

IDENTIFICATION LOCAL ISOLATES OF *Trichophyton mentagrophytes* AND DETECTION OF KERATINASE GENE USING PCR TECHNIQUE

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ABSTRACT

This study was aimed to identify dermatophytic selective isolate using PCR technique as a rapid molecular assay. The results of this study showed among 60 samples of patients suffering from ringworm disease. forty isolates (66%) were *Trichophyton mentagrophytes* which diagnosed as dermatophytosis according to morphological and cultural methods. In order to investigate the ability of isolates to keratin analyses using solid medium supplemented with keratin azure , the results revealed that 20 isolates appeared best ability to keratin analysis and nine isolates had best ability for keratinase production in submerged culture. According to this results *T. mantagrophytes* (K3) (had higher activity for keratinase) was chosen for molecular identification. The results of PCR revealed that primer for18S rRNA gene of *T. mentagrophytes* K1 isolate and specific primer for subtilisin like protease gene were amplified and appeared as single DNA band with a molecular base of 690 bp and 623bp respectively .The blast result of sample sequences of amplified fragment revealed that the isolate were 100% identical to reference sequence of *T.mentagrophytes* var. *interdigital* and depending on data base in NCBI The result of PCR product for enzyme showed new type named GBF60362 (402) subtilisin-like protease related to *T. mentagrophytes* 1354684064 BFB-SOLP00892.

Keyword:, molecular investigation, virulence factor dermatophytes, keratin analysis.

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تشخيص العزلة المحلية للعفن *Trichophyton mentagrophytes* والتحري عن جين انزيم الكيراتينيز بواسطة تقنية PCR

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المستخلص

هدفت الدراسة الحالية الى استعمال تقنية PCR لتشخيص احدى عزلات الفطريات الجلدية و التي اظهرت اعلى فعالية لانزيم الكيراتينيز وتشخيص وجود الجين المسؤول عن الانزيم. بينت النتائج امتلاك الفطر *Trichophyton mentagrophytes* اعلى نسبة عزل 66.6% (40عزلة) من بين العزلات الفطرية المعزولة من 60شخص مصاب بالفطريات الجلدية المشخصة بالفحوصات المظهرية و المجهرية. اظهرت 20 عزلة فقط قابليتها على تحليل الكيراتين من خلال انتاج الانزيم على وسط صلب مضافا اليه الكيراتين ازور كما بينت النتائج قابلية 9 عزلات فقط على انتاج الكيراتينيز في الوسط السائل فضلا عن ذلك اظهرت العزلة (K3) اعلى فعالية انزيمية والتي اختيرت لغرض التشخيص الجزيئي حيث كانت تتابعات العزلة مطابقة لتتابعات المصدر (*Trichophyton mentagrophytes* var *interdigital*) بنسبة 100% تبعا للمعلومات المسجلة بال NCBI كما اظهرت نتائج التحري عن جين الانزيم نوع جديد باسم GBF60362(402) 1354684064 BFB-SOLP00892 .

الكلمات المفتاحية: التحري الجزيئي, عامل ضراوة, فطريات جلدية, تحليل الكيراتين

INTRODUCTION

One of the keratinophilic fungi are Dermatophytes that infect keratinized tissues of human and animal origin. *Trichophyton mentagrophytes* is considered to be a species complex composed of several strains, which include both anthropophiles and zoophiles. Accurate discrimination is critical for comprehensive understanding of the clinical and epidemiological implications of the genetic heterogeneity of this complex(23,25,30). The conventional methods usually depending for Dermatophyte identification ,based on detection of phenotypic characteristics, such as direct microscopy and in-vitro culture . Morphological and physiological characteristics can frequently vary; but the phenotypic features factually can be easily influenced by external factors such as temperature and the medium used (6,21, 29). In the last few years, molecular approaches have been proven to be useful for solving taxonomic problems regarding dermatophytes. Genotypic differences are considered more stable and more precise than phenotypic characteristics(11). Molecular method such PCR, have brought greater efficiency in distinguishing between species and strains of dermatophytes(7,12,24). Dermatophytes are adapted to utilize keratin as a major nutritional source; hence, infection is generally cutaneous and restricted to the non-living cornified layers such as skin, stratum corneum, hair and nails of humans and animals(25) Keratinase is important virulence factors for the host invasion by the dermatophyte (31). Dermatophytes secrete keratinase to degrade human and animal keratin and invade the skin. Dermatophytes produce multiple endoproteases which fall into two large protein families, example the subtilisins (serine proteases), and the fungalysins (metalloproteases) (21,22). The objective of this study identification of *T. mentagrophytes* clinical isolate and detection of keratinase gene using molecular technique PCR.

