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Isolation and Identification of Dermatophyte Species from Iraqi Patients Using PCR-ITS Regions

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Abstract

Dermatophytes are the primary etiological agents responsible for superficial fungal infections worldwide. The current study, undertaken in Baghdad, Iraq, aimed to diagnose dermatophyte species by targeting the ITS (Internal Transcribed Spacer) region and employing phylogenetic analysis techniques. A total of 104 specimens from hair, nails, and skin scrapings of patients were examined microscopically and cultured on a specific medium SDA with addition of cycloheximide and chloramphenicol. PCR with ITS1 and ITS4 primers was employed for further characterization of dermatophytes. According to results, tinea corporis was the predominant infection (51.9%), and tinea cruris (18.2%). Molecular results revealed that *T. indotineae* (24%) is the predominant isolate, followed by *T. mentagrophyte* (5.7%), *T. simii* (2.8%), *T. rubrum*, and *M. canis* (3.8%, 1.9% respectively). This is the first record of *T. indotineae* affected by tinea corporis and cruris in Baghdad and Iraq. Twelve isolates, including nine *T. indotineae*, registered with new code in the NCBI database. The findings showed that PCR-ITS methods are essential for monitoring trends in dermatophytosis and detecting dermatophyte species in communities, given the presence of a complete database and operational taxonomic richness and specificity.

Keywords: Dermatophytes, Iraq, ITS region, Phylogenic tree, Sequencing, *T. indotineae*.

عزل وتشخيص أنواع الفطريات الجلدية من المرضى العراقيين باستخدام منطقة PCR–ITS

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الخلاصة

الفطريات الجلدية هي العوامل المسببة الأولية المسؤولة عن الالتهابات الفطرية السطحية على مستوى العالم. تهدف الدراسة الحالية، التي أجريت في بغداد، العراق، إلى تشخيص أنواع الفطريات الجلدية من خلال استهداف منطقة ITS (فواصل النسخ الداخلية) واستخدام تقنيات تحليل النشوء والتطور. من مجموع 104 عينة جمعت من شعر وأضافر وقشور الجلد من المرضى بالسعفة ثم فحصت المجهر الضوئي وزرعت على وسط SDA مع إضافة السايكلوهكسامايد والكلورومفينيكول. قد تم استخدام تقنية PCR مع البرايمرات ITS1 و ITS4 لغرض التشخيص الدقيق لأنواع للفطريات الجلدية. أظهرت النتائج ان الأكثر انتشارا هي سعفة الجسم (51.9%)، والسعفة الفخذية (18.2 %). أظهرت النتائج الجزيئية ان *T. indotineae* (24%) هي العزلة

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السائدة, تليها *T .mentagrophytes* (5.7%) و *T. simii* (2.8%) و*T. rubrum* و*M. canis* (1.9% ,3.8%على التوالي). يعد هذا اول تسجيل لنوع *T. indotineae* في بغداد/العراق. اثنتا عشر عزلة، منها تسع عزلات لنوع *T. indotineae* , تم تسجيلها برمز جديد في قاعدة بيانات NCBI. أكدت النتائج ان تقنية PCR–ITS تعتبر أداة أساسية لتحديد أنواع الفطريات الجلدية الشائعة عند استخدام قاعدة بيانات شاملة، بالإضافة الى مراعاة الغنى التصنيفي والدقة في تصنيف الفطريات.

1. Introduction

Dermatophytes are a group of pathogenic fungi that infect and damage keratinized tissues in humans, affecting the skin, hair, and nails of both fingers and toes, as well as in animals. This fungal infection is medically recognized as dermatophytosis, Tinea, or ringworm, and is primarily caused by fungi from the genera Trichophyton, Epidermophyton, and Microsporum [1]. Infection begins when arthroconidia adhere to the outermost layer of epidermis of skin (stratum corneum) then lyses keratin, a major part of this layer, because of the nutrients it contains [2, 3]. About 20% to 25% of humans are affected by Dermatophytosis [4]. A different form of clinical symptoms that is caused by dermatophyte spp. including, dermatophytic mycetoma (pseudomycetoma) and tinea infection (tinea corporis, tinea capitis, tinea pedis, tinea manuum, and tinea unguium) [5]. The main cause of transmission occurs when humans have direct or indirect contact with infected humans (anthropophilic), or with infected animals.

