Original Article

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Molecular and Phenotypic Identification and Speciation of Malassezia Yeasts Isolated from Egyptian Patients with Pityriasis Versicolor

Microbiology Section

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ABSTRACT

Introduction: Pityriasis Versicolor (PV) is a common health problem caused by genus *Malassezia*, a lipophilic fungi found as a part of the normal flora of skin. Although PV is common in Egypt, there is little information regarding the *Malassezia* species distribution in PV patients to date.

Aim: To spot a light on the distribution and clinico-epidemiological features of the *Malassezia* species in PV patients and healthy individuals that were established by conventional phenotypic and molecular techniques.

Materials and Methods: A cross-sectional study including 167 individuals; 137 clinically suspected PV patients attending Mansoura University Hospitals, Egypt and 30 healthy control individuals, was carried out. Characterization of *Malassezia* species was performed phenotypically by conventional, culture-based methods and biochemical tests. Genomic DNA was extracted from isolated colonies for PCR amplification of the highly conserved 26S rDNA region with further species level identification by Restriction Fragment Length Polymorphism (RFLP) using Hha1 and BstC1 enzymes. The association of *Malassezia* species with epidemiological profile and clinical characteristics was studied.

Results: A 94.2% of PV samples and 13.3% of control samples were positive by Potassium Hydroxide (KOH) while 71.5% of PV samples and 16.7% of control samples yielded growth in culture with high statistically significant differences (p=0.0001, for both methods). By phenotypic methods, only 75.5% of isolates from patients were identified as: *M. furfur* (51.4%), *M. globosa*, (29.7%), *M. restricta* (13.5%) and *M. pachydermatis* (5.4%) while by RFLP technique, six species were revealed: *M. furfur* (44.9%), *M. globosa* (24.5%), *M. sympodialis* (12.2 %), *M. restricta* (10.2%), *M. obtusa* (4.1%) and *M. pachydermatis* (4.1%). Most species were isolated from hypopigmented lesions of PV patients aged between 20-29 years. Neck and back were the most common affected sites. Only *M. furfur* (10%) and *M. globosa* (6.7%) were identified in healthy controls.

Conclusion: *M. furfur* and *M. globosa* are the commonly encountered species in both healthy and diseased human skin although other species were identified in PV patients. PCR-RFLP method represents a considerably accurate technique in identification of different *Malassezia* species for better understanding of their effect on the clinico-epidemiological characterization of PV patients in Egypt.

Keywords: Lipophilic yeasts, PCR-restriction fragment length polymorphism, Tinea versicolor

INTRODUCTION

Malassezia are lipophilic yeasts found as normal skin flora in 75%-98% of healthy adults, and is also responsible for skin-related diseases for example: PV, seborrheic/atopic dermatitis, dandruff and complicated systemic fungal infections especially in immunocompromised infants and young children [1,2]. They inhabit skin sites rich in sebaceous glands such as the forehead, the interscapular region and thorax as they produce lipase enzyme which is able to release fatty acids from the triglycerides present in the sebum [3,4]. Being a dimorphic fungi, the conversion of the *Malassezia* species from the yeast to the mold phase enables them to penetrate the corneocytes and invade the stratum corneum causing PV [5,6]. PV is a chronic cutaneous pigmentation disease, difficult to cure with high chances of relapse and recurrence [7,8]. Its current treatment is characterized by unpredictable clinical efficacy with emerging resistance and several side effects [1]. These drawbacks have increased interest in gaining an accurate identification of the causative *Malassezia* species to start prompt therapy without delay [3,8]. As *Malassezia* species have similar morphological and biochemical features, the currently used phenotypic techniques for diagnosis of PV usually do not allow rapid and exact characterization [5]. In addition, they are time-consuming, multi-step processes requiring several experimental methods. Therefore, several molecular typing methods have been used successfully, resulting in identification and classification of new *Malassezia* species [9].

