Molecular Identification and Epidemiological Aspects of Dermatophytosis in Tehran, Iran

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Abstract

Background: Dermatophytes are the most common fungal agents causing superficial skin infections in worldwide. Species identification of these fungi is important for therapeutic and epidemiological apects. The purpose of this study was to determine the epidemiology of dermatophytosis in patients referring to medical mycology laboratory of Razi hospital in Tehran, during 2014.

Materials and Methods: In this study, 610 clinical specimens were collected from patients with suspected dermatophytosis. Direct microscopy and culture examinations were performed for all samples. DNA was extracted from fungal colony using phenol chloroform. Then ITS1-5.8s-ITS2 region of ribosomal DNA (rDNA) was amplified by the universal fungal primers ITS1 and ITS4 and digested with enzymes *mva1*.

Results: In the present study, 236 subjects (38.6%) were positive for dermatophytosis. Of these, 64.8% were male and 35.2% female. The most frequent dermatophytes isolated were *Trichophyton interdigitale* (40.3%), *Trichophyton rubrum* (22.9%) and *Trichophyton tonsurans* (18.7%) respectivly. Also 58 samples were improperly diagnosed by morphological method, they were re-identified as *Trichophyton interdigitale* and *Trichophyton rubrum* by using PCR-RFLP.

Conclusion: The survey showed that PCR-RFLP is a rapid and reliable method for discrimination of dermatophytes. We suggest using of PCR-RFLP as a valuable method along with morphological examination for diagnostic dermatophytes particularly in clinical and epidemiological settings.

Keywords: Dermatophytosis; Identification; PCR-RFLP

Introduction

Dermatophytosis is a major public health problem, worldwide, caused by members of the genera *Trichophyton, Microsporum* and *Epidermophyton*, which include over 40 species, many of which infect humans. They can affect the keratinized tissues such as skin, nail and hair in human and a wide range of animals (1, 2). The infections caused by dermatophytes are generally superficial. However, in chronic conditions, the fungi may invade deeper tissues, particularly in simultaneous infections with other (4).

Epidemiology of dermatophytosis tend to alter due to factors such as socioeconomic, environment and climate, activity, migration and population density (3, 5). Some different studies have been conducted on pathogenic dermatophytes in different regions of Iran, but most of these studies used morphological based criteria which could not reflect the entire spectrum of dermatophytes species. Furthermore, epidemiology of dermatophytosis in Iran has shown significant changes in various regions

of the country over the last decades (6, 7, 8). The accurate identification of etiologic agents from suspected lesions is important for appropriate treatment and control of potential environmental sources of infection (9). The routine diagnosis of dermatophytosis is based on microscopic examination and culture. The microscopic identification from the lesion samples is rapid, but non-specific and is relatively insensitive. On the other hand, identification of some unusual and atypical isolates by in vitro culture method can be very slow and may take weeks to produce an exact result (10).

In recent years, Molecular methods such as RAPD-PCR, Nested-PCR, PCR-RFLP, PCR-EIA and Real-time PCR have been widely used for identification of dermatophytes (11). Some novel molecular methods have advantages such as rapid identification of dermatophytes at genus and species level either directly in clinical samples or in young non-reproductive fungal colonies (12, 13). The ITS regions of ribosomal DNA gene (rDNA) in the dermatophyte species were used as a reliable marker for species identification. Analysis of the ITS regions by PCR-RFLP has provided a simple and precise method for dermatophyte species characterization (11). Therefore in the present study, we conducted PCR-RFLP method to accurate identification of the dermatophyte strains isolated from Iran.

Materials and Methods

In this study, 610 suspected patients of dermatophytosis referred to Razi hospital in Tehran were examined in during March to September 2014. We recorded all the clinical signs and symptoms of the patients, and then samples were taken from different sites of patient's body based on clinical symptoms. Samples were examined with direct microscopic examination using 20% potassium hydroxide solution for the clearing of skin samples, KOH for nail clipping and simple lactophenol for hair. Another portion of specimens was inoculated into Mycobiotic agar plate (Sigma–Aldrich, St. Louis, MO, USA) and incubated at 28 °C for 4 weeks. Macroscopic and microscopic characteristics were studied and non-dermatophyte molds were excluded, then positive cultivated dermatophytes were maintained for further molecular analysis.

