarify the distribution of dermatophytosis agents in some parts of Iran.

Methods: A total of 603 clinical isolates was obtained from 500 patients in Tehran, Isfahan, Mazandaran and Guilan provinces. The isolates were identified using macro/micro-morphological criteria and electrophoretic patterns of PCR amplicons of BT2 after digestion with each of the restriction enzymes *FaI*, *HpyCH4V*, *MwoI* and *Alu21I*.

Results: Among the patients, 59.2% were male and 40.8% female. The most prevalent clinical form was tinea pedis (42.4%), followed by tinea cruris (24.2%), tinea unguium (12.3%), tinea corporis (10.8%), tinea faciei (4%), tinea manuum (3.14%), tinea capitis (3%) and tinea barbae (0.16%), respectively. *Trichophyton interdigitale* ranked the first, followed by *T. rubrum*, *Epidermophyton floccosum*, *Microsporum canis*, *T. tonsurans*, *T. erinacei* and *T. violaceum* (each 0.49%) and the less frequent species were *T. schoenleinii*, *M. gypseum* and *T. anamorph* of *Arthroderma benhamiae* (each 0.16%). A case of scalp infection by *E. floccosum* was an exceptional event in the study. No case of *T. verrucosum* was found.

Conclusion: *Trichophyton* species and *E. floccosum* are yet the predominant agents of infection in Iran, while *Microsporum* species are decreasing. *T. interdigitale* and Tinea pedis remain as the most causal agent and clinical form of dermatophytosis, respectively. It seems that BT2 can be a useful genetic marker for epidemiological survey of common pathogenic dermatophytes.

Keywords: Dermatophytes, Molecular epidemiology, Beta tubulin, PCR-RFLP, Iran

Introduction

The dermatophytes include closely related keratinophilic fungi in the genera *Trichophyton*, *Microsporum* and *Epidermophyton* that degrade the skin, nail and hair of mammalian and cause a cutaneous

infection known as "dermatophytosis" or "tinea" (1-3). Recent epidemiological studies have indicated that dermatophytosis has remained as a public health problem in the majority of communities,

even in developed countries (3-5). Epidemiology of dermatophytosis and dermatophytes is not static and may alter due to changes in factors such as socioeconomic, environmental and climate conditions, occupation, lifestyle, immigration-movements, travel activities and cultural practices (3, 6, 7). Large scale epidemiological data about diversity of pathogenic dermatophytes in different regions of Iran have rarely been documented, however dermatophytosis has increasingly been reported as the dominant superficial mycosis in the country (6, 8-12). Apart from the fact that previous studies used morphological-based criteria which could not reflect the entire spectrum of dermatophytes species, these studies were mainly focused on Tehran, capital of Iran (6,13-16) and other geographical area were subjected to narrow consideration. Recent advances in nucleotides sequencing and other DNA-based methods have promoted precise insight into the identification and epidemiology of dermatophytes (6, 17-20). The current study aimed to describe the spectrum of dermatophytoses and dermatophytes in some parts of Iran, with special emphasis on the species delineation of isolates implicated in infection using a newly described PCR-RFLP on β -tubulin gene (21).

Materials and Methods

Isolates

During June 2011 to June 2012, among outpatients referred to Medical Mycology laboratories in four provinces of Iran i.e. Tehran, Isfahan, Mazandaran and Guilan, the dermatophyte strains from 500 culture-proven cases of dermatophytosis were included in the study. Direct microscopic examination of skin scrapings, nails and hair shafts with 20% potassium hydroxide (KOH), as a routine screening method was used for verifying the presence of fungal elements. A portion of the specimens was inoculated onto Mycobiotic agar (Difco, USA) and the cultures were maintained up to 3 weeks at 28 °C. The cultivated dermatophytes were subjected to gross and microscopic assessment of colonies for preliminary identification and for further molecular analysis.

