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Phenotypic and Confirmatory Diagnosis by PCR Technique of Malassezia Fungi that Cause Tinea Versicolor among Young People in Kirkuk / Iraq

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Abstract:** This Study Was Conducted At The Biology Department, College Of Education For Pure Sciences, Kirkuk University. 200 Samples Were Collected From Different Parts Of The Human Body From People Who Had Signs Of Tinea Versicolor Disease And Their Age Groups Between 10-⩽40 Years And From Both Genders (Female And Male) From Some Cities Of Kirkuk Governorate, Including The Internal Departments Of Kirkuk University, Medical Laboratories, And Patients Attending Hospitals For Dermatological Consultations And Clinics Of Doctors Specializing In Dermatology, Hawija And Daquq Cities At A Period Between October 15, 2022, And May 13, 2023, The Number Of Pathological Samples Positive for Transplantation Was 87 Samples With A Rate Of 43.5%, And The Infection Rate In Males Was 56.3% And In Females 43.7%. Through The Study, Five Types Of Fungi Of The Genus Malassezia Spp. Were Isolated According To Their Appearance, Biochemical Tests, And Then PCR. They Are M. Furfur, M. Globosa, And M. Slooffiae; M. Restricta; And M. Obtusa. M. Furfur Was Most Common Among The Other Species, With 49.4% Of Species Records. M. Furfur Was Identified In Kirkuk Governorate As One Of The Causes Of Tinea Versicolor, As It Appeared In 43 Pathological Samples Taken From Different Sites Of The Skin, And Its Incidence Accounted For 49.4%. It Is The Most Affected Area Of The Body.

***Keywords:** Tinea Versicolor, Malassezia, Fungi, Skin Diseases.*

1. INTRODUCTION

Fungi are eukaryotic organisms that are similar to mammals in terms of structure and metabolic mechanisms in the manufacture of protein and nucleic acids[1][2]. The infection

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caused by fungi is known as fungal diseases (Augustine), which are usually chronic infections because they It grows slowly, and is divided into superficial and opportunistic diseases (opportunistic and systemic), and people who are immunocompromised are more susceptible to infection [3]. It contains the appropriate environment for the growth of these organisms, including moisture, heat, and keratinous materials [4]. Mycoses are common diseases in humans for a long time, as millions of people in the world are exposed to them [5]. They cause skin infections known as tineas, tinea, or dermatophytosis in humans and animals. It possesses important characteristics because it is keratinophilic and keratinolytic, but it does not have the ability to penetrate deep tissues below the stratum corneum, as most of them are unable to live at a high temperature such as body temperature [6]. Dermatophytes are fungi capable of attacking keratinous tissues of humans and animals such as skin, hair and nails [7]. As for fungal diseases, they are diseases usually caused by multicellular or single-celled eukaryotic microorganisms called fungi. Fungi, including saprophytic, symbiotic, and parasitic (opportunistic) pathogens infect humans and animals, causing superficial mycoses, skin fungal infections (mehmet), and subcutaneous infections. Cutaneous (Subcutaneous Mycoses) and Systemic (Systemic Mycoses) fungal infections [8]. They can invade different places of the human body, such as the lungs and skin, as well as other parts of the body. Fungi are usually found in the air, water, and soil, and some of their types cause human diseases, although most people are not affected by fungi, and if they are affected by them, this effect is temporary, as the human body has The ability to resist and overcome it, especially for people with high immunity. Some of the fungi that because fungal diseases are free-living, and cause disease in humans through inhalation or entry of their spores into the body through wounds or scratches, and some of them are considered part of the normal flora of the human body and are harmless unless the body is immunocompromised [9] . People who suffer from immunosuppression or healthy people are susceptible to infection with fungi. Tinea versicolor diseases are characterized by general pathological symptoms, which are itching and redness on the skin, and the appearance of spots consisting of a series of uniformly-centered circles spread on the borders of the infection, as ringworm infection appears in the form of spots with a clear center surrounded by scaly red edges, as the fungal infection is divided according to the area of the infection into tinea capitis That occurs in the hair follicles and ringworm of the beard that appears in the beard area in males and congenital ringworm or body ringworm whose locations are the smooth skin of the face (except for the beard area), trunk and extremities (including the back of the hand and foot) and thigh ringworm appears in the groin or groin area It is the area between the abdomen and the groin on both sides of the body, and on both sides of the pubic region, as well as tinea pedis, tinea cruris, palm tinea, tinea cruris, and facial tinea that occurs on the face except for the beard area[10] . Although the proportion of fungi pathogenic to humans and their importance is small compared to viruses and bacteria, their importance increases, especially in cases where the host is exposed to immunodeficiency that results from malignant tumors and the use of immunosuppressive chemical drugs, as well as the ability of fungi to produce mycotoxins that cause allergies and attack tissues. Directly, difficulties have also emerged in the use of antibiotics against skin fungi, and their treatment is not easy, as most of the used antibiotics have an inhibitory effect on the growth of microorganisms, in addition to their toxic effect on humans, as well as the emergence of multiple antibiotic resistance [11]. In addition, the

