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Original Article

Application of PCR-RFLP to Rapid Identification of the Main Pathogenic Dermatophytes from Clinical Specimens

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Abstract

Background: In the present study, a PCR-RFLP based molecular technique was designed to rapid identification of dermatophytes in clinical specimens. Skin scrapings obtained from human cases suspected to dermatophytosis were studied in order to identify involved etiological fungi.

Methods: In this experimental study, the specimens (skin scrapings) of patients referred to Mycology Department of Pasteur Institute of Iran were inoculated on Petri dishes contained selective agar for pathogenic fungi (SAPF) and incubated at 25º C until visible growth of fungal colonies. The colonies were examined for standard morphological characteristics after visible growth on the agar medium. A small portion of each fungal colony was further studied by restriction fragment length polymorphism (RFLP) analysis of the PCR-amplified internal transcribed spacer (ITS) region of ribosomal DNA (rDNA). PCR amplicons were electrophoresed on 2% agarose gel after digesting by different restriction enzymes including *Mva*I, *Hinf*I and *Hae*III.

Results: Among 160 clinical samples examined, 6 dermatophyte species including *Trichophyton mentagrophytes, T. rubrum, T. verrucosum, T. tonsurans, Microsporum canis* and *Epidermophyton floccosum* were finally identified based on the colony morphology and microscopic criteria. Specific PCR products and RFLP patterns for *Mva*I, *Hinf*I and *Hae*III enzymes allowed the rapid identification and reliable differentiation of isolated dermatophytes at the genus or species level for 5-10 day-old colonies.

Conclusions: The results showed that PCR-RFLP analysis of the ITS region of rDNA is a rapid and reliable tool which allows identification of major pathogenic dermatophytes isolated in this study at species level in young 5-10 day-old colonies.

Keywords: *Dermatophytosis, PCR-RFLP, ITS region, Identification, Dermatophyte species*

Introduction

Dermatophytes are a group of specialized fungi which affect keratinized tissue of skin, nail and hairs in human and a wide range of wild and domesticated animals all over the world (1, 2). These fungi are mainly classified in three major genera including *Microsporum*, *Trichophyton* and *Epidermophyton* based on the morphology of special reproductive structures named macroconidia (1). Dermatophytosis (ring worm; tinea) is referred to all skin disorders induced by the dermatophytic fungi. It is an important zoonotic disorder with worldwide distribution which received major consideration regard to its economic, and public health problems (2). The disease is reported to involve human and animals in different parts of Iran (3-6) with higher prevalence in warm and humid regions. Despite the high frequency of disease caused by different dermatophyte species in Iran, routine diagnosis based on the conventional methods is a time consuming procedure needed for even 30 d for final isolation and identification of etiologic agent at genus or species level. Likewise, in some instances, the causative dermatophyte fails to produce any obvious reproductive structure in culture (termed sterile mycelia) which makes it impossible for ultimate definitive diagnosis.

On the other hand, antifungal drugs are expensive, and they have many side effects in human

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and animals regard to the eukaryotic phenomenon of the members of fungal kingdom. Therefore, it is very important to select the choice therapeutic procedure for treatment of dermatophytosis based on the dermatophyte species involved (7).

In recent years, some investigators have focused on designing novel molecular methods for rapid identification of dermatophytes at genus and species level either directly in clinical samples or in young non-reproductive fungal colonies (8-12). However, there is no widely accepted molecular procedure which capable of differentiates all known dermatophytes from 3 major genera. Since many overlaps exist within and between genera and species in diagnosis of dermatophytic fungi, the demands for testing more samples from different geographic regions in order to establishing a high effective diagnostic procedure have been dramatically increased in recent years.

In the previous work, we had the opportunity to evaluate the quick and direct specification of these fungi by using PCR on patient's skin specimen (13). The results showed that this procedure could not be considered as a routine reliable method for accurate identification of dermatophytic fungi by direct examining of clinical materials.

In this communication, a PCR-RFLP procedure (Polymerase chain reaction-Restriction fragment length polymorphism) identified in the ribosomal-DNA (rDNA) repeat was established for molecular identification and further differentiation of the dermatophytic fungi isolated from human cases of dermatophytosis. Special consideration has been made to evaluate the efficacy of PCR-RFLP method for rapid and accurate differentiation of Iranian dermatophyte isolates in young (5-10 d) colonies compared with the routine time-consuming culture techniques.

