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RESEARCH ARTICLE

First identification of *Trichophyton rubrum* var. *raubitschekii* in Constantine (ALGERIA).

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Abstract

Onychomycosis is a nail infection caused by different fungal belonging to dermatophytes, yeasts and molds. Dermatophytes are the most incriminated. The aim of our study is to establish both conventional and molecular diagnosis for onychomycosis in Constantine.

Sixteen nail samples were collected from patients with nail lesions, clinically suspected of onychomycosis. Direct microscopic observation and culture on Sabouraud medium with and without cycloheximide were performed. The identification was based on macroscopic and microscopic features, and confirmed by real-time panfungal PCR analysis. The ITS1-5.8S-ITS2 rDNA region was amplified using the ITS1 and ITS4 primers; and sequenced. The 16 samples were positive on their KOH direct examination. On Sabouraud medium, only 5/16 gave positive culture. The urease test on Christensen medium performed on the 5 isolates revealed a positive urease for one isolate and showed numerous macroconidia in microscopic observation. This isolate showed 99 % of homology with both *Trichophyton raubitschekii JX827168.1* and *Trichophyton rubrum FM178326.1* in molecular diagnosis. Based on phenotypical characteristics and molecular analysis, this isolate was identified as a *Trichophyton rubrum* var. *raubitschekii*. The ITS phylogenetic tree, showed 100 % of homology in the ITS region of *Trichophyton rubrum*, and *Trichophyton raubitschekii* isolated. The systematic use of the urease test for *Trichophyton rubrum* could contribute to increase the prevalence of *Trichophyton rubrum* var. *raubitschekii* in the world.

Introduction:-

Onychomycosis or tinea unguium are the most common causes of nail abnormalities. They represent 50% of nail diseases with different clinical patterns (Nzenze Afène *et al*., 2011; Tanrıverdi and Özer, 2013). These infections are

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mainly caused by dermatophytes, sometimes by yeasts and rarely by environmental molds (Tanrıverdi and Özer, 2013).

Trichophyton rubrum and *Trichophyton mentagrophytes* are responsible for nearly 90% of toenail and at least 50% of fingernail onychomycosis (Tanrıverdi and Özer, 2013; Yadav *et al*., 2015). *T. rubrum* represents over than 80% of nail dermatophytes (Gupta and Nakrieko, 2015), and is considered as a complex of species including multiple morphotypes. Recently, several of these morphotypes, have been formally identified as variants of *T.rubrum*, including *T. raubitschekii*, usually found in Africa, Southeast Asia, Australia and South America (Hiruma *et al*., 2012) with tinea corporis and tinea cruris. However, it is weakly associated with tinea pedis and onychomycosis.

Identification of causative agents responsible for onychomycosis is usually based on direct microscopic examination of clinical samples and culture in order to determine the fungal features. The development of molecular biology techniques is a valuable addition to the detection and identification of dermatophytes.
In this context, we used conventional methods (direct examination and culture) and a real-time panfungal PCR assay for the diagnosis of onychomycosis in Constantine (Algeria) in the aim of deciphering *T. rubrum* complex looking for species closely related to this one.

Patients and methods

Nail samples:-
A total of 16 nails samples were collected from 16 patients oriented by their dermatologists, consulting at the Parasitology- Mycology Laboratory of Constantine Hospital (Algeria) for onychomycosis suspicion. Patients who received a local and/or a systemic antifungal treatment, during the three months before the consultation, were excluded from the study.
Nails were taken from the infected area, in the junction between infected and healthy nail zone (Chabasse and Pihet, 2014).

Clinical sample processing:-
Each sample was divided into two parts. The first part was treated with 30% KOH solution and observed directly under microscope for the presence of fungal elements. The second one was cultured on Sabouraud chloramphenicol medium with and without cycloheximide (Biorad, France) at 27 °C for up to 4 weeks (Dhib *et al*., 2014; Nzenze Afène *et al*., 2011). The identification was based on macroscopic and microscopic features, and confirmed, later, by RT-PCR analysis. The potatoes dextrose agar medium (PDA) was used to stimulate pigmentation and sporulation of the cultures.

