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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

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activity profiles of the different *Malassezia* species., and Polymerase chain reaction (PCR) – based methods ,this molecular technique is concerned with the use of the heterogenecity in chromosomal number and patterns to identify *Malassezia* species [4,5]. The probe used in these molecular analysis techniques is to identify the samples of *Malassezia* species weather obtained from culture media or be collected directly from non-culture 17 sample from skin before performing the molecular analysis or the analysis can be done directly from samples obtained from patient skin scales [6].

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 [7].

Molecular Identification test

Since the recognition of lipid dependent species, other than M.furfur [8], 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 1000C for 20 min and then mixed with 150 µl of 3M Sodium acetate. After that, kept at -20 0C for 10 min, and then centrifuged at 12000 g for 10 min at40C. The supernatant was extracted by mixing with the same volume of phenol- chloroform- isoamyl alcohol (25:24:1) [short vortex] then centrifuged at 12000 gfor 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 ofIsopropanol (2- propanol) at -200C for 10 min then centrifuged at 12000 g for 10min at 40C. Isopropanol was discarded and the DNA washed with 300 µl of 70% ethanol at12000 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.[Take a short vortex and microfuge].Aliquots of 1 Ml of the resultant solution are used as template in the PCR reaction. 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 thePCR reaction except DNA template and primer, it containing 25 µL of Taq DNAPolymerase 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 To distinguish those 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 ($\chi 2$) and Fisher's Exact tests were to determine the difference between the study groups. Comparisons of proportions were performed by crosstab using the $\chi 2$ 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 Discusion

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: 1Range 1: 1 to 848.

Strand	Gaps	Identities	Expect	**Score**
Plus/Plus	0/848(0%)	848/848(100 %)	0.0()	1567 bits(848)

```
Query
1GGAAGTAAAAGTCGTAACAAGGTTTCTGTAGGTGAACCTGCAGAAGGATCATTAGT
GAAA 60
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct
1GGAAGTAAAAGTCGTAACAAGGTTTCTGTAGGTGAACCTGCAGAAGGATCATTAGT
GAAA 60
Query                                                    61
GCAAGGGCCAGCCATACGGACGGCGCTACTCGCGTACAACGTCTCTGGCGCCCAAC
TTTA 120
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct                                                    61
GCAAGGGCCAGCCATACGGACGGCGCTACTCGCGTACAACGTCTCTGGCGCCCAAC
TTTA 120
Query                                                   121
CACAATATCCACAAACCCGTGTGCACCGTTTGGATGAGTAGGCCTCCTCGCGAGGCA
GAC 180
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct                                                   121
CACAATATCCACAAACCCGTGTGCACCGTTTGGATGAGTAGGCCTCCTCGCGAGGCA
GAC 180
Query                                                   181
TCTCCAATCCATTTCTACCAAACTCGTATGGTTGTATGAACGTGGAAATCGTTGGAC
CGT 240
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
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Sbjct 181

TCTCCAATCCATTTCTACCAAACTCGTATGGTTGTATGAACGTGGAAATCGTTGGAC

CGT 240

Query 241

AACTGGCCAACAACCAATAATACAACTTTCGACAACGGATCTCTTGGTTCTCCCATC

GAT 300

||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||

Sbjct 241

AACTGGCCAACAACCAATAATACAACTTTCGACAACGGATCTCTTGGTTCTCCCATC

GAT 300

Query 301

GAAGAACGCAGCGAAACGCGATAGGTAATGTGAATTGCAGAATTCCGTGAATCATC

GAAT 360

||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||

Sbjct 301

GAAGAACGCAGCGAAACGCGATAGGTAATGTGAATTGCAGAATTCCGTGAATCATC

GAAT 360

Query 361

CTTTGAACGCACCTTGCGCTCCATGGTATTCCGTGGAGCATGCCTGTTTGAGTGCCGT

GA 420

||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||

Sbjct 361

CTTTGAACGCACCTTGCGCTCCATGGTATTCCGTGGAGCATGCCTGTTTGAGTGCCGT

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. Sequence ID: KM454161.1 Length: 813 Number of Matches: 1Range 1: 49 to 813.

Strand	**Gaps**	Identities	Expect	**Score**
Plus/Plus	0/765(0%)	765/765(100%	0.0()	1413 bits(765)

