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Molecular Study of *Malassezia furfur* Isolated from Pityriasis Versicolor Patients

Abbas M. Ammari[1] , Saife D. Al-Ahmer[2] , Azhar Al Attraqhchi[3]

[1]Biology Department / College of Science / University of Diyala..
[2]Institute of Genetic Engineering and Biotechnology for Postgraduate Studies, University of Baghdad.
[3]Medical Microbiology Department / College of Medicine / Al-Nahrain University.

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Abstract: Humans´ skin is the largest organ of the integumentary system; it has multiple layers of ectodermal tissue and guards the underlying muscles, bones, ligaments and internal organs. Pityriasis versicolor is the prototypical skin disease etiologically connected to *Malassezia* species. The large sub unit (*lsu*) gene is now perhaps the most widely sequenced DNA region in fungi. To identify *Malassezia furfur* associated with pityriasis versicolor patients and healthy control by using molecular detection methods. Sixty patients suffering from pityriasis versicolor disease who attended Imammian kadhamain Teaching Hospital and one hundred control individuals were randomly selected from (entities, primary and secondary schools) for a period of six months. Clinical diagnosis was done by consultant dermatologist. Forceps and surgical blades were used for skin scrapings collection. Direct and indirect methods were applied for diagnosis. *Malassezia furfur* was not grown on Tween 60 esculin agar, whilst it was grown on assimilation test of Tweens (20, 40, 60 and 80) containing SDA and pigment induction medium. In successful singleplex PCR reaction, the *lsu* gene product of 580 bp molecular weight was observed. Upon stratification of the *M. furfur* according to the gender in pityriasis versicolor patients and control groups. *M. furfur* was the most frequently isolated in males, with a percentage of 65% and 73.10%, respectively. As a conclusions from these findings, it was suggested that pityriasis versicolor was more infection in male than female. Also the chest was the most infected lesions associated with *Malassezia furfur*.

Keywords: *Malassezia furfur*, Pityriasis versicolor, singleplex PCR.

Corresponding author: (Email: saifealahmer@yahoo.com).

Introduction:

Human skin is similar to that of most other mammals, except that it is not protected by a pelt, though nearly all human skin is covered with hair follicles, it appears hairless, the skin is divided into two general types, hairy and glabrous skin (1). Pityriasis versicolor is the prototypical skin disease etiologically connected to *Malassezia* species. (2). There are two main facts that permit an etiologic association of *M. furfur* with pityriasis versicolor; (i) it is more likely that a positive culture will be obtained from specimens taken from lesional skin than from macroscopically unaffected skin areas of either the same individual or matched healthy controls (3), and (ii) the hyphal state is connected to pityriasis versicolor lesions, independently of the *Malassezia furfur* isolated, and seems to play an important role in the pathogenesis of this disease. *Malassezia* species are lipophilic fungi that recovered in 75~98% of healthy adults (4). *Malassezia* species are non

mycelial, unipolar budding yeasts characterized by a thick cell wall (5). The genus of *Malassezia* are classified into 14 species; *M. furfur, M. pachydermatis, M. sympodialis, M. globosa, M. obtusa, M. restricta, M. slooffiae, M. dermatis, M. japonica, M. nana, M. yamatoensis, M. caprae, M. equina* and *M. Malassezia furfur* is a coccal, and their cells contain a plasma membrane, a thick and multicellular cell wall composed of chitin (6). The *lsu* gene is now perhaps the most widely sequenced DNA region in fungi. It has typically been most useful for molecular systematic at species level, and even within species (7).

Materials and methods:

Samples collection:

Sixty patients suffering from pityriasis versicolor disease who attended Imammian kadhamain Teaching Hospital and one hundred control individuals were randomly selected from entities, primary and secondary schools for a period of six months. Clinical diagnosis was done by consultant dermatologist. Forceps and surgical blades were used for skin scrapings. Direct and indirect methods were applied for diagnosis (8). Scale specimens were subjected for direct examination by placing on a clean slide mounted with a drop of 10 % KOH to dissolved keratinized material, covered with a cover slip, and then the slides were warmed gently and examined under light microscope (40X). To microscopic examination of the yeast cells, the suspension of yeast cells were prepared, loopful of this suspension was spread on sterile glass slide, and then stained with lactophenol cotton blue (9).