MATERIALS AND METHODS

Collection of samples

Sixty sample of skin scrapings were collected from patients admitted to Dermatology Department of Baghdad Educational Hospital suffering from ringworm disease.

Phenotypic identification

The fungal cultures of *T. mentagrophytes* were selected and maintained on Sabourouds Dextrose Agar medium(after identified isolates depending on conventional standard methods) (3,5).

Detection the Keratin lyses production on solid media by using Keratin Azure

According to Scott and Untereiner method(28), the basal medium (BM) was prepared in a final volume of 1 liter that contained 15 g Bacto Agar (Difco, Detroit, MI, USA) and 100 ml of each of the following solutions (prepared individually in a total volume of 1 liter): (i) major salts stock solution (5.0 g KCl, 5.0 g MgSO₄.7H₂O, 0.01 g CaCl₂.2H₂O); (ii) micronutrients stock solution (40 g NaH₂PO₄. H₂O, 20 g FeCl₃.6H₂O and 1 ml each of a solution containing 1000 mg/l MnCl₂.6H₂O, 1000 mg/l ZnSO₄.7H₂O, 100 mg/l Na₂MoO₄.2H₂O, 250 mg/l CuSO₄.5H₂O). The pH was adjusted to 9.0 by the addition of a solution of concentrated NaOH. Fifteen milliliter of BM were added to test tube (25 ml), then autoclaved at 121°C, 15 psi pressure for 15 min. after cooling 1 milliliter of Keratin Azure (4mg/ml of basal medium) added for each test tube . The test tubes inoculated with fungal inoculum which prepared according to Faraj method (8) by adding 200 µL of spore suspension (3*10⁶spore/ml) for each fungal isolate. The tubes incubated at 30°C for 1-3 weeks. The release of Azure pigment down the media indicate for Keratin lyses (28).

Determination the ability of fungal isolates to produce Keratinase in submerged culture

The keratinase production medium prepared according to Lin *et al.* method (18) ,the media contained ,per liter the following:(NH₄Cl 0.5g, NaCl 0.5g, K₂HPO₄ 0.3g, KH₂PO₄ 0.4g ,MgCl₂.6H₂O 0.1g, yeast extract 0.1g, chicken feather 10g) in 1000ml D.W, pH 7.5 , 250 ml capacity Erlmeyer flask containing 50 ml of sterilized media with 1% each of fungal suspension (3*10⁶spore/ml) that gave positive result in solid medium , flasks were incubated at 30 °C for 7 days. At the end of growth period the cultures were centrifuged at 6000 rpm for 15 min at 4 °C then the supernatant used for examined enzyme activity(18).

Keratinase assay: Keratinase activity was measured according to Gradisar *et al.* method

(10) .The reaction mixture containing keratin solution 5% (dissolved in 0.028M phosphate buffer pH 8) solution 0.5 ml ,crude enzyme solution 0.5 ml ,incubated at 45 °C in a water bath for 30 min. The reaction was stopped by adding 1ml of 10%(w/v) trichloroacetic acid and centrifuged (10000 rpm,15 min).The absorbance of the supernatant was measured at 280nm. The blank was treated in the same way except the addition of TCA which done before the initiation of enzyme reaction. The keratinase activity unit was expressed as amount of the enzyme corresponding to an increase in the obsorbance value (0.1) .

molecular identification

The isolate which appeared higher activity for keratinase is subjected for molecular identification.

DNA extraction: Genomic DNA was extracted from fungal growth according to the protocol of ABIO pure extraction kit (Promega / USA).

Table 2. The oligonucleotides primers sequences and PCR product for 18S rRNA and keratinase enzyme gene

Primer name	Primer sequence(5'-3')	PCRProduct size (bp)
ITS1 F	TCCGTAGGTGAACCTGCGG	591
ITS4 R	TCCTCCGCTTATTGATATGC	
subtilisin-like protease F	GGAATCGCTAAGAAAGCCAAAG	623
subtilisin-like protease R	CCCTCCAGTGCCATCAAATA	

Sequencing

The PCR product of 18s rRNA for *T. mentagrophytes* that amplified were send for Sanger sequencing using ABI3730XL, automated DNA sequencer, by Macrogen Corporation-Korea. The results were received then analyzed using genious software, the results of all sequences identified by basic local alignment search tool (BLAST) provided by National center of biological information (NCBI) at website (<http://www.ncbi.nlm.nih.gov>) to identify the homology with published sequences.

