Detection of Substitution Mutations *of Malassezia furfur* isolated from Iraqi Immunocompetent and Immunocompromised Patients with Seborrheic Dermatitis

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Abstract
Seborrheic dermatitis (SD) is a chronic widespread dermatological disorder, it is characterized with Scaling, redness and inflammation of infected site. Immunocompromised patients have immune systems deficient such as AISD, diabetes mellitus, organ recipients and chemotherapy. The causative agent of SD is unknown, but it has been related to the opportunistic yeast of Malassezia furfur isolated from both healthy skin and at sites affected with SD. Sequencing of LSU region represents the most useful techniques in Malassezia furfur identification. Eighty patients suffering from Seborrheic dermatitis disease were selected from two groups: (i) Those with seborrheic dermatitis and (ii) Immunocompromised patients with SD. The age of studied population was ranging from 1 to 60 years old.

Direct examination of specimens was performed using KOH (10%) and staining with lacto phenol cotton blue and indirect examination using culture media and molecular methods to differentiate for M. furfur from other species.

According to the gender, males were more infected with SD than female. According to age groups, the most frequent group was 41-60 years old. The percentage of positive results of M. furfur among immunocompetent and immunocompromised seborrheic dermatitis patients was 15% and 12.5% respectively. Upon sequencing of twelve isolates of M. furfur which sent for sequencing, 2 out of 12 isolates appeared as substitution mutations, the origin of these isolates are immunocompromised patients with SD disease. The substitution mutations may play an important role in the pathogenesis of immunocompromised with SD disease. In addition, molecular methods were more rapid and specific in comparison with cultural methods to identify M. furfur.

Keywords: *Malassezia furfur*, Seborrheic dermatitis, Immunocompromised patients and Molecular methods.

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Introduction

Seborrheic dermatitis (SD) is a common chronic, widespread dermatological disorder, it is characterized with scaling, redness and inflammation of an infected site. Mainly, the infection is occurring on the body areas which are rich in sebaceous glands such as the scalp, face and chest. SD affects different ages (infants, adolescents and adults), ethnicities and races[13]. The prevalence of SD increases from 1 to 5% in the immunocompromised patients comparing with immunocompetent, especially among patients with diabetes, chemotherapy and 30–85% of AIDS patients[22]. Immunocompromised patients have immune system deficient due to congenital or acquired immunologic problems such as human immunodeficiency virus [HIV] infection, diabetes mellitus, cancer, emphysema, or cardiac failure, ICU care, malnutrition and immunosuppressive therapy of other disorders like radiation, cytotoxic chemotherapy, corticosteroids, monoclonal antibodies directed against a specific component of the immune system.

The causative agent of SD is unknown, but it has been related to the opportunistic yeast of *Malassezia* genus which usually appears as normal flora on human skin and other warm-blooded animals´ skin. This genus includes 14 species: *Malassezia furfur, M. pachydermatis M. sympodialis, M. restricta, M. globosa, M. slooffiae, M. obtusa, M. dermatis, M. yamatoensis*, *M. japonica, M. caprae, M. eqina, MM. nana* and *M. cuniculi*[23].

The clinical responses of SD to antifungal therapy have led many studies to relate the role of *Malassezia spp.* in the pathogenicity of this disease. The distribution of *Malassezia spp.* on the skin is predominantly on the face, scalp and trunk, all of which are lipid-rich anatomic locations. Importantly, these locations also are sites of predilection for clinical involvement with SD[4].

Malassezia furfur was isolated from both healthy skin and at sites affected with SD. The researchers have revealed the density of *M. furfur* in correlation with the antifungal drugs. *Malassezia* cells are present on human skin in summer more than in winter, these changes due to humidity and temperatures could be predisposing factors to the development of SD[1,20].

In the beginning, *M. furfur* was identified by phenotypic methods, clearing the results is difficult and contributed to

variable results which eventually led to use more advanced methods such as molecular techniques. Thus, more studies of *M. furfur* are being undertaken to discover new sub-species and to make advances in terms of understanding the relationship between factors like gender, age, immune system, environmental circumstances and geographical location with SD.[6,8] The molecular researches could prove a high level of genetic diversity within *M. furfur*[9,12], the molecular markers i.e. Large Sub Unit (LSU) and Internal Transcribed Spacer (ITS) allow sub-species identification. The phylogenetic studies using these markers confirmed intra-species variation among some *Malassezia* species[11]. The use of these regions will have the most useful techniques in *Malassezia spp.* identification[3,18,19].