Genus *Malassezia* includes 14 species, namely *M. furfur*, *M. sympodialis*, *M. globosa*, *M. restricta*, *M. slooffiae*, *M. obtusa*, *M. dermatis*, *M. japonica* and *M. yamatoensis* associated with normal human flora but can also cause skin lesions, and *M. pachydermatis*, *M. nana*, *M. equina*, *M. caprae* and *M. cuniculi* are associated with animals. Few studies found that *M. pachydermatis* may be transmitted to humans from pets [7,10]. *M. pachydermmatis* is the only lipid independent species while others are lipid dependent [11].

In Egypt, among other skin diseases, the incidence of PV ranged between (10-14%) [12]. Other researchers reported that the incidence of PV increased markedly to about 40.8% and 30% among other dermatomycosis in Assiut Governorate and new valley governorate respectively [13]. Inspite of treatment, the recurrence rate of *Malassezia* is about 60% in the first year and 80% in the second year. So, accurate laboratory diagnosis of the *Malassezia* species causing PV disease is required [14,15].

So far, there is still debate on the specific types associated with the development of PV, with varying results from different countries. Little data about *Malassezia* species is available from Egypt [16,17].

Therefore, the focus of the present study was to identify and analyse the distribution and clinico-epidemiological features of the *Malassezia* species in both Egyptian PV patients and healthy

individuals by using of phenotypic and molecular methods.

MATERIALS AND METHODS

The study was conducted at outpatient clinics of Dermatology Department of Mansoura University Hospitals from October 2012 to September 2013. The principles outlined in the Declaration of Helsinki were followed and informed consents were obtained from all patients and healthy subjects [18]. The study protocol was approved by the local Ethics Committee of Mansoura University, Egypt.

Patients: A total of 137 clinically suspected PV patients (70 male and 67 female with the mean age 25.5±8.3 years) were included. Their characteristic lesions were scaly hypopigmented or hyperpigmented well-defined macules, with slight desquamation and colour ranging from white to brown [12]. Thirty healthy individuals of matched age and sex (21 male and 9 female with the mean age 21.5±9.2 years) were included as a control group. The samples were taken mainly from healthy skin of chest and back [10].

Inclusion criteria: Characteristic lesions of hypopigmented or hyperpigmented scaly macules and patches which may be associated with itching. The lesions lasting atleast one year were defined as longstanding forms and considered extensive if the achromic and/or hyperchromic lesions were distributed on at least four out of the following nine body sites (face, neck, back, chest, abdomen, axilla, arm, buttocks and genital area) and each site covered by more than 20 lesions [19]. The skin scales were collected where most of the lesions were evident with repeated scraping after asking patients not to wash themselves in the area of hypochromic lesions.

Exclusion criteria: Patients with skin lesions of extensive desquamation or inflammation that did not match PV. Patients who had received topical antifungal therapy within last three months or oral antifungal therapy within the last six months.

Specimen Collection: The lesions of PV patients were first cleaned with 70% ethyl alcohol prior to sampling. Skin scrapings were taken from most scaly site using sterile scalpel blade and transported in a sterile filter paper to the laboratory where further investigations were done. In addition, skin samples from healthy individuals were taken by means of sellotape from different healthy skin areas of chest and back.

Sample Processing

Direct microscopy: Microscopic examination of the scales was performed after treatment with 10-20% KOH. Characteristic spaghetti and meatballs appearance (short curved hyphae and round yeasts) confirmed the diagnosis of PV.

Culture [20,21]: All samples were inoculated in slants of Sabouraud's Dextrose Agar (SDA) and Sabouraud's Dextrose over laid with sterile olive oil (Oxoid) for isolation of lipid independent species and lipid dependent species, respectively and supplemented with cycloheximide and chloramphenicol. All the slants were incubated at 32°C and examined on days 3, 7 and then at weekly intervals up to three weeks for any developing colonies.

Smears from the colonies were stained with Gram's stain and examined under microscope for identification of *Malassezia*. Mixed cultures were excluded. After three weeks of incubation, the culture slants without growth were considered negative and discarded. In order to achieve pure cultures, for each positive sample five colonies were subcultured on SDA and stored at -20°C until PCR analysis.

