Morphological and Molecular Identification of *Trichophyton mentagrophytes* Isolated from Dermatophytes Patients in Garmian Area

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**ABSTRACT**

Conventional and molecular diagnosis considered as a complementary approach for making a final decision about causative agent of all microbial population, the present study was conducted in Kalar General Hospital, Dermatology unit and research laboratory center in Garmian University. Out of thirty clinically collected specimens only five samples displayed positive dermatophytic characters against microscopic and macroscopic as well as biochemical tests, such appearances of isolated colonies as white and brown color for both surface and reverse respectively, as well as, the colonies showed with a cottony texture which changed to powdery-granular colonies after two weeks incubation. Microscopic examination appeared numerous single-celled, spherical shaped microconidia were seen as clustered on both sides of hyphae, furthermore, multiseptate cigar shaped macroconidia and spiral hyphae were seen during the formation of granular colonies, biochemically analysis showed positive for urease, in addition, hair perforation testes were revealed positive results for isolates. Molecular identification carried out via conventional PCR protocol by using the primers set ITS1 and ITS4 which result in 700bp in agarose gel electrophoresis for all isolates. PCR-RFLP carried out by means of using *Bst*N1 digestion enzyme revealed four separated pattern bands 250,180,150 and 120bp. Sequencing of the ITS region in one of the isolated species revealed that similarity about 86% with *Trichophyton mentagrophytes* ATCC11481

**Keywords:** *Trichophyton mentagrophytes*, PCR, RFLP, dermatophytes, ITS region.

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1. **INTRODUCTION**

Skin infection by dermatophytes constitutes one of the health problems in the world (Kanbe et al., 2003). *Trichophyton mentagrophytes* is the most etiological agents of dermatophytosis in human and animal. In human *T. mentagrophytes* responsible for tinea pedis, tinea corporis, tinea unguium and tinea capitis (Symoens et al., 2011). *T. mentagrophytes* have the ability to invade the keratinized layer of the skin and causes hair, nail and skin infection which known as dermatophytosis...
Identification of dermatophytes performed by the combination of microscopic examination, in *vitro* cultural characteristic and other biochemical tests (de Hoog *et al*., 2000). Sometimes, identification of dermatophytes is complicated due to the similarities in the morphological characters between dermatophyte species which required a high level of scientific laboratory training (Nenoff *et al*., 2013). Molecular approaches have been used for the identification of dermatophytes at the level of species and strain (Gräser *et al*., 2008). The present study was aimed to investigate the conventional and molecular diagnosis of the causative agents of dermatophytes, as well as, characterizing their morphological and molecular analysis of genes and species.

2. MATERIALS AND METHODS

2.1 Samples Isolation

Thirty clinical specimens from infected hair, nail and frayed skin collected from suspected patients with dermatophytosis such (tinea pedis, tinea cruris, and tinea corporis) under the supervision of specialized dermatologist in the dermatology department of the General Hospital in Kalar district /Sulaimania Province. Specimens were collected from November to the end of December 2016. The specimens collected in sterile closed tubes and transferred to the research laboratory center of Biology Department at College of the Education University of Garmian for further mycological examination.

2.2 Phenotypic Identification

All isolates samples undergo two common tests, first as direct microscopic examination and the second through cultivating them on potato dextrose agar (PDA) and Sabouraud's dextrose agar (SDA) (at 25°C for 21 days and examined at intervals times each three days) to study the macroscopic examination for characterizing the surface and reverse color formation and pigmentation occurrence of the fungal colonies. Direct microscopic examination carried out by 10% KOH to describe the filamentous hyphae and arthroconidia states of the dermatophytes, as well as, Direct mount examination was carried out by Lactophenol Cotton Blue (LPCB) to analyze the fungal species, in addition to microscopic and macroscopic examination, biochemical tests were achieved by hair perforation test, growth on rice grain and urease test to determine extra characterization of the isolates (Forobes *et al*., 1998, Nenoff *et al*., 2007 and Kanbe, 2008).

2.3 Molecular Identification

The genomic DNA was extracted from the fungal colonies by using OMEGA Fungal DNA Mini Kit/USA based on the manufacturer’s instructions. Molecular identification of the pathogenic fungi was carried out by species-specific PCR which
conducted by amplification of the internal transcript spacer (ITS) gene of the rDNA in dermatophyte species and amplified by using one pair of primers (Table 1) symbolized ITS1 as forward primer and ITS4 as a reverse primer (built-up in University of Koea\ KRG) (Refai et al., 2013). PCR was performed in 25μL of PCR reaction mixture containing: 12.5 μL of master mix (GeNet Bio\Korea), 2μL of DNA template, 9.5μL of deionized water and 0.5μL from each primer (forward primer ITS1 and reverse primer ITS 4).

