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Rapid Identification of Dermatophytes Isolated from Clinical Specimens from Dermatophytosis Patients by Application of the PCR-RFLP method.

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ABSTRACT

Dermatophytes are a group of closely related fungi that invade keratinized tissue (skin, hair and nails). In the present study, a molecular technique was designed to optimize the polymerase chain reaction-based restriction fragment length polymorphism (PCR-RFLP) method targeting internal transcribed spacer (ITS) regions in *18S* ribosomal DNA (rDNA) of fungi for rapid detection and identification of dermatophytes. A total of 216 specimens from skin, hair and/or nail were collected from 150 patients with dermatophytosis. Specimens were inoculated on Sabouraud's Dextrose Agar (SDA). A small portion of each fungal colony was further studied by RFLP analysis of the PCR-amplified ITS region of rDNA. PCR amplicons and were visualized by electrophoresis on a 2% agarose gel after digestion by restriction enzymes including *HinfI* and *HaeIII*. Nine dermatophyte species including *M. canis, T. mentagrophytes*, *T. tonsurans*, *E. floccosum*, *T. rubrum*, *T. ajelloi*, *M. gypseum*, *M. audouinii* and *T. schoenleinii* were identified based on the colony morphology and microscopic criteria. Specific PCR products and RFLP patterns allowed for the rapid identification and reliable differentiation at the genus or species level. Our results showed that this technique is a rapid and reliable tool which allows identification of major pathogenic dermatophytes isolated at the species level.

Keywords: dermatophytes, PCR-RFLP, skin.

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INTRODUCTION

There are approximately 30 fungal species which act as etiologic agents of dermatophytosis. Of these, *Trichophyton rubrum* constitute the majority of isolates. *T. mentagrophytes* var. *interdigitale*, *T. tonsurans*, *Microsporum canis*, *M. gypseum* and *Epidermophyton floccosum* are also known as representative species of this infection (1).

Accordingly, identification of isolates at the species and strain levels is extremely important for accurate antifungal therapy as it reveals possible routes of infection and for prevention of infection to others, especially in immunocompromised individuals. Identification of dermatophyte species is essentially based on macroscopic and microscopic observation of their morphological features in culture media. However, the exact identification is a little bit difficult due to the morphological similarity, variability, and polymorphism shown by dermatophytes. Thus, accurate identification is time consuming and requires a significant level of knowledge and technical expertise (2).

Because of the difficulty to differentiate clinically, dermatophytosis from other non-mycotic dermatoses, particularly in the dystrophic nails, it is important to establish a biologically accurate diagnostic method. A definitive diagnosis of dermatophyte infection needs to be confirmed before the initiation of antifungal therapy because of the long duration of the treatment and its high cost, and of the potential side effects of the drugs. In addition, knowledge of the zoophilic or anthropophilic origin of the dermatophyte may allow for prophylactic measures to be carried out, such as treatment of pets whose owners develop skin disease (3). The present study was conducted to establish the use of polymerase chain reaction (PCR) to identify dermatophytes at the species level through the detection of internal transcript spacer (ITS) regions, *18S* rDNA genes and determine the dermatophyte species by digestion of dermatophyte amplified DNA by restriction enzyme digestion.

MATERIALS AND METHODS

Culture of fungal isolates

To determine the genotypes of the 28 dermatophyte isolates recovered from the 216 specimens, 170/216 (78.7%) isolates were considered positive specimens and their genomic DNA used as templates for PCR. For this, *18S* rDNA amplified DNA products of the ITS region from nine species isolated in the present study were used. A portion of specimens were cultured on SDA by spot inoculation. The cultures were incubated at 29±2 ºC until visible fungal growth occurred.

DNA extraction and purification: AnEZ-10 spin column fungal genomic DNA mini-prep kit (BioBasic Incorporation /Korea), was used for purification of DNA from young fungal colonies as per manufacturer's instructions. The genomic DNA of 28 dermatophyte isolates from young fungal colonies was extracted by using a grinder in the presence of liquid nitrogen to initially break up the mycelia. The concentration of extracted DNA was estimated by measuring its absorbance at (260 nm)*l* (4,5). Six microliters of DNA solution was used as a template in the subsequent PCR reaction and PCR products were resolved and quantified on 2% agarose gel.

Gene Amplification Reaction

Amplification of genes was carried out according to the experimental protocol of Accu power TLA PCR Premix tub under conditions of cycling as mentioned in cycling parameters of Table (3.8). The PCR reaction mixture was prepared as per manufacturer's instructions.