Species identification procedures by phenotypic methods [7,21]: Further species identification of *Malassezia* isolates was done according to their morphological features, physiological properties and conventional mycological methods. They include cultural characteristics, Gram staining for yeast phase, lactophenol cotton blue for mycelial phase and biochemical criteria as catalase test, urease test and Tweens assimilation test (Tween 20, 40, 60, and 80).

Morphological features [14]: *Malassezia* species were differentiated based on examination of colonial and microscopic morphologies i.e., colonies' colour, shape and texture on SDA medium, and cell shape, size and bud pattern in stained smears.

Biochemical tests [7,21]: The isolates of *M. restricta* were identified by the negative catalase test while, the isolates of *M. furfur* and *M. pachydermatis* were identified by the positive urease reaction. Moreover, the isolates of *M. pachydermatis* were identified by ability to grow on a lipid-free medium.

Tween assimilation test [22]: As reported by Kaneko T et al., some *Malassezia* species have the ability to utilize the different Tween compounds as a unique lipid supplement. From each isolate, a colony suspension (of at least 10^7cfu/mL) in 2 mL sterilized distilled water was made and poured onto SDA plate. The inoculums were then spread evenly. After solidification of each plate at 45°C, four wells were made and filled with 5 µL of a Tween compound (20, 40 and 80, respectively). These plates were incubated at 32°C for a week and the resulting growth was assessed around each well three times (after 2, 4 and 7 days).

Species Identification By Molecular Methods

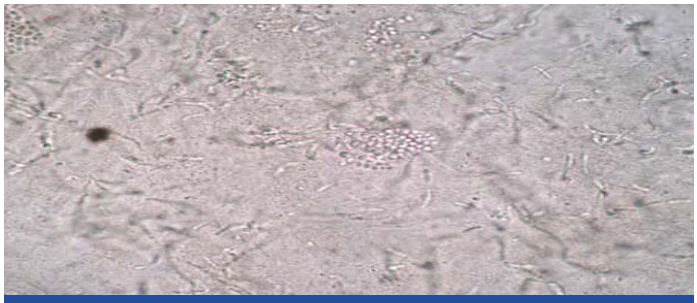
DNA extraction: Pure colonies obtained from SDA subculture were subjected to repeated freezing and thawing to dissolve their cell walls mechanically. The QIAamp tissue kit (Qiagen, Germany) was used for DNA extraction from yeast according to manufacturer instructions.

PCR-Restriction Fragment Length Polymorphism (PCR-RFLP) of 26S rDNA [23,24]: PCR-based RFLP technique using restriction enzyme digestion specific for the differentiation of the known 11 *Malassezia* species was used (*M. furfur*, *M. sympodialis*, *M. globosa*, *M. restricta*, *M. slooffiae*, *M. obtusa*, *M. dermatis*, *M. japonica*, *M. yamatoensis*, *M. pachydermatis* and *M. nana*).

For amplification of the highly conserved 26S rDNA region, specific primers were used. Their sequences were as following: forward, 5'-TAACAAGGAT-TCCCCTAGTA-3' and reverse, 5'-ATTAC-GCCAGCATCCTAAG-3'. Conventional PCR was performed in a 50 µL reaction volume using 25 µL master mix (Fermentas, Life Sciences) containing 25 mM of each deoxynucleoside triphosphate (equimolar concentration of dATP, dCTP, dGTP and dTTP), 1.25 U Taq DNA polymerase and 10x PCR buffer in addition to 2.5 µL each primer, 15 µL distilled water and 5 µL DNA template.

The amplification was performed in a GeneAmp PCR System 9700 thermal cycler (Applied Biosystems) and included initial denaturation step at 94°C for 5 minute, followed by 30 cycles of denaturation at 94°C for 45 seconds, annealing at 55°C for 45 seconds, and extension at 72°C for 1 minute, with a final extension step of 72°C for 7 minute. The expected size of the amplified PCR products were of 580 bp and visualized by1.5% (w/v) agarose gel electrophoresis in Tris Borate EDTA (TBE) buffer, stained with ethidium bromide (0.5 µg/mL), in comparison with DNA size marker (Thermo Scientific™ GeneRuler™ 50bp DNA Ladder, USA) and examined under UV trans-illumination.

