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Original Article

Unveiling *Talaromyces marneffei* emergence among HIV/AIDS patients: exploring phylogeny and molecular identification

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Abstract

Talaromyces marneffei is a pathogenic fungus that causes fatal health complications for patients who are infected with HIV. For the current investigation, sputum samples were collected from 19 immunosuppressed patients from two hospitals located in Baghdad, Iraq by which they were inoculated onto both Sabouraud dextrose agar (SDA) medium at 25 °C and BHI (brain heart infusion) agar at 36±1 °C for growth before being identified using single and nested PCR methods. The 18S rRNA gene sequence of *T. marneffei* was used to create two sets of oligonucleotide primers, RRF1 and RRH1 which are considered fungus-specific outer primers were employed. Both nested and solo PCRs using the *T. marneffei*-specific inner primers (Pm1 and Pm2) were carried out. To define the phylogenetic relatedness of this isolate, the MEGA X program was used to align the nuclear ribosomal DNA (rDNA) sequences of *T. marneffei*. Results showed that the wine-colored pigmented isolates in agar were dimorphic, exhibited bloom-like twigs and spore chains characteristic under the microscope, and were filamentous type colonies with light yellow villi. Finally, immuno-compromised patients in Iraq have *T. marneffei* in their blood cultures that will be induced to pathogenicity, and the PCR assay is valuable for *T. marneffei* identifying. Other results from nested PCR revealed that 8 human isolates, from 19, have specific fragments of about 400 bp on the agarose gel.

Keywords: *Penicillium marneffei*, 18S rRNA Gene, Nested PCR, Sequencing. Phylogenetic.

1. Introduction

Talaromyces marneffei (formerly *Penicillium marneffei*) is a pathogenic fungus that causes a life-threatening systemic mycosis for those who suffer from immunodeficiency, particularly patients infected with HIV(AIDS) [1]. Talaromycosis is an endemic disease that affects people with HIV/AIDS, cancer, organ transplantation, or immunodeficiency syndrome living in Southeast Asia, China, or northeastern India [2]. Talaromycosis disease may emerge after inhalation of aerosolized environmental fungal spores. In HIV patients, talaromycosis is more commonly spread through the blood and affects the whole body [3]. As a pathogenic and thermally dimorphic fungus, *T. marneffei* is the most extreme respiratory, cutaneous, and systemic mycosis. It is also more likely to cause fever and splenic swelling [4,5]. This fungus causes complex problems for HIV-infected patients and greatly threatens their lives, in addition to other symptoms such as weight, fever, anemia and skin lesions. Most patients are of low prognostics in antimicrobial therapy [6].

In collaboration with the Beijing Genomic Institute, the *T. marneffei* genome sequencing project was launched in 2002 to understand the project's pathogens and thermal dimorphism mechanisms. As with our predecessor, Larib-

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acter hongkongensis, the whole genome shotgun method determined the *T. marneffei* PM1 sequence [7]. PM1 was used for isolation in Hong Kong of a culturally recorded talaromycosis patient. A genomic DNA library was built using a physical screening method with inserts ranging from 3.0 to 5.0 kb. The sequence of 315,580 clones with a 6-fold coverage of the genome *T. marneffei* [7]. The PM1 draft is 2887, 485bp in total length, and consists of 2,780 adjacent sequences. Contiguities with an entire duration of 2.842 Mb have been ordered in 273 supra contiguities. Overall, 10,060, 9,257 (92 percent) longer protein-coding genes than 100 amino acids have been estimated. The mean gene density per 2.8 kb is one gene and 62.1 % of the genome section is the protein code sequence (51.2 % except for introns) [8]. The estimated total distribution in 91 percent of the marneffei genes is estimated to be 28,180 introns between 151,617 long and medium nucleotides. The TTAGGG is a well-known tandem repeat [9].