Phenotypic characterization:

Scales and swabs were inoculated into sabroud's dextrose agar (SDA) containing 0.5 ml/ L chloramphenicol, penicillin at concentration of 0.4 ml/ L and streptomycin at a concentration of 2 ml/ L overly with olive oil. The universals were incubated at 37°C for 1-2 weeks. (10). The suspension was obtained by inoculating 5 ml of sterile distilled water with a loopful of actively growing yeast and the concentration was adjusted to about 105 cell/ ml (11). Yeast cells were cultured on pigment production medium. After sterilization and cooling at room temperature, the suspension was smeared on the agar medium using sterile swab. The plates were incubated at 32°C for 2-4 weeks. Production of brown pigment was considered as a positive result for *Malassezia furfur* (12).

According to the method reported by Guillot *et al.*, (1996), yeast cells of 2x10 to 3x10 cfu\ml were suspended in 1 ml sterile distilled water and poured into plate containing SDA with 0.5 ml/ L chloramphenicol, penicillin at concentration of 0.4 ml/ L and streptomycin at concentration of 2 ml/ L cooled at about 50°C. The inoculum was then spread evenly. After solidification, four holes were made by means of a 2 mm diameter punch and filled with 5 µl of Tween 20, 40, 60 and 80, respectively. The plates were incubated for 1 week at 32°C. Utilization of Tween was assessed by the degree of growth and/ or precipitate reaction of *M. furfur* around the wells (13). Glucosidase activity was

assayed by using esculin agar tube. Using a loop, the yeast inoculum was deeply inoculated into the agar and incubated at 32°C for 5 days. The negative result revealed splitting of esculin into esculetin and glucose is revealed by darkening of the medium with liberation of soluble ferric salt incorporated in the medium (14).

Molecular characterization:

Brain heart infusion broth (100 ml) was inoculated with one loop full fungal growth and left for overnight incubation at 37 °C, broth was collected 3 ml of a culture grown for 20 hrs in brain heart infusion broth then added to two micro-centrifuge tubes (1.5 ml each). The cells were harvested by centrifugation at 13000 rpm for 3 minutes. DNA was extracted as described (15).

Conventional PCR technique:

The forward primer (TAACAAGGATTCCCCTAGTA) and reverse primer (ATTACGCCAGCATCCTAAG) were selected to allow the amplification of *lsu* gene in *M. furfur*. singleplex PCR amplification was carried out in a final volume of 50 µl. Each reaction contained 4 µl of template DNA, 2 µl of each primer, 20 µl of Go Taq green master mix and 22 µl of nuclease free water. An initial denaturation step at 94 ◦C for 5 minute was followed by 30 cycles of denaturation at 94 ◦C for 45 seconds. Annealing at 50 ◦C for 45 seconds, and extension at 72 ◦C for 1 minute with final extension step at 72 ◦C for 7 minutes. Amplified product was visualized by 1.5% (w/ v) agarose gel electrophoresis in TBE buffer, stained with ethidium bromide (0.5 µg/ ml) and photographed under UV transillumination (16).

Results:

A total of sixty patients had been included in the present study with ages ranging from 1 to 70 years, with a mean age of (28.63 ± 11.83) years for pityriasis versicolor patients, consisting of 40 (66.70%) males and 20 (33.30%) females. Control group included skin swabs collected from 120 apparently healthy volunteers, with ages ranging from 1 to 70 years with a mean of (30.03 ± 14.58 years). Males were 83 (69.20%) and females were 37 (30.80%) (Table 1).

Table (1): Age of individuals involved in the present study.

Study groups	Healthy control	Versicolor patients
Mean	30.03	28.63
St. Deviation	14.58	11.83
Median	29.50	28.00
Maximum	60.00	55.00
Minimum	9.00	6.00
P value	**<0.001***	

Isolated colonies on Sabouraud′s dextrose agar overly with olive oil were selected for this study. *Malassezia furfur* was identified according to their morphological features and physiological properties. The morphology of the yeast cells was studied by preparing lacto phenol cotton blue stained smears of the isolates from Sabouraud′s dextrose agar after one week incubation at 37 °C.

Based on the gross morphology of the colonies on culture media, the colonies were raised and smooth initially and get dry and wrinkled in time the color of *Malassezia* colonies was white to creamy (17). *Malassezia furfur* was not grown on Tween 60 esculin agar, whilst it was grown on assimilation test of Tweens 20, 40, 60 and 80 containing Sabouraud′s dextrose agar and pigment induction medium (18).

Macroscopic appearance:

Skin scraping and swapping samples were collected from different sites of patients and healthy control, with different characteristic features (Figure 1), wherease (Figure 2) shows different colonies which were appeared as white to creamy colored in different textures.