The restriction enzymes Hha1 and BstC1 (New England Biolabs, Hitchin, UK) were selected to obtain the best species-specific pattern. Digestion was performed by incubating 17 µL aliquot of PCR products with 10 U of the enzyme which sum up to 20 µL. After the reaction at 37°C for three hours, the electrophoresis was done as above and the restriction fragments were analysed with the size and number of DNA fragments.



[Table/Fig-1]: KOH examination of skin scales showing group of round yeast cells and short curved hyphae of *Malassezia* under x20 magnification.

Descriptive Data of the Studied Groups	PV Patients (n=137)	Healthy Individuals (n=30)	p-value
Gender Male (%) Female (%)	 70 (51 %) 67 (49%)	 21 (70%) 9 (30%)	0.070**
Age (mean±SD) years	25.5±8.3	21.5±9.2	0.197*
KOH results (no. %)	129 (94.2%)	4 (13.3%)	0.0001**
Culture isolates (no. %)	98 (71.5%)	5 (16.7%)	0.0001**
Identified *Malassezia* Species (By both phenotypic and molecular methods)	*M. furfur* (44) *M. globosa* (24) *M. restricta* (10) *M. sympodialis* (12) *M. obtusa* (4) *M. pachydermatis* (4)	*M. furfur* (3) *M. globosa* (2)	

[Table/Fig-2]: Demographic and microbiological data of studied PV patients and control individuals.
**calculated by Fisher-exact test
*calculated by Student t-test

[Table/Fig-3]: Sabouraud's dextrose agar slant covered with olive oil showing shiny and creamy coloured colonies of *Malassezia furfur*.

STATISTICAL ANALYSIS

The Student t-test and Fisher's-exact test were used to evaluate the differences in the frequency and distribution of *Malassezia* species between the PV patients and healthy subjects. A p-value ≤ 0.05 was considered statistically significant. A p-value <0.001 was considered as highly significant.

Malassezia species	Phenotypic Methods (no. of isolates)	PCR-RFLP (no. of isolates)	p-value
M. furfur	38 (51.4 %)	44 (44.9%)	0.008**
M. globosa	22 (29.7%)	24 (24.5%)	
M. restricta	10 (13.5%)	10 (10.2%)	
M. sympodialis	0	12 (12.2%)	
M. obtusa	0	4 (4.1%)	
M. pachydermatis	4 (5.4%)	4 (4.1%)	
Total identified	74 (75.5%)	98	0.0001**
Un-identified	24 (24.5%)	0	
Total	98	98	

[Table/Fig-4]: *Malassezia* species identification by phenotypic methods compared to PCR-RFLP in PV patients.
**= calculated by Fishers-exact test

RESULTS

A significantly higher direct KOH results among patients than healthy individuals (94.2% vs 13.3%; p=0.0001) [Table/Fig-1,2]. Also, a significantly higher isolation rate of *Malassezia* colonies was yielded among PV patients than healthy individuals (71.5% vs 16.7%. p=0.0001) [Table/Fig-2].

