http://garmian.edu.krd https://doi.org/10.24271/garmian.170

Isolation and Molecular Identification of *Trichophyton rubrum var. raubitschekii* from the Infant Groin.

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Abstract

Five clinical isolates of *Trichophyton rubrum* isolated from patients with dermatophytosis in the Dermatology unit of the General Hospital in Kalar distric/Sulaimani province/North region of Iraq. The isolates were identified according to the conventional laboratory methods and molecular methods, one of these strains was isolated from the groin of an infant with one year old and identifies as *Trichophyton rubrum var. raubitschekii*. This variety characterized by the production of cottony to a velvety colony. The front of the colony appeared brown color with brownish pigmentation at the reverse. Slide examination revealed numerous cylindrical shaped microconidia and few longer macroconidia. Molecular identification was conducted according to the conventional PCR by using set primers ITS1 and ITS4 and resulted in PCR product about 690bp. PCR-RFLP by using *BstN1* digestion enzyme revealed three pattern bands 380,180,100bp. While sequencing of the ITS region revealed high similarity with that of a number of uncultured endocytic fungus clone**.**

Key words: *Trichophyton rubrum var. raubitschekii;* ITS, PCR, RFLP, Sequencing.

I. Introduction

Trichophyton rubrum is the major causative agent of dermatophytosis all over the world which includes tinea pedis, tinea corporis and tinea capitis (Gräser *et al*., 2000). Different species of dermatophytes have similar signs which are difficult to distinguish during clinical examination. In *vitro* culturing of dermatophytes is sensitive, specific; but requires long incubation period represents a drawback for diagnosis and treatment. As well as microscopic examination to describe micro and macroconidia provides rapid identification and gives sensitivity about 85% to detect the fungal pathogen. The amplification of DNA by PCR in the recent years enhanced the phylogenic determination of dermatophyte species (Liu *et al*., 2002).The phenotypic variants of *Trichophyton rubrum* did not revealed DNA variability (Gräser *et al.*, 1999). *Trichophyton rubrum var. raubitschekii* belonging to

Trichophyton rubrum complex and distinguished byurease positive and the production of profuse microconidia in the culture. It is isolated classically from patients with tinea cruris and tinea corporis in Africa and South-east Asia (Arabatzis *et al*., 2005). This strain differs from the common strains of *T.rubrum* morphologically, clinically and physiologically despite homogeneity (Llkit *et al*., 2011). The present study was aimed to molecular identification of *Trichophyton rubrum var*. *raubitschekii* and comparison between the sequencing of the ITS gene in the locally isolate which was identified in the study and the recorded isolates according to the data that provided by NCBI.

II. Materials and Methods

The Subject Study

Twenty clinical specimens included infected nail, hair and skin scraping collected from suspected patients with tinea infection (tinea pedis, tinea cruris and tinea corporis) under the supervision of specialized physicians and attended to the Dermatology Department of the General Hospital in Kalar district /Sulaimani Province. Specimens collected during the period of three months from September to the end of November 2016. The specimens (nail and skin scraping) were collected aseptically into disposable containers, labeled and transported on time to the research laboratory of Biology Department at College of Education / University of Garmian, for mycological examination.

Identification of Dermatophytes

Each specimen was divided into two parts, one of them for direct microscopic examination and the other part for culturing. Microscopic examination by KOH was performed to show the filamentous hyphae and arthrospores of dermatophytes. The second part of the specimens were cultivated on PDA and SDACC incubated at 25 ^{o}C for 21 dyes and examined at regular intervals (each three days) (Rippon, 1988). The isolates were identified by cultural characteristic (colonial morphology) and fungal species were diagnosed by direct mount examination with was carried out by Lactophenol Cotton Blue (LPCB) (Forobes *et al*., 1998). Biochemical tests were performed by urease test, growth on rice grain and hair perforation test. The fungal genomic DNA was extracted from the fungal colonies by using OMEGA Fungal DNA Mini Kit\ (USA). Molecular identification was carried out by species-specific PCR by amplification of the internal transcript spacer (ITS) gene of rDNA in dermatophyte species. The amplification was carried out by using one pair of primers symbolized ITS1and ITS4 as forward primer and reverse primer respectively (Refai *et al*.,

2013). PCR was performed in 25 µL final reaction volume, of PCR reaction mixture containing which consisted of: 12.5 µL of Master Mix (X2), 2 µL of DNA template,9.5 µL of dDeionized water and 0.5 µL for from each primer (forward primer ITS1 and reverse primer ITS 4). The PCR conditions were made according to Ghojoghi, *et al*., (2015) included: Initial denaturation at 95 ^{o}C for one minute, followed by 35 cycles of denaturation at 95 ^{o}C for 30 seconds., annealing at 55 ^{o}C for 1 minute, extension at 72 ^{o}C for 2 minutes and final extension at 72 ^{o}C for 5 minutes. The amplified DNA fragment was visualized in 1.5% agarose gel. PCR-RFLP analysis was conducted by using the *BstN1* restriction enzyme (BioLab, UK) according to the protocol provided by the manufacturer. The PCR products were sequenced in South Korea (Macrogene lab\ as Standard Sequencing Service).

