Association of *Malassezia furfur* with Onychomycosis patients In Baghdad, Iraq

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Abstract

Background: Onychomycosis is a fungal nail infection. It infected about 10 % of the adults. The prevalence of onychomycosis is around 5% of fungal infections. Malassezia furfur is a basidiomycetous yeast and a part of normal human skin flora. By using biochemical, immunological and molecular methods on Malassezia yeast, this genus comprises 14 species. The higher detection rate of Malassezia furfur was observed by using molecular identification methods, the internal transcribed spacer (ITS) and large subunit (LSU) regions.

Objective: To detect of Malassezia furfur associated with Onychomycosis and evaluate the application of molecular method as rapid identification of *Malassezia furfur*.

Patients and Methods: Fifty samples have been collected from patients presented signs of onychomycosis in Al- Imammian Al-Kadhamain Teaching Hospital, Baghdad, Iraq for a period of six months (from 1st of October to the 30rd of April). Clinical diagnosis was done by consultant dermatologist. Twenty nine were males and twenty one were females, the mean age of studied group was (26.12 ± 9.89) years old, with age ranging from 10 to 60 years old. Fifty healthy volunteers as control includes nail clips, the range of ages was from 1 to 60 years old with a mean of (27.88 ± 12.03) years. Patients and control were investigated for M. furfur using phenotypic and molecular analysis.

Results: Males were more infected with onychomycosis than females, with a frequency percentage (58%). According to age, the most frequent among (31-40 years old) age groups. On the basis of Polymerase chain reaction (PCR) method it was showed a positive result in 6 (12%) out of 50 onychomycosis patients and a positive result in 1 (2%) out of 50 healthy volunteers.

Conclusion: From these findings, it was concluded that the males were most infected than females. PCR technique is sensitive, specific and less time consuming than cultural methods .

Key words: Malassezia furfur, Onychomycosis, Healthy Volunteers.

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Introduction

Onychomycosis is a fungal nail infection. It infected about 10 % of the adults [1] .The prevalence of onychomycosis is around 5% of fungal infections. This fungal infection constitutes about half of all nail problems[2].The most common symptom of onychomycosis is the nail becoming thickened ,and its vary in color. The appearance of infected nail become brittle, with removed from the toe or finger. If it left untreated, the skin around the nail become painful[3].

Malassezia furfur is a basidiomycetous yeast and a part of normal human skin flora [4;5].By using biochemical, immunological and molecular methods on Malassezia yeast, this genus comprises 14 species, with Malassezia furfur, M. pachydermatis, M. sympodialis, M. globosa, M. dermatis, M. restricta, M. slooffiae, M. japonica, M. nana,M. caprae, M. yamatoensis, M. equinaand M. Cuniculi have been identified, thus Malassezia spp. at present being agents of different dermatological diseases like pityriasis versicolor, seborrheic dermatitis and dandruff, atopic eczema, folliculitis, psoriasis and onychomycosis[6].

The causative fungi of Onychomycosis include dermatophytes, yeasts as Malassezia spp. and Candida spp., and molds[7].Dermatophytic fungi represent most causative agent fornail infectionparticularly in the western countries; while yeasts and non dermatophytes are most frequently responsible for this disease in the tropics and subtropics countries [8].

The important role of Malassezia furfur in onychomycosis is unknown because this fungus unableforkeratin degradation, the lyses of keratinis very importantfor this fungus to infect the nail [9].

Existence of Malassezia furfur in the sub ungula sites is necessary, because it is a source of systemic fungal infection[10]. Therefore, the patients who treated with immunosuppressive drugsmust be carefully noted for their primarily nail infection and subsequent with direct culture of infected nails [11].

Chowdhary et al., isolated Malassezia furfur from the nail clipping of a case 13 year old male patient[11].Crespo-Erchiga et al. ,said that the yeasts (Candida spp. And Malassezia spp.) as flora elements in subungual sites. Thus, because of its, the sub ungual sites are less infected[12].

The higher detection rate of Malassezia furfur was observed by using molecular identification methods, the internal transcribed spacer (ITS) and large subunit (LSU) regions are now perhaps the most widely sequenced DNA regions in fungi identification. DNA sequencing has been most useful for molecular detectionmethods. Polymorphisms in both of internal transcribed spacer (ITS) and large subunit (LSU) regions at inter- and intra-specific levels, guide agood practical markers for epidemiological studies[13].

