Genetic Variation of Sequencing *18srDNA* Gene of *Trichophyton mentagrophytes* Isolates.

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Abstract

Background: *Trichophyton* species are considered the most frequent causative agents in medical field.

Objective: The existing training designed to conduct the sequencing analysis of nucleotides of ITS1-5.8S-ITS2 for *Trichophyton mentagrophytes.*

Methods: The isolation and identification of pathogenic fungi, *Trichophyton mentagrophytes* from clinical specimens was done based on the standard morphological and molecular methods. The genomic DNA of fungal isolates were extracted and purified to amplify with primers of *18S rRNA* gene for detection and sequencing the nitrogenous bases to define the genetic variation among clinical isolates of *Trichophyton mentagrophytes* in compared with strains recorded in NCBI GeneBank.

Results:Eieght isolates of *Trichophyton mentagrophytes* were isolated and identified from clinical specimen of dermatophytosis The DNA sequencing analysis showed the presence of some genetic variation in nitrogenous bases between the local isolates and that recordred in locus of world NCBI GeneBank. Multiple Alignment sequence using NCBI BLAST revealed the recoding a new mutant isolates among the local isolates based on DNA homology percent.

Conclusion:The present results proved the successful the use of molecular methods in diagnosis the fungal isolates of *Trichophyton mentagrophytes* especially the sequencing analysis to find the genetically bases of these isolates.

Keywords:*Trichophyton mentagrophytes*; DNA Sequencing;Genetic variations

Introduction

Dermatophytoses are considered as one of common diseases in human which increased promptly in last decades , although these diseases are not life threatening but they are infected 10-20% of world populations (1). Dermatophytes are group of related fungi with morphological and functional characteristics and they capable to enter keratinized tissues of humanoid and animals causing cuteneous infections or tinea (2).Due to that Trichophyton species show a close and confuse phenotypic features on culture media, so these characters become a weak taxonomic tool in this state because it lacks or makes the difficulty the finding of telemorph state for many clinical isolates compained with anamorphic polymorphism phenomena.Thus, these factors make the taxonomy of these

dermatophytes so difficult especially the new species or strains that infect human which called anthropophilic dermatophyes.So,the diagnosis of these fungi comprise a taxonomic problem not only for species identification but for lackingthe macroscopic and microscopic of Trichophyton species (3). According to that, the determination of genotypes of dermatophytes is very important to evaluate the levels of genetic variations among species that habitat different ecosystems depending on the comparision of nucleotides sequences of target gene of fungal species with the reference isolates sequencing recorded in world GeneBank. Due to the close correlation among dermatophytes that infect human and animals, in addition to that, some isolates belong to some species of Trichophyton reveal different degrees of phenotypic similarities and at the sametimes they have different genotypes among one species of Trichophyton that named Trichophyton species complex.

Liu *et al*.(4) used the method of random amplified polymorphyic DNA (RAPD) for

analysis the evolutionary relation among *Trichopyton* species complex.Also, the real-time PCR and restriction fragment length polymorphism(REFLP) were used to detect of Trichophyton species (5,6). These species are unstable in taxonomy because of emergence of new species or strains that differed in many morphologic and microscopic features from the wild type that its evolved. So, the molecular methods began to solve this problem by using the sequencing analysis of nitrogen bases for ITS region in rDNA gene (7). So , the existing training designed to conduct the sequencing analysis of nucleotides of ITS1-5.8S-ITS2 for Trichophyton mentagrophytes anddetermine yhe DNA homology between them incomparison withreferenceisolates in GeneBank.