RESULTS AND DISCUSSION

Isolation and identification of *T. mentagrophytes*: In this study the most common

Detection of DNA by PCR: General primers (Internal transcribed spacer ITS1 and ITS4) was used for *T. mentagrophytes* identification and subtilisin like protease primer was used for keratinase gene detection as showed in Table1 (6). Amplification reaction were set up with 25 µl total reaction volume containing 12.5 µl of Taq mix (promega /USA) and 1 µl of each primers (1,6,13). And 4 µl of extracted DNA. All PCR amplification carried out in a Thermal cycler (Bio Rad, USA) Table1 .

Table 1 Temperature Cycling Program for PCR

Initial denaturation	Temp:95°C	Time:5min
No. of Cycles =35 cycles		
Denaturation	Temp:95°C	Time:30 sec
Annealing	Temp:55°C	Time:30 sec
Extension	Temp:72°C	Time:30 sec
Final Extension	Temp:72°C	Time:7min

The products were separated in 1% agarose gel stained with ethidium bromide,1xtris TBE buffer, and DNA ladder 100bp.The band DNA in gel visualized using gel documentation system (27).

isolates 66.6 % (40 isolates) were *T. mentagrophytes* From 60 samples which diagnosed as dermatophytosis ,which showed positive results with macroscopic and microscopic examination (Figure 1) . The higher frequency with *Trichophyton mentagrophytes* may be due to the direct or indirect contact with domestic animals such as cattle because they are zoophilic fungi and cause many ring worm infections. The investigation results of the ability of *T. mentagrophytes* isolates to produce keratinase using solid medium supplemented with keratin azure reveled that, out of 40 *T. mentagrophytes* isolates only 20 showed high ability to keratin hydrolysis, the release and diffusion of the azure dye into the lower layer indicated production of keratinase (Figure.2).

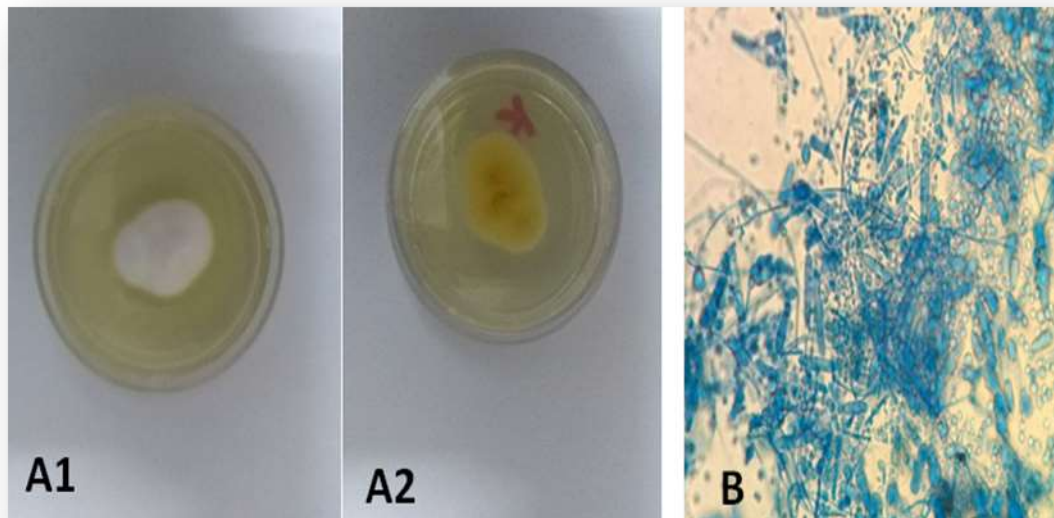


Figure 1. *Trichophyton mentagrophytes* grown on SDA at 30 C° for 14 days A1:surface view,A2:Revers side, B. Microconidia of *Trichophyton mentagrophytes* mounted with lacto phenol cotton blue(40X).



Figure 2. Production of keratinase by *T. mentagrophytes* on solid medium supplemented with keratin azure after incubation for 14 days at 30C°

The isolates which gave positive test on keratin azure medium showed response (dye release after 14 days), while the isolates with negative test only produced weak dye release after three weeks. Keratin azure is Azure dye-impregnated sheep's wool keratin which mainly includes alpha -Keratin as substrate (28), the diffusion of azure dye due to keratinase degradation action on keratin substrate which bend with azure dye resulting of release azure dye (9).