Molecular identification

The genomic DNA of all strains was extracted according to a previously described method (17). In brief, a piece of fresh mycelium was scraped from the surface of the plate and crushed with a conical grinder in an 1.5 ml Eppendorf tube containing 300 μ l of lysis buffer (200 mM Tris-HCl (pH 7.5), 25 mM EDTA, 0.5% w/v SDS, 250 mM NaCl). The suspension was mixed with phenol chloroform, vortexed shortly and centrifuged at 10,000 g for 10 min, mixed with chloroform and centrifuged again. DNA was precipitated with an equal volume of isopropanol and 0.1 volume of 3.0 M sodium acetate (pH 5.2) and the DNA pellet was washed with 70% ethanol, dried in air, suspended in 50 μ l of double distilled water and kept at -20°C till use.

Beta tubulin (BT2) region was partially amplified in a 25 μ l reaction mixture containing 2.5 μ l of 10x PCR buffer, 10 pmol of each forward T1 (22) and reverse Bt2b (23) primers, 1 μ l of genomic DNA, 1.5 μ M MgCl₂, 400 μ M deoxynucleotide triphosphates, 1.25 U of Taq DNA polymerase and enough sterile deionized water. The following conditions were set up for amplification: initial denaturation phase at 94°C for 6 min, 35 cycles of 94°C for 30 sec, 58°C for 30 sec and 72°C for 1 min, followed by an ultimate extension step at 72°C for 10 min. Species identification was performed in a two-step RFLP assay, preliminary by using the restriction enzyme *FatI*, (New England Biolabs Ltd, NHitchin, UK) and subsequently by *MwoI* and *HpyCH4V* (New England Biolabs Ltd, NHitchin, UK), and *Alu21I* (Fermentas, Vilnius, Lithuania) according to the manufacturer's instructions. All digestion reactions were performed in a 15 μ l mixture containing 1.5 μ l of 10 \times buffer, 0.5 μ l of each enzyme (5 U/ μ l), 8 μ l of BT2 amplicon and enough ultrapure water up to the final volume. DNA cleavage was performed at 37 °C for 2 h. To confirm the production of amplified and digested DNAs of the expected size, 10 μ l of each amplicons and digested fractions were respectively electrophoresed on 1.5 and 2 % gel agarose, stained with ethidium bromide (0.5 μ g/ml), visualized under UV illumination and the banding patterns were recorded by photography. Identification of all isolates was carried out through comparing the obtained banding profiles summarized in Fig. 1, with those deduced from the previous study (21).

Results

Totally, 603 dermatophytes strains were isolated from patients of which 394, 41 and 38 isolates were originated from three Northern provinces i.e. Tehran, Guilan and Mazandaran, respectively and 130 from Isfahan province, in the center of Iran. The patients from whom the strains were isolated included 245 (40.80%) females and 358 (59.20%) males (Table 1) and age range of 2-87 years (Table 2). Frequency of all infection forms was more in males than females except tinea corporis. In 93 patients who had lesions in more than one anatomical site (combined dermatophytosis), more than one strain was isolated. Amplification of the BT2 region in all strains yielded single band of approximately 770 to 800 base pair (bp), depending on the species. In the two-step BT2-RFLP by *FatI*, *HpyCH4V*, *MwoI* and *Alw21I*, 599 strains created banding patterns matched with expected species-specific profile, leading to the delineation of *T. interdigitale* as

the highest in terms of frequency, followed by *T. rubrum*, *Epidermophyton floccosum*, *M. canis*, *T. tonsurans*, *T. violaceum*, *T. schoenleinii* and *M. gypseum*. Four remaining strains which created restriction fragments for the *Arthroderma benhamiae* clade, were subjected to sequencing and identified as *T. erinacei* (n=3) and *T. anamorph* of *A. benhamiae* (n=1). Except in tinea cruris and tinea barbae, *T. interdigitale* was the dominant species isolated in all clinical conditions and all provinces. The frequency of dermatophytes isolated from different anatomic sites is summarized in Table 1. Clinical presentation of the infection revealed that feet involvement (tinea pedis) was the predominant clinical condition (42.4%) followed by tinea cruris (24.2%), tinea unguium (12.3%), tinea corporis (10.8%), tinea faciei (4%), tinea manuum (3.14%), tinea capitis (3%) and tinea barbae (0.16%), respectively. Regarding to the age distribution, the infection was manifested more in three age groups of 21-30, 31-40 and 41-50, respectively.