Four rodent species in Germany, including *Rhizomys pruinosus, Rhizomys sinensis,* and *Rhizomys sumatrensis*, were produced from *Marneffee marneffei*, isolated from *T. marneffei*, and samples from the soil surrounding the roots of the rats, which were built from bamboo [9]. *T. marneffei* originates from sources in humans and the environment and the culturing process takes approximately two weeks. The identity of this fungus can be determined based on the colony's morphology as well as the transformation and the mold eastward [10]. For *T. marneffei* detection in histological sections, a particular indirect fluorescent antibody test can be used. Also, immunodiffusion tests, latex agglutination testing, and immunosorbent assay were developed to detect *T. marneffei* antigens [11]. This mycosis can also be diagnosed using the indirect immunofluorescent antibody test [12], an immunoblot assay [13] or ELISA detecting Immune Globulin G antibodies in patient sera. To establish the phylogenetic relativity of this species and build the primers of oligonucleotides to identify the organism by PCR, the internal ribosomal (rDNA) transcribable space area of *T. marneffei* was sequenced. However, a single examination does not present the sensitivity and precision necessary to confirm a clinical diagnosis [8]. In the current study, we investigated isolation and using Nested PCR to determine the Genotype of local *T. marneffei* isolates based on PM1 and PM2 gene sequencing.

2. Materials and Methods

2.1. Specimens collection

T. marneffei was isolated from 19 sputum culture specimens from immunocompromised patients in Iraq hospitals. The specimens were collected for research purposes from the central labs of Al-Karama Teaching Hospital and Kadhimiya Teaching Hospital located in Baghdad during routine biochemical investigations. The research protocol was conducted in accordance with the Declaration guidelines of Helsinki's ethical principles. All patients gave their approval and the medical research committee's permission was achieved (approval number: 2019-SB-065).

2.2. Screening and isolation of the fungi

Using widely used standard plate culture methods, a total of 19 fungal isolates were found and identified. *T. marneffei* were cultured on SDA (Himedia, INDIA) for 7 days at 25±2°C and then transported to brain heart infusion agar (BHI; Difco, USA) for culturing at 35±2°C to achieve the yeast phase. Specimens that were collected have been inoculated into Sabouraud's dextrose broth (SDB, Himedia, INDIA) tubes. Then duplicate plates of SDA, with a Final pH of 5.6 ± 0.2 at 25±2°C, were employed to inoculate the broth SDB tubes [14].

2.3. Dimorphic features of *T. marneffei*

About dimorphic features, all reactions used yeast that was grown on BHI agar, a specific medium that is suggested for use in the culture of a wide range of microorganisms, including pathogenic fungi. On BHI, the isolates that were obtained from these cultures were incubated at 35±2°C for checking dimorphism [15].

2.4. Morphological examination

The lactophenol-cotton blue staining method was used to examine the morphological characteristics of the colonies, including the cultures' appearance, texture, growth rate, and color of the posterior and surface colonies. A small part of the fungal colony was taken and placed on a glass slide containing a staining solution drop, and the mycelial mass was carefully pulled apart by using two dissecting needles, then a clean slide was used to cover the mycelial mass to investigate it by the ordinary microscope [16].

2.5. DNA Extraction

The DNA was extracted according to the guidelines of the QIAamp FFPE Tissue Kit (Qiagen, Hilden, Germany). After that, the pellets were resuspended into 180 ml ATL (tissue lysis containing SDS and EDTA) buffer containing Proteinase K (Qiagen, Hilden, Germany) and then incubated at 55°C for 24 hours [17]. After the incubation, the samples were placed in boiling water for 5 minutes and then placed in a nitrogen liquid for 2 minutes to freeze the samples and then the samples were boiled again for 2 minutes to destroy the fungal cells. Last but not least, the remaining amount of DNA was determined using agarose gel electrophoresis.