III. Results

Among the twenty clinical specimens were isolated from patients with dermatophytosis, five of them identified as *Trichophyton rubrum* by conventional macroscopic, microscopic, and biochemical methods. The cultural characteristic of isolates has cottony to velvety colonies with white color at the surface and wine to brown pigmentation at the reverse. The microscopical method with 10% KOH shows fungal hyphae, numerous cylindrical, pyriform to oval shape microconidia born singly or along the hyphae and a large, smooth, multi septet and cylindrical shape macroconidia .

Out of five clinical isolates only one (20%) of them being from the groin of infant with one year old and identifies as *Trichophyton rubrum var. raubitschekii* which characterized by hardly furrowed with suede-like surface and raised at the center with faint pink color on the surface and dark brown at the reverse surrounded by halo with yellow color on SDADD (Fig 1). On PDA the front of the colony appeared with brown color and brownish pigmentation at the reverse (Fig 2). Microscopically, slide examination revealed numerous small rounded microconidia born along short branch or pedicles of hyphae (Fig 3A). Numerous cylindrical shaped macroconidia and few longer macroconidia were seen (Fig 3B and C). This isolate differs from the other clinical isolates of T.rubrum which was urease positive .

Molecular identification by species-specific PCR conducted by amplification of the ITS region on the ribosomal DNA by using a set of universal primers ITS1 and ITS4. Amplification of the ITS region was visualized in 1.5% agarose gel electrophoresis.

Amplicon size determined by comparison with 100 bp DNA ladder. A band size of 690bp confirms a positive result.

PCR-RFLP by *BstN1* restriction enzyme showed similarity with the results of PCR-RFLP of the rDNA in the other isolates of T.rubrum and revealed three patterns 380, 180 and 100 bp during the electrophoresis in 3% agarose gel (Fig 4). Sequencing of the ITS partial gene in the rDNA of the variety *Trichophyton rubrum var. raubitschekii* was differed completely from that of *T.rubrum* and showed high similarity to that of *Phoma spp*. and uncultured endocytic fungus (Fig 5).

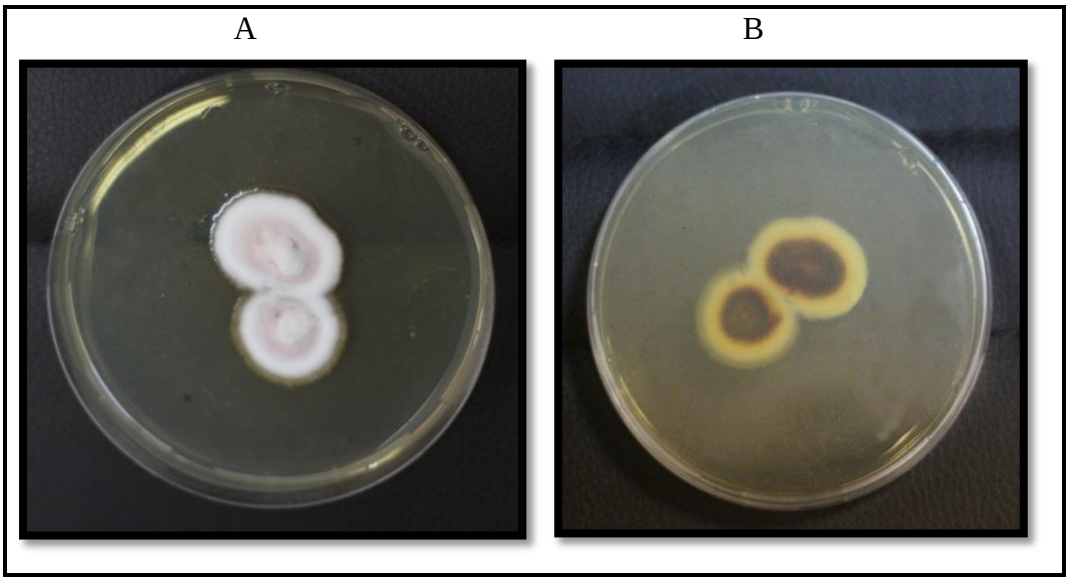

Figure (1): Colonial morphology of *Trichophyton rubrum var. raubitschekii* after three weeks of incubation at 25 °C on SDACC.

