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Isolation and molecular identification of dermatophytes causing ringworm in Anbar Governorate

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Abstract

In this study, 80 samples were collected from people infected with dermatophytes, clinically diagnosed by a dermatologist at Ramadi Teaching Hospital and Fallujah Teaching Hospital, in addition to private clinics from October 2022 to March 2023. The skin crust samples were taken with a glass slide. They were cultured on Sabouraud Dextrose Agar media for identification, relying on traditional methods and comparing them with molecular methods using primers specialized for dermatophytes and sequencing the nucleotides. Nine species of dermatophytes belonging to three genera were isolated after identifying them based on their morphological, microscopic, and molecular characteristics. The genus *Tricophyton* occupied the higher percentage, reaching 60.86%, followed in second place by the genus *Microsporum*, with a percentage of 31.88%, and then by the genus *Epidermophyton* which was less frequent, with a percentage of 7.24%. The study showed that the fungus Tricophyton mentagrophytes was the most frequent species, with a percentage of 39.13%, distributed into five clinical types: tinea corporis, tinea manuum, tinea cruris, tinea faciei, and tinea peadis. Molecular identification included the use of primers specific to dermatophytes, involving the primer pairs ITS1/ITS2, ITS3/ITS4, and ITS1/ITS4, derived from the nucleotide sequence in the ITS region. The sequence of the nucleotides of the dermatophytes was matched with the sequence of reference samples in Gen Bank. The results were identical to the morphological diagnosis for most study samples. This technique succeeded in diagnosing the isolate whose species we could not identify morphemically and microscopically.

Keywords: skin fungi, ringworm

عزل وتشخيص الفطريات الجلدية المسببة للسعفة في محافظة الانبار

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خلاصة

تم في هذه الدراسة جمع 80 عينة من أشخاص مصابين بالفطريات الجلدية، تم تشخيصهم سريرياً من قبل طبيب الأمراض الجلدية في مستشفى الرمادي التعليمي ومستشفى الفلوجة التعليمي، بالإضافة إلى العيادات الخاصة في الفترة من تشرين الأول 2022 إلى آذار 2023. وتم أخذ عينات القشرة الجلدية

بشريحة زجاجية. . وتمت زراعتها على وسط سابورو دكستروز أجار للتعرف عليها بالاعتماد على الطرق التقليدية ومقارنتها بالطرق الجزيئية باستخدام البادئات المتخصصة للفطريات الجلدية وتسلسل النيوكليوتيدات. تم عزل تسعة أنواع من الفطريات الجلدية التي تنتمي إلى ثلاثة أجناس بعد تحديدها بناء على خصائصها المورفولوجية والمجهرية والجزيئية. واحتل جنس Tricophyton النسبة الأعلى حيث بلغت 60.86%، يليه في المركز الثاني جنس Microsporum بنسبة 31.88%، ثم جنس Epidermophytonالذي كان الأقل تواتراً بنسبة 7.24%. أظهرت الدراسة أن فطر Tricophyton mentagrophytesكان أكثر الأنواع انتشاراً بنسبة 39.13%، موزعاً على خمسة أنواع سريرية هي: سعفة الجسم، سعفةmanuum ، سعفة الساق، سعفة الوجه، سعفة البازلاء. شمل التحديد الجزيئي استخدام البادئات الخاصة بالفطريات الجلدية، والتي تتضمن أزواج البادئاتITS1/ITS2 ، وITS3/ITS4، وITS1/ITS4، المستمدة من تسلسل النيوكليوتيدات في منطقة ITS. تمت مطابقة تسلسل النيوكليوتيدات للفطريات الجلدية مع تسلسل العينات المرجعية في Gen Bank. وكانت النتائج مطابقة للتشخيص المورفولوجي لأغلب عينات الدراسة. نجحت هذه التقنية في تشخيص العزلة التي لم نتمكن من تحديد نوعها شكليا ومجهريا.

