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Research Article

Determination of Metalloprotease Gene in a Few Dermatophyte Species and Its Relationship with Antifungal Resistance

Qadisiyah Hilal Hashoosh[1], Alaa Mohsen.Yaseen. Al-Aaraji[1]

[1]Department of Biology, College of Science, Baghdad University, Baghdad, Iraq.
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I N F O

Corresponding Author:
Qadisiyah Hilal Hashoosh, College of Science, Baghdad University, Baghdad, Iraq.
E-mail Id:
qads_hashoosh@yahoo.com
Orcid Id:
https://orcid.org/0009-0005-1398-4576
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A B S T R A C T

Introduction: Keratinophilic fungi called dermatophytes exclusively infect the stratum corneum, human hair, and nails. To infect host tissues and cause disease, dermatophytes create virulence factors such as keratinases and cellulase. Dermatophytes produce virulence factors such as keratinases and cellulase to infect host tissues and spread the disease.

Aim: To detect the metalloprotease gene *(MEP1–5)* by Real-Time Polymerase Chain Reaction analysis and its relationship with the resistance of dermatophytes against antifungals

Method: Eleven species of dermatophyte isolates obtained from Iraqi patients were diagnosed morphologically and molecularly [internal transcribed spacer (ITS) region], and were found to include the following types: *Keratinophyton indicum, Trichophyton interdigitale, Microsporum ferrugineum, Trichophyton rubrum, Trichophyton mentagrophytes, Arthroderma otae, Trichophyton simii, Microsporum canis, Trichophyton quinckeanum, Epidermophyton floccosum,* and *Trichophyton verrucosum*. All dermatophyte species were tested for antifungal susceptibility using the disk method. The Genomic DNA extraction kit was used to extract the genomic DNA from the fungal growth.

Results: Trichophyton simii was highly resistant to all antifungals. The percentages of genes found in the species were as follows: *MEP1* and *MEP2* (70%), *MEP3* (81%), *MEP4* (54%), and *MEP5* (36%). *Trichophyton simii* contained all five genes *MEP1–5*, while *Microsporum canis* and *Arthroderma otae* contained only one gene.

Conclusion: The study showed that higher the number of genes of a dermatophyte species, higher will be its resistance to antifungals. The presence of *MEP5* was found to increase the antifungal resistance. This study was considered to be the first study in Iraq to detect these genes using the qPCR method depending on the DNA extraction and the relationship with antifungal resistance.

Keywords: Quantitative PCR, Metalloprotease, Dermatophytes, Iraq

Introduction

It is common for pathogenic dermatophytes to invade keratinised tissues like the skin, hair, and nails, which can lead to superficial cutaneous infections. Human keratinised tissues are generally infected with dermatophytes from the genera *Trichophyton, Microsporum*, and *Epidermophyton,* some of which have shown to be members of *Arthroderma.* During the early stages of infection, dermatophytes use a few chemicals to mediate their attachment to the keratinised surface of the skin. The goal is to communicate with the epidermis. During the process of invading, and inducing host interaction, dermatophytes release a host of virulence factors to infiltrate the host tissues. In order to adhere to the surface of the keratinised tissue and penetrate the epidermis, dermatophytes require a few factors that mediate adhesion during the early stages of infection. Protease, lipase, and cellulase are a few of the various virulence enzymes that are secreted by dermatophytes.[1–3] Metalloproteases (MEP) are crucial for invasion of the host by dermatophytes. They release keratinase to infiltrate the skin and break down both animal and human keratin. From a feline clinical isolate of *Microsporum canis*, an extracellular keratinolytic metalloprotease (43.5 kDa) was isolated, and some of its features were found.[4] This metalloprotease's production was selectively stimulated by keratin, indicating that this enzyme may be one of the virulence-related elements contributing to dermatophytosis.[5] MEP3 was recently shown to encode the 43.5 kDa metalloprotease by Duek et al.,[5] who extracted three MEPs from an *M. canis* genomic library. Dermatophytosis is treated with a variety of antifungal medications.[6,7] Due to the concurrent rise in fungal infections and greater use of antifungal therapies, often for extended periods of time, resistance to antifungal medications has emerged.[8] Most epidemiological studies presently use in vitro assays for antifungal susceptibility. The optimum antifungal drugs for a particular fungal condition can be selected by doctors with the aid of antifungal susceptibility testing. A potent tool for microbiological diagnostics is the quantitative PCR (qPCR) technology. In the field of bacterial diagnostics, it can substitute culture techniques, particularly when rapid and sensitive diagnostic assays are required. This methodology is unquestionably suitable for viral, parasite, and gene identification, quantification, and type.[9] In light of this, the purpose of this work was to identify virulence genes in dermatophytes represented by a metalloprotease gene *(MEP1–5)* using Real-Time Polymerase Chain Reaction analysis (qPCR) and its relationship with the resistance of dermatophytes to antifungals.