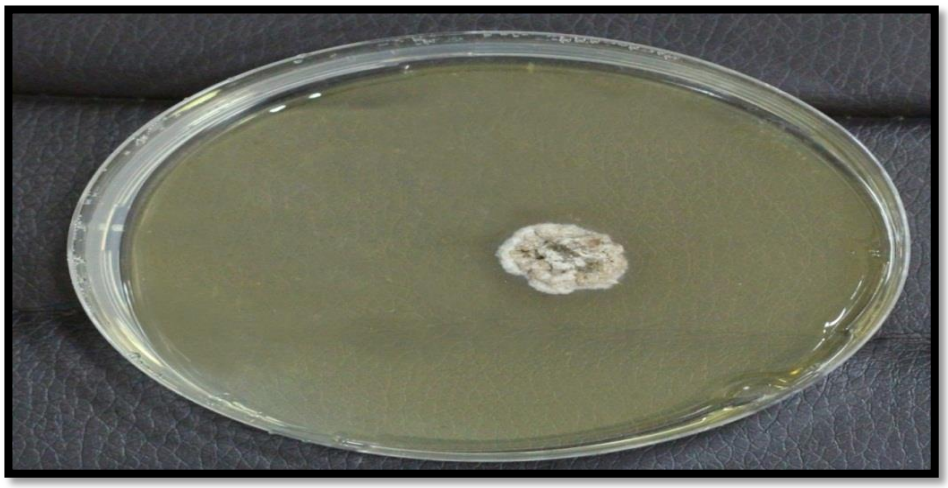

Figure (2): Colonial morphology of *Trichophyton rubrum var. raubitschekii* on PDA at 25 °C after one week of incubation.

