Tikrit Journal of Pure Science Vol. 29 (3) 2024

TJPS

https://doi.org/10.25130/tjps.v29i3.1596

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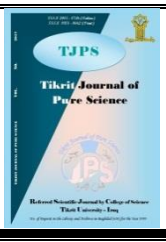
المجلات الاكاديمية العراقية

Tikrit Journal of Pure Science

ISSN: 1813 – 1662 (Print) --- E-ISSN: 2415 – 1726 (Online)

Journal Homepage: http://tjps.tu.edu.iq/index.php/j



Epidemiological and Molecular Characterization of *Malassezia species* from Patients with pityriasis versicolor in Erbil Province

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ARTICLE INFO.

Article history:

-Received: 19 / 12 / 2023

-Received in revised form: 14 / 4 / 2024

-Accepted: 1 / 5 / 2024

-Final Proofreading: 4 / 6 / 2024

-Available online: 25 / 6 / 2024

Keywords: Epidemiology, *Malassezia* species, 26SrDNA sequencing, Genotyping, Phylogenic diagnosis

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ABSTRACT

Background: Pityriasis versicolor (PV) is the most common chronic superficial infection of the stratum corneum. Pityriasis versicolor is the prototypical skin disease etiologically connected to Malassezia species. Malassezia furfur is the primary causative agent of pityriasis versicolor which causes either hyperpigmentation or hypopigmentation of the skin.

Material and Methods: Sixty patients suffering from pityriasis versicolor disease who attended Erbil Dermatological Center, from August 2021 up to July 2023. Clinical diagnoses were done by consultant dermatologist. Thirty nine were males and Twenty-six were females. DNA has been extracted from skin scraps isolated from various body areas using the DNeasy Blood & Tissue Kit (Qiagen, Germany) and it was amplified using specific primers for Malassezia strains. The amplified PCR products were sequenced commercially in both directions (Macrogen Inc. South Korea).

Results: The largest proportion of infections was reported by hyperpigmentation (64.6%), followed by hypopigmentation (44.5%). The rosy-coloured lesions were present only in 8 (12.3%) of patients. Applying of polymerase chain reaction is extremely critical to verify the diagnosis of Malassezia species. Ribosomal region of sequence analysis revealed that, the sequences of 14 isolates under the accession number (MT000715 and MT000716) were (99.9%-100%) homologous to M. furfur under the accession number NG_057730 and 6 isolates under the accession number (MT000717) were (100%) homologous to M.globosa (AY743604 and AJ249951). Phylogenetic analyzes were performed to assist investigate the relationship of M.furfur and M.globosa to support these results in Erbil province.

Conclusions: Phylogenetic analysis of the fourteen isolates (M. furfur) under the accession (MT000715 and MT000716) and the remaining six isolates belonging to the M. globasa (MT000717) 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 Erbil patients and that of the Gen Bank database.

Tikrit Journal of Pure Science Vol. 29 (3) 2024

TJPS

https://doi.org/10.25130/tjps.v29i3.1596

التوصيف الوبائي والجزيئي لأنواع الملاسيزية لدى مرضى النخالية المبرقشة في محافظة أربيل

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الملخص

الخلفية: النخالية المبرقشة (PV) هي العدوى السطحية المزمنة الأكثر شيوعاً في الطبقة القرنية. النخالية المبرقشة هي مرض جلدي نموذجي مرتبط من الناحية المسببة بأنواع الملاسيزية. الملاسيزية الفراء هو العامل المسبب الرئيسي للنخالية المبرقشة التي تسبب إما فرط تصبغ أو نقص تصبغ الجلد.

المواد والطرق: ستون مريضاً يعانون من مرض النخالية المبرقشة الذين راجعوا مركز أربيل للأمراض الجلدية، في الفترة من أغسطس 2021 حتى يوليو 2023. تم إجراء التشخيص السريري من قبل استشاري الأمراض الجلدية. تسعة وثلاثون من الذكور وستة وعشرون من الإناث. تم استخراج الحمض النووي من قصاصات الجلد المعزولة من مناطق مختلفة من الجسم باستخدام مجموعة DNeasy Blood & Tissue Kit (Qiagen، ألمانيا) وتم تضخيمها باستخدام بادئات محددة لسلالات Malassezia. تم تسلسل منتجات PCR المضخمة تجاريًا في كلا الاتجاهين (Macrogen Inc. كوريا الجنوبية).