Patients and Methods

One hundred samples were collected from patients presenting signs of onychomycosis and healthy volunteers in AL-Imammian Al-Kadhamain Teaching Hospital, Al-Obaidi primary and Secondary schools in Baghdad, Iraq, from 1st of October to the 30rd of April Studying groups aged ranging from 10 to 60 years old. A questionnaire was provided based on dermatological care program including name, age, demographic data, symptoms of disease, treatment history and disease complications. Nail clipper and scrapings were collected from 50 patients clinically diagnosis with onychomycosis by a sterile surgical blades and Nailclippings were obtained from 50 healthy volunteers (As control). All samples were stained with Lacto phenol cotton blue as direct preparation and then were cultured on Sabouraud's dextrose agar plus chloramphenicol (SC) (Merck, Germany) overlaid with olive oil and incubated at 37°C for 10 days and cultured on Chromagar *Malassezia* medium (Biomerieux, France) and incubated at 37°C for 2 days under aerobic condition. Finally, to differentiateM. furfur from other *Malassezia* spp., the samples were cultured on pigment induction medium and incubated at 32°C for 7days under aerobic condition .

***Malassezia furfur* DNA extraction**

Genomic DNA was extracted from *Malassezia furfur* isolates using wizard genomic DNA purificationkit according to manufacturer's directives (Promega, USA)with a few of modifications.In briefly, 1 ml of *Malassezia furfur* culture grown for 20 hours at 37°C in Yeast Potato Broth (YPB) broth (Sigma,USA) was transferred to a 1.5 ml microcentrifuge tube. The samples were centrifuged at 13,000 rpm for 3 minutes, then the yeast cells were re-suspended thoroughly in 300 µl of 50 mM EDTA (Sigma, USA).twenty µl of 20 mg/ml proteinase (Sigma, Missouri, USA) was added and carefully pipetted 3 times to mix, then samples were incubated at 37°C for an hour to digest the cell wall and cooled at room temperature. The samples were centrifuged at12,000 rpm for 3 minutes, the supernatant was removed and 300 µl of nuclei lysis solution was added to the pellet of cells, then mixed thoroughly. One hundred µl of protein precipitation solution (wizardgenomic DNA purification kit) was added and vortexed at high speed for 18 seconds, the samples were sit on ice for 5 mins. then centrifugated at 14,000 rpm for 2minutes. The DNA was added to clean microcentrifuge tube (1.5 ml)containing 800 µl of cold absolute ethanol and mixed by inversion until the DNA become visible agglomerate, then centrifuged at 14,000 rpm for 10 minutes. The supernatant wasdecanted in a carful, and the tubes were drained on cleanabsorptive paper and 300 µl of 70% ethanol was added, the tubes were ere shacked for mixing the content. The samples were centrifuged at 14,000 rpm for 3 minutes and all the ethanol was sucked out. The tubes were run out on absorptive clean paper and the pellet was dried for 10 minutes, fifty µl of DNA

rehydration solution (wizard genomic DNA purificationkit) was added. One point five µl of RNase solution was transferred to DNA sample was vortexed for 2 second, then centrifuged in a micro-centrifuge for 5 seconds to gather the liquid and incubated at 37°C for 15 minutes. The DNA sample was rehydrated by incubating at 65°C for 60 minutes and the solution was periodically mixed by carefully tapping the tube, then DNA sample was stored at -20°C until use.

DNA quantification

The extracted DNA was quantified spectrophotometrically at OD260/280 nm with ratios 1.4-1.5. The sensitivity of the ITS and LSU primers were assessed by PCR amplification (10ng-100ng) of purified *M.furfur* DNA.

Primers selection

The primers for ITSand LSU genes of M. furfur as the target genes were selected according to (14 and 15, respectively).These sets of primers were planned depend on the conserved sites in M. furfur; primers were synthesized byAlpha DNA, Kanda. The primers sequence of ITS and LSU genes and their product size are shown in Table (1).

PCR Master Mix

PCR reactions for detection of ITS gene of *M. furfur* were performed in 25 µl volumes containing 5.5 µl of nuclease free water, 12.5 µl of GoTaq Green Master Mix 2X containing (GoTaq DNA polymerase supplied in 2X Green GoTaqreaction buffer (pH 8.5), 400 µMdATP, 400 µMdGTP, 400 µMdCTP, 400 µMdTTP, 3 mM MgCl2, blue and yellow dyes which performed as loading dyes when reaction products are examined by agarose gel electrophoresis), 2. ITS-F1 primer and 2.5 µl of 20 pmol ITS-R4 primer and 2 µl of *M. furfur* DNA. PCR Master Mix for LSU gene of M. furfur was prepared as in ITS gene. All mixes were covered with 2 drops of mineral oil.