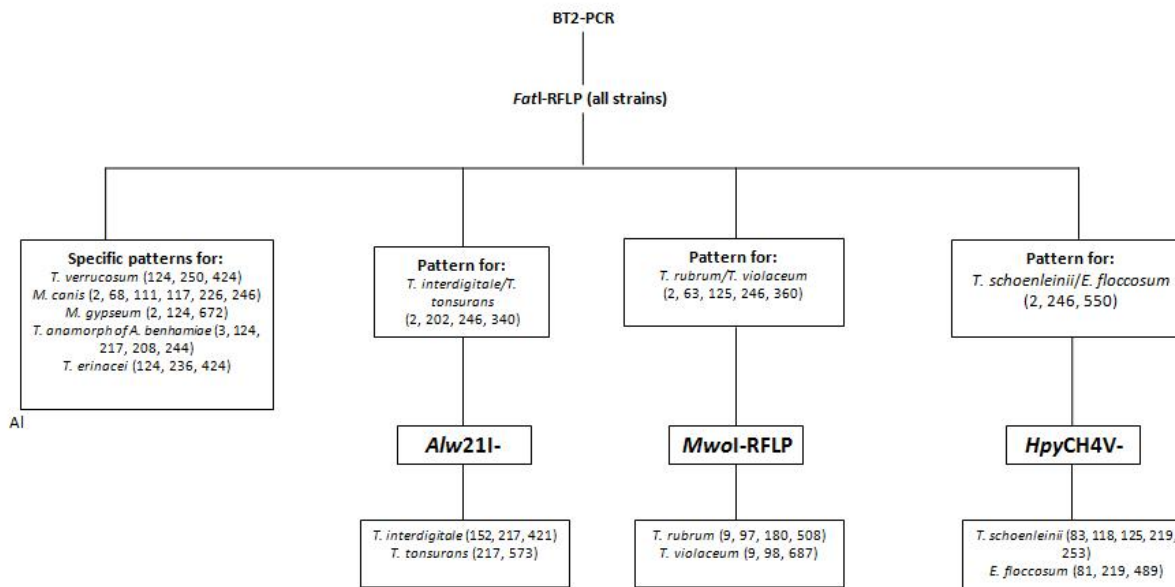


Fig.1: Flowchart of molecular identification steps for identification of dermatophytes in the study based on Beta-tubulin RFLP. (The numbers in parentheses are size of the fragments after digestion with each relevant restriction endonuclease)

Table.1: Frequency of dermatophytoses regarding to the dermatophyte species and patients gender