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diagnosis of fungal skin diseases, especially those that belong to the group (Normal flora), is very difficult and disturbing, due to the confusion and similarities in these fungi, in addition to the fact that their symptoms are similar to some other skin diseases such as psoriasis and eczema. The scientific progress in the field of technology is the use of the PCR technique, which provides reliable and rapid diagnostic methods. This study aims to isolation and identification of Malassezia spp from patients with tinea versicolor in the cities of Kirkuk province.

2. MATERIALS AND METHODS

Description of the Study Area and the Selected Sites

This study was conducted in the province of Kirkuk, which is located at latitude 28-35 north and longitude 19-44 east. It is an Iraqi governorate, located in northern Iraq. Its area is 9679 square kilometers (3737 square miles), and the governorate is divided into four districts. [12][13], the study included 200 persons (males and females) suspected of being infected with tinea versicolor collected from Kirkuk governorate and its surrounding areas. Their ages ranged 10 - ≤40 years. Work began from October 15, 2023 to May 13, 2023. Ethical consideration and approval were signed by people before sampling. Five sites were selected for the study, including:

- The first site is the internal departments at the University of Kirkuk (Al-Sayada).
- The second location is Al-Aman Medical Center (laboratory).
- The third location, Doctors Street (Old Discipline).
- The fourth site (Hawija).
- The fifth site (Daqouq).

Estimating the Infection Rate in the Sites Specified for the Study

The infections under study of those infected with tinea versicolor were estimated for the five sites specified for the study by taking samples from each site and placing them in Petri dishes. Sterile environments are poured into it, which are later used as fungi farms to detect infections from others.

Table 1 Study site of samples collected

Study site	Samples
internal departments at the University of Kirkuk	47
Medical laboratories	50
Clinics of doctors specializing in dermatology	45
Consulting clinic in Hawija city	38
Daqouq	20
Total	200

Samples Collection

During this study, samples were collected from skin scraping (200) people. Skin scraping was done for each person and from different parts of the body, including the shoulder, arm, foot,

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and neck of the volunteers included in the study, using a sterile scalpel, and they were divided according to the type of examination in Kirkuk governorate, and from the groups specified for the study. According to age, and comparing these totals with a group of non-infected people and those infected with other types, according to the table 2 showing the sample groups according to age and gender.

