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GENOTYPIC IDENTIFICATION OF *CANDIDA* SPP. ISOLATED FROM ONYCHOCANDIDIASIS PATIENTS BY PHENOTYPIC METHODS, PCR AND RAPD-PCR

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ABSTRACT

Candidal Onychomycosis (COM) is a common nail disease which plays as sources pathogenic reservoir giving a rise to repeated candidiasis infections. This study aimed to evaluate PCR assays and phenotypic tests for identification of yeasts isolated from COM patients. The study included 100 clinically suspected patients of COM attending the main hospital and clinics in Al-Dewania province in the middle of Iraq during September 2011 to April 2012. One hundred yeast isolates were identified morphologically by CHROMagar medium. DNA was extracted from 14 representative's isolates for accurate identification by PCR and fingerprinted by RAPD-PCR. Phenotypic examination of 100 yeasts isolates on CHROMagar revealed that these isolates were classified into 7 different species belonged to *Candida* form genus, PCR assay revealed that primer pair ITS1 and ITS4 was successfully amplified ITS1-5.8S-ITS2 rDNA region for 14 isolates of *Candida* spp. yielding a unique PCR products approximately 510-650bp in length. The results of RAPD-PCR assay showed that both primers (TAGGATCAGA and AGGTCACTGA) were genotyped 14 isolates of *Candida* into seven main genotypes; three of these genotypes had highly percentage of homologous (80-100%) among related isolates were studied in each *Candida* isolates, while the others four genotypes had 10-50% homologous. This study concluded that for accurate and prices identification must used PCR and fingerprinted by RAPD-PCR assays, the results of CHROMagar were correlated with gene expression for each *Candida* isolates, while the results of RAPD PCR assay were correlated with degrees similarity and difference of genotypes for *Candida* isolates under interest.

Keywords: Onychomycosis, *Candida* spp., Genotyping, PCR, RAPD-PCR, CHROMagar, Iraq

1. INTRODUCTION

Candidal Onycho Mycosis (COM) represents one of the nail diseases caused by Dermatophytes and non-dermatophyte mold and yeasts. COM representative bout 30%-50% of all nail diseases which caused by fungi (Midgley *et al*., 1994; Garg *et al*., 2004). *Candida* spp. Are composing a part of the normal flora that inhabits the human body included: Vagina, mouth, anal, skin and gastrointestinal tract. These fungi can invade skin and nail in immunocopennt and immunocompromised patients, diabetes, transplanted organs, highly antibiotic uses. *Candida* spp. was attacked individuals those exposed for nail damage or injury, hot and humid climates (Heikkila and Stubb, 1995; Rigopoulos *et al*., 1998). One of the risk factors that lead to nail yeast infection is frequent and prolonged exposure to water, mostly infected different age and sex groups of human those followed bad hygiene, share communal showers, farmers and hard workers (Davenport and Wilton, 1971; Jesudanam *et al*., 2002; Wang and Ching, 2005; Ching *et al*., 2005).

Previous studies refer that patients between 21 and 40 years old were had potential Onychomycosis (Vinod *et al*., 2000; Grover, 2003; Vijaya *et al*., 2004; Agarwalla *et al*., 2006). *C. albicans* and non-albicans species causes virulence such as *C. glabrata* have appeared as important

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pathogens (Odds *et al*., 1990; Canteros *et al*., 1994; Bokhari *et al*., 1999; Agarwalla *et al*., 2006).

Identified pathogenic yeasts based on biochemical tests and phenotypic characters Such as colonial colors on CHROMagar, show reliable methods for identification but not clear cut identification furthermore most of conventional approaches were time consuming (48 to 72 h) to perform identification task delayed performed, subjected to limitations, challenges because some of biochemical tests sometime fail to identify causing agents, many reports show that 12% of tested isolates need additional treatment and 0.8% are not well identified (Beighton *et al*., 1995; Tarini *et al*., 2010).

Recent studies reported that the identification the *Candida* spp. by utilized molecular methods such as PCR and RAPD assays fulfill rapid identification of yeasts with high level of sensitivity and specificity (Chang *et al*., 2001; Khlif *et al*., 2007) and have been more reliable and accurate yielded results and fulfilled in a short time about 3-5 h, compared with conventional approaches and aid in faster diagnosis and prescribe drugs for patients improved chance of treatment and survival (McCullough *et al*., 1994; Xiang *et al*., 2007). This study was aimed to evaluate using of PCR assays beside phenotypic tests for identification of yeasts isolated from Onychomycosis patients in Iraq.