Materials and Methods

Collection of Samples

Clinical samples of fungal isolates (nails, skin scrapings and hair clippings) were gathered from patients attending the Dermatology Department of ALEmamain AL-Kadhumain Teaching Hospital and AL-Zahraa Consulting Center for Allergy and Asthma, Baghdad between January 2021 and September 2022. The isolated dermatophyte-caused ringworm diseases were diagnosed using the internal transcribed spacer (ITS) region (Table 1).[10] The patient's or guardian's consent was obtained for the purpose of taking a sample. Ethical approval was obtained from the Ministry of Health, Al-Karkh Health, Baghdad, Iraq. (No. 148).

Table 1.Species Used in This Study

S. No.	Organism	Sequence ID
1	*Keratinophyton indicum*	OP821488.1
2	*Trichophyton interdigitale*	OP821449.1
3	*Microsporum ferrugineum*	OP821455.1
4	*Trichophyton rubrum*	OP821487.1
5	*Trichophyton mentagrophytes*	OP821489.1
6	*Arthroderma otae*	OP821470.1
7	*Trichophyton simii*	OP821471.1
8	*Microsporum canis*	OP821482.1
9	*Trichophyton quinckeanum*	OP821484.1
10	*Epidermophyton floccosum*	OP821485.1
11	*Trichophyton verrucosum*	OP821486.1

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Antifungal Testing Using the Disk Method

One cm^2 from a colony of dermatophyte species growing on a Sabouraud Dextrose agar (SDA) medium for 14 days was cut and poured into a tube with 10 mL of sterile distilled water, and the contents were mixed. 1 mL of the mixture was transferred using a sterile pipette and placed on the surface of SDA plates. It was then spread by loop and allowed to dry. The antifungal disks were uniformly distributed (one disk in the middle). The plates were incubated for 7 days at 30 °C. Zones of inhibition surrounding the disks were measured and recorded after the colonies had formed. Antifungal discs of itraconazole (10 mg/disk), ketoconazole (10 mg/disk), fluconazole (10 mg/disk), clotrimazole (10 mg/disk), and amphotericin B (100 mg/disk) were procured from Himedia Chemicals (all antifungals were applied to all species under this study).

Extraction of DNA

The Genomic DNA extraction kit was used to extract the genomic DNA from the fungal growth by the ABIOpure Extraction methodology, and the DNA was then kept at -20 °C until use.

Primer Preparation

The Macrogen Company provided these primers in lyophilised form. In a stock solution, lyophilised primers were dissolved in nuclease-free water to a final concentration of 100 pmol/L. To make a functional primer solution containing 10 pmol/L of these primers, 90 L of nuclease-free water was mixed with 10 L of primer stock solution, which was kept at -20 °C in the freezer. Table 2 lists the primers used and their sequences.

Real-Time PCR Programme

PCR was performed on the PCR system with a reaction volume of 10 µL, including 5 µL of Master Mix, 0.5 µL of each primer pair *MEP*, 1 µL of DNA, and 3 µL of water devoid of nucleases. In this study, real-time technology was used to identify genes according to the program of Real-Time PCR (Table 3).