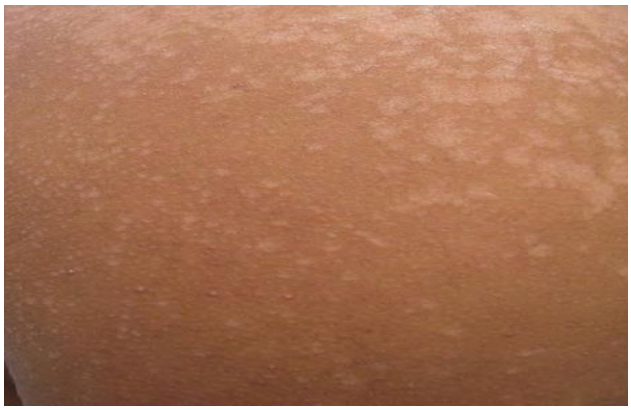

Figure (1): Gross appearance pityriasis versicolor in back.

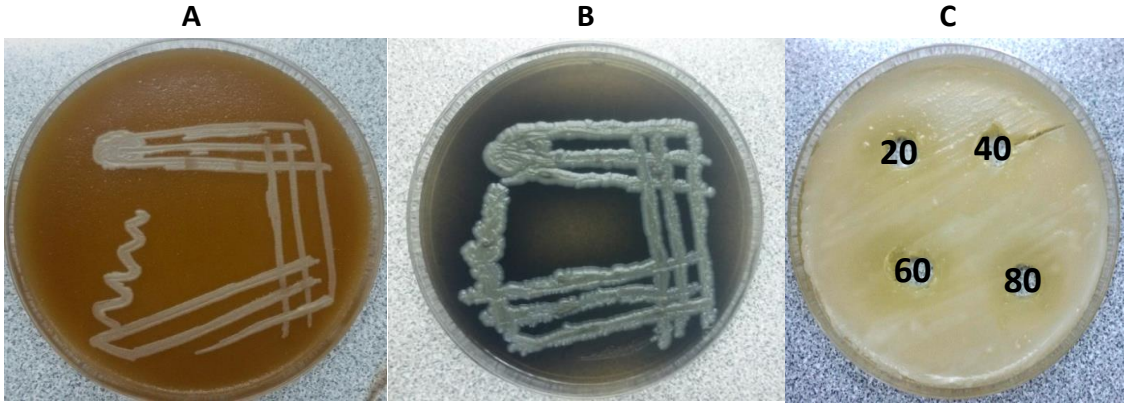

Figure (2): *Malassezia furfur* colonies cultured on: (A) Pigment induction medium incubated at 32°C for 2 to 4 weeks, (B) Esculin Tween-60 agar incubated at 37°C for 1 week. and (C) Tween assimilations incubated at 32°C for 1 week.

Microscopic appearance:

Direct microscopic examination shows short thick hyphae and spores were arranged mostly in grape like clusters. The number of spores and hyphae were changeable from lesion to another and in different patients. In some preparations they were very scanty and difficult to detect, in others the scraping seems to consist completely of hyphae and spores collections (Figure 3).

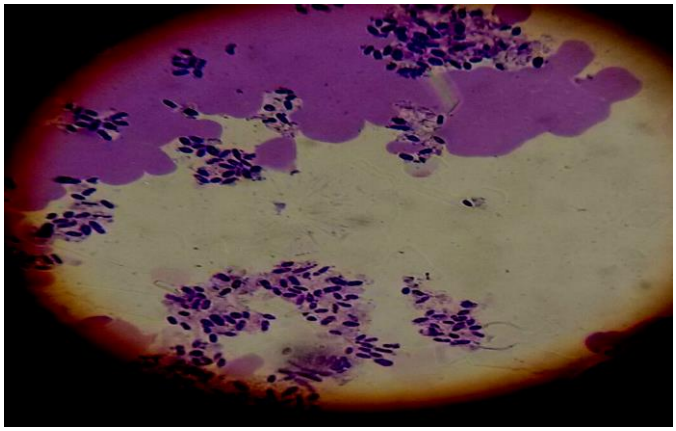

Figure (3): *Malassezia furfur* stained with Lactophenol cotton blue (X40).

Molecular identification of *Malassezia furfur* :

In successful singleplex PCR reaction, the *lsu* gene product of 580 bp molecular weight was observed. This was considered as a mandatory sign to successful reaction; upon agarose gel electrophoresis, its band was located between 500 and 600 bp bands of the 100 bp DNA ladder indicating the presence *lsu* gene. Upon stratification of the *M. furfur* according to the gender in pityriasis versicolor patients and control groups. *M. furfur* was the most frequently isolated in males, with a percentage of 65.0% and 73.10%, respectively (Table 2). No statistically significant difference was observed between males and females among pityriasis versicolor patients and control groups with a P value 0.459 and 0.884, respectively.