1.1. Patients

One hundred samples of nails had been collected from clinically suspected patients with Onychomycosis who attending private clinics in most regions of Al-Dewania province, Iraq and from outpatients attending Al- Dewania main hospital in the middle of Iraq. The period of sampling extended from between August 2011 to July 2012.

2. MATERIALS AND METHODS

1-Yeast isolates identification the samples were cultured on Potato Dextrose Agar (PDA) and incubated at 28°C for 2-14 days. After 24-48 h, yeast colonies isolated in pure cultures by streaking on PDA and on CHROMagar medium were subjected to preliminary identification assays (Pfaller *et al*., 1996).

A Tiny portion of each colony was mounted on slide with drop of lactophenole cotton blue stain and covered with slide cover and examined under high power for detection of the presence of chlamydospores. Fourteen representatives isolate were subcultured on Sabouraoud's dextrose agar for DNA Extraction.

2.1. DNA Extraction

For extracting genomic DNA of *Candida* isolates, a single colony for each isolate was taken by sterile loop and pick up in 600 µL of lysis buffer (200 mM Tris-Hcl pH 8,40 mM EDTA, 1.5% SDS and 150 mM NaCl) in a sterile tube. Few yellow sand was added to the cell suspension for disruption cell wall of yeast followed by heating for 85ºC and vortex. 600 µL of 25:24:1 volume of a mixture of Phenol: Chlorophorm: Isoamyl alcohol was added to cell suspension, vortexed for 5 min and centrifuged 5000 rpm for 3 min. The supernatant was transferred to a clean tube and 400 µL of chloroform was added. After centrifuging as the previous conditions, the aqueous phase was transferred to a clean tube and then 160 µL of 3M sodium acetate (pH 5.2) were added and was kept on -20°C for 10 min, centrifuged for at 10000 rpm for 5 min. The supernatant was transferred to a clean tube added and two volumes of isoprobanol were mixed gently and centrifuged 12000 rpm for 12 min. The supernatant discharged, the DNA pellet washed twice with 70% ethanol, dried and re-suspended within 50 µL distilled water and the sample was kept at -20°C until use (Mannarelli and Kurtzman, 1998).

2.2. PCR Conditions

The ITS1-5.8S-ITS2 rDNA regions was targeted PCR amplification using primer pairs ITS1 (forward, 5-TCC GTA GGT GAA CCT GCG G-3 and ITS4 (reverse, 5-TCC TCC GCT TAT TGA TAT GC-3). PCR amplification was performed in a final volume of 30 µL. Each reaction consists of 0.7 µL template DNA, 0.5 µl of each primer at 20 pemole, 5 µL master mix (Bionner Company, Korea). The amplification conditions were as follows: Initial denaturation cycle at 94°C for 5 min, followed by 35 cycles denaturation at 94°C for 1 min, annealing at 56°C for 1 min, extension at 72°C for 1 min and in the final cycle the final extension step was 72°C for 7 min. Eight µL of PCR products were visualized by 1% agarose gel electrophoresis in TAE buffer (pH 8). The gel was stained with 0.05% ethidium bromide premixed with agarose. The DNA bands were detected by Desk Gel imager scope 21 ultraviolet transilluminator.

2.3. RAPD-PCR

RAPD-PCR was accomplished with total volume 50 µL consist of 10 µL of 10x master mix (Bionner, Korea) with 37 µL DDW and 1.5 µL (50 pmole) for each random primers TAGGATCAGA and AGGTCACTGA (Valerio *et al*., 2006). The mixture was amplified using the following conditions: 95°C -4 min, 38 cycles 94°C 1 min -36°C 1.5 min 72°C 1.30-72°C 8 min (LABNET PCR System). The 8 µL of PCR products were run on

1.5% agarose gel at 55 volts for 1 h. The gel was stained with 0.05% ethidium bromide premixed with agarose. The DNA bands were detected by Desk Gel imager scope 21ultraviolet transilluminator.

RAPD-PCR products were subjected to Phylogenetic analysis and the phylogenetic tree denedrograms (UPGMA) were constructed by using UVI band software and evaluated the similarity coefficient factor according to (Mackenstedt *et al*., 1994).