Table 2 showing the sample groups according to age and gender

Groups	People status	10≤40years
Males and Females	Not infected	113
	Infected with M. furfur	43
	Infected with M. globosa	23
	Infected with M. slooffiae	14
	Infected with M.Restricta	7
	Infected with M.Obtuse	2

Identification and Examination / Direct Microscopic Examination

The diagnosis of fungal infections of Malassezia is one of the difficult problems because its symptoms may be similar to other skin diseases. Diagnosis depends on clinical symptoms. However, fungi respond to steroid examination to give an accurate diagnosis before giving appropriate treatment. Diagnosis can also be made by scraping the affected spots, preparing them and examining them with a microscope. In this study, 200 individuals from the control group were randomly selected from the areas of Kirkuk Governorate for a period of six months, and the clinical diagnosis was made by a consultant dermatologist. Forceps and surgical blades were used to scrape the skin. Direct and indirect methods have been applied for diagnosis. Scale samples were subjected to direct examination by placing them on a clean slide mounted with a drop of 10% KOH on dissolved keratinocyte, covered with a coverall, then the slides were gently heated and examined under a light microscope (40X). For the microscopic examination of mushroom cells, a suspension of mushroom cells was prepared, and this suspension was ringed under the microscope. In addition to that, in some fungal infections, we need to plant the mushroom on a suitable medium and then keep it at an optimal temperature for mushroom growth. Detection of Malassezia spp, for example, Pure and diagnosed fungal isolates were used, where the fungal isolates were initially diagnosed based on the external shape of the fungal growth and microscopic examination in the laboratory or by fungal culture analysis, to find out the presence of fungi in a specific area of the individual's body such as the skin, blood, or genitals, or nails, or wounds, affecting body systems [14], or by means of molecular diagnostics (Molecular Diagnostics).Where it is possible to detect fungal infection and determine the type of fungus causing it by performing a molecular diagnosis that is not based on cultivation, which is characterized by not requiring a sample of live fungal cells, as the principle of this test is based on the Polymerase Chain Reaction (PCR), which works even in Absence of live cells as long as typical DNA is available in the sample or a culture-based molecular diagnosis that requires a pure sample of fungal cells to be available in sufficient quantities for the examination.

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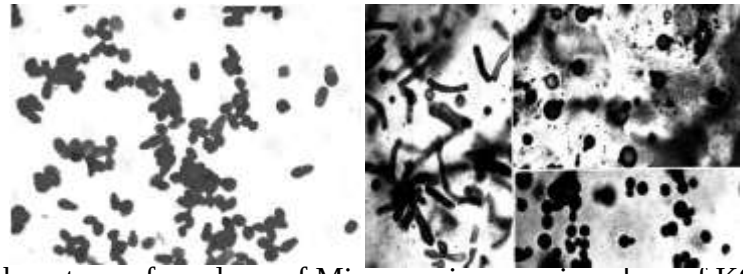

Fig.1 The phenotype of a colony of Microsporium spp in a drop of KOH grown on Sabouraud agar medium at a magnification of (40X).

Morphology and Microscopically Examination of the Colonies

The scraped were placed in sterile plastic Petri dishes. The sample was covered and transported to the laboratory for cultured on Sabouraud agar, and incubated at 37 ° for 7 days. By using a microscope, it is used to examine microorganisms that cannot be seen with the naked eye in the microbiology laboratory at the College of Education for Pure Sciences at the University of Kirkuk, which included examining the complete skin scraping image, which included measuring the fungus according to Single spore method As for the rest of the fungi, a part of the mycelium was taken from the side of the plate and transferred to the same culture medium at the top. All isolated fungi were also incubated for 14-21 days at 37°C. Fungi were identified according to the special taxonomic keys.