(zoophilic), or with soil contaminated with arthroconidia (geophilic) and is influenced by environmental factors, occupations, and lifestyles [6]. The extending of urban and rural populations, increased interest in raising pets in homes, variations in temperature and humidity, the use of immunosuppressive drugs, abuse and rise in antifungal resistance, misdiagnosis of infections, and other variables may all be used to explain the fluctuating frequency of dermatophytes, these factors affect the phenotypic and genotypic diversity of dermatophytes in various geographical regions [7]. Phenotypical identification of dermatophyte isolates is typically successful, but it takes a lot of time and requires a lot of knowledge from biologists due to differences may vary between individual isolates, and there may be similarities between different species, so identification is occasionally unreliable, for example, in the year 2020, *T. indotineae* was recently recognized as a separate species, different from *T. interdigitale* and *T. mentagrophytes* [8]. Consequently, clinical laboratories necessitate rapid and unbiased diagnostic methods for dermatophytosis to facilitate prompt administration of appropriate antifungal therapy and identification of the infection sources. To address this need, various molecular techniques have been developed, including multiplex PCR, nested PCR for dermatophytes, real-time PCR, PCR-RFLP, PCR-RLB, and PCR-ELISA [9], DendrisCHIP® [10], MALDI-TOF MS [11], SNP analysis [12]. The use of diagnostic techniques based on DNA sequences around 5.8S rDNA, specifically ITS1 and ITS2, is very sensitive and reliable in identifying different species of dermatophytes. This approach enhances diagnostic accuracy and ensures consistent results across different laboratories [13]. The process involves determining the sequence of amplified fragments of the target gene and aligning the resulting nucleotide sequences to an online database of DNA [14]. Thus, this study applies the PCR molecular identification technique, which includes studying the ITS1 and ITS4 sequences of clinical samples of dermatophytes obtained from tinea patients in Baghdad hospitals, as well as to perform genetic phylogenetic analysis of these samples.

2. Materials and Methods

2.1 Fungal specimens' collection and culturing

One hundred four clinical specimens were collected from patients who visited the dermatology consulting clinic at Al-Yarmouk Teaching Hospital and Al-Zahraa Consultative Center for Allergy and Asthma. Specimens included skin scrapings, nails, and hair clippings.

This study was approved by the ethics committee in the Department of Biology, College of Science, University of Baghdad Ref. No. CSEC/01223/00143 on December 20, 2023. The clinical identification of tinea was performed by a specialized dermatologist, before collecting the specimens, the patients were given a thorough description of the study and asked for their agreement and information on age, sex, origin of isolation, and other data was recorded. The specimens were examined microscopically after being treated with 5 drops of 10% KOH. The collected samples were then cultured on Sabouraud's dextrose agar (SDA) medium supplemented with cyclohexamide and chloramphenicol. The inoculated plates were incubated at a temperature range of 25-30°C and examined for growth after 7 to 14 days [15].

2.2 Molecular Characterization

2.2.1 DNA Extraction

Genomic DNA was isolated from positive fungal growth according to the protocol of ABIO pure Extraction (ABIOpure, USA) by following the manufacturer's guidance, DNA concentration values was detected by quantus fluorometer (Promega, USA) then stored at -20 °C.

2.2.2 PCR and sequencing

The PCR reaction was performed in a reaction volume of 25 µL, which included 12.5 µL of Master Mix,1 µL of each universal primers ITS1 (5`-TCCGTAGGTGAACCTGCGG-3`) and ITS4 (5`-TCCTCCGCTTATTGATATGC-3`) with annealing temperature (55C°) (Macrogen Company, Korea) [16], 7.5 µL of nuclease-free water, and 3 ng/µl of DNA. The PCR program was performed with 30 cycles as in Table1. The amplified DNA fragments were subjected to 1.5% agarose gel electrophoresis at 100V/mAmp for 60 minutes. Subsequently, the ethidium bromide-stained bands on the gel were visualized using a Gel Imaging System.