Species Clinical form	<i>T. interdigitale</i>	<i>T. rubrum</i>	<i>E. floccosum</i>	<i>M. canis</i>	<i>T. tonsurans</i>	<i>T. erinacei</i>	<i>T. violaceum</i>	<i>T. schoenleinii</i>	<i>M. gypsum</i>	<i>T. anamorph of A. ben-hamiae</i>	Total distribution of isolates in relation to sex (M/F)*
Tinea pedis	169	69	11	5	1	1	0	0	0	0	256 (165/91)
Tinea cruris	39	39	63	4	0	1	0	0	0	0	146 (81/65)
Tinea unguium	40	28	4	1	0	0	1	0	0	0	74 (50/24)
Tinea corporis	24	16	14	9	1	0	1	0	0	0	65 (28/37)
Tinea faciei	9	5	0	3	6	0	1	0	0	0	24 (12/12)
Tinea barbae	0	1	0	0	0	0	0	0	0	0	1(1/0)
Tinea manuum	7	6	1	3	0	1	0	0	0	1	19 (11/8)
Tinea capitis	6	1	1	4	4	0	0	1	1	0	18 (10/8)
Total (%) and geographical distribution of isolates in the study	294 (48.75)	165 (27.36)	94 (15.58)	29 (4.8)	12 (1.99)	3 (0.49)	3 (0.49)	1 (0.16)	1 (0.16)	1 (0.16)	603 (358/245)
	Maz: 21	Maz: 6	Maz: 7	Maz: 1	Maz: 1	Maz: 1	Maz: 1	Maz: 0	Maz: 0	Maz: 0	
	Gui: 15	Gui: 14	Gui: 9	Gui: 1	Gui: 2	Gui: 0	Gui: 0	Gui: 0	Gui: 0	Gui: 0	
	Isf: 66	Isf: 25	Isf: 24	Isf: 6	Isf: 2	Isf: 2	Isf: 2	Isf: 1	Isf: 1	Isf: 1	
	Teh: 192	Teh: 120	Teh: 54	Teh: 21	Teh: 7	Teh: 0	Teh: 0	Teh: 0	Teh: 0	Teh: 0	

* M/F: Male/ Female Maz : Mazandaran, Gui: Guilan, Isf: Isfahan, Teh: Tehran

Table.2: Distribution of dermatophytosis among patients of the study in regard to the age groups

Age group Clinical form	1-10 (%)	11-20 (%)	21-30 (%)	31-40 (%)	41-50 (%)	51-60 (%)	61-70 (%)	71-80 (%)	80< (%)	Total (%)
Tinea pedis	2 (0.33\)	10 (1.65)	31 (5.14)	54 (8.95)	61 (10.11)	57 (9.45)	37 (6.13)	2 (0.33)	2 (0.33)	256 (42.45)
Tinea cruris	1 (0.16\)	13 (2.15)	55 (9.12)	33 (5.47)	23 (3.81)	8 (1.32)	10 (1.65)	1 (0.16)	2 (0.33)	146 (24.21)
Tinea unguium	-	2 (0.33)	15 (2.48)	10 (1.65)	6 (0.99)	22 (3.64)	16 (2.65)	3 (0.49)	-	74 (12.27)
Tinea manuum	-	2 (0.33)	9 (1.49)	3 (0.49)	-	4 (0.66)	-	1 (0.16)	-	19 (3.15)
Tinea corporis	-	11 (1.82)	29 (4.80)	2 (0.33)	13 (2.15)	5 (0.82)	4 (0.66)	-	1 (0.16)	65 (10.77)
Tinea capitis	12 (1.99)	6 (0.99)	-	-	-	-	-	-	-	18 (2.98)
Tinea faciei	4 (0.66)	8 (1.32)	6 (0.99)	1 (0.16)	-	1 (0.16)	4 (0.66)	1 (0.16)	-	24 (4)
Tinea barbae	-	-	-	-	-	-	1	-	-	1 (0.16)
Total (%)	19 (3.15)	52 (8.62)	145 (24.04)	103 (17.08)	103 (17.08)	97 (16.08)	71 (11.77)	8 (1.32)	5 (0.82%)	603 (100)

