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Detection of Subtilisin (1-7) Genes and their Relationship to Antifungal Resistance in Several Dermatophyte Isolates

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Abstract

Dermatophytes secrete enzymes that allow them to invade keratin tissues, act as antigens, and trigger inflammatory responses. Subtilisin-like proteins degrade keratin structures, facilitating their infection of body tissues and the host. This study aimed to detect subtilisin genes (*SUB*1-7) through conventional PCR analysis and to investigate their relationship with the antifungal resistance of certain dermatophyte isolates. Twenty-two isolates obtained from Iraqi patients were identified both morphologically and molecularly, specifically using the internal transcribed spacer (ITS) region. A genomic DNA extraction kit was used to extract genomic DNA from fungal growth. The identified isolates were found to consist of the following species: *Trichophyton indotineae*, *Trichophyton mentagrophytes*, *Trichophyton rubrum*, *Trichophyton simii*, and *Microsporum canis*. Six antifungal agents were tested on the dermatophyte isolates using the disk diffusion method. All isolates from every species were found to be susceptible to voriconazole and fluconazole, while most isolates demonstrated resistance to nystatin. Virulence genes *SUB2* and *SUB5* were not detected in any of the dermatophyte isolates. *SUB1* and *SUB4* were the most frequently observed genes, while *SUB3* was the least frequent. This study is the first in Iraq to reveal subtilisin-like genes and their association with antifungal resistance, especially in *T. indotineae.*

Keywords: Antifungals, Conventional PCR, Dermatophytes, Iraq, Subtilisin-like genes, *T.indotinea*.

الكشف عن مورثات سبتيليزين (1–7) وعلاقتها بمقاومة مضادات الفطريات في بعض عزلات الفطريات الجلدية

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الخلاصة

تفرز الفطريات الجلدية الإنزيمات لغزو أنسجة الكيراتين، وتعمل كمستضدات وتسبب استجابات التهابية. انزيم البروتييز تعمل على تحلل الكيراتين، مما يسمح لها بإصابة أنسجة المضيف وأنسجة الجسم مما يسهم في نشر المرض .تهدف الدراسة الحالية إلى الكشف عن مورثات السبتليزين (1–7) عن طريق تحليل تفاعل البوليميراز المتسلسل التقليدي وعلاقته بمقاومة بعض عزلات الفطريات الجلدية ضد مضادات الفطريات. تم تشخيص اثنان وعشرون عزلة من الفطريات الجلدية التي تم الحصول عليها من مرضى عراقيين مورفولوجياً وجزيئياً [منطقة الفاصل الداخلي المنقول(ITS)] .تم استخدام مجموعة استخلاص الحمض النووي الوراثي

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لاستخراج الحمض النووي الوراثي من نمو الفطريات . وقد وجد أن العزلات التي تم تحديدها تتكون من الأنواع التالية: *Trichophyton indotinea* و *Trichophyton mentagrophytes* و *Trichophyton rubrum*و *Trichophyton simii* و *Microsporum canis* .تم استخدام ستة مضادات فطرية بين عزلات الفطريات الجلدية باستخدام طريقة القرص. كانت جميع العزلات بجميع أنواعها حساسة للفوريكونازول والفلوكونازول، في حين أبدت معظم العزلات مقاومة للنيستاتين. لم يتم الكشف عن نتائج لمورثات الضراوة *SUB 2* و*SUB 5* في أي من عزلات الفطريات الجلدية. *SUB1* و*SUB4* كان لهما أعلى تكرار وكان *SUB3*هو الأقل. تعتبر هذه الدراسة هي الدراسة الأولى في العراق التي تكشف المورثات الشبيهة بالسبتليسين وعلاقتها بمقاومة مضادات الفطريات خاصة في نوع*T.indotinea* .