2.6. Agarose gel electrophoresis

Electrophoresis was used to analyze DNA using 1% agarose gels (Hispanagar; Sphaero Q) with 0.5 gr/ml ethidium bromide in TAE (Tris-acetate-EDTA) buffer [40 mM Tris, 20 mM acetic acid, 1 mM ethylene diamine tetraacetic acid (EDTA), pH 8.0]. The sample was run for 2 h at 70V in the presence of a 100-bp DNA ladder (Gibco/BRL Life Technologies, Breda, The Netherlands), using a horizontal gel electrophoresis unit (Mini-Sub DNA cell, BioRad),

2.7. Oligonucleotide primers and Nested PCR assay.

In the presence of two sets of primers, nested PCR was achieved, including the inner primer and the outer primer. In this study, two series of nucleotide primers were utilized according to the instructions of Invitrogen company. The experiment was performed in the Molecular Dept. of the Institute of Liver Studies, King's College Hospital, and the College of London. Southampton, United Kingdom. The pairs of the outer and inner primers are recorded in Table 1.

The RRF1 and RRH1 universal primers were used for fungi identification and sequence of the 18S rRNA gene. While the inner primer pairs (Pm1 and Pm2) were specific

Table 1. The pairs of the outer and inner primers used for the detection of *Talaromyces marneffei* strain.

Primer	Nucleotide sequences(5'-3')	Ref.
RRF1	5' . . .ATCTAAATCCCTTAACGAG GAACA. . .3'	[18]
RRH1	5' . . .CCGTCAATTTCTTTAAGTTTCAGCCTT . . .3'	[18]
Pm1	5'...ATGGGCCTTTCTTTCTGGG...3'	[19]
Pm2	5' . . .GCGGGTCATCATAGAAACC..3'	[19]

and designed for the *T. marneffei* amplifying sequence of 18S rRNA gene by relating the 18S rRNA genes sequences of *T. marneffei* (GenBank; accession no. AF034197) [20] and other fungi in the Gen-Bank database. These two primer sets were utilized to perform the Nested PCR. The templates of DNA were amplified using the RRH1 and RRF1 primers. This phase was completed in a 50 µl volume comprising 5 µl of each primer, 0.5 µl of Taq polymerase (Thermofisher, USA), PCR buffer 5 µl and approximately 6 µl extracted DNA and molecular grade water (28.5 µl). The PCR amplification program of the first run of RRF1 and RRH was conducted using a thermal cycler (Perkin-Elmer, model 480, CA) programmed as follows: 95 **°C** for 5 minutes; 35 cycles of 95 **°C** for 30 seconds, 55 **°C** for 30 seconds, 72 **°C** for 45 seconds; and final extension at 72 **°C** for 10 minutes. The amplification program of the second run Pm1 and Pm2 was performed as follows: 95**°C** for 5 min; 30 cycles of 95 **°C** for 30 sec, 65 **°C** for 30 sec, 72 **°C** for 2 min; and final extension for 10 min at 72 **°C**. The single PCR amplification test was performed with Pm1 and Pm2 directly. The analyzing of PCR amplification products were analyzed via electrophoresis using 1% wt/vol of agarose gel as above [21].

2.8. Nucleotide sequence analysis

After staining with ethidium bromide, the amplified products that were gained from nested PCR were purified, separated, and irradiated with ultraviolet light (UV; 302 nm) to visualize them. Sequencing of the gene was performed by Macrogen, Korea. Homology searches were performed using the Basic Local Alignment Search Tool program (BLAST), available online from the National Center Biotechnology Information (NCBI) at (http://www.ncbi.nlm.nih.gov), and the BioEdit program. The products of PCR were purified and then sequenced in both directions using the Big Dye Terminator v3.1 Cycle Sequencing Kit on an ABI 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA). MEGA X software and NCBI's Basic Local Alignment Search Tool Bio ID were used to identify the sample, analyze the sequence in the nucleotide databases, and construct a phylogenetic tree [22].

3. Results

3.1. Isolation and colony morphology of the fungi strains

A total of 19 isolates of fungi were obtained and identified from sputum samples of immunocompromised patients. The result showed that the mold colony grew on the SDA medium at 25±2°C and exhibited white conidial heads with a suede-like to downy appearance. The colonies produce a pigment with brownish-red to wine color having the ability to be diffusible as they age, turning from greyish-pink to brown (Fig. 1).

