RFLP- PCR for identification of dermatophytes and Candida species from clinical isolates of hair and skin

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Abstract

The identification of hair and skin agents is important in order to define clinical treatment. However, in most cases conventional culture identification can be considered to be time-consuming and not without errors. The aim of the present study was to identify the dermatophytes and Candida spp. isolated from hair and skin lesions by using the polymerase chain reaction and restriction fragment length polymorphism analysis (PCR/RFLP). Fungal universal primer pair (ITS3/ ITS4) was able to amplify internal transcribed spacer 2 (ITS2) region of rDNA. However, three restriction enzymes were used to digest the PCR products: HaeIII, MspI and Corf. Twenty four isolates of dermatophytes and Candida spp. identified based on their PCR/RFLP pattern. Our study of PCR/RFLP method easily differentiated dermatophytes and Candida spp. and this result was also explained genetic intra-evolution for these isolates.

Key words: Dermatophytes, Candida spp., ITS, RFLP-PCR.

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Introduction

Dermatophytes are group of fungi that similar in morphology and physiology, they have the ability to invade keratinocytes tissue in humans and animals, causing cutaneous infections. They are also called Tinea or ring worm(1,2). The diseases caused by these fungi that in addition to the clinical symptoms of skin they can advance quickly, causing a systemic infections, especially in Immunocompromised patients (3,4).This disease is also a reservoir of opportunistic fungal and secondary bacterial infections(4,6).

Although the identification of fungal isolates by direct microscopy and mycological culture are called the golden standard, however, these methods require time and effort as well as the experience and high accuracy in diagnosis. In addition, often fungal strains can vary in phenotypic characteristics among strains. Recently, differentiating types of fungi depending on the molecular characteristics have been developed because the molecular methods are faster, more stable, more sensitive and not affected by environmental factors that may affect the phenotypic characteristics of the fungal isolates[7,8]. The
molecular methods could be useful for identification of young colonies, colonies that have lost of morphological features and dead strains [9].

The molecular techniques such as restriction fragment length polymorphism (RFLP) which used for fungal identification at the species level and restriction enzymes allowed the rapid identification and differentiation of fungal isolates at the genus or species level [10,11].

The aim of this study was evaluate the use of RFLP for identification and studies the genetic relationship between dermatophytic fungal isolates and Candida species.

Material and methods

Samples collection and fungal culture: A total of 60 specimens were collected from patients with dermatophytoses that were clinically diagnosed by the dermatologist in Unit of Mycology in Al-Marghan Hospital of Hilla (Iraq). Samples were 58.33% from male and 41.66% from female, which including 40 (66.66%) hair fragment and 20 (33.33%) skin scraps, for identification of dermatophytes and other fungi were cultured on Sabouraude's Dextrose Agar (SDA) with chloramphenicol and cycloheximide at 28°C. The identification of fungi based on morphological characteristics and Candida species identification on CHROM agar Candida medium, according to the mycological references [1,12].

Fungal genomic DNA extraction:-Genomic DNA of each fungal isolate was extracted by picking 1g of mycelia by using sterile loop and suspending into 300 μl of lysis buffer (10 mM Tris, 1mM EDTA (pH8), 1% SDS, 100mM NaCl, 300μl phenol-chloroform (1:1)) shaken for 5 min and added 10 μl proteinase K, then tubes were incubated in 65°C water bath for one hour, centrifuged at 1000rpm, the supernatant was transferred to new tube and equal volume of chloroform was added, mixed and centrifuged. The supernatant was transferred to new tube and equal volume of cool isopropanol was added and centrifuged at 1000 rpm for 10 min. the supernatant was poured out and added 500 μl of 70% ethanol alcohol s and centrifuged at 10000 rpm for 7 min, dry DNA pellet was re-suspended in 75 μl of TE buffer and stored at -20°C until use [13].