DNA extraction

Genomic DNA was extracted from fresh colonies using phenolchloroform method which had previously described. A small portion of fresh and pure of colony was placed in 1.5 ml tube containing 300 μl of lysis buffer (200 mM Tris-HCl (pH 7.5), 25mM EDTA, 0.5% w/v SDS (Sodium Dodecyl Sulfate), 250mM NaCl), and crushed with a grinder

and mixed with phenolchloroform (1:1), vortexed shortly, and centrifuged at 10,000 rpm for 10 min. Then the supernatant was mixed with chloroform and centrifuged. The DNA was precipitated with 0/1 volume of 3.0M sodium acetate and equal volume of isopropanol at -20 °C for 10 min, washed with 70% ethanol, then dried, suspended in 50 μl of double distilled water. The final solution was kept at -20 ºC until using as template for PCR (22).

PCR analysis

The ITS regions of rDNA gene of strains was amplified by the universal fungal primers, ITS1 (5´-TCCGTAGGTGAACCTGCGG-3´) and ITS4 (5´-TCCTCCGCTTATTGATATGC-3´). In a 25 ml reaction mixture, containing 3 ml of PCR buffer (Roche, Mannheim,Germany), 2.5 mM MgCl2 (25mM; Roche), 1ml dNTP (10mM; Roche), 15 pMol of each primer, 10 ng of genomic DNA and 0.5 units of Taq polymerase (GeNet Bio, Korea). The PCR conditions for each isolates were as follows: an initial denaturing (1 cycle 96 $^{\circ}$C for 6 minutes), followed by 35 cycles denaturation (94 $^{\circ}$C for 30 seconds), annealing (58 $^{\circ}$C for 30 seconds), extension (72 $^{\circ}$C for for 1 minutes) and final extension (72 $^{\circ}$C for 7 minutes).The PCR products were visualized by electrophoresis in a 1.5 % agarose gel, stained with sybergreen, visualized under ultraviolet transillumination.
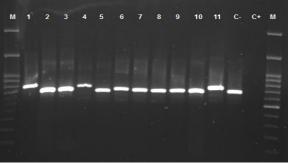

Figure 1. Agarose gel electrophoresis of ITS-PCR products of dermatophyte specimens. M: 100bp molecular size marker, Lane 1-4: samples, C-: negative control, C+: positive control

RFLP analysis

In order to generate species-specific patterns for dermatophytes identification, all PCR products were subjected to digestion with restriction enzyme *Mva*I (Fermentas Life Sciences, Lithuania) for 2h at 37 ºC. The reaction mixture contained 10 μl of PCR amplicons, 0.5 μl of the restriction enzyme, 1.5 μl of 10X buffer and 3 μl of water to a final volume of 15 μl. Finally PCR digested products were electro-phoresed by 2% agarose gel and identification of the isolates was carried out through comparing the electrophoretic RFLP patterns with those profiles described previously (11).

Ethics Statement

The protocol of this study was approved by Ethic Committee of Iran University of Medical Sciences. Every subject signed an informed written consent form.

Results

From 610 patients clinically suspected to dermatophytosis, 236 cases (38/6%) were positive for dermatophyte infections. These subjects included 153 (64.8%) males and 83 (35.2%) females. The patients were aged from 6 months to 71 years old with the mean of 27 years old. The dermatophytosis was observed more frequently in age group more than 50 years old while it was less prevalent among 0-10 year olds patients (Table 1).