The PCR mixtures were spun down shortly for 5-10 seconds then placed in thermal cycler (TCY, Crealcon, NL) and subjected to the following cycling conditions according to Ghjojghi, et al., (2015): initial denaturation at 95°C for 1minute, followed by 35 cycle of denaturation at 95°Cfor 30 seconds, annealing at 55°C for 1minute and extension at 72°C for 2minutes and a final extension step at 72°C for 5minutes. The amplified DNA fragments were visualized in 1.5% agarose gel electrophoresis containing ethidium bromide at 90 volts for 60 minutes at room temperature. Amplicon size determined by comparison with 100bp DNA ladder (GeNet Bio\Korea).

The PCR-RFLP analysis was conducted by using the BstN1 restriction enzyme (BioLab, UK) according to the protocol provided by the company. PCR product was sequenced in Macrogene lab\ South Korea as Standard Sequencing Service.

### Table 1- Primer sequences, product size and annealing temperature

<table>
<thead>
<tr>
<th>Primers</th>
<th>Primer sequence(5′-3′)</th>
<th>Product size(bp)</th>
<th>Ann. temp.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward primer ITS1</td>
<td>F (5′- TCC GTA GGT GAA CCT GCG C-3)</td>
<td>700</td>
<td>55°C</td>
</tr>
<tr>
<td>Reverse primer ITS4</td>
<td>R (5′- TCC TCC GCT TAT TGA TAT GC-3)</td>
<td>700</td>
<td>55°C</td>
</tr>
</tbody>
</table>

#### 2.4 Restriction Fragment Length Polymorphism (PCR-RFLP) Analysis

In order to the identification of dermatophytes at species level by species-specific PCR, all PCR products were subjected to digestion with the BstN1 restriction enzyme (BioLab, UK) according to the protocol provided by the manufacture. The mixture contained 10μL of PCR product, 1.5μL of 10 X NEB buffer and 0.5μL of the BstN1 enzyme, which recognizes the sequence 5′ CC (T/A) GG 3′ .Subsequently, reactions were incubated at 60°C in a water bath for one hour. 10 μL of each PCR digested products and 5 μL of DNA ladder were electrophoresed on 3% agarose gel at 90 volts for 90 minutes at the room. The gel was observed under a UV transilluminator and the DNA bands were visualized and photographed with a digital camera, then compared the electrophoretic RFLP pattern with those profiles (Jackson-Fisher et al., 1999; Rezaei-Matehkolaei et al., 2012; Elavarashi et al., 2013).

### 3. RESULTS AND DISCUSSION
The collected samples from (scraping skin, clipping nail and hair fragments) were treated with 10% KOH test and conventional laboratory identification which include macroscopic, microscopic and biochemical tests. Out of thirty collected specimens, only five of them (16.67%) displayed positive results against 10% KOH test, while the rest of all other isolates showed negative results.

Microscopically all five isolates were showed numerous single-celled and globose shaped microconidia were seen as a clustered on both sides of the hyphae, furthermore, Multiseptate cigar shaped macroconidia were seen during the formation of granular colonies (Fig. 1), as well as spiral hyphae were seen during the microscopic slide preparation under 40X. Singh and Beena, (2003) also conducting positive and negative results by direct microscopically examination.

Macroscopically analysis revealed that the growing colonies on PDA and SDA at 25 °C for the first week of incubation appeared as a flat with cottony texture which then produced powdery to granular colonies possessing radial margin, while in the study of Poluri et al., (2015) revealed that dermatophyte test medium (DTM) is better than SDA for screening and identification of dermatophyte species, on the other hands, the colonies pigmentation color appeared with white color in the surface side, while in the reverse side pigmentation revealed as brown color (Fig. 2). The biochemical analysis was done for the obtained colonies exhibit as positive results for urease test, as well as, also same results for hair perforation test were achieved, in addition, the biochemical identification system also can be useful to identified some of the fungus strains depending on their phenotypically and their biochemical characteristics as discussed by Maikhan and Mohammed-Amin, (2017) . The obtained results was in agreement with that discussed by Surendran et al., (2014), that the culturing may identify the fungus species till the species, moreover it will require other tests in order to determine their genus.