Fungal DNA Extraction:-
DNA extraction was carried out in laboratory of Parasitology and Medical Mycology (Claude Bernard University - Lyon 1). DNA was extracted using QiAamp DNA mini kit (Qiagen, Germany) according to the manufacturer's instructions with minor modifications. Small amounts of pure mycelia grown on PDA were transferred in 1.5 ml Eppendorf tubes containing 200 µL of physiological water then sonicated during 40 min. twenty µL of proteinase K and 200 µL of buffer ATL were added. Tubes were incubated for 10 minutes at 56°C and 200 µL of ethanol (96-100%) were added. The extracted DNA was recovered in 50 µl of Buffer ATE, centrifuged and stored at -20°C until use.

Polymerase chain reaction and DNA Sequencing:-
The real-time PCR has been realized by Light Cycler according to SYBR Green I technology (Roche Diagnostics Ref.: 2239264), in capillary tubes, using the primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3) and ITS4 (5'-TCCTCCGCTTATTGATATGC-3) (Jackson et al., 1999; Zhang *et al*., 2015). Real-time PCR technology was performed with fluorescent SYBR Green I. The extracted DNA (2 µL) was added to 18 µL of reaction mixture containing 10.4 µL H_2O, 1.6 µL $MgCl_2$, 2 µL of each primer (ITS 1 and ITS 4), 2 µL of Light Cycler Fast Start DNA Master SYBR Green I buffer (Roche Applied Science, Meylan, France). Conditions for cycling were 95°C for 10 min, followed by 44 cycles of 95°C for 10 s (denaturation), 58°C for 10 s (annealing), and 72 °C for 40 s (elongation), followed by a final extension step for 5 min at 72 °C. Fluorescence was monitored at the end of elongation step.

In order to sequence the fungal DNA, we purified the amplicon using a PCR purification kit (Qiaquick PCR Purification) and sequenced it using ITS1 and ITS4 primers, according to Sanger method. Results were compared on

a BLAST search via the National Center for Biotechnology Information (NCBI) nucleotide database (http://www.ncbi.nlm.nih.gov/BLAST).

A phylogenetic analysis of sequences representing the Internal Transcribed Spacer (ITS) region of rDNA amplified from *T. rubrum* isolated, and sequences of *T.rubrum* complex available in the GenBank database, were constructed with Neighbor joining method (Saitou and Nei, 1987) and with the substitution Kimura two parameters model and a 1000 replicates Bootstrap in Molecular Evolutionary Genetics Analysis- MEGA 6.0 (Kumar et al., 2008). Phylogenetic tree was produced using *Trichophyton interdigitale* (accession no KP308373.1) as an out-group.

Results:-

Patients' ages ranged between 23 and 75 years and the sex-ratio was 0,3. The 16 samples were positive on direct KOH examination and showed the presence of mycelium (Figure 2). On Sabouraud medium, only 5/16 samples led to positive culture. Patients had two clinical presentations: the distal lateral subungual onychomycosis (DLSO) and the DLSO hyperkeratosis with a thickened nail, brown yellowish color, peeling off at the distal portion of the nail (Figure 1). The cultures were flat to slightly raised, white to cream, sued-like to downy, with a yellow-brown to wine-red reverse. On PDA medium, the five colonies were fluffy disc-shaped with rounded edges and raised centers. Furthermore, they developed wine red pigment on their reverse (Figure 3A and B).