نتائج: تم الإبلاغ عن النسبة الأكبر من الإصابات عن طريق فرط التصبغ (64.6٪)، يليه نقص التصبغ (44.5٪). وكانت الآفات ذات اللون الوردي موجودة فقط في 8 (12.3٪) من المرضى. يعد تطبيق تفاعل البلمرة المتسلسل أمرًا بالغ الأهمية للتحقق من تشخيص أنواع الملاسيزية. كشفت منطقة تحليل التسلسل الريبوسومي أن تسلسل 14 عزلة تحت رقم الانضمام (MT000715 و MT000716) كانت (99.9٪ –100٪) متماثلة لـ M. furfur تحت رقم الانضمام NG_057730 و 6 عزلات تحت رقم الانضمام (MT000717). كانت (100٪) متماثلة لـ M.globosa (AY743604 وAJ249951). تم إجراء التحليلات التطورية للمساعدة في دراسة العلاقة بين M.furfur وM.globosa لدعم هذه النتائج في محافظة أربيل.

الاستنتاجات: تم تحليل التحليل الوراثي للعزلات الأربعة عشر (M. furfur) تحت المدخل (MT000715 وMT000716) والعزلات الست المتبقية التي تنتمي إلى (M. globasa (MT000717 بواسطة MEGA 5.05 ومقارنتها بتسلسلات أنواع الملاسيزية المختلفة المتوفرة في قاعدة بيانات بنك الجينات، أظهرت البيانات تقاربًا واضحًا بين عزلات الملاسيزية لدينا من مرضى أربيل وتلك الموجودة في قاعدة بيانات بنك الجينات.

1- Introduction

Pityriasis Versicolor should be a normal skin condition in tropical countries [1, 2] and are considered a superficial mycosis of the skin [2, 3]. The most prevalent species in *Pityriasis Versicolor* are *M.furfur*, *M.globosa* and *M. sympodialis* [4, 5]. The disease is mainly asymptomatic and the patient may suffer light itching in only some cases, especially in physical activity and sweating [6]. *Pityriasis Versicolor* can occur as macules or patches in affected areas with changing color (hyperpigmentation, hypopigmentation or rose-coloured). The lesions could have a light flour-like scale on its surface [3]. The illness may affect the skin of the face, neck, trunk, upper extremities and rarely the rest of the body [7]. The disease extension is variable, and therefore the recurrence occur in next summer and are quite common [8]. *Malassezia species* is diagnosed by clinical presentations which vary from person to person. However, the techniques will not be sufficiently selective. Besides, biochemical and phénotypical methods are not ready to be diagnosed immediately [9].

In recent years, molecular studies have changed the taxonomy of the genus Malassezia to a considerable extent[10]. Sequence variability between Malassezia species has been documented in rRNA genes. In 1996, Gueho et al. [11], classified the genus of Malassezia in seven distinct species, namely; M. furfur, M. pachydermatis, M. sympodialis, M. globosa, M. obtusa, M. restricta and M. slooffiae. Recently, based on DNA analysis, six new species have been introduced: M. dermatis, M. nana, M. japonica, and M. yamatoensis, M. equine, M. caprae. Of these M. caprae, M. equine and M. nana have only been isolated from domestic animals [4]. As well as, Molecular techniques like Polymerase Chain Reaction (PCR) of ribosomal 26S rDNA gene that have been used to identify *Malassezia spp* [12-15] and its simple, reliable, cost-effective and accurate method [4, 16, 17]. As a result, the categorization of the *Malassezia genus* was drastically changed [4, 18] Nine of the thirteen genus species, *M.furfur*, *M.sympodial*, *M.globose*, *M.restricta*, *M.slooffiae*, *M.obtusa*, *M.dermatitis*, *M japonica*, and *M.yamatoensis*, are related to human normal flora and pathogens. As well as, four species that are related to animals which include *M.pachydermatis*, *M.nana*, *M.equina*, and *M.caprae* [19-21]. The study's

https://doi.org/10.25130/tjps.v29i3.1596

objective is to determine the prevalence of specific Malassezia species using PCR technique among Pityriasis versicolor patients in the province of Erbil.