Material and Methods

Study population: Eighty patients suffering from Seborrheic dermatitis disease who attended medical city of El-Imammaine Kadhmain, Baghdad, Iraq. Patients were selected from two groups: (i) Those with seborrheic dermatitis and (ii) Immunocompromised (diabetes, long term of corticosteroids and chemotherapy) patients with SD. Clinical investigation was done by consultant dermatologist. Forty healthy individuals were randomly selected from entities, primary and secondary students for a period of 7 months. The age of studying populations was ranging from 1 to 60 years old. The study was under the agreement of Iraqi Ministry of Health.

The information form was concerned with patients´ name, age, infected site, treatment history and disease complications. The sterile scalpels were used for specimens collecting from patients while skin swabs were used to obtain specimen from health's skin. The direct examination of specimens using KOH (10%) and staining with lacto phenol cotton blue and indirect examination using two culture media (Sabouraud's dextrose agar plus chloramphenicol overlaid with olive oil and Chrom agar Malassezia medium) was performed to differentiate for *M. furfur* from other species.

Molecular characterization: Pure culture of *M. furfur* was grown overnight at 37°C in YPD broth, the genomic DNA was extracted by wizard genomic DNA purification kit according to manufacturer's constructions (Promega, USA) with few modifications and as described by Al Ammari et al[2]. The quality of DNA was evaluated spectro-photometrically at OD260/280 nm with ratios 1.4-1.5, To confirm the presence of extracted DNA, ethidium bromide staining in agarose gel (1%) at 7 volt \cm for 1 hour was used. LSU primers F- 5'- TAACAAGGATTCCCCTAGTA-3' and R- ATTACGCCAGCATCCTAAG 3' were used for PCR analysis of *M. furfur* [24].

PCR reaction was performed for detection and to amplify the LSU gene in 25 µl volumes containing 5.5 µl of nuclease free water, 12.5 µl of GoTaq Green Master Mix 2X, 400 µM dATP, 400 µM dGTP, 400 µM dCTP, 400 µM dTTP, 3 mM $MgCl_2$, yellow and blue dyes which function as loading dyes when reaction products are visualized using agarose gel electrophoresis, 2. LSU-F1 primer and 2.5 µl of 20 pmol LSU-R4 primer and 2 µl of the genomic DNA sample. The mixture was loaded with 2 drops of mineral oil. Amplification was carried out in a thermo-cycler with an initial denaturation 94ºC for 5 min. followed by 30 cycles of denaturation at 94ºC for 45sec, annealing at 53ºC for 45sec and extension at 72C for 1 min. PCR cycles were finished by a final extension for 7 min at 72°C. In addition, positive control and nuclease free water as a negative control were performed[24].

The size of PCR products was determined on 2% agarose gels by comparison with molecular weight standard 100 bp DNA ladder and the gel was run for 1 hour at room temperature. The PCR products were stained with ethidium bromide and visualized by an image analyzer. For detection of substitution mutation of LSU region of *M. furfur*, twelve amplified LSU regions (four isolates were choice from each study group) were sent to Microgen Inc., South Korea for sequencing in both directions[16]. The sequencing results were assembled as contig format using ContigExpress module of Vector NTI 9.0 program. To detect the point of mutation, a reference genome was downloaded from the NCBI database and aligned against the study genomes using ClustalW method of MEGA4 program.

Results and Discussion

Eighty seborrheic dermatitis patients including forty immunocompetent and forty immunocompromised patients were included in the study. In both groups, the range of their ages was from 1-60 years, with a mean age of 25.70±9.12 years old whereas the mean age of healthy volunteers was 27.42±11.20 years old. Males were more infected with SD than female as in table 1. According to age groups, the most frequent group was 41-60 years old as in table 2.

Culture and molecular methods showed the percentage of positive results of *M. furfur* among immunocompetent and immunocompromised seborrheic dermatitis patients was 15% (6 out of 40) and 12.5% (5 out of 40) respectively whereas healthy volunteers gave around 27.5% (11 out of 40).

Genomic alignment confirmed that only two isolates (isolated from immunocompromised with SD disease) have one point of mutation in different positions. The position of substitution amino acid is (F) in isolate number *M. furfur*[3] whereas another position of substitution amino acid is (I) in *M. furfur*[9].

In the recent years, *Malassezia* genus plays an important role in the etiology of different dermatological disorders. *M. furfur* remains the most common cutaneous infection, both immunocompetent and immunocompromised patient with seborrheic dermatitis may be affected by this fungus. In immunocompetent populations, *Malassezia furfur* are

implicated in the pathogenesis of seborrheic dermatitis whereas in immunocompromised patients (AIDS, Chemotherapy, Diabetes and Organ transplant recipients), Seborrheic dermatitis is suspected the most common problem associated with *M. furfur*. It believes that it increased infection in patients with immune suppression. In immunocompromised patients, lesions of SD may spread more rapidly than immunocompetent. Seborrheic dermatitis appears to be more frequent in tropical countries, but it also has been reported in countries with a temperate climate[12]. A higher prevalence of *M. furfur* (2.1%) caused hemolytic infections[15].