1. Introduction

Dermatophytes are a group of highly specialized pathogenic fungi that commonly cause infectious diseases. They have the ability to invade and reproduce in the keratinized layers of skin, as well as in human hair and nails. The first obstacle that dermatophytes encounter when infecting the skin and need to overcome is the protective barriers of the skin, which include morphological, chemical and physical barriers. Previous abnormalities and wounds in the stratum corneum also play a role in the occurrence of fungal infection [1]. The pathogens begin to colonize and adhere, followed by germination and invasion, and the first steps of infection are initiated by arthroconidia, which adhere to the host skin (animal or human) via surface proteins specific to each fungal species. Then, arthroconidia establish a favorable environment for growth as fungal hyphae and reactivate their metabolism. Finally, the epidermis (stratum corneum) is infected and spreads when the fungal hyphae produce germ tubes, which secrete important enzymes responsible for the degradation of keratin into peptides and amino acids, which are important as nutrients for the growth and reproduction of dermatophytes [2]. Keratin can then be further broken down by several enzymes secreted by dermatophytes, the main virulence factors of dermatophytes being proteases that are involved in infection and degradation of the host's stratum corneum [3]. Proteases can be classified as endoproteases or exoproteases based on their active sites, which include aspartic, cysteine, glutamic, metallo, serine, and threonine proteases. Serine-like proteases and metalloproteases are the main proteases secreted by dermatophytes. Endoproteases break peptide bonds within polypeptides, whereas exoproteases break only peptide bonds at the N- or C-terminus of polypeptides [4]. Dermatophytes were shown to possess a genome- encoding battery of secreted proteases [5]. The major dermatophyte endoproteinas are subtilisins (S8A serine protease family); and fungalysins (M36metallopeptidase family); however, five genes (mep1–5) encoding metalloproteases (Mep) and Subtilisin-like protease (*SUB*) are coded by seven genes (*SUB*1–7) [6]. In order to penetrate keratinized tissues, including skin, hair, and nails, and acquire nutrients needed to survive, dermatophytes excrete these digestive enzymes during the infection phases [7]. These enzymes have the ability to function as antigens and trigger different levels of inflammation [8]. Keratin-degrading enzymes known as subtilisin-like proteins (*SUB*) have been identified in *M. canis*, *T. rubrum*, and *T. mentagrophytes* [9,10]. Subtilisin genes (*SUB3* and *SUB4*) were the main proteases expressed by *Trichophyton* spp. during in vitro growth [11]. *SUB6* was also a reliable indicator of in vivo trichophytic and onychomycotic diseases caused by *Trichophyton* spp., and was also shown to be a major virulence factor for *T. mentagrophytes* [12,7]. *SUB3* is one of these virulence genes known to be involved in the adhesion of *M. canis* to the keratinized structures of the host. However, for *T. rubrum* to infiltrate the skin, *SUB3* and *SUB4* were required [13]. After cultivation in a medium containing powdered nail, hair, and skin chips, the expression of *SUB1*, *SUB6*, and *SUB7* increased significantly, indicating that these genes may also be involved in the pathogenesis of *Trichophyton* spp. [14, 2]. The pathogenicity of *T. rubrum* appears to be significantly influenced by the strong keratinase activities of *SUB*3 and *SUB4* [9]. *SUB6* was initially identified as the major allergen in *Trichophyton* species and shares a close relationship with *SUB7* [15,7]. Most people use medication to treat dermatophyte

infections, usually without a prescription [16]. Reports from South Asia, East Asia and the Middle East indicate a high level of virulence and low response to drugs commonly used in the treatment of dermatophytosis [17]. Since different species of dermatophytes may have different susceptibilities to different antifungals, prolonged exposure to azoles, amorolfine, and terbinafine at subtherapeutic levels has been associated with the development of resistant dermatophytes, leading to treatment failure and, ultimately, persistent infection [18]. Laboratory testing of dermatophyte susceptibility will not only help us understand the epidemiological pattern of antifungal resistance but may also help us prescribe the appropriate antifungal drug at the appropriate dose [19]. Clinically available antifungal drugs exclusively target specific biological targets. This research aimed to find virulence genes (*SUB*1–7) in dermatophytes and their association with their resistance to antifungals.

2. Materials and Methods

2.1 Collection of fungal specimens

Twenty-two dermatophyte isolates were used in this study, including *T. indotinea*, *T. mentagrophytes*, *T. rubrum*, *T. simii*, and *M. canis*. All isolates were obtained 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. Isolations were previously performed using the internal transcribed spacer (ITS) region by the same authors under the title (Isolation and identification of dermatophyte species from Iraqi patients using PCR-ITS regions). The manuscript was accepted for publication in Volume (66) Issue (4) and will be published in (April) 2025 in the Iraqi Journal of Sciences. 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.