Discussion

Dermatophytosis annually impacts millions of individuals and imposes a substantial cost to the societies for treatment (2, 24). The distribution pattern and causative agents of infection continuously change, therefore it is essential to update our knowledge in regard to the epidemiology of dermatophytes. To clarify the accurate epidemiology and to resolve the problems attributed to the morphology-based identification of dermatophytes, molecular biology methods focusing on genetic markers have met with significant successes over the recent years (18-21). In the current study, dermatophytes causing different clinical forms of infection were identified at the species level based on a set of BT2-RFLP profiles, which already was developed in our laboratory (21). Thus, this is the first application of PCR-RFLP on β -tubulin in a molecular epidemiological monitoring of dermatophytes worldwide. The distribution of various clinical manifestations and frequencies of isolates in four geographical areas of Iran was also determined. The frequency arrangement of causative species in the present study was similar to the recent molecular epidemiology report from Tehran (6), in which *T. interdigitale*, *T. rubrum*, *E. floccosum*, *M. canis* and *T. tonsurans* were the main agents of infection in decreasing order. However, currently *T. rubrum* is the most common species observed throughout the world (2, 4, 25). The incidence of infection by *T. interdigitale* (48.75%) as the most common species increased in this survey, but not significantly compared with that described by Rezaei-Matehkolaei et al. (6). Tinea pedis, the main clinical manifestation, was accounted for 42.4% of all infections followed by tinea cruris and tinea corporis and this finding was concordant with some reports from Iran (6, 16). Similar to the studies performed by Rezaei-Matehkolaei et al. (6) and Aste et al. (26) among the relative causatives *T. interdigitale*, *T. rubrum* and *E. floccosum* were the most frequent, whereas currently *T. rubrum* is the main species implicated in feet infection worldwide (2, 3, 7). Our study highlighted this fact that dermatophytosis of the feet is more common in adult-males and infrequent in females and children (16, 26, 27), because the in-

fection more occurred in age groups between 31-60 years, was nearly twice as frequent in men than women (64.4% vs 35.5%) and only 0.33% of the cases involved prepubescent patients.

It was noteworthy that we found a case of foot infection by *T. erinacei* among our samples, which was the first proven report of tinea pedis by this species from Iran. Heretofore, this zoophilic less-common dermatophyte was isolated in Tehran from two cases of tinea cruris and tinea manuum using DNA-based methods (6).

Similar to two recent studies conducted in Iran (6, 16) involvement of groin, perianal and perineal ranked second in prevalence and *E. floccosum* was the most common agent which was in accordance with the reports from the past till now in Iran (6, 8, 10, 12, 13, 16). While *T. rubrum* still remains the most frequent cause worldwide (2-4), *E. floccosum* seems to become more prominent as the main agent of tinea cruris in Iran.

Tinea unguium, an old unsolved problem, ranked third in frequency and accounted for 12.27% of all clinical conditions seen during the study, which was nearly equal to those observed in Qazvin and Ahvaz (10, 12). Overall, nail infection due to dermatophytes is known to predominantly occurs in males and older adults (25) and reports which found infection more prevalent in women are few (10). In agreement with this point, in current study it remarkably more affected men and the age group of 51-60 years. At present, *T. rubrum* is the most common dermatophyte causing onychomycosis throughout the world (2-4) and it also was addressed as the predominant agent of nail infection in some reports from Tehran, Qazvin and Babol (6, 10, 16, 28). However, in our study *T. interdigitale* was the main agent implicated and this result was in keeping with other investigations from Iran (8, 12, 29-30). As a whole, these three dermatophytes are the principal species responsible for dermatophytic nail infection in the world, though their frequency pattern varies geographically. The causative agents of tinea corporis, the fourth prevalent infection in the study, are extensively various in different areas and a wide range of species were reported from inside of the country and overseas. In previous reports from Ahvaz,