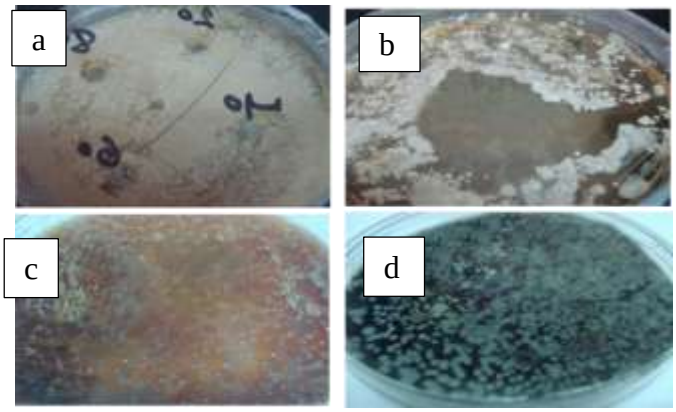

Fig. 2 Isolated of Malassezia spp on SDA and incubated at 37°C for 14-21 days. (a) incubated at 37°C for one week. (b) incubated at 37°C for 10 days. (c) incubated at 37°C for 15 days.ff` (d) incubated at 37°C for 3 weeks.

Identification / Species of the Genus Malassezia Spp

Malassezia spp. species appeared on SDA with different colors, and this is considered one of the phenotypic diagnostic characteristics, as Malassezia furfur (fig 3) on SDA presented a dark bluish-pink color and a velvety fluffy texture and clear zigzags appear on the colony and the back of the fungus is yellowish-white on the medium and the diameter of the colony ranges from 95-100 mm on SDA, microscopic examination showed conidia short, smooth-walled ending with a conical vesicle, on which are flask-shaped structures at the apex in a

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semi-circular shape, on which long chains of spherical conides are formed, with a diameter of 2.5-3 microns.

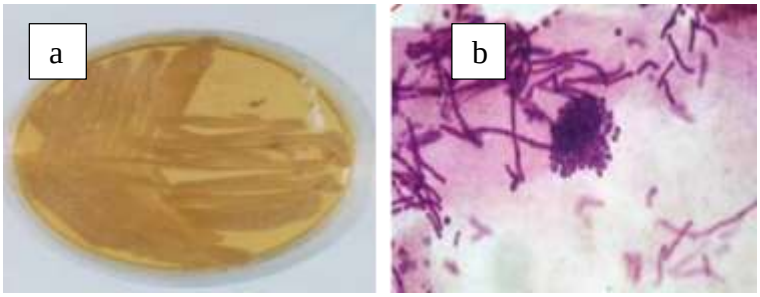

Fig.3 a- phenotype Malassezia furfur on SDA. b-under a microscope with a power of 400.

Figure 4 showed M. Globosa in violet color at the beginning of its growth on SDA with a microscopic circular shape. The mycelium appears divided and transparent conidia with thick walls arise from it and end in a spherical vesicle whose entire surface is covered by one row of flask structures that bear at their apex chains of conidia spherical.

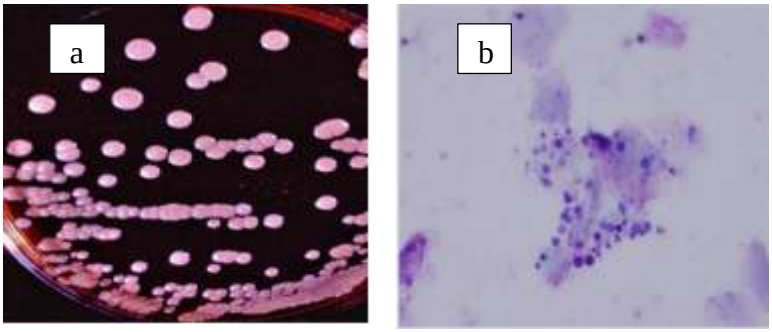

Fig. 4 a- phenotype M. Globosa on SDA. b- under a microscope with a power of 400.

Colonies of M. slooffiae (Fig. 5) appeared in a light pink color with flat surfaces divided by zigzag radial grooves, microscopic radial conidia heads and thick-walled conidia, topped by a vesicle that was completely covered by the phialids, conidia spherical or semi-spherical, pale pink, spiny, with a diameter of 3- 6 microns.