2.2 Susceptibility test (Antifungals Disk methods)

One cm2 from dermatophyte isolates growing on the SDA medium for 10-14 days was cut and poured into (10 ml) of D.W and mixed well. A sterile pipette was used to transfer (1ml) of the mixture to the plates containing SDA. It was then spread out by dabbing over the SDA surface and let dry overnight. The antifungal disks of itraconazole (30 mg/disk), ketoconazole (50 mg/disk), fluconazole (10 mg/disk), miconazole (30 mg/disk), voriconazole (10 mg/disk) and nystatin (50 mg/disk) are uniformly distributed to the surface of culture medium inculcated with the fungal suspension and a pair of forceps. The plates were incubated at 28°C for 7 days. Following colony formation, inhibition zones around the antifungal disks were measured and documented. Every isolate in the research received an application of every antifungal under study. Zones of inhibition are measured after visible fungal growth appears after 7 days [20].

2.3 Molecular Characterization

DNA Extraction and Primers

For all isolates under study, genomic DNA extraction and purification were carried out using the ABIOpure Extraction Kit protocol (ABIOpure, USA). The extracted DNA samples were stored at -20°C for future use. Following the method outlined in reference [21] for *SUB* (1-7), primers specifically designed for genes encoding secreted subtilisin virulence factors were provided by Macrogen (Korea) in lyophilized form, as detailed in Table 1.

Table 1: Specificity of primers used to detect *SUB* genes in dermatophyte species.

No.	Gene	Forward (5′ ➔ 3′)	Reverse (5′ ➔ 3′)
1	*SUB1*	TGGGTGTTTTCAGATTCATTTC	ACGGCGGGTGATGTTATGG
2	*SUB2*	AGAAGGCGTGTGGGCATAC	ACCCTTCCAGCATGACTTGA
3	*SUB3*	TCAAGGTTATCTCCGTCTTC	AAAGAGGACTTCTGGTCATC
4	*SUB4*	TCGCTGCTGGTAATGACAACG	GGAGCATAGATGTCAACTGAAG
5	*SUB5*	TCTGGCGGCATTGTCTGT	TGGGAGATGATAGCGTCTG
6	*SUB6*	GCTCATACAACTTGGCTTAG	TCAGAGGCAGGAGAAGAGT
7	*SUB7*	CGGCATCTGTCATCAACG	AGTGACCAGAGTATCCCTT

2.3.3 Conventional PCR program to detection of subtilisin genes (SUB)

A subtilisin gene (1-7) of dermatophytes was detected using conventional PCR. The PCR reaction was performed in a reaction volume of (25 µL), which included (12.5 µL) of Master Mix, (1 µL) of each primer pair *SUB* genes with annealing temperature degree (54C°), (7.5 µL) of nuclease-free water, and (3 ng/µl) of DNA. The PCR program was performed with 30 cycles, as shown in Table 2. The amplified DNA fragments were subjected to electrophoresis on 1.5% agarose gel at 100V/mAmp for 60 minutes. Subsequently, the ethidium bromide-stained bands on the gel were visualized using a Gel Imaging System [21].

Table 2: PCR amplification program to detection of *SUB* genes

No.	Phase	Temperature °C	Time	No. of cycle
1	Initial Denaturation	95	5 mints	1
2	Denaturation	95	30 second	30
3	Annealing	54	30 second	
4	Extension	72	30 second	
5	Final extension	72	7 mints	1
6	Hold	10	10 mints	

2.4 Statistical Analysis

Statistical analysis shows how different concentrations affected the research parameters. In this study, means were compared using the least significant difference (LSD) test.

3. Results

3.1 Antifungal Susceptibility

Six antifungals were used to determine the susceptibility test results of dermatophyte isolates. All isolates included in this study showed resistance to the antifungal nystatin, except for one isolate of *T. simii* and two isolates of *T.indotineae*, which exhibited susceptibility. In contrast, all isolates were completely susceptible to voriconazole and fluconazole, with no observable growth in the presence of these antifungal agents. Among the susceptible isolates, *T.indotineae* recorded the highest average inhibition zone diameter of 75 mm, whereas *T.simii* showed the lowest average inhibition zone diameter of 38 mm. The variations in inhibition zone diameters across the antifungal agents tested for dermatophyte isolates are comprehensively presented in Table 3.