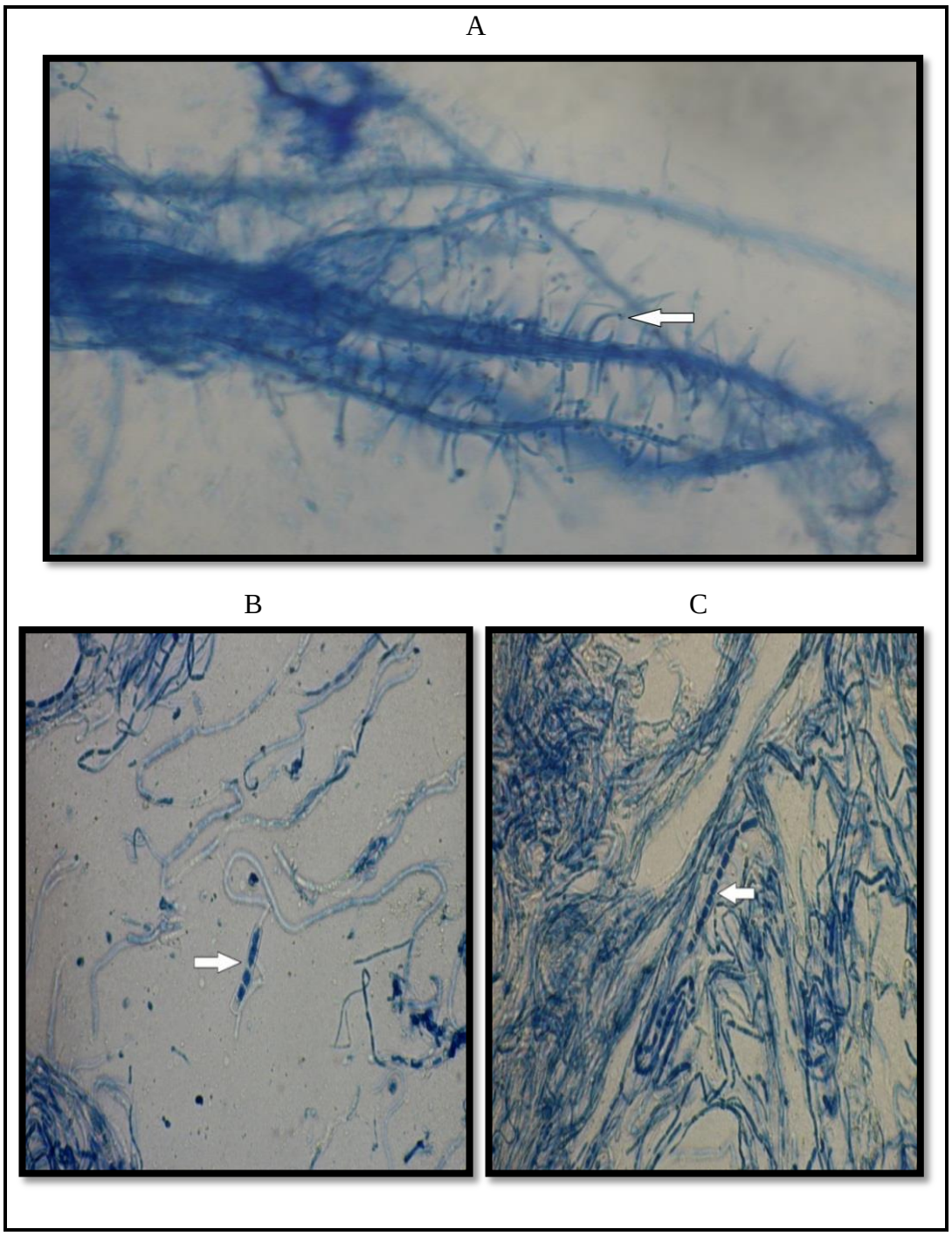

Figure (3): Microscopical morphology of *Trichophyton rubrum var. raubitschekii* colony mounted with LPCB stain showing: A) Microconidia bearing on branched hyphae. B) Cylindrical shaped macroconidia. C) Longer macroconidia (40X).

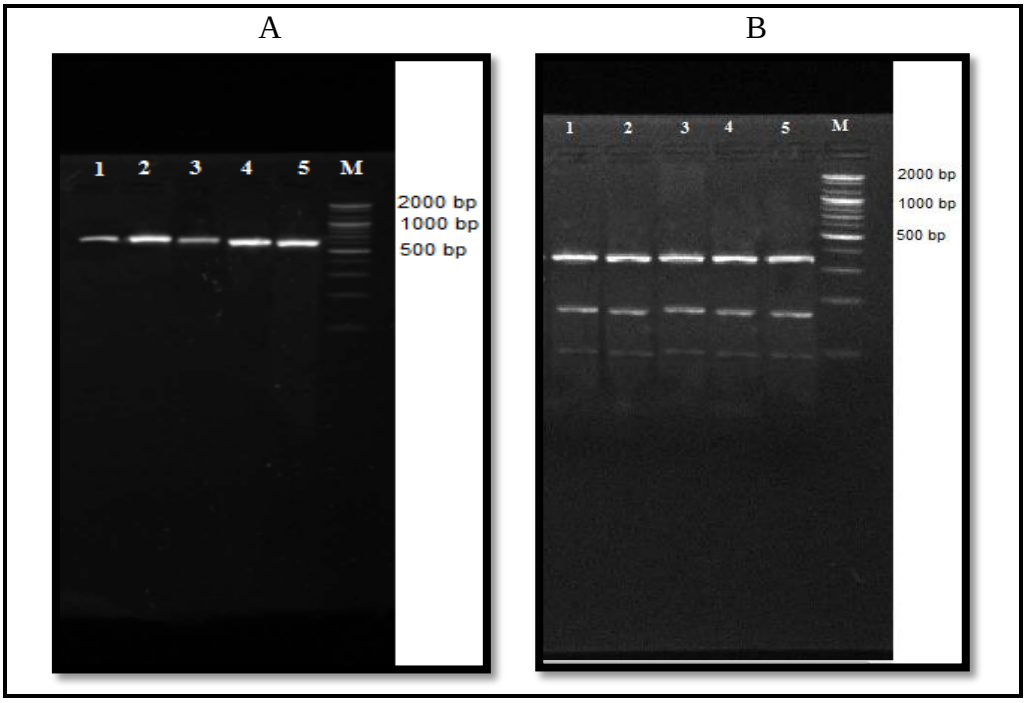

Figure (4): (A) Amplification of a 690bp fragment of ITS region in *Ttichophyton rubrum.* Lane: M, 100bp DNA Marker; lanes 1 to 5, PCR amplification of ITS region. (B) PCR-RFLP of ITS region in *Trichophyton rubrum*. Lane: M, 100bp DNA Marker; lanes 1 to 5, restriction analysis with BstNI (380bp, 180bp, and 100bp).