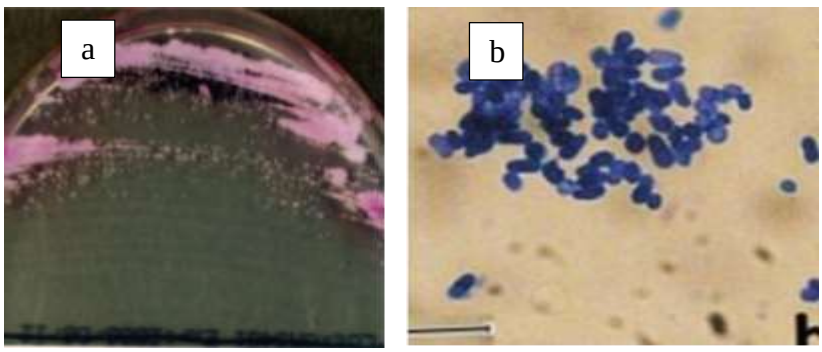

Fig. 5 a- phenotype M. slooffiae on SDA. b - under a microscope with a power of 400.

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Colonies of M. restricta appear in (Fig.6) with a fibrous shape topped by conidia, as the conidia are dark brown in color, while the conidia are in light brown color. Microscopically, the hyphae appear divided, and the conidial carrier is transparent with thin walls. As for the conidial head, it is spherical and large in size, on which the flask structures follow in a radial manner (resembling the sun) and in one row, at the end of which it bears long chains of conidia that appear yellow in color and are large in size, spherical or semi-spherical, with a diameter of 5-7 microns.

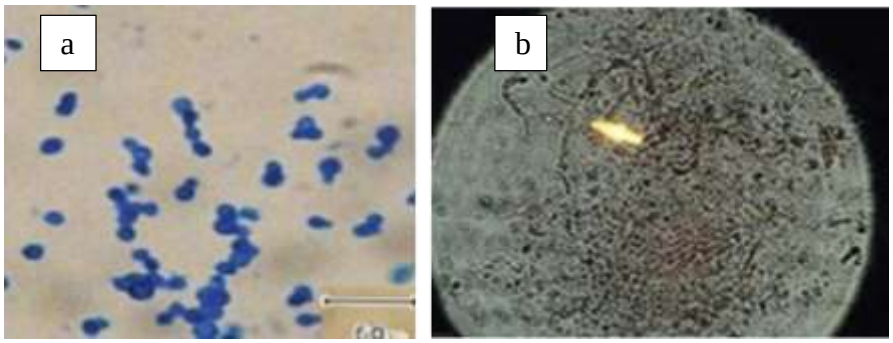

Fig. 6 a- phenotype M. restricta on SDA. b- under a microscope with a power of 400.

Colonies of M. obtusa appear (Fig.7) with a soft velvety surface and a light brown color. The back of the plate appears bright yellow. Microscopically, the conid heads look semi-spherical.

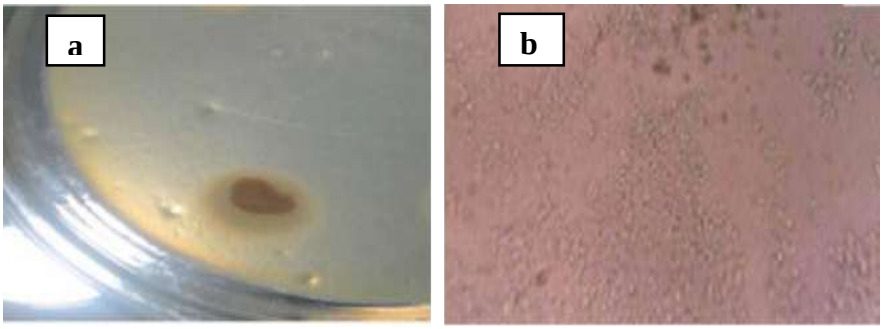

Fig. 7 a- phenotype M. obtusa on SDA. b - under a microscope with a power of 400.