Materials and Methods

One hundreds three specimens including hair fragments, nail clippings, and skin scrappings Were composed from sick confessed to Dermatology Department of AL-Diwaniya Teaching Hospital- Iraq during the period of start of December 2021 till finish of April 2022. Then all the samples were brought to lab and diagnosed by direct examination and laboratory culture(8).The typical medium for the isolation of pathogenic fungi from medical specimens is Sabouraud's dextrose agar (SDA)containing cycloheximide 0.5g/L to inhibit the growth of saprophytic

fungi and chloramphenicol 0.05g/L to inhibit the growth of fast growing bacteria. Cultures were incubated at 28+1 ºC. Cultures were firstly examined after(4-7) days, and then twice weekly for at minimum (3– 4)weeks formerly being deliberated negative . The distinctive characteristics of dermatophytes typically manifest within a span of 10-20 days. Many dermatophytes lose their unique cultural and microscopic traits when cultured for extended periods (Rippon, 1988). In this research, identification was primarily reliant on colonial and microscopic traits, employing specific media as outlined by Rippon (9) and (10). A segment of the colonies was cultivated on SDA through a spot inoculation technique. These cultures were incubated at a temperature of (29 ± 2)°C until visible fungal

growth occurred, or until they reached 5-10 days of age, for early PCR analysis. The genomic DNA of fungal isolates (young fungal colonies) was extracted using a grinder in the presence of liquid nitrogen to initially break down the mycelia. The final DNA extraction was accomplished using a specialized purification kit. The resulting DNA solution, totaling 60 µl, was stored at 20°C until it was needed as a template in subsequent PCR experiments. The PCR primers, designed for detecting Trichophyton sp. and based on the 18S rRNA gene, were developed in this study using the NCBI Gene Bank database and the Primer 3 online design tool. These primers were obtained from Macrogen company, Korea, as shown in Table 1.

Table (1): PCR Primers with their nucleotide sequence and amplicon size:

Primer	**Sequence (5'-3')**		**Product Size**
Trichophyton mentagrophytes *18S rRNA* gene	F	**GACGTTCCATCAGGGGTGAG**	584bp
	R	**CTGAATTGGCTGCCCATTCG**	

Fungal genomic DNA was extracted from fungal isolates using the EZ-10 Spin Column Fungal Genomic DNA Mini-Preps Kit, following the manufacturer's instructions. The quality and purity of the extracted genomic DNA were assessed using a Nanodrop spectrophotometer (THERMO. USA) by measuring absorbance at both 260 nm and 280 nm.

The primers were prepared as per the manufacturer's guidelines by reconstituting lyophilized primers with TE buffer to create a stock solution with a concentration of 100 pmole/ul. After centrifugation and an overnight incubation at 4°C, a working solution of the primers in TE buffer was prepared, achieving a final concentration of 20 pmole/ul for each primer, using the equation C1V1 = C2V2 (concentration versus volume).

The PCR master mix reaction was prepared using the AccuPower PCR PreMix Kit, following the manufacturer's instructions. Subsequently, these PCR master mix reaction components, as mentioned earlier, were placed in standard PCR tubes containing the lyophilized PCR PreMix, which includes all the necessary components for the PCR reaction, such as Taq DNA polymerase, dNTPs, Tris-HCl pH: 9.0, KCl, MgCl2, stabilizer, and tracking dye. The tubes were then centrifuged in an Exispin vortex centrifuge for 3 minutes before being transferred to a Mygene PCR thermocycler.

PCR thermocycler conditions for each gene were determined using a conventional PCR thermocycler system. The PCR products of each gene were subsequently analyzed using agarose gel electrophoresis, following the method outlined by Sambrook et al. (11).

The PCR products of ITS5-ITS4 primers were used to gain the nitrogen bases sequences of Trichophyton isolates which sent to Microgen company(USA).These sequences were compared with sequencing of reference isolates present in world Gene Bank by using NCBI blast nucleotide database program to get a high percent of compatibility for generic and specific name of anamorph or teleomorph state of each isolate.

Statistical analysis

The data underwent collection, summarization, analysis, and presentation through the utilization of SPSS version 25, along with Microsoft Office Excel 2010.

Results and Discussion

The results displayed that the positive amplification of the ITS region of *18s rDNA* gene for *Trichophyton mentagrophytes* had amplicon size(584bp) PCR product(fig. 1). The amplification of the ITS region of rDNA have been used by various workers (16).