Fungal sp. P5N27 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
Sequence ID: gi|573864655|gb|KF735014.1|

Alignment statistics for match #1

Score	Expect	Identities	Gaps	Strand
797 bits(431)	0.0	463/479(97%)	0/479(0%)	Plus/Plus

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Query  5    ACCCAGAGTTGTGAGCTTTGCCTGCTATCTCTTACCCATGTCTTTTGAGTACCTTCGTTT  64
            ||| |||||||  ||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  14   ACCTAGAGTTGTAGGCTTTGCCTGCTATCTCTTACCCATGTCTTTTGAGTACCTTCGTTT  73

Query  65   CCTCGGCGGGTCCGCCCGCCGATTGGACAATTTAAACCATTTGCAGTTGCAATCAGCGTC  124
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  74   CCTCGGCGGGTCCGCCCGCCGATTGGACAATTTAAACCATTTGCAGTTGCAATCAGCGTC  133

Query  125  TGAAAAAACTTAATAGTTACAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAA  184
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  134  TGAAAAAACTTAATAGTTACAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAA  193

Query  185  GAACGCAGCGAAATGCGATAAGTAGTGTGAATTGCAGAATTCAGTGAATCATCGAATCTT  244
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  194  GAACGCAGCGAAATGCGATAAGTAGTGTGAATTGCAGAATTCAGTGAATCATCGAATCTT  253

Query  245  TGAACGCACATTGCGCCCCTTGGTATTCCATGGGGCATGCCTGTTCGAGCGTCATTTGTA  304
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Sbjct  254  TGAACGCACATTGCGCCCCTTGGTATTCCATGGGGCATGCCTGTTCGAGCGTCATTTGTA  313

Query  305  CCTTCAAGCTCTGCTTGGTGTTGGGTGTTTGTCTCGCCTCTGCGAGTAAACTCGCCTCAA  364
            |||||||| |||||||||||||||||||||||| ||| |||||||||
Sbjct  314  CCTTCAAGCTTTGCTTGGTGTTGGGTGTTTGTCTCGCCTCTGCGTGTAGACTCGCCTCAA  373

Query  365  AACAATTGGCAGCCGGCGTATTGATTTCAGATCGCACTACATCTCGCGCTTTGCACTCAG  424
            |||||||||||||||||||||| || |||| ||||||||||||||||||
Sbjct  374  AACAATTGGCAGCCGGCGTATTGATTTCGGAGCGCAGTACATCTCGCGCTTTGCACTCAG  433

Query  425  AAAGACGACGTCCATAAGTAAATTTTGACACTCTTGACATCGGATCAAGTACGGATACC  483
            || |||||||||| ||||| ||||| |||||||||| ||||||| ||| |||||||
Sbjct  434  AACGACGACGTCCAAAAGTACATTTTTACACTCTTGACCTCGGATCAGGTAGGGATACC  492
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Figure (5): Sequencing of ITS region in *Trichophyton rubrum var. raubitschekii*

IV. Discussion

Trichophyton rubrum is an anthropophilic species and can be transmitted easily from infected person to another during simulation and using clothes. Venkatesan *et al.,* (2007) explained that tinea corporis was the most common type of dermatophytosis and *T. rubrum* was the major causative agent among dermatophytes in India. According to the morphological characters of the fungal colon on SDACC, two types of *T. rubrum* were identified and these included downy and granular types. Out of five clinical isolates of *T. rubrum*, three isolates were identified as downy types, one isolate was granular type and the other isolate was identified as *T. rubrum var. raubitschekii*. Morphologically, this variant differs from the other variants by producing reddish brown colony with hardly, glabrous furrowed suede-like texture. Microscopically, there are numerous microconidia carried on short branched hyphae, cylindrical macroconidia and few longer macroconidia with the ability to produce urease enzyme (urease positive) which is the important character for the identification process (Ellis *et al*., 2007). According to the molecular approaches which conducted by species-specific PCR revealed that the amplification of the ITS gene in the *T. rubrum var. raubitschekii* by PCR which result 690bp product, while the PCR-RFLP analysis by using single restriction enzyme *BstN1* provides three bands 380m 180 and 100bp and these results were similar with the results of the molecular identification of the other isolates of *T.rubrum* in the present study. The databases of ITS sequence of the variant *T. rubrum var. raubitschekii* (repeated three times) alignment and later calculated the similarity score between the query sequence and reference sequence by using BLAST sequence analysis tool ″http:www.ncbi.nlm.gov\BLAST″ from the National Center for Biotechnology Information. According to the sequence of ITS region in rDNA by using two universal primers ITS1 and ITS4 then alignment in NCBI this variant showed high proportions in the similarity with uncultured fungus reached to 97% and this variance between the results of this study with another study may be due to mistake in the identification process, technical errors during PCR amplification, a mutation in the ITS regions or mistakes in the sequencing process. So to make sure the diagnosis is accurate, we need to amplify other genes and re-analyze them by gene sequencing.

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