3. RESULTS

3.1. Isolation and Identification of *Candida* spp

Phenotypic examination of 100 yeasts isolates on CHROMagar revealed that these isolates were classified into 7 species belonged to *Candida* form genus. The percentages of the identified species were: *Candida albicans* 29%, *C. tropicalis* 25%, *C. krusei* 28%, *C. guilliermondi* 10%, *C.famata* 3%, *C. parapsilosis* 1%, while 4% of isolates were recognized as *C.kefyr* based on colors on CHROMagar medium (**Table 1**).

3.2. Germ tube and Chlamydospore Formation

Results showed that the chlamydospore was produced abundantly single, in Clusters, or contiguous pairs in *C. albicans* produced. Two species produce germ tube, the green and pale green to dark blue color of colonies on CHROMagar refer to *C. albicans*, *C. tropicals*, while *C.krusei*, *C. guilliermondi* and *C.kefyr* they show Pink to white, Green to Pink and Cream to pink color respectively (**Table 1**).

3.3. Simple PCR

The primer pair ITS1 and ITS4 was successfully amplified ITS1-5.8S-ITS2 rDNA region for 14 clinical *Candida* spp. isolates, yielded range of PCR products between 510-650 bp they are: Lanes (B-C) = *C.albicans* (529-535 bp), lane (E) = *C, prapsilosis* (511-520bp). Lanes (I, F, K) = *C. krusei* (500-510 bp). Lane (O) = *C.guilliermondi* (590-610 bp). Lanes (H, D, J, G, L) = *C. tropicals* (517-525 bp). Lane (M) = *C. famata* (630-640 bp). Lane (N) = *C. kefyr* (650 bp) (**Fig. 1**).

3.4. RAPD -PCR

The results showed that both primers (AGGTCACTGA and TAGGATCAGA) were successfully genotyped 14 isolates RAPD-PCR produced multiple bands with primer (AGGTCACTGA), the main band was consistently present in all isolates (Constant basal band 450-550 bp), but greatest variation occurred among the upper bands of constant single bands at 600-800bp while the lowest were at 200-400 bp (**Fig. 2**). While the primer (TAGGATCAGA) was successfully fingerprints were identified by two constant double bonds in the size range between at 1000-1500 bp. Lower bands at 150-250 bp, 150-300 bp and 350-500 bp with extra band 200 bp for some isolates (**Fig. 4**).

Table 1. Summarized main characteristics of *Candida* spp. isolates from infected nails of patients

Candida spp.	Percentage (%)	Germ tube Formation	Color on CHROMagar
C. albicans	29	+	Green
C. tropicals	25	-	Dark blue
C. krusei	28	+	Pink to white
C. famata	3	-	blue-pink
C. prapsilosis	1	-	Pink to White
C. kefyr	4	-	Cream to pink
C.guilliermondi	10	-	Green to Pink

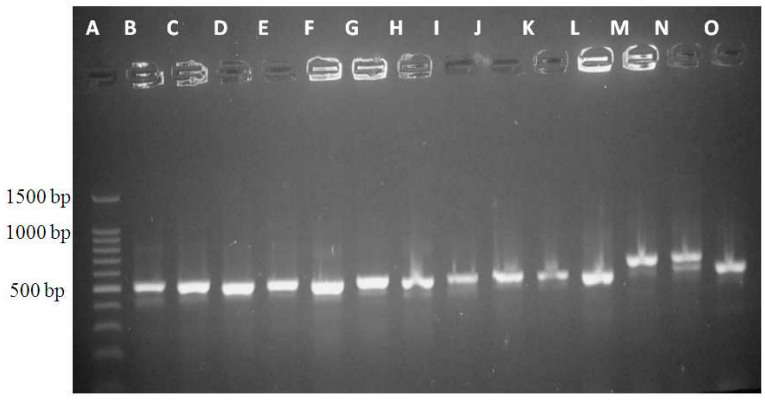

Fig. 1. Agarose gel electrophoresis of amplified PCR product for ITS1-5.8s-ITS2 rDNA of *Candida* spp. Primer pair ITS1/ITS4. Lane A = Molecular marker 100 bp; lanes B-O, clinical of *Candida* isolates