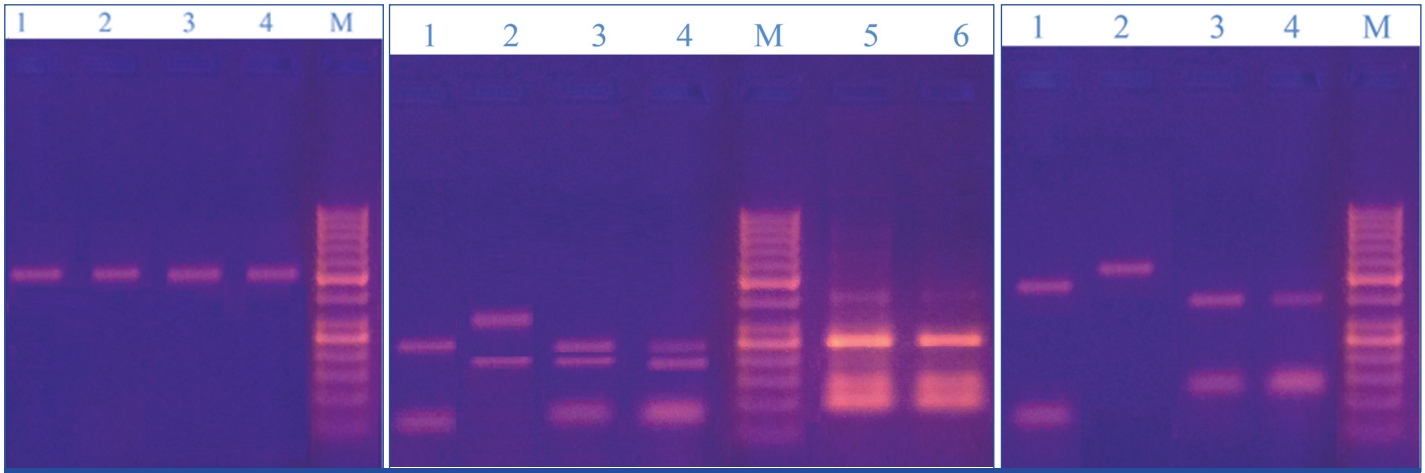
Mixed cultures were excluded. The positive growth for *Malassezia* was creamy, moist, pasty colonies on the SDA slants [Table/Fig-3]. Gram stained smears from the colonies were examined for unipolar budding yeast cells of *Malassezia*.

By phenotypic methods, five *Malassezia* isolates from healthy individuals were identified as *M. furfur* (10%) and *M. globosa* (6.7%) that were established by PCR [Table/Fig-2].

Out of 137 clinically suspected PV patients, 98 samples were confirmed PV by isolation of *Malassezia* isolates. Phenotypically, 74 (75.5%) isolates of *Malassezia* revealed four species as following: *M. furfur* 38 (51.4%), *M. globosa* 22 (29.7%), *M. restricta* 10 (13.5%) and *M. pachydermatis* 4 (5.4%) while 24 (24.5%) were unidentified isolates. By using PCR technique, all isolates (100%) were identified [Table/Fig-4].

The PCR method produced single PCR product of approximately 580 bp that was further studied by RFLP analysis using Hha1 and BstC1 restriction enzymes [Table/Fig-5].

Using Hha1, we could identify five different specie by their specific restriction bands including; *M. furfur* (bands of 250, (113,109 overlapping), 87 bp), *M. globosa* (bands of 455, 129 bp), *M. restricta*



[Table/Fig-5]: 26S rDNA PCR products of some isolated *Malassezia* strains (Lanes 1-4: *M. furfur*, *M. sympodialis*, *M. obtusa*, *M. pachydermatis* (580 bp) and lane M: DNA ladder 50bp, the bands were; 1000, 900, 800, 700, 600, 500, 400, 300, 250, 200, 150, 100, 50. It contains two reference bands (500 and 250 bp); **[Table/Fig-6]:** Restriction pattern of some isolated *Malassezia* strains digested by Hha1; Lane 1: *M. furfur* (250bp, 87bp), lane 2: *M. sympodialis* (357bp, 197bp), lanes 3&4: *M. pachydermatis* (250bp, 221bp &97bp), lanes 5&6; *M. obtusa* (250bp, 153bp& 109bp) and lane M: DNA ladder 50bp, the bands were; 1000, 900, 800, 700, 600, 500, 400, 300, 250, 200, 150, 100, 50. It contains two reference bands (500 and 250 bp); **[Table/Fig-7]:** Restriction pattern of some isolated *Malassezia* strains digested by BstC1 enzyme; Lane 1: *M. pachydermatis* (500bp, 70bp) lane 2: *M. obtusa* (580bp), lane 3: *M. furfur* (400bp, 180bp), lane 4: *M. sympodialis* (400bp, 180bp), and lane M: DNA ladder 50bp, the bands were; 1000, 900, 800, 700, 600, 500, 400, 300, 250, 200, 150, 100, 50. It contains two reference bands (500 and 250 bp).