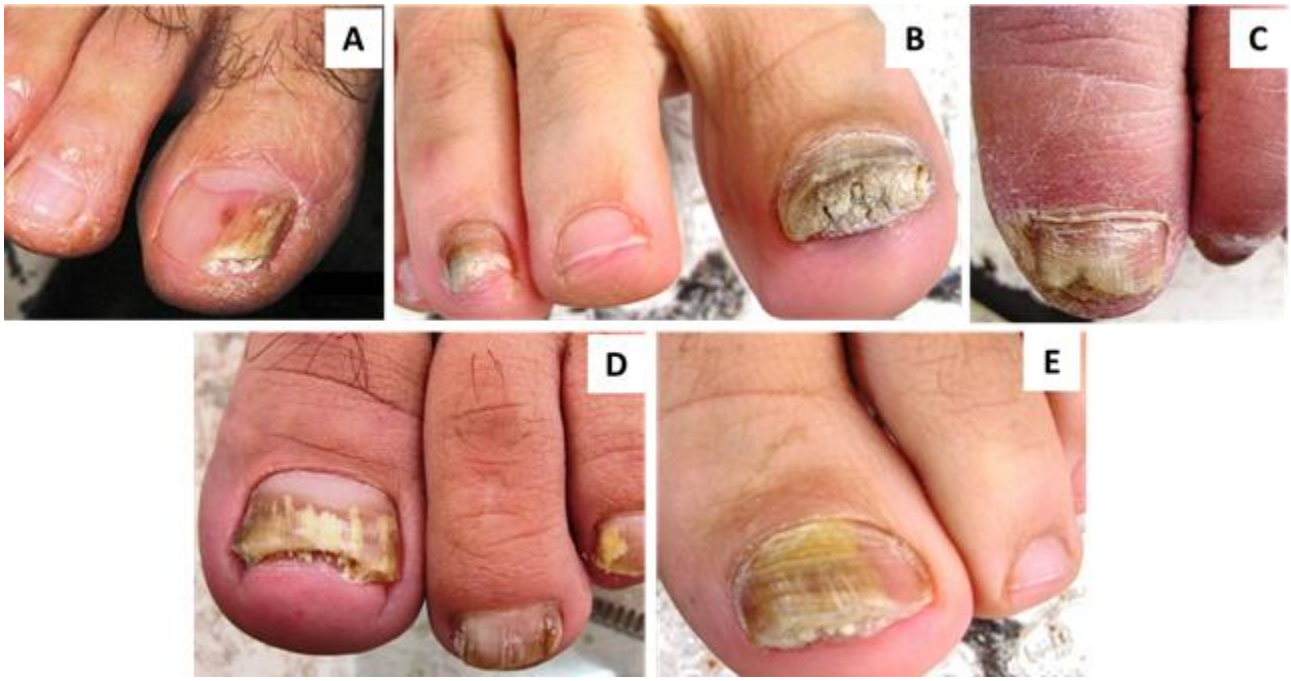

Figure 1:- Cases of onychomycosis caused by *Trichophyton rubrum.* A, C and D: Distal lateral subungual onychomycosis (DLSO); B and E: DLSO with subungual hyperkeratosis.

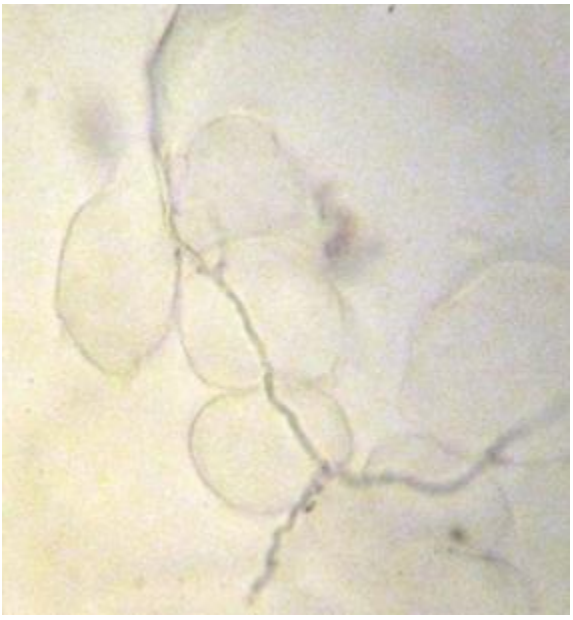

Figure 2:- Positive direct examination of nail scraping showing mycelium.

Microscopically, 4/5 isolates (*Tr.1, Tr.2, Tr.3* and *Tr.5)* produced scanty to moderate number of slender clavate microconidia and no or moderate macroconidia. ITS1 sequence of these isolates was 99 % identical to *T.rubrum* KC923433.1, AJ270802.1, JX122348.1 and AJ270793.1, respectively. These four isolates were urease negative. One isolate had numerous clavate or pyriform microconidia, abundant production of cylindrical or cigar shaped macroconidia and was urease positive within 6 days of incubation (Figure 3C and D) (Adamski et al., 2014; Arabatzis et al., 2005; Hiruma et al., 2012; Tietz et al., 2002; Zhang et al., 2015). ITS 1 sequence shared 99 % of homology with both *T. raubitschekii* JX827168.1 deposited by Liu and Zhang in 2012 (China) and *T.rubrum* FM178326.1. Based on phenotypical characteristics and molecular analysis, we concluded that *Tr.4* was a *T.rubrum* var. *raubitschekii*.