Table 1: Frequency of dermatophyte species isolated based on the patient's age

Age groups	*T.rubrum*	*T.tonsurans*	*T.interdigitale*	*E.floccosum*	*M.canis*	*T.violaceum*	*T.schoenleinii*	Total
0-10	2 (8.7)	5 (21.8)	12 (52.2)	2 (8.7)	1 (4.3)	0	1 (4.3)	(100) 23
11-20	0	33 (62.3)	13 (24.5)	6 (11.3)	1 (1.9)	0	0	(100) 53
21-30	8 (21.7)	4 (10.8)	17 (45.9)	7 (18.9)	1 (2.7)	0	0	(100) 37
31-40	10 (27)	0	18 (48.7)	7 (18.9)	1 (2.7)	1 (2.7)	0	(100) 37
41-50	12 (40)	2 (6.7)	9 (30)	5 (16.6)	2 (6.7)	0	0	(100) 30
51 ≥	22 (39.2)	0	26 (46.5)	8 (14.3)	0	0	0	(100) 56
Total	54 (22.9)	44 (18.6)	95 (40.2)	35 (14.9)	6 (2.6)	1 (0.4)	1 (0.4)	(100) 236

In our study, the most common type of dermatophytosis was tinea pedis (26.5%) and followed by tinea cruris (20.2%), tinea manuum (18.6%), tinea capitis (15.1%), tinea corporis (10.7%), tinea faciei (5.1%), tinea unguium (3.6%). Frequencies and relationships of clinical forms and causative species are summarized in Table 2.

Table 2. Frequency of dermatophyte species isolated based on the affected areas of patient's body.

Type	Foot	Groin	Trunk	Hand	Head	Face	Unguis
T.rubrum	21	12	4	13	0	4	6
T.tonsurans	1	0	4	10	27	3	0
T.interdigitale	44	13	8	19	10	2	3
E.floccosum	0	26	10	0	0	3	0
M.canis	1	0	1	4	0	1	0
T.violaceum	0	0	0	1	0	0	0
T.schoenleinii	0	0	0	0	1	0	0
Total	67 (26.6)	51 (20.2)	27 (10.7)	47 (18.7)	38 (15.1)	13 (5.1)	9 (3.6)

Moreover the prevalence of dermatophytes in patients and jobs were evaluated and significant relationship was found between distribution of *T. tonsusanse* and Sports, especially wrestling (p<0/25). Figure 1 shows agarose gel electrophoresis of the samples of PCR products of dermatophytes species isolated from different patients. Molecular examinations showed that *T. interdigitale* (40.2%) was the most frequently isolated species, followed by *T. rubrum* (22.9%), *T. tonsurans* (18.6%), *E. floccosum* (14.8%), *M. canis* (2.5%), *T. violaceum* (0.4%) and *T. schoenleinii* (0.4%). Figure 2 show electrophoretic patterns of

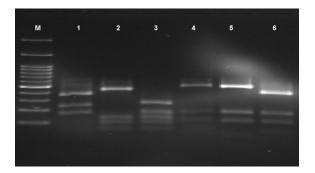PCR-RFLP for samples of dermatophytes isolates.

Figure 2. Restriction digestion of PCR products of dermatophyte strain with the enzyme *Mva1* (11). M: 100bp molecular size marker,Lane 1: *E.floccosum*, Lane 2, 3: *T.interdigitale*, , Lane 4, 5: *M.canis,* Lanes 6: *T.rubrum*

A comparison of morphology and molecular methods for identification of dermatophytes revealed that 4 cases were identified by morphological as *T. mentagrophytes* but these samples by PCR-RFLP were diagnosed as *T. rubrum*. Furthermore, 8 cases of *T. rubrum*, 35 cases of *T. mentagrophytes* and 11 cases of *T. verrucosum* which were identified by morphological method, whereas all the mentioned species were identfied *T. interdigitale* using PCR-RFLP method. (Table3).

Discussion
Dermatophytosis is common fungal infection of humans with wide spectrum of clinical appearance from mild to severe cutaneous mycosis (2). In our study, Frequency of dermatophytosis was more prevalent in men (64.8%) than women (35.1%). The high prevalence in males has been reported in several reports (6, 15, 16).