Molecular study of fungi / Genomic DNA extraction

DNA from 10-day-old fungi was extracted using Chelex®100 (BioRad Company, USA). A small amount of fungal colonies for each sample was taken and transferred into a 0.6 mL tube containing 200 µL of Chelex®100 and 100 µL of TE and the tubes were then placed in a water bath at 95 °C. After 10 minutes, samples were removed and centrifuged at 13,000 rpm for 10 minutes. Then carefully remove the upper aqueous layer containing the DNA from the samples and place them in 0.2 mL tubes. Then store in the refrigerator at -4°C.

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Table 1 Components and volumes of PCR mix

PCR Prod	Ml
Master mix	12.5
primer F	1
primer R	1
DNA	5
D.W	5.5
Total	25

Table 2 Steps of the program used in the DNA extraction

No.	Stages	Temp.˚C	Time	Cycle
1	Initial DNA Denaturation stage	95 ˚C	5 min	1x
2	DNA Denaturation	95 ˚C	40 s	35x
3	Annealing	58 ˚C	1min	
4	Extention	72 ˚C	40s	
5	The final elongation stage	72 ˚C	7min	1x

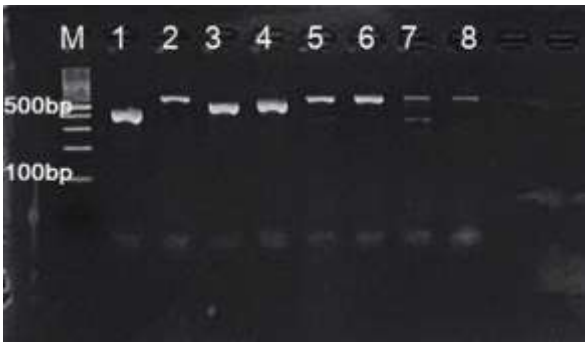

Fig 8 PCR products were separated on 1.5 % agarose Gel containing ethidium bromide and visualized under UV light.

Reaction Polymerase Chain (Pcr)

The method of [15] was followed to conduct this test, as the reaction materials described in the pcr amplification were mixed in an Eppendorf tube of 100 microliters according to the leaflet attached with the Green Master Mix manufactured by Promega. At the base pair 510-870 from the ladder table 3, the centrifugation process was performed at a speed of 10,000 rpm for half a minute to homogenize all the materials, after that the samples were placed in the PCR Sprint Thermal Cycler,and then it was run according to the program shown in Table4. The component and procedure are described according to [16] “Universal prefixes were used (5′-GCATCGATGAAGAACGCAGC-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) (QIAGEN, German) to amplify sequences of Malassezia species [17]

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Table 3 the sequence of nitrogenous bases in primers used in the amplification process

Gene	Primer Sequences '5→ ′3	Direction
ITS1	TCCGTAGGTGAACCTGCGG	Forword
	TCCTCCGCTTATTGATATGC	Reverse

Table 4 The used PCR amplification process program

Phase	**Time**	**Temperature (˚C)**	**Cycle No.**
Initial denaturation	1 min	96 ˚C	1
Denaturation	0.15 sec	96 ˚C	40
Annealing	0.30 sec	50 ˚C	
Extension	1.30 sec	72 ˚C	

Statistical Analysis
The data were analyzed statistically using a computer based on the software SAS, SPSS and ANOVA analysis of variance was used according to the F test to test the significance or non-significance of the groups used in the study for infected persons and the effect of plant extracts on mushrooms at the level of probability P≤ 0.01 and ≤0.05 Value and the mean were compared according to the Duncan multiple ranges test, standard deviation was used to determine the deviation of the values from the mean [18].

3. RESULTS AND DISCUSSIONS

Isolate Infected Individuals According to Gender and Age
The results showed that in 200 samples, 87 samples were infected, with a rate of 43.5% positive, and 113 samples were non-infected, with a rate of 56.5% negative for direct and indirect examination. 56.3% and for females 43.7%. The reason is attributed to the fact that the causative agent of tinea versicolor is a fungus of the genus Malassezia that is present on the surface of the body naturally for both sexes. It becomes a pathogen due to the influence of environmental conditions such as high air temperature, high humidity, genetic predisposition, pregnancy, oily skin, Hyperhidrosis, poor hygiene, poor nutrition, and weak immunity due to chronic

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infections or the use of immunosuppressive drugs, cortisone, and the use of greasy lotions and creams [19].