Figure3. Production of keratinase from *T. mantagrophytes* isolates in submerged culture using chicken feather 1% as keratin source, after incubation at 30C° for 7 days , pH 7.5 A. with culture *T. mantagrophytes* B. control .

Many researches of keratinase production were used the keratin azure test for keratinase activity assay, (2, 10, 20). Determination the ability of 20 fungal isolates to produce Keratinase in submerged culture using chicken feather (1%) as keratin source was carried out, the results as showed in Figure 3 and 4, indicated that only nine isolates had best ability for keratinase production (ability to degrade chicken feather).

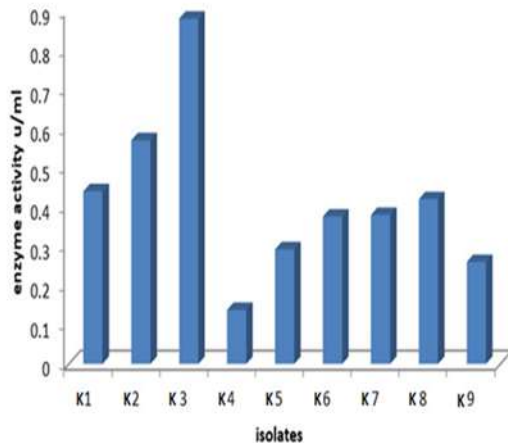


Figure 4. Keratinolytic activity of nine *T. mentagrophytes* (K) isolates after 7 days of incubation at 30°C in submerged culture

Keratinase activity in fungal filtrated (which give positive result in degradation of chicken feather) was measured. The result in Figure 4 reveal that high keratinase activity (0.88 U/ml) was appeared with *T. mentagrophytes* (K3) isolate so this isolate was chosen for complete the present study. The differences of keratinase production correlated to variety of nitrogen source and its concentration like feather meal, raw feather, chicken nails. In addition there are other physical factors affect the keratinase production such as temperature, inoculum density, aeration, pH and incubation time (26).

Molecular diagnosis of *Trichophyton mentagrophytes*

In order to confirm fungal identification, the primer for conserved region of 18S were used for amplification the DNA of *T. mentagrophytes* K3 isolate by PCR technique. The results of PCR revealed that primer for 18S rRNA gene of *T. mentagrophytes* K3 isolate and specific primer for subtilisin like protease gene were amplified and appeared as single DNA band with a molecular base of 690 bp and 623bp respectively Figure 5 and 7



Figure 5. Agarose gel electrophoresis of PCR reaction for 18S rRNA gene of *T. mentagrophytes* K3 isolate with product (690) bp . Bands were fractionated by electrophoresis on a 1% agarose gel (1.5 hr, 70V, 1XTBE buffer) and visualized under U.V. light after staining with ethidium bromide. Line (1) M. ladder of molecular size (100-1500bp).

submitted to Macrogen company /Korea for sequencing. The sequences were edited using Bioedit sequences software and compared with sequences reported in Gene bank National center for Biotechnology information (NCBI) by Blast analysis. The blast result of sample sequences of amplified fragment revealed that the isolate was 100% identical to reference sequence of *T. mentagrophytes* var. *interdigital* (Figure.6).

K3 Forward
 GATCCGAGGTACCTGA-
 TAAGTATAAAACAGATTTT-
 GAGGCCAGGGCGGCCGGTCTG-
 GAGGCGCTGGTTTGTGCCCATTGCCTAG
 GAAGCCGGAATCGCGGCCTGGCCAC-
 TGCTTTTCGGGCGCGTCCCGCACCCCAA-
 GACGGGGGCGCCGGAC-
 GGTCGTCCATCACACAA GCCGGGCTT-
 GAGGGGCTGAAATGAC-
 GCTCGAACAGGCATGCCCCCG-
 GAATGCCAGGGGGCGCAATGTGCGTTCAA-
 GATTCGATGA
 TTCACGGAATTCTGCAATTCACATTACTTATCG-
 CAATTCACATTACTTATCG-
 CATTTCGCTGCGTTCTTCATCGATGCCGGAAC-
 CAAGAGATCCGTTGTTGAAAGTTTTA
 ACTGATTTTTGCTTGCTAAC-
 GCTCAGACTGACAGCTCTTCTGAAA-
 GAATTTTTTT-
 GCGTCTGTCTCCGGCGGGCGCGGTCCAGCGTT
 TA
 GCCACTAAAGAGAGGCTCGCCGAAC-
 GGCTCTCTGGGA-
 GAGCGCGGCCCGCCGAGGCAACCGAG-
 TAAGGTAGACAAGAATGGGGCG
 GTACGGCCGGC