2- Methodology

2.1- Collection of Samples:

The research was carried out at the Erbil Dermatological Center, from August 2021 up to July 2023. Randomly 65 cases with *pityriasis versicolor* are involved. The patients diagnosed clinically by dermatologist upon their visit to the out-patient clinical center. Data were collected by direct interviewing with patient regarding socio-demographic information, disease history and symptoms. Then detailed physical examination is completed, and recorded. Later, the sample from the patients' skin lesions (5–10 mg) by scraping the skin with a sterile Forceps and surgical blades was collected in a clean Petri-dish, and it's preserved in 70% of ethanol for molecular analysis [12, 22].

2.2- Molecular Analysis:

DNA has been extracted from skin scraps isolated from various body areas using the DNeasy Blood & Tissue Kit (Qiagen, Germany) and it was amplified using specific primers for *Malassezia* strains, Both primers {forward (5'-TAACAAGGATTCCCCTAGTA-3') and the reverse (5'ATTACGCCAGCATCCTAAG-3') [23] are used . The mixture of amplification consist of 25µl which included (2X) Go-Tag Master Mix (12.5µl), 2µl of each primer (forward and reverse), DNA template (2µl) and nuclease-free water (6.5µl). The following conditions were used to carry out the amplification reaction: initially denatured (94^{o}C for 5min), Following, 30 cycles of denaturation (94^{o}C for 45s), annealing (55^{o}C for 45s), and elongation (72^{o}C for 1min), with a final extension step (72^{o}C for 7min). The results of amplification were examined by electrophoresis on a 1.5% agarose-gel and staining with ethidium bromide (0.5 μg/ml) in Tris-Acetate EDTA (TAEbuffer) 1X. DNA ladder (100 bp) makes by Qiagen in Germany, was used as the marker (molecular weight marker), and photographed under UV trans-illumination. The amplified PCR products (~580bp) were sequenced commercially in both directions (Macrogen Inc. South Korea).

3.2- Nucleotide sequence accession number:

The species of Malassezia was confirmed by analyzing the nucleotide sequences and the sequences were aligned through the ClustalW algorithm [24], provided by BioEdit v7.2.5 [25], with sequences available in the GenBank (NCBI) database. Nucleotide sequences were deposited in the GenBank database under accession numbers MT000715, MT000716 & MT000717 through the use of BLAST algorithm (http://www.ncbi.nlm.nih.gov/BLAST).

4.2- Phylogenic analysis:

Malassezia species under the accession number (MT000715 - MT000717) were compared with reference sequences (AJ249955, AY387131, AY743602, EU815319, EU815319, KJ425391, KX721515, KY108384, KY108389, NG_057730, AJ249951, AY387132, AY387133, AY743604, AY387136, AB070359, AB105064, AB105862, AB125263, AJ249950, AJ249952, AJ249953, AJ249954, NR_126107, KP825367, KP825375, KP825376, KP825377 and KT239958) in the GenBank database was supported in the species determination by utilizing the BLAST Algorithm (https://blast.ncbi.nlm.nih.gov/). Phylogenetic analyses were performed on individual partial gene sequences using MEGA-7 software (Molecular Evolutionary Genetics Analysis 3.1; (http://www.megasoftware) and the neighbor joining were used to build the tree.

4.2- Statistical analysis:

Through scatter plot software (GraphPad Prism v.7, CA, USA), data was analyzed in order to understand characteristics of the examined population with the prevalence of *Malassezia* species.