Table 5 The total number of positive and negative samples and their percentage distribution between males and females

Gender	No. samples	Positive samples	Percentage (%)	Negative samples	Percentage(%)
Male	125	49	39.2	76	60.8
Female	75	38	50.6	37	49.3
Total	200	87	٪43.5	113	56.5

Table 6 Distribution of infection percentage with Malassezia spp, according to sex and age

Variables	Age	Male positive samples	Male negative samples	Female positive samples	Female negative samples	P value
	10-20	23	30	16	18	0.095
	21-30	15	25	9	11	
	31-40	6	14	8	5	
	<40	5	7	5	3	
Total	200	49	76	38	37	

The distribution of patients in the age group 10-20 years with a total of 23 in males with a rate of 46.9% and 16 females with a rate of 42.1% was the highest compared to the rest of the age groups, followed by the age group 21-30 years 15 with a rate of 40.6% for males and 9 with a rate of 23.6% for females, then the age group 31-31 40 years, 6 with 12.2% for males, 8 with 21% for females, and finally the age group over 40 years, 5 with 10% for males and 5 with 13% for females, so the total number of infected males was 49 with a rate of 56.3%, and 38 with a rate of 56.3% for females, so the total for males and females was 87 infected And infected with fungi of the genus Malassezia spp, with statistically significant differences between them at the level of probability 0.01. These results are consistent with what was reached by [20], that the age group 10-20 years, (youth and adolescence), is the most susceptible to infection, and the reason In it is the high level of physical activity, hormonal changes and increased activity of sebum with the production of sebum (Sebum) glands. fatty acids in a lipid-rich environment in Malassezia spp [21], where the results of our study showed clinical characteristics and signs of the disease that tinea versicolor is more common among patients with oily skin, with significant differences from other skin types as well, as most patients with Tinea versicolor do not have a positive family history of the disease (negative history), that is, they are susceptible to infection. It was also noted that patients with tinea versicolor who do not suffer from concomitant diseases such as allergies and psoriasis have the highest incidence compared to patients with concomitant diseases of oily skin, because most of the Malassezia fungus They are lipophilic and have high enzymatic activity to digest these lipids and cause infection [22]. This disease is not contagious, and therefore infection may occur sometimes in more than one person within the same family, due to the presence of a genetic factor for infection [23].

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Diagnosis of Infected Individuals with Malassezia Spp

The results of Malassezia diagnosis for individuals who showed symptoms of tinea versicolor showed the presence of many fungal genera, the most common of which was Malassezia . furfur with an incidence rate of 49.4%, where some types of Malassezia spp isolated from individuals were diagnosed by methods for examining samples, as appropriate culture methods for samples and molecular methods were shown, with significant differences at the level of probability 0.01 and in 5 fungal isolates such as M. globosa In 23 isolates by 26.4%, M. slooffiae by 16.9% and 7 M. restricta cases by 8.4%. The number of samples that did not give positive growth was 2 samples, with a percentage of 16.9% and 2.29% of M.restricta and M.Obtusa from 87 isolates, as shown in Table 7.