PCR program

PCR was accomplished in a thermal cycler device (Applied Biosystem 9902, Singapore) according to the PCR program described by(14 and 15), with some modification. Briefly, the Amplification of ITS gene of M. furfur was carried out with initial denaturation 94°C for 5 min. was followed by 30 cycles of denaturation at 94°C for 45S, annealing at (53 °C for 30s for ITS gene and 55 °C for 45s for LSU gene), and extension at 72°C for 1 min. The thermal cycles were finished by a final extension for 7 minutes at 72°C. Positive result and Nuclease free water as a negative control were used too.

Statistical Analysis

PCR products analysis of each of ITS and LSU genes of M. furfur were performed on 2% agarose gels. The 100 bp DNA ladder(Promega, USA) was applied and the gel was take place at 100 volt for 1 hour at RT. The products of PCR were stained with ethidium bromide and seen by an image analyzer (ChemiImager 5500, Alpha Innotech, USA).

Results

Fifty patients with onychomycosis were enrolled in this study, the range of ages was from 1 to 60 years, with a mean age of

(26.12 ± 9.89) years old, consisting of 29 males and 21 females (58% and 42% respectively) the most frequent among (31-40 years old) age groups. Control group includes nail clipping composed of 50 healthy volunteers, the rang of ages was from 1 to 60 years and a mean of (27.88 ± 12.03) years old. Males were 30 and females were 20 (60% and 40%, respectively) Table (2).

Table (1): The primers sequences of ITS and LSU genes of M. furfur and their product size.

Name of Primer	Sequence of Primer (5'-3')	Size of Product
ITS F1 ITS R4	GCATCGATGAAGAACGCAGC TCCTCCGCTTATTGATATGC	~ 509bp
LSU F1 LSU R4	TAACAAGGATTCCCCTAGTA ATTACGCCAGCATCCTAAG	~ 580 bp

Table (2): Age of patients enrolled in this study.

Study groups	Healthy Control	Onychomycosis
Mean	27.88	26.12
Standard Deviation	12.03	9.98
Median	27.05	25.50
Maximum	55.0	50.0
Minimum	7.00	5.00
P value	<0.001*	

* Highly statistical significant difference

Lacto phenol cotton blue stain and culture methods using Sabouraud's dextrose agar plus chloramphenicol (SC), Chrom agar Malassezia Medium (Figure 1) and pigment induction medium yielded positive result of M. furfur as 6 (12%) out of 50 onychomycosis and 1 (2%) out of 50 healthy volunteers.

Figure (1): *Malassezia furfur* colonies cultured on CHROM agar Malasseziamedium incubated at 32°C for 2 days.

Analysis of extracted DNA of *M. furfur* isolates

After performing of the DNA extraction from isolates, agarose gel electrophoresis was accomplished to ensure the appearance of M. furfur extracted DNA using 1% agarose gel at 7volt /cm for 1 hour Figure(2).

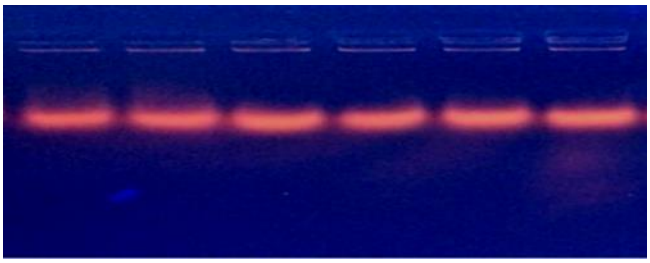

Figure (2) :Agarose gel electrophoresis of M. furfur extracted DNA using 1% agarose gel at 7 volt/ cm for 45 min. Lanes 1-10: extracted DNA.

Analysis of PCR products of M. furfur ITS and LSU genes.

Depending on the ITS and LSU sequence regions, a product of ~509 bp and ~580bp, respectively were amplified by PCR method showed positive results in 6 (12%) out of 50 patients with onychomycosis and one positive result as (2%) out of 50 healthy volunteers. (Figures 3 and 4, respectively).