كلمات مفتاحية : الفطريات الجلدية ، السعفة

Introduction

Dermatophytes are a group of fungi morphologically and physiologically similar to molds. Some of them cause well-known infections called tineas, dermatophytosis, or ringworm because of the inflamed circular scaly spots they cause (Jackson, 2022). Tinea is also called according to the site where the body is infected with this type of fungus, including tinea capitis, tinea peadis, tinea cruris, tinea corporis, tinea manuum, tinea face, tinea unguium, and tinea beard (Odom et al., 2000; Kheira et al., 2007). Soil and animals are the main sources of human infection with filamentous dermatophytes (Al-Khikani, 2020). Most diseases caused by dermatophytes are called ringworm infections because they expand circularly from their starting point in the skin to form an inflamed area on the skin (Kwon-chung and Bennett, 1992; Rippon, 1988).

Identification of filamentous dermatophytes that cause ringworm is very crucial. It has been noticed that traditional identification tests based on cultural and microscopic characteristics can be unstable and inaccurate in identifying and recognizing some species of dermatophytes due to differences from one isolate to another and the overlap of characteristics between the species (Perez-Rodriguez et al., 2023). Therefore, in addition to traditional methods, molecular methods have been adopted to identify filamentous dermatophytes, as molecular methods allow recognition of the pathogen at the genus and species level, facilitating and speeding up the prescription of treatment and controlling the disease spread (Hubka et al., 2014). Medical mycology has also recently witnessed a reclassification of different species as relying on genetics has become more widespread in species identification (De Hoog et al., 2017). The genome size of dermatophytes ranges from 2.25 Mb to 24.1 Mb, and complete genomes have been annotated for many species (Martinez et al., 2012).

Dermatophyte genomes are haploid and contain relatively little repetitive DNA (White et al., 2014).

There are several techniques for analyzing the dermatophyte genome, and DNA barcoding is very useful for accurate identification. Internal transcribed spacer polymorphisms (ITS1 and ITS2) surrounding the DNA sequence encoding the 5.8S ribonucleic acid (rDNA) are very sensitive and highly reliable for distinguishing between different types of dermatophytes (Tartor et al., 2019; Diongue et al., 2019).

Materials and methods

The samples were cultured directly in SDA media containing 250 $mg.L^{-1}$ of chloroamphenicol to prevent bacterial growth, and ammonium hydroxide (NH_4OH) was added to the culture medium instead of cyclohexamide to prevent the opportunistic fungi growth (**Al-Said** and **Al-Assawi**, 2019), as 4-6 drops of NH_4OH at a concentration of 30% were placed in 250 ml of culture media and stirred gently to mix with the solution, then poured into sterile dishes. The skin specimens were cultured on this media in dishes; next, the dishes were incubated at a temperature of 28°C for 1-4 weeks at least with checking constantly every 2–3 days to observe growth before being considered negative (Ndiaye et al., 2021).

1- Morphological examination of the colonies

Once fungal growth appeared on the surface of the media culture, it was necessary to investigate the cultural characteristics to properly identify dermatophytes. This involves examining the incubation period and characteristics of the colony, such as size, elevation, shape (whether it is flat or raised), color (on the upper and lower surfaces), and texture (whether it is powdery, cottony, or downy).

2- Microscopic examination of the colonies

This examination was conducted by placing a drop of the dye lactophenol cotton blue (LPCB) on a glass slide with a sterile inoculation needle; next, part of the fungal hyphae from the fungal colony was transferred to the glass slide, mixed with the dye, and then, the slide cover was placed on it and gently pressed and left for 2-5 minutes, after that, it was dried over a low flame (Forobes et al., 1998); finally, it was examined under a microscope with power (x10) and then power (x40) to recognize the shapes of the fungi, their sizes, spores, and the conidia type (Emmons et al., 1974; Kwon-Chung and Bennet, 1992).

3- DNA extraction of the dermatophytes

DNA was extracted from 12 isolates of dermatophytes that were identified morphologically and microscopically based on the extraction method using the modified method approved by the American company Geneaid.