Table 1: The optimum condition (PCR) of detection of gene

No.	Phase	Temperature °C	Time	No. of cycle
1	Initial Denaturation	95	5 mints	1
2	Denaturation	95	30 second	30
3	Annealing	55	30 second	
4	Extension	72	30 second	
5	Final extension	72	7 mints	1
6	Hold	10	10 mints	

In Korea, and by Macrogen Corporation, Sanger sequencing for PCR products was performed by using the ABI3730XL automated DNA sequences. Comparative sequence analyses were carried out using the Basic Local Alignment Search Tool (BLAST) on the National Center for Biotechnology Information (NCBI) website. Geneious software version 11.1 was used to generate phylogenetic trees from unambiguously identical sequences.

3. Results

The current study included the collection of 104 specimens, which included 82 skin scrapes, 16 hair clippings, and 6 nail specimens. The results showed that 65 specimens out of the 104 investigated specimens have positive fungal growth, giving a prevalence rate of (62.5%), while 39 specimens have negative fungal growth, giving a prevalence rate of (37.5%), where out of a total of 65 positive fungal growths, 40 (61.5%) isolates were dermatophytes, while 25 (38.5%) isolates were other fungi as in Figure 1. According to the results, tinea corporis had a higher prevalence at (51.9%), followed by tinea cruris at (18.2%), and a higher percentage found in females at (58.6%) than in males, The average age of patients in this study who are infected with tineas was ≤ 34 (3-65) years and other information such as animal and humans contact history as well treatment history found in Table 2. Direct examination, morphological

diagnosis of the colonies on the culture, and molecular diagnosis yielded the same results. *T. indotineae* isolates were found to be the most common fungus isolated, causing dermatophytosis at (24%), *T. mentagrophytes* at (5.7%), followed by *T. rubrum* at (3.8%), *T. simii* at (2.8%), and *M. canis* (1.9%). According to the SDA culture's results as in Figure 2, the morphology of the colonies was varied; colonies of *T. indotineae* were smooth and whitish; granular colonies had velvety nap and central rise-like umbo and reverse pigments that were yellowish to light brown; and *T. mentographytes* colonies surface was fluffy and white, their invert is commonly light brown or pallid, while *T. simii* isolates generated flat colonies that ranged in color from white to creamy and had an unpigmented or light yellowish reverse, while *T. rubrum* colonies are white to buff, fluffy, flat, and white or even colorless in reverse. *M. canis* colonies Surfaces are whitish, coarsely fluffy, with peripheral yellow pigment, in invert; the color is amber, which turned to brownish yellow over time and formed radial rings.

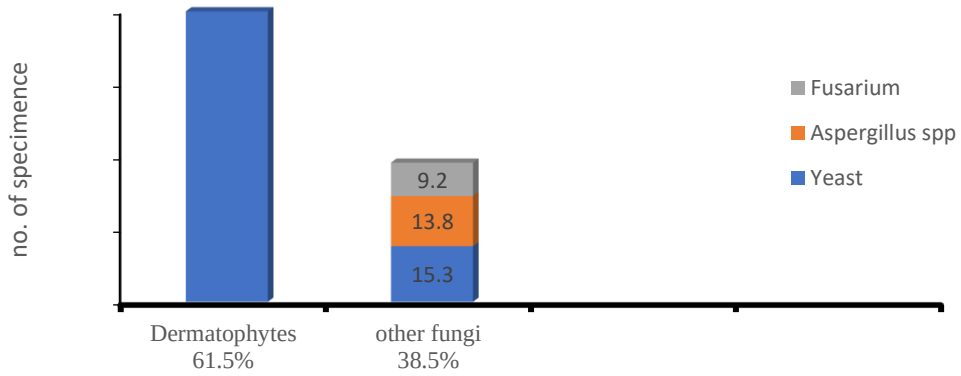

Figure 1: shows the cultivation results of Tinea cases: Dermatophyte and other opportunistic fungi.