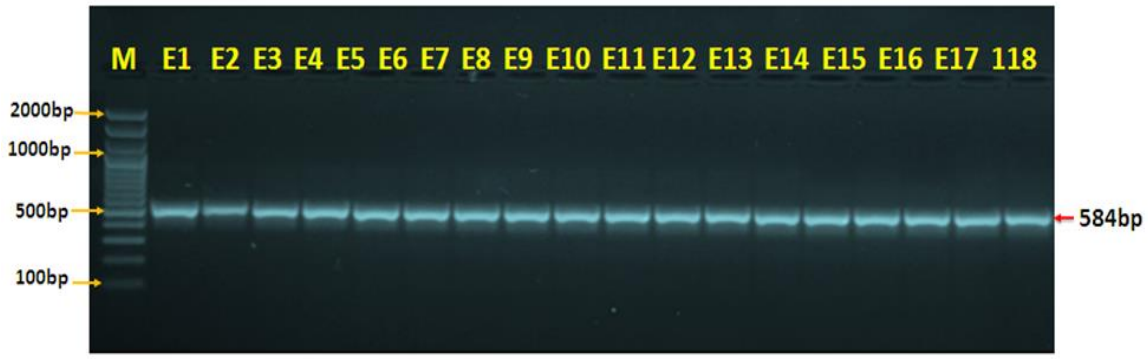

Figure (1): image of electrophoresis Agarose gel of PCR product investigation of *16S ribosomal* RNA gene of *Trichophyton mentagrophytes* isolates. (M=Marker ladder: 2000-100bp, lanes (1-18): positive (584bp) PCR product size.

Figures (2,3,4,5,6,7) show the Multiple Alignment sequence using NCBI BLAST of the partial 18S ribosomal RNA gene sequence of local isolates of *Trichophyton mentagrophytes* and NCBI Gene Bank .The results showed the close related of local isolates of *T,mentagrophytes* isolated from clinical specimens of dermatophytosis with reference strains NCBI Blast Trichophyton mentagrophytes : KX463658.1 **;**FJ746658.1; KJ606098.1 HQ014710.1; AF168125.1).

REF. NCBI TCGATGAAGAAC-GCAGC-GAAATGCG-ATAAG-TAATGT-GAATTGCAGAATTCCGTGA

Tm1 .....-.....-.....-.....-.....

Tm2 .....-.....-.....-.....-.....-.....

Tm3 ..... -.....-.....-.....-.....-.....

Tm4 .....- .....-.....-.....-.....-.....

Tm5 ...A.....A.....-.....G....-.....-.....-.....-.....

Tm6 ...A.....TC.....C.....- A.....C....-.....-.....-.....-.....

Tm7 .....A..G.....-.....-.....-.....-.....-.....

Tm8 ..... A.....-.....-.....-.....-.....-..... ....

Fig. (3): Multiple Alignment sequence using NCBI BLAST of local isolates of *T.mentagrophyes* isolated from Tinea capitis(No.Tm1-Tm8) in compared with reference strainFJ746658.1

REF. NCBI ATCATCGAATCTTTGAACGCACATTGCGCCCCCTGGCATTCCGGGGGGCATGCCTGTTCG

Tm1 .....

Tm2 .....

Tm3 .....

Tm4 .....

Tm5 ..... A……..T.....

Tm6 .....

Tm7 .....T.....

Tm8 G.....A.....G.....T……G.....G.....T..... T.....

Fig. (4): Multiple Alignment sequence using NCBI BLAST of local isolate of *T.mentagrophyes* isolated from Tinea pedis (No.Tm1-Tm8) in compared with reference strain no. KJ606098.1

REF.NCBI AGCGTCATTTC-AGCCCCT-CAAGCCCGGCTT-GTGTGATGGACGACCGTCCGGCGCCCC

Tm1. .....-.....-.....-.....

Tm2 .....-.....- .....

Tm3 .....-.....-.....-.....

Tm4 .....-.....-.....-.....

Tm5 .....-……T.....-…… .-.....

Tm6 … C.....T.....C-.....G.....-.....-……

Tm7 ..... A..... -.....A… A-.....T....T.....-……

Tm8 .....T-……..T.....-…. C...-C….-….A…..G.....G….-.. ..GA. A.....G....G……..G…….