3.2. Morphological analysis by optical microscopy

Mold colonies typically exhibit rapid growth, and an examination of their macroscopic morphology revealed that the fungus develops as a mycelium with septate hyphae containing conidiophores and conidia. The conidiophores had smooth walls, were hyaline, and had terminal verticils with 3-5 metulae and 3-7 phialides on each. The conidia were formed in basipetal succession from the phialides and were globose to subglobose with smooth walls (Fig. 1).

3.3. Dimorphic features of *T. marneffei*

The only Talaromyces species reported to possess a temperature-dependent dimorphic property was *T. marneffei*. The results showed that colonies on BHI agar are rough, glabrous, white or light brown, and yeast-like (Fig. 2). *T. marneffei* is characterized by a thermally controlled transformation from mold to yeast or a phase change, which is considered to be the main cause of its pathogenicity. When viewed under the microscope, *Talaromyces spp*. cells stained with lactophenol cotton blue are ellipsoidal or spherical and reproduce by division and do not reproduce by budding.

3.4. Molecular identification using 18S rRNA gene sequences

DNA was extracted from the isolates and analyzed by PCR. Using the two primer pairs (RRF1 and RRH1,

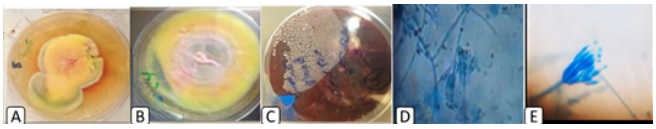

Fig. 1. (A) Pigment production of *T. marneffei* grown on SDA at 25 ^{0}C after 7 days. (B) pigment production of *T. marneffei* grown on SDA at 25 ^{0}C in the colony at 10 days, (C) pigment production of *T. marneffei* grown on SDA at 25 ^{0}C in the colony at 4 weeks (D) microscope morphology of mycelia phase staining by LPCP stain (40x)., (e). microscope morphology staining by Gram stain (40x).

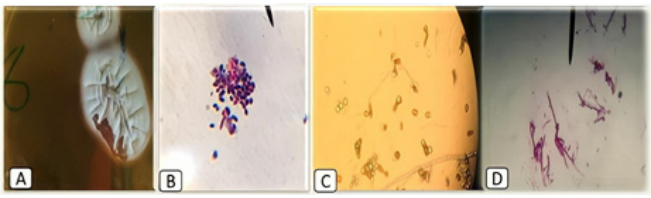

Fig. 2. (A), colony morphology of yeast phase grown on BHIA at 37 ^{0}C after 7 days (B). Microscope morphology of yeast phase staining by B: Gram stain(40x). (C) Microscope morphology of yeast phase staining by Gram stain (40x). (D) Microscope features short hypha staining by Gram stain (40x).

and Pm1 and Pm2), the system of nested PCR was verified to act specifically for *T. marneffei*, and other fungi, including *T. marneffei*, *Aspergillus fumigatus*, *Aspergillus flavus*, *Cryptococcus neoformans*, *Talaromyces spp*., *Histoplasma capsulatum*, *Candida krusei* and *Candida albicans*. Primers RRF1 and RRH1 amplified a region of the 18S rDNA sequences of *T. marneffei* and other fungi. However, the internal primers (Pm1 and Pm2) amplified a specific section of the 18S rDNA sequences of *T. marneffei*. In this study, all individual PCR products of 19 isolates using the RRF1 and RRH outer primers gave positive results for 18 isolated fungi, yielding approximately 631 bp in the first step of nested PCR (Fig. 3). In addition, the second step of nested PCR using the inner primer pair (Pm1 and Pm2) yielded approximately 8 positive isolates with a 400-bp fragment that was identified and sequenced; the consequences indicated that the fragment belonged to *T. marneffei* but was not specific for other fungi (Fig. 4).