Table 3. The results of culture and PCR-RFLP for detection of dermatophytosis in the study

Type	CnMn	CpMp	CmMn	CmMp	Positive specimens (%)
T. rubrum	178	50	8	4	54 (22.9%)
T. interdigitale	141	41	0	54	95 (40.3%)
T. tonsurans	192	44	0	0	44 (18.7%)
E. floccosum	201	35	0	0	35 (14.8%)
M. canis	230	6	0	0	6 (2.5%)
T. mentagrophytes	197	0	39	0	0
T. verrucosum	225	0	11	0	0
T. violaceum	235	1	0	0	1 (0.4%)
T. schoenleinii	235	1	0	0	1 (0.4%)

CnMn: Culture and molecular negative for dermatophytes negative; CpMp: Culture and molecular positive for dermatophytes positive; CmMn: Culture misidentification and molecular negative for dermatophytes negative; CmMp: Culture misidentification and molecular positive for dermatophytes positive.

It may be due to occupational contact in males as they more involved in outdoor activities. On the other hand, the highest incidence of of dermatophytosis was seen in age group of > 50 years old. Although Although dermatophytosis occurs in all ages, its frequency is variable and depends on the type of dermatophytes, hygiene status, occupational conditions and different climatic (17, 18). In our study, the common form of dermatophytosis was tinea pedis followed by tinea cruris and tinea manunm. Tinea pedis might result from wearing of socks and shoes for a long period especially in physical works that providing favorable conditions for fungal growth. Mahmoudabadi et al, (6) reported the common form dermatophytosis was tinea cruris followed by tinea pedis and tinea corporis in Ahwaz. Rezaei-Matehkolaei et al, (11) reported tinea pedis was the most prevalent clinical type of dermatophytosis in Tehran. Temperature and humidity are two important factors for tinea cruris and tinea pedis. Both diseases occur worldwide and are more prevalent in tropical countries (19). Identification of dermatophytes at the species level is essential for selection of the best therapeutic procedure and epidemiological goals. Conventional laboratory procedures for detection this group of fungi are based on phenotypic and physiological criteria (11, 20). But, due to the high degree of phenotypic similarity and cultural variability, identification is either slow or lack enough specificity.

The ability of molecular biology methods to identification of fungal pathogens is far superior to that of traditional phenotypic methods (12, 21). In this study, we identified dermatophyte stains by PCR-RFLP method by using universal fungal primers ITS1 and ITS4 and the restriction enzyme *Mva1*. This method is rapid, easy, and reliable. The method can also be used in clinical laboratories to identify clinically important dermatophytes strains. The results obtained in the present study were shown *T. interdigitale, T. rubrum, T. tonsurans E. floccosum* and *M. canis* were the main agents of infection in patients. The incidence of infection by *T. interdigitale* (40.3%) as the most common species increased in this survey. Mahoudabadi et al (6), have reported *T. mentagrophytes* was the most common dermatophyte isolated from disease in west Iran, followed by *T. rubrum* and *T. verrucosum*. Mirzahoseini et al (21), also reported *T. rubrum* (36.8%) the highest frequency in Tehran followed by *E. fluccosum, T. mentagrophytes*. Abbastabar et al in other study in Tehran reported (22) *T. interdigitale* as the highest in terms of frequency that it significantly related with our studies.

Our findings were compatible with the latest suggested changes in the classification of dermatophytes. For instance, *T. mentagrophytes var. mentagrophytes, interdigitale, granulosum* and *T. mentagrophytes var. goetzii* that in previously studies were known as *mentagrophytes* complex and *T. verrucosum var. autotrophicum*, recently based on new classification, these species are in the zoophilic and anthropophilic *T. interdigitale* sensu strict (23). According to the new classification of fungal strains, a comprehensive systematic classification is designed to dermatophytes. Therefore, it is essential to considering new changes in taxonomy and nomenclature dermatophyte groups for the accurate identification of causative agent of dermatophytosis (23).

In conclusion, use of molecular methods such as PCR-RFLP is more reliable than classical methods based on phenotypic features for identification of dermatophytes. Thus our data provide valuable information regarding the epidemiology of dermatophytosis in the capital of Iran which will likely be very favorable for management, control programs and help to develop the proper treatments for this disease.

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