Malassezia species	Age Groups (year)				
	< 20	20-29	30-39	40-49	≥50
M. furfur (n=44)	10	21	6	5	2
M. globosa (n=24)	3	13	4	1	3
M. restricta (n=10)	1	3	4	0	2
M. sympodialis (n=12)	2	5	0	5	0
M. obtusa (n=4)	0	2	0	1	1
M. pachydermatis (n=4)	2	1	0	1	0
Total (n=98)	18 (18.4%)	45 (45.9%)	14 (14.3%)	13 (13.3%)	8 (8.1%)

[Table/Fig-8]: Distribution of *Malassezia* species according to the age groups.

Malassezia species	Type of the Lesion			Gender	
	Hyperpigmented	Hypopigmented	Mixed	Male	Female
M. furfur (n=44)	10	30	4	16	28
M. globosa (n=24)	6	15	3	19	5
M. restricta (n=10)	5	5	0	6	4
M. sympodialis (n=12)	4	6	2	5	7
M. obtusa (n=4)	1	3	0	3	1
M. pachydermatis (n=4)	2	2	0	3	1
Total (n=98, 100 %)	27 (27.5%)	63 (64.3%)	8 (8.2%)	52 (53%)	46 (47%)

[Table/Fig-9]: Distribution of *Malassezia* species according to type of the lesion in both sexes.

Malassezia species	Site of the Lesion						
	Neck and Back	Neck and Chest	Neck and Face	Neck	Back	Back and Chest	Chest
M. furfur (n=44)	14	10	2	6	8	3	1
M. globosa (n=24)	7	6	0	2	3	5	1
M. restricta (n=10)	1	0	4	0	3	2	0
M. sympodialis (n=12)	1	5	0	0	4	2	0
M. obtusa (n=4)	0	1	0	1	2	0	0
M. pachydermatis (n=4)	2	1	0	0	0	1	0
Total (n=98, %)	25 (25.5%)	23 (23.5%)	6 (6.1%)	9 (9.2%)	20 (20.4%)	13 (13.3%)	2 (2%)

[Table/Fig-10]: Distribution of *Malassezia* species in different parts of the lesional skin of PV patients.

(No restriction bands, only band of 580 bp), *M. obtusa* (bands of 250, 153, 109 bp), *M. pachydermatis* (bands of 250, 221, 97 bp). On the other hand, colonies of 6 species were identified as either *M. sympodialis* or *M. dermatis* (they produced the same digestion pattern, 357, 197 bp) [Table/Fig-6].

They could be differentiated by using BstC1 enzyme where *M. sympodialis* produced restriction bands (400, 180bp) and other species were confirmed as following; *M. furfur* (bands of 400, 180 bp), *M. globosa*, *M. obtusa* (No restriction bands, only band of 580 bp), *M. restricta*, *M. pachydermatis* (bands of 500, 70 bp) [Table/Fig-7].

The tested 98 *Malassezia* isolates revealed six species: *M. furfur* 44 (44.9%), *M. globosa* 24 (24.5%), *M. sympodialis* 12 (12.2%), *M. restricta* 10 (10.2 %), *M. obtusa* and *M. pachydermatis* 4 (4.1% for each species) as shown in [Table/Fig-4] with significant higher identification rate of isolates by PCR-RFLP compared to phenotypic methods (p=0.008).

The highest frequency of PV (45.9%) was found in patients in the 20-29 age groups with predominant *M. furfur*, *M. globosa* and *M. obtusa* [Table/Fig-8].

The higher rate of infection was reported in males (53%) than that in females (47%) which was caused by all species except *M. furfur* and *M. sympodialis* which were predominantly seen in females. The most PV patients (64.3%) presented with hypopigmented lesions while only 8.2% patients presented with mixed hypopigmented and hyperpigmented ones. *M. furfur*, *M. globosa*, *M. sympodialis* and *M. restricta* were predominantly isolated from hypopigmented lesions [Table/Fig-9].