```
Query
1TCATTAGTGAAGATTCAAGGGCCAGCCATACAGACGTACAATAAGTGTGTCTCTGG
CGGC 60
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct                                                       49
TCATTAGTGAAGATTCAAGGGCCAGCCATACAGACGTACAATAAGTGTGTCTCTGGC
GGC              108              Query                     61
TCGTATCCACTATACATCCATAAACCCGTGTGCACTGTTAAGGAGTAAGAAAGAAG
GGGA 120
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct                                                      109
TCGTATCCACTATACATCCATAAACCCGTGTGCACTGTTAAGGAGTAAGAAAGAAG
GGGA 168
Query                                                      121
GGGAGAGAGTGCATGTGCTTTGCATATAACTCTCTCTCTTTCTCTTCCTTTCTCTCTCT
G 180
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct                                                      169
GGGAGAGAGTGCATGTGCTTTGCATATAACTCTCTCTCTTTCTCTTCCTTTCTCTCTCT
G 228
Query                                                      181
GTTAATTACACAAACTCGTATGGATTTGTATGAACGTGAGATATATCGTTGGACCGT
CAC 240
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||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct                                                    229
GTTAATTACACAAACTCGTATGGATTTGTATGAACGTGAGATATATCGTTGGACCGT
CAC 288
Query                                                    241
TGGCCAACAAATATAACACAACTTTCGACAACGGATCTCTTGGTTCTCCCATCGATG
AAG 300
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct                                                    289
TGGCCAACAAATATAACACAACTTTCGACAACGGATCTCTTGGTTCTCCCATCGATG
AAG 348
Query                                                    301
AACGCAGCGAAACGCGATAGGTAATGTGAATTGCAGAATTCCGTGAATCATCGAAT
CTTT 360
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct                                                    349
AACGCAGCGAAACGCGATAGGTAATGTGAATTGCAGAATTCCGTGAATCATCGAAT
CTTT 408
Query                                                    361
GAACGCACCTTGCGCTCTATGGTATTCCGTAGAGCATGCCTGTTTGAGTGCCGTGAA
TTC 420
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct                                                    409
GAACGCACCTTGCGCTCTATGGTATTCCGTAGAGCATGCCTGTTTGAGTGCCGTGAA
TTC 468
Query                                                    421
TCCCATCCCAAGCGGTTTTTATCAAAGAATTGCTAGGCGAAGGGGTTGAGATGGGCG
TTG 480
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
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Sbjct                                                          469
TCCCATCCCAAGCGGTTTTATCAAAGAATTGCTAGGCGAAGGGGTTGAGATGGGCG
TTG 528
Query                                                          481
TTATAACTGCTTTTCTTCTCTAGAAAAGCTCGCCCGAAAAACAGCTAACGCCTCTGG
GCC 540
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct                                                          529
TTATAACTGCTTTTCTTCTCTAGAAAAGCTCGCCCGAAAAACAGCTAACGCCTCTGG
GCC 588
Query                                                          541
ACTTTGCATCCGCTTCTCTGAGGGGAGAAGCGGCCAAGCGCGCTCTGATCATCAGGC
ATA 600
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct                                                          589
ACTTTGCATCCGCTTCTCTGAGGGGAGAAGCGGCCAAGCGCGCTCTGATCATCAGGC
ATA 648
Query                                                          601
GCATGATACGTCATTTGCTATGCTGTAGGAGAGCATTTGGTTGTGGTTATACCGCGT
GCG 660
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct                                                          649
GCATGATACGTCATTTGCTATGCTGTAGGAGAGCATTTGGTTGTGGTTATACCGCGT
GCG 708
Query                                                          661
TTATTTTTTTTTTGCAAACGCAAAGAAAAAAAAGCCCCCTTTCATTTCTGGTCTCAAA
TC 720
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct                                                          709
TTATTTTTTTTTTGCAAACGCAAAGAAAAAAAAGCCCCCTTTCATTTCTGGTCTCAAA
TC 768
Query 721 AGGTAGGATCACCCGCTGAACTTAAGCATATCAATAAGCGGAGGA 765
|||||||||||||||||||||||||||||||||||||||||||||
Sbjct 769 AGGTAGGATCACCCGCTGAACTTAAGCATATCAATAAGCGGAGGA 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

primers	**Sequences (5’-3’)**	**Size of Product**	**References**
TS1	(5´-TCCGTAGGTGAACCTGCGG-3´)	~509 bp	(White, *et al*., 1990)
ITS4	(5´-TCCTCCGCTTATTGATATGC-3´)	~509 bp	(White, *et al*., 1990)
V9G	(5′ TTACGTCCCTGCCCTTTGTA-3′)	~1700	(Ende & Hoog, 1999)
LS266	(5′ GCATTCCCAAACAACTCGACTC 3′)	~1700 bp	(Masclaux, *et al*.,1995)

Table (2) Components required for PCR reaction

No.	**Components**	**Volume(µl)**	**Concentration**
1	Red pre-mix*	12.5	1X
2	ITS1 or V9G primers(30 pmol)	1	0.5 µM
3	ITS4 or LS266 primers(30 pmol)	1	0.5 µM
4	Template DNA	1	<250ng
5	Nuclease Free water	Up to a final volume of 25 Ml	N.A