3.5. Nucleotide sequence analysis and phylogeny

To identify the sample, the sequence was examined in the nucleotide databases using the NCBI program Basic Local Alignment Search Tool Bio ID and uploaded to GenBank (ID). Related sequences from the sample or the NCBI nucleotide database (www.ncbi.nlm.gov/nucleotide) were identified using the Bio ID program [23]. In this study, analysis of the PCR products showed that the isolates in Iraq hospitals had a similarity of almost identical (99%) to Talaromyces, which had the numbers (PM1, PM2, PM3, PM4, PM5, PM6, PM7, PM8) with their respective accession numbers (MW332123.1, MW332124.1, MW332125.1, MW332126.1, MW332127.1, MW332128.1, MW332129.1, MW332130.1). Regarding phylogeny, the MEGA X program used the neighbor-joining approach to infer evolutionary history. The *T. marneffei* strains in the phylogenetic analysis of this study showed close association with *T. marneffei* strains from other regions of the world, with 99% genetic identity among those reported from China, Indonesia, India, Thailand, the USA, and other countries (Fig. 5).

4. Discussion

One of the most important emerging pathogenic fungi that can cause fatal mycoses in humans, especially in immunocompromised individuals with HIV, is *T. marneffei* of the family Trichocomaceae. Southeast Asia harbors a substantial population of this pathogenic fungus, which is thought to have originated there. However, there have been no documented examples of infection caused by this pathogen in Iraqi HIV patients. To type the genome of fungal isolates in immunocompromised patients in Iraq, this study used the regulatory genes PM1 and PM2. Through this study, 19 fungal isolates were isolated. Eighteen isolates yielded positive results after being isolated and identified from blood specimens collected from immunocompromised individuals in Iraq hospitals. These isolates were grown on SDA medium and BHI agar and incubated at 25°C and 37°C respectively. Similarly, Sun et al., 2011 [24] reported forty *T. marneffei* strains were isolated from screening patients. Using ITS sequencing, each isolate was identified and a selection was sent to GenBank. Isolates were grown on SDA plates at 25 °C for one week. Similarly, Uehara et al, 2008 [25] reported that three blood cultures were obtained from a Thai woman with AIDS

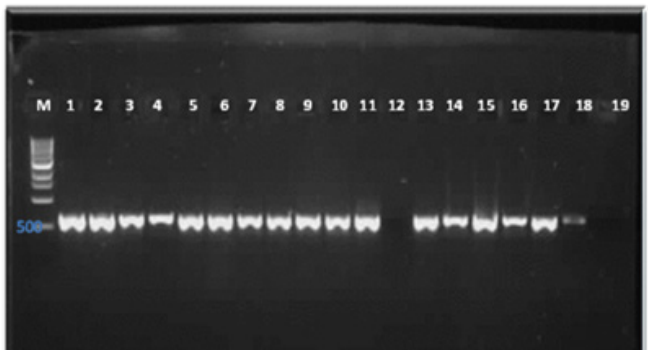

Fig. 3. Single PCR results of *Talaromuces marneffei* and other fungi by specific primer pair RRF1 and RRH1 (partial). Lanes: 1000-bp-ladder DNA; (2) *Talaromuces marneffei*; samples isolated from immuno-compromised patients in Iraq isolated from blood respectively.

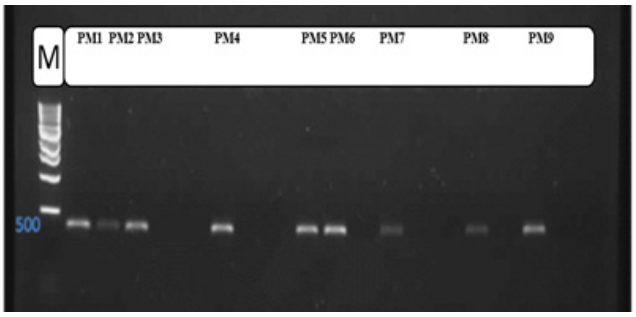

Fig. 4. 2 Nested PCR results of *Talaromuces marneffei* by specific primer pair Pm1 and Pm2 (partial). Lanes:(1), 1000-bp-ladder DNA of different isolates from Talaromuces marneffei (2 TO 9); ((PM1, PM2, PM3, PM4, PM5, PM6, PM7, PM8, PM9).