The most common site affected was the neck and back (25.5%), followed by neck and chest (23.5%), back (20.4%), and then back and chest (13.3%). *M. furfur*, *M. globosa* and *M. pachydermatis* had the most frequency on the neck and back, *M. sympodialis* on the neck and chest, *M. obtusa* on back and *M. restricta* on neck and face [Table/Fig-10]. Regarding healthy control group, all isolates were retrieved from back.

DISCUSSION

Pityriasis versicolor is a common recurrent chronic superficial dermatologic infection difficult to eradicate as its pathogenic agents i.e., *Malassezia* species is endogenous to the skin flora [3,25]. The isolated *Malassezia* species showed not only a noteworthy difference between healthy individuals and PV patients, but also showed a considerable geographic variation. The *Malassezia* species distribution among Egyptian population remains unclear.

The conventional methods for diagnosis of PV are direct microscopic examination of skin scrapings treated with 10% KOH and fungal culture. The present study revealed a mycological positivity of 94.2% of skin scrapings of PV patients and 13.3% from healthy control. Using culture, the recovery rate was 71.5% from PV lesions and 16.7% from control individuals. Although, direct microscopic examination of skin scrapings (KOH) is rapid, inexpensive and sensitive for preliminary diagnosis of PV, it lacks a colour contrast and requires a trained eye to interpret. Culture is highly important and specific to differentiate between the *Malassezia* species phenotypically [26].

As molecular techniques have become common tools to investigate *Malassezia* species rapidly, accurately and efficiently, we used culture dependent PCR-RFLP to successfully identify *Malassezia* species in PV patients and healthy individuals [20,27].

Our observations of *Malassezia* species are concordant with the previous studies that have isolated *M. furfur* as the predominant species followed by *M. globosa* and *M. sympodialis* from PV lesions in Egypt and Nigeria [16,17,28,29], while other studies reported that the most frequently isolated species from PV lesions were *M. globosa* (53.3% and 47.6%, respectively) followed by *M. furfur* (25.3% and 41%, respectively) [21,30]. Moreover, Gupta AK et al., found that *M. sympodialis* was mainly isolated from PV cases in temperate climate and *M. globosa* was the main species in tropical regions [31]. The distribution of *Malassezia* species varies with different geographical locations [4]. To our knowledge, this is the first report of the isolation of *M. restricta*, *M. pachydermatis*, and *M. obtusa* from PV in Egypt.

Each *Malassezia* specie has a specific biochemical and genetic characteristic and specific ecological niche. *M. restricta* showed a growing frequency as it was isolated from our patients in a higher percentage (10.2%) than others [21,32]. Gaitanis G et al., confirmed that *M. restricta* is a fastidious organism and is predominantly isolated in non culture based epidemiological studies [8].

In the current study, *M. obtusa* and *M. pachydermatis* were isolated from PV patients which is nearly comparable to the results reported

by Khosravi AR et al., [33]; whereas, Kaur N et al., [10] isolated them from healthy controls.

It is well known that *M. pachydermatis* is isolated from patients with close contact with animals like cattle and dogs, live inside or nearby their houses as commonly seen in rural areas [7,10]. Giusiano G et al., explained that *M. pachydermatis* has been considered only as a transient member of the human cutaneous biota; therefore, its association with PV deserves further investigation [32].

However, Romano C et al., and Rasi A et al., found that *M. globosa* is the predominant species in temperate climates due to its high lipophilic activity caused by lipases and esterases [19,34]. *M. furfur* is reported as the main species in Indonesia and Brazil [35,36]. Whereas *M. sympodialis* and *M. globosa* were the most frequently isolated species in Canada and Argentina, *M. slooffiae* and *M. restricta* were less common in Argentina [31,32].

The differences between the studies may not only be the result of geographical variation in species prevalence, but also due to racial differences, different experimental techniques, hygiene of the subjects, climate, and life styles [9,21].