3- Results and Discussion

Out of 65 patients with *Pityriasis Versicolor*, (39/65) were males and (26/65) were females, it means high frequency found in males than females (Table 1). These concurred with Ahmed *et al.*, [26] in Bangladesh, who showed that, the highest infection rate was discovered in males (89.11%) as compared to females (10.85%). Also, Jaffer *et al.*, [27] who represented that, the highest prevalence rate occurred in males more than females (M:F ratio is 2.1:1). While the result disagreed with Nikpoor *et al.*, [28], this infection incidence was higher in women. These indicated, the significant participation of men in outside activities with maximal exposure to heat and moisture, favoring the growth of Malassezian yeasts. Table 1 expressed that most of our patients were occupied as a government employee (33.85%) and students (32.31%). These agreed with Morais *et al.*, [29] who showed that the students are predominated 37.1% (43/116), liberal professionals 16.4% (19/116) and home professionals 12.9% (15/116) was second and third sectors in frequency. These could include the use of oil on the surface of the body, excessive sweat, low hygiene, malnutrition and systematic steroid medication. As well as, The results explained that, the frequency of *Pityriasis versicolor* is more predominant in patients with an adverse history of the disease by 78.46%.The result agreed with Jabry and Alsudani [30] in Iraq, which showed that the majority of patients (82.8%) with PV do not have a positive history of the disease (negative history), compared to patients with a positive history of the disease at 17.2%. There are little indications that the disease is highly contagious, since few cases are present in couple's cohabitation. Thus the varied factors due to the disease's existence within the family are variable [27].

https://doi.org/10.25130/tjps.v29i3.1596

Table1: General characteristics of patients

General characteristics of patients		Gender						P Value
		Male (39)	%	Female (26)	%	Total	%	
Occupations	Government employee	14	21.54	8	12.31	22	33.85	0.2935
	Private employee	9	13.85	11	16.92	20	30.77	
	Student	14	21.54	7	10.77	21	32.31	
	Retired	2	3.08	0	0.00	2	3.08	
Family history of *pityriasis versicolor*	Yes	10	15.38	4	6.15	14	21.54	0.3244
	No	29	44.62	22	33.85	51	78.46	

The disease mostly was asymptomatic among our patients (58.5%). Only 27 (41.5%) patients had mild itching during physical activities. Objectively, the skin lesions mostly affected the neck region (75.4%). The chest was second place in frequency (64.6%) of localization of *pityriasis versicolor* lesions. Both head (face) and abdomen regions were least (18.5%) affected areas of the body on our study sample (figure 1). These agreed with [15, 27, 30], who showed that the majority cases were asymptomatic and for cosmetic concern went to the hospital. The neck is the first section of the body to be affected, followed by the chest and then the back. The reason of these disease being more common in the upper body, which has a higher percentage of fat than the lower body parts, may be related to the pathogen's lipophilic nature, as these regions are characterized by thick and active sebaceous glands. The numerous of the skin lesions at the time of presentation were hyper-pigmented (64.6%). The rosy-coloured lesions were present only in 8 (12.3%) of patients (Table 3). The result agreed with Talaee *et al.,* [4] who revealed that 50% of patients with PV had hyperpigmentation lesion, while 37% of them had hypopigmentation lesion and 13% had both hyperpigmentation and hypopigmented lesion. Shah *et al.,* [31] revealed that, the shape, size, and color of the infection spots might vary from person to person, and they can be circular, oval, or irregular in appearance. Its color may range from yellow to brown or reddish-brown, but it usually takes a stain paler than the rest of the complexion. These spots are covered with a superficial layer of scales that resembles bran, making them appear light in color to those with dark skin and brown in those with white skin [30].

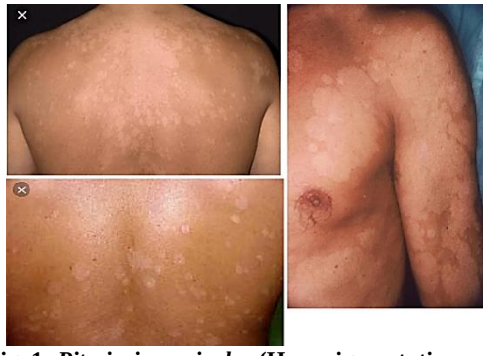

Fig. 1: *Pityriasis versicolor* (Hypopigmentation and Hyperpigmentation) over the body surface.