PCR assay: - The genomic DNA of the representative fungal isolates were amplified with fungal universal primer pair forward ITS3 and reverse ITS4 to amplify internal transcribed spacer 2 (ITS2) region of rDNA. The final volume of PCR reaction was 25 μL containing 1 μL of DNA (20 μg/ml) from each of isolates were mixed with PCR mixture consisted of 12 μl of 2x Master Mix (Promega), 2 μl of primers (10 pmole) and rest molecular-grade water. The PCR mixture was amplified by thermal cycler PCR System (Labnet, USA) with the following conditions: 1 cycle of 7 min at 94°C, followed by 32 cycles of 40 sec at 94°C, 45 sec at 55°C and 1.20 min at 72°C and a final extension step at 72°C for 7 min[14]. The PCR products were run on 1.2% agarose gel (Bio Basic Canada Inc.) with 0.5X EDTA buffer and stained with 0.05% ethidium bromide. Electrophoresis was performed at 100 V. in TBE buffer. The DNA bands were detected by Desktop Gel imager scope 21 ultraviolet transilluminator (Korea Com.).

RFLP-PCR assay: - 5μl of each PCR product were separately digested with 8 μl of each restriction enzyme (HaeIII, MspI and Corf) at 37°C for 3 hours. Restriction fragments were analyzed in 1.5% agarose gel whichstaining with 0.05% ethidium bromide at 80 V for 60 min and visualized by UV light and photographed by Desktop Gel imager scope 21 ultraviolet transilluminator (Korea Com.).

Results and Discussion

Fungal isolation and identification
Fifty four isolates identified with conventional method for dermatophytes and *Candida* species, the identification of dermatophytes isolates were diagnosed by macro and micro- morphological characteristics on SDA (Fig. 1 & 2), while *Candida* species isolates were identified by CHROM agar Candida medium (Fig. 3). The number and percentage of fungal species isolated from different site of sampling were summarized in table (1).

**Table (1): The number and percentage of fungal species isolated from different site.**

<table>
<thead>
<tr>
<th>Fungal species</th>
<th>Color in chromo agar</th>
<th>No. of sample</th>
<th>Percentage of frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Candida albicans</em></td>
<td>Light green</td>
<td>10</td>
<td>16.66</td>
</tr>
<tr>
<td><em>C. dubliniensis</em></td>
<td>Dark green</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td><em>C. glabrata</em></td>
<td>Purple</td>
<td>5</td>
<td>8.33</td>
</tr>
<tr>
<td><em>C. gulliermondii</em></td>
<td>Light purple</td>
<td>2</td>
<td>3.33</td>
</tr>
<tr>
<td><em>C. krusei</em></td>
<td>Light pink</td>
<td>5</td>
<td>8.33</td>
</tr>
<tr>
<td><em>C. parapsilosis</em></td>
<td>White to cream</td>
<td>5</td>
<td>8.33</td>
</tr>
<tr>
<td><em>C. tropicalis</em></td>
<td>Metallic blue</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td><em>Rhodotorula</em> sp.*</td>
<td>Dark orange (PDA)</td>
<td>2</td>
<td>3.33</td>
</tr>
<tr>
<td><em>Microsporum canis</em></td>
<td>White with orange Rv. P. (PDA)</td>
<td>12</td>
<td>20</td>
</tr>
<tr>
<td><em>Trichophyton mentagrophytes</em></td>
<td>White with pale brown Rv. P. (PDA)</td>
<td>7</td>
<td>11.67</td>
</tr>
<tr>
<td>Negative culture</td>
<td></td>
<td>6</td>
<td>10</td>
</tr>
<tr>
<td><strong>Total no.</strong></td>
<td></td>
<td>60</td>
<td>99.99</td>
</tr>
</tbody>
</table>

Ismael [15] isolated *Trichophyton* spp. and *Candida* spp. at 57.14% and 28.57% respectively, as other fungi as *Microsporum*, *Trichosoron*, and *Fonsecaea*. While Kannan [16], isolated *Trichophyton* spp. and *Candida* spp. with 66.3% and 100% respectively. Habib [17], diagnosis of *Candida* species causing vulvovaginal Candidiasis using some phenotypic experiments were carried out such as germ tube, from motion of pseudohyphae and clamydospores in CMA+TW80 medium, API20 candida and CHROM agar Candida.
Fig. (1): *Microsporum canis*, A: Colony morphology on SDA at 28°C., B: Micro and macroconidia.