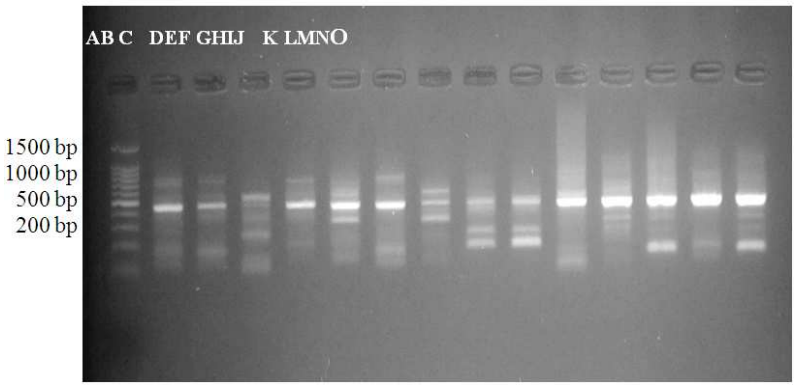

Fig. 2. Agarose gel electrophoresis of amplified RAPD-PCR products for *Candida* spp. Detection polymorphism of 14 clinical isolates of *Candida* isolates. Using primer AGGTCACTGA. Lane A = Molecular marker 100 bp; lanes B-O, clinical isolates of *Candida* spp.

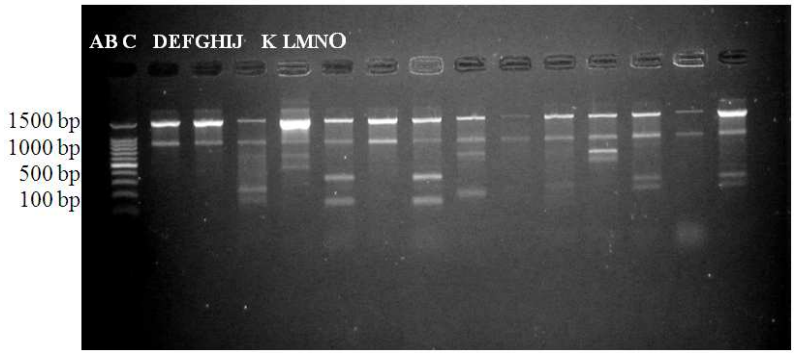

Fig. 3. Agarose gel electrophoresis of amplified RAPD-PCR products for *Candida* isolates. Detection polymorphism of 14 clinical isolates of *Candida* spp. Using primer TAGGATCAGA. Lane A= Molecular marker 100 bp; lanes B-O, clinical isolates of *Candida* spp.

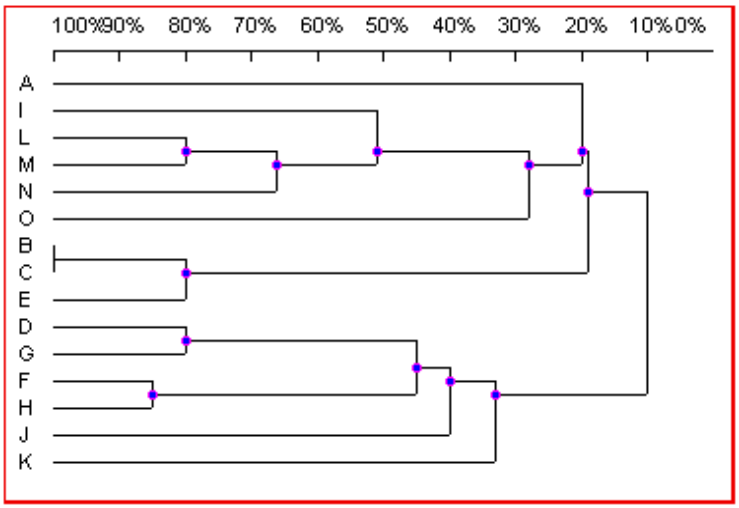

Fig. 4. Phylogenetic tree dendrogram (homologous coefficient %) between different isolates of *Candida*. Based on the RAPD–PCR by AGGTCACTGA primer. Was generated via UPGMA cluster analysis, lane A = Molecular marker, lanes I-K, clinical *Candida* isolates