Table 2.Primers Used Specifically for Genes that Encode Secreted Metalloproteases Genes[1]

Target	Sequence 5`-3`	Amplicon Size (bp)	Annealing Temp. (°C)
MEP1-F	CGCCACTGCTGTCCGTCTAA	83	60
MEP1-R	CCTTGGGGCTGTTGTTCACC		
MEP2-F	ATCCTCATCCACCATACCCTG	80	
MEP2-R	TCACCCTTGCTAATTCCCATT		
MEP3-F	AGCAGCACGCCAGCAACG	60	
MEP3-R	GCAGACGGAAGGACTCGATGT		
MEP4-F	AGTCGGGACACCATTCTTCAG	105	
MEP4-R	ATTTGGGCTTCTATGCTCTACG		
MEP5-F	GCCAGGGTGGTAAAGGCAATG	88	
MEP5-R	TCGGGAGGGGTAGCAAAATG		

F: Forward primer, R: Reverse primer

Table 3.PCR Thermal Cycling Program

Steps	°C	m: s	Cycle
Initial denaturation	95	10:00	1
Denaturation	95	00:15 Acquiring on green	40
Annealing	60	00:30	
Extension	72	00:30	

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Statistical Analysis

The statistical analysis system (SAS, 2012) program was used to study the effect of different concentrations in study parameters. The least significant difference (LSD) test was used for comparison between means in this study.

Results

Antifungal Testing

In the present study, amphotericin B, azoles (clotrimazole and ketoconazole), and triazoles (fluconazole and itraconazole) were used as screening drugs. Among the 11 species of dermatophytes, *Trichophyton simii* had no inhibition zone against all antifungals used in the current study and was resistant to them. *Trichophyton rubrum*, *Trichophyton mentagrophytes, and Trichophyton quinckeanum* were resistant to two antifungals, while *Microsporum canis* and *Arthroderma otae* were sensitive to all antifungals and had no growth. The inhibition zones of the various dermatophyte species have been shown in Table 4.

Results of Real-Time PCR

The presence of the most prevalent virulence genes, MEP1–5, was demonstrated by real-time PCR analysis (qPCR) of virulence genes. The qPCR results were used to detect the presence of virulence genes represented by metalloprotease genes (MEP1–5) in 11 types of dermatophytes. The results showed the following: *Keratinophyton indicum* and *Epidermophyton floccosum* were positive for *MEP1–3; Microsporum canis* and *Arthroderma otae* were positive for *MEP1; Microsporum ferrugineum* and *Trichophyton interdigitale* were positive for *MEP 1–4; Trichophyton verrucosum* was positive for MEP3,4; *Trichophyton simii* was positive for *MEP1–5; Trichophyton quinckeanum* and *Trichophyton mentagrophytes* were positive for *MEP2–5*; *Trichophyton rubrum* was positive for *MEP1,2,3,5* (Table 5).

The highest percentage of genes found in the 11 types of dermatophytes corresponded to *MEP3 (81%)*, and the lowest corresponded to *MEP5 (36%)*, as shown in Figure 1.

Table 4.Zone of Inhibition (in mm) of Dermatophyte Species

Species	Fluconazole F	Ketoconazole K	Itraconazole I	Clotrimazole CL	Amphotericin B (AMP)	Average
K. indicum	6.3	2.1	7.0	89.6	1.4	21.3
T. interditales	6.1	6.0	5.8	8.4	6.0	6.5
M. ferrugineum	12.0	5.8	8.7	62.3	11.0	20.0
T. rubrum	5.4	0.0	8	5.8	0.0	3.8
T. mentagrophytes	2.1	0.0	6.1	9.2	0.0	3.5
A. otae	90	89.6	90.0	89.6	89.6	89.8
T. simii	0.0	0.0	0.0	0.0	0.0	0.0
M. canis	89.6	1.1	90	90	89.6	72.1
T. quinckeanum	4.0	0.0	2.2	3.0	0.0	1.8
E. floccosum	3.8	8.7	89.6	63.3	6.0	34.3
T. verrucosum	90.0	7.1	63.3	89.3	5.3	51.0
Average	28.1	11.0	33.7	46.4	19.0	27.64
LSD	p = 0.05					-
Between antifungal	5.3 mm					-
Between fungal isolates	7.9 mm					-
Between interaction	17.7 mm					-

LSD: Least significant difference test (Analysis of Variation-ANOVA) was used for significant comparisons between means (three replicates). The chi-square test was used for significant comparisons between percentages (0.05 probability).