Molecular identification was by species-specific PCR carried out by amplification of the ITS region on the ribosomal DNA by using the set of universal primers ITS1 and ITS4. Amplification of the ITS region resulted in PCR product about 700bp when electrophoresed on 1.5 % agarose gel, while the digestion of the amplified ITS product by BstN1 restriction enzyme during the performing of PCR-RFLP revealed four patterns 250,180,150 and 120 bp through the electrophoresis in 3% agarose gel (Fig. 3). The profiles of electrophoretic analysis of patterns were obtained from ITS- RFLP in all clinical isolates showed similarity with those of Elavarashi et al., (2013) and Ahmadi et al., (2015); where they identified dermatophytes by PCR-RFLP and described the same patterns of ITS-RFLP. For species identification, ITS
sequence of clinical isolates aligned with the reference sequence recorded in the database by using BLAST sequence analysis tool "http://www.ncbi.nlm.gov/BLAST" from the National Center for Biotechnology Information. According to the sequence of the ITS region in rDNA of four clinical isolates were identified as *Trichophyton mentagrophytes* revealed that only one isolate showed similarity about 86% with *Trichophyton mentagrophytes* ATCC11481 (Fig. 4) and *Arthroderma simii* strain: CBS150.66 (Figure 5), and also showed 84% identity with a number of species such as *Trichophyton quinckeanum* strain ATCC 11480 and *Trichophyton schoenleinii* strain ATCC 22775. While the sequence of the ITS region in the other isolates of revealed uncultured fungus during the search process in NCBI database information. Relied on Yu *et al.*, (2004), the ITS regions have interspecies polymorphism and few variance at the level of intra species. Consequently, the sequencing of the ITS region in the rDNA in dermatophytes proved to be useful for the identification and resolving the phylogenetic relationship between dermatophyte fungal species (Dhieb *et al.*, 2014). The sequencing method of rDNA ITS fragments presently considered the gold standard for identification of strains in the *T. mentagrophytes* complex, (Li *et al.*, 2008)

FIGURE (1): Microscopic morphology of the obtained colony mounted with LPCB stain: 1 showing numerous single-celled, spherical to globose shaped microconidia were seen as clustered on both sides of hyphae. 2 showing Multiseptate cigar shaped macroconidia under (X40).

FIGURE (2): The colonies pigmentation color after three weeks of incubation at 25 °C on SDA, (panel A showing surface side pigmentation, panel B displayed reverse side pigmentation).
FIGURE (4): Partial Sequence of the Internal Transcript Spacer (ITS) in locally isolate of *Trichophyton mentagrophytes* with *Trichophyton mentagrophytes* ATCC11481
FIGURE (5): Partial Sequence of the Internal Transcript Spacer (ITS) in locally isolate of *Trichophyton mentagrophytes* with *Arthroderma simii*.

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<tr>
<th>Score</th>
<th>Expect</th>
<th>Identities</th>
<th>Gaps</th>
<th>Strand</th>
</tr>
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<td>383 bits(207)</td>
<td>5e-102</td>
<td>309/359(86%)</td>
<td>4/359(1%)</td>
<td>Plus/Plus</td>
</tr>
</tbody>
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**Query 86**

```
ACCTGCACC-TTCTCTGT-TTCCCTACTCGGATGTCCTCCGCCGGACGGCCTCTCTCTTG 143
```

**Sbjct 69**

```
ACC-GCCCCATTTCTTCTACCCCTCTGTTGCTTCGCGGCGGCGGCTTC-CAGGA 126
```

**Query 144**

```
GATTCGTTCCGAGGTCTCTCTCTTTTGGTCTCCACGTTGGACTTCTGCCCACCACGACGA 203
```

**Sbjct 127**

```
GAGCCGTTCCGCGAGCTTCTCTTTATGCTGCTCAAGCTGCGAAGCGCCGCCCGGAGGACA 186
```

**Query 204**

```
GACGCCTTTGATATTCTTCTACATTGCAATCTGACGCCTTAGATCTAGCAAAATCAG 263
```

**Sbjct 187**

```
GACGCAAAAAAAATTTCTTCTACAGAAGCTGCTAGTCTGACGATGCTAGAAAAATCAG 246
```

**Query 264**

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TTAAAACTCTTCTACAGGATCTCTTGGCCGGCATCGATGAAGAACGCTTCGAAATTGC 323
```

**Sbjct 247**

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TTAAAACTCTTCTACAGGATCTCTTGGCCGGCATCGATGAAGAACGCTTCGAAATTGC 306
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**Query 324**

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AATAAGTAATGTGAATTGCAGAATTCCCGAGAATCATCGAATCTTTGAACGCCACATTGCGC 383
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**Sbjct 307**

```
GATAAGTAATGTGAATTGCAGAATTCCGAGAATCATCGAATCTTTGAACGCCACATTGCGC 366
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**Query 384**

```
CCCCCTGTATTTCCGGGGGCAAGCTTCCGCTGTTGCTCTTACACCACTCAGATCTTTGAACGCCACATTGCGC 442
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**Sbjct 367**

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CCCCCTGTATTTCCGGGGGCAAGCTTCCGCTGTTGCTCTTACACCACTCAGATCTTTGAACGCCACATTGCGC 425
```
4. CONCLUSIONS

The following can be drawn out of this paper:

a. Conventional and molecular diagnosis considered as a complementary approach for making a final decision about causative agent of all microbial population

b. Microscopic examination and biochemical analysis together facilitated primary identification of the obtained dermatophytes genus

c. Molecular identification with exact set of primers ITS1 and ITS4, as well as by using BstN1 digestion enzyme revealed four separated pattern bands 250,180,150 and 120bp.

d. The ITS region in sequencing revealed that similarity about 86% with Trichophyton mentagrophytes ATCC11481.

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REFERENCES