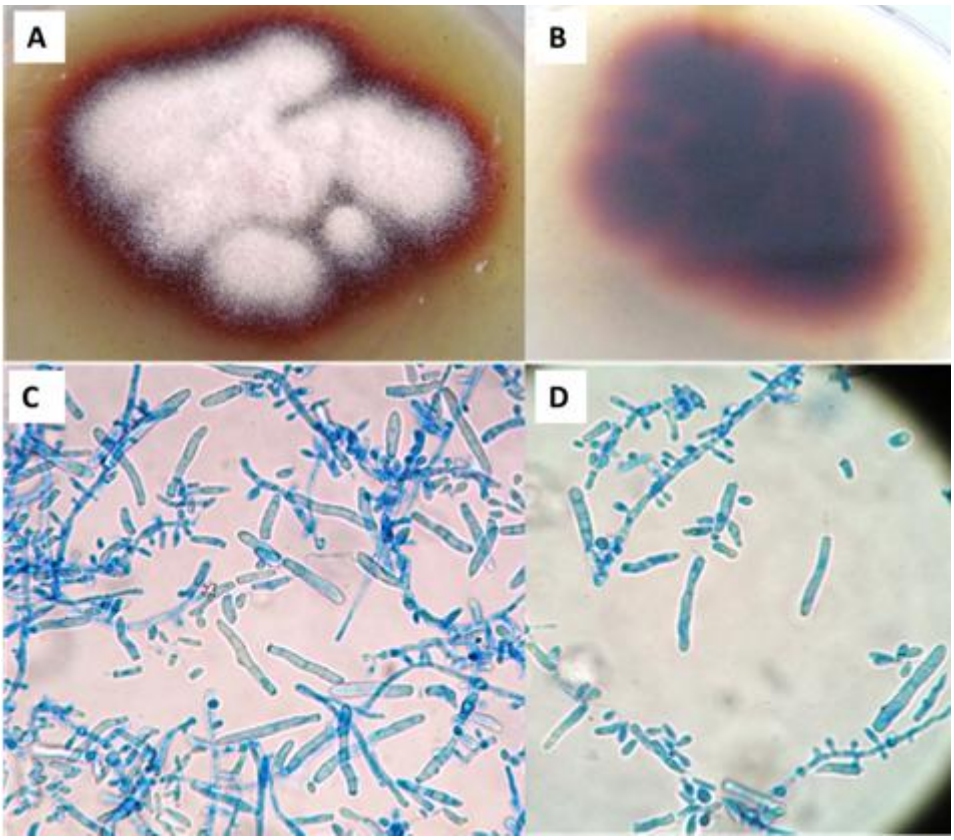

Figure 3:- *T. rubrum var. raubitschekii.* A: Colony on PDA after 10 days of incubation, B: Reverse of the colony, C and D: Microscopic morphological features X 400.

The ITS phylogenetic tree was composed of two separated clades with *T.rubrum* and *T. interdigitale* sequences clustering separately. All *T.rubrum* (*Tr.1*, *Tr.2*, *Tr.3* and *Tr.5*), as well as *T. raubitschekii (Tr.4)*, were grouped in the same branch with their related isolates which had 100% of homology in the 18S_ rDNA gene partial sequence ITS1, 5.8S_rDNA gene and ITS2 fragment (Figure 4).