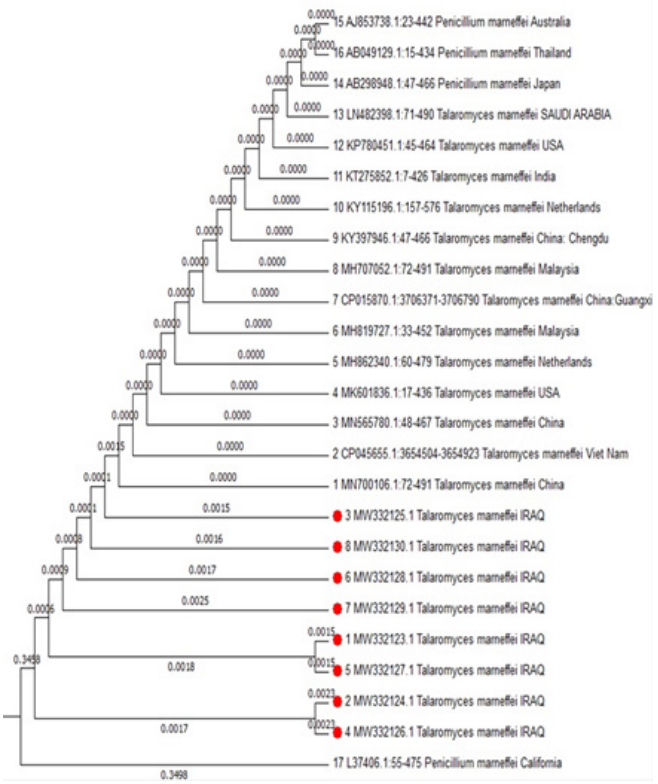

Fig. 5. Phylogenetic tree displaying the relationship between local *Talaromyces marneffei* isolates and other *Talaromyces marneffei*. The tree was constructed using a neighbor-joining algorithm in MEGA X software.

from which *T. marneffei* was isolated and identified as such. The patient, a 41-year-old Japanese bride originally from northeastern Thailand, came to Japan ten years ago. She was the third patient in Japan to contract this fungal species and is believed to be the first case in which the fungus responsible for the infection was successfully cultured, allowing the diagnosis of *T. marneffei*. The colony of the isolate was grown on BHI at 35 °C and SDA at 25-27 °C. Moreover, Hamza et al. 2022 [26], stated that out of 50 lung and 50 rumen samples from goats in Iraq, fungal isolates from goat lungs were grown on SDA media at 27 °C for 3-4 days and stained with lactophenol cotton blue at 25°C, yielding 41 and 35 isolates, respectively. According to the appearance (color, shape, and type of growth) and microscopic examination, the fungal isolates found in sheep lung samples included: Cryptococcus spp. followed by *Rhodotorula spp, Pencillium spp*. *Candida spp, Histoplasma spp, Aspergillus spp.,* and *Coccidioides spp.*, while those isolated from sheep rumen were, *Aspergillus spp.*, *Cladosporium spp.*, and *Coccidioides spp*. The study also revealed that *T. marneffei* is designated as one of the most important new pathogenic fungi that can cause fatal mycoses in humans, especially in HIV-positive individuals who are immunocompromised. However, there have been no documented examples of infection caused by this pathogen in Iraqi HIV patients. In this study, Nested PCR was used to type the genome of fungal isolates among immunocompromised patients in Iraq.