Fig. (5): Multiple Alignment sequence using NCBI BLAST of local isolate of *T.mentagrophyes* isolated fromTinea ungium(No.Tm1-Tm8) in compared with reference strain no. HQ014710.1

REF NCBI CGTTTTTGGGGGTGCGGGACGCGCCCGAAAAGCAGTGGCCAGGCCGCGATTCC-GGCTT-

Tm1 .....T.....T..... - …….-

Tm2 .....-.....-

Tm3 .....-.....-

Tm4 .....-.....-

Tm5 .....G.....G.....A.....G... G.....-...T….T.-

Tm6 .....T……C....C.....-.....-

Tm7 .....T.....G…….T. G... T…….-....T…….T……-.....-

Tm8 .....-...T... G.....T……T.....A.....T…….G……T.....G.... C……..T……...T.....-……..-

Fig. (6): Multiple Alignment sequence using NCBI BLAST of local isolate of *T.mentagrophyes* isolated from Tinea corporis (No.Tm1-Tm8) in compared with reference strain no.HQ223449.1

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REF. NCBI  CCTAGGGG-AATGGGCA
Tm1.       T....C…… .-.....
Tm2        ......C…… .-.....
Tm3        ......C……..-.....
Tm4        ......C……..-.....
Tm5        ........G……-….....
Tm6        ......C……. -.....
Tm7        ......C……..-.....
Tm8        ……. T-.... T.-.....
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Fig.(7): Multiple Alignment sequence using NCBI BLAST of local isolate of *T.mentagrophyes* isolated from Tinea barbae(No.Tm1-Tm8) in compared with reference strain no.AF168125.1

The PCR amplicons generated were sequenced with the same primers to obtain partial 18SrRNA gene sequences.The incomplete 18S ITS1-5.8S-ITS2 incomplete 28 rRNA sequences examined by nucleotide blast and the percentage similarity was calculated by using method of alignments explorer Clustal X Microsoft Mega 6.0 package.The gene sequence of local isolates of *T. mentagrophytes* showed 97-100% homology with sequence of T.mentagrophyes standard strain The DNA sequencing of the partial 18SITS1-5.8S-ITS2 partial 28S rRNA gene sequence data for each strain was compared to its nearest neighbor, and if the similarity score exceeded 97%, it indicated the presence of a new species (17).

In recent years, genotypic methods have demonstrated their utility in resolving identification issues related to dermatophytes. Genotypic variations have been deemed more stable and accurate compared to morphological characteristics (13). The detection rate of dematophytes by PCR assay showed a degree of variations due to that diagnosis of Trichophyton spp. By classic laboratory methods gave phenotypic variability and pleomorphism and the use of molecular methods give a rapid with high accuracy and specificity(14).

Comparing sequences within the ITS region is a common practice in taxonomy and molecular identification. It's favored because it can be easily amplified from small DNA samples, thanks to the high copy number of rRNA genes, and it exhibits significant variation even among closely related species. The ITS region stands out as the most frequently sequenced DNA region in the molecular diagnosis of fungi (15).

On the other hand, the results of analysis the sequencing of ITS1-rRNAgene of local isolates Trichophyton mentagrophytes isolated from different tinea lesions showed a high genetic variations in nitrogen bases of nucleotides when compared with some of world strains that recorded as reference NCBI in genebank. Table (2) shows the base sequences of some isolates under study and their percent of compatibility with reference isolates in world GeneBank. isolates.

Table (1):Type and position of mutation with genetic variation percent in local isolates of T.mentagrophytes in comparision with reference NCBI world strain.