Garcia Humbria et al[10] reported that no *M. furfur* was isolated from the patients with diabetic in Tunisia among 307 hospitalized diabetic patients. There was no difference found between two groups of patients[7] whereas the study in India hospitals could isolate 20% of *M. furfur*[17]. Another study could isolate *M. furfur* from 10% of the patients with diabetes[5].

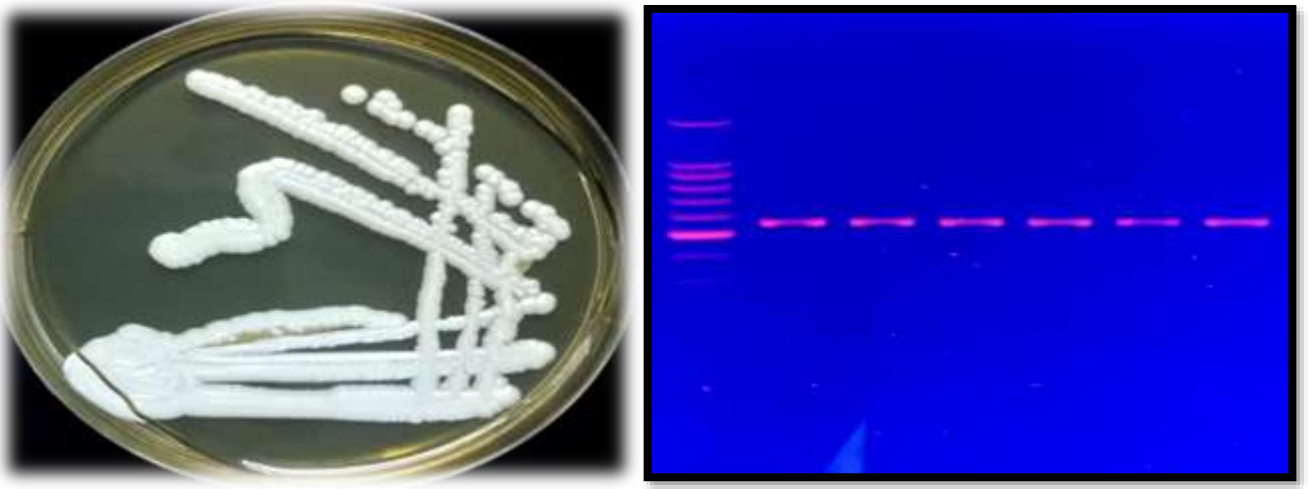
Table 1
Gender of individuals involved in the study

Gender		Study groups		
		HV*	**IC***	**IS***
Male	No.	20	24	29
	%	50%	60%	72.5%
Female	No.	20	16	11
	%	50%	40%	27.5%
Total	No.	40	40	40
	%	100%	100%	100%

*HV= Healthy Volunteers *IC= Immunocompetent *IS= Immunocompromised

Table 2
Ages of individuals involved in the study

Ages		Study groups		
		HV	**(IC)**	**(IS)**
1-20	No.	12	10	4
	%	30%	25%	10%
21-40	No.	16	15	12
	%	40%	37.5%	30%
41-60	No.	12	15	24
	%	30%	37.5%	60%
Total	No.	40	40	40
	%	100%	100%	100%



(a) **(b)**

Figure 1: (A) *Malassezia furfur* colonies cultured on Sabouraud′s dextrose agar over lied olive oil incubated at 37˚C for 1-2 weeks. (B) Gel electrophoresis using 1% agarose gel at 7volt /cm for 1 hour. Lane 1: 100 bp DNA ladder, lane 2-7: LSU PCR products of isolates

At the mycology laboratory at the National Skin Centre in Singapore, *M. furfur* infection constitutes 25.2% among a total of 12,903 cases of immunocompetent seborrheic dermatitis patients[21]. In contrast with our results, among the 300 patients with diabetes, *M. furfur* was seen more often (23%) than in the controls (8%)[14].

The differences in our findings and above studies may be due to difference in sampling techniques, control of diabetes, ecological factors (low temperature and skin´s pH), long term use of antibiotics and heredity and immunosuppressant factors.

The cultivated yeast showed colonies which appear creamy in color as in figure 1A. LSU gene, a product of ~580bp was amplified by PCR methods as in figure 1B.

Conclusion

According to the findings, this study concluded that molecular techniques are more rapid and specific to identify *M. furfur* in the immunocompetent and immunocompromised seborrheic dermatitis patients and the substitution mutations may play a vital role in the pathogenesis of immunocompromised with SD disease. Future researches should use other molecular methods with a large size of samples send for DNA-sequencing to determine the correlation between *Malassezia furfur* numbers and severity of seborrheic dermatitis disease.

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