Table 2: Incidence of Isolated *Malassezia Species* According to Symptoms

Characteristics		No	%
Symptoms	Itchy	27	41.5
	Asymptomatic	38	58.5
Location of Lesions	Head (face)	12	18.5
	Neck	49	75.4
	Chest	42	64.6
	Back	36	55.4
	Shoulders	19	29.1
	Abdomen	12	18.5
Colour of Lesions	White	29	44.5
	Dark (hyperpigmented)	42	64.6
	Rose	8	12.3

The PCR products are separated and electrophoresed to obtained ~580 bp band size on 1.5% agarose gel after staining with ethidium bromide as shown in Figure (2) which were the same bands generated by primer measured for DNA size marker 100bp DNA ladder [23]. As well as, the alignment of a partial nucleotide sequence of *Malassezia* species as compared with the previously published sequences under accession number NG_057730, AY743604 and AJ249951 [32, 33]. The result showed that the *Malassezia species* under the accession number MT000715 (8/20) was 100% and MT000716 (6/20) were 99.9% homologous to *M. furfur* under the accession number (NG_057730) due to nucleotide substitution (G → A) at the position of 514 as shown in Figure (3). On the other hand, Figure (4) revealed that *Malassezia* species under the accession number MT000717 (6/20) were 100% homologous to *Malassezia globasa* under the accession number AY743604 and AJ249951. The present study showed that; *M. furfur* 14/20 as the major species followed by *M. globosa* 6/20 from P.V. lesions in Erbil Province (Table 3). The outcome supported by Honnavar *et al.,* [34] in India who showed that; the commonly isolated species is *M. furfur* (50%), followed by *M.globose* (27.3%), both of *M. furfur* and *M. globosa* (15.9%), *M. sympodial*(4.5 %), and *M.slooffi*(2.3 %) and Elshabrawy *et al.,* [35] in Egypt. They revealed that, out of 98 samples, six species was isolated which include (44 (44.9%) *M.furfur*, 24 (24.5%) *M.globosa*, 12 (12.2%) *M.sympodialis*, 10 (10.2%) *M.restricta*, *M.obtusa* and 4(4.1%)*M.pachydermatis*). As well as, Diongue *et al.,* [14] in Senegal, showed that, the only

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M.furfur was discovered in 100% (39/39). Several factors play a major role in *M.furfur* pathogenicity such as high level of sebum production, stress, hormonal change, illness, allergic food, deficiency of vitamin B, unusual shampooing, curlers hair and blow dryers [21]. Other studies Talaee *et al.*, [4] from Iran, opposite to our results which is showed that, the higher infection rates is *M.globosa* (66%) as the major causes of pityriasis versicolor lesions followed by *M.furfur*(26%), *M.restricta*(3%), *M.sympodial*(3%), and *M.slooffi* (2%), respectively. On the other hand, Shokohi et al., [36] revealed that, *M.globossa* 29(47%) is the most prevalent while *M.furfur* 25 (41%) is the second most frequent agent. The molecular method used for isolation of *Malassezia* species to resolve the time consuming and the difficulties in interpreting some morphological, physiological patterns and confirmation of strains [13, 18, 34, 36, 37]. The different culture mediums and probably the ethnical, climatic and geographical elements and features of patients are responsible for factors determining this variation [14].

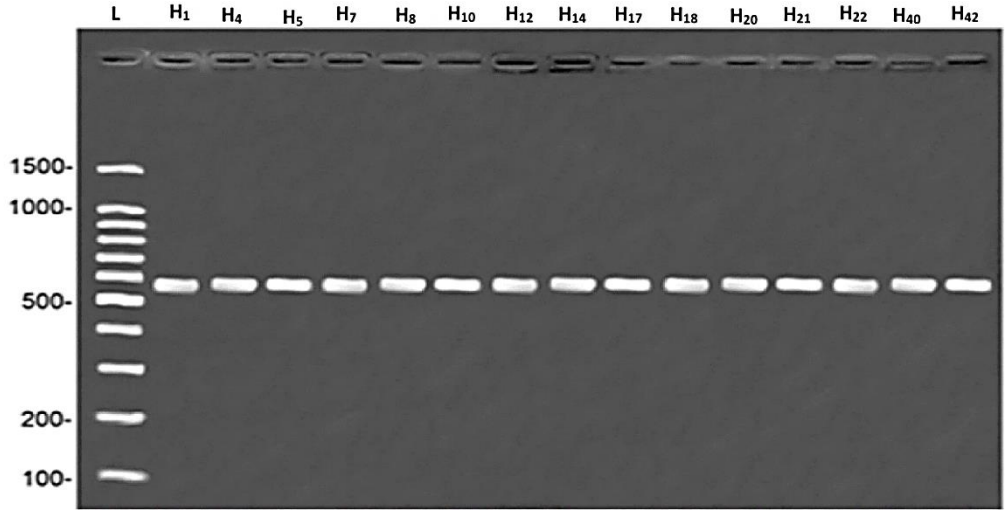

Fig. 2: Agarose gel electrophoresis of *Malassezia species* DNA isolated from human samples. DNA marker size (L: 100bp DNA ladder), lanes (1, 4, 5, 7, 8, 10, 12, 14, 17, 18, 20, 21, 22, 40 and 42–specific product for Malassezia species isolated from skin.