Screening of ITS regions by RFLP analysis

Preliminary screening by RFLP analysis of ITSs 1 and 4 was performed. The amplicon was digested with restriction enzymes *Hinf*I (Accu Cut ™ Restriction Endonuclease, USA Bioneer, Inc.) and *Hae*III (Promega Corporation Product, USA.). The digestion products were separated by electrophoresis on a 2% agarose gel, stained with ethidium bromide, and observed under UV light.

RESULTS

Genotype analysis of dermatophyte isolates

To determine the genotypes of the 28 dermatophyte isolates, the genomic DNA samples were used as PCR templates using *18S* rDNA amplified DNA products of ITS region from nine species isolated in the present study. All 28 isolates were characterized. Of these, 3/28 (10.71%) isolates were classified as having a genotype of both *T. rubrum* and *M. audouinii*, 2/28 (7.14%) isolates were classified as genotypes to each other of *T. mentagrophytes, T. schoenleinii, M. gypseum, E. floccosum* and *T. ajello,* 8/28 (28.57%) isolates were classified as genotype *T. tonsurans*, and 4/28 (14.29%) isolates were classified as genotype *M. canis*.

The ITS region amplified from the species *T. rubrum*, *T. mentagrophytes*, *T. tonsurans*, *T. schoenleinii*, and *T. ajello* were (~680 bp) in length, while the ITS region amplicons from *E. floccosum* was (~780 bp) and *M. canis, M. audouinii*, *and M. gypseum* were (~720bp) (Table 1 and Figure 1).

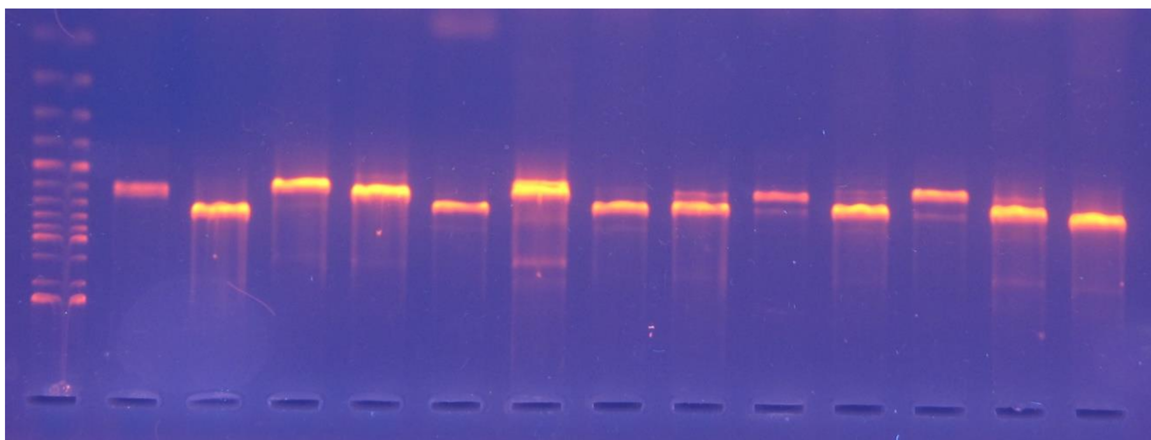
Table (1): The PCR product patterns of RFLP analysis of rDNA from fungal colonies using restriction enzymes *Hinf*I and *Hae*III.

Type of dermatophytes	PCR product (bp)	*Hinf*I (bp)	*Hae*III (bp)
Trichophyton mentagrophytes	680	370-160	400-100
T. rubrum	680-800*	380-157	320-100
T. tonsurans	610	380-160	400-100
T. ajelloi	580	370-157	400-100
T. schoenleinii	589	380-160	400-100
Microsporum canis	720	Negative	370-100
M. audouinii	700	370-157	365-100
M. gypseum	730	380-160	370-100
Epidermophyton floccosum	780	250-180-157	450-140

* Representative PCR products of patient No.98 and 101

Sub-genotyping RFLP analysis of dermatophyte isolates

In order to generate species-specific patterns to determine the sub-genotypes of PCR amplification products of the dermatophyte isolates, PCR products were screened by RFLP analysis with, *Hinf*I and *Hae*III restriction endonuclease digestion. Our results demonstrate that several isolates that showed similar amplification profiles had different restriction digestion profiles. In general, multiple isolates assigned to a single genotype by PCR amplification were assigned to distinct sub-genotypes by RFLP analysis (Table 1).