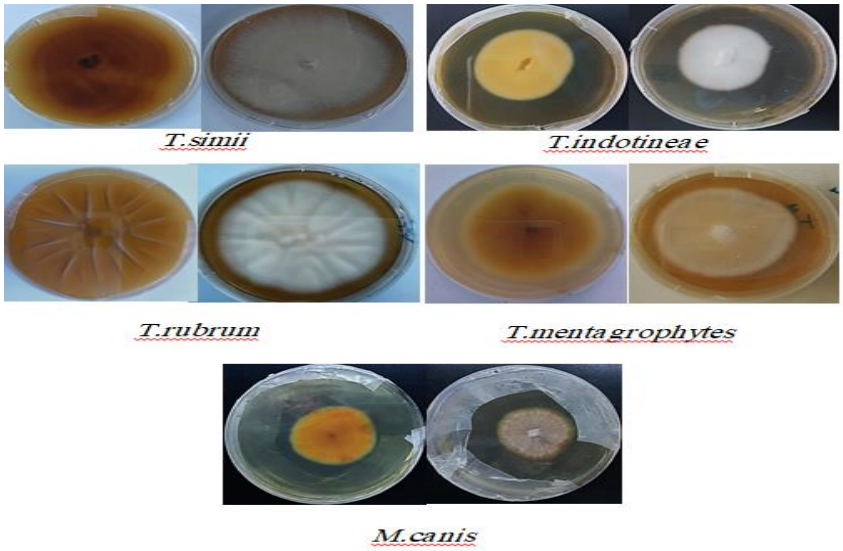

Figure 2: shows macroscopic view of dermatophytes colonies on Sabouraud's dextrose agar (SDA) containing cyclohexamide and chloramphenicol at 25-30 C°, checked after 7-14 days.

Table 2: Distributions and frequencies of dermatophyte isolates according to tinea forms concerning clinical information of patients

Clinical information		Dermatophytosis							
		Tinea corporis	Tinea cruris	Tinea pedis	Tinea manum	Tinea capitis	Tinea barbae	Tinea faciei	Total
Gender	Female	35	12	3	3	4	0	4	61(58.6 %)
	Male	19	7	5	2	3	5	2	43(41.3 %)
	Total	54 (51.9%)	19 (18.2%)	8 (7.6%)	5 (4.8%)	7 (6.7%)	5 (4.8%)	6 (5.7%)	
Age	Average (Min-Max)	24(3-46)	39(15-63)	45(30-65)	37(18-46)	32(21-43)	34 (23-52)	28(22-39)	
Animal contact history	Yes	16	9	0	2	2	0	0	
	No	38	10	8	3	5	5	6	
Patient contact history	Yes	37	11	3	1	2	3	3	
	No	17	8	5	4	5	2	3	
Treatment of illness before visiting clinic	+	4	15	1	2	1	5	4	
	-	2	10	2	2	1	0	2	

All PCR products obtained from dermatophyte samples were subjected to electrophoresis, revealing bands from the highly variable ITS regions of DNA with sizes ranging from 650 to 750 bp, as depicted in Figure 3. In this study, 13 isolates of *T. indotineae* exhibited a high degree of similarity (100% coverage, 100% identity, and 0% gaps) compared to reference strains in the GenBank database, while 8 isolates showed 99% identity. Additionally, *T. mentagrophyte* displayed 3 isolates with 100% similarity, 3 isolates with 99% similarity, *T. simii* had 3 isolates with 100% similarity, *T. rubrum* had 4 isolates with 100% similarity, and *M. canis* showed 2 isolates with 100% similarity. There were 12 new species registered in the NCBI's universal gene bank database, as shown in Table 3. ITS sequence analysis revealed that substitutions in the ribosomal RNA gene in 11 isolates are transitions; most of them at G/A and C/T, while other mutations only at G/A or C/T, and there was no transversion, insertion, or deletion mutation in any of the isolates. Only *T. mentagrophytes* (OQ787386.1) isolates show (100%) similarity and contain no mutation, but all other isolates have variations in the conformity percentage with a gene bank of (99%) similarity. Two *T. mentagrophyte* isolates have (99.80% and 99.85%) similarity and have mutation at G/A, while other *T. mentagrophyte* isolates also have a similarity in the conformity percentage with (99.84%) but a mutation at C/T. Four isolates of *T. indotineae* have (99.69%) similarity with mutations at G/A and C/T, while three isolates have (99.85%) similarity with mutations at G/A. *T. simii* has (99%) similarity have mutation at C/T.