Table 3: Isolated *Malassezia* species from Different Lesion Area

Malassezia Species	*Accession number*	Neck (6)	Chest (5)	Back (3)	Shoulders (2)	Face (2)	Abdomen (2)	Total	(%)
Malassezia furfur	MT000715	2	2	1	1	1	1	8	40
	MT000716	3	1	1	0	1	0	6	30
Malassezia globassa	MT000717	1	2	1	1		1	6	30

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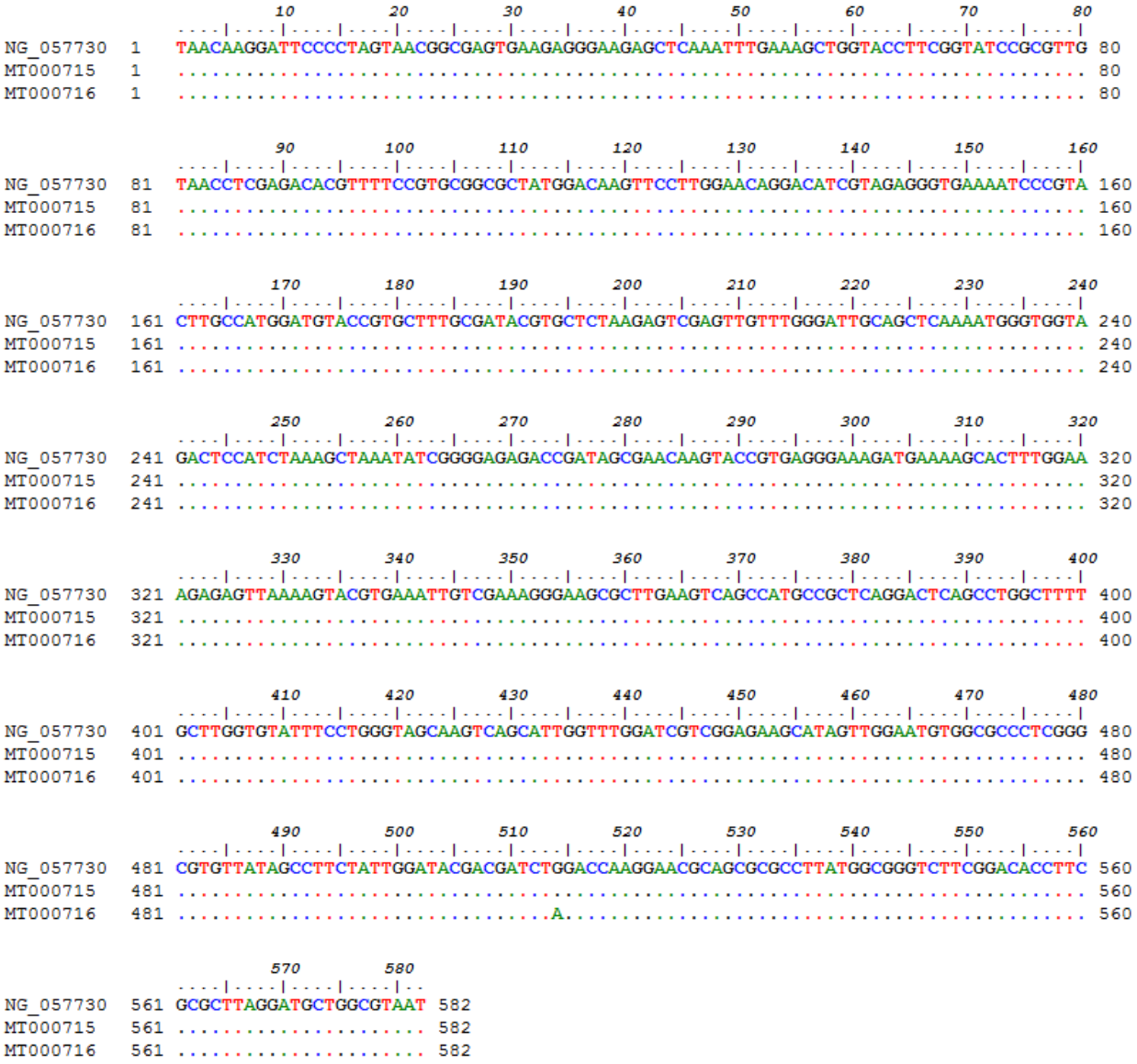