Hamadan, Qazvin, Isfahan and Tehran, *T. interdigitale* (12), *E. floccosum* (13), *T. tonsurans* (16) and *T. verrucosum* (8-10) were enumerated as the main agents involved in ringworm of the body. However, in agreement with the latest molecular epidemiological survey, performed in Tehran (6) the infection predominantly caused by *T. interdigitale* and *T. rubrum* in this study. It should also be noted that like the study of Rezaei-Matchkolaei et al. (6), among the strains of *M. canis* the most cases of isolation (31%) were correlated to tinea corporis. This zoophilic dermatophyte was cited as the dominant agent of tinea corporis in some European countries (3, 31) and currently is the most common *Microsporum* species causing dermatophytosis in Iran (6, 8, 9, 10, 12, 13, 16). Irrespective of all these, most cases of tinea corporis are currently associated with *T. rubrum* worldwide (3, 4, 25). Dermatophytosis of glabrous skin of the face has less extensively been studied, because it often not regarded as an independent entity, but as part of tinea corporis (2, 16, 32) or tinea gladiatorum (11). The infection is more frequently diagnosed in children due to contact with pets such as cat, dog and rabbit, and in adults as a result of contact with livestock or physical activities typical of this age group (32, 33). Different studies showed that the predominant species responsible for tinea faciei varies regarding to the geographic location and potential animal reservoir (32, 33). Studies performed in Ahvaz, Tehran, Qazvin and Hamadan showed that the infection was more common in males than females and the related frequent species were *T. interdigitale* (12), *M. gypseum* and *T. rubrum* (13, 14) and *T. verrucosum* (9, 10). In the studies carried out in Greece and Italy *M. canis* was mentioned as the prevailing agent of tinea faciei (25, 31, 32). In our study the infection was manifested as the same ratio in males and females and *T. interdigitale*, *T. tonsurans*, *T. rubrum* and *M. canis* were respectively the main corresponding species, which partially differed from the recent report from Tehran (6) that found *T. tonsurans* and *M. canis* as the dominant agents. Ringworm of the hand accounted for 3.14% of all infections and like the study performed in Tehran *T. interdigitale* and *T. rubrum* comprised the most frequent causes, respectively [6]. Similarly, these two species

were reported as the prevalent causes of tinea manuum in the majority of reports from Iran and Europe (10,12, 13, 25, 31, 34).

One of the remarkable aspects of the current survey was the first confirmed report of tinea manuum in an Iranian 60-year-old female due to *T. anamorph* of *A. benhamiae*. This *Trichophyton* sp. and *T. erinacei* are two zoophilic dermatophytes that formerly known as *T. mentagrophytes* var. *erinacei* and *T. mentagrophytes* var. *grannulosum*, respectively, however currently both fungi have species status and are considered as the anamorphic stages of the teleomorph *A. benhamiae* (35). Ecologically, both species adapted with house pets like hedgehogs, rabbits, guinea pigs, dogs and cats as the main hosts and the possible sources of human infections (35, 36). Since, pets are not well-liked in Iran (6, 16), incidence of infection by *A. benhamiae* is low and the sources of infection for such species are likely domestic or farm animals. Generally, the data pertaining to the epidemiology of taxa in *A. benhamiae* complex are limited, nevertheless in some recent reports the species was considered as an emerging pathogen of dermatophytosis in human and animals (36, 37).

A long time ago, infection of the scalp skin and hair shafts, which predominantly is common among preschool going and preadolescent populations (4) was the main form of dermatophytosis in Iran (8, 9). In contrary, recent studies showed a gradual decrease in the frequency of infection (6, 10, 12) and it also ranked the least in our study (2.8% of all infections), after tinea barbae. On the other hand, congruent with the observations made in Iran it was limited to the lower age groups of 1-10 and 11-20 year (6, 8, 9, 12, 15). Generally, the high prevalence rate of tinea capitis in some areas of Africa, especially in warm countries of the tropics was attributed to the inter-human transmission of infection among the school children and low level of lifestyle, however in Asiatic countries the prevalence of infection decreased due to improvement in socioeconomic and sanitary condition (4, 38, 39). Epidemiologically, different geographic regions maintain distinct species of dermatophytes as agents of tinea capitis. While, *T. tonsurans* represents most cases of infection in UK and North America, *M. canis* is the predominant species in