Figure6. The nucleotide sequence of amplified part of 18s rRNA gene aligned with up-loaded sequence of *T. mentagrophytes* K3

Sequencing of the internal transcribed spacer region (ITS regions) considered one of several alternative methods in molecular approaches developed to provide more accurate, rapid, and useful method for phylogenetic analysis of identification of dermatophytes. Due to small numbers of nucleotides differences in ITS regions considered as a strongly phenotypically and ecologically for separated *Trichophyton* species. Li (19) was found that the three *T. mentagrophytes* reference strains were identified as *T. interdigitale* by ITS sequence analyses. Many studies depend ITS region for dermatophyte species identification like Taha (29) who used ITS1 and ITS4 primers for 19

dermatophyte isolates resulted in amplified products of 690 bp specific for *T. rubrum*, *T. mentagrophytes*

Molecular detection of Keratinase (subtilisin-like protease) from *T. mentagrophytes*

Subtilisin-like protease is the primary virulence factors of *T. mentagrophytes* responsible for formation of dental biofilms and development of dental caries, so subtilisin-like protease gene amplified using PCR technique for detection keratinase enzyme. PCR product appeared as single band with molecular base 623bp (Figure 7). According to data base in NCBI The result showed new type named GBF60362 (402) subtilisin-like protease related to *T. mentagrophytes* 1354684064 BFB-SOLP00892 (with total sequence of this gene 1422 included 4 exon and 3 intron) (Figure8).

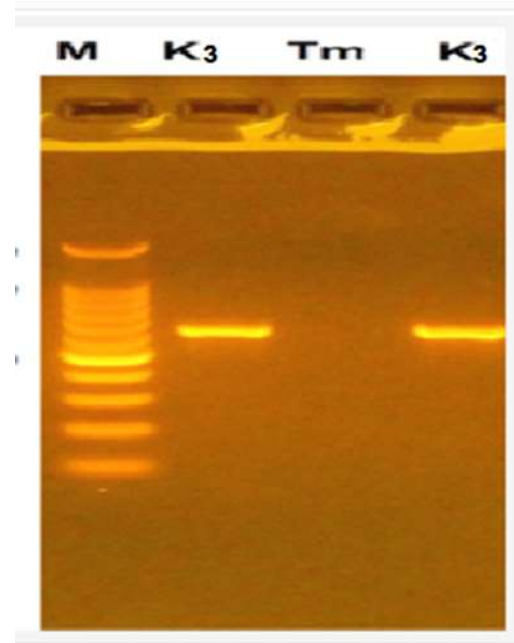


Figure 7. Agarose gel electrophoreses of PCR reaction for sub gene product (623) bp for DNA sample of *T. mentagrophytes* K3 isolate bands were fractionated by electrophoresis on a 1% agarose gel (1.5 hr, 70V, 1XTBE buffer) and visualized under U.V. light after staining with ethidium bromide. . line 1:M DNA marker (100-1500bp), line (2,4) isolate of *T. mentagrophytes* K3 . line 3:negative control
K3_Sub_P.ab1 >BFBS01015860.1:42681-44102 *Trichophyton mentagrophytes* TIMM 2789 DNA, Contig27724, whole genome shotgun sequence