4- Electrophoresis of the extracted DNA on agarose gel.

A volume of 100 ml of the TBE solution diluted at 1x (i.e., 900 ml of distilled water to 100 ml of TBE) was placed in a beaker, and 1.5 g of agarose was added to it; next, it was put in the microwave until boiling to dissolve all its components; then it was left it to cool a little; after that, five microliters of Ethidium bromide was added to it. An agarose support tray was prepared, and the well comb was placed at a 1cm distance from the tray end. Then, the agarose gel was carefully poured into the tray and left until it hardened. Then, the well comb was gently removed from the hardened gel. After that, the gel was transferred to the electrophoresis tank and immersed with TBE buffer solution until the surface of the gel was covered and 6 microliters of the DNA extracted with the kit as mentioned above was added to 4 microliters of the loading dye, then put it in the agarose gel wells. The electrophoresis was conducted for 30 minutes at a voltage of 100. The gel was then examined with ultraviolet light using a U.V. Transilluminator, and the gel was imaged with a digital camera to observe the DNA overlapped with the Ethidium Bromide dye as orange-colored bands.

5- Polymerase Chain Reaction (PCR)

Design of primers specific for dermatophytes

The specified primers were designed relying on the NCBI website, and the designed primer sequence information was sent to the Korean company BIONEER to prepare the lyophilized primers as listed in Table (1).

Table 1. Primers specified for the use in the study

	Primer	Sequence	Size
1	ITS1/ITS2	5′-TCCGTAGGTGAACCTGCGG-3	19bp
		5′-GCTGCGTTCTTCATCGATGC-3′	20bp
2	ITS3/ITS4	5′-GCATCGATGAAGAACGCAGC-3′	20bp
		5′-TCCTCCGCTTATTGATATGC-3′	20bp
3	ITS1/ITS4	5′-TCCGTAGGTGAACCTGCGG-3′	19bp
		5′-TCCTCCGCTTATTGATATGC-3′	20bp

6- Polymerase chain reaction (PCR) using dermatophyte-specific primers

PCR technology was used to amplify DNA using the primer pair (ITS1/ITS2, ITS3/ITS4, and ITS1/ITS4) derived from the sequence of nucleotides in the ITS region. After preparing PCR reaction tubes, the samples were vortexed and transferred to a PCR thermal cycler which was programmed

according to the required thermal cycle conditions determined as shown in Table (Abo El-Yazeed et al., 2013). The polymerase chain reaction results were tested by electro-forcing the samples on a 1.5% agarose gel prepared in paragraph (4).

Results and discussion

The three genera, *Micosporum, Tricophyton*, and *Epidermophyton*, were identified morphologically, microscopically, and molecularly. Their frequencies were in different proportions depending on the species. The highest frequency was for the genus *Tricophyton*, 60.86%, followed by the genus *Microsporum*, with a percentage of 31.88%, and the genus *Epidermophyton* had the lowest frequency, with a percentage of 7.24%. Nine species of dermatophytes belonging to the three genera were isolated. It was found that the most frequent species was *T. mentagrophytes*, with a percentage of 39.13%, and frequent in 27 samples distributed into five clinical types; 15 samples were in tinea corporis, three were samples in tinea cruris; one sample was in tinea facie, six samples were in tinea mnum; two samples were in tinea pedis. In contrast, the frequency of the *M. canis* fungus reached 11 samples, with a percentage of 15.94%, distributed into four clinical types: six were in tinea corporis, one sample was in tinea cruris, three in tinea manuum, and one sample was in tinea pedis, followed by the fungus *T. tonsurans*, frequented in seven samples, with a percentage of 10.14%. It was distributed into three clinical types: two samples for tinea corporis, three for tinea capitis, and two for tinea pedis. The frequency of the fungus *M. audoinii* was in six samples, with a percentage of 8.69%, distributed into three clinical types: two of them were in tinea corporis, three samples in tinea cruris, and one sample in tinea pedis. The fungus *M. gypseum* was frequented in 5 samples, with a percentage of 7.24%, distributed into two clinical types: one in tinea corporis and four in Tinea manuum. The *E. floccosum* fungus also reached a frequency of 7.24% in 5 samples, while the fungus frequency of the *T. mentagrophytes** reached 1.44% in one sample.