Material and Methods

Clinical materials

In an experimental study, a total number of 160 cases of human dermatophytosis referred to Mycology Department of Pasteur Institute of Iran during June 2005 to May 2006 12 months were examined (Table 1). Clinical specimens (skin scrapings) obtained from affected body areas including groin, feet, hand and chest (Table 2) were collected on sterile Petri dishes and maintained at room temperature until they were analyzed.

Culture conditions

A portion of skin scrapings was cultured on Petri dishes contained selective agar for pathogenic fungi (SAPF) medium by spot inoculation technique. The cultures were incubated at 25ºC until visible fungal growth for early PCR-RFLP analysis and then for maximum 4 wk in order to routine identification based on observation of reproductive structures by slide culture method.

Preparation of DNA

Genomic DNA was extracted from young fungal colonies, 5-10 day-old, grown on SAPF Petri dishes by using a grinder in presence of liquid nitrogen for initial breaking up of the mycelia. Final DNA extraction was achieved using a DNP ^{TM}KIT (CinnaGen Inc., Tehran-Iran) (8). The amount of 2 µL of DNA solution was used as a template in the following PCR:

Negative control DNA from prokaryote (*E. coli*) and eukaryotes (human and rabbit) were obtained from isolated colony and from peripheral blood mononuclear cells, respectively, by using the same kit. DNA from *Pichia pastoris* and *Candida albicans* were used as Positive controls. Both positive and negative control DNA samples were checked and quantified on 2% agarose gel and by using spectrophotometer.

PCR and preliminary screening by RFLP

The PCR-amplified internal transcribed spacer (ITS) region of ribosomal DNA (rDNA) was performed with primers ITS-1 (5'-TCCGTAG-GTGAACCTGCGG) and ITS-4 (5'-TCCTCC-GCTTATTGATATGC) under the following PCR conditions (8, 10, 11).

Amplification reactions were performed in final volumes of 50 µL containing 25 ng of template DNA, reaction buffer (10 mM Tris-HCl [pH 8.3], 50 mM KCl), 2.5 mM $MgCl_2$, 200 µM (each) dATP, dCTP, dGTP, and dTTP, 160 ng of primer, and 2.5 U of *Taq* DNA polymerase.

The samples were overlaid with sterile paraffin oil and PCR was performed for 35 cycles in a DNA Thermal Cycler (Bio-Rad) with 1 min of denaturizing at 93° C, 1 min of annealing at 58° C, and 1 min of extension at 72° C and then a final extension for 7 min at 72°C. PCR products (5 µL/sample) were separated by electrophoresis in 2% agarose gels for 2 h. Amplification products were detected by staining with ethidium bromide and were visualized under UV light.
In order to evaluate the specificity of the assay, successful amplifications of control DNA from other eukaryotes and prokaryotes were also carried out in a similar manner.
The amplicons were digested with restriction enzyme *Mva*I, *Hinf*I and *Hae*III (MBI Fermentas) and then electrophoresed in a 2% agarose gel, stained with ethidium bromide, and observed under UV light.

Results

Frequency of isolated dermatophytes

As shown in Table 1, a total number of 160 clinical materials (skin scrapings) obtained from patients, 97 males and 63 females, referred to Mycology Department of Pasteur Institute of Iran during 12 months were analyzed by both direct microscopic examination and cultural assays. Since PCR-RFLP was performed to analyse young fungal colonies, only culture positive specimens were considered in this study.
Table 1 and 2 illustrate the frequency and origin of the dermatophyte species isolated from Iranian patients. These fungi were identified based on observation of colony characteristics on SAPF medium as well as microscopic morphology of reproductive structures of mature colonies (2-4 week-old) by slide culture technique.
As indicated in Table 1, a number of 6 dermatophyte species were identified in clinical materials of examined patients, out of which *T. rubrum* (36.8%) showed the highest frequency followed by *E. fluccosum* (30.0%), *T. mentagrophytes* (24.3%), *T. verrucosum* (4.3%), *T. tonsurans* (3.1%) and *M. canis* (0.6%).
The frequency of dermatophyte isolates are shown in Table 2 based on the affected area of patient's bodies. The results showed that both *T. rubrum* and *T. mentagrophytes* species were mainly isolated from feet, while *E. fluccosum* and *T. verrucosum* mainly obtained from groin and hand areas, respectively.

