Molecular Characterization of *Malassezia* spp Isolated from Human Pityriasis Versicolor

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**Abstract**

The study was looking to isolation and identification of various species of *Malassezia* isolates associated with various clinical phenomena of pityriasis versicolor by molecular Techniques the results of DNA extraction showed that most of the isolates give just single bundle of DNA on agarose gel, and according to PCR results, thirteen DNA samples (43.3%) were amplified after PCR program for amplification by two sets of primers ITS1 - ITS4 3.2 and V9G - LS266, then all the thirteen isolates were subjected to DNA sequencing which showed the following results: eight isolates identified as *Malassezia furfur* and five as *Malassezia globosa* with the alignment bellow of each species.

*Malassezia furfur* isolate POL.10.11.IIIA 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence .Sequence ID: KC152898.1 Length: 848 Number of Matches: 1Range 1: 1 to 848. Phylogenetic analysis of the 13 *Malassezia* isolates were analyzed by MEGA 5.05 and compared with sequences of different *Malassezia* species available in Gen Bank database, the data showed a clear convergence between our *Malassezia* isolates from Basra patients and that of the Gen Bank database.

**Key words:** *Malassezia, pityriasis versicolor*;

**Introduction**

Pityriasis versicolor is *Malassezia* spp infection of the skin, it’s also sometimes called tinea versicolor, although the term tinea should strictly be used for dermatophyte fungus infections, Pityriasis versicolor most frequently affects young adults of both genders and is slightly more common in men than in women, it can also affect children, adolescents and older adults ¹. *Malassezia* species can be identified and diagnosed by methods based on their biochemical features, but such methods do not have enough guttered (discriminatory) power and because of that, cannot characterize the newly defined species. Add to that, the biochemical and phenotypical methods are not suitable to achieve an immediate way of diagnosis. Recently, molecular approaches and PCR methods for the accurate differentiation of *Malassezia* species are the most convenient way of identification and diagnosis ²,³. The taxonomy and nomenclature of *Malassezia* species was raped with confusion and chaos until 1995 when the molecular techniques shed lights and allowed physiological and ultrastructural studies to describe the characteristics of each species. Recently, using the combination of biochemical, physiological, morphological and molecular techniques seven additional species have been identified. There are four molecular techniques includes DNA sequence analysis , the first method implored in *Malassezia* species identification, it involves the nucleotide sequence analysis of the obtained ribosomal DNA gene of the yeast and the results are phylogenetically compared with distant or closely related *Malassezia* species, Biotyping using Api 20 NE and ApiZym enzymes, Chromosomal analysis using pulsed field gel electrophoresis (PFGE), molecular technique has received very little attention ⁴, but has so far being used to identify six of the fourteen species of *Malassezia* it depends on the use of enzyme
activity profiles of the different Malassezia species, and Polymerase chain reaction (PCR) – based methods, this molecular technique is concerned with the use of the heterogeneity in chromosomal number and patterns to identify Malassezia species. The probe used in these molecular analysis techniques is to identify the samples of Malassezia species whether obtained from culture media or be collected directly from non-culture sample from skin before performing the molecular analysis or the analysis can be done directly from samples obtained from patient skin scales.

### Materials and Method

#### Study group

Ninety five patients (40 females and 55 males) suffering from pityriasis versicolor skin disease who attended dermatology outpatient department (DOPD) of Al-Sader Teaching Hospital, Al-Basrah Teaching Hospital and Al-Faihaa Hospital were included in this study (from January 2016 to November 2018). Medical ethics requirements are fully followed during this study especially the collection of the samples under the supervision of the dermatologist and the approval of the patients. The demographic characteristics include gender, age, smoking, nutrition, marital state, education level and residency was recorded, clinical characteristics features of the disease sub divided into severity as mild with few macules (3-5) at one site, moderate more than 5 macules localized at certain area and sever that multiple patches involved large percentage of body surface.

#### Sample Collections

Ninety five samples were collected from patients with pityriasis versicolor in the form of skin scrapings took by sterile surgical blade, then transported in sterile containers and processed at the Mycology section of the Department of Microbiology. Direct and indirect methods were applied for diagnosis. Direct examination was done under microscope (40X) AL-Hammadani (1997). Indirect exam done with suitable steps depend on.

#### Molecular Identification test

Since the recognition of lipid dependent species, other than M.furfur, it became clear that molecular approaches are needed for better diagnostics, as well as our understanding of Malassezia community dynamics. It is noteworthy that the PCR- and sequence-based methods used for Malassezia biodiversity studies, and those employed to study Malassezia community structure on skin and molecular epidemiology are often similar and the distinction is not always clear. For instance, 13.8% of isolates identified by phenotypic means were found to be misidentified after molecular identification using sequence analysis of the D1/D2 domains of the large subunit ribosomal rDNA (LSU rDNA) and the ITS1+2 regions.