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Table 5.Results of Real-Time PCR (qPCR) to Detect MEP1–5 Genes in 11 Species of Dermatophytes

S. No.	Organism	Sequence ID	Name of Gene				
			MEP1	MEP2	MEP3	MEP4	MEP5
1	*Keratinophyton indicum*	OP821488.1	+	+	+	-	-
2	*Trichophyton interdigitale*	OP821449.1	+	+	+	+	-
3	*Microsporum ferrugineum*	OP821455.1	+	+	+	+	-
4	*Trichophyton rubrum*	OP821487.1	+	+	+	-	+
5	*Trichophyton mentagrophytes*	OP821489.1	-	+	+	+	+
6	*Arthroderma otae*	OP821470.1	+	-	-	-	-
7	*Trichophyton simii*	OP821471.1	+	+	+	+	+
8	*Microsporum canis*	OP821482.1	+	-	-	-	-
9	*Trichophyton quinckeanum*	OP821484.1	-	+	+	+	+
10	*Epidermophyton floccosum*	OP821485.1	+	+	+	-	-
11	*Trichophyton verrucosum*	OP821486.1	-	-	+	+	-

Using MEP1-5 Primers
+: Present gene, -: Absent gene

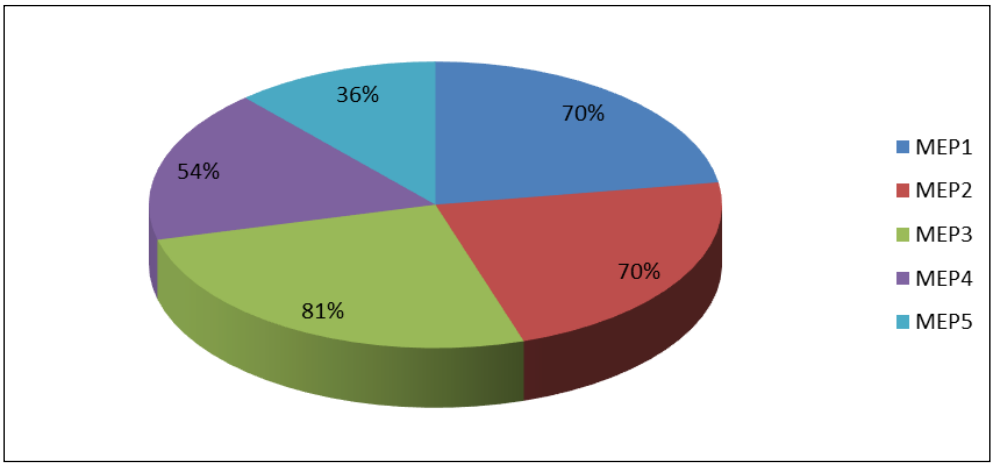
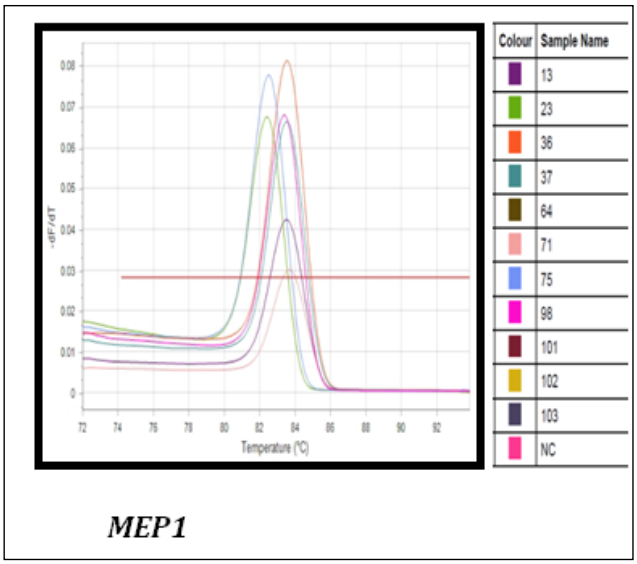
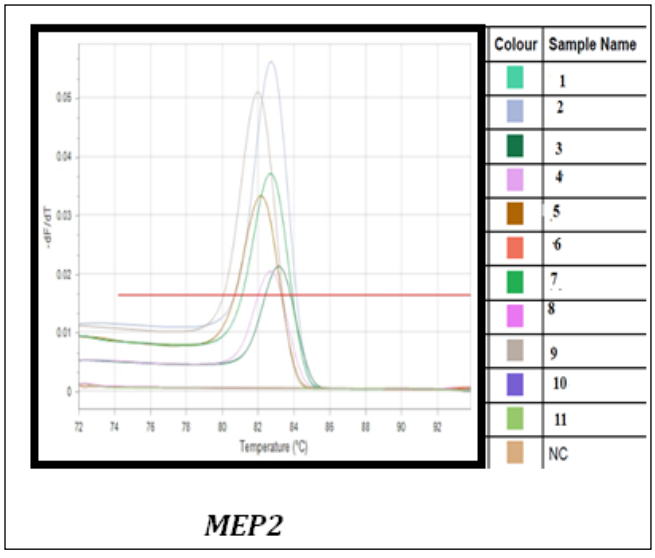