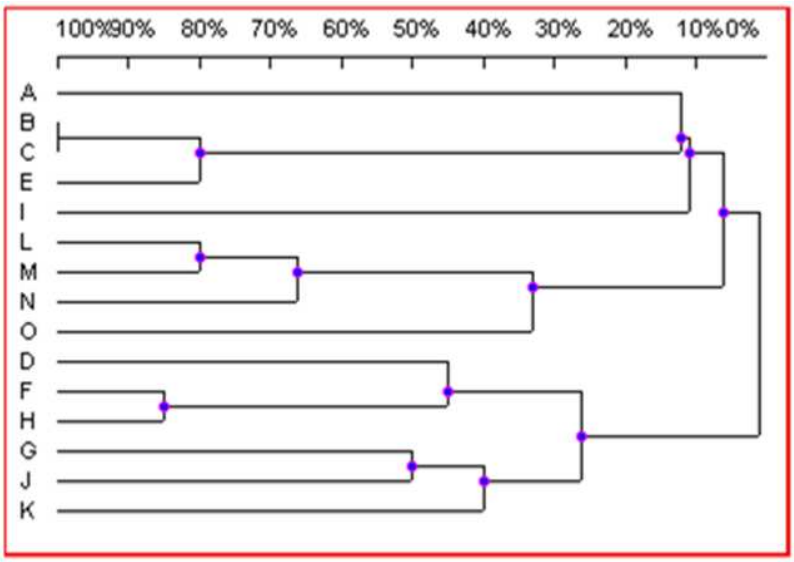

Fig. 5. Phylogenetic tree dendrogram of *Candida* isolates. Based on the RAPD-PCR by TAGGATCAGA primer. Was generated via UPGMA cluster, analysis, lane A = Molecular marker and lanes B-K, clinical *Candida* isolates

Phylogeny of RAPD-PCR produced created 3 main genotypes have homologous percentage with high of ranged between 80-100% (B&C, L&M and F&H genotype), while create 5 genotypes have homologous 10-50% (O, I, N, J and K genotype) (**Fig. 3 and 5**).

4. DISCUSSION

Candidal Onychomycosis is the most common diseases that widely prevalent in most sex and age's groups of patients that required for rapid presumptive identification by reliable and precise methods (Chang *et al*., 2001). CHROMagar was developed for presumptive identification of important *Candida* spp (Beighton *et al*., 1995). The identification of *Candida* species based on colony color on CHROMagar was showed some confusion between closely related species like *C. albicans* and *C. tropicalis*, no border line separated them because these isolates produced a wide spectrum in color from light blue-green, pale green to dark green color under the same incubation conditions, also *C.kefyr, C. guilliermondi* and *C. krusei* revealed pink-white color,. The CHROMagar medium was stilled an important test for pre-identifying many *Candida* species.

Molecular assay by Amplified ITS1-5.8S-ITS2 region was showed high distinctive species of *Candida*. The PCR products for 14 isolates of *Candida* which isolated from patient's nails were yielded genotypes had PCR products approximately ranged from 500-650 bp (included primer) and supported classified 7 species of *Candida*, *C. albicans*, *C. tropicalis*, *C. krusei*, *C. parapsilosis*, *C. famata*, *C. kefyr and C. guilliermondi* (**Fig. 2**) are rapid and sensitive alternatives for the identification of these species. Beighton *et al*. (1995) and Fujita *et al*. (2001) the amplified products by being coincident with the many previous studies (Tarini *et al*., 2010; Mousavi *et al*., 2012).

RAPD-PCR by two oligo-primers (AGGTCACTGA) and (TAGGATCAGA) showed special fingerprinting patterns for all *Candida* isolates. Phylogenetic tree denedrograms of *Candida* isolates showed variable degrees of homogeneous percentage from 100% in closed related isolates as in B&C (**Fig. 3 and 5**). Both primer gave similar patterns of RAPD PCR products in some isolates which appeared had the same fingerprinting genotypes. Our results were coincidence with the results of Tamura *et al*. (2001). The oligoprimers sometimes have great potential for detecting polymorphisms of many isolates within one species and in different species and removed the skeptical issue about discrimination potential of oligoprimers but the results of this study showed opposite to the idea that say oligoprimers may produced poorer at discriminating between the strains of another species (Roberts, 1992; Sullivan *et al*., 1995; Vinod *et al*., 2000).

5. CONCLUSION

This study concluded that identification of *Candida* spp. by phenotypic assays such CHROMagar and

culture characters were appeared less reliable comparing with results were conducted by genotyping by and PCR and RAPD-PCR assays, which facilitate identification of *Candida* spp. in clinical laboratories. The results of PCR and procedures described here allowed the identification of *C. albicans* and non-albicans. Also the genotyping of *Candida* isolates by RAPD-PCR revealed highly discriminated for genotypes of *Candida* and showed the precise similarity degrees of the isolates genotypes. Comparing with the results of CHROMagar which showed confusions in colors of some isolates of *Candida*.

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