Table 2. The isolated fungi according to the type of clinical infection.

Isolated fungi	Tinea corporis	Tinea cruris	Tinea capitis	Tinea facie	Tinea manuum	Tinea peadis	**Total**
M. canis	**6**	**1**	**-**	**-**	**3**	**1**	**11**
M. audoinii	**2**	**3**	**-**	**-**	**-**	**1**	**6**
M. gypseum	**1**	**-**	**-**	**-**	**4**	**-**	**5**
T. rubrum	**2**	**2**	**-**	**-**	**-**	**-**	**4**
T. mentagrophytes	**15**	**3**	**-**	**1**	**6**	**2**	**27**

T. verrucosum	**1**	**-**	**1**	**1**	**-**	**-**	**3**
T.tonsurans	**2**	**-**	**3**	**-**	**-**	**2**	**7**
E. fioccosum	**4**	**-**	**-**	**-**	**1**	**-**	**5**
T.mentacrophytes *	**-**	**1**	**-**	**-**	**-**	**-**	**1**
Total	**33**	**10**	**4**	**2**	**14**	**6**	**69**

The results of this study were consistent with the study of Garcia-Martos et al. (2004), conducted on dermatophytes in Spain, where *T. mentagrophytes* was the most frequent fungus, at a percentage of 24%. It was also consistent with the study of Hashoosh and AL-Araji (2023), where the isolates *T. mentagrophytes* were the most common fungus, with a percentage of 67.56%, indicating a high prevalence of this fungus causing dermatophytosis. Likewise, in a study in Iran, it was isolated by 48% of all ringworm types (Abastabar et al., 2013). In a study conducted by Singh et al. (2020), it was found that T. mentacrophytes was ranked first frequency, followed by *T. rubrum* in second place. Some references refer that *T. mentagrophytes* was the most frequent fungus of ringworm dermatophyte (Prasad et al., 2013; Jha et al., 2012), and a study conducted by Doddamani et al. (2013) reported that *T. rubrum* was the most common cause of tinea corporis.

Table 3. Isolated dermatophytes and their frequency

Isolated fungi	Frequency number	Frequency percentage	Male	Female
M. canis	**11**	**%15.94**	**(%8.69) 6**	**(%7.24) 5**
M. audoinii	**6**	**%8.69**	**(% 7.24) 5**	**(% 1.44) 1**
M. gypseum	**5**	**%7.24**	**(%4.34) 3**	**(%2.89) 2**
T. rubrum	**4**	**%5.79**	**(%4.34) 3**	**(%1.44) 1**
T. mentagrophytes	**27**	**%39.13**	**(% 30.43) 21**	**(% 8.69) 6**
T. verrucosum	**3**	**%4.34**	**-**	**(%4.34) 3**
T.tonsurans	**7**	**%10.14**	**(%2.89) 2**	**(%7.24) 5**
E. fioccosum	**5**	**%7.24**	**(%5.79) 4**	**(%1.44) 1**
T. mentagrophytes *	**1**	**%1.44**	**(%1.44) 1**	**-**
Total	**69**	**%100**	**(% 65.21) 45**	**34.78) 24 (%**

Molecular identification of dermatophytes

The current study included DNA extraction from the 12 study samples based on the DNA extraction procedure described in the materials and methods. The concentration and purity of the DNA samples were also measured with a NanoDrop Spectrophotometer, as the concentration and purity were within ideal limits and suitable for the PCR reaction. All samples were electrophoresed on a 1% agarose gel to confirm successful DNA extraction.