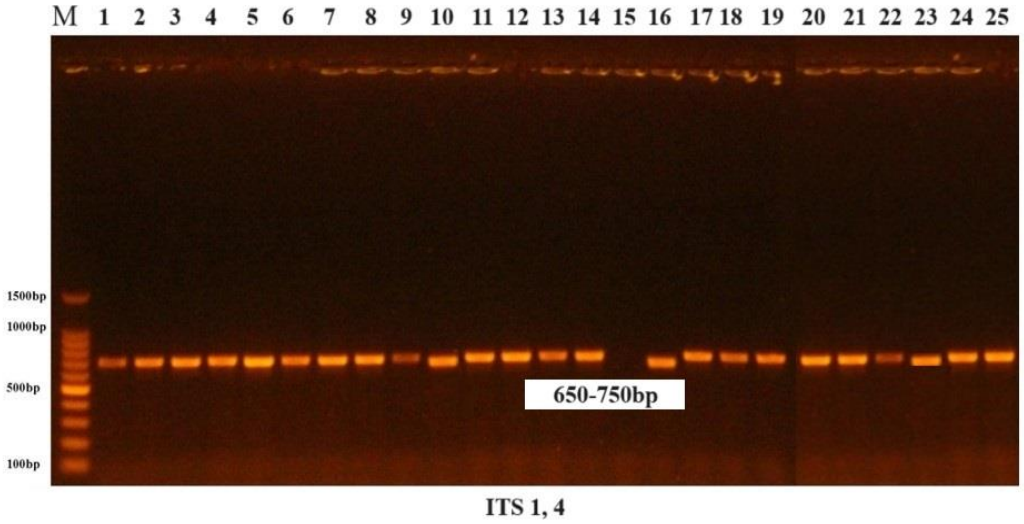

Figure 3: Agarose gel electrophoresis of PCR product for dermatophytes species isolates, the band size 650-750 bp. The product was electrophoresis on 1.5% L: DNA ladder (100), lane (1-25) PCR product of band size 650-750bp.

Table 3: Dermatophyte isolates are conventionally and molecularly characterized and given a new NCBI code.

Isolates	Compared Sequences ID In NCBI	New Accession	Identities	Type Of Substitution (Nucleotides)		Sex/Age (Year)	Source /Conventional Characterization
T. mentagrophytes	MT367568.1	OQ787076.1	99.80%	Transition	G/A	Male /18	Skin / *tinea corporis*
T. simii	OP391648.1	OQ787085.1	99.36%	Transition	T/C	Male/65	Skin / *tinea cruries*
T. indotineae	MT371234.1	OQ787108.1	99.69%	Transition Transition	T/C G/A	Male /35	Skin / *tinea corporis*
T. indotineae	MT371234.1	OQ789636.1	99.69%	Transition Transition	T/C G/A	Male /50	Skin / *tinea corporis*
T. mentagrophytes	MT367568.1	OQ787386.1	100%	----		Female/5	hair scalp/ *tinea capitis*
T. mentagrophytes	MW752112.1	OQ787419.1	99.84%	Transition	T/C	Female/45	Skin / *tinea corporis*
T. indotineae	MT371234.1	OQ787436.1	99.85%	Transition	G/A	Female/40	Skin / *tinea corporis*
T.indotineae	MT371234.1	OQ789643.1	99.69%	Transition	G/A T/C	Female/42	Skin / *tinea corporis*
T. mentagrophytes	MT367568.1	OQ787438.1	99.85%	Transition	G/A	Female/30	Skin/ tinea cruries
T.indotineae	MT371234.1	OQ789644.1	99.85%	Transition	G/A	Female/15	Skin / *tinea corporis*
T.indotineae	MT371234.1	OQ789635.1	99.85%	Transition	G/A	Female/29	Skin / *tinea corporis*
T. indotineae	MT371234.1	OQ789642.1	99.69%	Transition	T/C G/A	male/40	Skin / *tinea corporis*