Table (2): Rate of *Malassezia furfur* in patients with pityriasis versicolor in comparison with control group concerning gender.

Study groups				Results			Total	P value
				Neg.	*M. furfur*	*M.* spp.		
Healthy control	Type of gender	Female	Co.	18	7	12	37	0.884 * NS
			%	31.6%	26.9%	32.4%	30.8%	
		Male	Co.	39	19	25	83	
			%	68.4%	73.1%	67.6%	69.2%	
	Total		Co.	57	26	37	120	
			%	100.%	100.%	100.0%	100.0%	
PV. patients	Type of gender	Female	Co.	7	7	6	20	0.459 * NS
			%	43.8%	35.0%	25.0%	33.3%	
		Male	Co.	9	13	18	40	
			%	56.3%	65.0%	75.0%	66.7%	
	Total		Co.	16	20	24	60	
			%	100.%	100.%	100.0%	100.0%	

*** NS = No significant differences.**

Site of lesions and *Malassezia furfur* :

Upon stratification of the isolated *M. furfur* according to the site of lesions in patients with pityriasis versicolor. ***M. furfur*** was most frequently isolated from chest comparing with other sites, with a percentage of 35.0%. While,

among control groups, *M. furfur* and was the most predominant isolated from upper limbs and chest in equal proportion, with a percentage of 23.10%, (Table 3). No statistically significant differences were detected among site of lesions in both of patients with pityriasis versicolor and healthy control (p= 0.968 and p= 0.825, respectively).

Table (3): Percentage of *Malassezia furfur* isolated from pityriasis versicolor patients in comparison with control group concerning site of lesions.

Study groups				Results			Total	P value
				Neg.	*M. furfur*	*M.* spp.		
Healthy control	Site of lesions	Head	Co.	8	2	3	13	0.825 * NS
			%	14.0%	7.7%	8.1%	10.8%	
		Neck	Co.	8	3	4	15	
			%	14.0%	11.5%	10.8%	12.5%	
		Chest	Co.	6	6	9	21	
			%	10.5%	23.1%	24.3%	17.5%	
		Back	Co.	8	5	7	20	
			%	14.0%	19.2%	18.9%	16.7%	
		Lower limb	Co.	14	4	6	24	
			%	24.6%	15.4%	16.2%	20.0%	
		Upper limb	Co.	13	6	8	27	
			%	22.8%	23.1%	21.6%	22.5%	
	Total		Co.	57	26	37	120	
			%	100.0%	100.0%	100.0%	100.0%	
PV. patients	Site of lesions	Head	Co.	0	0	0	0	0.968 * NS
			%	0.0%	0.0%	0.0%	0.0%	
		Neck	Co.	5	6	7	18	
			%	31.3%	30.0%	29.2%	30.0%	
		Chest	Co.	4	7	9	20	
			%	25.0%	35.0%	37.5%	33.3%	
		Back	Co.	3	4	3	10	
			%	18.8%	20.0%	12.5%	16.7%	
		Lower limbs	Co.	0	0	0	0	
			%	0.0%	0.0%	0.0%	0.0%	
		Upper limb	Co.	4	3	5	12	
			%	25.0%	15.0%	20.8%	20.0%	
	Total		Co.	16	20	24	60	
			%	100.0%	100.0%	100.0%	100.0%	

*** NS = No significant differences.**

Discussion:

Laboratory detection methods are influenced by different conditions including temperature, incubation period, density of inoculums and nutrients concentration used in culture media. Thus, molecular detection methods become more important as a technique (19). Accurate identifications of the species are needed to obtain a better understanding of the role of each species in the etiology of disease, and to facilitate adequate treatment, this can be determined based on species-specific susceptibilities to antifungal agents (20).

Many factors play role in *Malassezia* pathogencity such as Sebum production, hormonal fluctuations,

illness, food allergies, vitamin D deficiency and cold weather (21). The LSU gene which was targeted in this study contains highly conserves bases sequences and sufficient sequence variations for interspecies specific identification (22). The high frequency of chest as sites of infection may be attributed to the fact that scalp, upper trunk and the face are rich in sebaceous glands compared to other parts of the body. However, our results in this regard are consistent with those reported by (23).

Conclusions:

According to the findings, it was suggested that pityriasis versicolor was more infection in male than female. Also the chest was the most infected lesions associated with *Malassezia furfur*.

Recommendations:

We recommended using immunological, histological and other molecular methods to study the pathogencity of *Malassezia furfur* and its role to other skin diseases compared with healthy control.

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