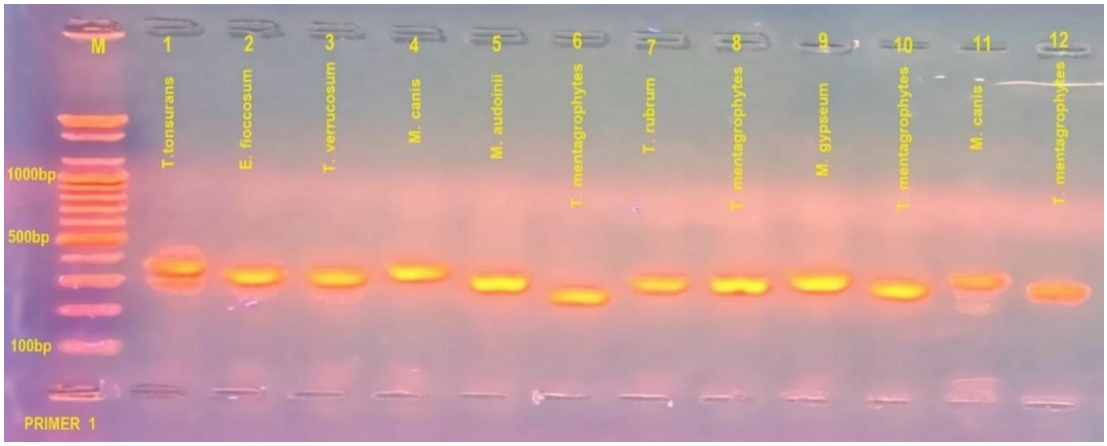
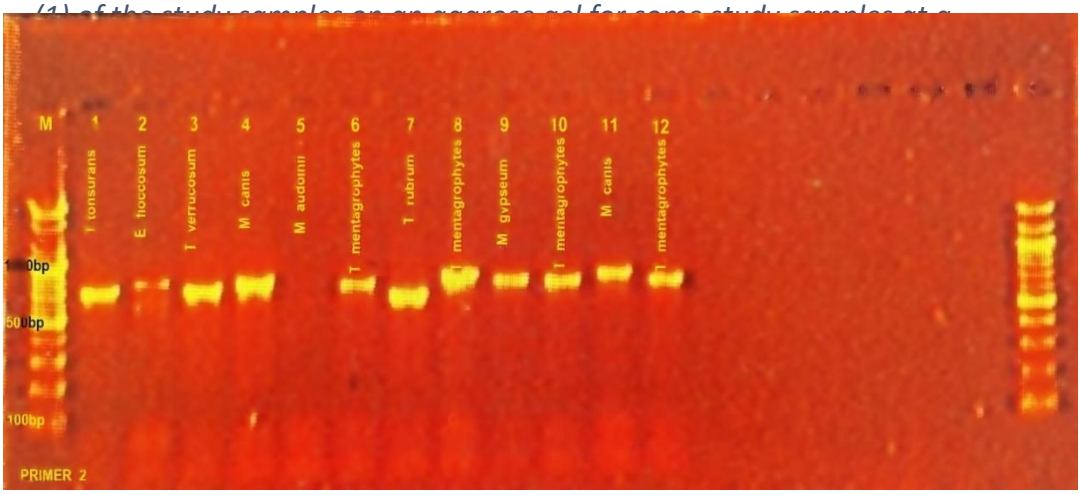


Figure 1. *Electrophoresis result of the polymerase chain reaction (PCR) for the primer (1) of the study samples on an agarose gel for some study samples at a*