Table 3: Inhibition zone diameter of antifungal susceptibility testing agents against isolated dermatophytes**.**

No.	No of isolation	NS	MIC	FLC	KT	VR	IT	Average
1	*T. indotineae*	0.00	35.5	90	14.5	90	23.5	42.25
2	*T. indotineae*	0.00	90	90	90	90	90	75
3	*T. indotineae*	0.00	90	90	90	90	26	64.33
4	*T.simii*	11.5	9	90	16.5	90	16	38.83
5	*T. indotineae*	0.00	16.5	90	37.5	90	22.5	42.75
6	*T. indotineae*	0.00	33	90	36	90	90	56.5
7	*T. indotineae*	0.00	90	90	90	90	31	65.16
8	*M.canis*	0.00	90	90	36	90	90	75
9	*T.mentagrophytes*	0.00	26.5	90	90	90	23.5	53.33
10	*T. indotineae*	0.00	90	90	35.5	90	32	56.25
11	*T. indotineae*	0.00	24.5	90	90	90	28	53.75
12	*T. indotineae*	0.00	18.5	90	90	90	38	54.42
13	*T.mentagrophytes*	0.00	19	90	90	90	90	63.17
14	*T. indotineae*	0.00	90	90	18.5	90	90	63.08
15	*T. indotineae*	19.5	28.5	90	34.5	90	90	58.75
16	*T. indotineae*	21.5	29.5	90	22	90	19.5	45.42
17	*T. indotineae*	0.00	90	90	90	90	90	75
18	*T. indotineae*	0.00	27	90	90	90	19.5	52.75
19	*T.mentagrophytes*	0.00	90	90	90	90	22.5	63.75
20	*T.indotineae*	0.00	90	90	36	90	90	66
21	*T.rubrum*	0.00	10	90	90	90	18.5	49.75
22	*T.simii*	0.00	20	90	11	90	21	38.67
	Avarage	2.4	50.4	90	61	90	48.25	-
	LSD			P = 0.05				-
	Species			0.53 mm				-
	antifungal			1.00 mm				-
	interaction			1.25 mm				-

FLC: Fluconazole, IT; Itraconazole, KT; Ketoconazole; MIC; Miconazole, NS; Nystatin; VR; Voriconazole.

3.2 Determination of Subtilisin Gene (SUB Genes)

DNA outputs of 22 dermatophyte samples were used to identify virulence genes represented by subtilisin genes (1-7) using conventional PCR. The results showed the presence of *SUB*1, 3, 4, 6 and 7 in dermatophyte isolates, while *SUB2* and *SUB5* were not detected in any of the isolates of dermatophytes under study. According to the results of agarose gel electrophoresis, some bands were present from *SUB1* (197 bp), *SUB2* (779 bp), *SUB3* (1072 bp), *SUB4* (269 bp), and *SUB5* (312 bp), in addition to specific bands detected from *SUB6* (909 bp) and *SUB7* (237 bp), as shown in Figure 1.