While El Fangary M and Taha M and Abo Zaid MH et al., found that 7.8% isolates were unidentified and the results of the conventional identification were in agreement with the pattern obtained from 26S rDNA PCR [28,37]. In our study, 24.5% of *Malassezia* species were not identified phenotypically but they had molecular pattern consistent mainly with *M. sympodialis*, *M. furfur*, *M. obtusa* and to less extent with *M. globosa*. This could be attributed to the high percentage of these species in our patients and the sensitive PCR-RFLP method we used.

Age group 20-29 years of patients was found to be the most commonly affected with predominant *M. furfur*, *M. globosa* and *M. obtusa*. This is in consensus with Giusiano G et al., who emphasized that the recovery rate of *Malassezia* in PV is known to be highest in the twenties of both genders when sebaceous glands show greatest activity [32]. Moreover, 21.4 % of PV cases were older than 40 years of age. It may be that subtropical climatic factors of Egypt enable a broader age range of PV [37].

The finding that *M. furfur* and *M. globosa* species were highest to be isolated from both healthy skin and PV patients in matched age groups confirms their potential ability to transform from commensal to pathogenic by factors to be further investigated on a large scale.

The effect of sex in predisposition to develop PV is still unclear. A higher rate of infection was observed in males than females with predominance of all species except *M. furfur* and *M. sympodialis,* which were predominant in females patients. Giusiano G et al., reported that only *M. globosa* was more common in female patients [32]. While both isolates of *M. furfur* and *M. globosa* were more common in male subjects of control group. Male preponderance seen in the present study may be related to either their higher exposure to factors like high temperature and humidity which are associated with outdoor activities or could be related to increased sebaceous gland activity in men [38].

Similar to other researchers like Ibekwe PU et al., the lesions of 64.3% of studied PV cases corresponded to the hypopigmented type and 8.2% had mixed hypopigmented and hyperpigmented types; whereas, 27.5% had hyperpigmented type [29].

Although Aljebre SH et al., denied any correlation between pigment variation and the type of skin in PV patients [39], Archana BR et al., reported that PV disease has a tendency to be hypopigmented in dark skinned individuals and hyperpigmented in fair skinned individuals [40].

In agreement with Archana BR et al., the present study showed that *M. furfur*, *M. globosa*, *M. sympodialis* and *M. restricta* were predominantly isolated from hypopigmented lesions [40], whereas *M. obtusa* and *M. pachydermatis* were the least.

In this study, we detected a high frequency of patients with multiple lesional sites as the most common site affected was the neck and back, in agreement with Shoeib MA et al., [16]. Most likely the high ambient temperature and humidity of region encourage PV spread [41]. Moreover, areas which remain covered by clothing encourage the development of lesions, supporting the concept that the occlusion of glands plays a role in this disease.

Epidemiologically, we would highlight the frequency of some species at certain body sites. *M. furfur*, and *M. globosa* and *M. pachydermatis* had the most frequency on the neck and back, *M. sympodialis* on the neck and chest, *M. obtusa* on back and *M. restricta* on neck and face. Similar distribution of *M. restricta* on face was reported by Ibekwe PU et al., study done in Nigeria but different for *M. furfur* and *M. sympodialis* which were isolated predominantly from face and back respectively [29]. The distribution of the lesions on various body sites in PV usually parallels the density and activity of sebaceous glands in these areas.

Current study can give an idea about the species present commonly in this locality that may have different sensitivities to antifungal drugs. Further research could facilitate the choosing of appropriate treatment options.

LIMITATION

We did not use lesional and non lesional samples from same PV patients; hence we could not study the possible effect of individual idiosyncrasy on the results. The relatively low sample size is another limitation. The subjects in this study only represent the population of certain locality in Egypt. A larger study covering all Egyptian Governorates is mandatory for studying the influence of regional factors on the distribution of *Malassezia* species.

CONCLUSION

Our results demonstrate that *M. restricta*, *M. pachydermatis*, and *M. obtusa* are pathogenic species for PV in Egyptian patients. The use of PCR-RFLP for identification of *Malassezia* species helps to deepen the knowledge of regional clinico-epidemiological features of the *Malassezia* strains that may be useful for selecting sensitive drugs and prevent the occurrence of recurrent or resistant PV infection.

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