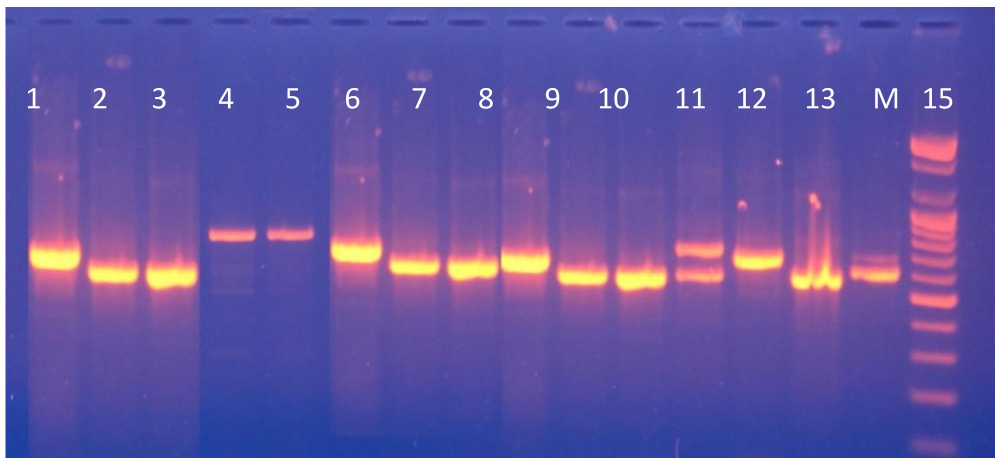


Figure (1): Agarose gel electrophoresis of ITS regions PCR product in *18S* rDNA of different dermatophytes. Lane (M): Size marker (100bp); Lane 1,17,18: *T.rubrum* (800bp); Lane 2,25: *E. floccosum* (780bp), Lane 4,12: *M.gypseum* (730bp); Lane 6,7,9,19: *M. canis* (720bp); Lane 3,5,10,13,15,16,23,28: *T. tonsurans* (610bp); Lane 8,11: *T. ajello* (580bp); Lane 14,22,26: *M. audouinii* (700bp); Lane 20,21: *T. mentagrophytes* (680bp); Lane 24,27: *T. schoenleinii* (589bp). (2% Agarose gel, 80 volts for 2 hours).

DISCUSSION

Molecular typing of an infectious agent is important for epidemiological and taxonomical studies, as well as for the development of appropriate infection control strategies. Because of the characteristics of the dermatophyte species and the need for better understanding of its epidemiology and taxonomy, molecular techniques are employed to provide characterization of isolates. We aimed to describe the distribution of genotypes within dermatophyte isolates from patients in Iraq. Although we identified different types, a phenotypic test cannot differentiate among them. Thus, the present study describes the use of conventional PCR techniques to identify the genotype of the isolate. Further, the study was conducted to determine the genotypic distribution and diversity of dermatophyte isolates recovered from dermatophytosis patients in the Al-Najaf government using *18S* rDNA primers (ITS region).

In this report, we demonstrate that PCR using *18S* rDNA amplified DNA products is a simple and reproducible method (6) that can be used as typing tool to supplement other typing methods, especially in laboratories where genomic based molecular typing is not available. This approach can also be used in an outbreak situation or to compare different isolates to establish identity or non-identity.

The detection rate of dermatophytes by PCR technique has shown a great deal of variation, and depends on the target gene used in the analysis (7). Because conventional laboratory procedures for the identification of dermatophytes are either slow or lack enough specificity, application of nucleic acid amplification technology has made rapid and precise identification of dermatophytes possible (8).

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 (9,10). As reported previously, sequence and length of ITS regions of ribosomal DNA in the dermatophytes have shown strong specificity among interspecies and conservatism among intraspecies, being suitable for typing and species identification. Additionally, this approach has proven to be useful for both resolving phylogenetic relationships between close taxonomic relatives and for species identification. From our results, we found that PCR products were close to each other among species. This is in line with the report of Wang *et al*, (11). Therefore, we can identify dermatophytes at the species level by PCR alone.

Although dermatophyte fungi are in great numbers, common pathogenic dermatophyte fungi are only few. Therefore, we focused on common dermatophyte fungi (e.g. *T. mentagrophytes*, and *M. canis*) in this study and we aimed to seek a rapid and simple method of identification for clinics that may not have adequate resources for traditional testing. Additionally, the reliable specificity among interspecies and conservation among intraspecies of the ITS region can ensure the sensitivity and specificity of identification.

Further, species identification has a wide role in monitoring the demographic distribution and changes in frequency of specific dermatophyte infections (12). Alternatively, we could obtain electrophoretic profiles in approximately 9 hours starting from initial acquisition of culture samples. In general, the multiple advantages of this approach provide the opportunity for dermatophyte identification at the species level and can be of great utility when traditional methods are not available.

In conclusion, this procedure can differentiate species of medically important fungi following necessary validation experiments, and can be used directly on clinical specimens to assist prompt diagnosis of systemic fungal infections.

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