PCR amplification profiles

DNA samples from young colonies (5-10 day-old) were amplified by PCR and detected on 2% agarose gel. In order to confirmation of identity, RFLP patterns of isolated dermatophytes were compared with those reported for standard dermatophytes by Mochizuki et al. (10). Likewise, for positive and negative controls, the DNA from organisms selected to assay primer specificity including two yeasts (*C. albicans* and *Pichia pastoris*), two mammals (human and rabbit) and one prokaryote, *E. coli* were also amplified (Fig. 1). The ITS region amplified from the species *T. rubrum*, *T. mentagrophytes*, *T. tonsurans* and *T. verrucosum* was about 680 bp in length. The ITS region amplicons from *E. floccosum* and *M. canis* were larger, 780bp and 720bp respectively (Table 3 and Fig. 1 & 2).

Preliminary screening of ITS regions by RFLP analysis

In order to generate species-specific patterns for fungal identification, PCR products of samples were screened by RFLP analysis with the restriction enzymes *Mva*I, *Hinf*I and *Hae*III. Digestion of the amplified ITS products with the restriction endonuclease *Hinf*I, for all tested dermatophytes except *E. floccosum* (250, 180, & 150 bp) produced unique and easily identifiable fragment patterns of 380 & 160 bp. For *M. canis*, *Hinf*I was not able to produce any obvious cutting pattern (Table 3).
The cutting pattern of ITS PCR products for *Hae*III was similar for *T. mentagrophytes*, *T. tonsurans*, and *T. verrucosum* (400 & 100 bp), weakly different with *M. canis* (370 & 100 bp) and significantly different compared to *T. rubrum* (320 & 100 bp) and *E. floccosum* (450 & 140 bp) (Table 3).

As shown in Table 3 & Fig. 3, digestion patterns obtained by the enzyme *Mva*I was quite different for all dermatophyte isolates except *M. canis* (was not checked). These patterns were as 250, 160 & 120 bp for *T. mentagrophytes*, 370 & 160 bp for *T. rubrum*, 360 & 250 bp for *T. tonsurans*, 450 bp for *T. verrucosum*, and 360, 230 & 170 bp for *E. floccosum.*

As shown in Table 3, among 59 isolates of *T. rubrum* and 7 isolates of *T. verrucosum* a different band-size PCR product obtained from only one *Trichophyton rubrum* isolate (800 bp related to patient No 101) and one *T. verrucosum* (550 bp related to patient No 137). RFLP pattern of patient No 137 was reported to be completely different from other patients infected with *T. verrucosum* (*Hinf*I 300 bp; *Hae*III 320 &100 bp; *Mva*I 350 & 200 bp). Furthermore, among 39 examined isolates of *T. mentagrophytes*, only two specific RFLP patterns were reported for patients' numbers 66 & 109 (Fig. 3).

Table 1: Total frequency of dermatophyte species isolated from cultures of clinical samples on SAPF medium based on the patient's sex

Type	n (%)	Male (%)	Female (%)
T. mentagrophytes	39 (24.3)	17 (17.5)	22(34.9)
T. rubrum	59 (36.8)	43 (44.3)	16(25.4)
T. tonsurans	5 (3.1)	3 (3.1)	2(3.2)
T. verrucosum	7 (4.3)	4(4.1)	3 (4.8)
E. floccosum	49 (30.0)	29 (29.9)	20(31.7)
M. canis	1 (0.6)	1(1.0)	_
Total	160 (100)	97 (100)	63 (100)

Table 2: Comparative frequency of dermatophyte species obtained from cultures of clinical samples based on the affected areas of patient's body

Type	Groin	Feet	Hand	Chest	Total
T. mentagrophytes	2	33	2	2	39
T. rubrum	6	49	1	3	59
T. tonsurans	_	1	3	1	5
T. verrucosum	_	2	5	_	7
E. floccosum	39	2	3	5	49

Table 3: The PCR product patterns of RFLP analysis of rDNA from fungal colonies using restriction enzymes *Hinf*I, *Hae*III and *Mva*I. Patient Numbers 101, 137 and also 109, 106 and 137 show different result for PCR and RFLP, respectively