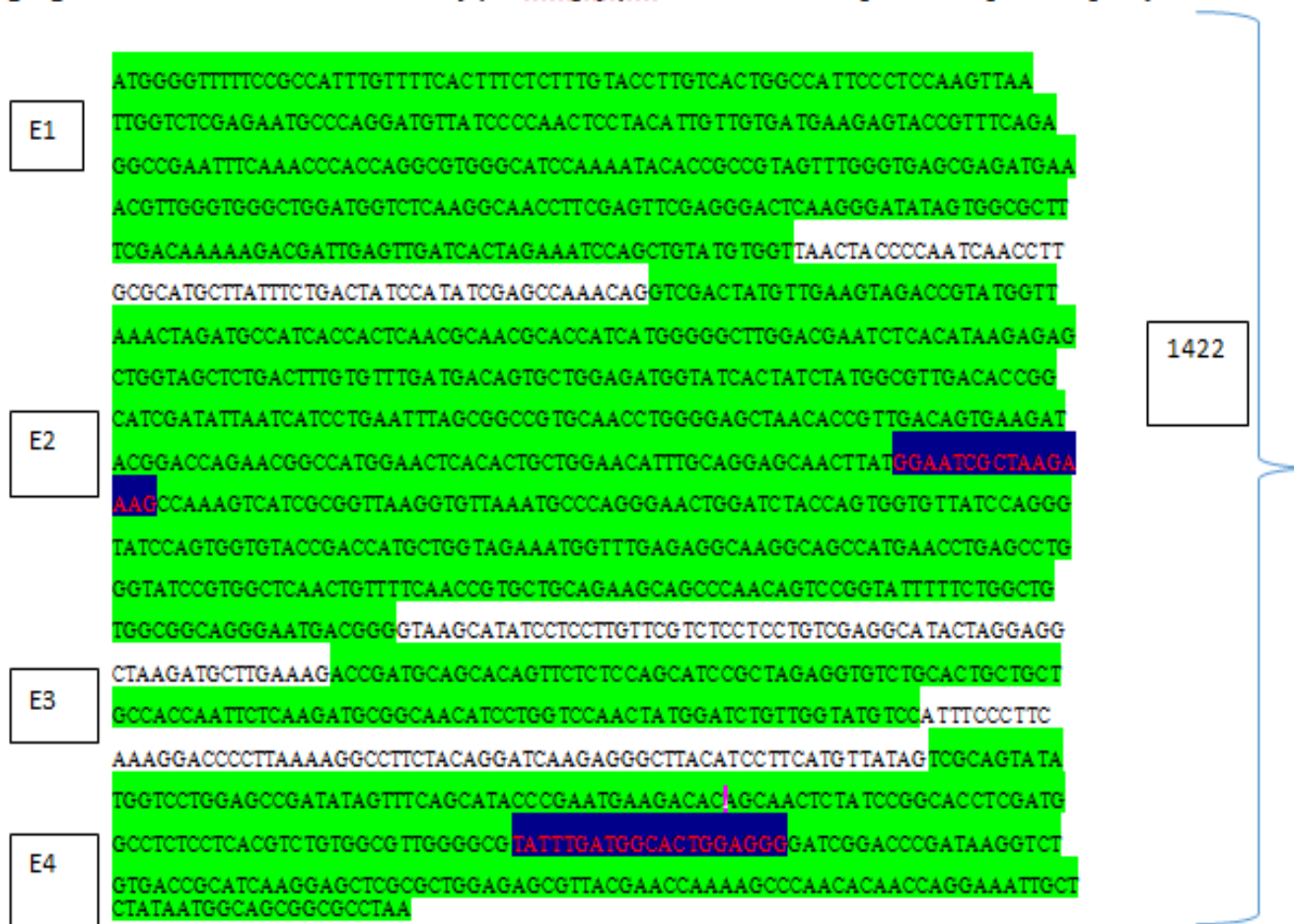


Figure8. The nucleotide sequence of amplified part of specific gene for keratinase (subtilisin-like protease) aligned : green line : exons , white line :introns, blue the primer

The presence or absence of gene was related with taxonomic affiliation and origin of isolates. Descamps with coworkers (4) studied gene encoding subtilisin- like protease in *Microsporum canis* , revealed that the entire gene encoding this protease named SUB3. In study by Jousson *et al.* (14), seven genes (*SUB1-7*) coding for subtilisin proteases were isolated in *T. rubrum*. Lemsaddek with coworkers (17) reported in his study that 7 types of sub (1-7) genes and all genes represented in all isolates of dermatophyte with different percentage ,but SUB3 like protease possess in all isolates which coded for a protease activity towards collagen, albumin, elastin and keratin. Recently Khalili *et al.* (16) studied subtilisin genes in *Microsporum canis* and referred that SUBs coding serine proteases on *M. Canis* give it the ability to digest keratinized tissues (skin and hair) in humans and animals and the absence SUBs in clinical isolate, indicates that they are indispensable for initial contact and adherence of fungi to host cells. Molecular detection methods proved to be highly sensitive and en-

able rapid and accurate detection of dermatophyte species from clinical specimens , Genotypic approaches have been proven to be useful for solving problems in dermatophyte taxonomy.

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