No	Sample	Mutation	Genetic variation %
1	Tinea.capitis_Trichophyton_ITS1-rRNA_gene	A -> T	50%
2	**T.capitis** Trichophyton_ITS1rRNA_gene	A -> T	100%
3	**T. capitis** Trichophyton_ITS1-rRNA_gene	C -> T	50%
4	**T. capitis** Trichophyton_ITS1-rRNA_gene	C -> A	0%
5	**T.capitis** Trichophyton_ITS1-rRNA_gene	C -> A	50%
6	**T. capitis** Trichophyton_ITS1-rRNA_gene	A -> C	50%
7	**T. capitis** Trichophyton_ITS1-rRNA_gene	A -> C	100%
8	**T. capitis** Trichophyton_ITS1-rRNA_gene	T -> A	50%
9	**T. pedis** Trichophyton_ITS1-rRNA_gene	G -> T	0%
10	**T. pedis** Trichophyton_ITS1-rRNA_gene	G -> T	0.25%
11	**T. pedis T**richophyton_ITS1-rRNA_gene	G -> A	0%
12	**T. pedis T**richophyton_ITS1-rRNA_gene	G -> A	100%
13	**T.pedis T**richophyton_ITS1-rRNA_gene	G -> A	50%
14	**T. pedis T**richophyton_ITS1-rRNA_gene	C -> T	8.18%
15	**T. pedis** Trichophyton_ITS1-rRNA_gene	C -> T	50%
16	**T. pedis** Trichophyton_ITS1-rRNA_gene	C -> T	50.19%
17	**T. corporis** Trichophyton_ITS1-rRNA_gene	C -> T	24.40%
18	**T. corporis T**richophyton_ITS1-rRNA_gene	G -> T	7.08%
19	**T. corporis** Trichophyton_ITS1-rRNA_gene	G -> T	49.97%
20	**T. corporis** Trichophyton_ITS1-rRNA_gene	G -> T	50%
21	**T. corporis T**richophyton_ITS1-rRNA_gene	G -> T	0%
22	**T. corporis T**richophyton_ITS1-rRNA_gene	G -> C	50%
23	**T. corporis** Trichophyton_ITS1-rRNA_gene	G -> C	0%
24	**T. corporis T**richophyton_ITS1-rRNA_gene	C -> T	100%
25	**T. ungium T**richophyton_ITS1-rRNA_gene	C -> T	100%
26	T. **ungium** Trichophyton_ITS1-rRNA_gene	C -> T	50%
27	**T.ungium T**richophyton_ITS1-rRNA_gene	C -> T	100%
28	**T. ungium** Trichophyton_ITS1-rRNA_gene	A -> G	11.25%
29	**T. ungium** Trichophyton_ITS1-rRNA_gene	A -> G	63.67%
30	**T.ungium** Trichophyton_ITS1-rRNA_gene	A -> G	50.46%
31	**T. ungium**Trichophyton_ITS1-rRNA_gene	A -> G	50%
32	**T. ungium**Trichophyton_ITS1-rRNA_gene	G -> T	50%
33	**T. barbae** Trichophyton_ITS1-rRNA_gene	G -> T	56.31%

34	**T. barbae** Trichophyton_ITS1-rRNA_gene	G -> T	14.98%
35	**T. barbae** Trichophyton_ITS1-rRNA_gene	G -> C	50%
36	**T. barbae** Trichophyton_ITS1-rRNA_gene	G -> C	0%
37	**T. barbae** Trichophyton_ITS1-rRNA_gene	G -> C	50%
38	**T. barbae** Trichophyton_ITS1-rRNA_gene	C -> T	99.20%
39	**T. barbae** Trichophyton_ITS1-rRNA_gene	C -> T	0%
40	**T. barbae** Trichophyton_ITS1-rRNA_gene	C -> T	2.48%

These results revealed the occurrence of point mutation either as miss matching or genetic gaps which differed in their number or types according to fungal species and some times based on isolate or strain of these species. This may be due to some stress of effect in their ecosystem or habitat of species which pay the fungus to form new strain and this is called eco-micro internal evolution(12) . The present results proved the successful the use of molecular methods in diagnosis the fungal isolates of *Trichophyton mentagrophytes* especially the sequencing analysis to find the genetically bases of these

Ethics Contemplation

This study adheres to the ethical guidelines set forth by the ethics committee of Al-Diwaniaya teaching hospital in Iraq. Prior to sample collection, verbal consent was obtained from the study participants or their relatives.

Conflict of interest: No recognized struggle of attention connected with this publication.

Conflict of interest: No identified engagement of attention linked with this publication.

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Availability of data and materials: The information used and/ or examined during this training are accessible from the consistent authors on sensible invitation.

Consent for publication: Not appropriate.

Competing interest: The authors professed that they take no challenging attention.

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