Fig. 3: Compared parts of the sequence results of cloned *Malassezia furfur* positive samples in humans with published sequences available in the NCBI database (NG_057730).

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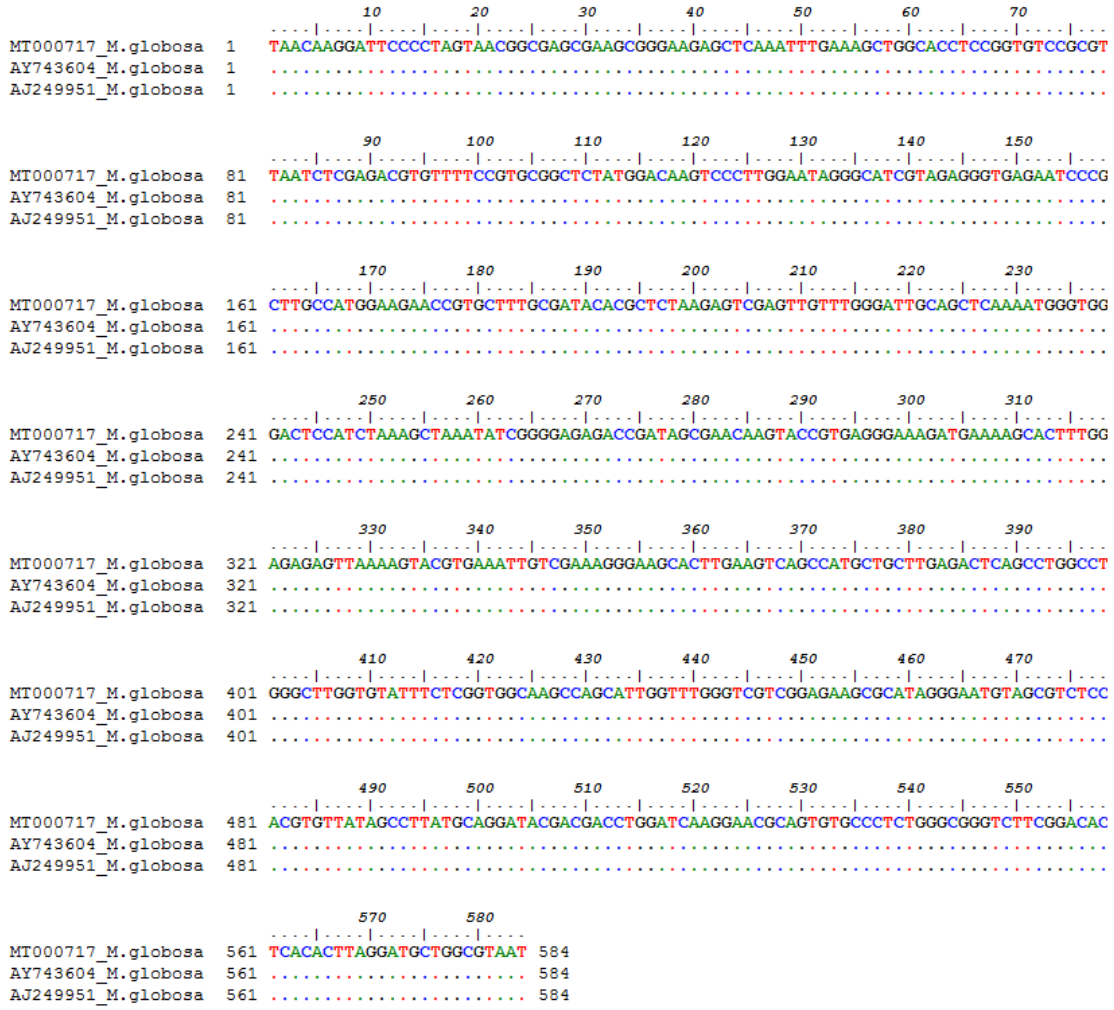

Fig. 4: Compared parts of sequence results of cloned *Malassezia globasa* positive samples in humans with published sequences available within the NCBI database (AY743604 and AJ249951).