#### Isolation of DNA

DNA was extracted from thirty isolates for extraction of yeast genomic DNA: A small amount of yeast colony grown on m Dixon’s agar with 300 mg glass beadsand 300μl lysis buffer (Tris-HCl 100mM Ph=8, EDTA 30mM, SDS 0.5%w/v) were placed in a 1.8ml cryotube then mechanically milled in a homogenizer for 1-2 minutes. It was then boiled at 100°C for 20 min and then mixed with 150 μl of 3M Sodium acetate. After that, kept at -20°C for 10 min, and then centrifuged at 12000 g for 10 min at 40C. The supernatant was extracted by mixing with the same volume of phenol- chloroform- isoamyl alcohol (25:24:1 ) [short vortex] then centrifuged at 12000 g for 10 min at 40C. The supernatant was extracted by mixing with the same volume of chloroform[short vortex] then centrifuged at 12000 g for 10 min at 40C. The supernatant (DNA) was precipitated by adding of an equal volume of isopropanol (2- propanol) at -200C for 10 min then centrifuged at 12000 g for 10 min at 40C. Isopropanol was discarded and the DNA washed with 300 μl of 70% ethanol at 12000 g for 10 min at 40C [ note at this step don’t vortex the tubes ]. Ethanol was discarded and DNA dried and suspended in 50 μl of ultrapure water. Aliquots of 1 Ml of the resultant solution are used as template in the PCR reaction. At last The extracted DNA detected by electrophoresis on agarose gel with ethidium bromide under the U.V. transiluminator.

#### PCR Amplification

The ITS1-ITS4 and V9G - LS266 primer pairs were used to amplify the inverting 5.8S ribosomal DNA( rDNA complex ) and the adjacent ITS1 and ITS2 regions.

#### Primer Preparation

The ITS1 and ITS4 as well as V9G and LS266 primers were provided by Cinna Gen Company/Iran.
in lyophilized forms, dissolved in sterilized deionized distilled water to obtain 10 ml as a final concentration and stored in deep freezer until using, as shown in table (1).

**Preparation of PCR Reaction Mixture**

PCR reactions were carried out in (Thermo-cycler and Flex Cycler) PCR machines and components requirements for PCR reaction are provided in Table (2).

**Red pre-mix**: The PCR master mix contained all the components needed for the PCR reaction except DNA template and primer, it containing 25 μL of Taq DNA polymerase 2× Master Mix Red (Ampliqon; Skovlunde, Denmark).

**Detection of PCR Products**

Amplified DNA was running by 1.5 % agarose gel electrophoresis in TBE buffer staining with ethidium bromide and visualized under UV trans illuminator (Gel Doc System) to be sure that the PCR amplicon were correct.

**Sequencing**

To distinguish these isolates where morphology and PCR were not helpful, and moreover to introduce the sequence data of Basra Malassezia strains, 13 isolates were subjected to sequencing. The ITS PCR products for each sample were transferred to a 1.5 μl microtube then were subjected to sequencing on an ABI Prism TM 3730 genetic analyzer (Microsynth, Switzerland) with the V9G and LS266 primers then the obtained sequences were edited and blasted against NCBI database using standard criteria for a significant match for species identification.

**Phylogenetic analysis**

To discuss the phylogenetic relationships, the nucleotide sequences of each ITS1 -ITS2 region of rDNA of the 13 Malassezia isolates were analyzed by MEGA 5.05 and compared with sequences of different Malassezia species available in Gen Bank database (http://www.ncbi.nlm.nih.gov/Genbank/index.html) for each area investigated. Phylogenetic analysis was performed using Un weighted Pair Group Method with Arithmetic Mean (UPGMA) considering ITS1-ITS2 sequence of Aspergillus niger.

**Statistical Analysis**

Statistical Package for Social Science (SPSS) version 24, 2016 was used for statistical analysis of the data. Chi-square (χ2) and Fisher’s Exact tests were to determine the difference between the study groups. Comparisons of proportions were performed by crosstab using the χ2 test to assess the significance of difference between groups. The significance level was set at P < 0.05, and the highly significance level was set at P < 0.001.

**Results and Discussion**

**DNA extraction**

The results of DNA extraction showed that most of the isolates give single bundle of DNA on agarose gel (Fig 1).

**PCR Amplification and Detection**

According to PCR results, thirteen DNA samples (43.3%) were amplified after PCR program for amplification by two sets of primers ITS1 – ITS4 (Fig 3.2 a and b) and V9G - LS266 (Fig2a).

**Sequencing**

The thirteen isolates were subjected to sequencing, show the following results: eight isolates identified as Malassezia furfur and five as Malassezia globosa with the alignment bellow of each species.

*Malassezia furfur* isolate POL.10.11.IIIA 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence.
Sequence ID: KC152898.1 Length: 848 Number of Matches: 1 Range 1: 1 to 848.