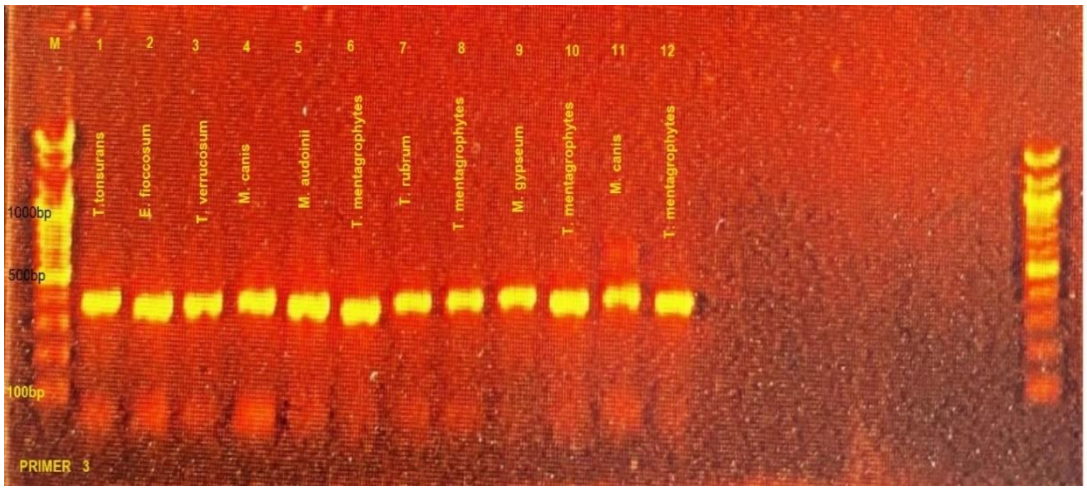


Figure 3*. Electrophoresis result of the polymerase chain reaction (PCR) for the primer (2) of the study samples on an agarose gel for some study samples at a concentration of % 1.5 5 vol. /cm for 1:15 hour.*

The molecular study of *T. tonsurans* displayed a single band with a 350bp molecular weight which is a stable molecular indicator for this species according to the primer (1). It has also been shown a single band with an 800bp molecular weight appears when the reaction is carried out with the primer (2), which is also considered a secondary indicator of the species. In contrast, the study showed that the primer (3) is considered non- diagnostic because it gave a single band with a weight of 850bp similar to the band produced for all species.

The molecular study of *E. fioccosum* also showed the appearance of a single band with a molecular weight of 320 bp, which is a stable molecular indicator for this species according to the primer (1). It also showed the appearance of a single band with a molecular weight of 900bp when the reaction was carried out with the primer (2), constituting a secondary indicator of the species; on the other hand, the study showed that the primer (3) is considered non- diagnostic because it gave a single band with a weighed 850bp like the band that resulted for all other types.

Studying *T. verrucosum* molecularly showed a single band with a molecular weight of 320bp, which is a stable molecular indicator for this species according to the primer (1). It also showed the appearance of a single band with a molecular weight of 800bp when the reaction is carried out with the primer (2), which also forms A secondary indicator of the species, while the primer (3) is considered non- diagnostic because it gave a single band with a weight of 850bp similar to the band that resulted for all other types.

The molecular study of the *M. audoinii* species also showed the appearance of a single band with a molecular weight of 250 bp, which is a stable molecular indicator for this species according to the primer (1). It also showed that there was no single band appeared when the reaction was carried out with the primer (2), which also constitutes a secondary indicator of the species, while it showed that primer (3) is considered non- diagnostic because it produced a single band weighed 850bp which was similar to the band produced for all other types.

As for *T. mentagrophytes*, the molecular study showed a single band with a molecular weight of 200bp, which is a stable molecular indicator for this species according to the primer (1). It also showed the appearance of a single band with a molecular weight of 900bp when the reaction is carried out with the primer (2), which also forms A secondary indicator of the species; however, the study showed that the primer (3) is considered non-diagnostic because it produced a single band with a weight of 850bp similar to the band produced for all other types.

The molecular study of M. gypseum demonstrates a single band with a molecular weight of 250bp, which is a stable molecular indicator for this species according to the primer (1). It also shows the appearance of a single band with a

molecular weight of 900bp when the reaction is carried out with the primer (2), which also forms a secondary indicator of the species, while the primer (3) is considered non-diagnostic because it gave a single band with a weight of 850bp which was similar to the band that resulted for all other types.