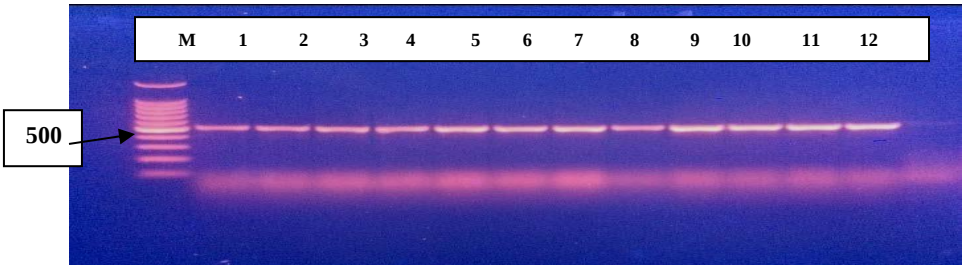

Figure (3): Agarose gel electrophoresis of singleplex PCR product of ITS gene for *M. furfur* using 1% agarose gel at 7volt/ cm for 1 hour. Site M: The DNA molecular weight marker (100 bp ladder), sites 1-12: singleplex PCR product of ITS gene.

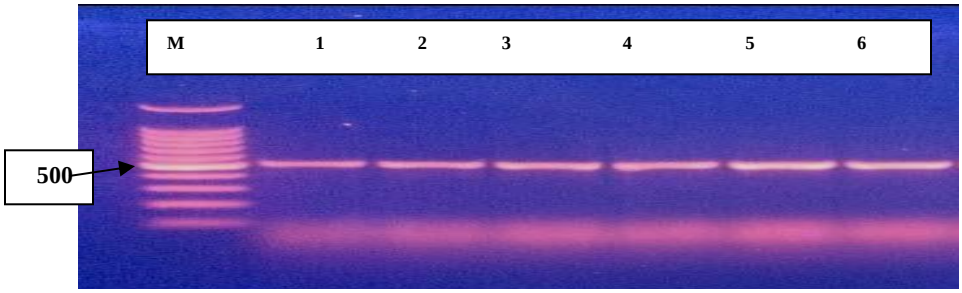

Figure (4): Agarose gel electrophoresis of singleplex PCR product of gene for *M. furfur* using 1% agarose gel at 7volt/ cm for 1 hour. Site M: The DNA molecular weight marker (100 bp ladder), sites 1-7: singleplex PCR product of LSU gene.

Discussion

Malassezia species, the causative agent of pityriasis versicolor, *Malassezia* (Pityrosporum) folliculitis, seborrheic dermatitis, dandruff, steroid acne, atopic dermatitis and psoriasis and less commonly with other dermatologic disorders such as confluent and reticulated papillamatosis, *Malassezia spp*. also found a causative

agent of onychomycosis, although it may exist as a colonizer of sub ungula sites of patients with nail disorder (16). In this study, 12% of onychomycosis caused by *Malassezia furfur* in comparison with healthy volunteers when M. furfur was positive in 2%. Prohic et al. (2015) *Malassezia furfur* was isolated in 1.8% (9), Escobar et al. proved the percentage of M. furfur was 30% in onychomycosis(17). Similarly, in a study conducted in Brazil, Malassezia spp. were isolated in 3.8% of onychomycosis. The important role of *Malassezia furfur* in onychomycosis is ideological because this species does not posses keratolytic capable therefore, it has not ability to invade nails, as they is not good source of lipids. Many factors play role in M. furfur pathogencity such as increased sebum production, hormonal fluctuations, stress, illness, infrequent shampooing, food allergies and vitamin B deficiency(18).

No guide of dermatophytic or non dermatophytic fungi to infect nail were examine by culture methods so the researchers went towards using Molecular methods that may be resolve the time consuming and the difficulties in interpretation of some morphological and physiological patterns.

This study agree with Chowdhary et al., (2005) while, disagree with Silva et al., (1997) and Prohic et al., (2015) who proved no significant statistically differences in the allocation of *Malassezia* furfur isolated according to epidemiological studies.

The differences in the number of enrolled cases, racial factor, habits of patients [20]. And the production of different enzymes such as phospholipase and lipase, all factors may play important roles in pathogencity of this yeast [21].