Type	PCR product (bp)	*Hinf*I (bp)	*Hae*III (bp)	*Mva*I (bp)
T. mentagrophytes	680	370 -160	400-100	250-160-120-360-250-160-120 (109)400 (66)
T. rubrum	680-800 (101)	380-150	320-100	370-160
T. tonsurans	680	380-160	400-100	360-250
T. verrucosum	680-550 (137)	380-150-300 (137)	400-100 320-100 (137)	450 350-200 (137)
E. floccosum	780	250-180-150	450-140	360-230-170
M. canis	720	uncut	370-100	--

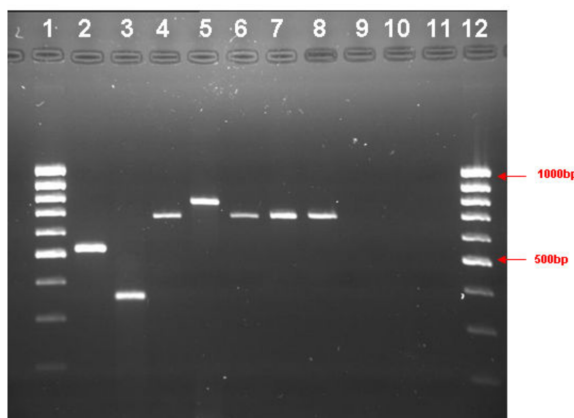

Fig. 1: Agarose gel electrophoresis (2%) of PCR product of different organisms. Lanes 1 & 12: Size marker; Lane 2: *Candida albicans*; Lane 3: *Pichia pastoris*; Lane 4: *T.tonsurans*; Lane 5: *E. Flucosom*; Lane 6: *T. verrucosum*; Lane 7: *T. mentagrophytes*; Lane 8: *T. rubrum*; Lane 9: *E. coli*; Lane 10: Rabbit; Lane 11: Human

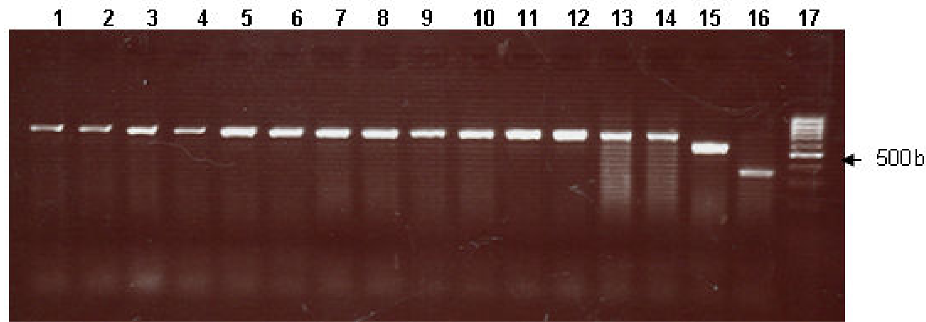

Fig. 2: Different band-size PCR products obtained from a few of *Trichophyton rubrum* specimens on 2% gel agarose electrophoresis. Lanes 1-14: different *T. rubrum* PCR product samples. Lane 15: *Candida albicans*; Lane 16: *Pichia pastoris*, lane 17: size marker.

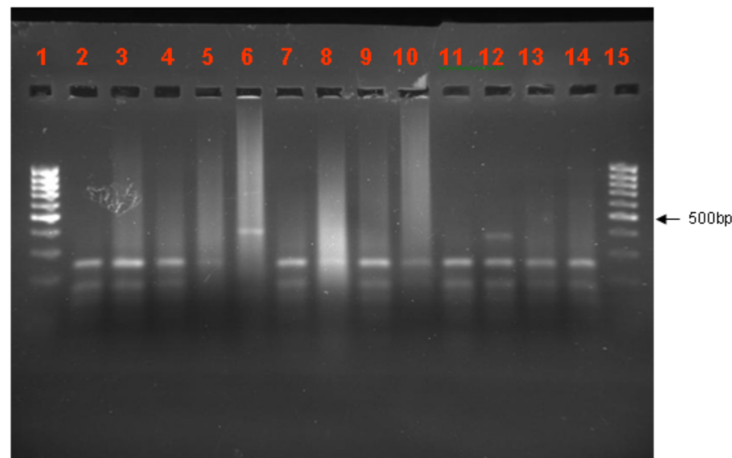

Fig. 3: Agarose gel electrophoresis (2%) of PCR product of *T. mentagrophytes* specimens digested with *Mva*I. Lanes 1 &15: Size marker, Lanes 6 (patient No 66) and 12 (patient No 109) show different pattern.