<table>
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<tr>
<th>Strand</th>
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GAAA 60

Sbjct

1GGAAGTAAAAGTCGTAACCAAGGGTTTCTGTAGGTGAACCTGCAGAAGGATCATTAGT
GAAA 60

Query 61

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TTTA 120

Sbjct

GCAAGGGCCAGCCATAACGGACGGCGCTACTCGGTACAACGTCTCTGGCGCCCAAC
TTTA 120

Query 121

CACAATATCCACAAACCCGTGTGACCCGGTTTGATGAGTAGGCTCTCTCGCGAGGCA
GAC 180

Sbjct

CACAATATCCACAAACCCGTGTGACCCGGTTTGATGAGTAGGCTCTCTCGCGAGGCA
GAC 180

Query 181

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CGT 240

Sbjct

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GAAT 360

Sbjct 301
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GAAT 360
Query 361
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GA 420

Sbjct 361
CTTTGAACGCACCGCTCGCTCCATGGTATTCCGTGGAGCATGCCTGTTTGAGTGCCGT
GA 420
*Malassezia globosa* strain 149.1 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence

Sequence ID: KM454161.1 Length: 813 Number of Matches: 1

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Sbjct 589
ACTTTGCATCCGCTTCTGTAGGGGAAGCGGCAAGCGCCTCTGTATCATCAGGC
ATA 600

Sbjct 648
GCG 660

Sbjct 709
GCG 708

Sbjct 769
GCG 765

Sbjct 813
**Phylogenetic analysis**

Phylogenetic analysis of the 13 *Malassezia* isolates were analyzed by MEGA 5.05 and compared with sequences of different *Malassezia* species available in Gen Bank database, show a clear convergence between our *Malassezia* isolates and that of the Gen Bank database. As we mentioned before the methods for identifying *Malassezia* species, several disadvantages are noted for demand the high similarity in physiological test results between some species. Moreover, obtaining specific environmental conditions, culture medium compounds and type of chemical materials determine the use of these methods to identify *Malassezia* species. The present study aimed to identify the predominant species in Basra/Iraq, using a single step PCR assay in patients with pityriasis versicolor. Most studies around the world used PCR techniques to identify *Malassezia* species isolated from culture medium, and this method was consistent with the present study that two sets of primers were used in 143, the identification of the *Malassezia* species. While most other studies on molecular assays used the scotch tape technique for sampling of skin lesions (DNA of *Malassezia* species was extracted directly from skin scrapings).

**Table (1) Oligonucleotide primers used in the PCR reaction**

<table>
<thead>
<tr>
<th>primers</th>
<th>Sequences (5’-3’)</th>
<th>Size of Product</th>
<th>References</th>
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<tr>
<td>TS1</td>
<td>(5’-TCCGTAGGTGAACCTGCGG-3’)</td>
<td>~509 bp</td>
<td>(White, et al., 1990)</td>
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<tr>
<td>ITS4</td>
<td>(5’-TCCTCCGCTTATTGATATGC-3’)</td>
<td>~509 bp</td>
<td>(White, et al., 1990)</td>
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<tr>
<td>V9G</td>
<td>5’ TTACGTCCCTGCCCTTTTGA-3’)</td>
<td>~1700 bp</td>
<td>(Ende &amp; Hoog, 1999)</td>
</tr>
<tr>
<td>LS266</td>
<td>(5’ GCATCCCCAAAACAAGTCGACTC 3’)</td>
<td>~1700 bp</td>
<td>(Masclaux, et al., 1995)</td>
</tr>
</tbody>
</table>

**Table (2) Components required for PCR reaction**

<table>
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<th>No.</th>
<th>Components</th>
<th>Volume(µl)</th>
<th>Concentration</th>
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</thead>
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<td>Red pre-mix*</td>
<td>12.5</td>
<td>1X</td>
</tr>
<tr>
<td>2</td>
<td>ITS1 or V9G primers(30 pmol)</td>
<td>1</td>
<td>0.5 µM</td>
</tr>
<tr>
<td>3</td>
<td>ITS4 or LS266 primers(30 pmol)</td>
<td>1</td>
<td>0.5 µM</td>
</tr>
<tr>
<td>4</td>
<td>Template DNA</td>
<td>1</td>
<td>&lt;250ng</td>
</tr>
<tr>
<td>5</td>
<td>Nuclease Free water</td>
<td>Up to a final volume of 25 Ml</td>
<td>N.A</td>
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</table>
Malassezia furfur isolate POL.10.11.IIIA 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence. Sequence ID: KC152898.1 Length: 848 Number of Matches: 1 Range 1: 1 to 848. Phylogenetic analysis of the 13 Malassezia isolates were analyzed by MEGA 5.05 and compared with sequences of different Malassezia species available in Gen Bank database, the data showed a clear convergence between our Malassezia isolates from Basra patients and that of the Gen Bank database.

**Financial Disclosure:** There is no financial disclosure.

**Conflict of Interest:** None to declare.

**Ethical Clearance:** All experimental protocols were approved under the Department of Microbiology, Collage of Medicine, University of Basrah, Iraq and all experiments were carried out in accordance with approved guidelines.

**References**