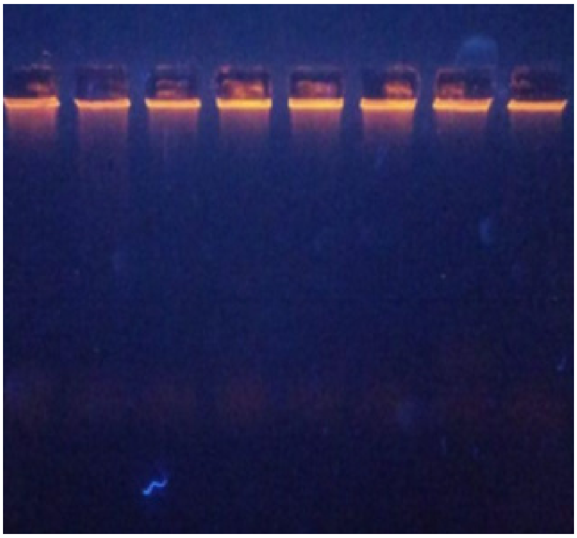

Figure 1: Single bands of DNA on Agarose Gel

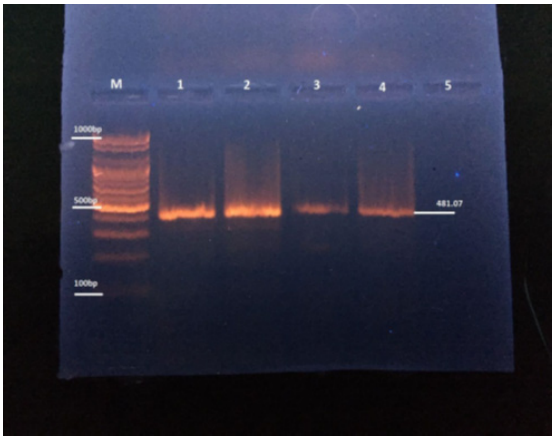

Figure 2 a: Electrophoresis of Agarose gel 1.2% (100 voltage for 60 minutes) with PCR products of *Malassezia* species (ITS1 - ITS4 primers), demonstrated by Gel Doc System, show that M: DNA Ladder (100-1000bp), 1-4 samples are *M.furfur,* 5 is a negative sample.

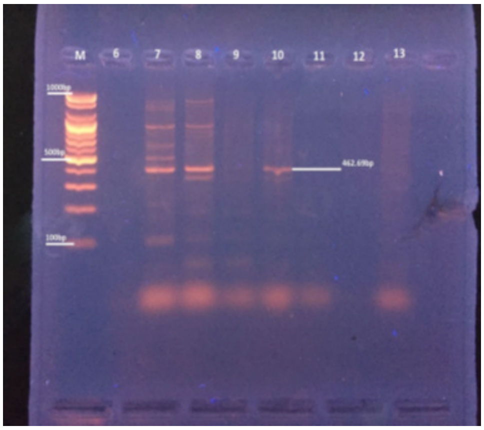

Figure 2 b: Electrophoresis of Agarose gel 1.2% (100 voltage for 60 minutes) with PCR products of *Malassezia* species (ITS1 - ITS4 primers), demonstrated by Gel Doc System, show that M: DNA Ladder (100-1000bp), 7,8 and 10 samples are *M. globosa,* 6,9,11-13 are negative samples.

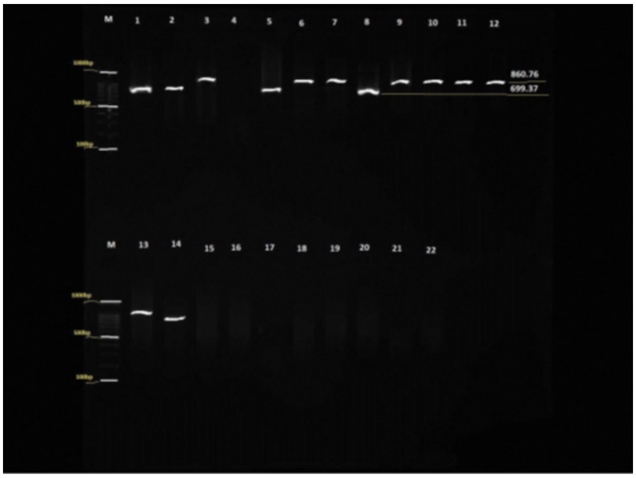

Figure 3: Electrophoresis of Agarose gel 1.2% (100 voltage for 60 minutes) with PCR products of *Malassezia* species (V9G - LS266 primers), demonstrated by Gel Doc System, show that M: DNA Ladder (100-1000bp),

Conclusion

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.

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.

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