most parts of continental Europe and Australia, and *T. violaceum* in South Asia and North Africa (4, 40). In studies performed in Tehran and Ahvaz, *T. violaceum* and *M. canis* were cited as the most common etiologic agents of tinea capitis (6, 12, 13, 15), but comparatively, in our study *T. interdigitale* surpassed over other species. A likely explanation for this transition may be that the distribution profile of dermatophytes involved in distinct type of dermatophytosis varies geographically, since the current survey was extended to four different provinces. Another salient finding, like the recent study from Tehran [6], was that no case of tinea capitis by *T. violaceum* was encountered. From the available data, it seems that dermatophytoses due to *T. violaceum* in general, and scalp infection in particular are eradicating in Iran. The most interesting finding in our survey might be an episode of tinea capitis (ectothrix type) by *E. floccosum* in a 6-year-old girl. *Epidermophyton floccosum* is an anthropophilic species with worldwide distribution (1,2, 5), however reports in relation to tinea capitis by this species are very rare. To the extent of our knowledge, there are sporadic reports from Palestine (41), Nigeria (42), Italy (43, 44) and Canada (45) regarding the isolation of *E. floccosum* from scalp lesions and our case is the sixth worldwide. Albeit rarely, this finding makes emphatic that this species has the potential to parasitize and infect the hair. In recent years, incidence of scalp lesions by *Trichophyton schoenleinii* (tinea favosa) has decreased worldwide (39) and there are evidences for eradication of this dermatophyte in developed nations (5, 7). In line with this fact, the isolation rate of this fungus in the present study and some recent surveys from Iran substantially decreased (6, 13, 14). Like the study of Aghamirian and Ghiasian (10) and Maraki et al.(25) a significant number of our patients (n=93) had mixed infections, consisting of concomitant tinea unguium/tinea pedis, tinea cruris/tinea pedis and tinea cruris/tinea corporis. Obviously, in these patients the same dermatophyte species was isolated from both skin and nail samples and the responsible agents, in order of frequency were *T. interdigitale*, *T. rubrum* and *E. floccosum*. In the few studies in Greece and Spain, which paid attention to this subject it was also found that *T. rubrum* and *T. in-*

terdigitale tend to establish co-infection in multiple organs (25, 27). Generally, derma-tophytosis simultaneously in more than one anatomical site is expected and frequently occurs due to scratching of the lesions by infected patient and mechanical spreading (7, 25).

Recently it was shown that infection of the beard and mustache took a decreasing trend in Iran since at the moment men pay more attention to self-care (6, 10). Similar result was found in our study and infection decreased to the minimum rate. Interestingly, the only observed case of tinea barbae was due to the anthropophilic species *T. rubrum*, while in many reports from Iran the dermatophytes other than *T. rubrum*, especially zoophilic species were listed as the infectious agents (8-10, 12). From the etiological point of view, while *T. verrucosum* constituted a notable portion of isolates in earlier studies in Iran, exceptionally no case of infection by this species were recognized in this investigation and the recent study conducted in Tehran (6). In other words, all referred strains from four provinces in which morphologically identified as *T. verrucosum*, in BT2-RFLP analysis were known as *T. interdigitale*. This discrepancy between phenotypic versus molecular identification was due to a 'species-to-species transition' in the dermatophytes taxonomy meaning that most of the strains which morphologically identified as *T. verrucosum* in many parts of the world (including Iran), are unambiguously the former *T. verrucosum* var. *autotrophicum* (an invalid name), which recently regarded to be conspecific with *T. interdigitale* based on new molecular analyses (35, 46).

Conclusion

T. interdigitale was the predominant agent of most infections in this study and given to the increase in occurrence of tinea pedis and tinea unguium, two old unsolved problems, it is likely to remain as the main pathogenic species in Iran. Although infection with *T. verrucosum* and some other species was not detected in recent molecular epidemiology surveys, even so this does not mean that the country is free of these species. Additional investigations, with emphasis to transition from phenotypic methods

towards molecular approaches relying on new perspective to concept of species, are required to elucidate the full spectra of dermatophytes specie in Iran, since the regional and sectional studies may not necessarily be the representative of overall pattern in the country.

Ethical considerations

Ethical issues (Including plagiarism, Informed Consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc) have been completely observed by the authors.

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