Regarding the molecular data and by using Geneious software, phylogenetic trees were performed to illustrate the evolutionary connections between the various dermatophyte species. In this study, 25 isolates were distributed among three subclades as shown in Figure 4. The first and second clades including the *T. mentagrophyte* clade are interfering with the *T.*

indotineae clade because *T. mentagrophyte* represents common ancestors of *T. indotineae* and is classified as new species and that is just now beginning to Iraq's recalcitrant dermatophytosis pathogen. The third clade included *T. simii* isolates.

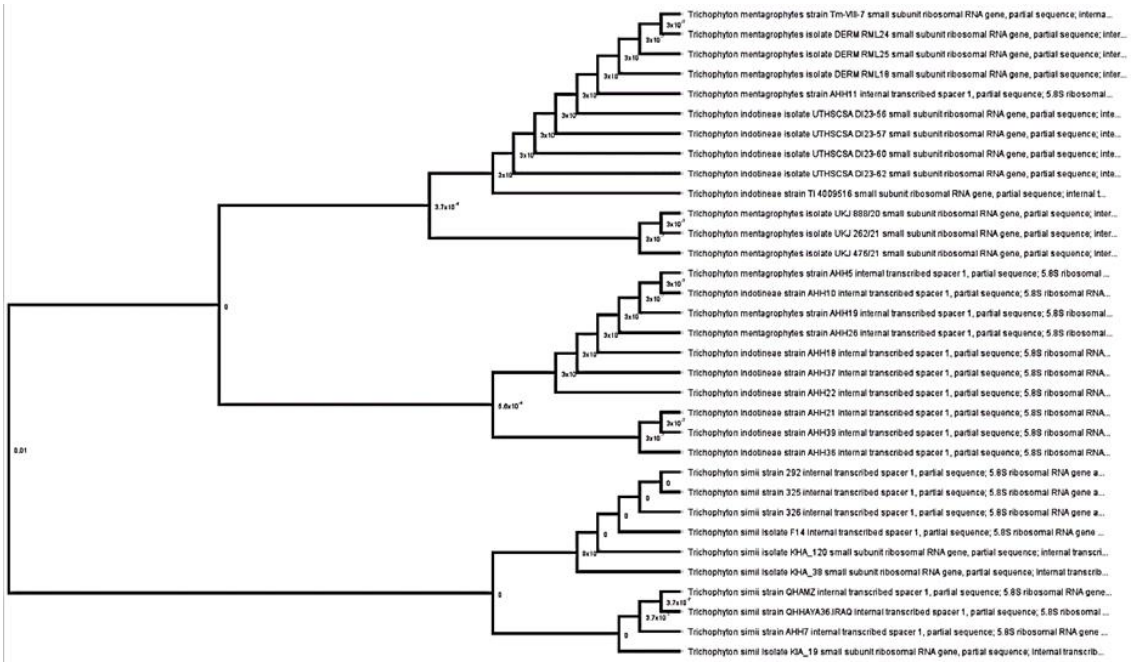

Figure 4: Phylogenetic tree with the highest level of likelihood for an Iraqi specimen based on their ITS sequences.