The phylogenetic tree was diagrammatic by (MEGA) software version 7.0 is shown in Figure (5). Fourteen isolates under the accession (MT000715 and MT000716) were *M. furfur* that had 99.5-100% similarity with the *M. furfur* under ID: AY743602, KX721515, KJ425391 and KY108389 [19, 20, 33, 38, 39]. The remaining samples (six isolates) belonging to the *M. globasa* was found, which showed 99.7-100% similarity with the sequence recorded for *M. globas*a under ID: AJ249951, KT239958, AY743604, KP825367, KP825376 and kp825375) sequence [19, 20, 32, 33, 38, 39]. The phylogenic tree confirms our results and the identity of the isolates are associated with *Pityriasis versicolor* which is the first report in Erbil Province, whereas *M.restricta* and *M.globosa* are generally related with seborrhoeic dermatitis/dandruff [40, 41].

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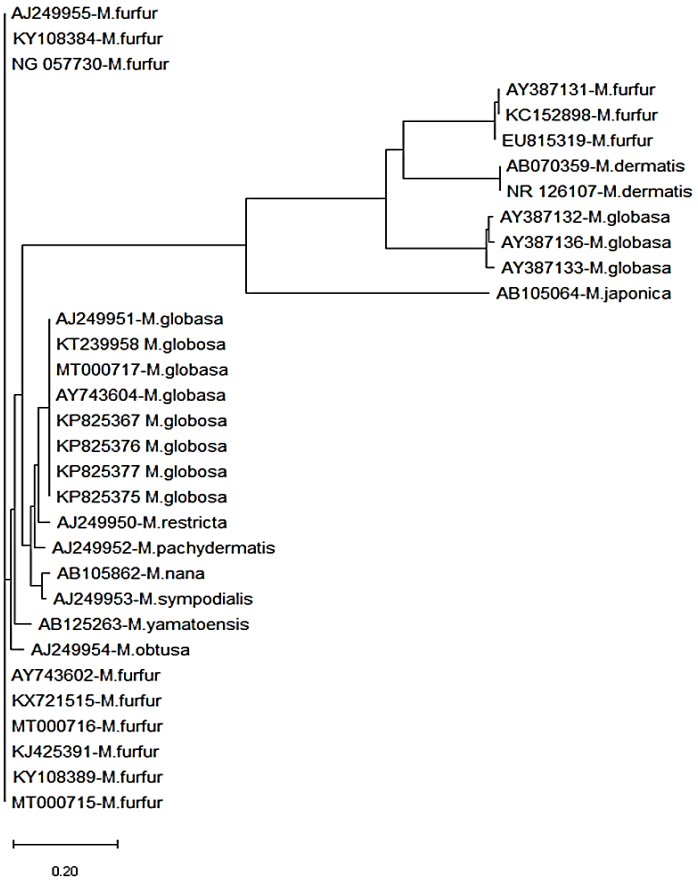

Fig. 5: Phylogenetic analysis of *Malassezia* genotypes by maximum likelihood, conducted the tree supported the multiple sequence alignment by MEGA ver.7.

4- Conclusion

Pityriasis versicolor was more prevalent among males than female in our study sample. The most frequent locations of the lesion were within the neck and chest. The hyperpigmented lesion was predominant presentation among the studied sample. *M. furfur* is dominant and *M.globosa* was the second species which are prevalent in our patients. Sequencing and phylogenetic analysis showed that, *M. furfur* and *M. globosa* in Erbil was not different from the pathogens in other humans from geographically distinct regions. These data provide important information about the incidence of *Malassezia* in humans and its benefit's for managing and controlling programs of the disease.

5- Acknowledgement's

The author would like to thanks Erbil Technical Health and Medical College for helping the works

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