Fig. (2): *Trichophyton* sp., A: Colony morphology on SDA at 28°C., B: Micro and macroconidia.

Fig. (3): Colonies color of *Candida* species on CHROMagar medium at 30°C for 24-48h. A: *C. albicans*; B: *C. glabrata*; C: *C. guilliermondii*; D: *C. dubliniensis*; E: *C. parapsilosis*; F: *C. krusei*.

### ITS-PCR assay

ITS3/ITS4 are universal primer pair that targeted the sequences place of the ITS2 gene of the yeast’s isolations used. Figure (4) shows agarose gel electrophoresis of PCR products for yeast species.
The molecular weights of the PCR products for the yeast isolations under study ranged from 400-500bp., while dermatophytes isolates ranged from 400-450bp. that agree with several sources which amplification of ITS region for most types of *Candida* yeast [18,19]. Our results are close to the results reached by many studies, Abdel-fatah [20], were amplified ITS gene by using the universal primers ITS1 and ITS4. Amplicons produced by *Trichophyton* spp. were about 680 bp- 780 bp in length, while the ITS region amplified from *C. krusei, C. tropicalis* and *C. albicans*, were 510, 524 and 550 bp, respectively.

![Agarose gel electrophoresis of PCR products for ITS2 gene of yeast and dermatophytes species by using ITS3/ITS4 primers pair.](image)

**Fig. (4):** Agarose gel electrophoresis of PCR products for ITS2 gene of yeast and dermatophytes species by using ITS3/ITS4 primers pair. Lane M= molecular marker 100 bp; Lane 1- 3: *C. tropicalis*; 4: *C. dubliniensis*; 5-6: *C. gulliermondii*; 7-8: *C. krusei*; 9: *C. parapsilosis*; 10, 11,13: *C. albicans*; 12: *Rhodotorula* sp.; 14-15: *C. glabrata*; 16-19, 21: *Microsporum canis*; 20, 22-24: *Trichophyton mentagrophytes*.

**RFLP-PCR assay**

PCR products were subjected to RFLP analysis by digestion with the restriction enzymes *HaeIII, MspI* and *Cof* in order to generate species-specific patterns for fungal identification. Digestion with these endonuclease enzymes produced different fragment patterns varying in number and size. Almost products were 2 fragments while with different size except isolate number 12 was 3 fragments in *HaeIII* and *MspI*. Also, these enzymes produced 5 RFLP patterns of all enzymes (Fig. 5-7). Specific PCR products and RFLP patterns for *MvaI, HinfI* and *HaeIII* enzymes allowed the rapid identification and reliable differentiation of isolated dermatophytes at the genus or species level and producing 1 to 3 fragments for different species[10]. Abdel-fatah [20], used *HaeIII* to digest the ITS region of rDNA gene and producing 2 to 3 fragments for different species of *Candida* and dermatophytes isolates. This PCR-RFLP strategy was also employed in the present study.

The only modification to the original procedure was the use of *MvnI* and *HinfI* instead of *MvaI*. Whereas digestion with *MvnI* allows discrimination between the three main dermatophyte genera (*Trichophyton, Microsporum*, and *Epidermophyton*) [21,22]. Although PCR/RFLP can be considered to be an “old tool” for PCR product analysis, it is still being used for studies of microorganism characterization because of its simplicity, reliability, easy adaptation for identifying several genera or species, and because it does not require expensive materials or equipment. These
characteristics are important in cost-effective studies and indicate that the use of this technique will probably become routinely accepted in mycology laboratories before the DNA sequencing or real-time PCR methods are used [23,24].

Fig. (5): Agarose gel electrophoresis of RFLP-PCR products for \textit{HaeIII} enzyme of yeast and dermatophtes species.

Fig. (6): Agarose gel electrophoresis of RFLP-PCR products for \textit{MspI} enzyme of yeast and dermatophtes species.

Fig. (7): Agarose gel electrophoresis of RFLP-PCR products for \textit{Corf} enzyme of yeast and dermatophtes species.
References

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