References

[1]Westerberg, D. and Voyack, M. (2013).Onychomycosis: current trends in diagnosis and treatment.". Am. F.J. 88 (11): 762–70.

[2]Ghannoum, M.and Nancy, I. (2014). Fungal Nail Infections (Onychomycosis): A Never-Ending Story?PLoSPathog. J. 10(6): 100-105.

[3]Abdullah,L. and Abbas, O. (2011).Common nail changes and disorders in older people.Can Fam Physician. 57(2): 173–181.

[4]Ashbee, H.; Evans, V. and Glyn, E. (2002).Immunology of Diseases Associated with Malasseziaspecies.Clin.Microbiol. Rev. J. 15 (1): 21-57.

[5]Gaitains, G.; Prokopios, M.; Markus, H.; Ioannis, D. and Aristea, V. (2012).The *Malassezia* Genus in Skin and Systemic Diseases.Clin.Microbiol. J. 25(14) : 106-141.

[6] Christopher, E.; Griffiths, M. and Peter, K. (2017).Psoriasis and Atopic Dermatitis.Dermatol T.J. 7(1): 31–41.

[7]Zareei, M.;Zibafar, E.;Daie, R.;Geramishoar, M.;Borjian, Z. and Hossein, L., (3013). Proximal onychomycosis due to *Malassezia furfur:* a case report. Teheran Univ Med J. 70(12):802–806.

[8] Chi, C.; Wang, Sh. and Chou, M. (2005). "The causative pathogens of onychomycosis in southern Taiwan". Mycoses.J. 48 (6): 413–20.
[9]Prohic A.; VlahovljakT. andSemra, C. (2015).The Prevalence and Species Composition of Malasseziaspecies in Patients with Clinically Onychomycosis.Med Arch. J. 69(2): 81–84. , S; Sadikovic.
[10]Gupta, A.; Batra, R.; Bluhm, R.; Boekhout, T. and Dawson, T. (2004).Skin diseases associated with Malasseziaspecies. Am. J. Acad. Dermatol.51:785-798.
[11] Chowdhary, A.; Randhawa, H.; Sharma, S.;Brandt, M. and Kumar, S. (2005).*Malassezia furfur* in a case of onychomycosis: colonizer or etiologic agent? Med. Mycol. J. 43:87-90.
[12]Erchiga, C. and Florencio, V. (2002).*Malassezia species* in skin diseases.CurrOpin Infect Dis J.15:133-42.
[13]Gupta, A.; Kohli, Y. and Summerbell, R. (2000).Molecular differentiation of seven *Malassezia species.J. Clin. Microbiol.* 38:1869–1875.
[14]Zunaina, E.; Wan, W.; Chan, Y.; Nur, H.; Balqis, K.; Siti, K.; Sabariah, O.;Zainul, F. and Manickam, R. (2008).Specific detection of fungal pathogens by 18S rRNA genePCR in microbial keratitis.BMC Ophthalmology, 8(7):1471.
[15] Lee, Y.; Ko, J.; Choe, Y. and Ahn, K. (2011). Epidemiologic study of Malassezia yeasts in patients with *Malassezia folliculitis by 26S rDNA PCR-RFLP analysis. Ann Dermatol. J.*, 23:177-184.
[16] Midgley G.(2000). The lipophilic yeasts: state of the art and prospects. Med Mycol.38:9–16.
[17] Escobar, M.; Carmona-Fonseca, J. and Santamaría, L.(1999).Onychomycosis due to Malassezia.Micol.J. 16(4):225-9.
[18] Laschinsky, T. (2010).Extreme Dandruff and Seborrheic Dermatitis Hair Conditions.
[19]Silva, V.; Moreno, G.;Zaror, L.; de-Oliveira, E. andFischman, O. (1997).Isolation of *Malassezia furfur from patients with onychomycosis. J* Med Vet Mycol.35(1):73–74.
[20]Buentke, E.; S. Zargari, L.;Heffler, J.; Avila-Carino, J.;Savolainen, C. and A. Scheynius (2000).Uptake of the yeast *Malassezia furfur* and its allergenic components by human immature CD1a+ dendritic cells. Clin. Exp. Allergy 30:1759-1770.
[21]Julian, R.; Stephen, J.;Challacombe, M. and Bernhard,H.(2003).Candida albicans Secreted Aspartyl Proteinases in Virulence and Pathogenesis.MicrobiolMolBiol Rev. 67(3): 400–428.