Table 7 Types of Malassezia and the number of infected individuals

Malassezia spp	Infected individuals	Percentage
Malassezia. Furfur	43	٪49.4
M. globose	23	%26.4
M. slooffiae	14	%16.9
M. restricta	7	%8.4
M. Obtusa	2	٪2.29
M. sympodialis	-	-
M. dermatis	-	-
M. japonica	-	-

Molecular Identification

DNA was extracted from five isolates of Malassezia spp. and it appeared in all isolates when conducting electrophoresis on a 0.8% agarose gel with an electric current of 60 volts, as shown in Figure 9 , then these isolates were diagnosed using PCR and sequencing techniques using the 18S rRNA gene, where ITS1 and ITS4 were able to amplify the ITS3- region 5.8S-ITS2 for all fungal isolates that were successfully tested [17] positive human samples were confirmed by PCR as shown in Table 8 as it appeared in all studied isolates and the size of the gene ranged between 510-871 depending on the type The fungus , and this is consistent with the findings of [24], as well as [25] when studying the genus Candida and agrees with the findings of [26] when studying species belonging to different genera of fungi as isolates were identified molecularly according to PCR products with ITS1-ITS4 sizes of Malassezia spp show that Malassezia furfur bp509, M. globosa bp430, M. obtusa bp483, M. Slooffiae bp410 and M. Restricta bp400.

Table 8 Malassezia spp., and DNA products generated through ITS3 and ITS4

Malassezia species	Sizes ITS1-ITS4 (bp)
M. furfur	509
M. globosa	430
M. obtuse	483
M.Slooffiae	410
M.Restricta	400

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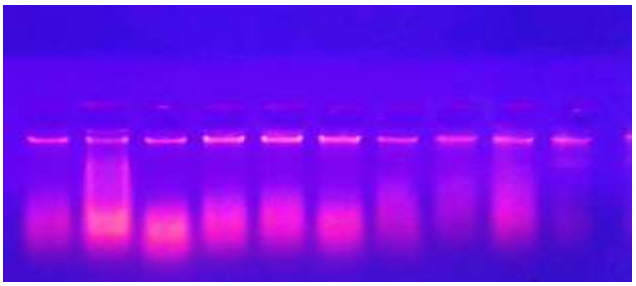

Fig. 9 Total DNA bundles on a 0.8% agarose gel at 60 volts for 60 minutes

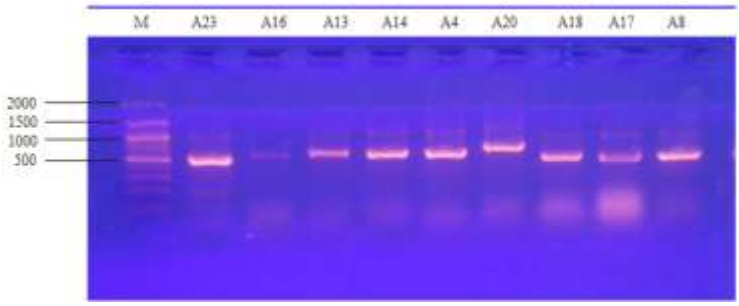

Fig. 10 PCR results representing 18SrRNA gene bundles on a 1% agarose gel at 85 volts for 35 minutes. M indicates a marker or a lader. A isolate symbol and the numbers represent the isolation number

Table 9 shows the names of the isolates, the percentage of similarity, the registration or accession number, and the nucleotide variation (mutation variation). Five isolates of fungi were isolated in our study

A2	MF327240	Malassezia. Furfur
A5	MF351537	Malassezia globosa
A6	MF373425.1	Malassezia slooffiae
A7	MF351538	Malassezia restricta
A8	MF351538	Malassezia Obtusa

4. CONCLUSIONS

Males were affected more than females, and according to the results of our study the percentage in males was 56.3% and females 43.7% There was no significant difference between the sexes exposed to the fungus Malassezia spp.The age group (20-10) years were more exposed to Malassezia infection, and by (44.8%) of the total infections. The most common fungus in skin infections is Furfur M. (49.4%) of the four isolated species.The best extracts in inhibiting the growth of the genus Malassezia spp tested are the hot alcoholic extracts of turmeric, garlic and licorice.The hot alcoholic extracts of the plants used in the study outperformed their inhibitory efficacy against the fungi Malassezia spp included in the research on hot and cold aqueous extracts.

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