Discussion

Infection of the keratinized tissues of skin, hair and nails in human and animals by a specialized group of keratinophilic fungi named, dermatophytes, results in dermatophytosis. For selection of the best therapeutic procedure, identification of dermatophytes at the genus or species level is very important. Because conventional laboratory procedures for the identification of dermatophytes is either slow or lack enough specificity, application of nucleic acid amplification technology, has made rapid and precise identification of dermatophytes possible, are required (14). PCR-RFLP provides a rapid and practical tool for identification of dermatophyte isolates that is independent of morphological and biochemical characteristics and thus enhances laboratory diagnosis of dermatophytosis (9-12).

The present study was conducted to evaluate the efficacy of restriction fragment length polymorphism (RFLP) analysis of PCR amplified ribosomal DNA (rDNA) including internal transcribed spacers (ITS) to identify dermatophyte fungi from clinical specimens at genus or species levels in early stage of growth when the reproductive structures are not produced to enable routine techniques to fungal identification. To provide the opportunity for comparing results as well as final conclusion, all clinical samples from suspicious lesions were also cultured and tested by conventional diagnostic methods.

The PCR product pattern was, in some cases, efficient to identify dermatoplyte isolates at the species level e.g. *E. floccosum* and *M. canis*, which produced a characteristically large ampli-

con (780bp and 720bp respectively), or to discriminate among species of the same genus.
As expected, fragment size of PCR products from different species showed different lengths. However, discrepancies with some other authors were obtained in the amplification pattern for a *T. rubrum* and a *T. verrucosum* species (Table 3). Based on the data reported by Mochizuki et al. (9), the PCR product of *T. rubrum* had a length of 692 bp which it is similar to those obtained for all our *T. rubrum* isolates (680 bp) except isolate No. 101 (800 bp). This unique isolate is probably an atypical strain of *T. rubrum* or it can be an intermediate strain between *T. rubrum* and *T. mentagrophytes*.
Regarding *T. mentagrophytes*, all isolates except isolates No.109 and No.66 showed a common pattern with *Mva*I that is similar to *T. mentagrophytes* var. *interdigitale* as reported by Mochizuki et al. (9) (Fig. 3). With respect to this data, it is seems that these two isolates, No.109 and No.66, are possibly *T. mentagrophytes* var. *erinacei* and *T. mentagrophytes* var. *quinckeanum,* respectively.
In the last few years genotypic approaches have proven to be useful for solving identification problems regarding dermatophytes; in fact, genotypic differences are considered more stable and more precise than phenotypic characteristics (9-12, 14, 15). In addition, species identification has a wide role in monitoring the demographic distribution and changes in frequency of specific dermatophyte infections (16).
The ITS regions were used in previous researches to differentiate yeasts to the species level by using PCR methods (17, 18).
This is highlighted by the study of Colin et al., which tested the length of the small-subunit (18S) rDNA and adjacent internal transcribed spacer (ITS) regions amplified with primers ITS1–ITS4. Digestion of the amplified ITS products with the restriction endonuclease *Mva*I produced unique and easily identifiable fragment patterns for a majority of species (9, 16).
This report describes the application of PCR-RFLP for the identification of species and varieties of common dermatophytes utilizing a primer pairs (ITS1–ITS4). The results showed (Table 3, Fig. 3) that the *Mva*I restriction patterns were highly reproducible and consistent for several species, indicating that the ITS regions in dermatophytes are conserved.
On the other hand, in about 9 h, we could obtain electrophoretic profiles starting from cultures, as the DNA extraction technique we used does not require more than 4 h and the amplification requires about 5 h.
In general, while the only disadvantage of the use of PCR-RFLP for identifying dermatophytes is the relatively higher cost in comparison to the classical method, the advantages of its use are many, so provide the opportunity for dermatophyte identification at species level Therefore, this method can be of great utility when it is not possible to use, for the above-specified reasons, the classical method, which is still valid and advisable for identifying species, with well-characterized morphological aspects (19).
It is concluded that this procedure can differentiate genera and occasionally species of medically important fungi and that following the necessary validation experiments; it can be used directly on clinical samples to assist prompt diagnosis of systemic fungal infections.

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