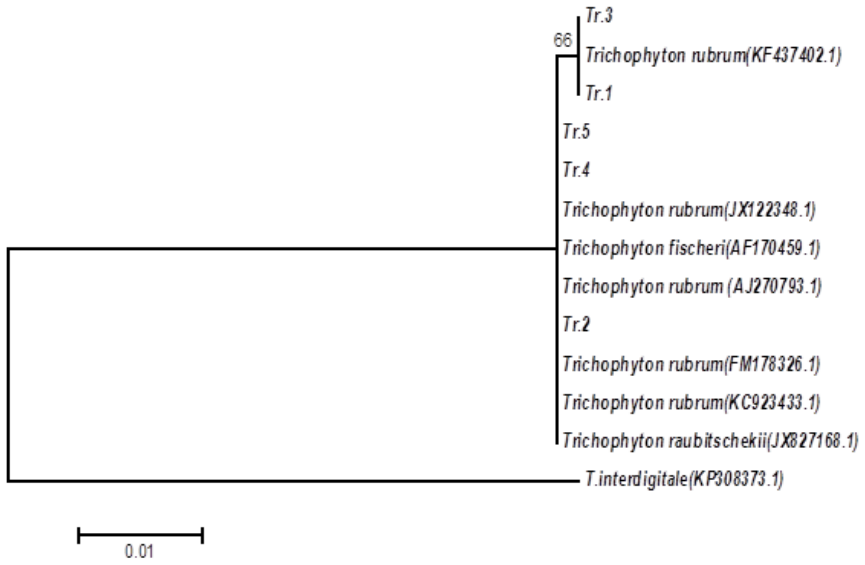

Figure 4:- Phylogenetic tree of *T. rubrum*, based on confidently aligned rDNA Internal Transcribed Spacer (ITS) sequences.

Discussion:-

We reported for the first time *T. rubrum* var. *raubitschekii* isolated from the toenail of a hypertensive 69 years old women living in Algeria presenting with an onychomycosis of the hallux. Microscopic examination of cultures and urease positive test were suggestive of *T.rubrum* var. *raubitschekii*. The molecular analysis of the isolate led to 99 % of homology with both *T. raubitschekii* JX827168.1 and *T. rubrum* FM178326.1. As previously reported, we demonstrated that *T. rubrum* var. *raubitschekii* was genetically identical to *T. rubrum*. In our study, this variant was isolated in one out of five *T. rubrum* positive cultures.

T. rubrum var. *raubitschekii* is an anthropophilic, and pathogenic dermatophyte, originated from Africa, Asia and South America (Brasch, 2007). It was lately found in Europe (Hiruma *et al*., 2012), but most of the patients involved were immigrants from subtropical and tropical areas. In latest studies, it was associated with tinea corporis, tinea manuum, tinea cruris and tinea unguium (Zhang *et al.*, 2015). Clinically, the lesions caused by *T. rubrum* var. *raubitschekii* were indistinguishable from those caused by *T. rubrum* (Figure 1C). This could explain the fact that no author described this variant before in Algeria. Djeridane *et al.* reported a global prevalence of toenail onychomycosis of 4.6 % in Algerian military. Onychomycosis were caused by *T. rubrum* in 35% of the cases (Djeridane *et al.*, 2006). This could be related to the misidentification of this variant. The colony is phenotypically closed to *T.rubrum*, *granular T.mentagrophytes* strains and *T. violaceum* (van Gelderen de Komaid and Borges de Kestelman, 2001; Zhang *et al*., 2015). The urease test is not systematically performed in the routine leading to misidentification of *T. rubrum* var. *raubitschekii*.

Initially, *T. raubitschekii* had been classified as a separate species (Adamski *et al*., 2014) but the molecular studies, such as the chitin synthase gene 1 (CHS1) and internal transcribed spacer (ITS) region (ITS1-5.8S-ITS2) sequences, recognized it as a variant of *T.rubrum*. Indeed, in our study, we analyzed a ribosomal DNA genomic fragment, consisting of internal transcribed spacer sequences (ITS) 1 and 2 and intermediary 5.8S rDNA, using universal ITS1 and ITS4 primers. We found that *T. rubrum* var. *raubitschekii* shares similarities with *T. rubrum* in sequence of ITS region, suggesting conspecificity as shown on the phylogenetic tree (Figure 4) where all the species are aligned in the same clade. This result can be explained by a genetic homology of both *T. rubrum* and *T. rubrum* var. *raubitschekii* in this genomic region, as previously demonstrated (Gräser *et al*., 2000). *T. rubrum* and *T.rubrum* var. *raubitschekii* are classified as the same species and considered as synonyms.

Conclusion:-

Using both conventional and molecular methods, we successfully isolated one *T. rubrum* var. *raubitschekii* out of five *T. rubrum* responsible for onychomycosis in Constantine. Due to genetic homology of the two variants, molecular analysis is not the panacea to differentiate *T. rubrum* var. *raubitschekii* and *T. rubrum senso stricto*. Systematic use of the urease test for *T. rubrum* producing numerous microconidia and macroconidia could contribute to increase the prevalence of *T. rubrum* var. *raubitschekii* in the world. To our knowledge, the role of this variant in onychomycosis physiopathology is not elucidated. If *T. rubrum* var. *raubitschekii* has a particular pathogenic profile, it could give a rational to implement specific mycological diagnosis to improve its detection.

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