Figure 1.Distribution of Genes Across 11 Species of Dermatophytes



MEP1



MEP2

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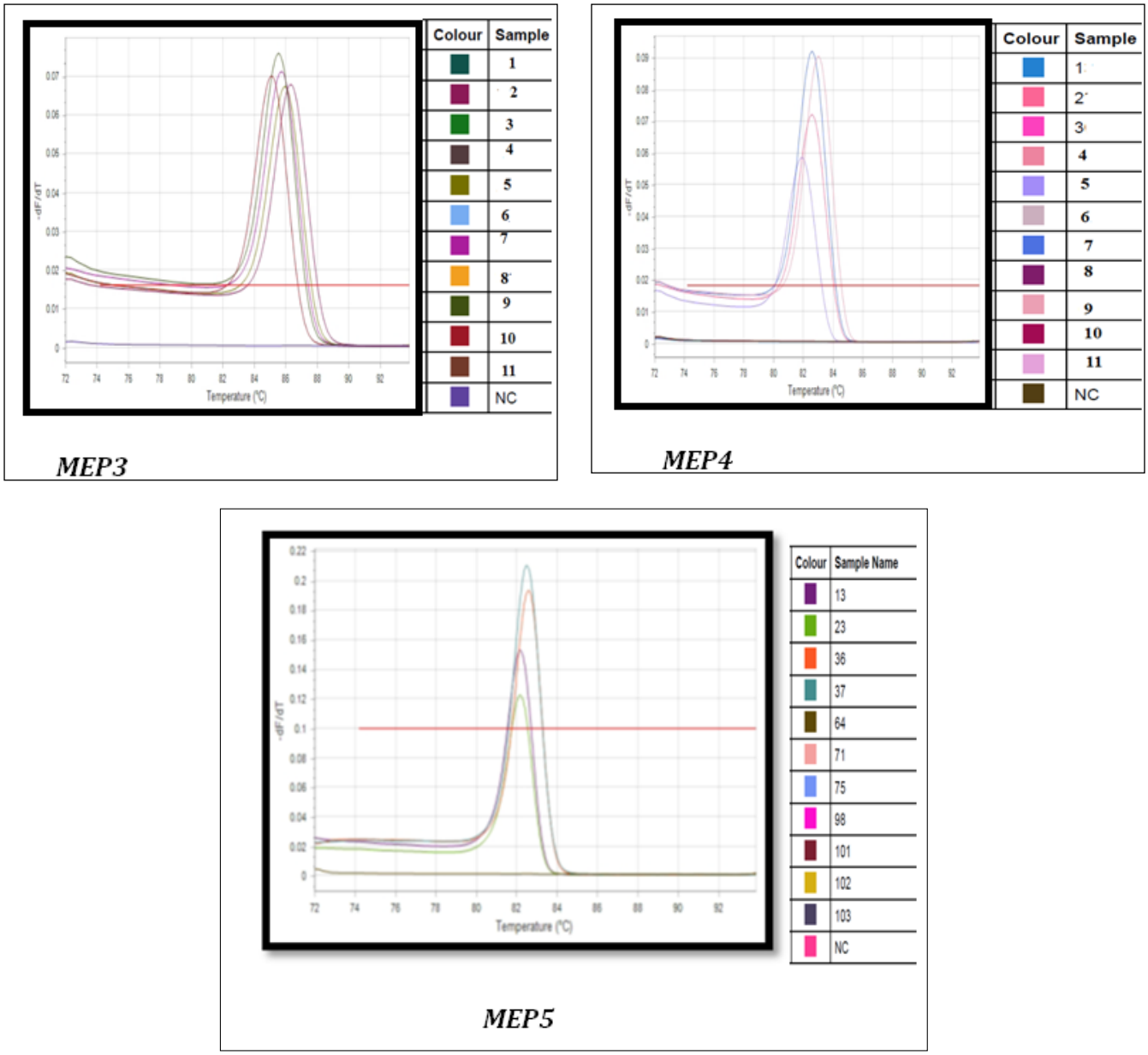