The primer sets used were the inner and outer primers, the universal fungal primers RRF1 and RRH1, which produced around 631bp PCR products, and the specific primers Pm1 and Pm2, which produced approximately 400 bp PCR products of *T. marneffei*, similar to the report by Vanittanakom et al. 2002 [8]. They reported whether the fungus was isolated from its original habitat or humans, the nested PCR system using two programs of reaction in the first and second phases with RRF1-RRH1 and Pm1-Pm2 primer pairs was confirmed to detect the *T. marneffei* strain. Amplification of part of the 18S rDNA sequences of *T. marneffei* and other fungal species by primers RRF1-RRH1 generated around 600 bp PCR products reportedly, the primer pair RRF1-RRH1 is fungal specific. A single PCR with the specialized primers Pm1-Pm2 produced successful results with *T. marneffei.* The length of the amplified product was approximately 400 bp which was not founded in other fungal species. Furthermore, Vanittanakom et al. (2006) [20] stated that the highly sensitive PCR technique was successfully used for the accurate detection of a wide range of fungi. For molecular diagnosis of *T. marneffei*, oligonucleotide primers (ITS1-5.8S-ITS2) specific for the internal transcribed spacer and the 5.8S rRNA gene are used. The fungal DNA initially was amplified with the primer pair ITS5 and ITS4, and then nested PCR was employed with the primer pair PM1 and PM4 or PM2 and PM4. This was done to evaluate the specificity of these *T. marneffei* primers. Using this technique, *T. marneffei* was successfully identified from a skin biopsy. The primer pair PM2 and PM4 were entirely amplified *T. marneffei* DNA and yielded a PCR result of 347 bp. Moreover, the results of this study are inconsistent with the report of Pongpom et al. 2009 [27], who stated that in the first step of PCR, nested PCR was performed using the outer primers (RRF1 and RRH1) and a separate PCR reaction (95°C for 5 min, followed by 35 cycles at 95°C for 30 sec, 55°C for 30 sec, and 72°C for 1 min, before a final extension at 72°C for 7 min). Using the particular and inner primer pair, 1 µL of the first PCR result was subjected to nested PCR amplification (Pm1 and Pm2) with the exception of the annealing temperature of 68 °C and the PCR cycle being either 15 or 30. The conditions and parameters of nested PCR were similar to those specified in the first PCR. The nested PCR and first products' final sizes were 400 bp and 630 bp, respectively.

5. Conclusions

There is no need to emphasize the significance of a thorough examination of *T. marneffei* human infections in environmental sources. Molecular genotyping of clinically identified *T. marneffei* is necessary to efficiently answer public health questions regarding sources and routes of transmission, pathologies and virulence, disease resistance, genetic variation, or relatedness of isolates. However, this pathogenic fungus has not yet been associated with HIV infection in Iraq. Accordingly, the nested PCR method would be useful for the isolation and detection of novel stains associated with *T. marneffei* and for prospective support of clinical diagnosis when archival material is available. Our results also suggest that immunocompromised individuals may act as *T. marneffei* reservoirs in Iraq hospitals.

Further studies may be done to determine the mechanism of *T. marneffei* pathogenicity in the immunocompromised host to point to a possible target for therapy. Resistance patterns in *T. marneffei* isolates need to be considered since this will provide a way of developing effective regimens for the treatment and management of patients. The molecular or environmental factors that may affect the dissemination or virulence of this fungus, particularly in populations from different geographical regions, will also yield important information.

Author contributions

Conceptualization, M.A.M., F.E., H.M.R.S. and H.A.A.; methodology, M.A.M., I.M.A., S.A. and M.S.B.; software, R.A. and M.A.; validation, A.A.M., M.A. and R.A.; formal analysis, M.A.M., F.E.; investigation, S.F.A., M.S.B.; resources, R.A., M.A. and I.M.A.; data curation, A.A.M., M.A. A.A.M., H.A.A. and H.M.R.S.; writing—original draft preparation, R.A., M.A., S.A. and I.M.A.; writing—review and editing, H.A.A., M.S.B., R.A. and M.A.; project administration, M.S.B.; All authors have read and agreed to the published version of the manuscript.

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Informed consent statement

Not applicable.

Data availability statement

The study did not report any data.

Conflicts of interest

The authors declare no conflict of interest.

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