4. Discussion

Among superficial mycoses, dermatophytosis are the most common, according to this study, dermatophytes are increasingly widespread in many countries as well as in Iraq and beyond. In India and most of Asia countries *T. mentagrophytes* (genotype VIII) has become rapidly widespread and common in recent years and has become a major problem [17]. A recent outbreak of dermatophytosis among Iraqi patients and difficulties in identification of dermatophytes by conventional methods, which largely based on clinical and morphology diagnosis, is usually less accurate because some skin diseases mimic symptoms of dermatophytosis [18-21]. Because DNA is an unequivocal marker and more accurate, it is possible to use sequencing of their more diverse ITS regions in diagnosis of dermatophytes because within the same species, these dermatophytes show significantly variable ITS regions [14]. The current study examined 104 patients, and a high percentage of females were found to be affected by most types of tinea infections. These findings are consistent with the results of other studies [22, 23], while in man the percentage are higher in tinea pedis and tinea barbae this could be attribute to some factors such as customary outdoor activities, direct contact with infected people or contaminated tools and this in consistent with [24, 25]. In younger age groups, dermatophytosis was found to be prevalent; the average age of patient in this study with dermatophytosis was ≤34.1 years, and these results were in consistent with result of [22, 23, 25] which concluded that the major of dermatophytosis infection within average 25-55 years, and this is due to physical activity as well as changing in hormones and the potential for exposure to infection are high [26]. Tinea corporis and tinea cruris were the predominant clinical diagnoses in this study, accounting for 51.9% and 18.2% of cases, respectively. These findings are consistent with previous research conducted in Iraq [27, 28] as well as several other countries, including India [10, 29], Iran [25], Lebanon [30], and China [31], where tinea corporis and tinea cruris have also been widely reported as common dermatophyte infections. In this study, this is the first of this species *T.indotineae* (In previous research before 2020 it

has been identified as *T. mentagrophytes* (genotype VIII)) were identified in Iraqi patients affected by tinea corporis and tinea cruris and it found to be the most common fungus isolated causing dermatophytosis at (24%), and this in consistent with [17, 32] and it identified as *T.indotineae* to avoided misidentification and confusion with other neighboring species such as *T. mentagrophytes* or *T. interdigitale* [31]. According to information collected from patients under study, the highest prevalence was observed in patients who had direct contact with infected people from human to humans by sharing items such as washing machines and clothes with infected people, using public Western-style toilets (anthrophilic), or raised animals (zoophilic). For species-level classification, dermatophytes' species with highly nucleotide pleomorphism in the ITS region of the rDNA have become the decisive criterion for identification and it is considered a powerful tool in DNA sequencing through which it can manage and analyze the phylogenetic relationships of ITS region inside the same genus as well as within the family Arthrodermatacae [27, 28]. Among 25 sequenced species of dermatophytes, 12 isolates were registered in the NCBI with new accession numbers. The substitutions in the ribosomal RNA genes are transitions in G/A or C/T or both. Substitutions such as transitions, transversion, or deletion in the ribosomal RNA genes may lead to an increase or decrease in antifungal resistance or pathogenicity of dermatophyte species [27]. A phylogenetic tree was designed using the ITS1 and ITS4 regions since these genes and species were comparable and alignable across all strains. By improving hygiene and generalizing animal quarantine, and as a result of migration and modern lifestyles, these dermatophyte spp. these lead to co-infection with other strains and shift from zoophilic (*T. mentagrophytes*, *M. canis*, and *T. simii*) to anthropophilic (*T.indotineae* and *T. rubrum*) and these lead to genetic shifting and formation of highly virulence strain [19, 33, 34].

5. Conclusions

Modern diagnostic methods demonstrated excellent performance characteristics for the detection and identification of dermatophytes. The ITS sequencing method provide effective, accurate, and realistic methods in identifying dermatophytes spp.. *T.indotineae* being the most common fungus isolated causing dermatophytosis in Baghdad. The transition of these dermatophyte species from zoophilic to anthropophilic forms has led to the spread of dermatophytosis, resulting in a rise in acute tinea infections in Baghdad. To address this escalating issue, urgent measures such as enhanced hygiene practices, strict animal quarantine protocols, minimizing direct contact with infected individuals, precise diagnosis of tinea infections in medical facilities including hospitals and private clinics, and the prescription of appropriate antifungal treatments have become imperative. The phylogenetic tree identifies most isolates from the same species, indicating significant genetic similarity, but indicating distinct differences from those from other species.

6. Acknowledgments

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7. Disclosure and Conflict of Interest

The authors declare that they have no conflicts of interest.

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