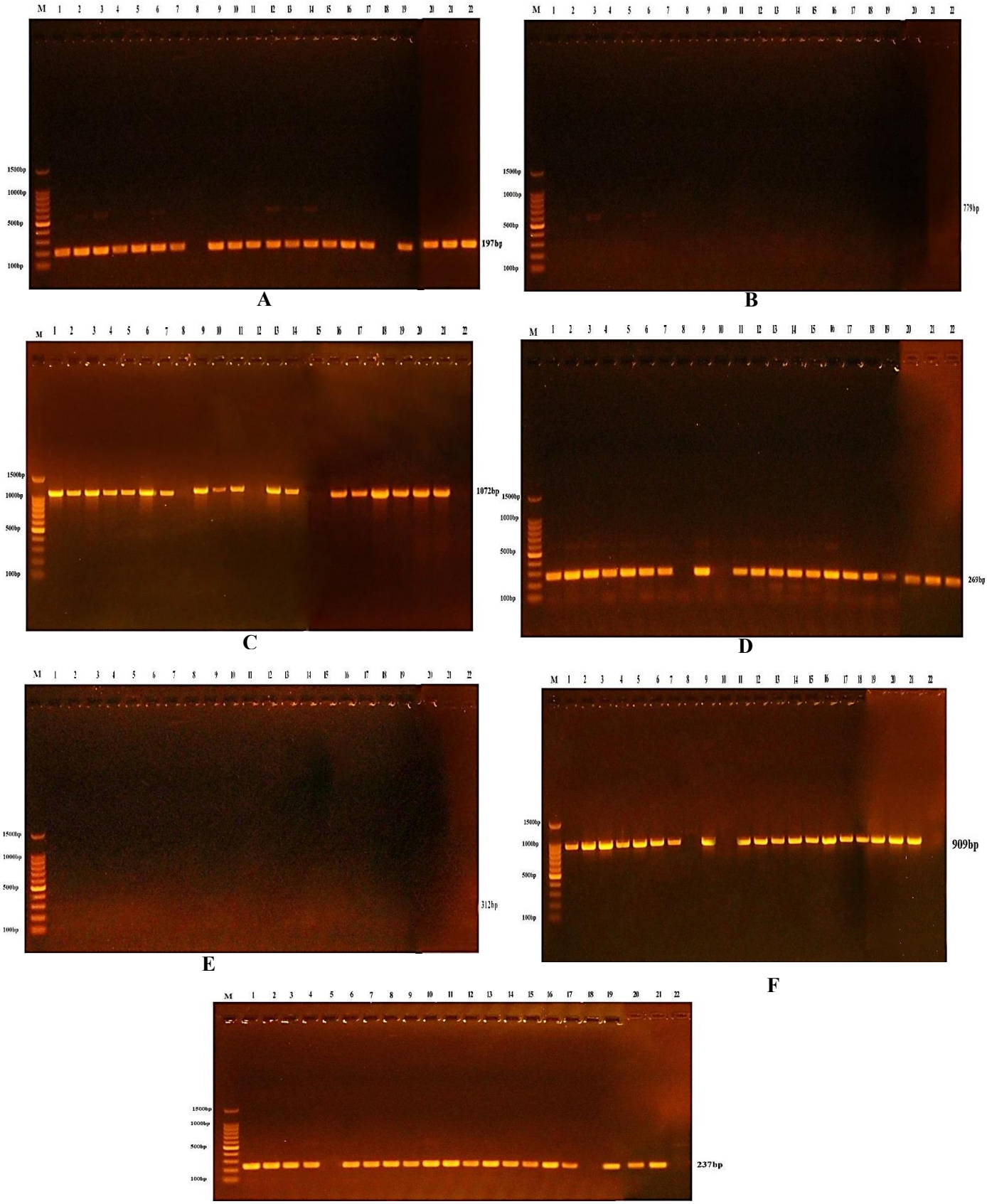

Figure 1: Results of *SUB* gene amplification of dermatophyte isolates fragmented on 1.5% ethidium bromide-stained agarose gel. M: 100 base pair ladder marker. Annealing temperatures in lanes 1 to 22 represent dermatophyte isolates. A: *SUB1*; B: *SUB2*; C: *SUB3*; D:*SUB4*; E: *SUB5*; F:*SUB6*; and G:*SUB7*.

Among the presented genes, the most prevalent genes, SUB1 and SUB4, were found in 90.9% of the isolates. SUB6 and SUB7 were observed in 86.3% of the isolates. SUB3 had the lowest frequency 81.8% of the isolates. The result showed *T. indotinea* was positive for SUB1, *SUB3*, *SUB4*, *SUB6*, and *SUB7*. While *M. canis* isolate was only positive for *SUB6* gene Table 4.

Table 4: Results of conventional PCR to detect *SUB*1–7 genes in dermatophytes isolates.

No.	Isolation	Subtilisin Genes							Percentage
		1	2	3	4	5	6	7	
1	*T. indotineae*	+	---	+	+	---	+	+	71.4%
2	*T. indotineae*	+	---	+	+	---	+	+	71.4%
3	*T. indotineae*	+	---	+	+	---	+	+	71.4%
4	*T.simii*	+	---	+	+	---	+	+	71.4%
5	*T. indotineae*	+	---	+	+	---	+	---	57.1%
6	*T. indotineae*	+	---	+	+	---	+	+	71.4%
7	*T. indotineae*	+	---	+	+	---	+	+	71.4%
8	*M.canis*	---	---	---	---	---	---	+	14.2%
9	*T.mentagrophytes*	+	---	+	+	---	+	+	71.4%
10	*T. indotineae*	---	---	+	---	---	---	+	28.5%
11	*T. indotineae*	+	---	+	+	---	+	+	71.4%
12	*T. indotineae*	+	---	---	+	---	+	+	57.1%
13	*T.mentagrophytes*	+	---	+	+	---	+	+	71.4%
14	*T. indotineae*	+	---	+	+	---	+	+	71.4%
15	*T. indotineae*	+	---	---	+	---	+	+	57.1%
16	*T. indotineae*	+	---	+	+	---	+	+	71.4%
17	*T. indotineae*	+	---	+	+	---	+	+	71.4%
18	*T. indotineae*	---	---	+	+	---	+	---	57.1%
19	*T.mentagrophytes*	+	---	+	+	---	+	+	57.1%
20	*T. indotineae*	+	---	+	+	---	+	+	71.4%
21	*T.rubrum*	+	---	+	+	---	+	+	71.4%
22	*T.simii*	+	---	---	+	---	---	---	28.5%
	Percentage	90.9 %	00.0 %	81.8 %	90.9 %	00.0%	86.3%	86.3 %	