Figure 2.Melting Peaks of MEP1, MEP2, MEP3, MEP4 and MEP5 Using Real-Time Analysis (qPCR) (Extensive with Fluorescence Cutoff of 5%)

Discussion

Metalloproteases (MEP) are crucial virulence components for the dermatophyte *Trichophyton mentagrophytes'* invasion of the host. Keratinase is secreted by dermatophytes in order to break down animal and human keratin and enter the skin. According to the findings of the present investigation, there is a correlation between the number of virulence genes represented by the metalloprotease genes *(MEP1–5)* and the degree of the target organisms' antifungal resistance. The relationship was direct, as the species containing a high number of MEP genes showed higher resistance to antifungals and *Trichophyton* species was found to be more resistant than *Microsporum* species and *Epidermophyton.*

MEP3 recorded the highest percentage of occurrence in the different types of dermatophytes, and the *MEP5* gene has a significant contribution to the antifungal resistance shown by *Trichophyton* species. Studies by Datt et al.[12] suggested that MEP4 may be the predominant metalloprotease among dermatophytes during the host infection stage and that *MEP4* and *MEP5* genes significantly contribute to the pathogenicity of *T. mentagrophytes* during host invasion. In a different investigation, five *MEP* genes were effectively transformed by *Agrobacterium tumefaciens* mediated transformation (ATMT), producing five *MEP* mutant strains. Among the mutant strains, *MEP3* demonstrated the highest levels of proteolytic activity, hair biodegradation potential, and animal pathogenicity. It was also found that the *MEP4*

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and *MEP5* mutants were the least harmful.[13] Real-time PCR technology is one of the most effective molecular tools, according to the study, which covered gene expression analysis, mutation detection, pathogen identification and quantification, GMO detection, susceptibility detection, microbial degradation monitoring, and spot tracking. This technology has been the *de facto* industry standard for diagnosing COVID-19. Common real-time PCR protocols have also been modified to meet specific needs.[14] The molecular technique was applied to different topics of biomedical research.[14–33] However, According to a study, the use of real-time PCR with pan-dermatophyte primers was more effective at detecting the presence of dermatophytes in the sample with a detection rate of 10.84% (45.00% vs 34.17%) as compared to direct analysis with light microscopy. Moreover, dermatophyte cultures were obtained from each sample that had a successful qPCR test.[34]

Superficial fungal infections are on the rise, and dermatophytes are the most typical culprits. Due to their ability to devour keratin, they result in a range of clinical symptoms. Although these infections often only affect the stratum corneum, they have the potential to spread throughout the body and should thus be treated carefully rather than being ignored. Skin infections caused by dermatophytes are frequently mistaken for other non-fungal skin conditions.[35] Therefore, accurate knowledge of the etiologic agents is essential for starting treatment right away. An individual's social, psychological, and professional well-being may be impacted by dermatophytosis. Since dermatophytosis cannot be vaccinated against, spreading awareness of the condition, quick diagnosis, and effective treatment can help to contain its spread.

Conclusion

We conclude from the data obtained in this study that there is a relationship between the numbers of *MEP* gene and antifungal resistance in the dermatophyte species, and the presence of *MEP5* increases its resistance. *Trichophyton* species have higher resistance than other species. The *MEP3* gene recorded the highest percentage of appearance in the species of dermatophytes, and the *MEP5* gene contributed to the antifungal resistance of *Trichophyton* species. Real-time PCR is currently one of the most widely used molecular methods and it is frequently employed in biological sciences and medicine because it is quantitative, precise, sensitive, and quick.

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Conflict of Interest: None

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