It has been shown through the current study that the primer (ITS1/ITS2) was more accurate in diagnosing dermatophyte species and a stable diagnostic indicator, that it can be used for dermatophytes quickly and accurately, especially in samples that are contaminated with other fungi. It is also evident that the primer (ITS1/ITS2) is more accurate than the primer (ITS3/ITS4) and that the primer (ITS1/ITS4) is no longer a diagnostic primer, neither at the genus nor species level, since it exhibited a single band in all isolated types of fungi with a weight of (850bp).

The study by Li et al. (2008) reported that identification percentage recorded by ITS1 and ITS2 sequences are higher than 97% based on the reference sequences of the new species of strains. It was also noticed that most *Trichophyton mentagrophytes* strains were misdiagnoses of *Trichophyton interdigitale*. The results of the study conducted by Salehi et al. (2020) revealed that conventional and PCR-RFLP techniques were not able to accurately identify all dermatophyte species and differentiate between closely related species such as T. interdigitale and T. mentagrophytes, while ITS rDNA and TEF-1α gene sequence analyzes provided an accurate diagnosis for all dermatophyte species (isolates at genus and species level). The study conducted by Kaur et al. (2020) in Punjab, India, referred to identify 40 isolates of the fungus *Trichophyton spp* out of 48 isolates of dermatophytes that showed distinct bands after amplification using the random primers OPAA17 and OPD18. However, the study showed that using the two primers OPAA17 and OPD18 is efficient, rapid, and accurate for diagnosing dermatophytes in atrophic onychomycosis using AP-PCR. Data analysis in a study revealed that the primers OPAA17 and OPD18 were inefficient in recognizing some isolates or even detecting their presence (Ibrahim et al., 2022). In the study of Naseif et al. (2020) using primers ITS1 and ITS4, PCR amplification of dermatophytes showed the identification of distinct species and molecular weights, including 550bp for *T. tonsurans* and *T. equinum*; 650bp for *T. verrcossum*, *T. Bullosum*, and *M. appendiculatum*; 690bp for *T. Rubrum* and *T. mentagrophytes*; 700bp for *T. interdigitale*; and 740bp for *M. canis* and *E. floccosum*. In a study conducted in Tunisia, Neji et al. (2010) identified three dermatophytes (*T. mentagrophytes*, *T. Rubrum*, and *M. Ferrugineum*) with a 450bp obtained from the CHS1 gene. In another study, polymerase chain reaction with primers ITS1 and ITS4 was used to confirm positive dermatophyte cultures, as the ribosomal RNA (rRNA) database was used to perform sequence analysis and verify homogeneous data for microorganisms in comparison with (NCBI), where 37 dermatophyte isolates were found, 8 of them contained variances in the

identification percentage with Gen Bank by 99%, which were also registered in Gen Bank (Hashoosh and AL-Araji, 2023). Differences in result can be explained by geographical differences and the fact that nucleotide substitutions in PCR primer regions (especially the 3' ends) prevent primer annealing and PCR amplification (Mohammed and Kasim, 2020; Ramaraj et al., 2017; Tiba et al., 2020). In the study of Abdel-Fatah et. al. (2013), the primers ITS1 and ITS4, specific to the ITS1, 5.8S, and ITS2 regions, were used and were able to distinguish between some species of dermatophytes whose weights ranged between 680bp and 780bp. Hence, simultaneous sequence analysis of these genomic regions (ITS) is very useful for confirming the identification of dermatophyte species.

Conclusions

The highest prevalence in this study was recorded by the fungus *T. mentagrophytes*, with 27 isolates with a percentage of 39.13%. During this study, morphological methods were confirmed by DNA sequencing of the ITS regions up to 88% for some species. However, it seems insufficient in the case of polymorphisms, and thus, it is recommended that molecular techniques be used in our laboratories to improve the accuracy of diagnoses.

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