4. Discussion

Serine protease inhibitors (*SUBs*) significantly reduce the adhesion of arthropods to the stratum corneum, and the production of subtilisin protease is a major virulence factor for dermatophytes. Expression of *SUB* plays an important role in the pathophysiology of dermatophytes and may be essential for early adhesion and invasion [12]. These fungi are also among the most common causes of fungal infections in humans; by some estimates, dermatophytes affect 25% of the world's population. Despite the high incidence of dermatophytes, some species in this group are among the few fungi that can be spread from person to person. Dermatophytes can be treated in several ways, ranging from short-term topical treatment of skin infections with over-the-counter antifungal preparations to long-term oral treatment with prescription medications for onychomycosis, which may not be successful because antifungals do not penetrate the nails well [22].

The current study documented a correlation between the number of virulence genes represented by the Subtilisin–like proteas (*SUB*1-7) and antifungal resistance in dermatophytes. *Trichophyton* species was found to be more resistant than *Microsporum* species. Even though resistance in *Trichophyton* spp. is increasingly reported worldwide [23]. Most of the isolated under study show high resistance to antifungals (nystatin). The long-term use of nystatin for dermatophytosis likely reflects a lack of knowledge, as the drug has not been effective in treating tinea for many years [24, 25]. While the fields of dermatology in Iraq have not applied this information in their practices, the specialized medical fields must have the opportunity to learn more about the best methods for treating tinea, which contributes to reducing the rate of development of resistance to antifungals

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However, the current study's findings demonstrated that no isolates possessed the *SUB2* and *SUB5* genes. This is consistent with the study of Khedmati *et al.,* [21]. While *SUB1* and *SUB 4* had the highest frequency, this may indicate that these genes play an important role in virulence and contribute to the secretion of specific proteases in patients with acute inflammatory skin infections. As in the studies of Ortiz *et al*., and Naeimipour *et al.,* [11, 26], the result of our study showed the presence of *SUB6* and *SUB7*, and this gene among the protease genes is an important allergenic gene that increased strongly during infection. Nevertheless, rather than the capacity of dermatophytes to induce infection, subtilisin could be associated with the intensity of infection [27]. *SUB* genes may be important in the early stages of dermatophyte infection as they play a role in the attachment of dermatophytes to the host stratum corneum. However, some studies revealed that the absence of some virulence genes responsible for severe infections was not necessary for dermatophyte invasion [15].

The current study shows that the relationship was direct, as the species containing a high number of *SUB* genes showed higher resistance to antifungals, most of *Trichophyton* species had a high number of genes and more resistance to antifungals especially the new species detected in Iraq, *T.indotineae*, a hypervirulent species responsible for outbreaks of dermatophytosis in parts of South Asia, Iran, India, and China [22, 28-30]. The emergence of the pathogen has been associated with misuse of creams containing topical steroids and antifungal/antibacterial medications, accompanied by a very sudden development of previously uncommon symptoms, widespread chronic fungal skin infections potentially leading to treatment resistance, development of antifungal resistance, and increased virulence factors [8].

5. Conclusions

The results of this study highlight the correlation between susceptibility patterns of clinically derived dermatophyte isolates and the presence of *SUB* genes, suggesting that the prevalence of these species is linked to their enhanced virulence. Accurate identification of the causative fungi is crucial for selecting effective antifungal treatments early in the course of infection. Among the *SUB* genes, *SUB1* and *SUB4* were the most prevalent in dermatophyte isolates, while the absence of *SUB2* and *SUB5* suggests that they may not be essential for fungal invasion. Misdiagnosis of tinea infections and inappropriate use of antifungals contribute to high resistance rates. Therefore, enhancing specialized medical training on optimal treatment strategies is vital to reduce the development of antifungal resistance in Iraq.

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Disclosure and Conflict of Interest

No conflicts of interest are disclosed by the authors.

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